Paul Shapshak · Andrew J. Levine
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Global Virology II – HIV and NeuroAIDS

Springer

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Preface

The spread of HIV-1 and associated NeuroAIDS commenced in earnest during the last few decades of the twentieth century, and although better characterized by the start of the twenty-first century, the spread of HIV continues in the current decade. The characteristics of NeuroAIDS and treatments have come within reach of practitioners, clinics, research, and development. Presentations in this book include HIV global view, global views in NeuroAIDS, descriptions of NeuroAIDS in several countries, CNS HIV entry, neuropathology, peripheral nervous system illness, genetics, neuroimmunology, perinatal NeuroAIDS, neurocognition in children, human and drug trafficking, drug abuse, oral manifestations, socioepidemiology, HAND and HAART, chemotherapy, genetics and epigenetics, gene expression, multiscale oscillatory biology, new tools and data mining, DNA sequence and database errors, amyloidogenic pattern prediction, miRNAs, neuronal apoptotic pathways, humanized mouse models, psychiatric comorbidities, cardiovascular disease, HCV, Chagas disease, TB, opportunistic infections, Zika virus, Ebola virus, biostatistics, HIV and SIV evolution, and vaccines.

The presentations in this book are a fraction of all that is being done. In addition, there are many books that review many topics during prior years of which a few are mentioned [1–9].

With all this progress, why produce a book such as this? The progress needs to be summarized and described for the global audience: the "cures" for HIV and NeuroAIDS. Moreover, worldwide, with the plethora of various strains of HIV, the work that has been done serves as a paradigm for the continued quest against HIV disease spread and pathogenesis.

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Acknowledgements

This book, for professionals, students, faculty, and interested readers, brings to bear snapshots of where we are with HIV and NeuroAIDS. Clearly, it took many years of work and debate among physicians, scientists, researchers, and other practitioners, including clinicians, interventionists, virologists, immunologists, and epidemiologists—from molecular levels of analysis to patients and clinics—for progress to occur and thus allow us to comprehend and attack the scope of infection, spread, and damage caused by a 9000 nucleotide-base microorganism, a small virus.

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Contents

Chapter 1
Multidisciplinary Approach to HIV/AIDS: Historical Perspective

Clyde B. McCoy, A. Jeanene Bengoa, Duane C. McBride, Brian T. Foley, Shikha Puri, Alejandro J. Mendez, and Paul Shapshak

Keywords HIV/AIDS • Epidemiology • Risk activity • Sex • Drug abuse • IDU • NIDA • Transmission • Multidisciplinary • Historical perspective

Core Message

The HIV/AIDS epidemic has persisted over the past three decades due to its adaptability, deleterious effect on the immune system, and various modes of transmission. A multi- and interdisciplinary collaborative approach is one of, if not the best, method in combating the HIV/AIDS epidemic. Specific demographics and high-risk groups dominate this epidemic and are a driving force. Therefore, collaborative approaches must continue to evolve to reduce and prevent the spread of HIV/AIDS. Although the HIV/AIDS epidemic has shown signs of improvement, trends continue in the United States, highlighting the overall global impact of this virus.

Clyde B. McCoy and A. Jeanene Bengoa contributed equally to this work.

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1.1 Introduction

The Body Snatchers, a science fiction novel written in 1954 by Jack Finney set the precedence in portraying the mindset surrounding a plague. Throughout the years, the original novel evolved into subsequent literary works and cinematic movies. In 1994, *Body Snatchers*, a film that starred Meg Tilly and Gabrielle Anwar strangely but accurately portrayed the HIV/AIDS epidemic through a unique perception. The graphic display of individuals who were "normal" and had not yet been subject to the alien seed "pods" clearly depicts the experience of the population throughout the HIV/AIDS epidemic. Those who had not been exposed to those alien "pods" would peer intently at one another, attempting to decipher if they had become alien and fatal. *Body Snatchers* mirrored the feelings and unfortunate fear experienced by those in the forefront of the HIV/AIDS epidemic.

The forefront of the HIV/AIDS epidemic in the Americas was seen in the manifestations of diseases in unusual populations and with no clear explanations of Kaposi's sarcoma and *Pneumocystis carinii* pneumonia (PCP) in the populations that were being seen in major cities such as New York, Los Angeles, and Miami. It is hard to imagine how quickly this epidemic spreads, and it is even more difficult to understand what would have happened if not for global scientific efforts that quickly launched research on these populations using multiple strategies. Among institutes commending such works were the Centers for Disease Control (CDC) and the National Institutes of Health (NIH). The NIH included the National Institute on Drug Abuse (NIDA), which had only become an institute in 1974. NIDA quickly established several research centers that happened to be in the very cities where these populations were being studied clinically. Miami received one of these first centers beginning in 1974 [1].

Furthermore, beginning in 1974 in Miami, there was an additional funded NIH institute center, the Comprehensive Cancer Center. At the advent of the study of this epidemic, there were multidisciplinary and interdisciplinary research teams, including some of this chapter's authors, who worked at both NIH-sponsored centers.

It is unfathomable to imagine the rate at which the HIV/AIDS epidemic would have spread if it were not for the creation of research organization such as NIDA under the umbrella of NIH in 1974 [1]. The first part of the twentieth century saw little to no drug abuse research until the 1960s and 1970s, when the drug epidemic ran rampant [1]. The abuse of drugs provided the need and platform to develop strategies, establish metrics, and institutionalize drug abuse research and prevention to promote multidisciplinary collaboration both domestically and abroad.

The multidisciplinary and interdisciplinary foci that developed throughout the institutionalization of drug abuse research assisted in implementing research priorities within both clinical and research settings [1]. Through this approach, disciplines such as demography, psychiatry, neurology, pediatrics, psychology, sociology, immunology, viral epidemiology, molecular biology, anthropology, ethnography, biostatistics, and public health provided the basis for providing the highest quality of research regarding HIV/AIDS among drug abusers. In addition, development and testing the efficacy of interventions to reduce high-risk behaviors included field

studies regarding drug abuse paraphernalia, efficacy of bleach in eliminating HIV and HCV from needles, as well as many other pertinent vectors of this epidemic [4–8]. This evolutionary process provided the means for extensive community outreach and the acquisition of funding that would allow for crucial information regarding the pathways of transmission, acquisition, and proliferation that manifest within the individual and society [1].

NIDA and other similar agencies at the local, state, and national level through funded infrastructure provided the sustainable foundation for research to progress structure and collaborative support. Unbeknownst to researchers globally, developing NIDA and other scientific oversight would allow for research to develop an understanding of this "strange and pernicious disease" [4, 9]. Although this virus was not studied until the 1980s, the foundation to reduce the impact of the HIV/AIDS epidemic began a decade earlier.

1.1.1 The Initial Years of Fears

Retrospectively, HIV infected humans in the 1920s in the Kinshasa region of what became the Democratic Republic of the Congo and Zaire. In the 1950s and 1960s, the virus embarked on its path as a global pandemic [2, 3]. Once reaching the United States, the HIV/AIDS epidemic forever changed the landscape of the United States. Attempting to understand this inexplicable disease and phenomenon created a sense of fear and apprehension. This virus was first presented to the University of Miami School of Medicine Grand Rounds in 1981, which puzzled physicians as to the cause and effect as well as the potential outcomes and risks associated therewith.

The first reported case in Florida involved a young Caucasian man of Northern European descent who was diagnosed with Kaposi's sarcoma. The physician reporting this case noted how unusual and unique the symptoms were as Kaposi's sarcoma was not known to exist in the age group of the patient or among those of Northern European descent. A second unusual case was also introduced. This time, the young man had been diagnosed with PCP – a disease that, according to the attending physician, usually occurred among the very old who were living in a nursing home. These cases puzzled the group in attendance at the University of Miami School of Medicine Grand Rounds in 1981, and many questions arose from these two cases at hand such as where the patients lived, where they had been, what acute or chronic diseases they had, and with whom they interacted [4]. It was further reported that there was little in the patients' background information that indicated any major acute or chronic diseases that would suggest they would be susceptible to these illnesses.

In the search for the explanation of "this strange and pernicious disease," the University of Miami research centers, departments, and schools had attracted and organized a very effective, multidisciplinary, and interdisciplinary group [1]. This allowed for a very synergistic approach to determining what the origins, consequences, and interventions should be for this "new thing" that initially went by several nomenclatures, such as [1] Gay-related immune deficiency (GRID) and [2] Gay disease. Further, the virus causing AIDS was given various names such as lymphade-

nopathy-associated virus and HTLV-III and even the 4H club which led to increased stigmatization and unnecessary stereotyping [4, 9]. Soon after, AIDS was used to describe this new syndrome that moderately caused a defect in cell-mediated immunity within individuals that had no other known diminished resistance to opportunistic infections such as organ transplant or inherited immune deficiency [10].

The misconception and misinterpretation of the HIV/AIDS epidemic was one of the main reasons for such a rapid increase in the proliferation of this disease. Immediately after this virus surfaced, immunologists were confident that HIV/AIDS could be managed through a simple vaccine and eventually as treatable as other chronic diseases such as asthma and diabetes [4]. Unknown to these scientists was that the AIDS virus was not capable to be overcome by a vaccine that would attempt to trick the immune system into producing effective antibodies [4].

1.1.1.1 AIDS Cases 1981–1991

Misdirection and a lack of scientific understanding led to the rapid increase in the prevalence of HIV/AIDS not only among individuals in the United States, but globally. The exponential rise of HIV/AIDS cases during the first decade of this epidemic shows that although agencies such as NIDA were available to combat drug abuse, the specificity and means by which HIV progresses and becomes AIDS were a mystery to all researchers and healthcare practitioners. Four cases of AIDS were documented in 1981, and by 1991, there were over 261,159; 23,162 and 7488 cases in the United States, Florida, and Miami-Dade County (MDC), respectively [11].

The surveillance systems that had been established in MDC prior to the rise of the HIV/AIDS epidemic enabled vital data to be collected regarding this virus from the first documented cases. Beginning with four AIDS cases that were presented at the University of Miami Medical Grand Rounds in 1981, there was an exponential increase in the number of HIV/AIDS cases in the following decades [12]. By 1991, nearly 7500 cases were identified in Miami, Florida, which more than tripled to 25,000 in the next decade [7]. The cumulative incidence of AIDS in MDC during 1981–1991 was 85 per 100,000 individuals, the highest rate reached in any decade (see Fig. 1.1) [7].

The first decade (1981–1991) of the HIV/AIDS epidemic in MDC, with 7488 AIDS cases, resulted in ranking only behind the much larger Los Angeles and San Francisco metropolitan areas [12]. Further, MDC was ranked second among US metropolitan areas, with 706 cases attributed to heterosexual contact, third in cumulative AIDS cases among women, and second in the cumulative AIDS cases among pediatric cases [12] (Table 1.1).

The rankings and impact of the HIV/AIDS epidemic within MDC were driven by specific demographics and risk groups which were unknown during the advent of this disease [7]. Men had a higher cumulative incidence when compared to women, and when observed by race/ethnicity, Black/African American had the highest cumulative incidence followed by White/Caucasian and the Hispanics [7]. Although understanding the demographics was essential to understanding and

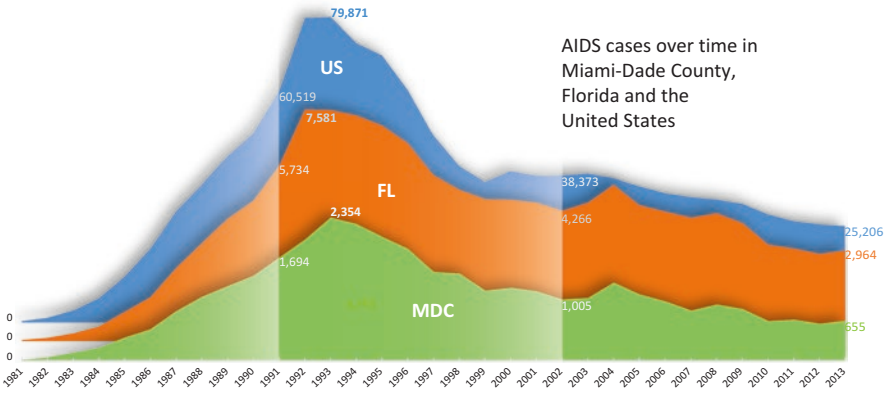

Fig. 1.1 Miami-Dade County vs. State of Florida vs. United States of America. This chart represents the number of AIDS cases per year from 1981 to 2013 [14, 33]

Table 1.1 Cumulative AIDS cases by year for United States, Florida, and Miami-Dade County [14, 33]

		Year		
		1991	2002	2013
Geography	United States	261,159(23)	866,590(16)	1,214,305(10)
	FL	23,162(43)	87,666 (26)	130,099(15)
	MDC	7,488(85)	25,347(43)	34,903(25)

developing methodologies to combat this epidemic, gaining valuable data regarding specific risk groups served as a major contributor in preventing the uncontrollable spread of HIV/AIDS, especially before serological testing for asymptomatic infection became available.

However, the category that accounted for the largest risk group was male-to-male sexual contact (MSM), which represented 45% of the cases irrespective of race/ethnicity [7]. Injecting drug users (IDU) were the next highest group at 23%, followed by heterosexual contact at 20%, unrelated/other causes at 6%, MSM/homosexual/bisexual/IDU at 4%, and blood transfusions at 2% [7]. Once the race/ethnicity was incorporated into the major risk groups, the percentage of high-risk practices differed. Like the overall trend, Hispanics primarily contracted AIDS through MSM at 70% followed by IDU at 10%, while Black/African Americans contracted AIDS predominantly through heterosexual contact and IDU at 37% [7]. Southern Florida was particularly influenced by its proximity to Haiti and thus its influx of Haitian immigrants. It is now well understood that the HIV-1 subtype B epidemic was simmering in Haiti for at least a decade before spreading to the United States and Europe [13].

MDC is an indicator, reflecting the total number of AIDS cases and the impact that HIV/AIDS had in the state of Florida. Although MDC has been one of the major epicenters for the HIV/AIDS epidemic, Florida has experienced its own

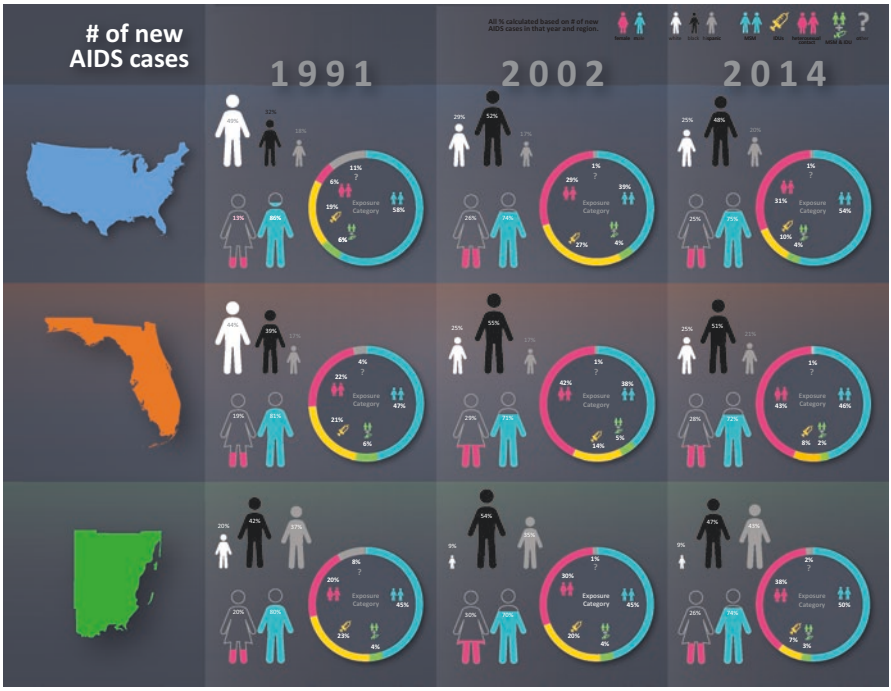

Fig. 1.2 Demographic breakdown of AIDS cases by decade and location [14, 33]

unique challenges distinct from MDC. Like the impact of this epidemic on MDC, individuals who were not white, male, and/or gay had a false sense of security that this virus could not affect them [14]. This misconception, coupled with the initial lack of scientific information, provided the mode for this virus to proliferate among risk groups that were thought to be safe from this epidemic.

The state of Florida saw a consistent increase in the total number of AIDS cases from 0.1 per 100,000 people in 1981 to 43 per 100,000 in 1991 [14]. Like MDC, men dwarfed women in the total number of AIDS cases (see Figs. 1.1 and 1.2) [14]. Further, the greatest number of AIDS cases occurred within White/Caucasian, followed by Hispanics and African Americans [14]. Also, MSM and IDUs served as the largest cluster among all high-risk groups [14]. The proliferation of this virus in MDC and Florida and the widespread increased incidence of AIDS signaled Florida as a major epicenter in the HIV/AIDS epidemic within the United States.

The impact and fear because of the HIV/AIDS epidemic was daunting nationwide. The total number of AIDS cases reported during the first decade showed the proliferation of this virus throughout the United States [15]. The trends experienced within MDC and Florida were also seen in the United States regarding gender. Men accounted for 86% of the total number of AIDS cases when compared to women, which was slightly a larger gap when compared to both MDC and Florida. Further, White/Caucasian individuals accounted for 49% of the total AIDS cases from 1981 to 1991 followed by Black/African Americans with 32% and Hispanics with 18%

[15]. The greatest impact of this epidemic within the United States was among MSM (58%), followed by IDUs (19%) and heterosexual contact (6%) [15].

1.1.1.2 Interventions and Progressive Movements 1981–1991

Social structures within the United States were not necessarily supportive of practices that could help combat an emerging epidemic. Indeed, increased urbanization reduced social structures that constrained sexual behavior [7, 5]. During this initial period of this epidemic, prostitution and venereal diseases increased alongside the abuse of hypodermic needles which became more widespread [1]. Further, changes in sexual mores in the 1960s reduced barriers within the heterosexual population, as also the Gay Liberation Movement in the 1970s presented new and unexpected losses of barriers and an increased severity of this new epidemic [9]. In addition, civic and legal policies produced severe limitations of the availability of clean legally acquired hypodermic needle/syringes, enhancing the conditions for acquiring and transmitting HIV/AIDS among IDUs [4, 5, 16, 17].

Prior to 1981, surveillance tools and agencies such as the Florida Department of Health had been established both within MDC and Florida, which allowed for the prompt documentation of the first AIDS cases. The first AIDS surveillance began within the Florida Department of Health and Rehabilitative Services, and the Centers for Disease Control and Prevention (CDC) declared AIDS a reportable disease in 1983, resulting in a lapse in the reporting of crucial information in the initial years [11]. The delay in recording AIDS as a reportable disease missed the opportunity in documenting the initial cases of IDU, heterosexual contact, transfusions, and infants [11]. In 1983, the HIV/AIDS epidemic was declared a "public health emergency" by the Florida State Health Officer which enforced the responsibility of physicians to report diagnosed cases, which actively began in 1984, mostly in South Florida [11].

In 1984, HIV was determined to cause AIDS with a major impact on the way hospitals, clinics, public health practitioners, and various other healthcare entities in Florida approached this epidemic. This fundamental knowledge directed active surveillance within Florida, and especially in South Florida, which was one of the epicenters for this epidemic [11]. The progression of programs and initiatives continued, and in 1985, the Food and Drug Administration (FDA) approved the first HIV antibody test, and national screening of blood commenced [11]. Further, 26 anonymous HIV counseling and testing sites were established with a statewide toll-free AIDS hotline [11].

After implementing these programs, Jackson Memorial Regional Medical Center, serving as a public hospital and the medical center for the University of Miami, was the primary hospital to deliver comprehensive care to HIV/AIDS patients [11]. Because 75% of all AIDS cases in Florida, health education, and risk reduction programs were established in Miami-Dade, Broward, Monroe, and Palm Beach counties [11]. Statewide public information programs were established to provide vital information and education regarding the HIV/AIDS epidemic to all households in Florida [4]. Six years after the first cases of this epidemic, the first antiretroviral drug, Retrovir, was developed to assist in combating this disease [12].

The expansion of the initial programs and availability of antiretroviral therapy used in AIDS treatment and prevention, coupled with the required notifications to partners of individuals with HIV/AIDS, began the movement in working to contain the impact of this virus.

As the late 1980s approached, new laws, interventions, and programs surfaced and were implemented statewide. In 1987, the World Health Organization (WHO) launched the Global Program on AIDS [11]. The Joint United Nations Program on HIV/Acquired Immune Deficiency Syndrome (UNAIDS) was developed and resulted in more impactful initiatives such as the Multi-Country AIDS Program (MAP) launched by the World Bank, which gave rise to most nations worldwide agreeing to global goals to reverse the spread of HIV [18]. As a result, 700 individuals were enrolled to receive antiretroviral therapy in 1988, which saw one of the first prominent movements in preventing the excessive spread of this virus [11]. In 1989, Project AIDS Care was established by Medicaid, and the FDA approved the use of antiretroviral therapy in mothers, which provided a new mode of preventing the transmission of HIV/AIDS to infants [18]. Although an overall improvement in treatment options occurred just before the start of the 1990s, the 1990s also brought the epidemic of "crack" cocaine in South Florida which led to a substantial increase in the risk factor for AIDS and other related sexually transmitted infections, such as needle-sharing and prostitution in exchange for drugs [19–21].

The Ryan White Comprehensive AIDS Resource Emergency Act was passed in 1990 by the US Congress; this Act provided essential services to those infected with HIV who lacked health coverage. This resulted in Florida obtaining seven million dollars to provide care and support to those living with HIV/AIDS [12]. Also, the second antiretroviral drug was approved by the FDA, and soon after, a combination antiretroviral drug was created that would provide a mechanism that would further assist in preventing the uncontrollable spread of HIV/AIDS. The introduction of Videx in 1991 further enhanced the ability to reduce the impact of the HIV/AIDS epidemic nationwide [12].

The AIDS epidemic provided the platform for many initiatives to be developed prior to all the factors associated with HIV/AIDS. For example, the CDC established the National AIDS Information Line (1983), National AIDS Clearinghouse (1987), institution of the nationwide America Responds to AIDS public information campaign (1987), and distributed *Understanding AIDS* (1988), which was the first mailed information regarding a major public health problem delivered to every residential mailing address in the United States [20]. Throughout the advent of these successful programs, the identification of HIV as a root cause of AIDS created the platform to develop serological tests that could detect HIV in the blood [20, 21].

1.1.1.3 Molecular Epidemiology

Beginning with the first infectious molecular clone of HIV-1 to be sequenced in 1985, many more clones from the United States, Europe, and Africa were also sequenced soon, and it became obvious that HIV was a diverse and rapidly evolving

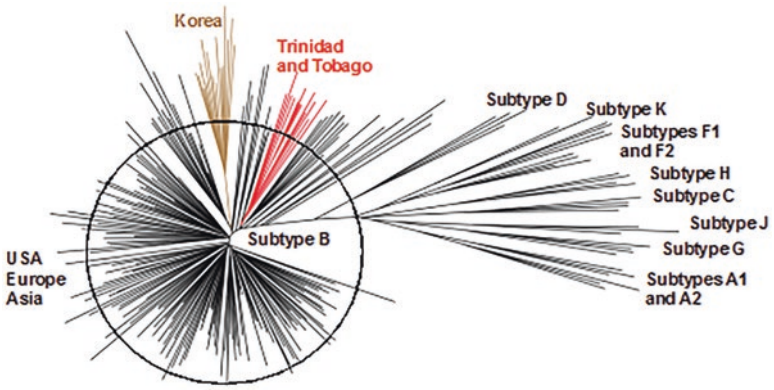

Fig. 1.3 Maximum likelihood phylogenetic tree constructed from HIV-1 subtype B [23]

virus [20, 22]. The rapid rate of evolution of HIV coupled with the knowledge that the virus spreads by direct intimate contact with an infected person allowed the development of a new field now known as molecular epidemiology. Many studies showed that analyzing the virus gene sequences could provide an accurate reconstruction of the spread of HIV within a local group or transmission cluster, and that the same methods can be applied to larger data sets and the study of larger portions of the pandemic [23]. One of the first widely publicized transmission cases solved in part by molecular methods is known as "the Florida dentist case," in which one dentist evidently infected six patients [24].

Misreporting and the resulting lack of public understanding of HIV and AIDS in general, especially during the early years of the pandemic, have resulted in some misreporting and confusion. Although the Florida dentist case was a doctor-to-patient spread of HIV, it was equally clear that this case was very highly unusual, and that dentists and other doctors could continue to work with reduced risk to their patients, assuming appropriate medical precautions.

Although HIV-1 subtype B was present in Haiti before it arrived in the United States and Europe, neither Haiti nor individuals from Haiti are to blame for the US epidemic. Figure 1.3 is a maximum likelihood phylogenetic tree constructed using HIV-1 subtype B complete envelope gene sequences sampled before 1999. Although there are some local subepidemics detectable in such a data set, for the most part the United States and European sequences are intermingled with each other.

Although it is possible to use molecular epidemiology to study HIV transmission patterns in geographical regions such as Florida, the process is fraught with many problems. One of the main problems is detecting and sampling recently infected individuals if the purpose is to study the current trends rather than infections that may have taken place a decade or more prior. Another problem is patient confidentiality and privacy, and the possibility of doing more harm than good by discouraging people from seeking diagnosis and treatment if they fear invasion of privacy or being identified as an IDU.

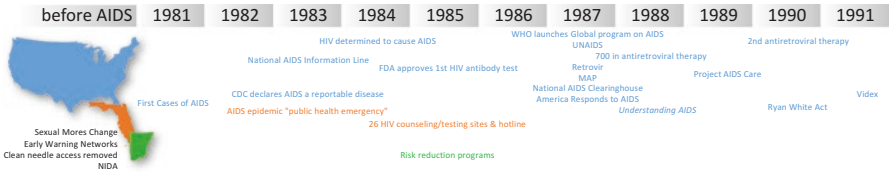

Fig. 1.4 First 10 years of AIDS timeline for key moments [11]

1.1.2 AIDS Cases 1992–2001

The second decade (1992–2001) of the HIV/AIDS epidemic not only saw an increase in the total number of AIDS cases but also new programs, interventions, and initiatives while obtaining crucial knowledge to combat this disease. Although different programs and laws were enacted during this epidemic, comprehensive approaches were not established effectively for targeting and serving the highest risk groups to stem the peaking of the epidemic. This misstep led to an increase in the total number of AIDS cases in the United States, Florida, and Miami-Dade County to more than 260,000; 23,000; and 7500, with new cases peaking: new 80,000; 7500; and 2500 [11, 14].

The total number of AIDS cases within Miami-Dade County continued to rise at an exponential pace throughout the time from 1982 to 1994, with a leveling off in the number of new AIDS cases diagnosed each year in the 1994–2001 time period, mainly due to the development of highly active antiretroviral therapy cocktails. Although Miami, Florida, only accounted for less than 1% of the US population in the early 1990s, it contributed over 3% of all AIDS cases [25]. Miami-Dade County continued to serve as one of the major epicenters during the 1992–2001 time period. AIDS and its impact were ever so present among South Florida and its respective risk groups (see Figs. 1.2 and 1.4). The cumulative HIV incidence of men was 1,703 and for women, 445 per 100,000 [25]. These rates rose exponentially for the first ten years after 1981. Further, African Americans accounted for the largest cumulative incidence of AIDS in MDC at a rate of 2,647 per 100,000, followed by Hispanics at 696 per 1000,000 and White/Caucasian at 657 per 100,000 now had a larger incidence of AIDS when compared to White/Caucasian [25].

As more data was acquired and a better understanding of the epidemic emerged, an understanding of the major risk factors among the demographics in South Florida became more evident. As seen during the first decade, MSM accounted for the largest percentage of AIDS accumulative cases [25]. Further, IDUs followed with the next highest percentage of cases attributed to these high-risk practices at 23% (3,745/19,864) followed by heterosexual risk at 20% [25].

The largest increase in the number of AIDS cases in Miami-Dade County occurred from 1993 to 1996, with the total AIDS cases averaging more than 2000 new cases peaking at 2354 [26]. Following this substantial increase of AIDS cases, a reduction in the total number of new cases began in 1997. From then on, the number of AIDS cases continued to decrease yearly and fell to 1,136 total cases in 2001

[26]. It took nearly two decades to observe a prominent decrease in the number of AIDS cases in the late 1990s, and this was primarily due to effective antiretroviral therapies making HIV treatable, rather than a large reduction in new infections.

Yet, even with the reduction in the total number of AIDS cases that occurred during the latter part of the 1990s, MDC still ranked in the top ten of nearly every AIDS statistic. MDC ranked second in per-capita MSM AIDS cases, seventh among IDU, and fourth in total per-capita AIDS cases among adults/adolescents [25]. As stated above, MDC accounted for <1% of the US metropolitan population yet ranked excessively high among the nation and major metropolitan areas regarding the HIV/AIDS epidemic.

The impact of MDC on Florida exemplified the influence a region can have on its respective state. The number of AIDS cases in Florida rose each year *vis-a-vis* MDC rates of 36.7 per 100,000, 55.1 per 100,000, and 63.7 per 100,000 in 1992, 1993, and 1994, respectively [14]. Males continued to dwarf women in the total number of AIDS cases from 1992 to 2001. For example, in 1994, men had a cumulative incidence of 101.3 per 100,000, whereas women only had an incidence of 28.3 per 100,000. These rates increased during the first half of the 1990s and then saw a prominent reduction in the total number of AIDS between men and women (Fig. 1.4). The epidemics as reviewed by the number of new AIDS cases each year peaked in the early 1990s for the United States, Florida, and MDC. There was a sharp and continuous decline, except for a smaller peak in early 2000s, especially for Florida and MDC (Fig. 1.4).

1.1.2.1 Interventions and Progressive Movements 1992–2001

There were major improvements in both prevention and treatment during the first decade of this epidemic. The evolution of prevention programs continued to progress to combat this epidemic. Further, it was through all these programs and the acquisition of information and knowledge that helped assist in the development of a combination antiretroviral therapy in 1992, and protease inhibitors in 1997, which led to a substantial decrease in AIDS-related deaths while improving the quality of life with those infected with this virus [12]. Preventive strategies continued to be developed, and the fight against this disease had immense momentum as seen by the CDC Serostatus Approach to Fighting the HIV Epidemic (SAFE), providing a framework for improving the health of persons living with HIV and preventing transmission to others [26]. Additional initiatives continued to advance the treatment and prevention of the HIV/AIDS virus [4, 5, 16] (Fig. 1.5).

Finally, in 1993, the CDC recognized and expanded its AIDS case definition to include a CD4 count of <200, and having diseases such as tuberculosis (TB), cervical carcinoma, and bacterial pneumonia if HIV were present [11]. This change in definition resulted in a great increase in reported cases in MDC, Florida, and the United States [11]. The OraSure saliva HIV test was approved by the FDA and supplemented the existing blood tests that provided crucial information regarding the epidemic [11]. The Comprehensive Drug Research Center (CDRC) took a lead

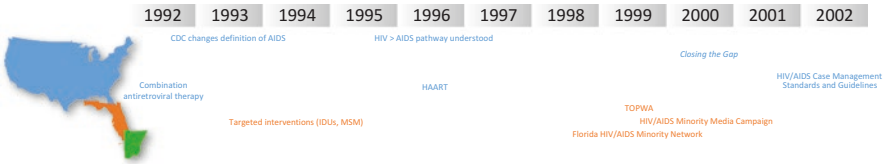

Fig. 1.5 Timeline of the second decade of AIDS [11]

in testing this new tool in our outreach programs. The success of surveillance tools coupled with an extensive classification of this virus provided vital epidemiological profiles that assisted in developing community and specific high-risk group prevention initiatives [11].

Continued acquisition of comprehensive epidemiological profiles nearly 15 years after the first AIDS cases provided the ability to create unique and specific interventions. Combating the impact of IDU within this epidemic was vital as this high-risk group continued to expose themselves to lifestyles that enhanced their probability of acquiring and transmitting AIDS [19–21]. Prevention protocols were then developed regarding the cleansing of needles shared and used by IDUs with bleach in MDC [17, 21].

It was through programs such as these that helped decontaminate needles in an effective and efficient manner. Exemplifying the effectiveness of such programs was highlighted by a study conducted by the CDRC and Health Services Research Center (HSRC) in which needle/syringes from shooting galleries around the Miami, Florida, area were collected and tested without and with cleaning by bleach treatment [4–6, 18, 19]. The study indicated that use of household bleach and proper cleaning techniques by laboratory studies proved that bleach-treated needle/syringes tested negative for HIV whereas those that were not cleaned by bleach tested positive [4–6, 18, 19].

It was innovative and continually developing interventions such as needle/syringe-cleansing programs that promoted cleaning of paraphernalia with bleach and sustained the momentum positively impacting this epidemic. In addition, ancillary paraphernalia were tested for the presence of HIV that included cottons, cookers, and wash-waters used at shooting galleries, which were shown to contain the presence of HIV [17]. In addition, laboratory results indicated that bleach should be utilized for 30 s, which was added to the intervention protocol [6, 7]. Programs and preventive services such as the bleach cleaning of used needle/syringes demonstrated the utility and effectiveness of science-based interventions.

In 1996, the FDA approved a new regiment of antiretroviral therapy that ushered in the era of highly active antiretroviral therapy (HAART) [11]. The implementation of HAART coupled with innovative programs and initiatives resulted in the first major decrease in the prevalence of AIDS nationwide. Continual efforts promoted development of new therapeutic options including Nelfinavir (Viracept), Delavirdine (Rescriptor), Saquinavir (Fortovase), and Zidovudine (AZT), which provided powerful pharmaceutical and clinical options for combating HIV/AIDS [11].

It was also during this time that the HIV/AIDS pathways were established and explained in a detailed and scientific manner, which allowed for a more precise understanding of the epidemiology and pathogenesis of HIV/AIDS [27, 28–31]. The HIV/AIDS pathways were distinctive; understanding their complexity allowed for a more comprehensive epidemiological profile to be developed of all demographics and high-risk groups. This crucial scientific data further promoted the development of new programs such as Florida's Targeted Outreach for Pregnant Women Act (TOPWA) program (1999), Florida HIV/AIDS Minority Network (1999), and "Closing the Gap" (2000). Additionally, media campaigns were developed to promote and increase awareness of HIV/AIDS throughout the county, state, and national level [5].

A significant shift in the HIV/AIDS epidemic occurred post-1990s. Prevention became more prominent than treatment, which had driven the approach for the first 20 years of this epidemic. HIV/AIDS information, protocols, and interventions were then translated in multiple languages, while increasing the networks and programs for all individuals exposed to this epidemic. Challenges continued to arise and cause barriers within the county, state, and national level, but there was an increasing scientific platform to overcome obstacles. The wealth of scientific information of the HIV facilitates current and future policies and programs.

1.1.3 AIDS Cases 2002–2013 (Figs. 1.1, 1.2, and 1.4)

The trends experienced during the 2002–2013 period of the HIV/AIDS epidemic were evidence that the epidemic was not over and was to be very resistant to the remarkable decline that started in the last half of the 1990s. As matter of fact, both Florida and MDC witnessed increases in new cases at various times during the decade (Fig. 1.2).

The impact of AIDS within the county, state, and national level continues into the third decade (2002–2013) of this epidemic. Even with the newly implemented programs, interventions, and access to antiretroviral drugs, the number of new AIDS cases continued. The total decline in AIDS cases in MDC began in the late 1990s and fell under 1,000 total cases in 2007 [14]. The total number of AIDS cases increased in 2008, but then, once again, fell under the 1,000 mark and has continued this trend ever since. Compared to the previous two decades, MDC continues to experience new cases ranking among the highest in the country [14].

Addressing the major demographics and risk groups has served as one of the most important epidemiological tools. Males continued to have a higher incidence of AIDS when compared to females. The trend persisted throughout the first two decades of this epidemic with men accounting for more than 70% in each decade compared to women [14]. Further, Black/African Americans accounted for the largest percentage of AIDS cases followed by Hispanic/Latino [14].

The incidence of specific high-risk groups, such as MSM and IDU, continued into the third decade of the HIV/AIDS epidemic, with MSM as the largest group

battling AIDS with a total 50% cases, followed by adult heterosexual contact with 38% of these cases [14]. The major shift was that IDU decreased to 7% from more than 20% in the first two decades. Although the overall change was not enormous, occurrences such as these indicate the potential success of current interventions and programs while highlighting the need for implementing new initiatives that adapt to the current needs. With the declines, in new AIDS cases over the third decade, especially in the IDU category, we undoubtedly see the results of the role that NIDA and drug researchers and drug interventions have had in developing the science and interventions directly targeting this major risk group.

However, Florida continues to rank in the top ten in nearly every category regarding HIV/AIDS. As of 2013, Florida ranked first in both newly diagnosed HIV infections and AIDS cases (110,000) [14]. Also, Florida ranked third in the nation for people living with HIV through 2014. Further, consistent with the two prior decades, men accounted for the clear majority of AIDS cases with more than 70% of all cases [32]. Black/African Americans were the largest race/ethnic group living with AIDS at 51% of all cases, followed by White/Caucasian at 25% and Hispanics at 21% [32]. Further, of those living with AIDS in Florida, the greatest transmission mode occurred among MSM (46%), heterosexual contact (43%), and IDU (8%) [32].

The trends experienced during the period between 2002 and 2014 in MDC and Florida differed from that of the United States. That is, the total number of AIDS cases within the United States has continued to see a rise throughout this period and culminated with 1,200,000 estimated cases in 2014 [32]. African Americans account for approximately 48% of all AIDS cases followed by Hispanics at 25% [33]. The rate of new cases for Hispanic males was 2.9 and 4.2 times that for White males and females, respectively [32]. Further, MSM were most severely impacted by AIDS at 54% of all cases followed by heterosexual contact at 31%, IDU at 10%, and MSM + IDU at 4% [33].

The earliest association of AIDS resulted in a stigmatization that those with this disease were considered “untouchable” due to misinformation and prejudice [4]. Irrational fear and paranoia often led to an increase in the discrimination of those battling HIV infection and AIDS throughout the United States. The rapid progression of AIDS led to greater uncertainty and uneasiness among the populations combating this new epidemic. The health inequities present among the different race/ethnicities and risk groups within the United States were the result of the misunderstanding and lack of treatment and care for HIV/AIDS during the decades of this epidemic.

1.1.3.1 Interventions and Progressive Movements 2002 to Present

There were major improvements and progress during the first three decades of the HIV/AIDS epidemic; the evolution of prevention programs must continue to ensure a strong platform to combat this epidemic. Many interventions, testing, and medical advances were and are essential for national initiatives to be effective in treating and managing those who have contracted HIV/AIDS [10, 14] (Fig. 1.6).

Fig. 1.6 Timeline of the third decade of AIDS [11]

After three decades in battling the HIV/AIDS epidemic, complacency settled in among individuals, communities, and the media at the county, state, and national levels. The first HIV/AIDS Case Management Standards and Guidelines were developed with the CARE Act Titles in 2002 [14]. Additionally, the first "entry inhibitors" were introduced into the HIV/AIDS epidemic. Nearly 25 years after the advent of this epidemic, over 500 case managers, supervisors, and other interested staff from the Ryan White Titles I, II, III, IV, and HOPWA were trained in the first statewide HIV/AIDS Case Management Training referred to as the "Nuts and Bolts" [34, 35]. The time it took to fund and conduct scientific research, and turn the science to policy statewide initiatives across the nation highlights one of the main reasons the prevalence of AIDS has continued over time.

These prominent and impactful programs that took nearly 30 years to develop assisted in combating this devastating epidemic [14]. The CARE Act Title program, which was passed by the US government provided substantial support to public programs that combat chronic diseases through various laws and legislation. Additionally, the Ryan White Titles (I, II, III, and IV) provide funding for healthcare entities to provide care and treatment. Initiatives such as these provide those with AIDS or HIV infection the ability to obtain the treatment required to prevent the spread and eventually diminish the prevalence of AIDS worldwide.

Early in 2001, one-on-one capacity building activities and initial prevention programs for individuals testing positive were funded that minimized the unnecessary transmission of HIV. Rapid HIV testing was then developed, and the AIDS Drug Assistance Program (ADAP) continued to proliferate and provide funds for drugs and other essential services. The success of ADAP led to new initiatives which targeted the HIV/AIDS crisis among MSM and other high-risk groups in Florida [14].

Community-based organizations and other similar entities continued which assisted in reducing the prevalence of AIDS and the health gaps present within different race/ethnicities and risk groups. Extensive community mobilization began throughout the nation which provided individuals with the care and access to treatment that could assist in preventing the continual spread of this disease. Further, media and social outreach strategies were improved which provided a means that would contribute to spreading vital information regarding AIDS. Also, programs such as Out in the Open (2007), Organizing to Survive (2008), and Man Up (2009) delivered interventions that assisted groups at the highest risk for contracting and transmitting AIDS [14]. Although there were substantial increases in programs and interventions to combat the HIV/AIDS epidemic, funding constraints

and accessibility prevented critical services to be efficiently and effectively delivered to all needed persons. For example, beginning in 2010, the ADAP saw its first wait list created for services and continued to exist until 2012 when the list was eliminated.

In 2011, world leaders adopted a new declaration that reaffirmed commitments and called for an intensification of efforts to combat the epidemic through new commitments and targets through the United Nations General Assembly and a Special Session (UNGASS) [36]. Most recently, UNAIDS set specific goals and targets to control the HIV/AIDS epidemic by 2020; by 2030 to ensure that 90% of people living with HIV know their HIV status; that 90% of people who know their HIV-positive status are on consistent treatment; and that 90% of people on treatment have suppressed viral loads [36].

Over $30.0 billion is being allocated to AIDS research and programs, prevention initiatives, housing assistance, care, and many other components essential in reducing the impact of the HIV/AIDS epidemic [36]. The momentum to eliminate this disease is in full force and must continue to proactively and aggressively reduce the incidence of new HIV infections. The major impact of AIDS has been consistent among US metropolitan areas with New York, Los Angeles, and Miami topping the list, stressing the importance of improving current programs, initiatives, and interventions to eliminate the spread of the disease [37].

1.1.4 Global Movement

The combined evolution and improvement of the world economy with subsequent increased efficiency and development of the international transport and travel technology has been a major driving force in the global spread of HIV/AIDS [4]. The HIV/AIDS epidemic has affected global populations through many avenues, with MSM, IDU, and heterosexual contact being the predominant risk groups. In addition, newborns and children suffer as well with this disease [38]. This epidemic has spread rapidly among the risk populations around the world, although in some areas it has spread slower and without such a profound impact [36].

Different parts of the world have experienced substantial discrepancies on the progression of HIV/AIDS. Moreover, 95% of HIV cases worldwide are concentrated in developing countries [38]. Further, HIV/AIDS disproportionately impacts distinct demographics and social groups throughout the world. Of the 36.9 million people living with AIDS around the globe, Sub-Saharan Africa accounts for nearly 70% of the cases [39]. Understanding the global network and its unprecedented reach provides the framework for approaching the HIV/AIDS epidemic by developing interventions by focusing on the highest risk networks [40].

Population-based interventions have been very effective in combating the HIV/AIDS epidemic globally [40]. Developing global population-based initiatives through international collaboration has provided a comprehensive approach that recognizes different lifestyles and information regarding this disease which continues to spread, albeit at a slower rate [40]. The specific programs developed throughout

the world provide an expansive resource of enlightened scientific information regarding risk factors such as sex and drug practices, provides better education, health and economic access, and other service components that could have a profound global impact on this epidemic [40]. The success of international programs depends upon a concise understanding and sustainability of modifying interventions to local institutions and communities with distinct cultures and lifestyle practices [40]. It has been consistent international collaboration in striving to break down all the barriers associated with the HIV/AIDS epidemic that led to improved programs and influencing global initiatives. Further, these initiatives have been a major portion of the fight against HIV/AIDS since 1987, when the World Health Organization (WHO) launched the Global Program on AIDS. UNAIDS developed the UN Millennium Development Goals (MDGs) which gave rise to every nation agreeing to global goals to reverse the spread of HIV and AIDS [41]. In the early years of HAART, there was much concern about detrimental side effects of long-term treatment, and thus much study of delaying treatment until CD4 counts fell below some threshold. Recent studies such as the START trial have now proven beyond doubt that treatment should begin as soon as possible [41].

1.1.5 Conclusion

Acquiring a comprehensive scientific understanding of the HIV/AIDS epidemic from a county, state, and national level leads to scientific progress in reducing the prevalence of this disease worldwide. Incorporating a global approach as detailed in this chapter provides a well-defined basis for understanding global epidemics.

Without continued and sustainable funding, initiatives cannot progress, and the HIV/AIDS epidemic will continue to unnecessarily impact millions of people worldwide. Much increased funding at all levels is needed to ensure preventive and treatment services for those affected by HIV/AIDS [36]. The progress of scientific knowledge and a thorough understanding of this epidemic and solid actions taken around the world will be needed to reach the future goal of eliminating this destructive disease, as we have done with other diseases, such as polio and small pox, with global consequences.

Conflict of interest The authors report no conflicts of interest.

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Chapter 2
Global Issues in NeuroAIDS

Jennifer Massey and Bruce Brew

Keywords HIV • Neurology • Immunology • Autoimmune • Infectious disease • Blood borne virus • Dementia • Neuropathy • Meningitis • Myelopathy • Myopathy • HIV associated neurocognitive decline • Immune reconstitution inflammatory syndrome • HIV associated peripheral neuropathy

Core Message
Neurological manifestations and *sequelae* of HIV infection are extensive both as direct and indirect manifestation of the disease, with associated infective, hematological, malignant, vascular, and immune-mediated processes occurring with differing prevalence at different stages of HIV disease and with independent host factors. Additionally, combination antiretroviral therapy (cART) is associated with a multitude of neurological ramifications. As the global burden of HIV continues to predominate in developing nations, neuroHIV and neuroAIDS issues in these settings vary from those seen in developed countries; however, the spectrum of disease remains unchanged and awareness of progress in this field is essential for adequate neurological care for HIV patients universally.

2.1 Direct Neurological Effects of HIV: Primary Infection

Symptomatic primary human immunodeficiency virus (HIV) infection (PHI) will occur in up to 90% of infected patients, with no clear "at risk" groups, or nations identified [1]. Onset of disease following exposure to HIV is typically within 2–4 weeks with the duration of symptoms lasting a median of 18 days [2]. Systemic illness manifests

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as fever, lymphadenopathy, headache, rash, myalgia, or arthralgia, with neurological symptoms classically occurring within a fortnight. (Throughout this chapter, the viral type is HIV-1 unless otherwise specified. Viral subtypes are not reported.)

PHI can manifest as a multitude of neurological syndromes ranging from headache, myalgia, cranial nerve palsies, myelopathy, radiculopathies, acute demyelinating neuropathy, and rarely, an acute encephalopathy. The most typical form however is meningioencephalitis, with generalized symptoms including seizures. There are reports of PHI initiating a myopathic process, including cases of florid rhabdomyolysis [3, 4].

Factors influencing the prevalence and severity of primary HIV infection likely relate to the tropism and virulence of HIV and the degree of host immune response. It has been shown that patients with highly replicative viral strains with multitropism are more likely to develop symptoms, particularly if they are unable to mount humoral and cellular HIV-specific immune responses [5]. Host HLA-haplotypes are well recognized to impact on the rate of disease progression, and this is thought to also influence the degree of PHI [6].

Diagnosis is based predominately around a high index of clinical suspicion, as many symptoms are nonspecific. Host immunodeficiency may be reflected through a drop in lymphocyte count. Transient thrombocytopenia is frequently seen, along with elevated serum hepatic transaminases, which normalize within weeks. Evidentially, serum must be tested for HIV RNA, as HIV serology during this time is often indeterminate. In regard to neurological PHI, cerebrospinal fluid (CSF) very often shows a lymphocytic pleocytosis reflecting CD4:CD8 ratios in blood [7], with raised CSF protein and HIV RNA.

Early diagnosis of PHI theoretically allows an opportunity to lower the viral set point, which is determined following established infection. Following initial infection, the HIV viral load rises steadily; however, following immune activation, this falls to a steady-state point of viral replication. Modulation of this set point determines the future progression of the disease, and doing so could suppositionally be established with the use of antiretrovirals. Unfortunately, early trials did not seem to consistently support this [8–10] with only a single study involving zidovudine showing a short-term benefit and others showing no change in this regard; however, emerging trials are pointing toward a beneficial effect of early therapy during acute infection, with early combination antiretroviral therapy (cART) limiting the reservoir size of latently infected cells. To date, however, these benefits appear transient [11, 12].

No study has specifically addressed gross neurological outcomes in this regard; however, increasing work is being done in regard to prevention or improvement of HIV-associated neurocognitive disorders (HAND), which is discussed later. Current WHO guidelines recommend commencement of cART at the point of diagnosis.

It has been postulated that patients' neurological symptoms of PHI may benefit from early cART commencement although again there is no clear evidence base for this. Given the possible effect on viral set point, lower risk of developing both AIDS and resultant benefits with regard to cognitive impairment, most neurologists would at present recommend early commencement of cART [13] preferably using drugs that reach efficacious concentrations in the CNS but avoiding a triple nucleoside combination due to the increased rate of early virologic nonresponders [14]. The

role of drug holidays is no longer supported, and as such, treatment is lifelong. The utility of immunomodulatory therapies such as mycophenolate mofetil (MMF), cyclosporine, or interleukin-2 (IL-2) remains under investigation with no clinically beneficial studies reported [15].

2.2 Direct HIV Neurological Effects of HIV: Meningitis

Aseptic meningitis may develop in the early to medium term following HIV infection, and can be present in patients with otherwise well-controlled HIV disease, with a stable or only mildly decreased CD4 count. There is now evidence that it does however correlate with an elevated HIV viral load in CSF [16], although this was seen nonspecifically in all patients with neurological symptoms.

The clinical picture may be acute, over a period of weeks, and associated with systemic symptoms of fevers, confusion, and seizures or cranial neuropathies. The chronic variant develops over a period of months, with the predominant features being headache or features of raised intracranial pressure. Typically, symptoms are self-limiting, but may recur throughout the course of infection.

Diagnosis on CSF can be made on the basis of a mild mononuclear leukocytosis, although this will vary with differing degrees of immunodeficiency. In patients with greater degrees of immunosuppression, consideration must be given to opportunistic infections. Antiretrovirals including zidovudine, efavirenz, delavirdine, and nevirapine have been associated with nonspecific features of meningitis including headache, malaise, confusion, and stupor [16]. However, these are rare complications and in some cases may relate to immune reconstitution syndrome as discussed later.

Given the nonspecific nature of symptoms and risk of confounding coinfection, correlation of symptoms with HIV viral load is important. Advice regarding how this information should influence treatment is challenging, although commencement of cART with good CNS penetration appears intuitive, and certainly this is recommended for patients with HIV RNA in CSF that is disproportionate to serum levels. Of note, there are limited trials to support this, and given the condition's self-limiting nature, the majority of management is symptomatic. A small trial of zidovudine in 11 patients did not improve symptoms [17]. Anecdotal use supports short courses of steroids for headache if opportunistic infection is excluded.

2.3 Direct HIV Neurological Effects of HIV: Myelopathy

An acute HIV myelitis can occur with primary infective illness, or more commonly with the development of AIDS.

Clinical features relate to the location of the lesion, the typical sites for involvement being the thoracic cord and the conus. Treatment lacks evidence but should be based around the commencement of cART with good blood-brain barrier penetration. The role of steroids is unclear.

Vacuolar myelopathy, as opposed to HIV myelitis, may develop in HIV at any stage but is more common in advanced disease. While the condition is associated with HIV disease, the precise role of HIV in the pathogenesis is unclear. A similar if not identical illness has been described in systemic lupus erythematosus. Vacuolar myelopathy has an estimated symptomatic prevalence in AIDS of 5–10% but is seen in 50% of cases on autopsy [18]. Progression occurs over months typically with spastic paraparesis, hyperreflexia, extensor plantar responses, impaired proprioception, and sensory ataxia. The disease often eventually stabilizes and may not have significant impact on time to death [19]. MRI is typically normal or nonspecific. Further investigations should be performed to exclude other causes of myelopathy, and the diagnosis can only be truly confirmed at autopsy, where intralamellar vacuolation in spinal white matter is seen, typically in the posterior columns of the thoracic cord [18]. There is no known effective treatment [19], although high-dose methionine seems to have modest benefits [20].

2.4 Direct HIV Neurological Effects of HIV: Myopathies

Myopathies in HIV may be secondary to inflammatory conditions related to cART (as discussed in further detail later) or exacerbated in the context of other medications use.

Polymyositis is quoted to occur in up to 2% of HIV patients [21]. The disease occurs with moderately advanced HIV, although rates have not been clearly correlated with CD4 levels in previous reviews [21]. The inflammation is driven by a CD8 cytotoxic T cell response. As more advanced immunosuppression occurs with disease progression however, the condition appears to "burn out," correlating with the declining ability to mount an inflammatory immune response [22].

Creatinine kinase (CK) will be elevated, although this is nonspecific, and myositis antibodies appear to be of lower yield in these cases. EMG will support a myopathic process with small duration, low-amplitude polyphasic motor unit potentials, fibrillations, and positive sharp waves. Muscle biopsy remains the definitive investigation, showing CD8+ driven endomysial inflammatory infiltrates [22]. Treatment should be with intravenous immunoglobulin (IVIg) to minimize immune compromise; corticosteroids and other immunosuppressants are reserved for more refractory cases. The role of cART in treatment of this condition is not established, though theoretically at least it is likely to be beneficial [21].

Inclusion-body myositis has also been associated with HIV infection [20]. The HIV-associated variant differs from the nonimmunocompromised form of the disease, with earlier age of onset and higher CKs. It has been established that the virus does not replicate within muscles but is present in endomysial macrophages, although the autoimmunity occurs as a result of specific CD8+ T cells invading muscle fibers, recognizing muscle antigens and inducing inflammation. Again, trials of IVIg can be considered [23]. There is no clear role for steroids, but up to 25% of patients have been shown to transiently respond to IVIg, and T cell immunotherapy remains under investigation [24].

As seen in the general population, drug-induced myopathies remain the most common cause of raised CK in the developed world. With increasing understanding of long-term vascular effects of HIV and hyperlipidemia, and treatment of this, statin-associated myopathy prevalence is increasing. This can be exacerbated by interactions with protease inhibitors, particularly ritonavir.

2.5 Direct Neurological Effects of HIV: Peripheral Neuropathy

Peripheral neuropathies can occur as a direct consequence of HIV infection early or medium term into the disease, typically when immunodeficiency is significant [25]. Peripheral neuropathy also occurs in conjunction with HCV in the context of coinfection, medication side effects, or other associated conditions.

Lumbosacral polyradiculopathy, which develops subacutely, typically manifests as asymmetric leg weakness, saddle anesthesia, bladder and/or bowel dysfunction, and sensory loss. Most commonly, this is secondary to cytomegalovirus infection, suprainfection, or reactivation in the setting of immunosuppression. However, it can occur with other viral coinfections, particularly herpes simplex. Nonetheless, 30–50% of patients have no coinfective illness, and radiculopathy is thought to be mediated by HIV itself [26].

Plexopathies characteristically occur as part of a seroconversion illness or during seroconversion of a coinfection with another blood-borne virus. Mononeuropathies of cranial (typically facial) and peripheral nerves may occur as isolated events, or progress to a mononeuritis multiplex syndrome. These may occur as a consequence of coinfections including varicella zoster virus, syphilis, and hepatitis C or secondary to HIV alone.

Mononeuritis multiplex can also be directly related to HIV through vasculitis. Other causes include cryoglobulins, lymphoma, and the coinfections previously mentioned. Interestingly, HIV patients may develop an early hyperlymphocytic reaction to the virus with proliferation of CD8 cells resulting in a Sjogren's-like neuropathy and associated sicca symptoms [27].

Patients are also susceptible to Guillain-Barre syndrome as well as a chronic inflammatory demyelinating polyneuropathy. This is characterized by weakness, areflexia, and an elevated CSF protein, and typically occurs in a bimodal pattern either at seroconversion or chronically, later in the illness [28]. It may also occur with immune reconstitution. First-line therapy is based around IVIg or plasmapheresis.

Distal symmetrical polyneuropathy is the most common peripheral nerve complication of HIV. Typically, it occurs late in the disease; there appears to be an increased incidence in patients with lower CD4 counts (30% of patients with CD4 <200).

Overall, the incidence has declined in the cART era (despite certain patient cohorts showing the opposite) [29]. Contributing factors to disease include vitamin deficiency, alcohol abuse, medications (particularly D4T as will be discussed), glucose intolerance/diabetes, and renal impairment.

Clinically, the neuropathy is symmetrical, predominantly sensory, and distal, rarely extending above the knees. Ankle jerks are absent or depressed as are knee jerks later in the illness. Weakness is very unusual, and when present is confined to the feet. Pain is a common feature. An autonomic neuropathy may occur in conjunction, typically manifesting as postural hypotension and gastroparesis [29].

HIV neuropathy is a length-dependent axonal degeneration. Differential diagnosis should include coinfection with other blood-borne viruses including hepatitis C and HTLV, hematological conditions including lymphoma, autoimmune conditions such as Sjogren's disease, and sarcoidosis or medication side effects. There is an inflammatory monocytic infiltrate associated with decreased numbers of dorsal root ganglion neurons. Treatment in addition to cART is symptom-based management [29].

2.6 Direct Neurological Effects of HIV: HIV-Associated Dementia and Lesser Forms of Impairment

HIV-associated dementia (HAD) or AIDS dementia complex (ADC) is a well-recognized condition often described under the subheading of HIV-associated neurocognitive disorders "HAND." HAD presents as a subacute dementing illness in patients usually with advanced HIV disease. Milder variants as per the HAND classification are minor neurocognitive disorder and asymptomatic neurocognitive impairment.

Overall, it is thought that approximately 40% of HIV-infected patients have some form of HAND [30, 31], not excluding the confounding effects of psychiatric disorder, substance use, coinfection, etc.; if the latter are taken into account, the percentage drops to approximately 20–25% [32, 33]. As life expectancy of HIV patients increases with advances in treatment regimens, prevalence continues to rise [34]. Incidence appears to be stable, though rigorous prospective data from the current HIV population are lacking. The development of HAND is not universal and appears to relate to both host and viral factors. Of note, HAND is a distinct entity from the disease HIV encephalitis, which should be reserved for patients who have pathologically proven features of a multinucleated giant cell encephalitis with HIV in the brain parenchyma [35].

HAD is typically subcortical, lacking features of aphasia, alexia, and agraphia. Motor dysfunction is frequently seen early in the disease [36]. Progression previously occurred over weeks to months, but this has now typically extended to months to years in the cART era. The three classically affected cognitive domains include cognition with changes to memory, concentration and reasoning, and motor function specifically gait unsteadiness, clumsiness, and a decline in fine motor skills as well as behavioral change most classically progressive apathy or disengagement from daily activities. Primitive reflexes may be elicited on examination [36].

In patients on cART, the disease can be tentatively classified into active or inactive states with the defining feature being clinical, immunological (CSF viral immunological activity), or radiological (on MRI spectroscopy) progression of the disease

Table 2.1 Classification of HAND [37]

Inactive	The disorder has not changed clinically or neuropsychologically for more than 6 months
	The CSF has no evidence of viral or immunological activity
	MR spectroscopy does not show evidence of increased glial turnover
Active	Progressive: the disorder is worsening clinically or neuropsychologically
	Stable: the disorder has not changed for more than 6 months, but there is activity in the CSF (viral or immunological) or by MRS
	Regressive: the disorder is improving

(Table 2.1) [37]. Validation of activity status by these measures, while intuitively reasonable, awaits large studies. Currently, it appears the disease phenotype is much the same between treated and untreated patients; however, with increased life expectancy, more "typical" causes for dementia may overlap with HAND, causing a confounding clinical picture. The increased prevalence of CNS vascular disease in the HIV population may also contribute to the clinical overlap syndrome. Interestingly, the only clear risk factor associated with HAND progression in the recent MACS study was hypercholesterolemia [33].

Not only does HAND contribute to a growing burden of HIV-associated neurological complications, but increasing evidence exists that HIV may facilitate other neurodegenerative conditions.

Common understanding regarding neurodegeneration relates to a breakdown of protective cellular mechanisms resulting in accelerated neurotoxicity. Alzheimer's disease and other neurodegenerative conditions are understood to occur subsequent to aggregation of misfolded proteins, which in turn activate cellular defense mechanisms. Subsequent activation of inflammatory pathways includes excitotoxicity, oxidative stress, and mitochondrial dysfunction. In CNS HIV infection, activated microglia and astrocytes contribute to neurodegeneration via upregulation of proinflammatory molecules, including TNF-α, IL-6, and MCP-1, resulting in a neurotoxic environment. Oxidative stress is thought to be the final common pathway in the majority of neurodegenerative diseases including HAND. HIV also affects normal aging processes. Aging results in frontal and hippocampal neuronal loss and less efficient myelination. Similar regions are affected by HIV. Aging disturbs cellular disposal of toxins via increased abnormal ubiquitin inhibiting protein degradation. Inhibition of the ubiquitin-proteasone complex, as is seen in AD, Parkinson's disease, and motor neuron disease also occurs in HIV [38].

Investigations are predominately focused around exclusion of differential diagnoses. Routine serum testing for metabolic causes of dementia should be performed including thyroid function, B12, and folate levels. Neuroimaging should include CT or preferably MRI to assess the degree of atrophy, exclusion of mass lesions, and exclusion of progressive multifocal leukoencephalopathy. MRI may show periventricular T2 changes in the white matter, which are associated with HAND. MRI spectroscopy can contribute to diagnosis by showing reduced levels of N-acetyl aspartate and increased levels of choline and myoinositol in the deep frontal white

matter and especially the basal ganglia. CSF should be performed to assess for HIV RNA, exclude coinfection, test for drug resistance of the virus, and assess for immune activation markers or other features of CNS inflammation.

While HIV-1 is the cause of HAD in patients with unsuppressed HIV, the cause in milder forms of HAND in the context of viral suppression is still unclear. Evidence now shows that HAND continues to be a progressive neurological syndrome in the cART era, even as frequency declines.

Potential explanations include poor CNS drug penetration and a dissonance between serum and CSF virologic control; the "legacy effect" where cognitive dysfunction has occurred in the pretreatment period; CNS toxicity from antiretrovirals; and possible contribution by immune restoration in an IRIS-type phenomenon [39]. Normal aging degenerative changes as outlined earlier are also relevant. HIV presumably accesses the CNS within infected mononuclear cells [40]. HIV strains vary in their degree of neurotropism and neurovirulence, likely explaining at least in part why only some HIV-infected patients develop HAND [41].

Studies that have attempted to assess the "legacy effect," specifically neuropsychological status early in infection, and whether cognitive function can be altered by early commencement of cART have been limited by access to populations at this stage of disease. One small study reported approximately 25% of newly diagnosed HIV patients had impaired neuropsychiatric performance at a median of 19 days post infection, and that this correlated with CSF HIV RNA. These outcomes were not reversed by cART introduction at the time of diagnosis on testing at 3 and 6 months, suggesting limited reversibility in this group [42]. Another study showed slightly more promise, indicating initiation of cART can improve but not restore brain integrity [43]. Adding weight to the evolving picture of early neurological complications of HIV is a report of perinatal HIV children with adequate viral suppression on cART having lower brain volumes, more white matter disease, poorer brain structural integrity, and poorer cognition compared to age-matched controls on neuropsychological performance and imaging [44].

Much of HAND pathogenesis is still to be elucidated. It is recognized that the inflammatory cytokines released in the brain and spinal cord in response to viral infection play a more dominant role than direct virally induced brain damage; however, the detailed pathogenetic pathways including host genetic influences remain only partially answered [45].

cART should be commenced if there is evidence of HAND. If the patient is already on cART but not suppressed (either in the blood or the CSF), then antiretroviral (ARV) resistance studies should be done to determine which ARVs should be included in a revised cART regimen. If the patient is virally suppressed in both blood and CSF, then the management path is unclear. Theoretically at least, it would be reasonable to alter the cART regimen if there was evidence of inflammation in the CSF, for example, an elevated neopterin. If there is no inflammation in the CSF, then management is even less clear – the patient may have inactive or "burnt out" disease in which case a wait-and-see approach would be reasonable [45].

Another approach would be to intensify cART by the addition of another ARV known to be effective in HAND. This would be reasonable if there was clinical

suspicion of progression of HAND despite any other evidence of activity. There is some emerging evidence for this [91]. The role of MRI spectroscopy in guiding management in such scenarios is still in evolution. Adjunctive therapy in the form of neuroprotectant medication has no clear role currently. The exact ARVs that should be included in a cART regimen for the treatment of HAND is controversial, with some studies favoring ARVs with higher CNS penetration and efficacy while others find no superior benefit [46]. There are methodological issues with most of these studies; when these are taken into account, the evidence does seem to favor ARV regimens with better CNS penetration and efficacy, though not conclusively. A review of observational studies investigating this question concluded that in well-conducted trials neuroHAART was effective in improving neurocognitive function and decreasing viral load, but large randomized trials are required in this field [47].

2.6.1 Indirect Effects of HIV: Infective – Tuberculosis

Tuberculosis (TB) coinfection occurs predominantly in the developing world where it remains a leading cause of death in patients with HIV. Not only does HIV increase the risk of disseminated tuberculosis, but coinfection poses challenges to treatment and is known to be a risk factor for poor clinical outcomes. Central nervous system (CNS) TB can manifest as tuberculous meningitis, tuberculoma, and tuberculous abscesses.

Tuberculous meningitis (TBM) may occur in up to 10% in coinfected patients where TB is endemic [48]. The majority of patients who develop TBM have a CD4 count <500 cells/μL, and more frequently <200 cells/μL. Common systemic features include fever, headache, and altered mental state. Two-thirds of patients will have meningeal signs and approximately a third of these, focal neurology [49]. TBM has the highest morbidity and mortality compared with other end-organ TB infection. Early diagnosis is critical and notoriously challenging due to the relatively nonspecific signs and the fastidious nature of the organism. Diagnosis of TB from the CSF has improved significantly with the use of PCR, which has a sensitivity and specificity of 70.5 and 87.5%, respectively [49]. Treatment for tuberculosis should be commenced immediately. In patients who are not already on cART, this should ideally be delayed until after TB treatment is complete, if not 6–8 weeks underway to prevent immune reconstitution inflammatory syndrome (IRIS); however, the safety of doing so will depend on the CD4 count: in patients with CD4 counts <50, delay in antiretroviral therapy should be minimized [50]. The routine use of steroids is not currently advocated for HIV-infected patients with TBM. However, corticosteroids are the treatment of choice in patients with TBM and HIV who develop IRIS. Drug interactions must be carefully considered, particularly in regard to Rifampicin. Prognosis is poor, and 30–50% of patient with TBM will die, although outcomes appear similar to those in the non-HIV population.

Both tuberculoma and tuberculous abscess typically present with a constellation of systemic features, seizures, and focal neurological deficits related to the site of the lesion. A more fulminant course is expected in abscesses. Imaging confirms a solid-

enhancing or ring-enhancing lesion which may mimic the imaging appearances of cryptococcomas, proving to be a management challenge with regard to empiric treatment [49]. Confirmatory tests include screening for pulmonary TB with chest x-ray, CSF (where safe to do so), MRI possibly with MR spectroscopy. In developed nations where tissue biopsy is more readily attainable, PCR and culture of tissue will be beneficial. In developing nations, it is reasonable to commence empiric TB treatment in patients with respiratory symptoms, negative serology for toxoplasmosis, and recent co-trimoxazole treatment on the basis that this argues against CNS toxoplasmosis [49].

2.6.2 Indirect Effects of HIV: Infective – Toxoplasmosis

Toxoplasmosis remains the commonest cause of focal cerebral lesions in the developing nations' HIV population. The infective agent is *Toxoplasmosis gondii*, an intracellular protozoa. The tachyzoite represents the acute infective organism and the bradyzoite the organism responsible for latent disease. Both cellular and humoral immunity are required to prevent parasitic proliferation. CD4 T cells and gamma interferon are essential in this process, and when CD4 counts fall in HIV, latent toxoplasmosis can be reactivated [51].

The majority of patients with toxoplasmosis have a CD4 count <100 cells/uL. Clinical manifestations reflect a focal lesion and include headache, focal neurological symptoms and signs, and sometimes seizures. Disseminated infection, a much less common form of the disease, leads to headache and confusion usually. Diagnosis can be made on the grounds of a typical history, imaging features, and serological testing, given that the majority of HIV-related cases are reactivation. Antibody-negative cases can occur with advanced immunodeficiency and loss of prior antibody, and rarely, disease may be a result of primary infection. If CSF can be safely obtained, it will typically show a pleocytosis, raised protein, and normal to low glucose. Culture takes weeks in duration, and PCR is the test of choice on CSF; however, it is only positive in about 60% of patients, dependent on the proximity of the lesion to the ventricles [51]. Imaging with contrast CT or MRI is beneficial with lesions showing heterogeneous contrast enhancement. Typically periventricular lesions are less likely to represent toxoplasmosis. In HIV-positive patients, MRI spectroscopy has limited benefit in differentiating toxoplasmosis from CNS lymphoma; however, FDG PET-CT may be beneficial in this situation [51].

A provisional diagnosis of cerebral toxoplasmosis can be made when there is no history of toxoplasmosis prophylaxis (low-dose co-trimoxazole), a CD4 count <200, particularly <100, positive toxoplasmosis serology, and brain imaging consistent with multiple enhancing mass lesions.

Empirical therapy with pyrimethamine, sulfadiazine, and folinic acid often results in clinical response in 1–2 weeks. cART should probably be delayed for several weeks to decrease the risk of IRIS which may result in clinical deterioration [52]. Primary therapy is given for 6 weeks followed by long-term maintenance therapy at reduced doses, with duration determined by response to cART. Therapy can be discontinued when there is a persistent CD4 count >200 [52].

2.6.3 *Indirect Effects of HIV: Infective – Cryptococcus*

Currently, cryptococcal meningitis is the largest single cause of neurological infectious death worldwide [53]. Consistent with other CNS infections in immunosuppressed patients, cryptococcal disease may manifest as either meningitis or mass lesions, cryptococcomas. Crytpococcal CNS disease is particularly prevalent with a CD4 count <100cells/μL [54]. Cryptococcal meningitis is almost exclusively caused by *Cryptococcus neoformans* var *neoformans* serotype. Meningitis is a much more common clinical presentation compared to cryptococcomas.

Patients with meningitis typically present with headache and sometimes fever [54]. Generalized symptoms of drowsiness, visual obscuration, cranial neuropathies, and seizures may occur over time if untreated and typically reflect raised intracranial pressure. Cryptococcomas typically present with relevant focal neurological changes. Serum cryptococcal antigen is almost always positive in meningitis, but it can often be negative in cryptococcomas. Imaging should always be performed prior to lumbar puncture to assess the presence of raised intracranial pressure and other potential diagnoses. CSF cryptococcal antigen has a sensitivity of 88–90% in HIV-positive patients [54]. Treatment with amphotericin and flucytosine as induction therapy for 2 weeks is followed by fluconazole. The duration of therapy varies according to clinical response and CSF but is of the order of a year [55]. Recent studies have demonstrated the balance between a CNS response facilitating fungal clearance and an excessive damaging response. The Cryptococcal Optimal ART Timing trial found that antiretroviral therapy given later in the course of meningitis (>5 weeks after diagnosis) had better outcomes than treatment in the initial fortnight [56].

2.7 Indirect Effects of HIV: Progressive Multifocal Leukoencephalopathy

Progressive multifocal leukoencephalopathy (PML) continues to be a major contributor globally to neurological disease in patients with HIV and remains a treatment dilemma for clinicians. Although the use of cART worldwide has resulted in a decline in the incidence of the disease in the HIV population, it remains a condition without specific treatment.

PML is a subacute demyelinating disease of the CNS affecting oligodendrocytes and astrocytes. It is a consequence of reactivation of latent infection with the polyomavirus, John Cunningham (JC) virus. The site of latency is controversial with most considering it to be in the bone marrow, kidney, and possibly brain. JCV is a ubiquitous organism with antibody prevalence in the general population of approximately 50–60% [57]. The features of primary infection are not known to cause clinical disease. Viremia recurs in cases of immunodeficiency and correlates with CD4 count, but does not appear to correlate with HIV plasma viral load [58]. PML is the AIDS-defining illness in approximately half of HIV-positive patients. Average survival is 3–4 months in the absence of cART [58].

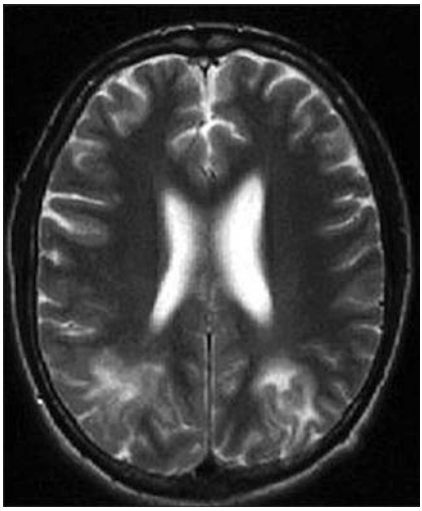

Fig. 2.1 T2-weighted magnetic resonance image shows hyperintense signal abnormalities in the white matter of the parieto-occipital lobes due to progressive multifocal leukoencephalopathy [60]

Clinically, PML is defined by subacute focal neurological deficits in the infected region. Fever is absent. Localizing features include hemiparesis, speech and language disturbance, cognitive dysfunction, headache, and ataxia. Serum JCV DNA is not useful as it does not correlate with PML. The best diagnostic test is CSF JCV DNA, which is detectable in 80–90% of cases who are not on ARVs [59]. Although diagnostic sensitivity has declined in the cART era, it remains useful. Brain biopsy for immunohistochemical detection of JCV is the gold standard but rarely required. MRI will show subcortical and deep white matter signal change on FLAIR and T2 sequences, typically with a ribbon of cortical sparing. Lesions typically do not enhance with gadolinium (Fig. 2.1).

In the HIV-positive PML population, treatment is centered on cART with response rates of approximately 50% likely relating to the degree of immune recovery achieved by cART. Cytosine arabinoside, cidofovir, and other agents have been trialed without benefit. Initiation of cART may result in IRIS, which can cause life-threatening cerebral edema, and steroids may be required [61].

2.8 Indirect Effects of HIV: Hematological

Primary CNS lymphoma and Kaposi's sarcoma, which were commonly seen in HIV patients in the pre-cART era, are now becoming increasingly rare. Challenges remain however in recognition, diagnosis, and management of both diseases.

Primary CNS lymphoma (PCNSL) is a diffuse non-Hodgkin's lymphoma that, in contrast to the nonimmunosuppressed population, develops secondary to reactivation of latent Epstein Barr virus (EBV) in HIV-positive patients. All cases contain the EBV viral genome [62]. Incidence differs depending on the presence of cART and remains higher in the developing world. The benefit of

cART on incidence is reflected in the decrease of PCNSL from 313.2/100,000 person-years to 77.4/100,000 person-years after 1996 [63].

Typically, the disease develops as in intracranial mass, but it can also involve the spinal cord, meninges, and eyes. Symptoms relate to the focal location of the lesion, in addition to the generalized symptoms of mass lesions, headaches, cognitive changes, altered alertness and less commonly seizures, which are seen in approximately 10% of cases [64]. Given radiographic features of PCNSL are nonspecific; differential diagnoses for consideration include other demyelinating or inflammatory conditions such as sarcoidosis or, in cases of single lesion disease (which makes up between 60 and 81% of cases), other space-occupying lesions [64].

Diagnostic work-up involves MRI brain with gadolinium. Stereotactic biopsy is the only certain means of diagnosis, although CSF for flow cytometry in case of leptomeningeal spread, or micro-RNA, can be useful, though the yield is low [65]. MR spectroscopy may aid diagnosis if it shows changes of metabolites, specifically phosphorylethanolamine, consistent with lymphoma [66]. SPECT in HIV patients is beneficial as the absence of Thallium-201 uptake on early images at the site of a CT/MRI abnormality excludes the diagnosis of lymphoma with a high degree of confidence [66].

Once diagnosis is confirmed, patients should have ocular assessment for involvement, as well as systemic staging, because they have a higher incidence of extranodal lymphomas. Although survival outcomes are improved in the cART era, overall prognosis is poor [67]. The AIDS population has a median survival of 2–5 months following commencement of treatment which typically involves combined chemoradiotherapy as well as commencement/continuation of cART [67]. In patients with poor functional status, treatment is typically limited to cranial radiotherapy; in patients with more aggressive treatment aims, high-dose methotrexate is used.

It should also be noted that HIV patients are at greater risk of metastatic lymphoma invading both the CNS and PNS. There may be an increased incidence on patients with advanced HIV/AIDS of cerebral gliomas, although there is no clear correlation with CD4 count. Rarely, Kaposi's sarcoma may cause intracerebral mass lesions associated with hemorrhage.

2.9 Indirect Effects of HIV: Cerebrovascular Disease

In comparison to other neurological complications of AIDS, cerebrovascular events are relatively uncommon, although HIV is recognized as an independent risk factor for stroke [68–70]. Typically, stroke occurs in the context of aforementioned opportunistic infections and associated metabolic syndrome, linked with certain antiretroviral regimens.

HIV predisposition to stroke is thought to be predominantly driven by atherosclerotic mechanisms. The prolonged life expectancy seen in accordance with the introduction of cART has correlated with not only higher rates of hyperlipidemia and hypertension but also the translation of these risk factors to predominately isch-

emic stroke. Both the older generation protease inhibitors and nucleoside reverse transcriptase inhibitors may be associated with hyperlipidemia. Protease inhibitors have been associated with the development of the metabolic syndrome although a causal relationship has not come to fruition in larger studies, with the caveat that patients responding well to treatment might have increased risk of atherosclerosis-related strokes after prolonged exposure [71]. Hypercoagulability may also be an important consideration, with protein S deficiency being frequently encountered in the HIV stroke population but rarely the cause of stroke [71].

It is now well recognized that HIV creates a chronic inflammatory state with increased rates of atherosclerotic disease compared with age-matched population. Both HIV and immunosuppression contribute to atherogenesis, and it remains unclear which correlated greater with risk: viral load or immunosuppression.

Studies of carotid artery atherosclerosis show HIV is an independent risk factor for arterial stiffness, with similar magnitude of risk to smoking. An inverse relationship has also been shown with CD4 count and carotid distensibility. Probable mechanisms include both procoagulant and endothelial dysfunction in response to inflammatory cytokines. This inflammatory response is modulated by host factors, specifically genetic variants that increase activation of monocytes and macrophages that accelerate atherosclerosis [72, 73].

Although immunodeficiency (as measured by CD4) count) influences stroke risk [74, 75], a direct virotoxic effect of HIV on arteries cannot be excluded. A recent study in sub-Saharan Africa not only demonstrated risk of stroke associated with HIV infection but notably higher risk in patients commencing treatment in the preceding 6 months [70]. Although this may be explained by a greater degree of immunosuppression seen with lower CD4 counts in this population, an immune reconstitution phenomenon against target antigens from HIV-mediated cell damage or the virus itself is plausible [76].

Differential consideration in patients presenting with stroke-like symptoms must include cerebral vasculitis/HIV vasculopathy, occurring as an inflammatory response to the viral antigen, although this is rare. If this is the cause of vascular events, treatment with cART alone is unlikely to be adequate and immunosuppressive agents are typically required [77]. Tuberculosis can cause an invasive/obliterative arteriopathy, either directly or in the context of meningitis. VZV recurrence/reactivation can also cause a large vessel vasculopathy.

2.10 Indirect Effects of HIV: Seizures

There is an increased seizure frequency in the HIV population, which is likely to be multifactorial. Not only do coinfections and associated lesions, as well as inflammatory states cause seizures, but the direct neurotropic effect of HIV lowers seizure threshold [78]. Around 10% of patients will develop epilepsy without an associated cause [78]. Confounding factors include substance abuse, trauma, and coinfection. cART which crosses the blood-brain barrier including efavirenz has also been

anecdotally associated with seizures in the pediatric population. It is also important to consider interactions with protease inhibitors and anticonvulsants in known epileptic patients commencing treatment for HIV [79].

2.11 cART and the Neurological System: IRIS

Immune reconstitution inflammatory syndrome (IRIS) was first clinically recognized in the context of advancements in HIV treatment in the late 1990s. Successful treatment and rising CD4+ve lymphocyte counts were associated with a paradoxical clinical deterioration, an immunological reaction to opportunistic infection or tumor. IRIS in the settling of PML, crytpococcal meningitis, and HIV encephalitis is of relevance in neurology.

IRIS classically develops in the most immune-deficient patients who experience the greatest immune reconstitution following commencement of ARVs. HIV treatment induced IRIS is particularly important in the developing world, given the frequent delay to commencement of cART and high opportunistic infection burden. It is also frequently underrecognized, being difficult to differentiate from the underlying infection or inflammatory process itself.

PML is characterized by minimally enhancing MRI lesions (Fig. 2.2). As the immune system is restored, PML lesions in IRIS frequently contrast enhance and result in significant amounts of edema, although this is seen less reliably in milder lesions. The presence of punctate or linear enhancement is notably consistent with inflammatory PML [61]. MRS can be used to show elevated myoinositol and lipid/

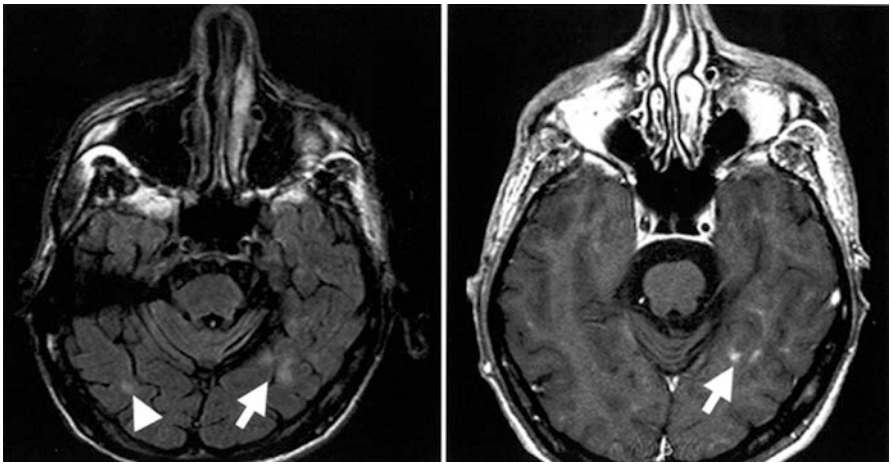

Fig. 2.2 Contrast enhancement of PML lesions in a patient with IRIS. A left temporal lobe lesion seen in FLAIR (*arrow, left panel*) minimally enhances after gadolinium injection in T1-weighted image (*arrow, right panel*), whereas a right temporal lobe lesion is only detected in FLAIR sequence (*arrowhead, left panel*) [80]

creatinine peaks in lesions with inflammation [61]. Prognosis is usually reasonable. PML with cART immune reconstitution can be treated with high-dose corticosteroid therapy – typically 1 g for 3–5 days, occasionally repeated fortnightly to monthly. Steroids should only be used in conjunction with ongoing cART. Cryptococcal meningitis and IRIS are a common clinical scenario. Increasingly, it is recommended that cryptococcus treatment precedes HIV treatment by at least 5 weeks [56]. Treatment efforts include removing the antigen burden with large-volume lumbar punctures and steroids to "dampen" the immune response. Case reports also exist of a syndrome in treated HIV patients of severe CD8 infiltration to the brain, essentially an IRIS to HIV itself. IRIS and the balance between inadequate and overactive immune response to neurological complications of HIV, particularly opportunistic infections, will continue to challenge clinicians, and early awareness/recognition of this entity is likely key to ongoing management.

2.12 cART and the Neurological System: Adverse Effects – PNS Toxicity

In addition to the direct HIV-related distal sensory polyneuropathy seen in the HIV population, cART is also associated with a toxic neuropathy and may cause over a third of cases of DSPN in patients with HIV on treatment [81]. This may be distal, affecting the arms and legs and resulting in pain, paresthesia, and gait instability, or involve the autonomic nervous system.

It largely relates to the use of nucleoside reverse transcriptase inhibitors: didanosine, zalcitabine, stavudine [81]. There have been reports of protease inhibitors inducing neuropathy although evidence remains insubstantial. The clinical presentation is frequently indistinct from that due to direct HIV toxicity, and as such the importance of early recognition lies with the physician who must be aware of the association with certain antiretrovirals. Drug toxicity neuropathy is typically more painful, has a more abrupt onset, and progresses rapidly [82]. Nerve excitability studies can aid in differentiating direct viral effects versus antiretroviral drug therapy neuropathy; one report described normal properties of the axonal membrane in sensory and motor nerves demonstrated in patients with viral neuropathies with excitability changes indicating pathology of the internode in nucleoside neuropathies [83].

The mechanism of action relates to mitochondrial DNA damage and is typically dose-dependent. There is classically partial to complete reversibility with cessation of the offending agent. Although serum acetyl-L-carnitine levels are decreased in cART-related neuropathy, there is no good evidence for replacement/supplementation [84]. The mitochondrial haplogroup T has also been associated with an increased risk of development of antiretrovirus-induced neuropathy.

cART-induced myopathy with zidovudine can also occur during treatment for HIV. Again, the effect is dose-dependent, with lifetime doses >200 g being the highest risk, but the incidence also appears to increase with advancement of disease [85]. The frequency of AZT-induced myalgias is 8–10%, and myopathy is up to 18% [85]. Presentation is with proximal weakness and atrophy predominantly involving the buttocks. CK is typically elevated, and a biopsy may be required to delineate the cause of the myopathy. Termination and substitution with another antiretroviral is indicated; typically, there is a period of 1–2 months of improvement followed by plateau [86].

The integrase inhibitor raltegravir is also reported to have caused a handful of cases of rhabdomyolysis with significant CK elevations and has subsequently been associated with symptomatic myopathy manifest as myalgia and proximal myopathy with normal CKs [87]. This does not seem to be a concentration or time-dependent effect, and the mechanism is unknown, but in cases where the antiretroviral agent was changed due to intolerance, objective improvement occurred indicating reversibility [87]. Implications for therapy remain unclear; myalgia alone, if tolerable, would not routinely necessitate change of agent, but change may be required by weakness or rising CK.

2.13 cART and the Neurological System: Adverse Effects – CNS Toxicity

ARVs have variable CNS penetration. Recognition of this heterogeneity has resulted in measurements such as the CNS penetration effectiveness score (CPE) and the observation in some studies of worse neurocognitive function with certain ARVs with higher CPE, contributing to HAND in the cART era [88]. There has been some in vitro and in vivo support for this concept. NRTIs are believed to cause mitochondrial toxicity, and this appears targeted, with lower NAA levels in MRI spectroscopy in patients on stavudine and didanosine [89]. Efavirenz is known to damage dendritic spines in neuronal culture [90], and has been well described as causing generalized neurobehavioral side effects. Management is on an individual case-by-case basis, but if patients are clearly intolerant, adjustment of regimen is recommended.

2.14 Conclusions and Future Directions

HIV-related neurological complications continue to be a challenging field for neurologists in the developing and developed world. While CNS opportunistic infections and early HIV-related complications such as HIV myelopathy and encephalitis are increasingly rare in the developed world, they continue to burden patients

globally. In the developed world, neurologists are frequently called upon to investigate the causes of cognitive impairment, peripheral neuropathy, and immune-mediated syndromes related to HIV, as well as complications of treatment. Early recognition and intervention remain key to impacting ultimate patient outcome and quality of life, and future progress in immune-based therapies will likely broaden the field of HIV neurology in the coming years.

Conflict of interest The authors report no conflicts of interest.

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Chapter 3
HIV-1 Related Central Nervous System Diseases: Pathogenesis, Diagnosis, and Treatment – An Indian Scenario

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Keywords India • HIV • NeuroAIDS • Neurological complications • Dementia • Antiretroviral therapy • Adherence to treatment • Follow-up • Opportunistic infections • Toxoplasmosis • Cryptococcal meningitis • TB • PML • Seizures • Central nervous system lymphoma • Non-Hodgkin's lymphoma • Hospital-based reports

Core Message
The advancement of medical sciences in India has recognized an increase in the prevalence of neurological complications associated with HIV infection. Infection with HIV continues to be a major contributing risk factor for the development of neurological complications. The limitations of the studies from India primarily are attributed to the lack of a complete knowledge of the disease, a lack of adherence to treatment, as well as loss during follow-up. In the current scenario, early diagnosis of HIV and access to care and treatment are most vital.

3.1 Introduction

3.1.1 AIDS in India

India ranks third in its HIV-infected population of around 2.1 million people (UNAIDS Gap Report 2016[1]). India has different epidemics in various parts of the country. The epidemic episodes in the southern and western states are primarily

[1] http://www.unaids.org/sites/default/files/media_asset/2016-prevention-gap-report_en.pdf.

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heterosexual. The northeastern states initially experienced HIV in the injection drug user population and sexual partners; however, the distribution due to heterosexual population has been increasing. In 2015 alone, around 87% of new infections were reported. Five states with highest prevalence, which includes Manipur, Mizoram, Nagaland, Andhra Pradesh, and Karnataka, are in the east and south of the country. Few states in the north and northeast India also are contributing now to high prevalence. The Indian population is primarily infected with subtype "C" HIV-1. Broad intervention programs were launched by the government of India along with international, nongovernmental, and community-based organizations. The main barriers to effective control are the low literacy levels, insufficient resources, stigma, and widespread migrations [1].

3.1.2 NeuroAIDS: An Overview

HIV-1 infects the nervous system of almost all the patients with systemic infection leading to disorders related to central and peripheral nervous systems (CNS and PNS). Autopsy studies revealed that around 75% of patients dying with AIDS had neurological abnormalities [2]. The virus is neuroinvasive (enters the CNS), neurotropic (replicates and survives in neural tissue), and neurovirulent (causes nervous system diseases) [3]. The presumed overall mechanism of CNS invasion is that virus-infected monocytes cross the blood-brain barrier (BBB), mature, and infect perivascular macrophages, choroid plexus, and capillary endothelial cells [4]. Oligodendrocytes and neurons are rarely infected, and indirect mechanisms are postulated for their damage [5]. BBB breakdown and persistent infection lead to neurotoxicity, axonal and neuronal injury, and clinical symptoms. Damage includes immune system dysfunction and paves the way for the development of opportunistic infections [6]. The CNS is permeable to antiretroviral drugs to various degrees, making the management and eradication of reservoirs of virus infection difficult. The virus can evade the immune system creating an environment to replicate, mutate, and reinfect via the circulation. The persistent inflammatory response may induce pathways leading to other neurodegenerative changes and diseases.

During early stages of infection, HIV-1 induces polyclonal hyper-gammaglobulinemia leading to demyelinating diseases of the CNS and PNS. The virus induces progressive multiple symptoms of motor, behavioral changes, and cognitive impairment. The development of these neurological syndromes in HIV-positive patients is consequent to a chain of events, which is determined by the properties of the virus itself, genetics of the host, changes in gene expression, and interaction with the environment. The neurological syndromes associated with HIV can be categorized into three classes: (i) primary HIV neurological disease (HIV is both required and sufficient to cause illness), (ii) secondary/opportunistic neurological disease (where HIV infection leads to infection by other pathogens – opportunistic infections), and (iii) treatment-induced neurological diseases [1–7].

3.1.3 NeuroAIDS in the Developing World

Prior to the availability of highly active antiretroviral therapy (HAART), neurological complications were heralding manifestation of AIDS in 7–20% of the patients, and the prevalence rate varied between 39% and 70%. The widespread use of HAART resulted in the decline of neurological diseases including opportunistic infections. Nonetheless, AIDS-associated neurological diseases continue to contribute a significant burden for developing countries. In a 2007–2008 study conducted in Thailand, the most common CNS disorders were cryptococcal meningitis (37.8%), tuberculosis (TB) (35.8%), toxoplasmosis (12.8%), progressive multifocal leukoencephalopathy (PML) (4.1%), varicella zoster virus meningitis (2.7%), pneumococcal meningitis (1.4%), herpes simplex virus meningitis, and Epstein-Barr virus–related primary CNS lymphoma [7]. Similarly, in South Africa, observed CNS disorders mirrored those seen in the pre-ART era in developed countries [8]. In a study from South Africa, paradoxical neurological worsening was observed in 28% of patients who had been started on HAART. Studies from sub-Saharan Africa indicate that both HIV-related dementia and peripheral neuropathy were significant problems [9]. For example, in a study from Uganda in 2005, 31% of HIV-infected patients had dementia and 47% had peripheral neuropathy [10]. Regional differences in both the prevalence and rates of progression of HIV-associated dementia were attributed to differences in the neuropathogenicity of different HIV subtypes. Prior to HAART usage in the United States, around 20% of the HIV-positive patients had HIV-associated dementia (HAD) and 40% had milder HIV-associated neurocognitive disorders (HAND) [11–13]. However, the incidence of HAD is decreasing, while the prevalence is still increasing slowly. Within HIV-infected populations of sub-Saharan Africa (e.g. Uganda), the prevalence of HAD in a cohort was estimated at 31%. It was noted that 72% in this cohort were HAART-naïve, which is near the pre-HAART prevalence of HAD in the United States [14]. Improvement in HAND with HAART administration has been documented in cohort studies in North America, Europe, Australia, and elsewhere [15–20]. In India, the patient profile and risk factors differ from the industrialized countries. In the pre-AIDS era, toxoplasmosis was the most frequent CNS opportunistic infection. Similarly, CNS tuberculosis was also frequent in Indian AIDS cases when compared to other countries [21]. As we observe, limited studies are available from India focusing on the neurological manifestations of HIV infection.[2]

[2] https://www.ncbi.nlm.nih.gov/pubmed/?term=neuroaids++india.

3.2 Prevalence of HIV-Associated CNS in India

3.2.1 Meningitis

During acute HIV infection, the CNS infection remains asymptomatic; however, the investigation of cerebrospinal fluid (CSF) and imaging studies can detect abnormalities during this period, which presage later neurological sequelae. During seroconversion, patients may show aseptic meningitis. The most common neurological syndrome associated with infection is acute meningitis (meningoencephalitis). This type of meningitis resolves within 2–4 weeks by itself without any sequelae. Mild lymphocytic pleocytosis is noted in CSF accompanied by normal glucose and slightly elevated levels of total protein. A retrospective observational study involving 81 HIV-positive patients with neurological manifestations from Western India reported the prevalence of aseptic meningitis in 9.87% of cases [22].

Subacute and chronic meningitis are the results of tuberculous infection or cryptococcal infection. Tuberculous meningitis (TB meningitis, TBM) is the most common neurological manifestation of *M. tuberculosis* infection. This subacute meningitis evolves over several days or weeks, with signs of meningeal irritation, headache, altered mental status, irritability, and fever. In a study, CT scans showed hydrocephalus in two-thirds of the patients. Meningeal enhancement is the most characteristic feature of TB meningitis [23]. CSF investigation revealed the presence of lymphocytic pleocytosis, with increased proteins and diminished glucose levels. Antitubercular treatment is prescribed for the patients along with a short-term corticosteroid therapy as an adjuvant treatment in complicated situations. It should be noted that TB meningitis is the highest reported type of meningitis across India, irrespective of patient age and gender [23].

Cryptococcal meningitis, caused by *C. neoformans*, is the second most common opportunistic infection associated with AIDS patients. One study reported a prevalence rate of 2.79% among 573 HIV seropositive cases [24]. Patients with this type of meningitis experience subacute to chronic headaches, with and without fever. In addition, in severe cases, adverse changes in mental status endure as well. CSF investigations reveal moderate mononuclear pleocytosis, increased protein, and normal to decreased glucose levels. CSF from the HIV-positive patients should be subjected to fungal culture to determine the cryptococcal infection type and to ascertain its drug sensitivity. Treatment for cryptococcal infection involves IV Amphotericin B at a dose of 0.75 mg/kg daily, with a total cumulative dose of not more than 1.5–2 g, with flucytosine 25 mg/kg for 2 weeks, or fluconazole, 400 mg/day for 10 weeks. This is followed by fluconazole, 200 mg/day until the CD4 T cell count has increased to >200 cells/ml for 6 months [24]. Different studies across India reported that cryptococcal meningitis infection rates varied between 19.8% and 45.8%. In northern India, the prevalence of HIV is lower, compared to southern or western India, where HIV infection is higher. However, there were more cryptococcal meningitis cases in northern India compared to the other areas. This discrepancy was ascribed to case misdiagnosis and underreporting [25].

3.2.2 Seizures

Seizures constitute an important manifestation among various neurological disorders associated with HIV infection. Based on the data derived from hospital-based reports, new-onset seizures were observed in 20% of 500 HIV seropositive patients [26]. Opportunistic infections are the major component cause of seizures. In addition, metabolic and electrolyte disturbances also play a crucial role in recurrent seizures. The prevalence of seizures in HIV patients with meningoencephalitis (induced by opportunistic infections) was approximately 12–16% [27]. Another important factor responsible for seizures is toxoplasmosis. In a retrospective study conducted in south India, seizures were observed in 35% of cases and were the prime manifestation in 11% of total patients with CNS toxoplasmosis. In this study, approximately 25% of patients with cryptococcal meningitis manifested with seizures as well [28]. CNS tuberculosis and progressive multifocal leukoencephalopathy (PML) are rarely associated with seizures. Seizures are usually focal or generalized and have a high recurrence rate. Patients are usually evaluated with MRI of brain to exclude focal lesions. CSF analysis is done if magnetic resonance imaging (MRI) is unremarkable, to rule out cryptococcal and tuberculous meningitis. Long-term antiepileptic drug therapies are considered for treating seizures.

3.2.3 Dementia

A spectrum of neurocognitive impairment disorders is associated with HIV which includes subsyndromic cognitive deficits and mild-to-severe cognitive impairment. The latter is termed as AIDS dementia complex (ADC) or HIV encephalopathy, or HIV-associated dementia (HAD). During the advanced stage of infection, when the immune system dysfunction is moderate-to-severe HAD, the subcortical dementia is observed. The incidence of HAD declined after the commencement of antiretroviral therapies in the affected patients [29]. The HAD incidence in India is less than that in western populations, due to differences in viral strains. A study from south India disclosed that the prevalence of drug-naïve HIV patients infected with clade C was like that of clade B infections from western countries [30]. The affected individuals often exhibited poor concentration and memory, behavioral disturbances along with bradykinesia, tremor, and seizures. Fewer studies from India have studied HAD along with other neurological complications. The prevalence rates varied between 1% and 8% [22]. The reason for a dearth of studies may include shorter life expectancy, briefer survival due to opportunistic infections, or due to underdiagnosis. A recent study adopting the International HIV Dementia Scale (IHDS) to assess the levels of cognitive dysfunction, as suggested by previous investigators, reported that 32.50% of the study population showed cognitive dysfunction, 30% showed asymptomatic neurocognitive impairment, and 2.5% exhibited mild neurocognitive disorder [31]. Approximately, half of the HAD patients improve with antiretroviral treatments. For extrapyramidal symptoms, low doses of dopaminergic drugs and atypical antipsychotics for behavioral disturbances are suggested.

3.2.4 Space-Occupying Lesions

Toxoplasmosis, tuberculoma, PML, and lymphomas may lead to focal space-occupying lesions in the brain [32]. Toxoplasmosis is observed at the advanced stage of HIV infection when the CD4 count is <200 cells/μl. The rates declined with the advent of the antiretroviral therapy. The patients with CNS toxoplasmosis possess fever, headache, and seizures along with focal neurological deficits [33]. Patients with toxoplasmosis have multiple ring enhancing lesions with special predilection to basal ganglia or posterior fossa with evidence of blooming on FLAIR.[3] Studies from south India reported the prevalence rate of 34.78% and 15% in the HIV-positive patients [34, 35]. Prevalence of 67.8% was reported in the seropositive HIV-infected patients when compared to 30.9% in immunocompetent adults from Maharashtra [36]. Standard treatment for toxoplasmosis consists of sulfadiazine (3–4 g/day) and pyrimethamine (75 mg/day) with leucovorin for a minimum of 4–6 weeks. The drugs are prescribed until CD4 counts exceed 200 cells/μL. Tuberculomas are encapsulated and will show a solid-type or ring enhancement. A recent hospital-based study from south India investigating 30 patients with CNS tuberculoma revealed that convulsions were the common clinical presentation. Patients in age group between 20 and 40 were highly affected. The treatment included antituberculous treatment along with oral steroids [37]. Primary central nervous system lymphoma (PCNSL) is a malignant non-Hodgkin's lymphoma and accounts for 1% of all intracranial tumors. PCNSL accounted for 1.2% in a hospital-based study from India. Lymphomas are solitary and periventricular. The association of PCNSL with HIV infection has been low in India due to early death in AIDS because of opportunistic infections [38]. PML (caused by JC polyomavirus) is a demyelinating disease of the CNS. Multifocal scalloped white matter lesions are considered characteristic of PML. There is sparse literature on HIV/AIDS with PML from developing countries including India. A retrospective study from north India revealed 1.2% of the patients attending the antiretroviral treatment (ART) clinic had PML [39]. Moreover, PML was the first AIDS-defining illness in 56% of the cases. Majority of the deaths occurred in the initial few months. Various treatment regimens against JC virus were unsuccessful; however, highly active antiretroviral therapy (HAART) remains the only successful therapy [29]. Literature from India is limited to a small series of case reports. Three cases have been reported from a tertiary care center in southern India. Two patients died within 45 days of diagnosis, and the third patient was lost to follow-up, limiting the data on long-term outcome. Limited access to diagnostic facilities is a major drawback for diagnosis of PML. This important factor results in underdiagnosis or underreporting from resource-constrained settings.

[3] https://radiopaedia.org/articles/blooming-artifact.

3.2.5 Stroke

Ischemic and hemorrhagic strokes and transient ischemic attacks are associated with HIV infection. HIV infection itself is the prime cause of stroke and occurs at the advanced stage of the infection, when the CD4 count is <200 cells/μl. The mechanism of thrombosis is not known; however, decreased S protein levels and increased anticardiolipin antibodies may contribute to the development. A study from northeast India investigated the nature of stroke in 19 HIV-positive patients. CT scans of the patients' brains showed 89.47% had infarction, 5.26% had hemorrhage, and 5.26% had both hemorrhage and infarction. In 78.94%, stroke was the first presentation of HIV infection and 46.67% had recurrent cerebrovascular accidents [40]. Antithrombotic treatment (ATT) along with low-dose antiplatelet agents may be given to these patients with ischemic strokes (after excluding thrombocytopenia). Anticoagulants are required for the management of venous thrombosis [41].

3.2.6 Extrapyramidal Syndromes

Movement disorders such as hemichorea, Parkinsonism, choreoathetosis, dystonia, and hemiballism are associated with HIV-positive patients, as lesions are more commonly observed in the basal ganglia [42]. Patients with AIDS have been proven to have toxoplasmosis. The patients under ATT may develop low-level immune activation which may facilitate the development of neurodegeneration and accelerate the development of Parkinson's disease. Management of these disorders is similar to that in non-HIV-infected counterparts with lower doses of drugs in the patients [43].

3.2.7 Myelopathy

Spinal cord disease occurs in 20% of the individuals who have HIV encephalopathy. Myelopathy can be induced by HIV infection itself or by opportunistic infections such as tuberculosis, toxoplasmosis, herpes zoster, herpes simplex, syphilitic infections, or cytomegalovirus [43]. Human T-lymphotropic viruses (HTLV) I and II can also lead to myelopathy in endemic areas. HTLV1 and II coinfections have been reported for HIV-infected patients from south India, especially in Tamil Nadu [44, 45]. HTLV-related myelopathy occurs during the early period of HIV infection. Myelopathy due to HTLV1 can be treated by ATT and corticosteroids; however, myelopathy induced by HTLVII has no therapeutic options.

Vacuolar myelopathy is caused by HIV itself.[4] It mimics the subacute combined degeneration of spinal cord, pure sensory ataxia secondary to dorsal column involve-

[4] http://library.med.utah.edu/WebPath/AIDS2016.PDF.

Table 3.1 Additional studies on AIDS CNS complications from India

CNS complications	Description	Reference
Meningitis	Comparison of clinical, radiological, and pathological features of TBM with and without HIV reported that despite similar clinical features, cognitive dysfunction was more common among the HIV group	[46]
Cryptococcal infection	In an autopsy investigation of a series of 170 cases (between 1990 and 2009), the cryptococcal infection of the nervous system was observed to be more frequent (31.3%) than toxoplasmosis (20.8%) and TBM (20.1%)	NIMHANS [47]
PML	The advancement in molecular testing technologies and neuroimaging facilities suggested that the incidence of PML was on the rise as its higher incidence rate (2.8%) was reported in a study from Bangalore	[48]
Neuropathies	The incidence of peripheral neuropathy in HIV/AIDS patients from Hyderabad was higher (20.4%) when compared to the incidence from Goa (4.6%) Neuropathies including herpes zoster were the commonest manifestation in a study from Pune (28.7%) Of non-OIs, the peripheral neuropathies constituted 19.3% and 35.8% in a recent study from Mumbai	[49–52]
Stroke	A study from Bangalore reported ischemic stroke in 2.9% and cortical venous thrombosis in 0.5% in the infected patients, whereas higher incidence was reported from a study series from Mumbai (29.8% ischemic stroke and venous thrombosis in 4.4%)	[28, 53]

NIMHANS National Institute of Mental Health and Neurosciences, *TBM* Tuberculous meningitis, *PML* Progressive multifocal leukoencephalopathy, *OI* Opportunistic infection

ment, or HIV myelitis. Vacuolar myelopathy is rarely reported from India. MRI of the spinal cord is done on patients with myelopathy to rule out opportunistic infections. MRI for patients with vacuolar myelopathy may show spinal cord atrophy or symmetric hyperintense signals on T2-weighted sequences. Primary HIV-related spinal cord syndromes do not respond well to ATT; however, such therapy may help patients with HIV myelitis. A study from Maharashtra, India, investigating the neurological manifestation in HIV patients reported 2.46% of the study sample had vacuolar myelopathy. Similarly, a hospital-based study from south India reported myelopathy in 33 patients out of 411 [22].

Additional studies in India of HIV-associated neurological complications are summarized in Table 3.1.

3.3 Conclusion

Despite the decline in incidence, HIV remains the sixth leading cause of death, globally. HIV-associated neurocognitive impairments comprise primary illnesses including meningitis, tuberculoma, and myelopathy. Infections with HIV continue

to be a major contributing risk factor for the development of the neurological complications as they lead to increased morbidity and mortality in HIV-infected patients. Hence, it is crucial to identify the primary causative factor to alter the morbidity rates and improve the quality of life. A high index of suspicion and routine surveillance are required to assist in an early diagnosis and appropriate therapy, as most patients respond to therapy. Investigation of various hospital-based retrospective studies from India reveal that most patients do not arrive for treatment and that they are lost during routine follow-up. There is still a lack of education among patients. If these impediments are addressed, there is indeed hope to win the battle against AIDS and NeuroAIDS in countries that require these advances.

Conflict of interest The authors report no conflicts of interest.

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Chapter 4
HIV-1 Diversity in Brazil

Rodrigo Pessôa and Sabri Saeed Sanabani

Keywords Human immunodeficiency virus type 1 (HIV-1) • Genetic diversity • Molecular epidemiology • HIV subtypes • Recombinant forms

Core Message

Genetic variation in human immunodeficiency virus (HIV)-1 poses significant public-health and clinical challenges. In Brazil, several subtypes cocirculate which are diversely distributed in different geographical regions. The continuous molecular evolution of these subtypes, together with the introduction of new subtypes as well as intermixing of HIV-1 variants, is inevitable and may have significant implications for diagnosis of infection, quantification of viral loads, drug-resistant pathways, transmissibility, response to therapy, and challenges to development of vaccine. Thus, understanding the genetic diversity and geographical distribution of HIV is significantly important for planning effective intervention measure to prevent infection, either through educational programs, therapy interventions, or vaccine strategies.

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4.1 Introduction

Acquired immune deficiency syndrome (AIDS) was first recognized as a cluster of symptoms when an increasing number of young homosexual men in New York City and San Francisco died from opportunistic infections and rare malignancies. Eventually, similar symptoms were found among intravenous drug users, hemophiliacs, and other recipients of blood transfusions. In 1984, the human immunodeficiency virus (HIV) was isolated and subsequently assigned as the causative agent of AIDS. Since then, HIV/AIDS has become one of the most devastating epidemics in humans in recent history. At the end of 2014, the WHO global health report showed that about 36.9 million people were living with HIV and tens of millions of people had died of AIDS-related causes since the beginning of the epidemic (http://www.who.int/gho/hiv/en/). Over 12 million children have been orphaned by AIDS and about 1600 babies acquire HIV from their infected mothers everyday [1]. In the past three decades, the HIV pandemic has caused a great burden on global wealth and health, especially in sub-Saharan Africa where the highest rate of HIV infection has been recorded (http://www.unaids.org/).

As the prospects of an effective vaccine and curative treatments remain uncertain, HIV/AIDS will continue to be a significant threat to public health in the coming years. As of 2014, the Joint United Nations Program on HIV/AIDS (UNAIDS) reported an estimated 87,000 new infections in Latin America, bringing the number of people living with HIV to an estimated 2 million (http://files.kff.org/attachment/fact-sheet-the-global-hivaids-epidemic). The Caribbean has been hardest hit by the epidemic and has the second highest HIV prevalence rate in adult subjects in the world after sub-Saharan Africa. Six countries in Latin America and the Caribbean have generalized epidemics, with Haiti having the region's highest prevalence rate (1.9%), and Brazil the greatest number of people living with the disease (approximately 730,000–1,000,000) and home to roughly half of all new HIV infections in the region (http://www.unaids.org/sites/default/files/media_asset/MDG6Report_en.pdf).

The immense genetic variability of HIV-1 viruses is considered the key factor that frustrates efforts to halt the virus epidemic and poses a serious challenge to the development and efficacy of vaccines. Like other human positive-sense RNA viruses, HIV has a high mutation rate as a result of the error-prone nature of the reverse transcriptase (3×10^{-5} mutations per nucleotide per replication cycle) [2, 3]. This high rate of mutation, coupled with the increased replication capacity of the virus (10.3×10^9 particles per day) [4], allows for the accumulation and fixation of a variety of advantageous genetic changes in a virus population, which are selected for by the host immune response and can resist newly evolving host defenses.

Recombination is another potential evolutionary source that significantly contributes to the genetic diversification of HIV by successfully repairing defective viral genes and by producing new viruses [5]. The most recent common ancestors (MRCA) of HIV-1 groups M and O were estimated to have existed around 1920 [6, 7] and became a worldwide public health threat 60 years later. To date, HIV-1

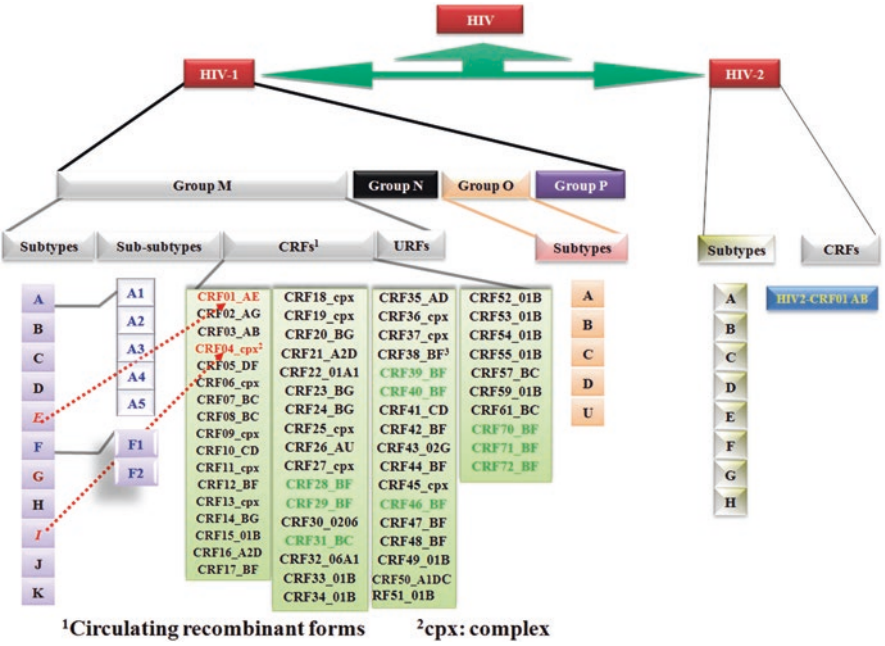

Fig. 4.1 Classification of HIV-1 and HIV-2 variants: The vast majority of HIV-1 strain belong to group M which has further been classified into subtypes, sub-subtypes, circulating recombinant forms (CRFs), and unique recombinant forms (URFs). In contrast, HIV-2 has been classified into eight different groups. Among these, only group A and B are epidemic. The full-length genome sequencing of subtype E and I determined that they are actually CRF01_AE and CRF04_cpx, respectively. The CRFs written in green color have been identified to circulate in Brazil

viruses are classified into four phylogenetic groups: M, O, N, and P, which most likely reflect four independent events of cross-species transmission from chimpanzees [8, 9], as shown in Fig. 4.1. The M group (for main), responsible for the majority of viral infections worldwide, is further subdivided into nine subtypes (A–D, F–H, J, and K), among which subtypes A and F have been further classified into two sub-subtypes [8]. Moreover, early sequencing studies have provided evidence of a recombination between genomes of different HIV subtypes [10, 11]. Such interclade recombinant strains are consistently reported from regions where two or more clades are predominant. Recombinant strains from at least three unlinked epidemiological sources, which exhibit identical mosaic patterns, have been classified separately as circulating recombinant forms (CRFs) [12, 13]. Currently, there are more than 70 defined CRFs http://www.hiv.lanl.gov that are epidemiologically important as subtypes. In addition to the known CRFs, a large number of unique recombinant viruses, which are called unique recombinant forms (URFs), have been characterized worldwide [14].

Together, CRFs and URFs are estimated to account for 18% of incident infections in the global HIV-1 pandemic [15]. It is likely that novel subtypes and CRFs

will be isolated in the future as recombination and mutation continue to occur. HIV-1 subtypes, CRFs, and URFs show considerably different patterns of distribution in different geographical regions [15, 16]. This diversity has an impact not only on diagnosis, replication, development of mutations, and therapy response, but also on the ability of distinct viruses to recombine during coinfection [17].

Available epidemiological data indicates that subtype B is the major clade in the Americas, Western Europe, Japan, and Australia [15, 18]. Subtype A is the main genetic form in Russia and the former Soviet Union countries, but it is also commonly observed in several African countries such as the Democratic Republic of Congo (DRC), Kenya, and Tanzania [19]. Subtype C is prevalent in South Africa, Somalia, Djibouti, Ethiopia, as well as in India, while subtype D is the dominant form in other regions, such as Uganda, Sudan, and Libya. In some geographic areas, recombinants are the most widely distributed forms, as is the case with CRF01_AE in Southeast Asian countries, CRF02_AG in West African countries, and CRF07_BC and CRF08_BC in China [20]. Subtypes A1, B, C, and the CRF02_AG are the most prevalent HIV-1 group M genetic clades and are responsible for the infection in more than 75% of AIDS cases worldwide. Molecular epidemiological studies have, thus, made significant contributions to our understanding of the molecular diversity of HIV-1, enabling the detection of emerging HIV-1 subtypes, and improving the tracking of the epidemic worldwide. Of note, the transmission of the CRFs and URFs further complicates efforts toward the development of effective vaccines due to existing diversity [21].

In this chapter, we review the diversity of HIV-1 in Brazil, the largest country in South America, and the fifth largest nation in the world [22]. The first case of AIDS was recorded in the country by retrospective analysis in late 1981. At that time, Brazil was in the midst of a transition from military rule to civilian government and was facing a devastated economic and social welfare system [23]. This process of transformation would drastically affect not only the immigration pattern and spread of infection but also the overall national strategy to curb the spread of HIV/AIDS.

While the AIDS-related illnesses during the early years of the epidemic posed an unprecedented challenge to the health authorities, Brazil today is considered a model of a successful response to the epidemic, particularly for countries of low- and middle-income status. It has been reported that since the 1990s, the number of Brazilians dying from AIDS-related illnesses has fallen by 50%. The antiretroviral program currently reaches 80% of the infected population, which is similar to the coverage in wealthier, more developed nations. From 1980 to June 2014, 757,042 new AIDS cases were reported in Brazil. The HIV-1 incidence has been increasing rapidly and progressively in Brazilians exposed to HIV through heterosexual sex since the beginning of the epidemic, because of a process of feminization of the AIDS epidemic: in 1985, the male per female new AIDS case ratio was 26.7, while in 2014 it had decreased to 2.0 (http://www.aids.gov.br). The prevalence of AIDS in the adult population is 0.4%, and the incidence of new cases was 78/1000, 000 inhabitants in 2014.

Currently, the HIV-1 epidemic in Brazil is dominated by subtype B, followed, to a lesser extent, by BF1 URF subtypes, particularly in the north, northeast, west central, and southeast of the country. In contrast to these regions, subtype C is the

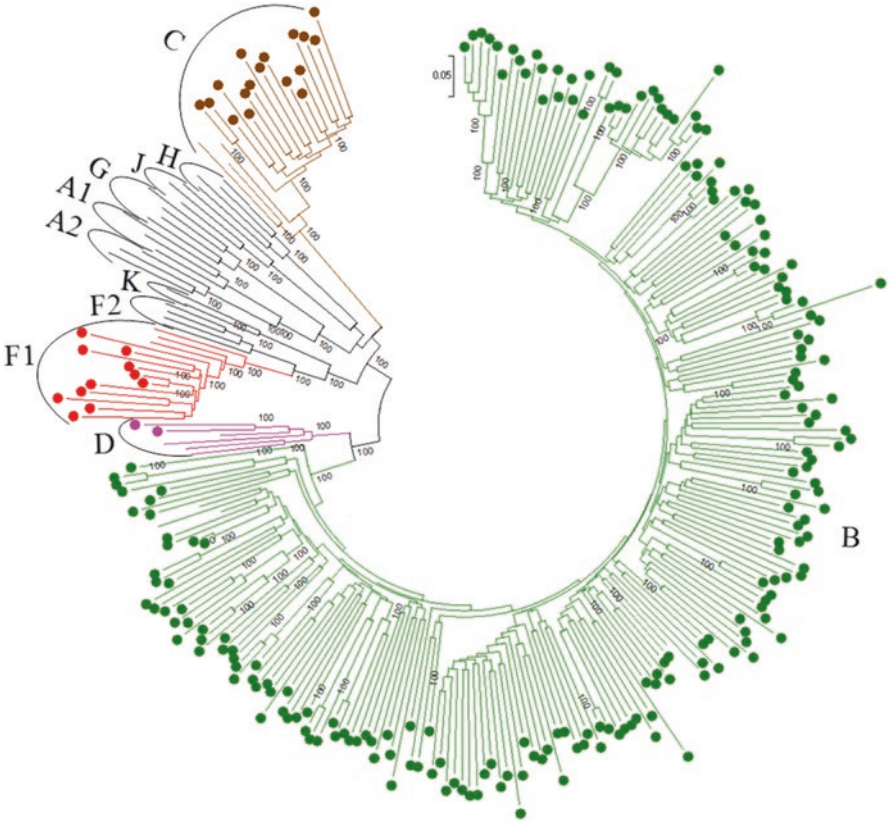

Fig. 4.2 Phylogenetic analysis of all Brazilian nonrecombinant HIV-1 near full-length (published and unpublished) genomes and 37 HIV-1 reference sequences from the Los Alamos HIV-1 database (http://hiv-web.lanl.gov) representing 11 genetic subtypes. Annotation of samples is as follows: symbol-green circle (subtype B), symbol-brown circle (subtype C), symbol-red circle (subclade F1), and symbol-pink circle (subtype D). The tree was constructed using the PHYML v.3.0 package. For clarity purposes, the trees were midpoint rooted. The approximate likelihood ratio test (aLRT) values of 100% are indicated at nodes

second most prevalent genetic subtypes in the southern region. The distribution of these variants is based on published studies, which often used different molecular techniques including the heteroduplex mobility assay, and partial or full-length genome sequencing. Figure 4.2 shows the results of a phylogenetic tree analysis of all Brazilian HIV-1 full-length nonrecombinant genomes available in the database and other unpublished sequences from our group [24]. In this chapter, we present a detailed revision of the literature of the HIV-1 epidemic in the five main geopolitical regions. The molecular data of subtype distributions were obtained from published national studies for the last 15 years. The proportions of HIV-1 subtypes and recombinants in each region were combined in a similar way to what has been described before [15].

4.2 Molecular Epidemiology of HIV-1 Infection in Brazil

4.2.1 North Region

The north region is the largest region of Brazil, which borders six different South American countries. The cumulative number of HIV-1 cases by the end of 2014 was 41,036, resulting in an incidence rate of 122.8 per million population in 2014 according to data from the Brazilian Ministry of Health (http://www.aids.gov.br). A broad diversity of HIV-1 subtypes has been detected; however, nucleotide sequence data are still scarce in comparison to other regions of Brazil [25–29]. A molecular epidemiological survey of HIV-1 from this region revealed that the majority of the HIV-1 variant characterized between 2000 and 2011 ($n = 332$) belonged to subtype B (82.8%), and the second most prevalent strain was subtype C (5%), F1 (4.8%), BF1 recombinants (4.5%), CF1 (0.9%), and other different subtypes that circulate in small proportions (0.3% each) including CRF02_AG, as shown in Fig. 4.3.

In a recent study including 377 individuals with virologic failure, the genotypic profile indicated that the majority of the analyzed variants (90.7%) were of subtype B, followed by subclade F1 (5.7%) [30]. The same study reported the circulation of subclade A1 for the first time in the north region. The evaluation of temporal distribution of B and non-B subtypes over the 10 years failed to reveal significant differences, providing evidence of a stabilized HIV-1 epidemic in terms of diversity in this region [30]. Notably, the subtype definition of HIV-1 sequences reported in the later studies was solely based on small fragments of viral genomes, which are not sufficient to identify all recombinant viruses, and thus, it likely underestimates the complexity of these divergent strains.

4.2.2 Northeast Region

The northeast is the Brazilian region with the largest coastline in the country, which makes the region very appealing to tourists around the world. The cumulative number of reported HIV-1 patients in this region was 108,599, resulting in an incidence rate of 66 per million of population in 2014 (http://www.aids.gov.br). The molecular epidemiological data from ten published studies and others from our own laboratory (Pessoa et al. 2016) derived from the northern region of the country during 2006 and 2015 revealed the circulation of four genuine subtypes (B, F1, C, and D), four URFs (BF1, BC, DF1, and BCF1), and two novel CRF70 and 71 BF1 strains [31–40]. Overall, subtype B accounted for most of the HIV-1 infections (78%) in this region followed by a large variety of BF1 strains (11.5%) and F1 subclade (5.7%).

A recent REDS-II (Retrovirus Epidemiological Donor Study) study of HIV-1 molecular epidemiology analyzed the partial *pol* gene of 110 samples from seropositive blood donors in the state of Recife, capital of the state of Pernambuco (PE), and the principal port city of the northeast [41]. The study reported a relatively high

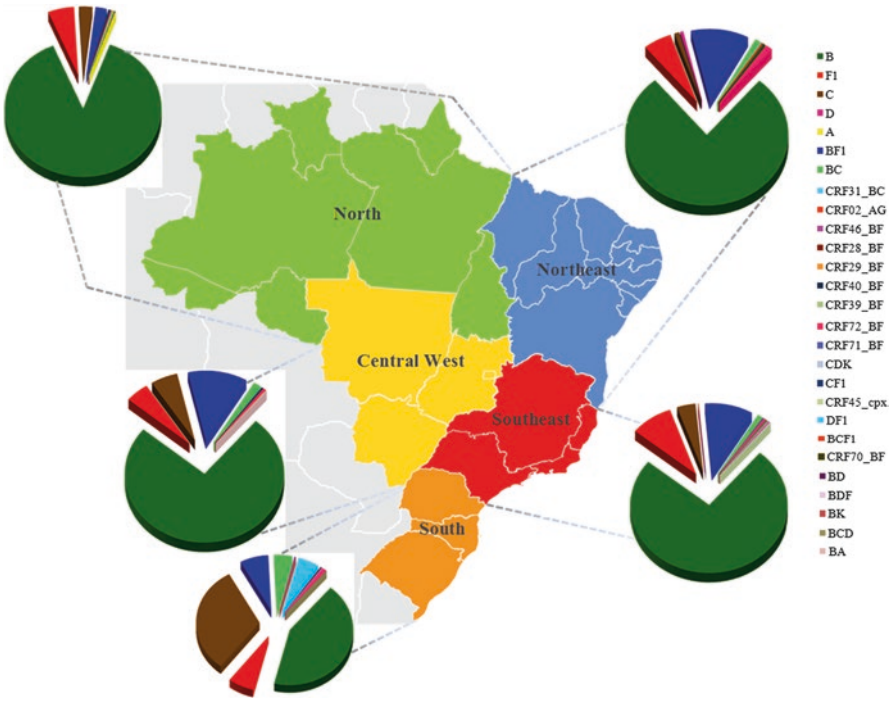

Fig. 4.3 General distribution of HIV-1 subtypes and recombinants in the five geopolitical regions of Brazil. Briefly, the subtype profile was determined by pooling data from molecular epidemiological surveys published for the last 15 years, and the resulting aggregates in each region were used to determine the overall proportions of HIV-1 subtypes and recombinants [90–113]

prevalence of subclade F1 (26 [24%] of 110) and only one case of BF1 recombinant among blood donors in this region. These findings contrast with those from the previous studies of HIV-1 NFLGs in Brazil [42–46]. Resequencing of the HIV-1 viruses from the same samples was performed to determine whether the classification of these strains extends to the NFLG and partial larger fragment [39]. The results revealed 23 of the 24 donors in whom genotyping was successful were infected with HIV-1 BF1 recombinant variants including CRF70 and 71 BF1 strains. The same study estimated the prevalence of BF1 recombinant variants at 20.4% and CRF71_BF1 at 10.2% (11/108) of HIV-1 strains circulating among blood donors in the northeast region. Again, this study showed a surprisingly low prevalence rate of 0.9% of F1 viruses, suggesting that, in previous studies, the occurrence of subclade F1 was overestimated due to partial genome sequence data. The apparently low prevalence of subclade F1 is ecological and may not be due to inherent properties of the virus itself but rather to the chance results of subtype B (a founder virus in Brazil), where it was introduced and consequently established into our HIV-1 infected population before the other subtypes were introduced [45].

4.2.3 Center-West Region

The center-west is the second largest region of Brazil by area, but in terms of population it is the least populated with 14,400,000 inhabitants. According to the current data provided by the Brazilian Ministry of Health, the reported number of HIV-1/AIDS cases by the end of 2014 was 44,112, with 1161 newly diagnosed cases during that year (http://www.aids.gov.br). In 2014, the estimated incidence rate of HIV-1 infection was 81 per million. The data from 11 studies published between 2000 and 2013 in this region showed that subtypes B and BF1 recombinants account for more than 87% of the viral subtypes circulating in this region (Fig. 4.3) [47–57]. Almost an equal prevalence was detected for subtype C (5.5%) and F1 (4.8%). Other subtypes including D, BC, CF1, BCF1, and DF1 recombinants have also been found in this region but at a low prevalence. Cardoso et al. [47] assessed the molecular diversity of HIV-1 *env*, *gag*, *protease*, and *reverse transcriptase* from 77 HIV-1 infected pregnant women. The results indicated that 66.2% of the isolates were subtype B, 6.5% subclade F1, and 3.9% and approximately 25% B and F1 virus subtypes.

4.2.4 Southeast Region

The southeast is the vital center of the country, where the largest cities and the highest population density are concentrated. It is estimated that more than 70 different nationalities compose the southeastern population of 84,400,000. According to the data from the Brazilian Ministry of Health, a cumulative total of 411,800 cases of HIV/AIDS were reported from January 1980 to 2014 (http://www.aids.gov.br) resulting in an incidence rate of approximately 68 per million of population in 2014. Of note, most studies conducted to assess the diversity of HIV-1 in Brazil have been performed using samples from the southeastern region, mainly from the states of Rio de Janeiro and São Paulo. Twenty-six studies and other HIV-1 NFLG diversity from unpublished survey (Pessoa et al. 2016) reported a circulation of five genuine subtypes (B, F1, C, D, and A), ten CRFs (CRF28_BF, CRF29_BF, CRF31_BC, CRF39_BF, CRF40_BF, CRF71_BF, CRF72_BF, CRF45_cpx), and six URFs composed with subtype B, F1, C, D, and K [42–45, 58–78].

The overall distribution indicates a high prevalence of subtype B (74.8%), followed by URF BF1 (9.8%), and subclade F1 (8.7%). Subtype C and BC recombinants represent 3.8% and 1% of the HIV-1 viruses circulating in this region, respectively.

The recent characterization of 233 HIV-1 NFLGs from infected blood donors in this region revealed the circulation of 13 different HIV-1 subtypes, including four pure ones that are responsible for 73% of infections, five different URF variants responsible for 24%, and four CRF strains responsible for 3% of infections [39, 59, 74, 79] (Pessoa et al. 2016). The results from the same study indicated that 67.4%

of the isolates were of subtype B, 18.9% BF1 URF, 3.9% subtype C, and 0.8% non recombinant F1 subclade and D subtype each.

In another study of 77 NFLGs and 32 partial sequences from the recently HIV-1 infected Brazilian subjects in the state of São Paulo, the phylogenetic analysis showed that subtype B is largely dominant (79.8%), followed by BF1 URF [43]. All along, previous studies suggested a consistent and continual spread of subtype B and BF1 URF variants over time in this region [43, 80, 81]. This dynamic factor together with the elevated number of dual and super-infections in this region [79, 82] may lead to the complete disappearance of subclade F1. It has been shown that the heterogeneity within the global B subtype is equally reflected within the Brazilian subtype B viruses, which seemed to have had multiple HIV-1 introductions from North America [43].

Previous studies aimed at reconstructing the past population dynamics of subtype B in Brazil via the coalescent theory have indicated that the epidemic growth of this clade started in the late 1960s, when it grew exponentially over the first two decades [83]. The element that initially contributed to and fueled the exponential growth of subtype B viruses in this region may have been primarily the transmission from acutely infected men who had sex with men and intravenous drug users [84, 85]. Based on the revision of the data from the previous studies (depicted in Fig. 4.3), it seems that there is a lack of substantial spread of subtype C and its recombinants in the southeast region. It is possible that the high prevalence of subtype B viruses and other recombinant variants saturating the HIV-1 infected population coupled with effective behavior changes may be responsible for the differential spread of these strains.

4.2.5 South Region

The south region of Brazil is the smallest area of the country, but its population of 25,800,000 is twice as large as the number of inhabitants in the north and center-west regions. The states of Rio Grande do Sul, Santa Catarina, and Paraná compose the southern region, which shares borders with Uruguay, Argentina, and Paraguay, as well as with the Brazilian states of São Paulo in the southeast and Mato Grosso do Sul in the center-west region. The epidemiological data from this region indicates a progressive increase of the AIDS-related mortality since the beginning of the 2000s and is currently considered one of the Brazilian regions with the highest AIDS incidence reaching 131.5 per million of population in 2014. The cumulative number of HIV-1 notified cases by the end of 2014 was 151,495. The southern region is also characterized by a distinct subtype profile in that the spread of HIV-1 subtype B is matched by HIV-1 subtype C [86]. These features have attracted various studies to investigate the history and dynamics of subtype C in the country, which estimated an origin between the 1960s and the 1970s [87, 88].

Several studies either based on partial or NFLG sequencing showed subtype C from different regions in Brazil to form a tight monophyletic group, indicating a

founder effect [89, 90]. The results by pooling data from 24 molecular epidemiological surveys published between 2005 and 2015 indicate a cocirculation of 14 distinct subtypes consisting of 42.8% subtype B, 31.6% subtype C, 7.2% BF1 recombinants, 6.5% subclade F1, 5.5% CRF31_BC, 4.6% BC recombinants, and 1% subtype D, as shown in Fig. 4.3.

4.3 Conclusions

This review of HIV-1 subtype diversity showed an unprecedented diverse picture of the subtype distribution in Brazil, indicating that the epidemic in this country is old and mature. Evidently, the circulation of diverse subtypes and the evolution of new recombinants with different and complex mosaics will continue. Thus, there is need for regular investigation and information updates to assist the concerned organizations in the country to plan for effective preventive measures.

Conflict of interest The authors report no conflicts of interest.

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Chapter 5
HIV and SIV Evolution

Brian T. Foley

Keywords Immunodeficiency • Evolution • Selection • Phylogenetics • Epidemiology • AIDS

Core Message
Human immunodeficiency virus type 1, the virus responsible for the AIDS pandemic, is just one of a large group of primate lentiviruses. Although many aspects of the evolution of these viruses is known in great detail, other aspects remain shrouded in mystery due to large timescales and a lack of fossil records. Retroviruses in general are not primarily noted for their neurological effects, but a great many, including the lentiviruses, cause neurological problems in at least a subset of infected hosts. The lentiviruses are primarily noted as the cause of immunodeficiency, but they also cause neurological damage. Despite the vast genetic distances between the groups of primate lentiviruses, many aspects of their biology remain remarkably similar.

5.1 Introduction

The primate immunodeficiency viruses (PIVs) comprise a diverse group of lentiviruses, all derived from a single common ancestor, which infect old world monkeys and apes. Many, but not all, species of old world monkeys each carry their own lineage of PIV and have apparently coevolved together for as much as several million years. The evidence suggesting such an ancient origin includes the fact that some PIV lineages recapitulate their host evolution. For example, the African green monkeys (**family**, Cercopithecidae; **genus**, *Chlorocebus*; **species**, *Ch. aethiops*, *Ch.*

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cynosurus, *Ch. djamdjamensis*, *Ch. pygerythrus*, *Ch. sabaeus*, and *Ch. Tantalus*) are known to carry at least three lineages of PIV (SIV-sabaeus, SIV-tantalus, and SIV-vervet) which are more closely related to each other than to lineages of PIVs found in other primates. Likewise the chimpanzees and gorillas carry PIVs that are related to each other and different than those carried by other primates [1–3] (Fig. 5.1).

The origins and evolution of retroviruses are not yet known. Mammals and many other vertebrates carry dozens of endogenous retroviruses in their germ line DNA. Nearly all endogenous retroviruses have a simple LTR-*gag-pol-env*-LTR genome, whereas the T-cell leukemia viruses and the lentiviruses have accessory genes such as *tat, rev, vif*, and *nef*. Several mammal species have now been found with endogenous retroviruses that appear to be ancestral to the lentiviruses, but they all lack most of the accessory genes [4]. Thus, the origins of lentiviruses are also somewhat unknown, but the evidence suggests that they have been infecting mammals for more than 20 million years [4–6]. In addition to primates being infected with primate immunodeficiency viruses, the felines, bovines, equines, ovines, and caprines each have lentiviruses which also infect T cells and cause immunodeficiencies and other pathologies.

Retroviruses in general tend to be quite host specific, and the lentiviruses are typical in this regard. Most primate lentiviruses cannot replicate in human cells primarily due to the host APOBEC enzymes but also due to other innate host defense mechanisms

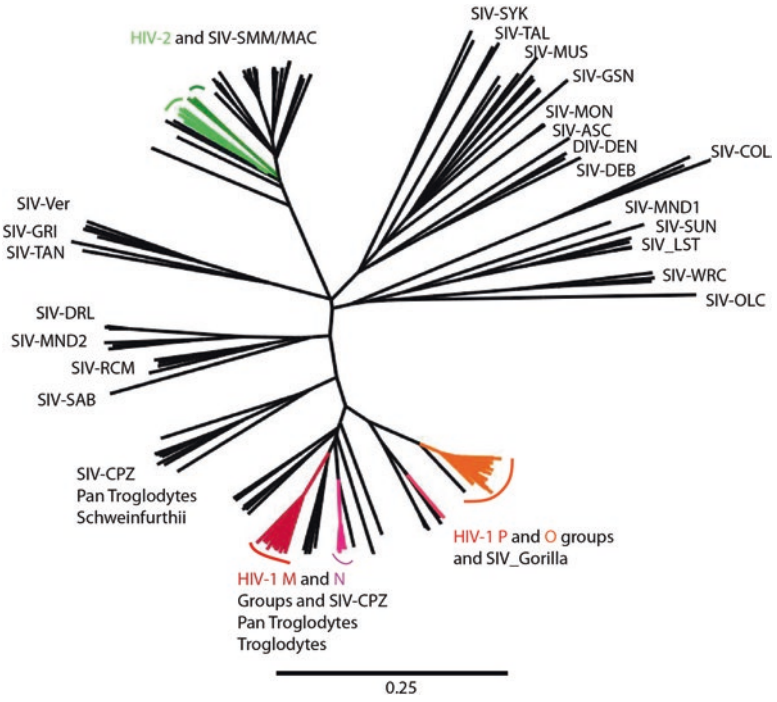

Fig. 5.1 Phylogenetic tree of primate lentiviruses. Phylogenetic tree constructed by maximum likelihood method using complete genomes of primate lentiviruses. Viruses infecting and spreading in humans are colored, shades of red and orange for HIV-1 viruses derived from chimpanzees and gorillas, and shades of green for HIV-2 viruses derived from sooty mangabeys. Several other viruses were transferred from sooty mangabeys to humans but have not been noted to spread in humans

[7, 8]. Other factors above the cellular level also influence the relationship of a virus to a particular host species or geographical location. One factor is the frequency of interaction between infected individuals of one species with other species. It is quite likely that increased hunting of chimpanzees and gorillas with modern weapons lead to transfers of simian immunodeficiency viruses (SIVcpz and SIVgor) from these species into humans. Another possibility is that humans have been exposed to these SIVs many times in the past, but it took higher human population densities and/or blood exposures via transfusions and needle reuse to jump-start the human epidemic [9, 10]. Iatrogenic transmission of HIV has been greatly reduced in the developed world, but unsafe medical practices continue in some developing nations [11].

After a cross-species transfer event takes place, such as transfer from chimpanzee into humans, the virus evolves from the single point source of the transfer into lineages that diversify from that single common ancestral virus to form what is known as a "star phylogeny" except when multiple viruses infect any one individual and then recombine. Inter-lineage recombination creates a network or web relationship between lineages rather than a perfect star or tree. Within any one infected individual, recombination within the viral swarm or population is a highly frequent occurrence because each virion packages two ssRNA viral genomes and the reverse transcriptase skips between the two genomes when synthesizing the first strand complementary DNA. There is some, but not a complete, blockage of multiple infections of a single cell with more than one virion, but many of the billions of infected cells in an individual will be multiply infected. The vast majority of humans infected sexually are singly infected from a single-donor sex partner. In IV drug user communities, multiple infections from more than one donor are common. Within a single community, whether sexual or IV drug user, it is more common for a set of highly related viruses within a single subtype than for multiple subtypes of virus to be circulating (Fig. 5.2).

Selection pressure on virions within each infected individual is quite extreme. Both CTL-mediated and antibody (B-cell-mediated) immune responses vastly reduce the viral load from as high as the tens of millions of virions in the first weeks of infection to a "set point," a typical reduction four orders of magnitude lower than the peak. That is, 99.99% of virions are being removed, and only 0.01% survives to reproduce in each round of replication. This can result in nearly complete replacement of one population, be it one that is sensitive to attack of a single CTL epitope or one that is sensitive to a drug, by a mutant-resistant population, in a matter of weeks [12, 13]. The host immune system attacks many virus epitopes at once, some CTL and some antibody, and also evolves over time to become more efficient at attacking the virus. Drugs on the other hand can only target one viral factor per drug and do not evolve over time. Using multiple drugs simultaneously is thus critical to success. Combination antiretroviral therapy (cART) goes by various names such as highly active retroviral therapy (HAART). The most common combinations include drugs that target two sites in the reverse transcriptase enzyme and one target in the protease enzyme.

Selection pressure driving the virus to change over time such as to evade the host immune system or to evade (become resistant to) drugs is known as positive selection. The other major selection pressure on the virus is for the virus to be "highly fit" in terms of replication rate within each individual and in terms of being transmissible to other individuals.

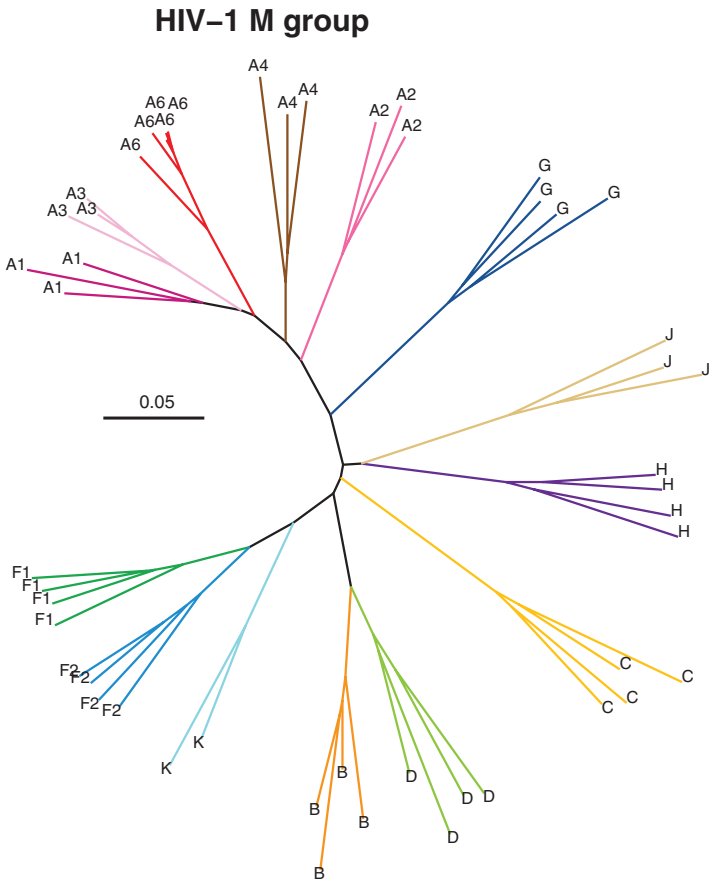

Fig. 5.2 HIV-1 M group phylogenetic tree. Phylogenetic tree constructed by maximum likelihood method using complete genomes of HIV-1 M group major subtypes. CRF26_AU is included because the authors defined the A-like regions as subsubtype A5. It is noteworthy that subtypes B and D are related to each other, and recent standards of nomenclature and classification would be subsubtypes (as are F1–F2, A1–A6) rather than full subtypes, but they were classified very early in the study of HIV-1 as subtypes

5.2 Mutations

5.2.1 Bias

Retroviruses are observed to evolve at a rate nearly ten million-fold faster than mammalian nuclear genes. The exact rate of evolution depends on the region of the genome, with the envelope gene evolving faster than the polymerase gene, for example, due to differences in selection pressures. Moreover, the rate of evolution

is a function of both the mutation rate and the selection pressures on the genes under study. It is generally accepted that mutations occur at random and selection creates differences in the observed patterns of change over time [14], but there are several biases in the mutation step of the process as well. Transition mutations (A <-> G and C <-> T) occur much more frequently than transversion mutations (A <-> T, A <-> C, G <-> T, and G <-> C) for a number of reasons, and for retroviruses, the G to A mutation rate is far higher than any other rate including A to G. Also, the context of bases surrounding a given base can influence the mutation rate. One well-known example is that C followed by G is prone to mutation to T because of DNA methyltransferases. Methylcytosine can deaminate to uracil, which then pairs with adenosine so that the cytosine is replaced by thymidine in the next round of replication. Most mammalian DNA is depleted of CpG dinucleotides except for regions known as CpG islands [15], and many viruses also have a lack of CpG.

With human infections we can almost never know the exact sequence of the infecting virus, and we therefore always observe changes over time that are at least partially influenced by selection as well as the underlying mutations. In experimental infections of macaques, a single infectious molecular clone with a known genome sequence can be used, but even in this case, lethal mutations are eliminated, and with sampling over time, we observe evolution that is influenced by selection. Other ways to study the fidelity of HIV reverse transcriptase and thus mutations without selection include using single-round replication vectors in vitro or in cell cultures and sequencing cDNA made from RNA templates of known sequence after a single round of reverse transcription.

The base composition of lentiviruses is A-rich and C-poor with the A:C ratio close to 2:1. The base composition bias is fairly uniform across the genome and even more uniform between different viruses. The same regions that are less A-rich in one primate virus are also less A-rich in others. The result of this is that many so-called “silent” sites in the genome, third codon positions where any of the four bases can be used to encode the same amino acid when translated, appear to be under strong purifying selection. Many phylogenetic analysis programs can be set to assume that silent sites are neutral and freer to change than nonsilent sites in protein coding regions.

For most purposes, there is no need to separate mutation rate from evolution rate, and in fact it is counterproductive to do so when the observed virus sequences are the product of both mutation and selection over time. Likewise, selection processes vary slightly from one patient to another or even within a single patient over time, but the average behavior over many sites in the genome and over larger timescales can be remarkably consistent. Conversely the study of very small regions of the genome and/or using very small or not well-chosen data sets (not randomly selected from the population under study) can result in very poor estimations of rates and/or patterns of evolution [16–19].

5.2.2 Hypermutation

A large source of mutation in retroviruses is the activity of APOBEC enzymes. The APOBEC enzymes recognize DNA/RNA heteroduplex molecules in the cytoplasm and deaminate cytidines in the RNA strand. The result is that many stop codons are introduced into the genes, and functional proteins can no longer be produced by the mutated viral genome [8, 20]. Because the retrovirus brings some reverse transcriptase and integrase protein into the cell, the mutated nonfunctional provirus can be integrated into the host genome, and it is not uncommon to find hypermutated viral sequences when proviral DNA is amplified and sequenced. Although dead or nonfunctional genomes cannot continue to replicate and evolve, each one is just one replication round away from its parental virus and so can contribute valid information about the host virus population.

5.2.3 Insertions/Deletions

Duplications of motifs such as NK-kappa-B binding site, PSAPP/PTAPP motif in gag. Duplications of nearby DNA RNY in env. Length of Env loops. Vpr/Vpx duplication.

In addition to point mutations, insertions and deletions contribute to the evolution of DNA. Insertions and deletions are usually not counted in phylogenetic analyses of evolution because there are many different types of insertions and deletion events, and it is not possible to directly compare them to rates of single base mutations. One common type of insertion and deletion event is known as variable numbers of tandem repeats, where a simple repetitive element increases or decreases in copy number. Gene duplication events are also relatively common in most organisms, but in retroviruses there are constraints on genome size, which would limit the viability of most such events. One gene duplication event in the primate lentiviruses is hypothesized to have created the Vpx plus Vpr gene pair in some lineages, while other lineages have Vpr plus Vpu genes [21, 22].

5.3 Selection

There are many different types of selection forces acting on viral genome sequences, usually with overlapping and either conflicting or supporting roles. The most obvious selection forces on protein coding regions of the virus are conserving the amino acid codons needed for a given protein function and changing the surface of the envelope protein to evade the host antibody immune responses [23].

5.3.1 Positive by CTL

Cytotoxic T lymphocytes possess HLA (system?) that cleaves viral proteins into primarily 9-mer peptides which are then presented on the cell surface of infected cells by xxx and recognized by killer T cells. Viral protein cleavage is not random, but dozens of 9-mers per viral protein can be presented by the average infected cell. Not all epitopes are equally effective at eliminating virus-infected cells, so there are dominant epitopes responsible for the majority of viral reduction plus many more weak epitopes that are much less effective. Different human HLA genotypes tend to target different viral epitopes such that the dominant epitope in one individual is not dominant in most others.

5.3.2 Positive by Antibody

Infected individuals produce antibody responses to nearly all viral proteins, with a general trend in the order of appearance of strong antibody responses appearing in the first weeks of infection. Most antibodies do not neutralize the virus and have little impact on viral load and thus have little impact on viral evolution. Neutralizing antibodies, which bind to the viral envelope glycoprotein, prevent the virus from binding to cell surface receptors on uninfected cells (CD4 and either CCR5 or CXCR4 coreceptor). Neutralizing antibodies are often highly specific for only a single lineage of virus. In relatively rare cases, a single antibody can bind to and neutralize a wide variety of lineages, and these are called broadly neutralizing antibodies (BNabs).

Elimination of virions that are bound by neutralizing antibodies drives the evolution of escape mutants. The most common mechanism of escape is addition and/or subtraction of glycosylation sites, which are highly prevalent and variable on the surface of the envelope glycoprotein. The glycosylation of envelope is referred to as "the glycan shield" [24].

5.3.3 Positive by Drug

In contrast to host immune responses, which are variable between infected individuals and even variable over time within each individual, an antiretroviral drug is always the same and thus exerts the same selection pressure in all individuals who take the drug. Detection of immune system escape mutations is complex and requires many viral sequences and immune reaction tests for each individual. Detection of drug resistance mutations can be done with as little as one viral sequence per individual and rather simple tests for viral replication rate in the presence or absence of each drug.

For one example, the change of wild-type methionine to valine at amino acid 184 (M184V) in the reverse transcriptase protein results in nearly complete resistance of the virus to azidothymidine (AZT) and some related nucleoside analog reverse transcriptase inhibitors. The M184V mutation also partially cripples the reverse transcriptase enzyme such that although the virus is resistant to any level of AZT, it replicates slower than wild-type virus. Maintaining AZT therapy in the presence of M184V mutant virus can be of benefit to infected people because the drug still suppresses all of the wild-type viruses; thus viral load is reduced. Also, each drug resistance mutation first occurs in a single virion, so that populations of drug-resistant virions, which descend from that mutant, have low diversity and greater chance for control by the individual's immune responses.

A major concern with drug resistance mutations is the transmission of drug-resistant virus from one infected person to others such that whole populations of people are infected with drug-resistant viruses, and the effectiveness of treatment and especially the ability to prevent mother-to-child transmissions with simple drug regimens proven to be safe for fetuses and infants is reduced. Surveillance for transmitted drug resistance (TDR) is thus an important public health issue as well as being beneficial to individuals who will get a report of which drug combinations are most suitable for treating the particular virus they are infected with.

5.3.4 Negative by Replication Rate

Although Darwin's theory of evolution is most often said to be "survival of the fittest," it is actually more accurately described as "elimination of the weakest" for most living things. It is not typically the single most fit individual that survives each generation and huge advancements in fitness are rarely realized in a single round of replication. Highly unfit or lethal mutations on the other hand occur very frequently and are eliminated from the gene pool nearly instantly. The dominant population of any group of organisms is generally referred to as "wild type," and the vast majority of mutants are observed to be less fit than wild type. When nonlethal but slightly less fit mutants arise, they can be eliminated from the population over time either by being outcompeted by wild-type individuals or by reverting to wild type. They can persist in the population by acquiring compensatory mutations, which allow them to regain fitness, or by spreading into habitats where they are no longer in competition with wild-type individuals.

Nearly all organisms have many stages in their life cycles where changes in fitness can influence the longer-term evolution of the population or species as a whole. Although a fast replication rate will benefit a lineage of virus within an individual in the short term, killing the host or making the host too sick to interact with potential new hosts to pass on the infection leads to an evolutionary dead end. Replication rate within an individual can thus be in conflict with overall epidemic growth rate. The lentiviruses require close physical contact for host to host spread, and tend to have long asymptomatic periods during which an infected individual can pass the

virus on to others. The term "lenti" in *Lentivirus* is Latin for slow, and these viruses were named long before the primate immunodeficiency viruses were discovered [25, 26].

One important factor in controlling HIV viral replication rate seems to be the very poor codon use in the viral genome. The genetic code is redundant, with 61 codons (plus 3 stop codons) for 20 amino acids, and different organisms have different levels of transfer RNAs matching the various synonymous codons. The lentiviral genomes have a very high frequency of codons that are read by low-level tRNAs in mammals. When synthetic DNA encoding HIV proteins but with high-level "fast" mammalian codon use is transfected into mammalian cells, protein production can be nearly 200-fold higher than transfection with DNA using the lentiviral codons.

5.3.5 Negative by Infectivity

To be passed from cell to cell within an individual and to be transmitted between host individuals, a virus must bind to cell surface receptors and maintain an ability to penetrate the host cell membrane. Although the virus evolves rapidly, host cell receptors are nearly invariant within any mammalian species. For the lentiviruses, the CD4 T-cell receptor, which is also expressed on macrophages, dendritic cells, and several other cell types, is the primary host cell receptor. The CD4 binding site on the envelope protein is thus quite highly conserved (Fig. 5.3).

5.3.6 Negative by Codon Use Bias

Codon use bias is a conundrum in HIV-1 M group and most other lentiviruses. The synonymous codons selected for use by the virus are very slow for translating the viral messenger RNA to protein in human cells. In the study of protein production levels, changing the codons from native HIV-1 sequences to codons optimized for mammalian expression, results in as much as a 200-fold increase in the level of protein produced [28, 29]. Lentiviral genomes are nearly uniformly A-rich and C-poor with the A:C ratio in any given region of the genome close to 2:1.

5.3.7 3.8 Negative by RNA Secondary Structure

In addition to encoding all of the proteins needed for viral replication and defeat of host immune defense mechanisms, the virus must also provide mechanisms of packaging viral genomes into virions and regulating gene expression levels and

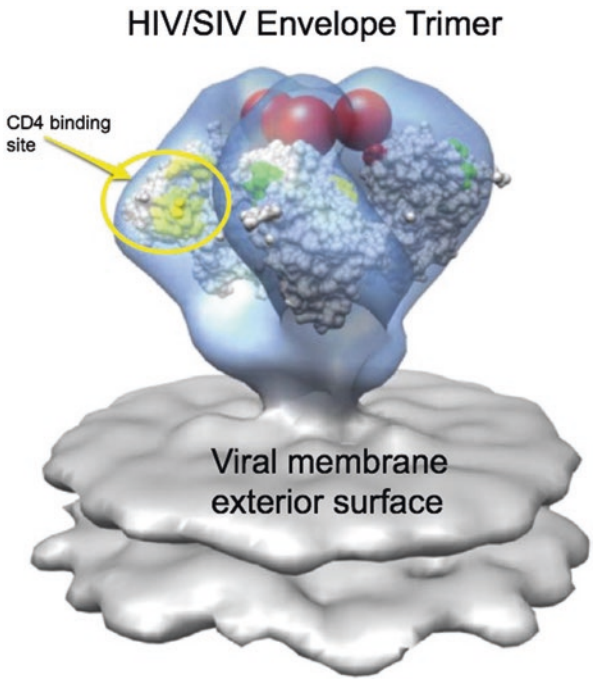

Fig. 5.3 Three-dimensional rendering of HIV/SIV envelope protein structure. Red balls illustrate the V1–V2 hypervariable loop region, green highlights the V3 loop region, and yellow highlights the relatively conserved CD4 cell surface receptor binding site (The figure is adapted from Ref. [27])

timing, and several other functions, which are known to involve self-complementarity and RNA secondary and tertiary structures [30, 31] (Fig. 5.4).

5.4 Recombination

Retroviruses package two copies of the viral RNA in each virion. During reverse transcription, the viral reverse transcriptase enzyme can switch between the two template RNA molecules and thus produce a complementary DNA that is partially derived from each of the two template genomes which were packaged in the virion. Although many or most cells are infected with only one virion such that the progeny viral genomes being packaged are identical to each other, multiple infections of a single cell are common and result in progeny virions with two different genomes packaged. Dual infection of a single individual with two different subtypes of HIV-1 can thus result in intersubtype recombination (Fig. 5.5).

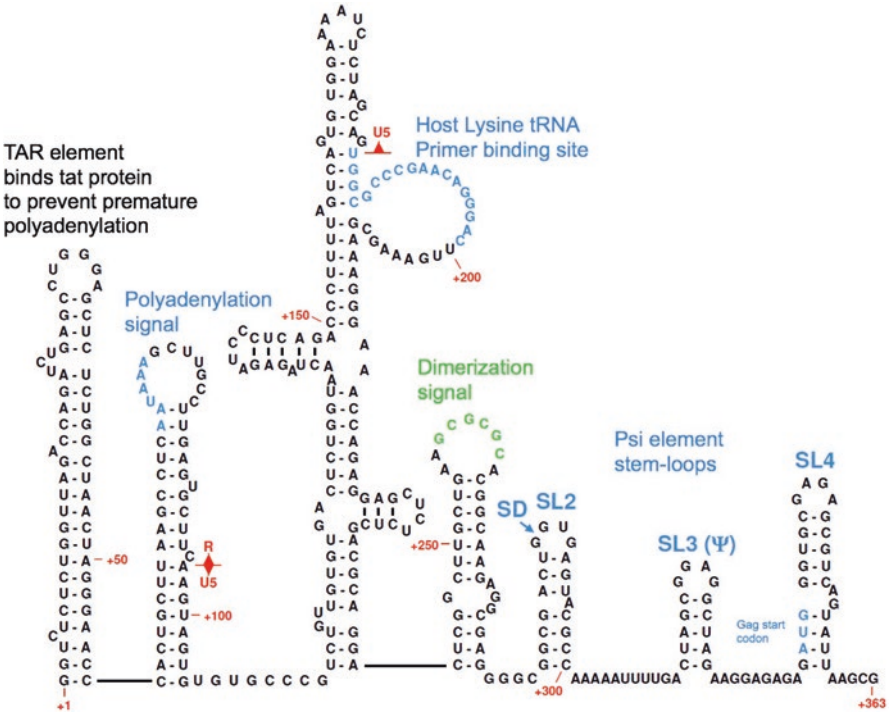

Fig. 5.4 Viral RNA secondary structure stem-loops in the 5′ long terminal repeat (LTR)

5.4.1 Recombination Within an Individual Virus Lineage

The vast majority of HIV-1 infections are derived from a single virus. This is known as the transmission bottleneck. It remains unproven whether the transmission bottleneck is the result of a single virion crossing the mucosal surface at the time of transmission or alternatively whether several viruses typically are transmitted but one of them rapidly outgrows the others in the initial days of infection. Multiple infections with more than one strain of virus are somewhat rare, but in populations of people at high risk of infection, such as commercial sex workers and IV drug users, it is not unusual to find people infected with more than one strain. If a person is infected with a second virus before seroconversion to the first virus has taken place, it is termed a dual infection. Infection with a second strain after seroconversion is known as superinfection.

Within an individual that was infected with only one virus, recombination happens, but the recombinants are derived from two nearly identical template genomes such that detecting the recombination events is often impossible. However, after many years of infection, the viruses within an individual have acquired some diversity, and it then becomes possible to detect the recombinant genomes [33, 34]. Although intrapatient recombination in a singly infected individual does not have

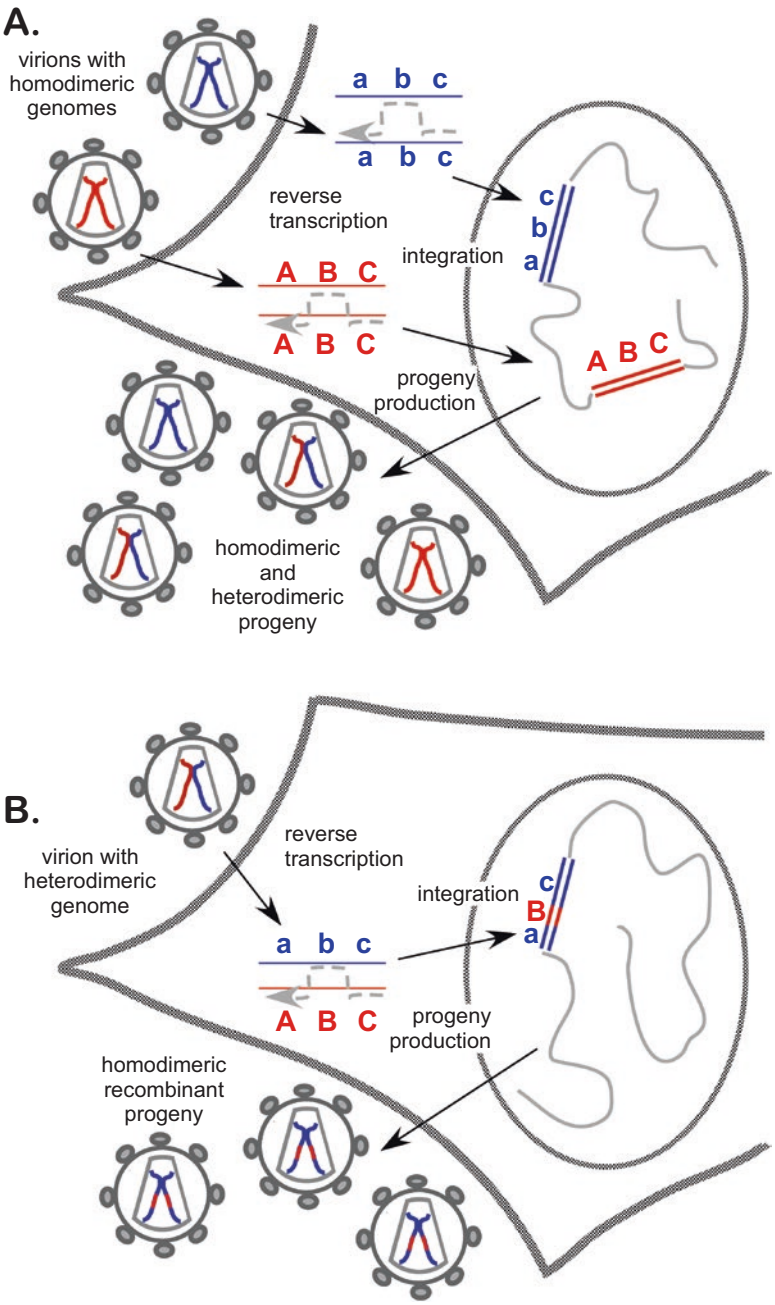

Fig. 5.5 Generation of recombinant genomes. (**a**) Coinfection of a single cell with two genetically distinct viruses can, if both proviruses are activated to production at the same time, lead to production of some virions containing one genome from each, depicted as red and blue here. (**b**) The recombinant genomes are generated during reverse transcription in a cell infected with a virion containing two different genome templates. The recombinant genomes are generated by template switching during the reverse transcription and do not require any nucleic acid strand breakage or repair (Artwork kindly provided by Alice Telesnitsky [32])

any significant impact on virus evolution for vaccine design or phylogenetic analyses of the overall epidemic, it does have large impacts on the ability of the virus to escape numerous selection pressures. For example, recombination between a virion with a drug resistance mutation in the pol gene and another virion with a mutation allowing it to escape immune selection in the env gene can produce progeny viruses that are resistant to both of these selection pressures.

5.4.2 Intrasubtype Recombination

Virus recombination is typically only noted when the two participating viruses are of different genetic subtypes. Intrasubtype recombination is of course far more frequent, but it is usually difficult to detect and so commonplace that it is usually not of interest to report on it. Dual infections and superinfections with the same subtype of virus but from a different donor are more common than infection with multiple subtypes, because in most communities in the world, only one subtype of the virus is present [34, 35]. Intrasubtype recombination has little or no impact on epidemiology, vaccine design, and many other aspects of HIV biology, but it can be a driving influence for recombining different regions of the viral genome carrying different selective advantages within an individual such as antibody escape mutations in the envelope gene and CTL escape mutations in the *gag* gene [33].

5.4.3 Intersubtype Recombination

Multiple infections with different subtypes soon result in intersubtype recombinant viruses. Although in theory recombination could happen often enough within such an individual to soon generate scrambled genomes with only very short regions derived from each of the parental viruses, in practice the observed intersubtype recombinants are not scrambled and usually have fewer than ten recombination breakpoints [36, 37]. It is possible that genetic distance between the subtypes has resulted in genomes that are not fully compatible with each other, and thus not all recombinants are equally fit [38]. This would prevent many intersubtype recombinant virions from thriving in an individual and prevent the scrambling of genomes over time. It is also possible that recombination just does not happen as often as theory would predict [39].

5.5 Phylogenetic Reconstruction

Phylogenetics is the study of the evolutionary relationships between organisms. Although the evolutionary histories of many plants and animals can be accurately inferred by analyzing phenotypic traits such as leaf structures or wing and beak shapes, the use of DNA sequences is much more common today and more accurate [3, 40]. Reconstructing the evolutionary tree or ancestral history of viruses has many uses, and there are many data sets available in which the biology is very well known such that the theory and practice can be tested [16, 41, 42].

5.5.1 Data Set Choices

Several factors influence the types of information that can be gained by phylogenetic analysis of virus genetic sequences. When analyzing the evolution of the viruses within a single patient, the most common type of sample is a blood sample, but the viruses present in the blood at any one moment in time may not be an accurate representation of the viruses present in lymph nodes, central nervous system, or other sites in the body. Several studies have attempted to assess the degree to which different sites in the body tend to host their own sublineages of virus, which is known as compartmentalization [43–46]. Within a population of people, it is similarly difficult to obtain a truly random sample of infected people. In addition to the choice of biological samples to use, there are choices to be made about which region(s) of the genome to sequence and how many sequences will be needed to obtain the desired statistical power.

In all cases, some compromise must be reached between the theoretically ideal data set and the data that can actually be obtained given biological, ethical, funding, and other constraints. In many cases it is possible to enhance the statistical power of a given study by supplementing the new data from a given study with data obtained by other studies and available in the genetic databases such as GenBank and the HIV Databases at the Los Alamos National Laboratory [47, 48].

5.5.2 Method and Model Choices

The simplest model of evolution assumes that all DNA base changes are equally likely and that there is no selection pressure or other influence on the rate of evolution of different sites in each gene. The Kimura two-parameter model adds just one factor, stating that transitions and transversions have different rates [49]. More complex models can evaluate a different rate for each base change and also allow each site in a gene (column in a multiple sequence alignment) to have a different rate of evolution. There are programs such as ModelTest and PartitionFinder to assist in the

rational decision of which model of evolution to use on a given data set [50, 51]. As the size of the data set grows, the computational resources needed to perform the most complex analyses increase factorially with the number of sequences and linearly with the length of the sequences. Although it is impossible to compute the absolutely correct or best tree from large data sets, there are heuristics employed to greatly reduce the number of computations needed to arrive at a very reasonable result. Poor choice of samples, sequencing errors, and other problems with the input data sets are far more often the cause of serious problems than suboptimal choices of computational methods.

5.5.3 Recombination Detection

Phylogenetic reconstruction of evolutionary history in general assumes that the sequences being analyzed are not recombinant and that each sequence has one history. Although in practice HIV does undergo recombination, the scope of the recombination is limited. The viruses in one infected individual are not recombining with viruses in any other infected individual. Recombination can confound or invalidate phylogenetic analyses, but it is not always a problem, and there are many methods available for detecting recombination [34, 40, 52].

5.5.4 Alignment

Almost any analysis of multiple sequences from the same organism requires that all of the sequences be aligned to one another in a multiple sequence alignment. Pairwise alignment of any two sequences, or of each sequence in a set to one reference sequence, is usually simple using the Smith-Waterman algorithm [53]. Aligning many sequences to each other, when each of the sequences has insertions and deletions relative to other sequences, becomes a much more difficult problem, but many programs have been written to automate the task [54]. The HIV Databases at Los Alamos National Laboratory have developed tools specifically designed for aligning HIV sequences, which take into account the multiple overlapping reading frames used by the virus [47, 55].

Obtaining a very good multiple sequence alignment often involves iterations of producing a multiple sequence alignment, analyzing the alignment by methods such as building a phylogenetic tree and using Simplot to look for uniform diversity between sequences, and then adjusting the alignment if the analyses indicated any region of the alignment or sequences in the alignment were aberrant.

5.6 Rates and Dates

5.6.1 Molecular Clock Tests

The gene sequences of viruses and other organisms change over time due to mutations and selection pressures. The consistency of the rate of change over time is known as the molecular clock hypothesis. Individual mutations happen in a stochastic manner with little predictability, but the sum of changes over large regions of the genome and longer timescales tends to be more uniform. Many factors including population sizes, selection pressures, generation times, and fidelity of replication influence the clock rate; thus most data sets do not show evidence of a strict molecular clock. However, given large data sets, the average behavior is clocklike enough to allow many inferences about the past history of populations such as effective population sizes and dates of divergence from a common ancestor [3, 40, 56].

5.6.2 Examples

Very early in the study of the HIV/AIDS pandemic, it was noted that there was a great diversity between HIV isolates in comparison to the diversity observed in most other viruses [57, 58]. The first estimates to estimate the rates of evolution and to use the rate to date the origin of the pandemic were hampered by small sample sizes and by missing information about the natural history of the primate lentiviruses. Very good estimates have now been made by many groups, using independent methods and sample collections, with very high levels of agreement between them [3, 9, 59–62].

Within the HIV-1 M group, many studies have analyzed the growth of subsets of the AIDS pandemic using sequences from viruses collected over time in various parts of the world. Several studies, for example, have attempted to pinpoint the time and location of the beginning of the HIV-1 subtype B epidemic in the USA [63–65]. Molecular clock analyses of HIV-1 subtype B in the USA and Europe agree that the date of the common origin of subtype B was between 1960 and 1970, and many papers speculate that HIV was incubating in the USA for nearly 20 years before being detected in 1981. However, it is also possible that the subtype B viruses were evolving in other parts of the world, and then multiple introductions of HIV-1 subtype B entered the USA in the late 1970s and early 1980s [66]. Analyses of virus sequences can provide accurate information on the date of the common ancestor of the viruses, but this does not provide information on the geographic location of the ancestor.

5.6.3 *Saturation Effects*

The DNA bases thymine and cytosine are pyrimidines with one ring, while guanine and adenine are purines with two rings. Because of the size difference and other factors, substitutions of one base for another do not all happen equally. Transitions far outnumber transversions, with the lentiviruses being especially prone to G to A transitions. Rather than simply counting all point differences between sequences equally, models of evolution can calculate different rates for different types of mutations and attempt to correct for multiple mutations at a given site. One method of

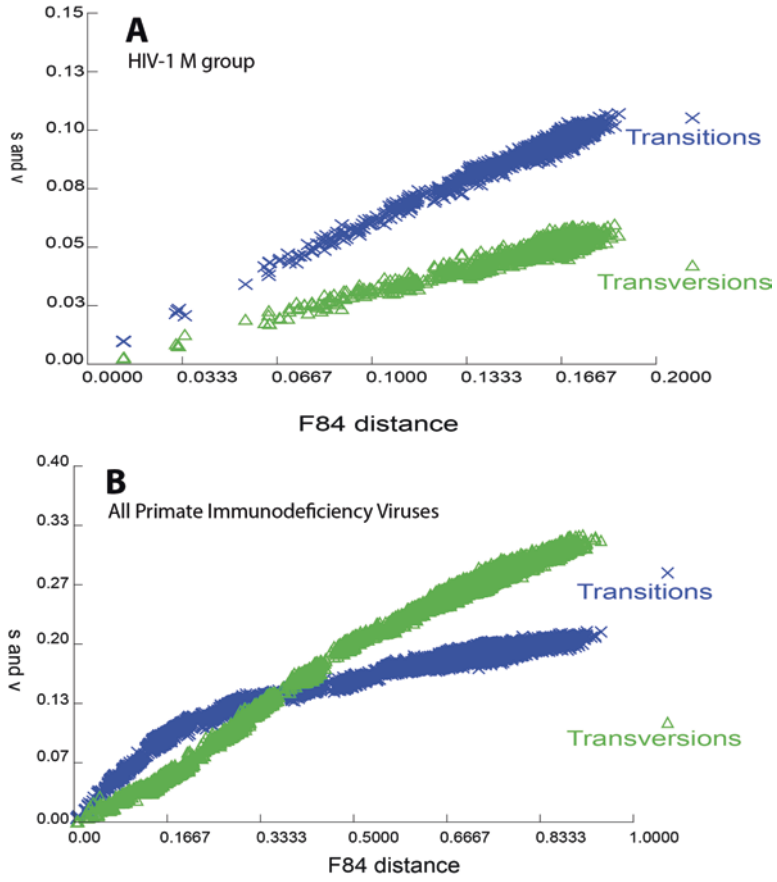

Fig. 5.6 Saturation of sequences, multiple hits per site. Transitions (G <-> A and C <-> T) outnumber transversions (A <-> C, A <-> T, G <-> C, and G <-> T) when distances are relatively small, but as mutations accumulate such that more variable sites have mutated more than once, saturation is reached, and computation of the phylogenetic or molecular clock time distance from the observed distance becomes difficult to impossible. (**a**) complete genomes of HIV-1 M group viruses were analyzed. (**b**) the complete genomes of all primate immunodeficiency viruses were analyzed

testing for saturation of mutable sites is to calculate the transition to transversion ratios of all pairs of sequences in the data set. The DAMBE phylogenetic analysis package [40] provides a tool for producing a graphical plot of transitions and transversions versus pairwise distances. Figure 5.6 shows the results of analyzing the data sets used to make Figs. 5.1 and 5.2.

Most phylogenetic tree building programs also calculate a matrix of substitutions observed in the data and have an option for outputting the full matrix. However, this matrix is an average over all comparisons and will not show whether or not saturation is observed in the data.

Conflict of interest The authors report no conflicts of interest.

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Chapter 6
Human Brain Imaging in HIV and NeuroAIDS

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Keywords HIV • NeuroAIDS • HAND • Brain • Imaging • MRI • MRS • DTI • PET • Biomarkers

Core Imaging Modalities
Structural changes in HIV
fMRI and brain connectivity in HIV
MRS at work: a lens of brain metabolites changes in HIV
DTI: white matter fibers integrity in HIV
Imaging cerebral blood flow in HIV
PET and SPECT in HIV

6.1 Introduction

More than 25 years have passed since the description of neurological dysfunction, HIV-associated dementia. A plethora of research done before and after the era of combined antiretroviral therapy (cART) focused on the role of immune responses and their neurotoxic effects on cognition in HIV infection. However, more work is still ahead of us to explain unanswered questions regarding the pathogenesis of NeuroAIDS.

HIV infection usually progresses to AIDS. Barely a few percent of infected patients can control the infection without treatment due to a diminishing T cell response during infection. cART suppresses viremia; however, chronic activation of

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the immune system is the major factor behind the progression of the disease and not the HIV replication [1].

Postmortem brain tissue studies suggest trafficking of bone marrow-derived monocytes into the deep white matter during late stage of infection [2].

The pathogenesis of AIDS dementia complex is still not well understood. Once HIV-infected monocytes and macrophages cross the blood–brain barrier, mediators and neurotoxins are released with development of neurological dysfunction. Brain macrophages, neurons, astrocytes, and glial cells act as chronic HIV reservoirs [3].

Neurocognitive deficits in motor, reaction time (speed), visuospatial reconstruction, memory encoding, attention, and executive functions are common symptoms in NeuroAIDS. Ruling out other causes of dementia can be challenging to the clinician as there are no clinical markers for HIV-associated cognitive impairment. Neurocognitive deficits are measured by neuropsychological tests using either the Memorial Sloan Kettering (MSK) AIDS Dementia Scale [4–6] or more commonly today using the Frascatti criteria [7]. HAND diagnosis is based on neuropsychometric testing and includes asymptomatic neurocognitive impairment (ANI), mild neurocognitive impairment (MCI), and the most severe form, HIV-associated dementia (HAD) [7]. Correlation of neurocognitive tests with imaging markers is a common tool used to help understand the pathogenesis of NeuroAIDS.

Currently, there is significant reduction of HIV-associated brain disease, specifically HIV encephalitis and CNS opportunistic infections, due to effective combination antiretroviral therapy (cART) [8].

An emerging body of evidence from neuroimaging, clinical, and pathological studies suggests that despite remarkable advances in treatment, central nervous system dysfunction in the context of chronic and stable HIV persists despite successful systemic therapy [9].

Little was known about the anatomical and structural alteration caused by HIV infection. Currently, with advanced neuroimaging techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET), using different brain radioligands, the structural, functional, and metabolic brain changes in the brains of HIV patients are being unveiled. These alterations, described in the literature, although nonspecific, are considered new lenses, which help understanding the pathogenesis of NeuroAIDS. This technology ultimately helps monitor disease progression, treatment effect, and development of new means of therapy [10].

6.2 Brain Imaging

Brain imaging techniques include those which show brain structure (structural imaging) and those which show function or brain metabolism (functional imaging) of the regions of interest in normal and pathological conditions. Structural techniques include X-rays (including angiography), computed tomography (CT), and magnetic resonance imaging (MRI). Functional procedures include functional MRI (fMRI), magnetoencephalography (MEG), magnetic resonance spectroscopy (MRS), magnetic resonance spectroscopic imaging (MRSI), positron emission

tomography (PET), electroencephalography (EEG), and single-photon emission computed tomography (SPECT).

6.2.1 Structural Imaging

Although computed tomography (CT) involves ionizing radiation, it is still often used for many clinical HIV brain imaging studies, as it is more commonly available in hospitals, and it is less costly than magnetic resonance imaging (MRI). Besides being safe and nonionizing, MRI is a better imaging modality for anatomical and structural mapping, with great tissue resolution and contrast. The MR signal is from water and fat which are of different precession frequencies and have different T1 relaxation times. Chemical shift is the difference between water and fat magnetic moment or spin frequency of the atom or energy level (in Hz per pixel). Clinically, 1.5 and 3 Tesla (T) machines are used. High magnetic fields such as 7 T are used for research studies. MRI is based on nuclear magnetic resonance in which, by using head coils, radiofrequency pulses are sent to the examined organ. The protons from the various types of tissues (from water content to fat content with different relaxation times) will absorb radiofrequency and will then spin. When the radiofrequency pulse is turned off, relaxation of tissues at different rates will emit radio wave energy indicators that are captured as MRI signals.

Structural changes are sometimes better enhanced when chemical agents such as gadolinium are injected to improve tissue contrast. Using T1 images, findings include increased post-contrast enhancement on contrast-enhanced magnetic resonance imaging (CE-MRI), which can reflect breakdown of the microvascular integrity of the BBB in HIV neuronal inflammation [11]. This measure can be correlated with viral load and neurocognitive impairment and can predict progression of HIV-positive patients and the development of HIV-associated dementia [12].

Diffuse cortical and subcortical brain atrophy in HIV infection is well known even before the cART era [13]. Example of HIV brain atrophy is shown in Fig. 6.1.

Corresponding T2 and FLAIR sequence nonspecific hyperintense focal lesions may be seem in the frontal and parietal white matter, suggestive of gliosis [14, 15]. Voxel-based morphometry (VBM) is an MRI technique, which measures brain region volumes. VBM is associated with changes mainly frontal white matter, subcortical, and striatal regions including the basal ganglia. Structural MRI shows significant reduction in volume of the frontal white matter with dilation of the ventricles due to atrophy. This cortical atrophy is usually progressive even if the HIV patients are under treatment [16].

Although age-related GM atrophy is primarily seen in the superior temporal and inferior frontal regions, HIV-related GM loss includes the posterior and inferior temporal lobes, parietal lobes, and cerebellum. The interaction of age and HIV status has a significant effect on brain volume [17, 18].

New imaging techniques using multiatlas-based segmentation of multiple brain areas according to patient age group [19–22]. Example of automated multiatlas brain segmentation is shown in Fig. 6.2.

Immunodeficiency is a risk factor for opportunistic infections in NeuroAIDS. Using computerized tomography (CT) and MRI, especially in T1, T2, and FLAIR images, HIV-1 and AIDS-associated CNS pathologies are visualized. These neuropathologies include encephalitis, primary CNS lymphoma (enhancing parenchymal nodular lesions in the frontal lobe and basal ganglia with perilesional edema, and restricted diffusion [23]), cerebral toxoplasmosis (single or multiple focal brain lesions with ring enhancement and possible "eccentric target sign"),

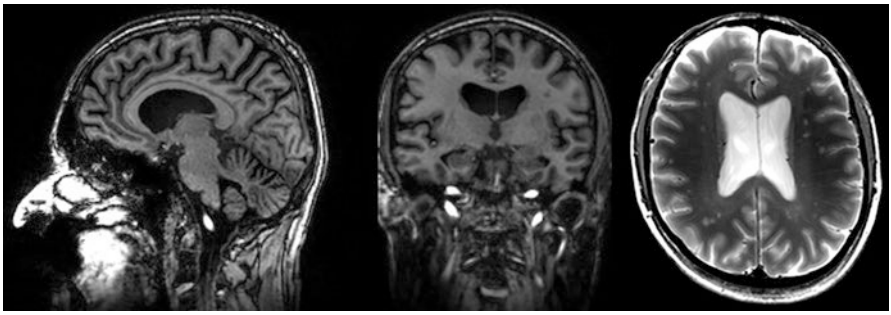

Fig. 6.1 Sagittal T1, coronal T1, and axial T2 images of the brain of an asymptomatic neurocognitive impairment (ANI) HIV+ patient showing signs of brain atrophy (dilated lateral ventricles and wide *sulci*). Multiple small T2 hyperintensities are seen in the periventricular white matter bilaterally

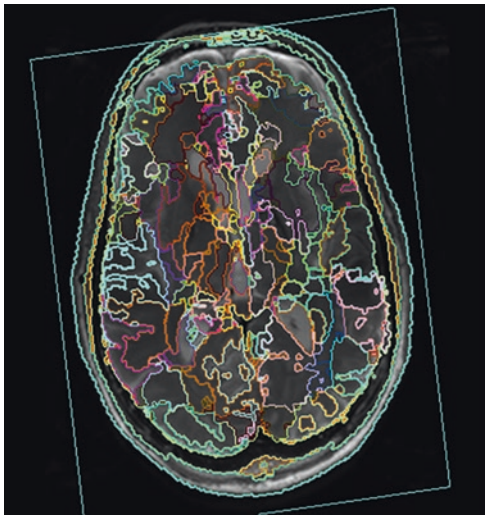

Fig. 6.2 Atlas-guided brain MRI segmentation of 286 regions of interest (ROIs) of an HIV+ subject's brain using BrainGPS: A multiatlas cloud-based processing pipeline

cytomegalovirus (CMV) and Epstein-Barr (EB) virus encephalitis [24], progressive multifocal leukoencephalopathy (PML) (as ill-defined hyperintense edge and hypointense core on diffusion-weighted imaging (DWI) in the white matter and U fibers) [25], tuberculoma (focal brain lesions), neurocysticercosis, and CNS fungal diseases, which include cryptococcal meningitis, aspergillosis, and mucormycosis [26, 27]. Toxoplasmosis may be difficult to distinguish from cerebral lymphoma [28]. Associated hydrocephalus or ophthalmic involvement with the various opportunistic CNS infections and inflammations can also be detected using CT or MRI. These opportunistic infections and pathologies were more prevalent before the cART era compared with today. Example of opportunistic infections (toxoplasmosis and herpes) and PML in HIV brain are shown in Figs. 6.3, 6.4, and 6.5.

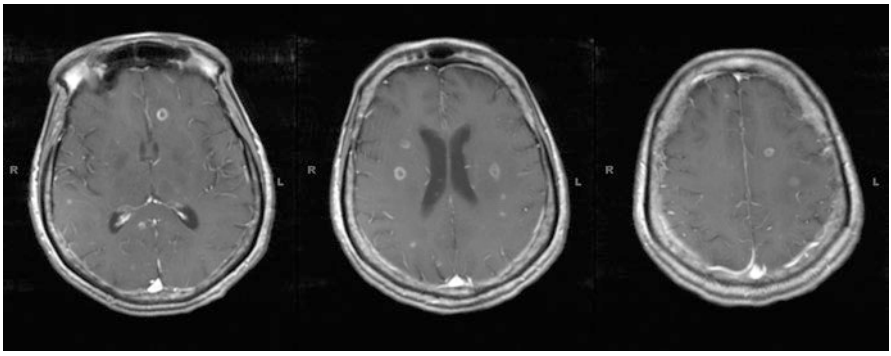

Fig. 6.3 T1W image showing bilateral, multiple ring enhancing lesions in the periventricular region consistent with toxoplasmosis in an HIV+ immunocompromised patient (Images are the property of Dr. Haris Sair and the Johns Hopkins School of Medicine Neuroradiology teaching file)

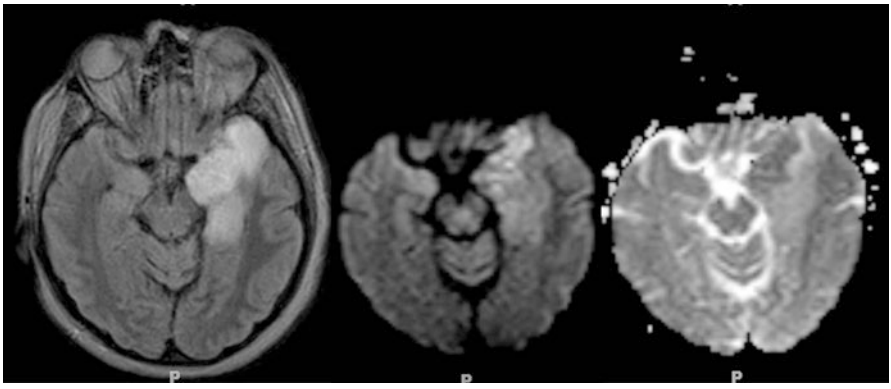

Fig. 6.4 T2-FLAIR image (*left*) demonstrating edema in the left anterior and mesial temporal lobe. DWI-trace and ADC images (*middle* and *right*) demonstrate areas of restricted diffusion consistent with Herpes infection in HIV+ patient (Images are the property of Dr. Haris Sair and the Johns Hopkins School of Medicine Neuroradiology teaching file)

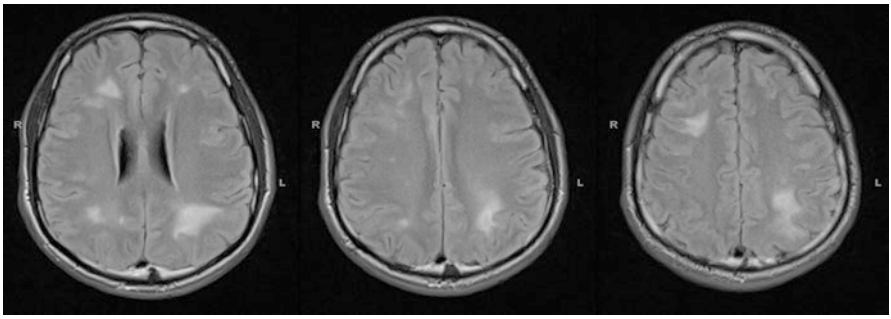

Fig. 6.5 Multifocal confluent T2-FLAIR hyperintense white matter lesions including the subcortical U fibers consistent with progressive multifocal leukoencephalopathy (PML) (Images are the property of Dr. Haris Sair and the Johns Hopkins school of medicine neuroradiology teaching file)

6.2.2 Functional Neuroimaging

6.2.2.1 Magnetic Resonance Spectroscopy (MRS) and Magnetic Resonance Spectroscopic Imaging (MRSI)

HIV has been extensively studied using magnetic resonance spectroscopy (MRS) at field strengths of 1.5 and 3.0 Tesla (T) [29, 30]. Proton magnetic resonance spectroscopy (MRS) is a noninvasive and safe imaging modality that profiles brain metabolism at a molecular level. It allows mapping of the chemical composition of the tissue. It can therefore give information on the human brain pathophysiology, which may appear normal to the neuroradiologist through measurement of these brain metabolite levels that may show neuronal or glial impairment [31]. It utilizes a continuous band of radiofrequencies that excite hydrogen atoms in different chemical compounds other than water, thus, allowing an understanding of chemical changes of the brain at the molecular level. The tissue chemical composition determines the frequency of the metabolic peak on the MRS spectrum. It is a spectrum of the amount of metabolite peak concentration against the chemical shift or frequency (in ppm). This enables identifying the metabolites in a voxel (large voxel ranging from 1 to 10 cm^3) on the spectrum as well as quantifying it. In MRS, the chemical shift is the difference between metabolites spin, the magnetic moments in part per million (PPM) which is the Hz multiplied by million since the frequency difference is very small. PPM is a locator number on the spectrum that reflects the spin difference and thus gives the information about the molecular group carrying the proton. MRS can, therefore, help identify metabolites, which is measured to help early detection, progression, and in monitoring responses to therapeutic interventions.

Magnetic resonance spectroscopic imaging (MRSI) is similar to MRS in measuring brain metabolites; however, despite requiring a longer scan time, it has the advantage of mapping the metabolic profile of the whole brain, i.e., exploring regional metabolic variations in one session. It acquires data from small voxels ranging from 1 to 4 cm^3 covering the whole brain. Factor analysis is one statistical

technique, which looks at multiple-dependent variables and reducing them to fewer factors to evaluate the metabolite levels across several brain regions. One study showed identified "NAA factor" that differentiated between patients with HIV dementia from those without dementia and correlated best with psychomotor and executive function tests. The "Cr factor" was increased in HIV dementia. The Cr factor correlated with the patients' CSF monocyte chemoattractant protein-1 levels. Both NAA and Cr factor scores were strongly weighted to white matter neuronal impairment in HIV dementia. Regional Cho elevations reflected glial turnover early in the disease differentiating HIV+ from HIV- [32].

Measuring brain metabolites can reflect neuronal integrity and gliosis in early and late stages of HIV and HIV-associated dementia (HAND). These metabolites can be used as biomarkers for seroconversion as well as neurocognitive impairment. In HIV, infected host immune cells cross the BBB early in the early stage of infection activating microglia and infect other immune cells adding to the spread of infection. Neuronal toxicity and inflammation lead to neuronal and glial dysfunction and further disruption of the BBB [12]. Previous MRS studies showed previous brain magnetic resonance spectroscopy (MRS) studies at 1.5 and 3 T that have shown alterations in the neurometabolic profile in HIV+ patients. One study showed impaired neuronal integrity across the white and gray matter regions as reflected by the decreased NAA/Cr and Glu/Cr in HIV patients with symptomatic cognitive impairment which correlate with neuropsychiatric tests [31, 33]. Example of single voxel brain spectrum is shown in Fig. 6.6.

Other MRS studies showed improvement of MRS values after treatment suggesting neuroprotective value of cART in HIV infection [34]. Another study, case control study using 3 T MRS, showed that increased Glx in FWM may suggest excitotoxicity in HIV-positive patients [35].

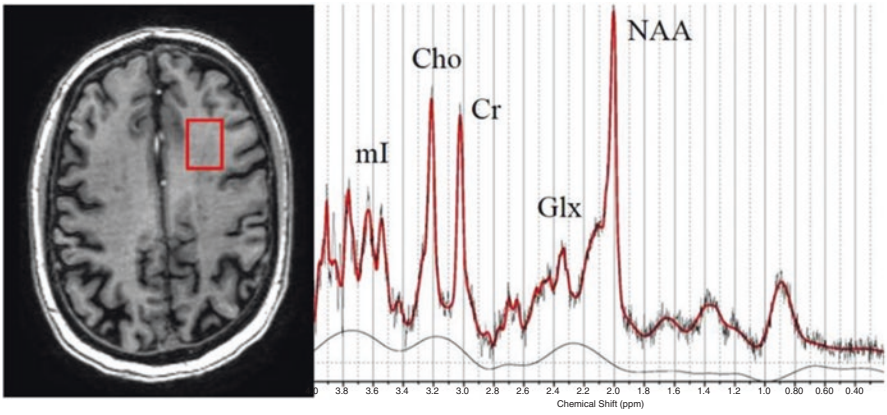

Fig. 6.6 Left frontal white matter (FWM) magnetic resonance spectroscopy (MRS) voxel (*red box*) and its corresponding spectrum in a 55 years old white HIV+ male patient with asymptomatic neurocognitive impairment (ANI)

6.2.3 MRS Metabolites

6.2.3.1 N-Acetyl Acetate (NAA)

NAA is only found in the neurons. Its concentration is about 7.8 millimolar (mM). They provide a measure of neuronal integrity. NAA decreases in neuronal and axonal loss as well as decreased neuronal function. The concentration also decreases in case of brain edema. Mitochondrial energy correlates with the synthesis of NAA in the neurons and axons. Decreased NAA concentration represents loss of neuronal integrity and is therefore a marker for neuronal dysfunction in NeuroAIDS [36].

6.2.3.2 Creatine (Cr)

Cellular oxidative metabolism is represented by the spectral resonance of Cr and phosphocreatine (PCr). It reflects the energy metabolism. The concentration of Cr is about 4.5 mM and is generally stable in the healthy brain. Thus, constant cellular metabolism in the healthy brain is reflected in the stability of the concentration of Cr. Stability in the concentration of Cr indicates constant cellular energy metabolism; therefore, it is used as a reference or control metabolite when assessing other brain metabolites level. Besides measuring the metabolites concentrations, calculating the ratios of brain metabolites to Cr is another common measure used by researchers to assess brain metabolites. In the brain, reductions in the concentration of Cr indicate deficient cellular energy metabolism [37]. Cr may decrease if the HIV patient has an associated hepatic encephalopathy.

6.2.3.3 Choline (Cho)

Phosphoryl choline and glycero-phosphoryl choline, components of the manufacture and the breakdown of cellular membranes, make up the largest part of the total Cho resonance. It is about 1.3 mM of concentration. Increments in Cho likely represent a greater cell growth or cellular proliferation and gliosis. Thus, Cho is a marker of cell membrane turnover. Increase in its concentration indicates active myelin breakdown [37, 38]. Choline (Cho) concentrations and ratios to creatine (Cr) in the basal ganglia are found to be elevated early in HIV infection but then stabilize after treatment [34]. One study showed that when cART is started during chronic HIV, it was associated with reduced neuronal glia and inflammatory markers. Alterations in Cho were noted among individuals who remain cognitively impaired after 12 months of cART as compared with those without HIV-associated neurocognitive disorders (HAND) [39].

6.2.3.4 Myoinositol (MI)

MI is a cerebral osmolyte, which regulates osmolar states and cellular volume. Its concentration in the brain is about 3.8 mM. It represents gliosis in demyelination and in AIDS dementia. Additionally, MI takes part in the second-messenger system of phosphoinositide [37]. Myoinositol (MI) is a surrogate marker of glial activation as measured by magnetic resonance spectroscopy (MRS) [12].

One study showed that the ratio of myo-inositol to creatine is increased in white matter of HIV+ patients early in the disease compared with normal controls, which then normalizes. In AIDS dementia complex, MI is at normal levels, whereas NAA levels decrease [40].

6.2.3.5 Glutamate (Glu), Glutamine (Gln), and Glx

Glutamate receptor stimulation is one mechanism by which gp120 and TAT exert their neurotoxic events through glutamate receptor stimulation. Glutamate excitotoxicity plays a role in TAT-induced toxicity and peroxynitrite, nitric oxide, and reactive nitrogen species [41–44] which causes inflammation and neuronal damage can be a target for therapeutic approaches targeting glutamate receptors [45], other report that glutamate is protective to neurons [46]. TAT-induced neurotoxicity is also induced via the dopaminergic system in the striatum [47].

Reduced Glu uptake has previously been demonstrated to occur in vitro in astrocytes exposed to HIV possibly through reduced expression of the glutamate transporter, excitatory amino acid transporter 2 (EAAT2) [48]. Using 3 T MRI scanner, one MRS study showed decreased frontal white matter (FWM) Glu and Glx (the summation of Glu and glutamine) in HIV-associated dementia (HAD) patients compared with HIV patients with mild cognitive impairment group (MCI) and normal controls (NC). Their findings were positively associated with impaired performance in neurocognitive domains, such as fine motor, executive functioning, working memory, and attention [31].

6.2.3.6 Lactate (Lac)

Lactate is usually not normally detected in the brain tissue due to its very low concentration. If detected, it is usually increased in anaerobic nonoxidative glycolysis indicating ischemia.

6.2.3.7 Glutathione (GSH)

Glutathione (GSH) is a protective antioxidant against the increased reactive oxygen and nitrogen species which occurs in HIV infection. Oxidative stress is usually measured by invasive technologies [49]. Due to its very low concentration, it is difficult

to detect GSH by conventional MRS. Today, novel MEGA PRESS MRS techniques can detect GSH. Low GSH concentration reflects oxidative stress. In HIV, activated macrophages release free radicals which cause disruption of the blood–brain barrier and neuronal synapses [46], leading to functional impairment in the brain of individuals with HIV [50]. GSH can, therefore, be a predictive marker in identifying individuals at risk of oxidative stress in HIV dementia.

Using MRS and MRSI, predictive models for the associated predictors of CNS injury are useful in identifying human risk factors and their interaction in the progression of HIV. For instance, risk factors such as age, race, and gender; disease-specific factors such as HIV RNA concentration, ADC stage, duration of HIV infection, nadir CD4, current CD4 count, plasma RNA, and treatment-specific factors such as CNS penetration and effectiveness of cART can all be in the same predictive model. Using MRS, one study scanned 260 HIV+ subjects with various neurological stages and looked at all these risk factors. They measured and calculated 12 MRS brain metabolites ratios in 3 brain regions: the basal ganglia, the frontal white matter, and gray matter (MFC = midfrontal cortex). Their study showed that in chronic HIV, the host and viral factors which are related to both the current and past HIV statuses contribute to persisting abnormalities in the cerebral metabolite levels [9].

One study sought to investigate premature aging and cardiovascular disease (CVD) as two risk factors to HIV-associated neurocognitive disorder (HAND) in virally suppressed clinically stable HIV-infected adults. They aimed to know if brain MRS metabolites abnormalities in HIV adults involve only the frontal white matter (FWM) and caudate (the two typically affected brain regions) or do they extend to other brain region affected in pathological aging such as the posterior cingulate cortex and wanted to assess age and CVD as additional independent risk factors of brain injury in HIV+ adults. They also looked at immune recovery (measured by CD4), HIV duration, and inflammatory markers (CSF B2 microglobulin and neopterin) and its association with brain neurochemical injury (measured by metabolites abnormalities using MRS) in HIV adults, and if MRS are predictive of lower neuropsychiatric (NP) performance and if age, CVD and HIV status may moderate this relationship. They included 92 HIV-positive patients and 30 HIV-negative individuals in their study. They showed that (1) CDV risk factors were associated with lower NAA in PCG and *caudate*, (2) past acute events were associated with increased MI in PCG, (3) HIV duration was also associated with lower NAA in caudate, (4) high CNS cART penetration efficiency was associated with lower MI in PCG, (5) the degree of immune recovery on cART was associated with higher NAA in FWM, and (6) CSF neopterin was associated with high MI in PCG and FWM, (7) HIV and age interaction was associated with lower FWM NAA and (8) the increased Cho/NAA ratio was associated with decreased NP performance. The authors concluded that in long term virally suppressed, clinically stable HIV-infected adults, HIV duration, age, and CVD (past or recent events) are independent risk factors for brain inflammation [51].

MRS provides quantitative estimates of the concentration of cerebral metabolites in the various regions of the brain. Furthermore, normal cerebral metabolism is

characterized by the ratios of the concentrations of specific metabolites including NAA/Cho, NAA/Cr, Cho/Cr, and MI/Cr. The normal values for individuals at specific ages and gender provide guidelines to assess deviations from the normal and healthy concentrations and ratios.

6.3 MRS Protocols

Magnets of 1.5 T and 3.0 T are common clinical scanners. Scanners of 3.0 T provide a higher resolution. Magnet of 7.0 T is used for HIV research and not in the clinic. Using a head coil, the subject is scanned in the supine position. Structural imaging typically includes T1, T2, and fluid attenuation inversion recovery (FLAIR) in sagittal, oblique coronal, and oblique axial planes. Spectroscopic data are acquired with a single voxel technique (MRS) or multiple voxel imaging (MRSI) techniques. The signals are obtained for specific regions of interests (ROIs). In HIV, frontal white matter (FWM), basal ganglia (BG), anterior cingulate (ACG), posterior cingulate gyrus (PG), and hippocampus (if researchers are interested in aging studies in HIV) are the commonly used ROIs.

To minimize the artifacts of blood and cerebrospinal fluid (CSF), VOIs commonly exclude vasculature structures containing CSF. A point-resolved spectroscopy sequence with long and short echo times of short or long TE is usually obtained after shimming, fat, and water suppression. Sequences are run to estimate the concentrations of relevant metabolites and pertinent metabolic ratios, particularly NAA, Cho, and Cr. Ratios to Cr or to Cho can also be calculated: NAA/Cho, NAA/Cr, and Cho/Cr. The estimation of metabolites with a small brain concentration is facilitated with sequences with short echo times. MEGA-point-resolved spectroscopy (PRESS) editing (MEGA PRESS) techniques are novel techniques, which can detect brain metabolites which are difficult to resolve, such as GSH, N-acetyl aspartate glutamate (NAAG), or gamma amino butyric acid (GABA) [52–57]. Recently, novel MRS MEGA PRESS editing techniques are now available to measure multiple brain metabolites in one session, thus, decreasing the scan time especially if the patients cannot tolerate long scan times inside the MR machine [57–59].

6.3.1 Functional MRI (fMRI)

Assessment of the functional connectivity of brain regions is one area that was unveiled by functional MRI (fMRI). It examines the network dysfunction and coordination between the different parts of the brain needed for complex tasks done through fMRI experiments inside the magnet. These tasks can be correlated with tasks done outside the magnet as well as serological and neuropsychiatric tests and reaction times to investigate the level of neurocognitive impairment.

In HIV, it is known that there is injury and structural changes in the subcortical and *frontostriatal* brain regions; therefore, exploring the association of HIV serological and neurocognitive status with structural and functional resting stated fMRI data is of great importance [60–63] . One study suggested that HIV-associated dopaminergic abnormalities could be related to impaired frontostriatal connectivity which can be explored using PET imaging modality [62]. Another fMRI study showed that HIV+ showed reduced connectivity in the left caudate and the ventral prefrontal cortex and global pallidus as compared with seronegative controls which explained the impaired sequencing task in HIV+ patients [63]. Another fMRI study showed that HIV+ scored worse in facial recognition task as compared with seronegative controls which also correlated to brain volume reduction in HIV+ patients [60, 61].

6.3.2 Diffusion Tensor Imaging and DWI

The introduction of diffusion-weighted imaging had led to fiber tracking which helps the clinicians and surgeons to see damaged or displaced brain tissue.

The technique depends on measuring the molecular diffusion of water molecules in the brain tissue depicting macro- and microscopic anatomies of the various brain regions [64]. This anatomy can be disrupted in pathological conditions. Quantification of the degree of white matter (WM) abnormalities in chronic and virally suppressed HIV-infected (HIV+) persons can be achieved. Increased mean diffusivity reflects increased neuroinflammation which can be used to follow up progression of the disease [65]. White matter DTI measures like fractional anisotropy (FA) can be normal except for patients with longer HIV disease duration and who developed neurocognitive impairment [66]. Furthermore, restricted diffusion within the brain lesion on diffusion-weighted sequences was especially seen in focal lesions in HIV opportunistic infections or primary lymphoma.

6.3.3 Perfusion-Weighted Imaging (PWI) and Arterial Spin Labeling (ASL)

Perfusion-weighted imaging (PWI) technique using intravenous contrast injection allows mapping of hemodynamic changes based on the perfusion of the injected contrast material. This technique is used if there is suspicion of stroke in HIV patients.

Arterial spin labeling (ASL) is a safe MRI technique which provides a rapid, noninvasive, and quantitative measure of the cerebral blood flow (CBF) in various brain regions without intravenous injection. It can be used as a biomarker of the effects of HIV on the brain perfusion. Studies showed that the CBF is reduced in the early stages of HIV seroconversion even before they show neuropsychological impairment [67, 68].

6.3.4 Quantitative Susceptibility Mapping (QSM) and Susceptibility-Weighted Imaging (SWI)

Three-dimensional gradient echo (GRE) is a novel MRI-based sequence mainly used for susceptibility-weighted imaging (SWI) and quantitative susceptibility mapping (QSM) [69]. Quantitative susceptibility mapping (QSM) relies on the magnetic field distribution estimates of the examined brain tissue [70].

T2 GRE and SWI hypointensities can be seen in the frontal white matter and the U fibers and cortex adjacent to the white matter lesions in the demyelinating progressive multifocal leukoencephalopathy (PML) [71].

6.3.5 Positron Emission Tomography (PET) and Single-Photon Emission Computed Tomography (SPECT)

PET is an imaging technique, which quantitatively evaluates the metabolic processes in the living organisms. PET provides the ability to estimate the rate at which the brain utilizes nutrients. Measurement of endogenous compounds in specific regions of the brain reflects dysfunction of the regional brain metabolism which can help understand the pathogenesis of NeuroAIDS [8].

PET and SPECT would discriminate the cases as inflammatory versus noninflammatory or neoplastic. Thallium-201 SPECT can be used in cerebral opportunistic infections in NeuroAIDS [28].

Administration of 2-deoxy-2-[18F] fluoro-d-glucose ([18F] FDG) is a safe and effective tool, which is performed widely in hospitals to examine glucose metabolism by the different body organs. Since glucose is metabolized by rapidly growing cells, the application of this procedure for whole-body tomography is suited for people with primary malignancies like lymphomas and opportunistic infections which may develop in advanced cases of HIV.

Studies using Technetium-99 m hexamethyl-propyleneamine oxime-single-photon emission computed tomography were used to evaluate brain perfusion in HIV infection. Studies showed global and focal decrease in blood flow to the brain in the early stages of infection without showing any signs of dementia or neuropsychiatric impairment [68]. Today arterial spin labeling is safe and noninvasive and can provide the same information without exposing the patient to radiotracers or ionizing radiation.

High active antiretroviral therapy (HAART) has lengthened the life expectancy of patients infected with human immunodeficiency virus (HIV); the risk of cognitive impairment in the aging HIV+ subgroup has continued to increase. Premature aging has been hypothesized as a risk factor for HIV-associated neurocognitive disorders (HAND) and is of great interest to researchers [51, 66, 72, 73]. The questions are: Does premature aging occur in HIV dementia? Is aging a risk factor to early development of HIV dementia in HIV+ individuals?

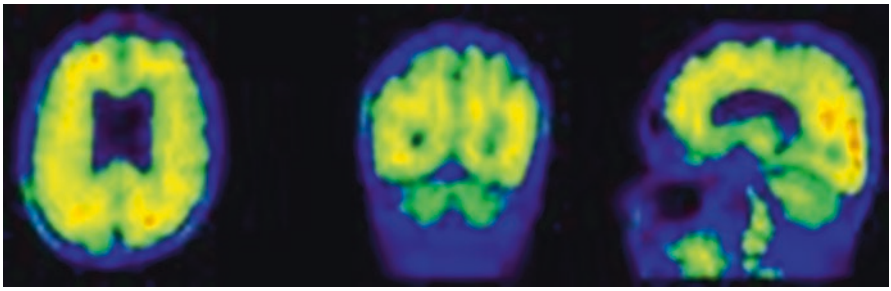

Fig. 6.7 Axial, coronal, and sagittal PET images showing ^{18}F AV45 standardized uptake value ratio (SUVR) image of an HIV+ individual showing high AV45 uptake in the *Precuneus* and the occipital region

PET imaging using ^{18}F AV45 (a specific tracer targeting amyloid plaques) is a new tool to investigate the association of increased amyloid deposition (as measured by standardized uptake value ratio (SUVR) reflecting AV 45 uptake) and aging in HIV. Example of ^{18}F AV45 PET scan in HIV brain is shown in Fig. 6.7.

6.4 Conclusions

Novel neuroimaging techniques are rapidly advancing allowing us to better understand the pathophysiology of the HIV-associated brain changes down to the molecular level. Multiparametric imaging is on the rise where fusion of multiple techniques and modalities would increase the sensitivity of MRI to detect subtle brain changes.

To date, pathological analysis, after a brain biopsy of the human living brain, remains the gold standard to establish the diagnosis of vast brain pathologies; however, nonspecific inflammatory pathology noted in probable cases has been often attributed to sampling of brain tissue [14]. With advanced imaging modalities allowing us to noninvasively diagnose brain pathologies at the molecular level, without resorting to stereotactic brain biopsy, we expect that in the future, neuroimaging will become the "gold standard" for definitive diagnosis of brain pathology.

Conflict of interest The authors report no conflicts of interest.

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Chapter 7
HIV Neuroinvasion

Suzanne Gartner and Yiling Liu

Keywords HIV • SIV • Neuroinvasion • Brain • Macrophage • Monocyte • Trafficking • HIV neuroinvasion • HIV encephalitis • HIV brain infection • SIV brain infection • HIV macrophage infection • Monocyte trafficking • Cerebrospinal fluid (CSF) • Blood-brain barrier (BBB) • Perivascular space (PVS) • Choroid plexus • Transendothelial migration

Core Message

HIV can enter the brain during all stages of systemic infection. Entry during the acute phase may occasionally be accompanied by encephalitis or meningitis and high viremia in the cerebrospinal fluid; however even in these cases, brain infection is curtailed by an immune response. Still unclear is whether this initial entry establishes indefinite HIV persistence within the brain.

During the asymptomatic stage of infection, in the absence of compromise of the blood-brain barrier, HIV appears to enter the brain primarily via transendothelial migration of monocytes, a normal physiological process characterized by the continual entry of low numbers of monocytes; these entering monocytes take up residence within the perivascular space. HIV entry into the brain during late-stage infection,

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when AIDS is present, appears more flagrant, is likely mediated by the entry of infectious virions and infected cells, and is likely enhanced by the presence of neuroinflammation and opportunistic infections. HIV-associated clinical neurological disease remains a problem, even in this age of effective anti-HIV drug therapy and thus, efforts to develop new therapies aimed at keeping HIV "silent," in the absence of continuous antiretroviral drug therapy, should not disregard infection in the brain.

7.1 Introduction

More than 30 years have passed since HIV was identified as the etiological agent of acquired immunodeficiency syndrome (AIDS). Much has been learned about how the virus causes disease, persists, and establishes latency. Effective antiretroviral drugs, and measures to prevent transmission, have been developed, and the standard-of-care in developed countries enables infected individuals to live relatively normal lifespans. Unfortunately, many aspects of HIV infection in the brain remain unclear, and there is considerable controversy as to its significance. Certainly, antiretroviral therapy (ART) has reduced the incidence of HIV encephalitis, and the more severe forms of cognitive impairment and dementia. Yet, clinically detectable neurological impairment, termed HIV-associated Neurocognitive Disorder (HAND), still affects many individuals, even after years of ART, and HIV RNA can be detected in the cerebrospinal fluid (CSF) of individuals experiencing virologic failure during treatment. These facts alone compel us to both revisit the existing knowledge regarding infection in the brain, and to continue to pursue new investigations. In recent years, the emphasis on T-cell infection has intensified to the point where a number of HIV/AIDS researchers have come to consider macrophage infection essentially irrelevant. This perspective derives in part from technical issues like the greater accessibility of T-cells for sampling, the fact that T-cells are easier to culture---they proliferate and can easily be expanded--- and the fact that they appear to be infected in vivo in greater numbers, compared to macrophage lineage cells. Thorough study of HIV infection in macrophages requires examination of tissues. While not a perfect mirror of HIV-associated events occurring within the brain parenchyma, the CSF, which is relatively accessible for sampling, can provide some indication of viral activity there, and hence, is a valuable source of information. The CSF "window," and the fact that HIV infection in brain is relatively free of T-cell infection, makes this tissue particularly attractive for the study of HIV infection in macrophages. Also, while a number of features of Simian Immunodeficiency Virus (SIV) infection in macaques differ from those of HIV infection in humans, the SIV system, nonetheless, can provide considerable insight into HIV infection, particularly within the brain and other difficult to access tissues like the gut. Its value and importance should not be overlooked.

The subject of this chapter is HIV neuroinvasion. Included will be discussion of the timing, modes and sites of HIV entry into the brain, and the potential impact of ART on this entry. The question of when HIV enters the brain is particularly important to the issues of viral latency and persistence, and the potential for the brain to serve as a viral reservoir of significance. Knowledge regarding HIV neuroinvasion

can also advance our understanding of the relationship between infection and replication within the brain, and the development of cognitive impairment.

Throughout this chapter, unless otherwise indicated, we will use the term "HIV," in reference to Human Immunodeficiency Virus Type 1. Human Immunodeficiency Virus Type 2 (HIV-2) is a genetically related, less-prevalent lentivirus that can also cause immune deficiency and neurological disease, but information about HIV-2 is quite limited, especially information pertaining to neuroinvasion and neuropathogenesis. HIV-2 infection is seen primarily in West Africa, where it typically exhibits a milder course of disease than HIV-1 [1]. There are also cases of individuals co-infected with both of these viruses [2–4]. Although few in number, case reports and patient-based studies of HIV-2-infected individuals manifesting neurological disease have been published, and in some cases, the neurological presentation led to identification of infection with HIV-2 [5]. These reports include examples of patients exhibiting encephalitis [6], demyelinating encephalomyelitis [7], dementia [8], progressive multifocal leukoencephalopathy (PML) [9, 10], symptomatic cerebral toxoplasmosis [5], and lower motor neuron disease [11]. Of relevance, cognitive and motor deficits have been observed in infant pigtailed macaques infected in utero with HIV-2 [12]. (This in utero infection occurred via inoculation of the mothers during the third trimester of pregnancy.) Also, Mörner and colleagues evaluated brain tissues recovered at autopsy from HIV-2 infected patients and found that the productively infected cells were exclusively either microglia or infiltrating macrophages [3]. Thus, the limited data available suggests that HIV-2 likely follows a course similar to HIV-1 with respect to entry into the brain and consequent development of neurological disease, but that these events may occur at a considerably lower frequency than in the case of HIV-1.

7.2 Timing of HIV Entry into the Brain

An overview of the timing and modes of HIV entry into the brain is presented in Table 7.1.

7.2.1 Viral Entry Early in Infection

HIV DNA has been detected in the brains of asymptomatic infected individuals, as well as those who experienced HIV encephalitis [13–15]. Abundant evidence indicates that HIV can enter the brain during the acute (primary) infection [16–21]. During this phase and/or seroconversion, some patients develop neurological symptoms. These are typically seen in association with systemic symptoms of infection such as fever, myalgia and truncal rash [20, 22]. Acute encephalopathy, presenting with cognitive and motor impairment, has also been reported [18, 22]. In some individuals, this was accompanied by seizures. Importantly, in essentially all cases, the neurological symptoms resolved and often, this occurred within days of presentation. The kinetics of this resolution suggests an association with the development of an anti-HIV immune response within the central nervous system (CNS), which has

Table 7.1 Timing and mode of HIV entry into the CNS

Stage of infection	Frequency of entry	Clinical and pathological associations	Blood-brain barrier compromise?	Mode of entry
Acute	HIV neuroinvasion likely occurs in most individuals, but the abundance of entry events within an individual is variable and related to features of the systemic infection	Often clinically silent; more rarely, associated with meningitis or encephalitis and high viremia in CSF	Brain inflammation may occur, but frank BBB compromise is minimal except in cases of meningitis or encephalitis	Free virions and infected cells[a]
Asymptomatic	Ongoing, occurs at low levels	No noticeable associations	Typically minimal and not required for entry of infected monocytes	Primarily via transendothelial migration of blood-borne monocytes, a normal, ongoing, physiological process hijacked by HIV
Late-stage	Increased	Enhanced by neuroinflammation and opportunistic infections; may be accompanied by encephalitis and/or dementia	Often present, but not necessarily severe	Infected cells[a] and free virions

[a]Although evidence is lacking, it is conceivable that during acute infection, HIV-infected CD4+ T-cells, in addition to infected monocytes, may enter into the brain, whereas in late-stage infection, when few circulating CD4 + T-cells exist, the entering infected cells are almost exclusively monocytes

been documented in some cases [23], and more definitively, in the SIV model system [24, 25]. An unfortunate case of accidental inoculation with HIV has provided additional insight into neuroinvasion during acute infection [17]. Although the individual received zidovudine within 45 min of the infusion error, he experienced acute HIV infection and died 15 days later. Other therapeutic interventions included treatment with di-deoxy-inosine and interferon-α. HIV DNA was detected in several regions of his post-mortem brain by PCR, and the virus was isolated from the parietal lobe (the only region investigated in this way). Interestingly, however, immunostaining for HIV p24 yielded negative results in the brain, although there were rare infiltrating gp41-positive cells present within the perivascular and sub-pial spaces, and also around the site of a past infarction. Since gp41 has been shown to

cross-reactive with a nonviral protein associated with macrophage activation [26], the positive staining may represent artifact. Mild perivascular cuffing and mild lymphocytic meningitis were seen during histological evaluation of the brain, but there were no giant cells, glial nodules or white matter abnormalities. The infectious dose was determined to be 600–700 tissue culture infectious doses ($TCID_{50}$). Neurological symptoms were present and progressed; these were thought to reflect hepatic encephalopathy, although the patient, who was 68 years old, had prior evidence of a mild dementia and a history of severe alcoholism. Of relevance, he never developed pharyngitis, myalgia, a rash, generalized lymphadenopathy, headache or any other conditions indicative of a symptomatic primary HIV infection. Clearly, HIV entered the brain of this individual during an asymptomatic acute infection. The paucity of detectable HIV expression, however, is somewhat unexpected, and suggests that significant viral replication may not necessarily follow entry into the brain, even during systemic primary infection. This has implications for the issues of viral latency and the mode of viral entry, be it cell-free or cell-associated. These will be discussed in greater detail later.

HIV neuroinvasion during primary infection in the setting of meningitis or meningo-encephalitis also occurs. A particularly interesting case presenting as fulminant meningoencephalitis has been described [27]. At presentation, the plasma HIV viral load in this patient was 1.4×10^6 copies/ml, and the CSF viral load exceeded the upper limit for quantitation (10^7 copies/ml), presumably reflecting high level HIV replication in the CNS. The patient recovered and viral suppression in plasma and CSF was achieved after 5 months of ART, however, 6 months after presentation, upper neuron signs remained. Additional cases of meningoencephalitis in association with HIV primary infection have been reported, and of relevance, this condition has also been seen following the withdrawal of ART [28]. Further evidence demonstrating early HIV entry into the brain comes from a study reported by Valcour and colleagues, describing 20 individuals experiencing acute HIV infection; HIV RNA in CSF, parameters of intrathecal immune activation, and magnetic resonance spectroscopy (MRS) images were examined [29]. (The 20 study subjects were classified as acute infection, Fiebig stages I-IV [30], with an average of 15 days post virus exposure.) No neurological signs or symptoms other than headache were present. HIV RNA was detected in the CSF in 15 of 18 subjects, as early as 8 days after estimated transmission. Some study subjects manifested evidence of intrathecal immune activation, and MRS indicated the presence of brain inflammation. Other studies utilizing neuroimaging techniques also indicate early entry of HIV into the brain. Recently, Cao and colleagues reported findings from a neuroimaging study of 56 HIV-infected individuals and 26 seronegative controls that included cases of (1) primary infection (HIV seronegative), (2) individuals at 4–12 months post infection, and (3) individuals at >12–24 months post infection [31]. Plasma viral load was undetectable in 11 of the 56 subjects, including 10 receiving ART. The researchers identified neuroimaging changes present during the primary infection stage that most probably reflect acute neuroinflammation, a situation that would either derive from, and/or, be accompanied by, HIV entry into the brain. They also noted that the changes they observed in primary infection, such as

atrophy, and striatal and callosal injury, were comparable to those seen in advanced HIV infection [32, 33]. Further indicative of HIV neuroinvasion during primary infection, Helleberg and Kirk studied a patient who presented with acute psychosis and magnetic resonance imaging (MRI) findings suggestive of vasculitis and multiple infarctions [34]. He was seronegative for HIV antibody, but HIV RNA was detected in his plasma and CSF. A brain biopsy 6 weeks later revealed HIV encephalitis with microglial nodules.

SIV infection in macaques is an excellent model for HIV infection and disease, including the development of neurological disease [24, 35–39]. Several reports have detailed early SIV neuroinvasion [24, 35, 37, 40]. The processes and events associated with SIV neuroinvasion appear quite similar to those observed with HIV. In a study of early SIV infection in rhesus macaques inoculated intravenously with uncloned SIVmac251, Chakrabarti and colleagues found that CNS infection was a frequent and early event, and that virus-expressing cells were detectable within the brain as early as 7 days post inoculation [35]. These cells were mainly CD68+ and located within the perivascular space, bringing the authors to conclude that they were likely blood-derived monocytes that had crossed the blood-brain barrier (BBB). While the virus-expressing cells were confined to the perivascular space (PVS) at 7 days after inoculation, at 1, 2, and 3 months later, they were also seen beyond the PVS, within macrophages associated the microglial nodules, as well as scattered throughout the brain parenchyma. This could reflect spread of the virus infection or migration of infected cells from the PVS into the parenchyma. A similar study by the same group, in which a significantly higher viral inoculum was used yielded comparable results [41]. Here, quantitation of the virus-expressing cells was performed. At day 7, the average number of virus-expressing cells was 8 per cm^2 area, while at day 30, it was only 3 per cm^2 within the same region of brain. In situ hybridization for viral mRNA was performed using probes for both an SIV regulatory (nef) and structural (env) gene and comparison of the two indicated that the low level of virus expression seen was likely not a consequence of a block at the level of viral transcription.

Early dissemination of SIV into the brains of rhesus macaques infected orally with a high dose inoculum has also been observed [42]. SIV gag DNA was detected in the brains of 3 of 4 inoculated animals at 1–2 days post infection. (The animals were perfused, thereby excluding the possibility that virus detected in brain was associated with contaminating blood.) These and the other findings discussed clearly indicate that both HIV and SIV are capable of entering the brain during primary infection.

7.2.2 Viral Entry Throughout the Course of Infection

In addition to HIV entry into the brain during acute infection, there might also be continual entry of infected monocytes or free virions throughout the course of infection. Monocyte trafficking into the brain occurs at low levels under normal

conditions [43, 44], in the absence of any compromise of the BBB. These cells enter the brain via transendothelial migration and take up residence within the PVS, at least initially [45]. They act as scavengers and are thought to play a role in immune function [46, 47]. Their turnover rate is thought to be on the order of 3 months, whereas the resident microglia persist for years [45, 46, 48]. Information regarding the level of HIV brain infection during the asymptomatic stage is limited; however, available information indicates that the levels are typically low [49, 50]. For example, Bell and colleagues performed quantitative DNA PCR on brain tissues from 13 HIV+ asymptomatic subjects who died suddenly. No HIV DNA was detected in 6 of the brains, and only very low levels were seen in the other 7 [49]. Similarly, Sinclair et al. detected HIV DNA in only 2 of 8 brain specimens taken from asymptomatic individuals [50]. Moreover, as evidenced by the lack of viral RNA and proteins, HIV expression is typically absent from the brain during the asymptomatic stage [50, 51]. These low levels could reflect a continual reseeding and/or the persistence of HIV genomes that entered during acute infection. Phylogenetic analyses might provide insight into this question, but more directly relevant studies are required. Many of the studies of HIV DNA sequences present in brain have been limited to examination of the V3 hypervariable region of the gp160 envelope gene [52–54]. This region is relatively short and tends to be significantly conserved in brain, owing to its relationship to macrophage-tropism, a typical characteristic of HIV brain tissues. Hence, while it can provide important information regarding HIV tissue and cell compartmentalization, it cannot provide the level of subspecies discrimination needed. In an analysis of HIV gag (p17) sequences, and sequences spanning the V1-V2 region of env, Hughes and coworkers found that genetic diversity among gag sequences from lymphoid tissues was consistently lower than that present in brain [55]. The calculated age of the viral populations in lymphoid tissue ranged from 2.65–5.6 years, compared with 4.1–6.2 years for the populations in brain, leading the authors to conclude that in two of the study subjects, HIV genetic evolution may have occurred early, several years prior to the onset of AIDS. Moreover, in a study of V1-V5 sequences from multiple regions of brain, Shapshak and colleagues observed regional clustering, suggestive of independent evolution of HIV species within different regions of the same brain [56]. These findings are intriguing, and demonstrate the potential of HIV genetics to help answer questions relating to both time and mode of viral entry into the brain.

Limited SIV viral genetics studies have also addressed the question of timing of viral entry. Using their SIV rapid progressor model, which features accelerated development of SIV encephalitis and disease progression in general, Clements and colleagues observed “steady-state” levels of viral DNA throughout the first 56 days following inoculation, whereas viral RNA, which was detectable at significant levels at day 10, was absent from the brain at day 21 [57]. The authors also examined sequences of the V1 hypervariable region of the envelope gene recovered at day 56 from brain DNA and RNA, and from PBMC RNA from a macaque with widespread viral replication and mild SIV encephalitis. The neurovirulent molecular clone, SIV/17E-Fr predominated in both the brain DNA and RNA, but was not represented among molecular clones recovered from the PBMC RNA. This lack of sequence

homology between the brain- and circulating leukocyte-derived viral species suggests that trafficking cells may not be entering the brain during the asymptomatic stage of infection, or near the late stage. However, in this study, only PBMC RNA, and not DNA, was examined, so only viral strains actively replicating within the PBMC would be detectable. Reported studies, as well as unpublished work from our laboratory, suggest that circulating monocytes are often quiescently infected [58]. HIV and SIV replication and particle production in monocyte/macrophages is tightly linked to the state of differentiation of the cell [59–61], and blood monocytes typically require some level of maturation before they can support active viral replication [62, 63]. SIV/17E-Fr replicates well in macaque blood monocytes [64]. Also, monocytes generally represent only about 5–10% of the PBMC population. It is conceivable, therefore, that in contrast to what was found in PBMC RNA, the SIV subspecies present within DNA recovered from circulating purified monocytes might include a predominance of SIV/17E-Fr. This would reflect latent infection of the monocytes. More information is needed, however, to determine if there is low-level seeding of the brain with HIV (or SIV) throughout the asymptomatic stage. Such seeding would more likely be mediated by infected monocytes taking advance of a normal physiological process.

7.2.3 Viral Entry Late in Infection

Previously, we have proposed that an initial critical step towards the development of HIV dementia is an increase in the trafficking of blood monocytes into the brain, and that this increase is a consequence of systemic events that occur with, and after, the onset of AIDS [65]. In a study of HIV gp160 sequences recovered from DNA prepared from lymphoid tissues and multiple regions of brain from a patient with HIV dementia, we observed high homology between sequences from brain parenchymal deep white matter and bone marrow, and moreover, sequences recovered from DNA from blood monocytes collected 5 months earlier were even more genetically similar [66]. These findings support the idea that bone marrow-derived monocytes traffic into the brain. They also suggest that bone marrow may be a site of events that ultimately lead to the enhanced trafficking of monocytes into the tissues. We believe that significant neuroinvasion occurs in most infected individuals late in the course of infection, during AIDS. One of the best correlates of HIV dementia is encephalitis [67, 68]. HIV encephalitis is characterized by the presence of macrophage-derived multinucleated giant cells, along with increased numbers of monocyte/macrophages, within the PVS. Thus, we reason that while significant neuroinvasion occurs in most infected individuals during AIDS, the process is even more amplified in those who go on to develop HIV encephalitis and cognitive impairment. The AIDS stage of infection is characterized by both functional immune suppression, and immune activation. Our model links neuroinvasion with events occurring outside of the brain during end-stage disease. Some of these events likely take place within the bone marrow, given that marrow is the site of monocyte

production, as well as HIV infection [69]. Activation of monocytes prior to their exit from marrow, and/or during the short time they spend in the blood, may occur and could serve to prime them for transendothelial migration. This increase in monocyte trafficking is likely not limited to brain. Donaldson and colleagues demonstrated that HIV disease progression---movement from the asymptomatic stage of infection to AIDS---is associated with the appearance of infection in nonlymphoid tissues, including the brain [70]. End-stage SIV infection is also characterized by widespread infection of nonlymphoid tissues, the target cell in these being macrophages [71]. Regardless of whether more of these monocytes are infected that in the pre-AIDS situation, the net result, because of their sheer numbers, is likely to translate into an increase in the number of infected cells entering the brain. This could explain why HIV-associated dementia typically does not develop until frank AIDS is present [72]. Otherwise, it is necessary to explain why, if the virus is responsible for the development of dementia, it can take 10–15 years or more from the time of viral entry during acute infection, to the development of detectable clinical disease. It is critically important, however, to avoid assuming that this increase in the level of HIV infection in brain is directly responsible for the development of neurological disease, particularly cognitive impairment. Monocyte/macrophage numbers, in general, are increased as a consequence of this enhanced trafficking, and based on what is known about the number of these cells harboring genome, it is likely that most of those invading the brain are not infected. Also, the abundance of macrophages, appears to be a better correlate of HIV-associated dementia that the presence or extent of HIV infection [67, 73]. Ultimately, the infiltration of uninfected monocytes into the brain may prove to be as important to the development of HIV dementia, or HAND, as HIV infection and/or replication.

7.2.4 Role of Strain Specificity in Viral Entry

It is conceivable that the timing of neuroinvasion could depend on the viral strain. In a serial sacrifice study, Smith and colleagues compared SIVmac1A11, a non-pathogenic molecularly cloned strain, with [1] uncloned SIVmac251, and [2] a molecular clone derived from this swarm (SIVmac239), both pathogenic [24]. Animals were followed for up to 23 weeks. SIV was recovered from the plasma of animals inoculated with SIVmac1A11 at weeks 2 and 4, but not later, and was never recovered from CSF. Also, no intrathecal immune response was detected in the SIVma1A11 infected animals, and the neuropathological changes characteristic of SIV brain infection were not apparent. In contrast, based on these same measures, inoculation with SIVmac251 or SIVmac239 resulted in infection of the brain. These findings are somewhat puzzling in that the primary host for infection within brain is the macrophage, and while SIVmac1A11 replicates well and induces cytopathicity in both T-lymphocytes and macrophages, SIVmac239 has a limited ability to infect macrophages, at least in vitro. However, the SIV-expressing cells in the brains of the SIVmac239 and SIVmac251 inoculated animals appeared to be macrophages. This

could reflect a change in chemokine coreceptor usage during acute infection. The high level of virus replication during this time could likely support the random generation of mutations associated with receptor usage, followed by selection of such mutations. SIVmac1A11 has delayed replication kinetics, relative to Sivmac239, so possibly, the immune response initiated by infection was sufficient to prevent development of the events required for neuroinvasion, such as transendothelial migration of infected monocytes.

Less is known about the genetic and biological features of HIV strains that can enter the brain early. However, it has been reported that the strains typically transmitted, and therefore present during acute infection, are macrophage-tropic [74]. Thus, it is likely that the HIV strains entering the brain during acute infection are macrophage-tropic. Because the term "macrophage tropism" appears to have lost some of its identity in this age of focus on T-cell infection, mention of the historical record appears warranted. Macrophage tropism of HIV was originally described as a *biological* phenomenon of HIV isolates recovered from cultures of macrophages [75, 76]. Subsequently, macrophage tropism and T-cell tropism---the ability to infect macrophages versus neoplastic T-cell lines, respectively---were shown to correlate with chemokine coreceptor usage, macrophage-tropic HIV using CCR5, and neoplastic T-cell-tropic HIV using CXCR4. (Normal T-cells can use either coreceptor.) The relevant fact to this discussion is that macrophage-tropic strains of HIV are present within macrophage lineage cells within the brain, and are most probably carried there by infected monocytes, including during acute infection. It has been proposed that macrophage-tropic strains "evolve," from strains that infect CD4+ T-cells [77], but this seems a nonessential mechanism relative to brain infection, since early HIV neuroinvasion is well documented, and the infected cells which appear in brain are macrophages. The factors that determine whether HIV neuroinvasion elicits meningitis or meningoencephalitis are unknown. While determinants are likely to reside with the host, it is conceivable that viral strain specificity could also play a role. Many of the initial reports demonstrating early HIV neuroinvasion were studies of individuals who experienced symptomatic acute infection [16, 20]. At present, there is insufficient evidence to determine the frequency of this event in the setting of asymptomatic acute infection. Specific characteristics of HIV strains, including features such as replication kinetics, could impact the potential for neuroinvasion. In a recent study, Arrildt and colleagues examined paired M- and T-tropic viruses to identify characteristics of the HIV env gene that distinguish tropism, and might also contribute to HIV genetic evolution in macrophages [77]. They put forth the concept that the emergence of M-tropic HIV strains is a multistep process that includes subtle changes in the conformation of the envelope protein, and enhanced usage of CD4.

In their study of SIV neuroinvasion following high dose oral inoculation with SIVmac251, Milush and colleagues found genetic similarity within the V1-V2 region between the SIV variants present in brain and lymph nodes, suggesting little or no brain-specific selection during acute infection [42]. Some strains of SIV, however, are more neurovirulent, so initial entry of a particular viral strain may not necessarily lead to the establishment of a persisting infection in the brain. The SIV

strain, SIV/17E-Fr, is well known for its ability to invade the CNS during acute infection and induce neurological disease in pigtail macaques [64]. This strain was selected for these capabilities, and serves as the basis for the "rapid progressor" model developed by Clements and colleagues [78]. More recently, a strain of SIVsm that is neurovirulent in rhesus macaques, and can establish infection in the CNS during the early phase of infection, was obtained after a series of 4 in vivo passages [40]. Interestingly, host major histocompatibility complex class I (MHC-1) and TRIM5α genotypes appear to influence neurological disease progression in animals inoculated with this strain.

7.2.5 Role of the Immune Response in Viral Entry

The immune response may play a role in the timing of HIV neuroinvasion. HIV entry during acute infection would necessarily occur in the absence of a host immune response. This absence, could, in a sense, facilitate neuroinvasion since, presumably, both humoral and cellular immune responses could act to some degree as a barrier. The targets--- free virions or virus-expressing cells--- could effectively be neutralized or destroyed prior to entry into the brain, for example, within the circulation. It is also conceivable that virions and/or infected cells could cross the BBB and be confronted by specific antibody or cytotoxic lymphocytes, particularly within the PVS, and subsequently be eliminated. Immune-mediated viral clearance within the brain has been shown to occur in several virus infections in humans. Also, evidence from the SIV system suggests that the immune response is primarily responsible for diminishing the productive infection in brain [24, 79]. This could reflect the elimination of many infected cells from the brain. A decline in the level of viral DNA following acute infection was not seen in the SIV rapid progressor model [57], but this may not be the case in other SIV systems in which the time between acute infection and disease is considerably longer. As noted earlier, the levels of HIV DNA present in brain during the asymptomatic stage are either very low or undetectable. Presumably, the intrathecal immune response would play a predominant role in this immune control, but the systemic response might also contribute by limiting plasma viremia and infected cells.

One would expect that the immune response could participate in controlling subsequent HIV entry into the brain, particularly during the asymptomatic stage of infection. However, while free virus particles might be neutralized and virus-expressing cells killed, latently infected monocytes could remain free of attack within the circulation and continue to cross the BBB uninterrupted, again making use of a normal physiological process. Once latency is broken and they begin to replicate and express HIV, they could, however, become targets of destruction. The timing of their eradication relative to completion of the viral life cycle is difficult to predict. Potentially, infectious particles could be released at levels sufficient to provide for spread of the infection, prior to eradication of the cell. Equally important are the questions of how long a macrophage can live in the brain, and how long HIV

latency can be maintained in this cell. The turnover rate for perivascular macrophages (PVM) appears to be on the order of 3 months, but it is not clear if these cells leave the brain or die. Resident microglia represent a very stable population with a very low turnover rate. Potentially, latently infected macrophages could persist for months or even longer, thereby evading any immune response. Then, when the response wanes or replication is initiated, for whatever reason, the infection could spread to other cells; some of these might also be capable of establishing a latent infection. Thus, there could be a dynamic situation between virus expression and the immune response.

Long-term immunity in the setting of chronic virus infection usually requires occasional low-level virus expression. That is, immunity cannot be maintained indefinitely under conditions of complete viral latency. This is likely to be the case in HIV infection, as well, although the timing and other particulars of the situation may be more complicated, owing to the fact that this infection leads to destruction of the immune system over time. Also, in the case of HIV, as with other lentiviruses, antigenic drift occurs, so that neutralizing antibody is able to remove virus particles bearing envelope proteins that reflect replication of prior members of the quasispecies, but not those species currently being produced [80, 81]. Thus, an important question relates to the level of HIV replication within brain during the asymptomatic stage of infection. As noted earlier, for HIV and SIV, the level of virus replication within brain during this stage appears to be minimal.

7.2.6 Timing of Viral Entry and Establishment of Latency

One of the most important questions regarding early HIV neuroinvasion, is whether this initial entry is able to lead to establishment of a permanent infection within the brain. This has often been assumed to be the case, although evidence is lacking. A persistent infection could either be latent or productive, with virus expression occurring continuously, or possibly even sporadically. In the few studies reported, no, or only very low levels of HIV DNA were detected in brain parenchymal tissues taken from HIV-infected individuals who died during the asymptomatic stage of infection [49, 50]. Although several studies of early SIV infection have examined viral RNA loads, few have assessed viral DNA levels. An exception is in the case of the rapid progressor model developed by Clements and colleagues, in which pig-tailed macaques are simultaneously inoculated intravenously with both a neurovirulent molecular clone and an immunosuppressive viral swarm [57]. These animals experience an accelerated course of immune deterioration and more than 90% develop SIV encephalitis. The animals are at the terminal stage of disease by 3 months. It is conceivable that 3 months is too short an interval for macrophages infected during acute infection to drop out. This model is also characterized by the development of low levels of SIV-specific binding antibody, but no neutralizing antibodies [37]. Evidence from other SIV infection systems strongly suggests that it is the immune response that curtails and diminishes the initial infection within the brain [24, 82].

Thus, this lack of a significant response in the rapid progressor model may also help to explain the persisting SIV DNA levels in this system.

One additional very intriguing study of relevance comes from Hurtrel and coworkers, in which they compared intracerebral versus intravenous SIV inoculation of macaques [36]. Intravenous, but not intracerebral inoculation led to brain pathology characterized by numerous multinucleated giant cells, glial nodules and areas of demyelination. This suggests that following its direct introduction into the brain parenchyma, sustained spread of the virus infection may not readily occur in the absence of immune suppression. A consistent finding in the IV-inoculated animals was an increase in the number of PVM; lymphocytic infiltrates were minimal. These investigators also found that resident microglia were only weakly susceptible to infection. Furthermore, Baskin and colleagues found that intrathecal inoculation of SIV does not result in an increased of brain disease [83], suggesting that the CSF compartment does not provide the virus access to the parenchyma in such a way that sustained infection can result. HIV infection of the brain parenchyma has been reported to be independent of infection of the meninges [84]. However, these processes were shown to occur simultaneously in macaques inoculated intravenously with SIV [24].

Whether the initial entry of HIV into the brain results in establishment of a lifelong brain infection is still unknown. Furthermore, although the brain is frequently considered a reservoir for HIV, the ability of this potential reservoir to reseed peripheral lymphoid tissues also remains in question. It is possible that several factors, both viral- and host-derived, converge to determine the final outcome. Obviously, these questions have considerable clinical significance, and their answers could impact the direction of treatment options. Therapeutic targets could include residual virus in brain, as well as the trafficking monocyte population. Although ART has clearly decreased the incidence of the more severe forms of HIV-associated neurological disease, levels of motor and cognitive impairment persist in some patients, and some experience virologic failure which includes the presence of HIV RNA in CSF. More studies to address these issues are urgently needed.

7.2.7 Timing of Entry and Compromise of the Blood-Brain Barrier

Compromise of the BBB could obviously contribute to the ability of HIV to enter into the brain. (The reader is referred to reviews by Atluri et al. [85] and Ivey et al. [86] and Annunziata [87].) An issue of importance regarding the timing of HIV entry into the brain relates to whether compromise of the BBB occurs during neuroinvasion, or is even necessary. The frequency and extent of this compromise is likely to vary, depending on the stage of HIV infection. More frequent and more extensive disruption of this barrier is probable during endstage disease [88], when opportunistic infections may be present, whereas during the asymptomatic stage, the endothelium is more likely to remain unperturbed. Acute infection, particularly that

accompanied by meningitis or meningoencephalitis, could be associated with disruption of the BBB. Interestingly, however, in their serial sacrifice study of neuroinvasion during acute and early SIV infection, Smith et al. found that BBB integrity was maintained throughout the course of study (up to 23 weeks), as determined by the CSF albumin/serum albumin ratio [24]. This method, of course, cannot detect more subtle barrier compromise. In contrast, Stephens et al. did detect BBB disruption during acute SIV infection [89], and Andersson and colleagues found evidence of disruption during the asymptomatic stage of HIV infection [90].

Whether or not BBB disruption facilitates HIV neuroinvasion may also depend on the mode of viral entry. Monocyte transendothelial migration is a normal physiological process, so HIV entering as infected monocytes, particularly latently-infected ones, would not require any alteration in the endothelium. Neuroinflammation, however, involves changes in the BBB, including endothelial cell alterations, and can be characterized by leukocyte infiltration [91]. Consequently, its presence could permit the entry of greater numbers of HIV-infected monocytes or even infected T-lymphocytes. Of note, high levels of monocyte chemoattractant Protein-1 (MCP-1) have been found in the CSF of patients with AIDS dementia, which could, as has been suggested, amplify the recruitment of additional HIV-infected monocytes into the CNS [92]. In addition, increased levels of MCP-1 precede and predict the development of SIV encephalitis.MCP-1 [93]. It seems likely that free HIV particles could enter the brain, along with other serum constituents, at sites of barrier disruption. The subsequent fate of these particles is, however, uncertain. Also, evidence from the SIV model suggests that activation of the BBB requires cell-associated virus [94]. Thus, while compromise of the BBB could facilitate or enhance HIV neuroinvasion, it does not appear to be essential.

7.3 Modes and Sites of Viral Entry

As mentioned earlier, HIV could enter the brain as either free virus particles or infected cells. These infected cells could be either monocytes, or CD4+ lymphocytes, the two primary hosts for productive infection. There are a few reports of HIV infection of other types of leukocytes, including CD8+ T-cells and NK cells, but the significance of these is unclear, and they will not be considered further. While CD4+ T-lymphocytes could conceivably transport HIV, either as a product of active replication, or in a latent form, evidence supporting this possibility is lacking in both studies of humans and SIV-infected macaques. The cells actively expressing HIV or SIV within the brain parenchyma, including within the PVS, have been shown by numerous studies to be macrophages [35, 41, 95, 96]. Moreover, CD4+ lymphocytes are themselves rarely seen in the brains of HIV-infected individuals [96], arguing against the possibility that their entry as latently-infected cells is a significant event. The transendothelial migration of infected blood-borne monocytes clearly appears to represent a primary mode of HIV neuroinvasion. As noted above, the infected monocytes could cross the BBB making use of a normal physiological

process, and/or take advantage of conditions such and neuroinflammation and/or the presence of opportunistic infections, which could facilitate transendothelial migration. Moreover, alterations integral to the monocytes themselves, such as the presence of a state of cellular activation, could enhance their transmigratory capabilities. The factors and processes that set the stage for monocyte transendothelial migration, and participate in its execution, will be considered here. Similarly, information regarding the mechanisms involved in the movement of HIV particles across the BBB will be discussed. Lastly, some viruses appear to be able to enter the CNS via axonal transport, a classic example being poliovirus [97]. Although HIV can be detected near peripheral nerves, there is no evidence to suggest that it can travel along nerves to the brain.

7.3.1 Viral Entry, the Blood-Brain Barrier, and the Perivascular Space

The microvascular endothelium that lines blood vessels within the brain differs significantly from that found elsewhere in the body. The brain microvascular endothelial cells, along with other elements, form a diffusion barrier, the BBB, which prevents most substances in the blood from entering into the brain [98]. Tight junctions that form between endothelial cells are a key element of this barrier, and thereby restrict diffusion. Pericytes and the foot processes of astrocytes are also components of the barrier, a primary role for astrocytes being to maintain tight junctions. Pericytes appear to contribute to structural support, and also play a role in angiogenesis and formation of the tight junctions [99]. Obviously, disruption of the barrier, or perturbation of the endothelial cells, could increase the opportunity for HIV to enter into the brain.

Regardless of whether HIV crosses the BBB in infected cells or as free virions, once it completely traverses the endothelium, it encounters the PVS, formally referred to as the Virchow-Robin space. This is a unique neuroanatomical structure, in that it is contiguous with the sub-pial space and partially filled with CSF [100]. Under normal conditions, small numbers of perivascular macrophages reside within these spaces, where they are thought to perform immune surveillance. For the entering virions to be able to establish infection, they must encounter a susceptible target cell and infect it. Given the fragile nature of these particles, and their apparent short half-life, this event most likely must occur within hours of entry. Macrophages are normal residents of the PVS, could encounter cytotoxic T-cells, and also could be eliminated. Little is known about HIV-specific CTL within the brain, including within the PVS. However, such cells have been detected in CSF, even without in vitro expansion [101]. The entry of latently infected cells might represent a particularly attractive strategy for the virus, permitting initial evasion of the immune response, while preserving the potential for virus replication and expression later, when a less hostile microenvironment might be present.

7.3.2 Viral Entry via Infected Monocytes

Not surprisingly, a number of viruses infect monocytes and macrophages. Monocytes are particularly attractive hosts for viral latency because of their longevity, their generally quiescent nature, their particular roles within the immune response, and their ability to traffic into almost every tissue and organ within the body under normal circumstances. Important to keep in mind is the fact that HIV is a lentivirus, and what characterizes lentiviruses is their ability to infect and replicate in macrophages. Perivascular macrophages are bone marrow-derived, found adjacent to blood vessels, turn over continuously within the CNS, and are primary targets for HIV-1, HIV-2 and SIV infection [3, 102]. Examples of HIV-expressing perivascular macrophages are shown in Fig. 7.1.

Although the evidence suggests that both PVM and resident microglia serve as hosts for productive HIV and SIV infection in the brain, the relative contributions of each cell type remains in question. This issue impacts our understanding of the potential for HIV latency and persistence in the brain. Is the continual, albeit low level, influx of HIV-infected monocytes into the PVS, vital to maintaining the infection in brain? Is this process halted or altered by systemic suppression of viral replication via ART? Normally, in brain, CD163 is expressed by PVM but not microglia [103]. In a study of human and macaque specimens, infected with HIV and SIV, respectively, Fischer-Smith and colleagues demonstrated productive viral infection in CD163+ cells in brain in humans and macaques with encephalitis, colocalization of CD163 and CD16 gene expression, and in macaques, a correlation between increased numbers of circulating CD163+/CD16+ monocytes, and increased viral load and CD4+ T-cell decline [104]. (Increased numbers of circulating CD16+ monocytes correlates with clinical HIV dementia [105].) Moreover, in an elegant study tracing the trafficking of fluorescein-labeled monocytes from the blood into the CNS in macaques infected with SIV, Clay and coworkers found dye-positive monocytes in the choroid plexus and perivascular spaces between days 12 and 14 post infection [106]. The dye-positive monocytes present in the brain expressed CD16, as well as the macrophage marker CD68. Monocyte infiltration correlated with the presence of SIV in brain tissue and CSF, and induction of the proinflammatory molecules, MCP-1 and CXCL9/MIG. These and other findings indicate that blood monocytes can carry HIV and SIV into the brain. They also indicate a link between increased monocyte trafficking into the brain and the development of encephalitis, which is the pathological correlate of clinical HIV dementia [67, 68].

In the natural history setting, the incidence of HIV neurological disease appears to be significantly higher in children. Rates ranging from 12–90% have been reported [107–110]. Encephalopathy may even be the initial manifestation of the presence of AIDS [111]. HIV DNA has frequently been detected in post-mortem brain tissues from children who died with AIDS [84, 112], with a significant amount present as unintegrated molecules [113]. HIV expression has also been detected in pediatric brains, the productively-infected cells being limited to macrophages and microglial cells [114]. In addition, these investigators demonstrated expression of

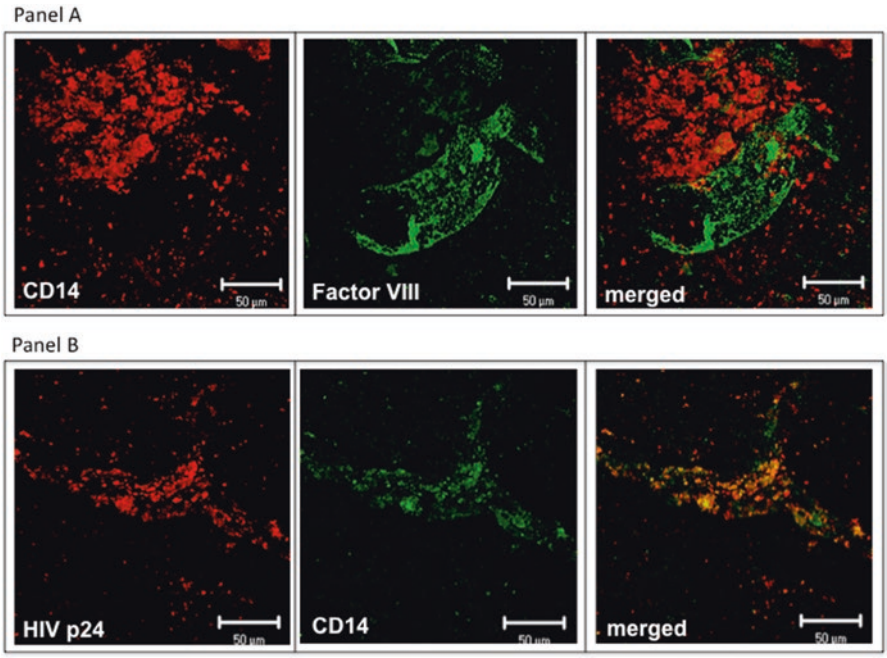

Fig. 7.1 Confocal microscopy images of immunostained sections of mid frontal gyrus taken from the brain of NeuroAIDS case #507, autopsied at Johns Hopkins Hospital. This patient exhibited HIV dementia, and HIV encephalitis was present. Panel (**a**) shows the accumulation of large numbers of macrophages (CD14+ cells) adjacent to a blood vessel stained with anti-Factor VIII antibody. Panel (**b**) demonstrates that the HIV p24-expressing cells are primarily macrophages (CD14+), as evidenced by colocalization of the *red* and *green* signals, which appears as a *yellow* color

the HIV nonstructural protein, nef, within astrocytes, suggesting HIV infection, but restricted replication, in this cell type. As with HIV encephalopathy in adults, however, there is not a clear correlation between the level of HIV expression, and the presence of neurological disease [115].

Although in utero transmission of HIV has been documented, infants usually acquire the infection perinatally [116]. It is difficult to determine the timing of HIV entry into the brain in these situations, and few studies have attempted to do so. Early in the epidemic, children also acquired HIV infection from blood products or transfusions [117]. There are also significant differences between the course of infection in adults and children, and these are particularly apparent in brain, partly owing to the fact that the brain is still developing in children [118, 119]. Discussion of these issues is beyond the scope of this chapter. However, relative to the question of the mode of HIV entry into the brain, it may be important be consider the fact that infants and children have significantly higher levels of circulating monocytes compared to adults [120, 121]. While the absolute monocyte counts in adults range between 285–500 cells per mm^3, in children counts are up to 750 cells per mm^3.

Moreover, during the first 2 weeks of life, absolute counts of 1000–1200 are normal [120]. If, as data from the SIV model suggest, initial entry of the virus into the body is followed by seeding of the bone marrow by day 3 [122], and subsequent trafficking of infected monocytes into the brain soon after, then this normal relative monocytosis in infants may facilitate HIV seeding of the brain. Interestingly, high levels of circulating monocytes, along with low levels of CD8+ T-cells, have been shown to be a predictor of progressive encephalopathy in HIV-infected children [123]. Of course, other unique features of the developing brain may also play a role.

A number of factors have been identified which appear to enhance monocyte migration across the BBB. These include adhesion molecules [124], chemokines [125–127], and HIV products [128]. Interestingly, Koedel and colleagues found that the nef protein, but not tat, gp120 or gp160, was chemotactic for leukocytes in vitro [129].

7.3.3 Viral Entry via Cell-Free Virions

Based primarily on studies using in vitro models, a number of molecules have been implicated in the transport of HIV particles across the BBB, typically via perturbation of the BBB. These include cell-derived products [130, 131], drugs [132], and HIV-specific proteins [133–135]. Some of these appear to act by directly perturbing the endothelium, for example, by eliciting neuroinflammation. Others may act in more subtle ways, relying on natural processes to orchestrate the effects. In all of these instances, however, a common consequence could be enhancement of the potential for HIV particle transmigration, and perhaps also cell migration. Also, although they lack CD4, the primary receptor for HIV, there is evidence suggesting that endothelial cells themselves can serve as hosts for HIV infection and replication [136]. Endothelial cell infection has also been observed in the SIV system [137]. This infection could promote direct entry of HIV virions into the PVS, the particles being derived from endothelial cells actively replicating and expressing the virus.

Transport of HIV particles through endothelial cells, with subsequent release on the parenchymal side, has also been proposed as a mechanism by which HIV virions can cross the BBB [138, 139]. In this case, the particles are taken up from the blood by means of adsorptive endocytosis or macropinocytosis. In an elegant study using human brain microvascular endothelial cells as a model of the BBB, Liu et al. demonstrated that this process involves lipid rafts, MAPK signaling and glycosylaminoglycans, and proceeds without any disruption of the endothelial cell tight junctions [139]. Importantly, they also found that while most of the virion-containing vesicles went on to fuse with lysosomes, resulting in the degradation of the particles, approximately 1% of the particles found their way to the abluminal side.

Paracellular entry via the endothelial cell tight junctions has also been observed, and suggested as a mode of HIV virion transport across the BBB [140]. TNFα appears to participate in this process. HIV Tat-induced apoptosis of the endothelial cells has also been proposed as a paracellular route, the consequence of this apoptosis being the creation of perforations within the BBB that can permit virus particle entry [141].

7.3.4 Entry via the Choroid Plexus

The choroid plexus (CP) is a highly vascular structure located within the ventricles of the brain. It is composed of a polarized single epithelial layer overlaying a stroma containing fenestrated capillaries. The epithelial cells are joined by tight junctions located on the apical side and hence, they form a "blood-CSF" barrier. These cells secrete the CSF and provide selective active transport of micronutrients, metabolites and drugs into the CSF [142]. They also absorb material from the CSF and transport it to the blood, thereby providing a cleansing function [143, 144]. Moreover, the CP epithelial cells have been shown to take up virus-sized particles (100 nm microspheres) injected into the ventricles, and they also bear Fc receptors [145]. The fenestrated capillaries allow the passage into stroma of not only macromolecules, but also, unfortunately, microorganisms, which helps to explain why this tissue is a frequent site of microbial infection, including opportunistic infections in AIDS patients [146]. Immune complexes are also very commonly observed within the CP in patients with AIDS or autoimmune diseases, the likely origin being the bloodstream [147]. Thus, the CP is not protected by a BBB analogous to that within the brain parenchyma.

Macrophages are present within the CP stroma and these appear to have a more activated phenotype, compared to the parenchymal microglial cells [148]. A distinct population of monocyte-derived macrophages, referred to as epiplexus macrophages, is also present [149, 150]. These are located on the apical side of the epithelium, and their numbers increase significantly following injury or an inflammatory challenge to the brain [151]. The migration of blood-derived monocytes to the apical side of the CP epithelium, however, has been shown to be a frequent, ongoing process, even under normal conditions [149]. Owing in part to their predilection for macrophages, a number of lentiviruses infect the CP; these include VISNA, CaEV, SIV and FIV. This infection occurs early, and it appears to play a critical role in maintaining viral persistence within the CNS [152]. The CP has also been shown to be a site of HIV infection. For example, in a study of 25 AIDS cases, Falangola et al. found HIV infection within the CP in 44% of cases, and determined that the virus-expressing cells were macrophages [153]. In a separate study, HIV+ cells exhibiting a dendritic morphology and also strongly immunoreactive for HLA-DR were observed, indicating that CP dendritic cells might also be targets for productive infection within this tissue [154]. Of relevance here, HIV infection may be present within the CP during the asymptomatic stage of infection [51, 155]. Whether or not it becomes infected during acute HIV infection is unknown. However, in a serial sacrifice study of SIVmac251-inoculated macaques evaluated at 7 days and also 1, 2 and 3 months following inoculation, infected cells were rarely detected within the CP [36] This led the authors to conclude that the CP is not a major site of viral entry during *early* systemic SIV infection.

7.4 Influence of Antiretroviral Therapy on HIV Neuroinvasion

The BBB is designed to tightly control the passage of molecules and other substances from the blood into the brain. Specific transporters facilitate entry into the brain of such simple molecules as glucose and amino acids. Larger, yet still relatively simple molecules, such as insulin and transferrin, cross the BBB via receptor-mediated endocytosis [156]. Many pharmacologic agents, including antiretroviral drugs, cross the BBB by means of transport systems. The nucleoside reverse transcriptase inhibitors (NRTIs) use probenecid efflux transport mechanisms, which do restrict brain entry, and the protease inhibitors (PIs) appear to use the efflux transporter, P-glycoprotein, and also perhaps multi-drug resistance protein (MRP) [157, 158]. P-glycoprotein appears to severely limit brain uptake of the PIs, in that these drugs cannot be detected in the CSF of patients using them [157]. A comprehensive summary of the transport of anti-HIV drugs across blood-CNS interfaces has been published by Varatharajan and Thomas [159].

The incidence of HIV-associated dementia (HAD) has declined since the introduction of highly active antiretroviral therapy (HAART) [160, 161]. HAART is nowadays simply referred to as ART, and typically includes a PI. This class of drugs appears to play a prominent role is slowing the course of HIV disease progression and similarly, in lowering the incidence of HIV neurological disease. Since the PIs do not appreciably cross the BBB, exactly how they act to prevent or retard the development of dementia is unclear. It would seem that they do not exert their influence on dementia by acting directly to inhibit HIV replication within the brain parenchyma. We have proposed that PIs might act to decrease the activation of monocytes, thereby lowering their ability to traffic into the brain [162]. This decrease in monocyte trafficking would likely lead to a decrease in monocyte-mediated HIV entry into the brain. How PIs might interfere with monocyte activation is an issue for speculation, but their interference is likely mediated by a series of events that take place within the bone marrow, and involves both HIV replication and immunological responses.

ART has also dramatically improved the longevity and health of HIV-infected individuals and in many, some degree of immune restoration occurs. However, a small number of patients develop an inflammatory reaction in response to this treatment, or experience reactivation or exacerbation of opportunistic infections [163]. This condition is referred to as “immune restoration disease (IRD) or “immune reconstitution inflammatory syndrome (IRIS). The pathogens most commonly seen in the situation are Mycobacterium avium complex, Mycobacterium tuberculosis, Cryptococcus, Herpes zoster and Hepatitis B and C [164–166]. In some cases, these infections extend to the brain, where they can be extremely damaging and difficult to treat [167]. The development of a severe, demyelinating leukoencephalopathy, in the absence of any detectable opportunistic infections, has also been observed in patients on ART [168]. High levels of HIV RNA were detected in post-mortem brain tissues recovered from these individuals, and intense perivascular infiltrates

with HIV gp41+ macrophages were observed, along with widespread myelin loss, axonal injury, microgliosis and astrocytosis. In a separate report, two cases of encephalopathy in association with ART were described, one acute and the other a worsening of a pre-existing condition [169]. Both patients died. Autopsies were performed and revealed the presence of massive and diffuse perivascular and intraparenchymal infiltrates of CD8+ lymphocytes within the brain. The patient with pre-existing encephalopathy had classic HIV encephalitis with multinucleated giant cells and HIV p24+ macrophages, but in the other (acute presentation), there was no evidence of replicating HIV. The mechanisms underlying these reactive syndromes are unknown. Likewise, it is not clear if this response can lead to significant enhancement of HIV neuroinvasion. Obviously, opportunistic infections, both at the systemic level and within the brain, could perturb the BBB and lead to HIV entry. More significant, however, could be the enhancement of infected monocytes trafficking into the brain. The limited evidence available supports this possible scenario. Thus, ART could, in some individuals, facilitate HIV neuroinvasion. Typically, this would be expected in patients with end-stage disease, but because the triggers for IRD have not been conclusively identified, and since some patients use ART prior to the presence of clinical AIDS, IRD might possibly arise during the asymptomatic stage of infection. Fortunately, the response can sometimes be controlled by withdrawal of ART and/or treatment with steroids.

7.5 Concluding Remarks

Although our understanding of HIV infection within the brain continues to grow, significant gaps remain. Still unclear is whether the brain, particularly in the presence of continual ART, represents an HIV reservoir of significance, and with the capability to reseed lymphoid tissues with infectious virus. While speculative at this time, such reseeding is not beyond possibility. In Fig. 7.2 we present our model for HIV entry into and exit from the brain. Studies in animals demonstrate that cerebral interstitial fluid moves from the brain parenchyma along channels comprised of perivascular spaces, into the subarachnoid space, where it becomes contiguous with the CSF. From the subarachnoid space, CSF and interstitial fluid can (1) make their way into the venous circulation via drainage into the dural sinus, or (2) pass through subarachnoid spaces along nerves that join with cervical lymph nodes [170, 171]. Thus, infectious HIV particles, or possibly even infected cells, could leave the brain and enter either the bloodstream or cervical lymph nodes. Hopefully, future viral genetics studies, and research using the SIV and other animal models, will evaluate this hypothesis.

The evidence currently available indicates that HIV can enter the brain during all stages of the systemic virus infection---the acute (primary) phase, the asymptomatic phase, and the end stage, when immunodeficiency and its consequences are present. Neuroinvasion during acute infection may or may not be accompanied by the manifestation of neurological signs and/or symptoms, and in some cases, encephalitis,

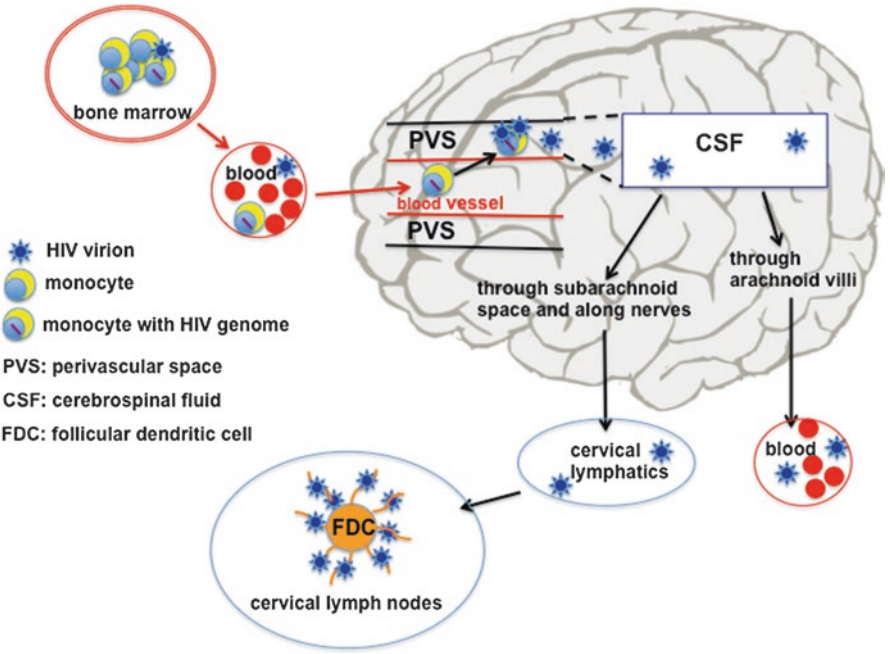

Fig. 7.2 Proposed route of HIV entry into and out of the brain. Highlighted is HIV entry mediated by latently infected monocytes trafficking from the bone marrow, through the blood, and into perivascular spaces within the brain, where HIV replication can be instigated. Because interstitial fluid within the perivascular spaces ultimately joins the CSF, virions produced by perivascular macrophages can find their way into the CSF. Once within the CSF, virions can move through the arachnoid villi and end up in the blood, or move through the subarachnoid space or channels alongside nerves, and enter the cervical lymphatics. From cervical lymphatics, the virions have access to cervical lymph nodes, where they can infect susceptible target cells, or become trapped on the follicular dendritic cell network

meningitis or meningoencephalitis may develop. High-level viremia is often present in these patients, and given the setting of inflammation, cell-free virion-mediated HIV neuroinvasion seems probable. This early neuroinvasion appears to be curtailed by the development of an immune response. Inflammation accompanying this immune response might also, however, contribute to enhancing HIV entry into the brain, particularly by perturbing the BBB. Still unclear is whether early neuroinvasion establishes lifelong HIV persistence in brain. Studies using the SIV rapid progressor model demonstrate decreased viral replication in brain, as evidence by viral RNA levels, but "steady-state" levels of viral DNA, as the acute phase of infection wanes. While suggestive of viral persistence following early initial entry of virus into the brain, the nature of this model—accelerated development of disease—may not accurately reflect events in humans, where the course of disease is more prolonged.

There is little doubt that in the natural history setting, HIV enters the brain during late-stage infection. This is part of a systemic process that, as noted earlier, involves the seeding of many nonlymphoid tissues with HIV. Immune control of the infection has been lost. Opportunistic infections frequently develop within the brain at this time, which results in further perturbation of the BBB and induction of additional processes that can further enhance HIV entry, and likely even HIV replication within the brain. Neuroinflammation appears to be an important contributor to HIV neuroinvasion, and also perhaps HIV replication in brain. A more mechanistic understanding of how molecules such as viral regulatory gene products and immune regulatory molecules associated with neuroinflammation and/or loss of BBB integrity, is needed.

In the absence of perturbations in the BBB, HIV entry into the brain during the asymptomatic stage of infection is likely mediated primarily by monocyte transendothelial migration. This is a normal physiological process, it occurs at low levels, and would involve migration of both infected and uninfected monocytes. HIV-infected monocytes can be present in blood even during ART. The degree to which these are latently infected is unknown, but either way, they represent a means for the continual introduction of HIV into the brain and therefore, controlling monocyte trafficking into the brain may represent an important target of therapeutic intervention. When, how and in what anatomical locations monocytes with migratory potential become infected remains a matter of speculation at this time, but the identification of markers such as CD163 and CD16 that link circulating monocytes with virus-infected PVM in brain, points to a direction for further research.

There is no doubt that some HIV-infected individuals on long-standing ART still experience HIV-associated neurological disease. Accurate diagnosis of more minor impairment can be confounded by factors such as drug and alcohol use, but still, virologic failure in CSF occurs, and indicates a link between the presence of HIV within the brain and clinical disease. Efforts to develop new therapies aimed at keeping HIV “silent” in the absence of continuous ART should not disregard infection in the brain.

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Conflict of interest The authors report no conflicts of interest.

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Chapter 8
Neuropathology of HIV-1 Disease

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Keywords Cerebral β-amyloidosis • HIV-associated neurocognitive disorders (HAND) • HIV encephalitis • Multifactorial • Opportunistic diseases • Pathophysiology • Vascular pathology • Cerebral β-amyloidosis • Cryptococcosis • Cytomegalovirus disease • Gliosis • HIV-associated neurocognitive disorders • HIV encephalitis • Primary central nervous system lymphoma • Progressive multifocal leukoencephalopathy • Synaptodendritic degeneration • Tau pathology • Toxoplasmosis • Vascular pathology

Core Message

Mild forms of HAND remain common despite widespread use of cART.

HAND is not consistently correlated with HIV encephalitis.

HAND is a multifactorial syndrome attributed to the extent of brain HIV infection and shaped by a variety of comorbid factors.

The pathophysiology of HAND may vary from case to case, representing the combined effects of multiple contributing factors.

8.1 Introduction

In the typical course of HIV-1 disease prior to the era of combination antiretroviral therapy (cART), almost all HIV-infected adults eventually succumbed to advanced immune suppression and were affected by a variety of AIDS-defining conditions, including opportunistic diseases of the central nervous system (CNS) [1–11]. With

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regard to the neurobehavioral complications, HIV-associated neurocognitive disorders (HAND) of varying severity were found in more than half of patients with AIDS [12, 13]. The incidence rates of HIV-associated dementia (HAD, also called *HIV encephalopathy*), the severest form of HAND, were up to 14% in reports from North America and Europe [14–17].

Widespread implementation of cART in HIV-infected populations has markedly reduced the incidence of opportunistic diseases and HAD [18–21]. Effectively cART-treated patients can achieve systemic viral suppression, maintain immune function, and expect to live to old age [21]. However, cART neither cures HIV disease (i.e. viral eradication) nor restores full health [22]. The milder forms of HAND, that is, asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND), remain prevalent in people living with HIV [23–26] and can affect even individuals with undetectable or low plasma viral loads and high CD4 T-cell counts [24, 26–28]. HAND is a multifactorial syndrome attributed to the extent of CNS HIV infection and shaped by an array of comorbid factors, such as HIV-1 genetic variants, host genetic polymorphisms, chronic cART-related neurotoxicity, substance use, hepatitis C virus (HCV) coinfection, brain aging, and systemic chronic inflammation and metabolic disturbance [29].

New clinico-neuropathological entities have emerged in the cART era, including immune reconstitution inflammatory syndrome (IRIS), severe inflammatory HIV leukoencephalopathy, HIV-associated CD8 T-cell encephalitis, and burnt-out lesions. In addition to CNS parenchymal pathology, HIV-infected persons may be at increased risk of cerebrovascular diseases as a result of the metabolic and systemic effects of HIV and cART on vascular cell components (e.g. endothelial cells, smooth muscle cells, and pericytes) [29–42]. As people receiving effective cART live to older age, they will be exposed to a greater extent to conventional vascular risk factors (e.g. aging, hypertension, diabetes mellitus, hypercholesterolemia, and cigarette smoking) [29].

The prevalence of older HIV-infected individuals is increasing. In the United States, persons aged 50 years or older accounted for 42.4% of all people living with HIV in 2013 and constituted 16.8% of new HIV diagnoses in 2014 [43]. In addition, older age may be associated with delayed diagnosis of HIV infection. In a retrospective study of individuals newly diagnosed with HIV infection from 2006 to 2011 in New York City with an estimated HIV prevalence of 1.7% [44], adults aged ≥50 years constituted 21.3% of all newly diagnosed individuals. Among these older adults, 70% were diagnosed as in-patients and 68.9% concurrent with AIDS, compared to 41.7% and 38.9% of younger adults, respectively.

This chapter describes parenchymal and vascular pathological changes commonly found in the CNS of HIV-infected adults, including HIV-specific pathology and nonspecific pathology. Opportunistic CNS diseases are also included as they likely continue to affect patients who eventually succumb to advanced immune suppression due to either lack of access to cART or treatment failure [45]. The potential effects of comorbid factors on HAND pathogenesis are discussed.

8.2 HIV-Associated Neurocognitive Disorders

Neurocognitive disorders specifically associated with HIV disease range from mild degrees of neurocognitive and motor impairment to frank dementia. In research settings, seven domains of neurocognitive functioning are typically assessed using a comprehensive battery of standardized neuropsychological tests [23, 46, 47]: information processing speed, attention/working memory, learning, recall memory, verbal fluency, abstraction/executive functioning, and motor/psychomotor speed, with statistical correction for demographic variables (i.e. age, sex, ethnicity, and education).

According to the Frascati research terminology and criteria [48], HAND is categorized into three disorders of increasing severity of neurocognitive impairment: ANI, MND, and HAD. HIV-infected individuals who have mild impairment (one standard deviation below the mean) in two or more neurocognitive domains are diagnosed as having ANI if the impairment does not affect their daily functioning (as determined by self-reporting or by observation of others) or MND if the impairment interferes modestly with daily functioning. HAD is characterized by severe impairment (at least two standard deviations below the mean) in two or more neurocognitive domains with marked interference with daily functioning. By definition, a diagnosis of HAND is made only after excluding delirium and preexisting causes of neurocognitive impairment, such as other CNS infections, CNS neoplasms, cerebrovascular diseases, preexisting neurological diseases, and severe substance abuse compatible with CNS disorder [29, 48].

Prior to the cART era, neurocognitive impairment of varying severity was observed up to 55% in patients with AIDS who had no opportunistic CNS diseases [12]. The incidence of HAD in patients with AIDS was up to 14% in North America and Europe [14–17, 20]. Patients with low (<200 cells/μL) or even intermediate (200–349 cells/μL) CD4 T-cell counts, previous AIDS-defining conditions, longer duration of HIV infection, and older age at seroconversion were at increased risk of HAD [20]. In Kampala, Uganda, a survey of 117 antiretroviral-naïve HIV-infected persons with moderate to severe immune suppression during 2009–2010 [49] showed that all categories of HAND were highly prevalent (ANI 18.8%, MND 32.5%, and HAD 41.0%).

In populations with ready access to cART, the incidence of HAD has markedly decreased [19, 20], but the overall prevalence of HAND has not declined [23, 24, 26, 47, 50–52]. For instance, in a cross-sectional study from six university HIV clinics across the United States (*CNS HIV Antiretroviral Therapy Effects Research* [CHARTER]) during 2003–2007 [25], among 1316 HIV-infected individuals who did not have major comorbid risks for CNS dysfunction, 32.7% had ANI, 11.7% MND, and 2.4% HAD. The pattern of neurocognitive impairment in the cART era may more commonly involve memory, learning, and executive functioning domains, in contrast to that in the pre-cART era with predominant impairment in motor skills, cognitive speed, and verbal fluency [47, 53].

Further, HAND can affect even individuals with undetectable or low plasma HIV-1 RNA loads and high CD4 T-cell counts [24–28]. These findings suggest that successful control of systemic HIV replication and restoration of cellular immunity

may not fully protect against HAND. Some cART regimens may be unable to effectively control HIV replication in the CNS due to their poor CNS penetration [51, 54] and/or the presence of independent genetic evolution and development of antiretroviral drug resistance of HIV strains within the CNS (i.e. viral compartmentalization) [55–62]. In case CNS HIV replication is well controlled in response to effective cART, HAND can be attributed to various combinations of comorbid factors that may additively or synergistically cause neural injury [29, 63].

More variability in the clinical course of HAND has been observed in the cART era, that is, neurocognitive deficits may progress, improve, fluctuate, or remain static over time [26, 52, 53, 64]. In a longitudinal CHARTER study of 436 HIV-infected persons with mean follow-up of 35 months [65], neurocognitive improvement or decline was driven by a variety of metabolic factors (e.g. hematocrit, blood albumin, and aspartate aminotransferase levels), antiretroviral treatment, and comorbid factors. MND and HAD adversely affect adherence to antiretroviral treatment, daily life activities, and employment [66–68] and are independent risk factors for AIDS-associated death [69, 70]. Also, patients with ANI may be at increased risk of earlier development of MND and HAD [52, 71]. Even in the acute and early stages of HIV infection, HIV-infected persons are found to be at elevated risk of neurocognitive impairment (particularly in the functioning domains of learning and information processing speed) and dependence in real-world functioning (including cognitive symptoms in daily life, basic and instrumental activities of daily living, clinician-rated global functioning, and employment), when compared to the non-HIV control participants [72, 73].

The longer duration of HIV infection has been shown to associate with poorer neurocognitive performance even in untreated HIV-infected persons with CD4 T-cell counts above 500 cells/μL [74]. However, if HIV replication is effectively controlled early in the clinical course, the HIV infection duration may no longer be a risk factor for HAND [75]. Early diagnosis of HIV infection followed by immediate initiation of cART (before the vulnerable organ systems are irreversibly damaged) may prevent the complications of HIV disease, especially HAND and serious non-AIDS-related conditions [47, 66, 75–77]. The International AIDS Society announced in 2015 the Vancouver Consensus [78], calling on the leaders of the world to implement HIV science, commit to provide access to immediate antiretroviral treatment to all people living with HIV, and support the UNAIDS 90/90/90 goals. The goals state that by the year 2020, 90% of all people living with HIV will know their HIV status, 90% of all people with diagnosed HIV infection will receive sustained cART, and 90% of all people receiving cART will have viral suppression. Still, the long-term effects of earlier cART initiation on neurocognitive functioning need to be assessed in longitudinal studies since certain antiretroviral drugs may be neurotoxic [29, 66, 79–83].

Recently, the Frascati criteria [48] have been challenged with regard to the apparent over-diagnosis of ANI (i.e. high false positive rates) [29, 84]. In a study of 103 HIV-infected persons in comparison to 74 well-matched non-HIV control participants [85], neurocognitive impairment based on Frascati criteria was present in 48% and 36%, respectively. In contrast, based on a statistical method referred to as *multivariate normative comparison* [86], neurocognitive impairment was diagnosed in 17% in the HIV-infected group and 5% in the control group.

8.3 Challenges to Establish Neuropathological Correlates of HAND

Although the clinical manifestations of some opportunistic CNS diseases might mimic dementia, a causal relationship between HIV and HAD was first suggested in 1985 by Shaw et al. [87]. Using Southern blot analysis and in situ hybridization, they detected HIV-1 DNA and RNA, respectively, in brain tissue specimens from a small group of patients with HAD. In the same year, Levy et al. [88] and Ho et al. [89] successfully isolated HIV from the neural tissues and cerebrospinal fluid (CSF) of patients with HAD by cocultivation with peripheral blood mononuclear cells.

Is productive HIV infection of the CNS (histopathologically represented by HIV encephalitis [90]) a neuropathological correlate of HAD (or HAND)? Since the early years of NeuroAIDS research in the mid-1980s, investigators have been aware of a *far from exact* clinicopathological correlation between the severity of HIV-associated neurocognitive impairment and that of brain pathology (HIV encephalitis in particular) [91–94]. For example, Wiley and Achim [95] found that all AIDS patients with HAD carried high brain burden of HIV-1 gp41 (envelope) protein assessed by immunohistochemistry. Many nondemented patients had lower brain viral loads; however, there were a subset of patients who had high brain viral loads without clinically documented neurocognitive impairment. In contrast, Brew et al. [96] found that only 61% of patients with HAD showed HIV-1 p24 (capsid)-immunoreactive cells in the brain. A similar clinicopathological correlation was not observed in milder forms of HIV-associated neurocognitive impairment [90, 97, 98]. Other studies suggested that high levels of microglial activation [99] and aberrant cytokine expression [100] in the CNS (markers of neuroinflammation) were more closely related to HAND diagnosis. Based on a comprehensive battery of neuropsychological tests, Cherner et al. [101] reported that HIV encephalitis was found at autopsy in all 14 patients with syndromic neurocognitive impairment and in four of five patients with subclinical neurocognitive impairment. However, in this study, nine of 20 neuropsychologically normal patients also had HIV encephalitis. Recent autopsy studies further showed that HAND was not consistently correlated with HIV encephalitis [42, 102–106].

There are potential reasons for the apparent inconsistency in the correlation between HAND and HIV encephalitis in clinicopathological analyses reported in the literature. First, productive HIV infection is not uniformly distributed in the CNS [107]. The extent and regional distribution of lesions may determine the clinical manifestation [106, 108, 109]. However, there are no consensus criteria for grading the severity of HIV encephalitis on standard histopathology [106, 109], although grading has been attempted on immunohistochemistry for HIV-1 gp41 in brain tissue sections [94, 110–112]. Quantitative (in addition to qualitative) assessments of both HIV burden (e.g. HIV-1 RNA assays) in tissue homogenates [101, 107, 113] and relevant pathological changes (e.g. glial fibrillary acidic protein [GFAP]-immunoreactive astrogliosis and ionized calcium-binding adaptor molecule-1 [Iba1]-immunoreactive microgliosis) in tissue sections [114–116] sampled from multiple CNS regions may be a better approach in an attempt to explain the variation in both the pattern and severity of HIV-associated neurocognitive impairment [91, 117].

Second, HAD in the pre-cART era was a progressive disorder leading to death within months [53]. Since the advent of cART, HAND of varying severity has been treatable in most instances [21, 118], so affected patients will not die shortly after the onset of HAND (or even HAD). In this scenario, there may be a significant temporal discrepancy between the occurrence of HAND and the brain pathology shown at autopsy. In an opposite scenario where HIV encephalitis or high CNS viral burden was found at autopsy without clinically documented neurocognitive impairment [95, 101], it is possible that HIV encephalitis occurred shortly (within a few weeks or months) before death due to treatment failure and neurocognitive assessment was not feasible. Third, in subsets of patients, HAND may occur in the context of low-level or negligible HIV replication in the CNS and as a result of comorbid factors contributing to parenchymal and vascular injury in the CNS.

In general, postmortem neuropathological descriptions in HIV-infected individuals are naturally biased toward advanced stages of the disease (e.g. full-blown AIDS and serious non-AIDS-related conditions). In a given patient, merely a history of receiving cART does not guarantee success in suppressing systemic and CNS viral replication. Reported changes of the neuropathological patterns in the cART-era literature appear to reflect a mixture of findings in HIV-infected patients who had differentially succeeded in suppressing viral replication initially and failed antiretroviral treatment eventually, or in those patients who died from serious non-AIDS-related conditions (e.g. cardiovascular disease and liver failure), or illicit drug intoxication, with different combinations of comorbid conditions [102, 106, 119–127].

It is important to note that the profiles of HIV-infected autopsy materials currently available for NeuroAIDS research may not reflect the profiles of people who live with HIV in the same population in the cART era. Whereas some previous studies addressing the molecular mechanisms of HAND were based on autopsy cases clinically diagnosed with HAND [63, 94, 105, 128–130], other studies were based on autopsy cases neuropathologically diagnosed with HIV encephalitis [131–140]. The work using HIV encephalitis-based approach may not be advantageous to the clinical translation in the cART era [117, 141] because (a subtype of) HAND that is associated with HIV encephalitis is potentially treatable with CNS-effective cART [21, 118].

8.4 CNS Pathology in Pre-AIDS

Pathological changes in the CNS of pre-AIDS patients are generally minimal. The description of neuropathological features has been derived from the relatively small number of HIV-infected autopsy cases mostly related to illicit drug intoxication and fatal trauma [142, 143]. Common neuropathological changes include low-level leptomeningeal infiltration by CD8 T lymphocytes predominantly and CD20 B lymphocytes, perivascular lymphocytic cuffing (particularly in the white matter), subtle microgliosis and astrogliosis, and myelin pallor [106, 122, 142, 144, 145]. There is no definite evidence of HIV replication in the CNS of pre-AIDS patients [142, 143]. In a study of eight pre-AIDS brains [146], only two brains were shown to contain HIV-1 proviral DNA by polymerase chain reaction assay of cerebral cortical tissue specimens.

8.5 HIV Encephalitis and Leukoencephalopathy

HIV encephalitis and leukoencephalopathy are neuropathological changes considered to be specific to HIV disease and commonly affecting the CNS of late-stage AIDS patients [8, 90]. Histopathologically, multiple foci of diffuse and nodular microgliosis, perivascular macrophage infiltration, multinucleated giant cells (of microglia/macrophage origin), and astrogliosis characterize HIV encephalitis (Fig. 8.1a, b). HIV leukoencephalopathy is characterized by myelin loss, microgliosis, perivascular macrophage infiltration, multinucleated giant cells, and astrogliosis, with little or no lymphomonocytic infiltrate in the deep white matter [5, 9, 90, 109, 147]. In both entities, if multinucleated giant cells are not found, evidence of robust HIV replication in the CNS (i.e. immunohistochemistry for HIV-1 structural proteins gp41 or p24, or in situ hybridization for HIV-1 RNA) is required [108]. The immunohistochemical method (HIV-1 gp41 or p24) is more sensitive than the

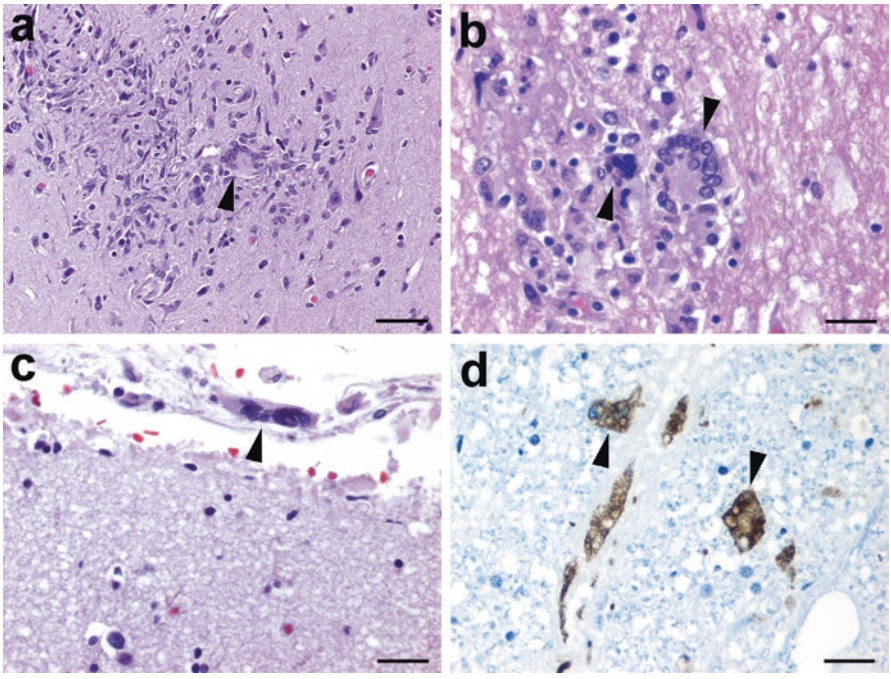

Fig. 8.1 HIV encephalitis and myelitis. (**a**) A (loose) microglial nodule with a microglia/macrophage-derived multinucleated giant cell (*arrowhead*) in the cerebral cortical gray matter, scale bar: 100 μm. (**b**) Multinucleated giant cells (*arrowheads*) in the cerebral subcortical white matter, scale bar: 50 μm. (**c**) A multinucleated giant cell in the cerebral leptomeninges (*arrowhead*), scale bar: 50 μm. Hematoxylin–eosin staining (**a**–**c**). (**d**) Multinucleated giant cells with intracytoplasmic CD68 immunoreactivity (a marker of activated microglia/macrophages) in the spinal cord (*arrowheads*), scale bar: 50 μm, immunohistochemistry with a monoclonal antibody against CD68 (clone KP1) and counterstaining with hematoxylin

lone standard histopathology (multinucleated giant cells) in making a diagnosis of HIV encephalitis in CNS tissue sections [110, 111].

HIV encephalitis and leukoencephalopathy denote productive HIV infection of microglia and perivascular macrophages, as well as multinucleated giant cells [113, 148–150]. Although there are no consensus criteria for grading the severity of HIV encephalitis on standard histopathology [106], grading has been attempted on immunohistochemistry for HIV-1 gp41 in brain tissue sections [110–112]. Quantitative assessment of CNS HIV burden can be performed in tissue homogenates using HIV-1 RNA assays [94, 107, 113]. Productive HIV infection in AIDS brains is not uniformly distributed, the level of which, for instance, has been shown to be higher in the caudate nucleus and hippocampus than in the middle–frontal cortex and cerebellar cortex [107]. In addition, productive HIV infection may extend to the leptomeninges [109, 151] (Fig. 8.1c) as well as involve the spinal cord (i.e. HIV myelitis) [109, 152] (Fig. 8.1d), usually occurring concurrently with HIV encephalitis that extensively affects the cerebrum, cerebellum, and brain stem (personal observation). The severity, extent, or distribution of lesions may determine the clinical manifestation [106, 108, 109].

In a series of 160 HIV-infected brains in the pre-cART era [153], HIV encephalitis alone was seen in 11% and a combination of HIV encephalitis and HIV leukoencephalopathy in additional 13%. There was a significant increase in incidence of HIV encephalitis from 1983 to 1985 [1], which was likely related to increased survival in later years of the AIDS epidemic [90]. In support of this notion, a clinicopathological study [110] showed that survival after the initial diagnosis of AIDS was longer in patients diagnosed with HIV encephalitis at autopsy. It might be interpreted that most patients with AIDS would develop HIV encephalitis if they lived long enough with progressive immune suppression and did not succumb earlier to other severe AIDS-defining conditions (e.g. opportunistic diseases).

8.6 Vacuolar Myelopathy

Vacuolar myelopathy is common in untreated patients with AIDS [1, 106, 154]. The prevalence of vacuolar myelopathy in AIDS autopsy cohorts varies markedly with geographical regions [90, 109, 155]. Histopathologically, vacuolar myelopathy is basically vacuolar myelinopathy characterized by intramyelinic and periaxonal vacuoles with some macrophage infiltration, which is pathologically reminiscent of *subacute combined degeneration of spinal cord* associated with vitamin B12 deficiency [108, 152]. The (ascending) posterior and (corticospinal) lateral columns of the spinal cord are predominantly affected although the involvement is not limited to any particular white matter tracts. Vacuolar myelopathy usually starts in the mid-thoracic cord with rostral and caudal spreading with increasing severity. The clinical manifestation includes leg weakness, spastic paraparesis, sensory ataxia, and incontinence [156]. The association between vacuolar myelopathy and productive HIV infection has been inconsistent among several AIDS autopsy studies [109, 155].

Vacuolar myelopathy is not considered to be HIV-specific pathology as it can be seen in the context of immune deficiencies other than HIV disease [93, 109, 155].

8.7 Emerging HIV-Related Cliniconeuropathological Entities in the cART Era

There are emerging HIV-related cliniconeuropathological entities reported in small subsets of cART-treated patients, which are clinically and pathologically distinct from classic HIV encephalitis and leukoencephalopathy. These entities include IRIS, severe inflammatory HIV leukoencephalopathy, HIV-associated CD8 T-cell encephalitis, and burnt-out lesions of HIV encephalitis and various opportunistic infections [156].

8.7.1 Immune Reconstitution Inflammatory Syndrome

IRIS is clinically characterized by paradoxical neurological deterioration despite successful HIV suppression and cellular immune recovery in HIV-infected persons following cART. IRIS affects up to a quarter of patients usually within a few months of commencing cART. IRIS is mediated by activated CD8 T lymphocytes trafficking into the CNS, inducing an inflammatory and edematous flare due to improved host immune response to antigens of infectious agents. Infectious diseases that commonly prompt IRIS include cryptococcosis, progressive multifocal leukoencephalopathy (PML), and tuberculosis [157, 158]. IRIS is clinically classified into *paradoxical* IRIS, where the underlying infection is known and being treated prior to starting cART, and *unmasking* IRIS, where the causative infection is not known before starting cART. Further, it has been proposed that HIV itself in the CNS can induce IRIS in a form of HIV-associated CD8 T-cell encephalitis [159, 160] (See Sect. 8.7.3).

Histopathologically, there are intense parenchymal and perivascular infiltration by lymphocytes (predominantly CD8 T cells) and necrosis [161]. These histopathologic changes correlate with the presence of contrast-enhancing lesions with edema and mass effect on magnetic resonance (MR) imaging, and deterioration of the neurological symptoms [162].

8.7.2 Severe Inflammatory HIV Leukoencephalopathy

Langford et al. [163] described in 2002 a severe inflammatory form of HIV leukoencephalopathy in patients with AIDS who failed cART. All seven patients had advanced immune suppression and six of them were diagnosed with HAND (five

with HAD and one with ANI). All the patients had high HIV-1 RNA burden in the brain and no opportunistic CNS diseases. Neuropathological changes included white matter rarefaction, myelin loss, intense perivascular infiltration by macrophages and lymphocytes, microglial nodules, multinucleated giant cells, abundant HIV-1 gp41-immunoreactive cells, and astrogliosis. It is the presence of intense perivascular mononuclear inflammatory cell infiltration, severer white matter damage, and higher levels of HIV replication that makes these reported cases distinct from classic HIV leukoencephalopathy originally defined in the pre-cART era. Subsequently, Gray et al. [125] reported a similar case of severe demyelinating HIV leukoencephalopathy.

8.7.3 HIV-Associated CD8 T-Cell Encephalitis

A new form of encephalitis so called *HIV-associated CD8 T-cell encephalitis* has been reported in the cART-era literature [157, 159, 164–166]. Small subgroups of HIV-infected patients on cART developed acute or subacute encephalopathy, which responded variably to corticosteroid treatment. CSF analysis showed CD8 T-cell predominant pleocytosis. Neuropathological changes were characterized by perivascular and intraparenchymal infiltration by CD8 T lymphocytes, microgliosis, and astrogliosis, variably associated with evidence of HIV replication and myelin loss. This clinicopathological entity was proposed to be included in the framework of IRIS associated with HIV itself in the absence of opportunistic infectious agents in the CNS [159, 160].

8.7.4 Burnt-Out Lesions

In subsets of HIV-infected patients who develop opportunistic CNS infection or HIV encephalitis and are successfully treated with cART and (if available) specific antimicrobial therapy, the restoration of cellular immunity and resolution of the infection are not accompanied by the neurological improvement. This phenomenon can be explained by the presence of residual irreversible tissue damage within the CNS [125]. The so-called *burnt-out lesions* seen at autopsy are essentially devoid of detectable infectious agents. Opportunistic CNS infections reported to leave burnt-out lesions after being successfully treated include PML, varicella zoster encephalitis, and toxoplasmosis [125, 167]. The cliniconeuropathological diagnosis of burnt-out lesions is made on the basis of clinical and radiological features, prior detection of causative infectious agents in the CSF, and presence of pathological changes compatible with particular infections.

8.8 Nonspecific CNS Pathology in HIV Disease

Nonspecific pathological changes commonly found in the CNS of HIV-infected adults include gliosis, white matter lesions, synaptodendritic degeneration, neuronal loss, and vessel mineralization [7, 109]. Additionally, age-related pathological changes reported include cerebral β-amyloidosis [106, 168], Tau pathology [169], and arteriolosclerosis [42]. It remains to be determined to what extent these CNS pathological changes play a role in the clinical spectrum of HAND.

8.8.1 Gliosis

Gliosis occurs commonly in the CNS of HIV-infected adults, including astrogliosis, microgliosis, microglial nodular lesions, and Alzheimer's type II astrogliosis. Specifically in the cerebral cortex of AIDS brains, *diffuse poliodystrophy* is characterized by GFAP-immunoreactive astrogliosis, microgliosis, and neuronal loss [108, 109, 155]. Immunohistochemistry for GFAP is useful for highlighting astrogliosis especially in the cerebral cortex because GFAP-immunoreactive astroglia are sparse in the normal cortical gray matter (personal observation). Using GFAP immunohistochemistry, whole-slide digital microscopy, and computer-assisted quantitative image analysis, Soontornniyomkij et al. [115] showed that levels of astrogliosis were three-fold and four-fold higher in brains of patients with AIDS (free of HIV encephalitis) than in age-matched non-HIV control brains in the cortical gray matter and subcortical white mater of the middle frontal gyrus, respectively.

In the brains of cART-treated patients with systemic viral suppression, Anthony et al. [122] observed markedly increased microglial activation by immunohistochemistry for CD68 in the hippocampus and basal ganglia, when compared to non-HIV control brains. Especially in the hippocampus, microglial activation in the cART-treated group was higher than in pre-AIDS brains from the pre-cART era. The cART-treated patients did not have neurocognitive impairment, and there was no HIV-specific pathology, HIV-1 p24 immunoreactivity, or opportunistic diseases in the brain. It is possible that some antiretroviral drugs in cART regimens might induce microglial activation seen in this study. Still, the possibility of confounding effects of HCV coinfection on gliosis could not be excluded [170] as eight of ten cART-treated patients were HCV-seropositive, as compared to none of nine non-HIV control participants and two of 11 pre-AIDS patients from the pre-cART era. In another study, Tavazzi et al. [63] reported immunohistochemical evidence of CD68-immunoreactive microglial activation and GFAP-immunoreactive astrogliosis in the frontal white matter and basal ganglia of HIV-infected patients with varying degrees of neurocognitive impairment but without HIV encephalitis, as compared to non-HIV control brains.

Microglial nodular lesions are defined as scattered microglial nodules in the absence of evidence (e.g. standard histopathology, immunohistochemistry, and in

situ hybridization) for specific etiologies, such as cytomegalovirus (CMV) disease, HIV encephalitis, herpes simplex encephalitis, and toxoplasmosis. Microglial nodular lesions can be seen in subsets of HIV-infected brains [1, 42, 102, 108, 109]; however, their clinical significance remains unclear.

Alzheimer's type II astrogliosis has been variably reported in HIV-infected brains [1, 4, 102]. The presence of Alzheimer's type II astrogliosis denotes chronic metabolic encephalopathy (e.g. hepatic and uremic). Alzheimer's type II astroglia are enlarged protoplasmic astroglia having round to oval vesicular watery nuclei and scant cytoplasm, identified within the gray matter, such as the putamen and cerebral cortex. In a cART-era clinicopathological study from the National NeuroAIDS Tissue Consortium (a consortium of four HIV brain banks in the United States) [102], Alzheimer's type II astrogliosis was observed in 15% of 589 autopsy brains reviewed. Of interest, the frequency of Alzheimer's type II astrogliosis was 25% in patients with clinically diagnosed HAD and, in contrast, ranged from 8% to 11% in patients with normal cognition, ANI, or MND.

8.8.2 White Matter Lesions

White matter lesions are defined as foci of myelin pallor or white matter rarefaction, with or without macrophage infiltration and astrogliosis, in the absence of specific etiologies, such as HIV leukoencephalopathy and PML [1, 42]. Perivascular white matter lesions are commonly associated with hemosiderin deposition [109], denoting remote micro-hemorrhage [171, 172].

In a clinicopathological study, Power et al. [173] detected myelin pallor of the cerebral subcortical white matter (stained with Luxol fast blue) without evidence of active demyelination in eight of 15 demented patients with AIDS, three of 13 non-demented patients with AIDS, and none of two pre-AIDS and nine non-HIV control participants. There was also immunohistochemical evidence of serum protein accumulation more frequently in the cortex and subcortical white matter of demented patients with AIDS. These findings suggest an association between alterations in the blood–brain barrier and dementia in a subset of patients with AIDS.

8.8.3 Synaptodendritic Degeneration

Evidence suggests that synaptodendritic degeneration is a neuropathological substrate of clinical neurocognitive impairment observed in patients with AIDS [94, 114, 174, 175]. Masliah et al. [94, 116, 174] have optimized a quantitative image analysis method following immunofluorescence confocal microscopy for synaptophysin (SYP, a marker of presynaptic terminals) and microtubule-associated protein-2 (MAP2, a marker of neuronal soma and dendrites) to assess

synaptodendritic integrity in vibratome-prepared gray-matter tissue sections. The extent of synaptodendritic degeneration (based on SYP and MAP2 immunoreactivity density), particularly in the putamen and hippocampus, was shown to correlate with the degree of neurocognitive impairment in HIV-infected patients [176, 177]. In AIDS brains, the HIV-1 RNA burden was found to correlate with synaptodendritic degeneration [114, 174], as was the burden of HIV-1 gp41 protein assessed by immunohistochemistry [111].

It is likely that synaptodendritic degeneration is a neuropathological feature that precedes neuronal loss [178] and is shared by a variety of chronic neurodegenerative processes, including mild cognitive impairment and Alzheimer's disease [179–184]. Regardless of the specific pathogenic mechanisms, the final common pathway leading to HAND is likely to be damage to neuronal integrity. Loss of specific neuronal populations in the cerebral cortex [178, 185–190], limbic system, and basal ganglia [190, 191] has been reported in AIDS brains. For instance, a decrease in the number of calbindin-1-immunoreactive neurons in the frontal cortex was found to correlate with the HIV burden [178].

8.8.4 Cerebral β-Amyloidosis

Several autopsy studies reported cerebral β-amyloid plaque deposition in the neocortex (Fig. 8.2a) [106, 168, 192–196] and hippocampus [169] of HIV-infected patients. For instance, Esiri et al. [192] found that the frequency of brains showing β-amyloid plaques rose from 18% in the fourth decade of age to 50% in the seventh decade in the HIV-infected group, as compared to that in the non-HIV control group

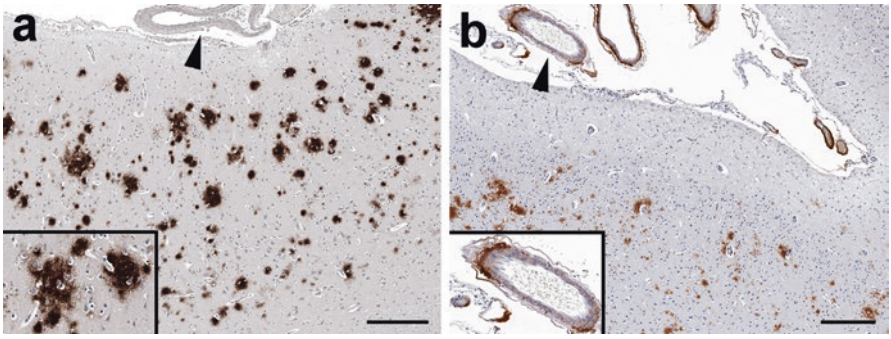

Fig. 8.2 Cerebral β-amyloidosis. (**a**) Diffuse (extracellular) β-amyloid plaques are widespread in the cerebral cortical gray matter, showing perineuronal and perivascular localizations, scale bar: 300 μm (*inset*: higher magnification). (**b**) Cerebral β-amyloid angiopathy affects leptomeningeal vessels (*arrowhead*), scale bar: 300 μm (*inset*: higher magnification), in comparison to an unaffected leptomeningeal vessel (**a**, *arrowhead*). Immunohistochemistry with a monoclonal antibody against β-amyloid peptide (amino acid residues 17–24, clone 4G8) and counterstaining with hematoxylin

from none to 36%. Soontornniyomkij et al. [168] reported that the occurrence of β-amyloid plaques in the middle–frontal cortex was 29.5% of 105 HIV-infected brains and 22.7% of 22 non-HIV control brains. The apolipoprotein E (*APOE*) ε4 genotype and older age (≥50 years) independently predicted cerebral β-amyloid plaque deposition in the HIV-infected group [168]. In contrast, Khanlou et al. [197] found no β-amyloid plaques in the cerebral neocortex or hippocampus of 36 brains of older HIV-infected patients (≥50 years). Cerebral β-amyloid angiopathy (Fig. 8.2b) was observed in small subsets of HIV-infected brains [168, 193, 197]. Whether HIV-infected persons are at increased risk of developing cerebral β-amyloidosis (as either extracellular plaques or vascular deposits) at the earlier age or greater extent than the general population remains to be determined in large autopsy cohorts of HIV-infected and non-HIV control brains, with necessary statistical adjustment at least for age and *APOE* genotyping.

When present in the brains of HIV-infected individuals, how does β-amyloid plaque deposition play a role in the development of neurocognitive impairment? Neocortical β-amyloid plaques found in HIV-infected brains are mostly of diffuse type (Fig. 8.2a, inset) and rarely associated with Tau pathology [168, 192, 194, 195], similar to those seen in elderly individuals with normal cognition [198]. Diffuse β-amyloid plaques likely represent the earliest stage of temporal progression of β-amyloid plaques [199], in contrast to neuritic cored β-amyloid plaques characteristically present in symptomatic Alzheimer's disease brains. Nonetheless, Soontornniyomkij et al. [168] found that the presence of β-amyloid plaques in the middle–frontal cortex (regardless of the density) was associated with clinically diagnosed HAND among *APOE* ε4 carriers.

Different APOE isoforms, synthesized by astroglia under normal physiological conditions and by neurons in response to neuronal stress or injury, may differentially regulate the clearance of soluble β-amyloid at the blood–brain barrier and influence the propensity for β-amyloid to aggregate [200–203]. For instance, when the brains (frontal cortex) of patients with Alzheimer's disease were matched for the total β-amyloid plaque burden, the level of β-amyloid oligomers was nearly three times higher in those with the *APOE* ε4/ε4 genotype than the *APOE* ε3/ε3 genotype, suggesting that the APOE4 isoform impacts the metabolism of β-amyloid oligomers [204]. Accumulating evidence from in vitro experiments and animal models suggests that soluble oligomeric β-amyloid species, instead of or in addition to fibrillar β-amyloid forms, are crucial for synaptotoxicity, neurodegeneration, and neurocognitive impairment [205]. Moreover, the APOE4 isoform may have direct toxic effects on the blood–brain barrier and neurons through various molecular pathways independent of β-amyloid [203, 206].

Post-translational modifications of β-amyloid peptides result in the production of N-terminal truncated β-amyloid with pyroglutamate formation [N3pE-β-amyloid(3–40/42)] and phosphorylated β-amyloid [pSer8-β-amyloid(1–40/42)]. Both modified β-amyloid forms may accelerate the speed of β-amyloid aggregation into oligomers and protofibrils and are detectable in human brains in later stages of regional β-amyloid plaque expansion [207]. It is of interest to investigate whether

the presence of N3pE-β-amyloid and/or pSer8-β-amyloid in HIV-infected brains is associated with neurocognitive impairment.

Intraneuronal β-amyloid accumulation was reported in HIV-infected brains [193, 196, 197]. Of note, all these studies used the anti-β-amyloid(17–24) antibody (clone 4G8), which might cross-react with amyloid precursor protein or its other derivatives [208]. In subsets of HIV-infected brains and non-HIV control brains, Soontornniyomkij et al. [168] observed intracellular β-amyloid immunoreactivity in neuronal soma in the middle–frontal cortex more frequently with anti-β-amyloid(17–24) antibody (clone 4G8) than with C-terminal-specific antibodies against β-amyloid(1–40) and β-amyloid(1–42). This intraneuronal β-amyloid immunoreactivity was focally present with no consistent co-occurrence of β-amyloid plaques in the same tissue section. In Alzheimer's disease brains, it was β-amyloid(1–42) immunoreactivity that was especially apparent within neurons [208, 209]. The pathogenic significance of intraneuronal β-amyloid accumulation in HAND remains to be determined.

Clinical studies by Clifford et al. [210] and Brew et al. [211] showed that β-amyloid(1–42) levels in the CSF were reduced in patients with HAND compared to those in cognitively normal participants. In a CHARTER study of 183 HIV-infected adults [212], family history of dementia (defined as having a self-reported first- or second-degree relative with dementia) appeared to moderate the relationship between CSF β-amyloid(1–42) levels and HAND. That is, lower CSF β-amyloid(1–42) levels were associated with HAND in those patients with family history of dementia. In addition, higher baseline CSF β-amyloid(1–42) levels were associated with a lower risk of neurocognitive decline during follow-up. Nonetheless, *APOE* genotyping was not taken into account in these three reports. In a small clinical study by Ances et al. [213] with the assessment of cortical Pittsburgh compound B (PiB) retention on positron emission tomography (PET), none of five HAND and 11 cognitively normal HIV-infected patients had increased PiB retention in contrast to symptomatic patients with Alzheimer's disease. On the other hand, β-amyloid(1–42) levels in the CSF were decreased (<500 pg/mL cutoff value) in two of four HAND and three of eight cognitively normal HIV-infected patients, but in only one of eight non-HIV control participants apparently matched for the *APOE* ε4 genotype and age.

Decreases in β-amyloid(1–42) levels in the CSF correlate generally with increases in cortical PiB retention on PET, indicating the presence of cerebral β-amyloid deposition [214, 215]. However, changes in the CSF begin at earlier ages than changes in cortical PiB retention [216, 217]. As PiB (a derivative of thioflavin-T [218]) binds to β-pleated sheet aggregates of peptides (i.e. amyloid, including fibrillar β-amyloid), PiB readily marks cored β-amyloid plaques (whether or not they are neuritic) [217, 219]. Due to its higher affinity to fibrillar β-amyloid compared to the affinity of thioflavin-S [220], PiB may also mark diffuse β-amyloid plaques [217, 219], characteristically composed of small amounts of fibrillar β-amyloid [221].

Collectively, it is likely that HIV-infected patients with reduced β-amyloid(1–42) levels in the CSF have cerebral β-amyloid deposition, which (depending on the

fibrillar β-amyloid load) may or may not be detected by PiB PET [216, 217]. Accordingly, measurement of β-amyloid(1–42) levels in the CSF may be more sensitive than PiB PET for the detection of cerebral β-amyloid deposition in HIV-infected persons. It is of interest to study the relationship between CSF or plasma levels of certain *synaptotoxic* β-amyloid oligomer species and neurocognitive impairment in HIV-infected persons [205]. β-Amyloid oligomer-targeted MR imaging of the brain may be available in the near future using molecular MR contrast probes, such as β-amyloid oligomer-specific antibodies conjugated to Fe_3O_4 magnetic nanoparticles [222].

8.8.5 Tau Pathology

In an autopsy study, Anthony et al. [169] reported that the extent of Tau pathology in the hippocampal formation was increased in cART-treated patients with systemic viral suppression, when compared to that in the age-matched non-HIV control participants. These cART-treated patients did not have cognitive impairment, HIV encephalitis, HIV-1 p24 immunoreactivity, or opportunistic CNS infections. Although the Tau pathology appeared more widespread, extending beyond the entorhinal cortex, in the HIV-infected groups when compared to that in the non-HIV control participants, only occasionally did it involve the temporal neocortex. The extent of Tau pathology tended to increase with age in HIV-infected patients, similar to that seen in the non-HIV control participants. Pre-cART HIV-infected patients with cognitive impairment did not show elevated levels of hippocampal Tau pathology. Collectively, these findings suggest that cART might play a role in the accelerated progression of Tau pathology. Of note, the Tau pathology, which was still confined to the hippocampal formation, was not associated with cognitive impairment in this study [169], in agreement with the observation in elderly individuals with intact cognition [198]. It is of interest to further analyze whether certain antiretroviral drugs or combination regimens are most robustly associated with the accelerated progression of Tau pathology in HIV-infected persons.

With regard to the cerebral neocortical involvement of Tau pathology, Soontornniyomkij et al. [168] reported that in the middle–frontal cortex phospho-Tau-immunoreactive neurites (Fig. 8.3a) were sparse in 32.4% and of moderate density in only 1.9% of 105 HIV-infected patients. There were rare neurons with diffuse soma immunoreactivity (Fig. 8.3b), neurons with neurofibrillary tangles (Fig. 8.3c), and clusters of dystrophic neurites (consistent with neuritic plaques).

In a cross-sectional clinical study by Cysique et al. [223], higher phospho-Tau (phosphorylated threonine-181 residue) levels in the CSF were found to correlate with greater degrees of current neurocognitive impairment in 43 adults (mean age 57 years) with chronic cART-treated HIV disease and more than 95% having viral suppression in the plasma and CSF.

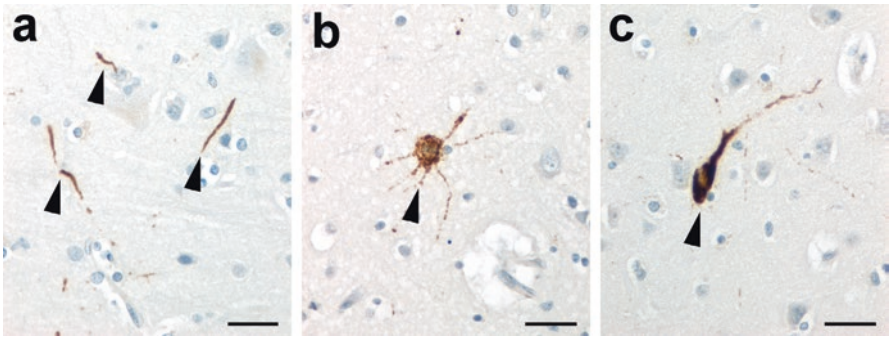

Fig. 8.3 Phospho-Tau-immunoreactive neuronal pathology in the cerebral cortical gray matter. (**a**) Neurites (*arrowheads*), scale bar: 50 μm. (**b**) Neuronal soma (*arrowhead*), scale bar: 50 μm. (**c**) Neurofibrillary tangle (*arrowhead*), scale bar: 50 μm. Immunohistochemistry with a monoclonal antibody against phospho-Tau (phosphorylated serine-202 residue, clone AT8) and counterstaining with hematoxylin

8.8.6 Vascular Pathology

HIV-infected persons are at increased risk of cerebrovascular diseases, potentially caused by infective vasculitis, opportunistic CNS diseases, cardiac embolism, hypercoagulopathy, or HIV infection itself [30–41]. Among a wide range of CNS vessel pathological changes found in this context [30], arteriolosclerosis was shown to associate with ischemic stroke during life [34] and cerebral infarction at autopsy [36]. In a community-based study [224], the presence of punctate white matter hyperintensities on MR imaging was found to correlate with older age and higher systolic blood pressure in HIV-infected persons. Whereas in the general population, white matter hyperintensities are thought to represent ischemic lesions caused by arteriolosclerosis [225], the similar lesions in HIV-infected persons may also reflect foci of HIV leukoencephalopathy or nonspecific white matter lesions [226–229]. Vascular pathology described here includes arteriolosclerosis and vessel mineralization.

Arteriolosclerosis is associated with systemic arterial hypertension, diabetes mellitus, and aging in the general population [230]. Histopathologically, arteriolosclerosis is defined as concentric intramural hyalinization of small arteries or arterioles (Fig. 8.4a) [171, 230]. Severer forms of arteriolosclerosis may cause neurocognitive impairment via cerebral blood flow restriction sufficient to produce microinfarcts or white matter lesions. In mild arteriolosclerosis, neurocognitive compromise may be related to disturbance of cerebrovascular autoregulation (in response to fluctuations in systemic arterial pressure and in blood/tissue gas partial pressure and pH) and deficiency in functional hyperemia (adjustment of regional cerebral blood flow in response to local neural activity), which together could impair new protein synthesis in neurons required for synaptic plasticity and memory formation [230–234].

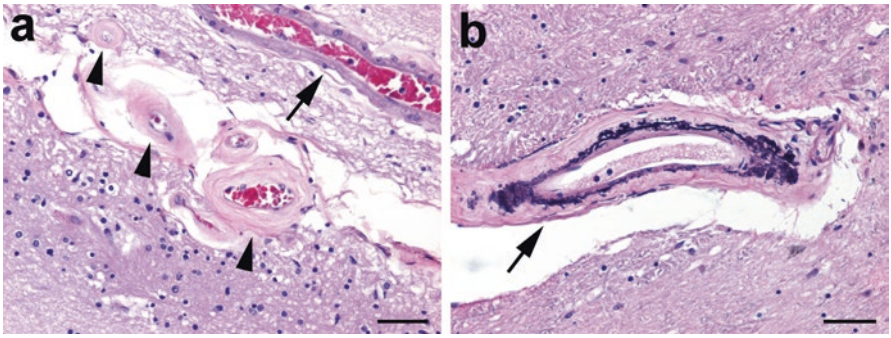

Fig. 8.4 Vascular pathology. (**a**) A small artery and arterioles in the cerebral white matter are affected by severe arteriolosclerosis (*arrowheads*), in comparison to an unaffected small artery (*arrow*), scale bar: 100 μm. (**b**) A small artery in the globus pallidus is affected by vessel mineralization (*arrow*), scale bar: 100 μm. Hematoxylin–eosin staining

A clinicopathological study of HIV-infected adults by Soontornniyomkij et al. [42] revealed direct associations between protease-inhibitor-based cART exposure (at the last follow-up visit) and higher likelihood of both mild and moderate/severe arteriolosclerosis (in the forebrain white matter) after statistically adjusting for diabetes. Moderate/severe arteriolosclerosis was associated with diabetes after statistically adjusting for cART exposure, hypertension, and older age (≥50 years). Of clinical significance, the presence of mild arteriolosclerosis was associated with clinically diagnosed HAND, which remained significant after statistically adjusting for HIV encephalitis or older age. These findings suggest that protease-inhibitor-based cART exposure increases the risk of arteriolosclerosis and thereby neurocognitive impairment.

An autopsy study by Morgello et al. [235] included both HIV-infected patients and non-HIV control participants. In the HIV-infected group, protease-inhibitor-based cART exposure (at death) was found to associate with severer arteriolosclerosis than non-nucleoside reverse-transcriptase inhibitor-based cART. Interestingly, HCV coinfection was the strongest risk factor for arteriolosclerosis. In the entire cohort, arteriolosclerosis was found to associate with HCV seropositivity, hypertension, and African American race, but not with HIV status, diabetes, or hyperlipidemia.

As the retina is an extension of the diencephalon embryonically, changes in the retinal vasculature may reflect similar changes in the cerebral microvasculature. Specifically, retinal vessel calibers and other aspects of vascular geometrical topography, which are readily assessed through noninvasive fundoscopic examination and retinal digital image analysis, may serve as markers of cerebral microvascular pathology [236, 237]. Nonetheless, whereas in hypertension the pathological microvascular changes in the retina and brain are very similar, in diabetes the changes are significantly different [236]. In a clinical study of 1250 HIV-infected patients without opportunistic ocular infections at baseline examination, Gangaputra et al. [238]

found smaller central retinal artery equivalent to be associated with increasing age, hypertension, and history of ever cART exposure, based on semiautomated evaluation of fundus photographs.

Vessel mineralization is defined as intramural deposition of basophilic amorphous material in small and medium-sized arteries (Fig. 8.4b) [4, 7]. Vessel mineralization is observed in subsets of AIDS brains [4, 7, 36, 42, 155]. It is found primarily in the globus pallidus; in some brains, it is also present in the cerebellar white matter, thalamus, hippocampus, dentate gyrus, and cerebral peduncle [42]. The pathogenesis of vessel mineralization in AIDS remains unclear. The presence of vessel mineralization does not appear to be associated with that of arteriolosclerosis [42].

8.9 Opportunistic CNS Diseases

According to autopsy studies in both the pre-cART era [1–11] and cART era [102], opportunistic diseases commonly affecting the CNS of patients with AIDS include CMV disease, PML, cryptococcosis, toxoplasmosis, and primary CNS lymphoma. More than one opportunistic disease, as well as HIV encephalitis, may coexist in the CNS of a given patient with AIDS. There are also other infections affecting the CNS with lower frequencies, such as herpes simplex virus encephalitis, varicella zoster virus encephalitis, bacterial cerebritis, tuberculosis, histoplasmosis, candidiasis, and aspergillosis [7, 9, 155].

Opportunistic diseases usually occur when advanced immune suppression takes place (e.g. CD4 T-cell count <200/μL) due to uncontrolled systemic HIV replication [19]. In patients who are receiving effective cART, viral replication is suppressed to undetectable levels, CD4 T-cell counts return to physiologic levels, and consequently opportunistic diseases are prevented. It is noted that restoration of cellular immunity may not be complete until CD4 T-cell counts increase to >750 cells/μL [239]. In geographical regions where cART is readily accessible, the incidence of opportunistic diseases has dramatically decreased, including CMV disease [240], PML [19, 241, 242], cryptococcosis [19, 158, 243], toxoplasmosis [19, 244, 245], and primary CNS lymphoma [246, 247]. Antibiotic prophylaxis is also effective in preventing some opportunistic infections, such as toxoplasmosis [244].

8.9.1 Cytomegalovirus Disease

Seroepidemiological studies show that most adult humans worldwide are infected with CMV (human herpes virus 5, family Herpesviridae, subfamily betaherpesviridae, enveloped spherical linear double-stranded DNA virus). However, only hosts with cellular immune suppression develop symptomatic CMV disease after reactivation of latent CMV infection. In the pre-cART era, CMV disease was

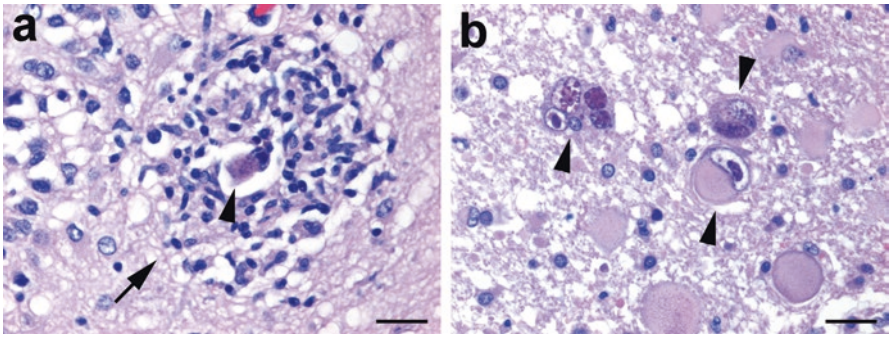

Fig. 8.5 Cytomegalovirus (CMV) encephalitis. (**a**) A (compact) microglial nodule (*arrow*) with an infected cell containing intranuclear and intracytoplasmic CMV inclusions (*arrowhead*), scale bar: 50 μm. (**b**) Cytomegalic infected cells with intranuclear and intracytoplasmic CMV inclusions (*arrowheads*), scale bar: 50 μm. Hematoxylin–eosin staining

the commonest opportunistic viral infection and affected several organ systems (e.g. CNS including retina, esophagus, colon, adrenal gland, liver, and lung) [248]. CMV has been implicated in five neurological syndromes in AIDS: retinitis, diffuse microglial nodular encephalitis with dementia, ventriculoencephalitis, myelitis/polyradiculopathy, and mononeuritis multiplex [10, 249–252].

Latent CMV infection is established in myeloid progenitor cells in the bone marrow. Unlike varicella zoster virus and herpes simplex virus (subfamily alphaherpesviridae), CMV is not transported axonally and does not establish latent neuronal infection. CMV infection in the CNS is caused by entry into the CNS of infected hematogenous cells, and subsequent infection of microvascular endothelial cells and CNS resident cells [253, 254].

Histopathologically, CMV diffuse microglial nodular encephalitis is characterized by a wide distribution of microglial nodules with or without cytomegalic cells bearing intranuclear and intracytoplasmic CMV inclusions, and isolated cytomegalic cells (Fig. 8.5a, b). CMV inclusions are identified in astroglia, endothelial cells, neurons, and ependymal cells [155, 253–256]. In CMV ventriculoencephalitis, cytomegalic cells largely replace the ependyma, with necrosis of subependymal parenchyma, suggesting dissemination of CMV through the CSF pathway [253–255]. Immunohistochemistry for CMV-specific antigens identifies more CMV-infected cells than standard histopathology.

8.9.2 Progressive Multifocal Leukoencephalopathy

PML is a demyelinating disease of the CNS that occurs almost exclusively in the context of advanced immune suppression [161]. PML is caused by productive infection of oligodendroglia by the neurotropic polyomavirus JC (genus

Orthopolyomavirus, family Polyomaviridae, nonenveloped icosahedral double-stranded circular DNA virus) [161, 257].

Asymptomatic primary JC virus infection occurs in childhood, and JC virus establishes latent or low-grade persistent infection in B lymphocytes, CD34 hematopoietic progenitor cells, tonsillar stromal cells, renal epithelial cells, and CNS glial cells [161, 241, 257–259]. Seroepidemiological surveys in many countries showed the prevalence of JC virus infection in adults ranged from 66% to 92% [257]. The immune competence, including immune surveillance in the CNS, is necessary for the prevention of JC virus reactivation and replication.

Symptomatic JC virus infection of the CNS is associated with disturbances of adaptive immunity affecting B lymphocytes, antibodies, and CD4 and CD8 T lymphocytes [259, 260]. It remains to be determined whether the development of PML involves CNS access of infected B lymphocytes or reactivation of JC virus in situ within the CNS [161, 257]. Due to the absence of effective JC-virus-specific antiviral therapy, the only way to eliminate the virus from the CNS is to reconstitute general immune competence [259, 261, 262].

The transcription of early and late JC viral proteins is regulated by the noncoding control region, which requires cell-type-specific transcription factors present in glial cells as well as transcription factors that are expressed in all cell types [241]. There are two main genetic forms of JC virus, dictated by the sequence in the noncoding control region: the archetype (typified by the CY strain) and the PML-associated prototype (typified by the Mad-1 strain) [257]. Oligodendroglia support productive infection by JC virus, leading to cell lysis and consequently demyelination. Infection of astroglia by JC virus leads to cellular morphological transformation. Productive infection can also occur in cerebellar granule cells, leading to cerebellar atrophy; this pathological entity is termed *JC virus granule cell neuronopathy* [161, 263].

PML lesions are asymmetrical, multifocal, and bilateral in the subcortical white matter and later involve the deep periventricular white matter, cerebellar peduncles, thalamus, and basal ganglia [241, 258]. Histopathologically, PML lesions show loss of myelin, with relative sparing of axons. However, in larger lesions, axonal loss may be evident. There are numerous foamy macrophages laden with myelin debris, reactive astroglia, and only scanty perivascular lymphocytes in classic PML. At the margins of the lesions, infected oligodendroglia have enlarged nuclei containing homogeneous amphophilic inclusions, which displace the chromatin toward the nuclear membrane (Fig. 8.6a). There are *bizarre* astroglia having hyperchromatic, irregular, lobulated enlarged nuclei, and abundant eosinophilic cytoplasm (Fig. 8.6b) [155]. Viral DNA can be demonstrated in oligodendroglia and astroglia in CNS tissue sections using in situ hybridization with JC-virus-specific nucleic acid probes. By immunohistochemistry, JC viral proteins can be detected in infected cells including enlarged oligodendroglia and bizarre astroglia. The early gene product T-antigen is found in the nuclei. The capsid protein VP1 (an indicator of active viral replication) is found in both the cytoplasm and nuclei of infected cells. The late accessory regulatory gene product agnoprotein is located mostly in the perinuclear cytoplasm. By electron microscopy, crystalline arrays of icosahedral JC virus particles are present primarily in the enlarged nuclei of oligodendroglia [257, 260].

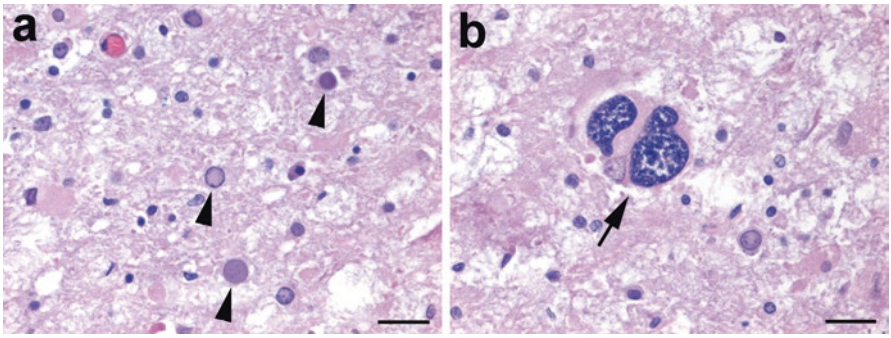

Fig. 8.6 Progressive multifocal leukoencephalopathy (PML). (**a**) In the cerebral white matter with myelin loss, infected oligodendroglia have enlarged nuclei containing JC virus inclusions, which displace the chromatin toward the nuclear membrane (*arrowheads*), scale bar: 50 μm. (**b**) An astroglial cell has bizarre giant hyperchromatic nuclei (*arrow*), scale bar: 50 μm. Hematoxylin–eosin staining

8.9.3 Cryptococcosis

Cryptococcus neoformans and *C. gattii* are fungi (encapsulated saprophytic yeasts) harbored in soil contaminated with pigeon droppings and in the heartwood of several tree species. CNS cryptococcosis is mostly caused by reactivation of latent pulmonary infection (via inhalation) and hematogenous spread upon cellular immune suppression [158, 264]. The great majority of patients with *C. neoformans* CNS infection are immunocompromised, particularly AIDS patients with blood CD4 T-cell counts <100 cells/μL. *C. gattii* can infect both immunocompetent and immunocompromised hosts [243]. The prevalence of cryptococcal meningitis in patients with AIDS is especially high in sub-Saharan Africa and Southeast Asia [264, 265]. Cryptococcal antigen is detectable in serum at least 3 weeks before the onset of symptoms, allowing screening and early treatment. Cryptococcal antigen detection in the CSF and serum is more sensitive than light microscopy of the yeasts in the CSF using India ink background contrast [243].

Histopathologically, cryptococcal meningoencephalitis in AIDS is characterized by numerous pleomorphic budding yeasts (5–10 μm), extracellularly dispersed or residing within the cytoplasm of enlarged macrophages, and macrophage-derived multinucleated giant cells within the leptomeninges and subarachnoid space (Fig. 8.7a, b), with extension into the expanded perivascular Virchow–Robin space in the CNS [155]. Cryptococcomas are mass-like collections of yeasts and macrophages containing yeasts within the CNS parenchyma. No or minimal lymphocytic infiltration is present, except in case of IRIS. Characteristically, the yeast cells have a polysaccharide capsule (up to 5 μm thick) that stains positive (red) by mucicarmine histochemistry [264].

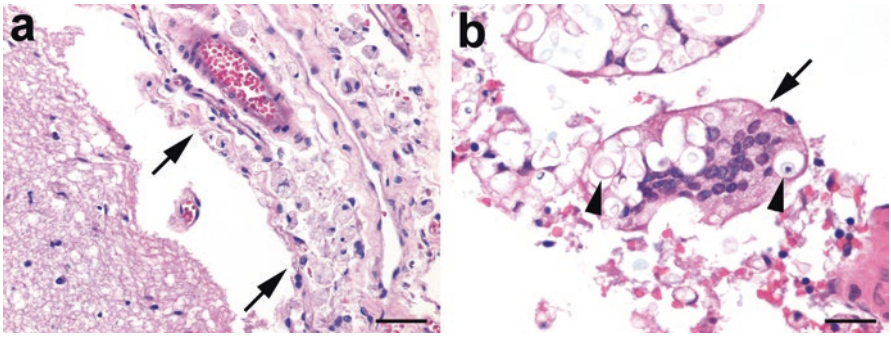

Fig. 8.7 Cryptococcal meningitis. (**a**) The leptomeninges (*arrows*) are expanded by macrophages containing cryptococcal yeasts, scale bar: 100 μm. (**b**) In the leptomeninges, macrophage-derived multinucleated giant cells (*arrow*) contain cryptococcal yeasts (*arrowheads*) in the cytoplasmic vacuoles, scale bar: 50 μm. Hematoxylin–eosin staining

8.9.4 Toxoplasmosis

Toxoplasmosis is caused by *Toxoplasma gondii* (subclass coccodia, phylum *Apicomplexa*) protozoa infection [266]. Seroepidemiological studies showed the prevalence of toxoplasmic infection varied markedly in different geographical regions of the world, ranging from 5% to 90%.

The cat (feline) family is the definitive host and virtually all warm-blooded animals can be intermediate hosts of *T. gondii* [266, 267]. *T. gondii* is an obligate intracellular parasite capable of infecting almost all nucleated host cells. Infection can be acquired in utero, with severe damage to the developing brain and eye. Most people become infected after childhood through eating raw or undercooked meat from intermediate hosts containing viable tissue cysts (bearing bradyzoites) or by ingesting infective oocysts (containing sporozoites) shed in cat feces. Following ingestion of tissue cysts or oocysts, the parasites invade through the gut wall and enter the circulation where they undergo an acute phase of rapid division of tachyzoites within several host cell types, predominantly macrophages. Repeated division can occur before the host cells rupture and the parasites are released. Tachyzoites spread through the circulation to many organs, including skeletal muscle, cardiac muscle, smooth muscle, eye, and CNS.

Primary toxoplasmic infection is usually asymptomatic or alternatively presents with an acute febrile illness with (cervical) lymphadenopathy and a rash [267]. This acute phase becomes latent following effective cellular immune response. In the CNS and muscle, acute-phase tachyzoites can undergo conversion into bradyzoites, which divide slowly within intracellular cysts, each containing a few to thousands of bradyzoites. Bradyzoite-bearing tissue cysts are latent in the CNS, skeletal muscle, cardiac muscle, and eye [266].

CNS toxoplasmosis occurs by reactivation of latent infection upon cellular immune suppression. Most patients affected by CNS toxoplasmosis have underlying

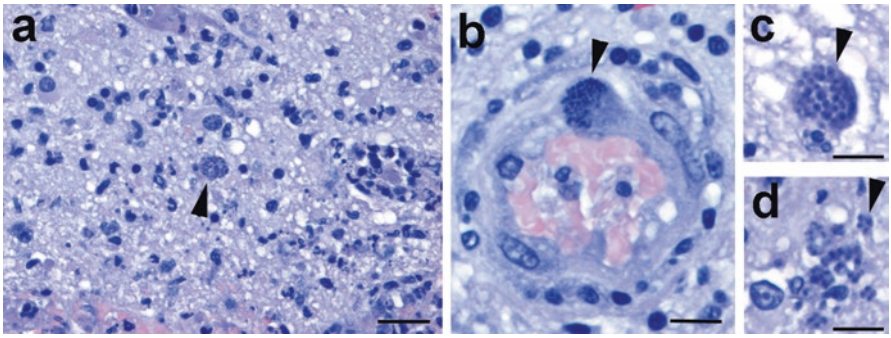

Fig. 8.8 Cerebral toxoplasmosis. (**a**) Necrotic cerebral tissue with polymorphonuclear and mononuclear inflammatory cell infiltration and a toxoplasma cyst (*arrowhead*), scale bar: 50 μm. (**b**) A cerebral microvessel with an intracellular cluster of toxoplasma organisms (*arrowhead*) within a parasitized endothelial cell, scale bar: 25 μm. (**c**) A toxoplasma cyst (*arrowhead*), scale bar: 25 μm. (**d**) An aggregate of extracellular toxoplasma organisms (*arrowhead*), scale bar: 25 μm. Hematoxylin–eosin staining

AIDS. Multiple space-occupying lesions in CNS toxoplasmosis cause seizures and focal neurological signs [268, 269]. In the diffuse microglial nodular encephalitis form of toxoplasmosis, however, there are no localizing neurologic deficits and so the premortem diagnosis is challenging [268, 270].

In the CNS, *T. gondii* can multiply in neurons, glial cells, ependymal cells, and vascular cells. Histopathologically, necrotic space-occupying lesions in CNS toxoplasmosis show parasitized host cells with intracellular tachyzoites in cytoplasmic vacuoles (pseudocysts), as well as extracellular tachyzoites (oval or crescent-shaped cells, measuring 2–4 by 4–8 μm), in a background of parenchymal necrosis, mixed polymorphonuclear and mononuclear inflammatory cell infiltration, microgliosis, astrogliosis, vasculitis with parasites in vascular endothelial or smooth muscle cells, and hemorrhage (Fig. 8.8a–c). Parasitized host cells with intracytoplasmic bradyzoite-containing cysts (true tissue cysts, 12–100 μm in diameter) are found at the inflamed periphery of the necrotic lesions as well as in the distant areas devoid of tissue reaction [7, 155, 271]. In toxoplasmic-diffuse microglial nodular encephalitis, microglial nodules with intracellular bradyzoite-bearing cysts and extracellular tachyzoites are widely distributed in the CNS parenchyma [268, 270]. Immunohistochemistry for *T. gondii* protein antigens (specific to tachyzoites or bradyzoites) is sensitive for identifying parasites within CNS tissue sections.

8.9.5 Primary CNS Lymphoma

Almost all primary CNS lymphomas (PCNSL) are of diffuse large B-cell phenotype. AIDS is associated with a 3600-fold increased incidence of PCNSL compared to the general population. Characteristically, PCNSL manifests in advanced stages

of AIDS when blood CD4 T-cell counts are extremely low. Epstein-Barr virus is detected in virtually all AIDS-related PCNSL, in contrast to PCNSL in immunocompetent hosts and systemic lymphomas in patients with AIDS [272, 273].

Histopathologically, PCNSL is characterized by a highly cellular infiltrate of large lymphoma cells with perivascular cuffing and angiocentric growth consisting of concentric layers of lymphoma cells within spitting vessel walls. There are frequent mitotic figures, apoptotic cells, and geographic necrosis [246]. Immunohistochemistry reveals that lymphoma cells are of mature B-cell phenotype expressing pan-B-cell markers (CD19, CD20, CD22, and CD79a) often with light chain (kappa or lambda) restriction, intermingled with small reactive T and B lymphocytes and macrophages. IgM and IgD, but not IgG, are expressed on the surface of lymphoma cells. The proliferative (Ki-67) index is extremely high, usually exceeding 70%. Reactive astrogliosis and microgliosis are present in the adjacent parenchyma [246].

8.10 Potential Effects of Comorbid Factors on HAND Pathogenesis

Increasing evidence has suggested that HAND in the cART era represents *deficits of multiple etiologies* [48, 51]. In addition to HIV replication in the CNS, there are comorbid factors that are probably of biological relevance, such as CNS HIV-1 genetic variants, host genetic polymorphisms, chronic cART-related neurotoxicity, substance use, HCV coinfection, brain aging, and systemic chronic inflammation and metabolic disturbance [29].

8.10.1 HIV-1 Genetic Variants

More than 90% of HIV-1 infections worldwide are caused by group M (major), consisting of nine clades (or subtypes) as well as interclade recombinants. In a given HIV-infected individual, there are swarms of HIV consisting of related but nonidentical viral genomes, called quasi-species [274]. Clinical evidence suggests that persons infected with clade D HIV have the highest risk of developing HAD, followed by clades B, C, and A in a decreasing order [275]. Nonetheless, the impact of different HIV clades on the risk of HAND remains controversial [274, 276]. For instance, a study of antiretroviral-naïve HIV-infected persons with advanced immune suppression (mean blood CD4 T-cell count 127 cells/μL) in Uganda [277] revealed that 89% of nine clade D-infected individuals were affected by HAD, compared to 21% of 33 clade A-infected individuals. In a subsequent Uganda study from the same group [49], HAD was found in 41% of 117 antiretroviral-naïve HIV-infected individuals with moderate to advanced immune suppression (mean blood CD4 T-cell

count 254 cells/μL); no significant association of HAD with HIV-1 clades (A versus D) was identified. A study in Southern Brazil [278] showed no significant difference in the frequency of HAND between clade B (65% of 26) and clade C (57% of 21) HIV-infected individuals from the same geographic region. In a study of HIV-infected persons in two geographic regions in China [279], HAND was found in 32% of 111 clade B-, 22% of 122 clade C-, and 25% of 75 clade B/C-infected individuals, the difference that did not reach statistical significance. Moreover, it remains unknown as to whether the clade status determines how soon one develops HAND after primary HIV infection and the rate of progression from ANI to MND to HAD [275].

In largely clade C HIV-infected populations, several surveys found the prevalence of HAD in south African countries was much higher than that in south Asian countries, although overall the milder forms of HAND were common in both distantly geographic regions [275]. Intraclade differences in clade C Tat and gp120 proteins were proposed to account for this difference in HAD prevalence [280, 281]. Specifically, substitution of cysteine by serine at position 31 (C30S31) in clade C Tat protein was shown in in vitro experiments and mouse models to associate with lower neurotoxicity compared to the intact dicysteine (C30C31) motif [275, 280]. Rao et al. [280] reported that the proportion of clade C variants with C30C31 Tat protein was much higher in South Africa (26% of 47 clinical isolates) and Zambia (20% of 45) than in India (3% of 101) and Bangladesh (2% of 56), which appeared to parallel the prevalence of HAD in these countries. However, Paul et al. [282] directly examined within the same cohort in South Africa the impact of the C30S31 variant in clade C Tat protein and found no significant differences between individuals with the C30S31 motif ($n = 46$) and those with the C30C31 motif ($n = 128$) with regard to the performance on a battery of neuropsychological tests; no analysis with respect to HAND categories was reported.

In a population-based study in India, Tilghman et al. [283] identified two genetic signatures in the exon 1 of clade C *tat* sequence to be associated with neurocognitive impairment. Arginine (rather than lysine) at position 29 and proline (rather than leucine) at position 68 were associated with global neurocognitive impairment. No unique genetic signatures were identified in the C2−V3 coding region of clade C *env* sequence.

HIV enters the CNS via infected leukocytes early in the course of infection [284]. Evidence of independent genetic evolution and development of antiretroviral drug resistance of HIV strains in the CNS has supported the concept of viral compartmentalization [56, 57, 59, 60]. The factors that drive this evolution may include limited viral exchanges across the blood–brain barrier and limited antiretroviral drug bioavailability in the CNS leading to a lower selection pressure on HIV replicating within the CNS [55, 274]. In some instances, compartmentalization can occur in the CNS of persons during primary HIV infection [59]. In a cross-sectional study of 66 HIV-infected persons representing all major clinical stages in relation to neurocognitive impairment [58], increased CSF compartmentalization of the HIV-1 *env* sequence, which might reflect independent HIV replication and evolution within

the CNS, was found to specifically associate with HAD but not with the milder forms of HAND. In contrast, a study of 117 antiretroviral-naïve HIV-infected individuals with moderate to advanced immune suppression in Uganda [49] showed no significant association of HAD with compartmentalization of the HIV-1 *p24* sequence between the CSF and peripheral blood.

8.10.2 Host Genetic Polymorphisms

Host genetic polymorphisms may play a central role in HAND pathogenesis, especially those variants involving neurodegenerative processes and innate and adaptive immune responses [117, 168, 285–288]. A number of candidate-gene association studies have identified host genetic variants as risk or protective factors for HAND. However, few of these genetic polymorphisms have reliably been replicated in later studies, and none of prior associations have been replicated in the single published genome-wide association study [117]. A comprehensive review of genetic, transcriptomic, and epigenetic studies of HAND was published by Levine et al. [141].

The *APOE* ε4 allele correlates with the earlier onset and greater extent of cerebral β-amyloidosis [216, 289–291] and is a major risk factor for sporadic Alzheimer's disease and cerebral β-amyloid angiopathy [292] in the general population. The major codominant alleles (i.e. ε2, ε3, and ε4) in the human *APOE* gene are associated with differential biological activities of their protein products [200]. The APOE protein is a β-amyloid-binding molecule that may influence the clearance of soluble β-amyloid at the blood–brain barrier and affect β-amyloid seeding and aggregation [200–202]. Across several clinical studies in HIV-infected adults, it remains controversial as to whether the *APOE* ε4 allele increases the susceptibility to HAND [223, 286, 287, 293–301]. For instance, Valcour et al. [298] reported that the *APOE* ε4 allele conveyed the risk of HAD within the older group but not in the younger group. In a study of 201 HIV-infected adults (mean age 40 years) in China, Spector et al. [287] found the *APOE* ε4 allele to associate with higher likelihood of HAND at baseline evaluation. In contrast, Morgan et al. [299] reported no significant association between the *APOE* ε4 carrier status and HAND in a cross-sectional CHARTER study of 466 HIV-infected adults (mean age 44 years) in the United States. Cysique et al. [223] found the *APOE* ε4 carrier status was not associated with HAND in a cross-sectional study of 43 cART-treated adults (mean age 57 years) in Australia.

A clinicopathological study by Soontornniyomkij et al. [168] showed an interaction effect of the *APOE* ε4 status and cerebral β-amyloid plaque (mostly of diffuse type) deposition on HAND. That is, the presence of β-amyloid plaques was associated with HAND among *APOE* ε4 carriers, but not in non-ε4 carriers. In agreement with this finding [168], a clinical study in the older population by Kantarci et al. [302] revealed that higher brain β-amyloid loads detected by PiB PET correlated with poorer neurocognitive performance among *APOE* ε4 carriers.

Differential susceptibility to HAND may also depend on host genetic polymorphisms in the cytokine regulatory response to HIV replication or viral proteins [177]. For example, the G/G homozygosity for CC chemokine ligand-2 (*CCL2*, also known as monocyte chemoattractant protein-1 [MCP1]) promoter at position −2578 was associated with the increased risk of HAD [285] and the elevated levels of MCP1 in the CSF [303]. The tumor necrosis factor (*TNF*)-alpha-308 allele 2 was also shown to associate with HAND [286]. The mannose-binding lectin-2 (*MBL2*) O/O (any combination of B, C, and D alleles) genotype (relative to A/A, exon 1) was associated with increased risk of neurocognitive decline over the 12-month period [287].

In a genetic–pathological analysis of HIV-infected brains by Levine et al. [114], the dopamine receptor D3 (*DRD3*) C allele (rs6280C/T) and CC chemokine ligand-3 (*CCL3*, also known as macrophage inflammatory protein-1 [MIP1]-alpha, a natural ligand for CC chemokine receptor-5 [*CCR5*, the major coreceptor for HIV-1 cell entry]) A allele (rs1719134A/G) were associated with microgliosis (Iba1 immunoreactivity density in the cerebral gray matter). The interleukin-1-alpha (*IL1A*, a proinflammatory cytokine) T allele (rs17561G/T) was associated with astrogliosis (GFAP immunoreactivity density in the cerebral gray matter).

The *DRD3* C/C (glycine/glycine) genotype conveys the increased binding affinity of DRD3 to dopamine compared to the other two variants (glycine/serine and serine/serine) [304, 305]. Gupta et al. [306] found the *DRD3* C (glycine) allele to associate with neurocognitive impairment in executive functioning in HIV-infected adults with lifetime methamphetamine dependence. In a study of Caucasian adolescents, Bombin et al. [307] reported the *DRD3* C/C (glycine/glycine) genotype was associated with poorer executive functioning performance in cognitive paradigms related to the concept of cognitive flexibility or set-shifting compared to the *DRD3* T/T (serine/serine) genotype.

In addition to nuclear DNA polymorphisms, patterns of mitochondrial DNA variants (i.e. haplogroups, defined by accumulations of specific mitochondrial DNA single nucleotide polymorphisms) may represent a maternal ancestry-specific factor that influences neurocognitive impairment in HIV-infected persons. Several studies have explored associations between mitochondrial DNA haplogroups and clinical outcomes of HIV disease (e.g. AIDS progression, CD4 T-cell recovery, and HCV-associated hepatic cirrhosis) and antiretroviral treatment (e.g. lipodystrophy, insulin resistance, and hyperlipidemia) in populations of European or African descent; however, the findings have been inconsistent [308]. In an observational CHARTER study of 1027 ambulatory HIV-infected adults, Hugan et al. [309] reported that persons of genetically determined admixed Hispanic ancestry had a greater likelihood of neurocognitive impairment (based on the cross-sectional global deficit score or HAND diagnosis) than persons of European or African ancestry. In multivariable models, among persons of genetically determined admixed Hispanic ancestry, those persons with mitochondrial DNA haplogroup B had a lower likelihood of neurocognitive impairment than those persons with other haplogroups, after statistically adjusting for the comorbidity status (incidental versus contributing), current cART status, plasma HIV-1 RNA levels, reading ability, and nadir blood CD4 T-cell counts.

8.10.3 Chronic cART-Related Neurotoxicity

In long-term cART, certain antiretroviral drugs may cause chronic toxicity to parenchymal and vascular components of the CNS, potentially leading to neurocognitive impairment [42, 66, 79–83].

Do antiretroviral drugs with higher CNS Penetration Effectiveness (CPE) ranks confer higher risks of inducing neurotoxicity? The aim of using cART regimens with higher CPE scores is to achieve HIV suppression in the CNS. The CPE system has been designed to predict antiviral efficacy, not toxicity [82]. The relationship between drug concentrations and toxicity can be completely different from the relationship between drug concentrations and antiviral efficacy [29]. A low CPE rank does not mean that the drug is absent from the CNS but simply implies that its levels seem to be below those needed to inhibit HIV replication. These low levels, however, could still result in other effects, such as neuronal injury and gliosis. In addition, not all drug adverse effects are dose-dependent. The adverse effects of some drugs are idiosyncratic, that is, they occur for a nondose-dependent reason, such as vulnerability of particular patients to the drug (Dr. Scott L. Letendre, personal communication).

Efavirenz (a non-nucleoside reverse transcriptase inhibitor) is a common component of first-line cART regimens and found to associate with CNS adverse effects. After treatment initiation, acute neuropsychological adverse reactions occur in a substantial proportion of individuals but generally resolve after a few weeks of continued use [310]. CNS toxicity related to long-term efavirenz exposure was suggested in a cross-sectional study of asymptomatic HIV-infected patients by Ciccarelli et al. [311], showing that efavirenz use was associated with increased risk of the milder forms of HAND. In an observational cohort of 445 HIV-infected patients in the United States [312], long-term efavirenz users had poorer neurocognitive performance in speed of information processing, verbal fluency, and working memory domains, when compared to long-term lopinavir–ritonavir users, even though lopinavir–ritonavir users had worse HIV disease characteristics known to increase the risk of neurocognitive impairment. In the subgroup of patients with undetectable plasma HIV-1 RNA loads, efavirenz users had poorer speed of information processing and executive functioning compared to lopinavir–ritonavir users. Interestingly, there was an interaction with HCV serological status. Among HCV-seronegative patients, efavirenz use was associated with poorer neurocognitive performance particularly in the speed of information processing and executive functioning domains. In contrast, among HCV-seropositive patients, lopinavir–ritonavir use was associated with poorer performance particularly in learning, recall, and motor functioning.

The potential adverse effects of efavirenz and ritonavir on the brain were studied in 11 healthy HIV-seronegative individuals (mean age 31.5 years) [313]. In this small sample study using resting-state functional connectivity MR imaging and pulsed arterial spin labeling, short-term exposure to either efavirenz (14 days) or ritonavir (3 days) was not associated with significant alterations in functional connectivity or cerebral blood flow.

The neurotoxicity of individual antiretroviral drugs and cART regimens was studied in primary rat cerebral neuronal cells in culture [80, 314, 315]. For instance, in a survey of 15 antiretroviral drugs by Robertson et al. [80], the reduction in MAP2 immunoreactivity in neurons was used to indicate the neuronal toxicity of 1-week exposure to antiretroviral drugs. Experimental concentrations of antiretroviral drugs were in a range of at least one order of magnitude above and below the therapeutic plasma concentrations used in patients to suppress HIV replication. The levels of neuronal toxicity of antiretroviral drugs tested were found to range from undetectable to moderate, without significant cell death. This in vitro neurotoxicity study provides preliminary information on potential neurotoxic risks of antiretroviral drugs in the CNS; however, further in vivo animal models and clinical studies are needed.

An in vivo study addressed the potential neurotoxic effect of cART on synaptodendritic integrity in two animal models [315]. In the simian immunodeficiency virus (SIV)/pig-tailed macaque (*Macaca nemestrina*) model, cART-treated SIV-infected animals were found to have lower SYP immunoreactivity levels in the hippocampus compared to untreated SIV-infected or uninfected animals, whereas no significant difference in hippocampal MAP2 immunoreactivity levels was observed. However, cART-treated SIV-uninfected macaques were not included in this study. In the adult rat model, both SYP and MAP2 levels in the hippocampus were lower in animals with 1-week exposure to cART compared to control animals.

In addition to neuronal toxicity, the potential toxic effects of antiretroviral drugs on astroglia and oligodendroglia were demonstrated in cell systems and animal models [316, 317]. Vivithanaporn et al. [317] investigated the toxic effects of protease inhibitors on astroglial function. Primary human fetal astroglia in culture exposed to lopinavir or amprenavir for 2 days showed decreased excitatory amino-acid transporter-2 (EAAT2) expression, accompanied by reduced expression of cell proliferation markers Ki-67 and PCNA, without significant cell death. In lopinavir-treated astroglia, intracellular L-glutamate levels were decreased, while intracellular gamma-aminobutyric acid (GABA) levels were increased. In adult HIV-1 Vpr transgenic mice (expressing Vpr protein selectively in myeloid-derived cells), 3-week exposure to lopinavir/ritonavir was associated with reduced total levels of L-glutamate, L-aspartate, and L-serine in the cerebral cortex. Jensen et al. [316] reported that primary mouse oligodendroglia precursor cells exposed to ritonavir or lopinavir for 3 days displayed reversible dose-dependent decreases in oligodendroglia maturation, whereas those cells exposed to zidovudine (a nucleoside reverse transcriptase inhibitor) showed no significant changes. Adult mice with 14-day exposure to ritonavir showed decreases in myelin oligodendrocyte glycoprotein (MOG) and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase) levels in the frontal region. In addition, cART-treated patients with HAND were found to have lower myelin basic protein levels in the prefrontal cerebral region compared to cART-naive patients with HAND or non-HIV control participants.

The use of nelfinavir (a protease inhibitor) may promote cerebral β-amyloid accumulation because this drug can inhibit the insulin-degrading enzyme, a β-amyloid degrading enzyme in the CNS [318]. Nelfinavir and saquinavir (a protease

inhibitor) at therapeutic dosage can inhibit proteasome peptidase activity and lead to intracellular accumulation of polyubiquitinated proteins [319]. Nucleoside reverse transcriptase inhibitors (e.g. didanosine, stavudine, and zalcitabine) can induce mitochondrial dysfunction, resulting in oxidative stress and neuronal injury [320, 321].

In patients receiving cART, certain antiretroviral drugs may directly cause injury to vessel walls or indirectly induce metabolic abnormalities (e.g. insulin resistance and hyperlipidemia) that accelerate the development of atherosclerotic large vessel disease [322, 323]. Nonetheless, the potential impact of cART exposure on the development of arteriolosclerotic small vessel disease remains controversial [30, 33, 34, 37, 39–41]. Specifically, some drug components of protease-inhibitor-based cART may contribute toxicity to cell components of small vessels, leading to arteriolosclerosis [42, 235] (see Sect. 8.8.6). Different antiretroviral drugs even in the same class may carry differential degrees of toxicity to small vessels. Also, the toxic effects of antiretroviral drugs may vary with the duration of drug use and drug metabolism. As arteriolosclerosis is associated with aging in the general population [230], it is possible that certain antiretroviral drugs or combination regimens affect the integrity and function of small vessels by inducing premature senescence of vascular endothelial cells, smooth muscle cells, and pericytes [324–326].

8.10.4 Substance use

A range of substances of abuse, including opiates, cocaine, methamphetamine, and alcohol, are known to induce immune suppression and enhance HIV replication [327, 328]. Accumulating evidence from neuropsychological, neuroimaging, and neuropathological studies suggests that substances of abuse accentuate the adverse effects of HIV on the CNS [329–333]. Additionally, some clinical studies showed the impact of methamphetamine [334] or alcohol [335] on neurocognitive impairment in HIV-infected adults with a history of lifetime dependence might be more obvious in older age.

Interactions between substances of abuse and HIV-1 gp120 and Tat proteins facilitate disruption of the blood–brain barrier, release of TNF-alpha and other neurotoxic cytokines, upregulation of CCR5 expression, and induction of oxidative stress [327]. The toxic effects of methamphetamine or HIV-1 Tat or both on the blood–brain barrier, neurons, and glial cells have been investigated extensively in animal models and in vitro cell systems (for review, see [336]). Both methamphetamine and HIV-1 Tat were shown in vitro to inhibit astroglial Wnt/β-catenin signaling [337, 338]. In astroglia in culture, negative regulators of Wnt/β-catenin signaling increased the degree of permissiveness to HIV replication, upregulated the expression of transcription factors involved in the robust production of pro-inflammatory cytokines and chemokines, and downregulated glutamine synthetase (catalyzing the conversion of the excitatory neurotransmitter glutamate and ammonia into glutamine) [339].

Addictive psychostimulants increase extracellular dopamine in select dopaminergic brain regions [340]. Circulating CD14+/CD16+ monocytes play a key role in HIV neuropathogenesis [341–343]. An in vitro study by Coley et al. [344] demonstrated that dopamine, as well as D1-like dopamine receptor agonist SKF38393, increased migration and adhesion of CD14+/CD16+ monocytes (expressing all five dopamine receptors). In earlier reports, dopamine increased the number of HIV-infected cells in primary cultures of human monocyte-derived macrophages [345] and production of MCP1 by macrophages [346]. Taken together, these findings suggest that once CD14+/CD16+ monocytes cross the blood–brain barrier into the CNS parenchyma in response to chemokines, elevated extracellular dopamine in the brain (inherent to the setting of psychostimulant abuse) can promote the accumulation of CD14+/CD16+ monocytes in dopaminergic brain regions, thereby facilitating HIV persistence in the CNS and local neuroinflammation.

Methamphetamine use in particular is a common comorbidity in HIV-infected persons [347]. The pharmacokinetics of methamphetamine in the human brain is characterized by relatively fast uptake, widespread distribution, and slow clearance, as shown in PET studies with [^{11}C]-d-methamphetamine [348, 349]. Consequently, the brain undergoes extended exposure to the sympathomimetic and toxic effects of methamphetamine. The widespread distribution of methamphetamine was also observed in the postmortem brains of chronic methamphetamine users with methamphetamine present in their blood [350]. Accordingly, chronic methamphetamine use may induce long-lasting injury involving broad brain regions and not limited to structures containing presynaptic monoaminergic nerve terminals [347, 351]. In support of this notion, a PET study with [^{11}C](*R*)-PK11195 (a radiotracer targeting the 18-kDa translocator protein enriched in activated microglia) showed higher activated microglial density in multiple brain regions of abstinent methamphetamine users compared to age-matched control participants [352]. In abstinent methamphetamine-dependent women, the glial ^{13}C–bicarbonate production rate from [1-^{13}C]acetate (equating with the glial Krebs cycle rate) in the frontal region on ^{13}C MR spectroscopy was much lower than that in normal control participants [353]. In addition, evidence of increased white matter hyperintensities on MR imaging, suggestive of ischemic lesions caused by cerebral small vessel disease [225], was reported in men with lifetime methamphetamine dependence [354].

Methamphetamine dependence was found to increase the risk of cognitive impairment in HIV-infected individuals [355], particularly those who had advanced immune suppression [356]. Even in the acute and early stages of HIV infection, methamphetamine use was associated with neurocognitive impairment and an increased risk of clinically significant challenges in real-world daily functioning [72, 73].

Additive effects of chronic methamphetamine use and HIV infection were documented on cerebral metabolite abnormalities indicating neuronal injury (lower *N*-acetylaspartate levels in the frontal cortex, frontal white matter, and basal ganglia) and glial activation (higher *myo*-inositol and choline levels in the frontal white matter), measured with proton MR spectroscopy [357]. In another study [358], the effects of methamphetamine on cerebral metabolite changes were not evident, but

methamphetamine seemed to modify the effects of HIV to the extent that plasma HIV-1 RNA levels correlated inversely with *N*-acetylaspartate levels and directly with *myo*-inositol levels in the frontal white matter only in the HIV-infected methamphetamine-dependent group. In a structural MR imaging study [359], HIV infection was found to associate with decreased volumes of cortical, limbic, and striatal structures, whereas methamphetamine dependence was associated with increased volumes of the basal ganglia and parietal cortex. Neurocognitive impairment was associated with decreased cortical volumes in HIV-infected participants but increased cortical volumes in methamphetamine-dependent participants.

The effects of chronic methamphetamine use on neuropathological changes have been investigated in postmortem HIV-infected brains [112, 137, 360]. Langford et al. [112] reported that among HIV encephalitis brains, those of chronic methamphetamine users had higher degrees of CD45-immunoreactive microgliosis and SYP immunoreactivity loss in the frontal cortex. Selective loss of calbindin-1-immunoreactive interneurons in the frontal cortex was described particularly in the brains of HIV-infected methamphetamine users with evidence of HIV encephalitis [112], which correlated with memory impairment [360]. Everall et al. [137] showed that transcript expression of select interferon-inducible genes in the frontal cortex was upregulated in the HIV encephalitis brains of methamphetamine users compared to HIV encephalitis brains and HIV-infected brains (without discernable pathology) of individuals with no history of methamphetamine use. In all these studies, the association of methamphetamine use with greater neuropathological changes was reported in HIV encephalitis brains. Hence, the findings might not be applicable directly to virally suppressed persons in the cART era.

Recent reports using postmortem HIV-infected brains have described nuclear DNA methylation, mitochondrial DNA injury, and cerebral gliosis [116, 361, 362]. Epigenetic mechanisms were studied in the frontal cortex of HIV-infected individuals with or without methamphetamine dependence [361]. Methamphetamine was associated with increased levels of *DNMT1* gene (DNA (cytosine-5)-methyltransferase-1 enzyme, involved in the maintenance of DNA methylation) expression and global DNA methylation that correlated directly with HIV-1 RNA levels. Genome-wide profiling of DNA methylation in a subset of cases showed differential methylation in select host genes involved in cellular pathways, such as dopamine metabolism and transport. These findings suggest that HIV and methamphetamine may act together to alter expression of select genes by regulating DNA methylation in the brain.

In a clinicopathological study of 78 HIV-infected adults, Soontornniyomkij et al. [116] showed that lifetime methamphetamine dependence (18 past and two current dependence at the final follow-up visit) was associated with focal cerebral Iba1-immunoreactive microgliosis, even after statistically adjusting for biologically-relevant covariates including HIV encephalitis, white matter lesions, opportunistic diseases, HCV seropositivity, and lifetime dependence on alcohol, opiates, and cannabis. Nonetheless, there was no significant association of methamphetamine with other brain pathological changes including cerebral GFAP-immunoreactive astrogliosis, frontal cortical synaptodendritic loss, cerebral β-amyloid plaque deposition,

and arteriolosclerosis in the forebrain white matter. These findings suggest that some of the pathological changes in certain brain regions might be reversible and have gradually diminished following prolonged abstinence from methamphetamine [352, 363–367]. It is also possible that some of the pathological changes examined in this study [116] might not have been induced by methamphetamine in the first place [368, 369]. The differential susceptibility to methamphetamine neurotoxicity might vary from region to region in the brain [351, 364].

Although a large number of animal studies were reported addressing the effects of acute or chronic methamphetamine exposure on various brain structures [370–374], to date only a few studies have focused on the combined effects of methamphetamine and HIV in animal models [375–377]. For example, Hoefer et al. [376] reported evidence of long-lasting synaptodendritic loss in the cerebral cortex and hippocampus of HIV-1 gp120-transgenic mice exposed to a chronic escalating-dose multiple-binge methamphetamine regimen that mimicked a pharmacokinetic profile of methamphetamine use in humans.

8.10.5 Hepatitis C Virus Coinfection

Approximately 20–30% of people living with HIV are coinfected with HCV. The blood-borne transmission of HCV is the commonest route, whereas transmission via sexual intercourse occurs less frequently and vertical transmission is uncommon [378]. This observation explains the high rates of HIV/HCV coinfection among intravenous drug users [327]. Nonetheless, increases in sexually acquired HCV seroconversion have recently been documented in HIV-infected men who have sex with men [379]. Following acute HCV infection, approximately 90% of HIV-infected patients develop chronic HCV disease. This rate of chronicity is significantly higher than that in patients infected with HCV alone (<70%) [378]. A positive anti-HCV antibody test should be followed by a sensitive quantitative assay for plasma HCV RNA [380, 381]. The presence of anti-HCV antibody in the absence of detectable HCV RNA in blood indicates spontaneously resolved or treated infection. Accurate genotyping of HCV is also important as various HCV genotypes (1 to 7) respond differently to, and need different durations of, treatment with pegylated interferon and ribavirin [382].

Coinfection with HIV is associated with higher HCV RNA loads, persistent HCV viremia, and reduced response to interferon-based HCV therapy. HIV/HCV coinfection with low CD4 T-cell counts, together with alcohol use, is commonly associated with rapid progression of hepatic fibrosis to cirrhosis and increases in morbidity and mortality [378, 383]. Early initiation of cART is recommended for HIV/HCV coinfected patients although it may confer a higher risk of hepatotoxicity than in patients with HIV infection alone. Currently, efficacious HCV therapy with combination direct-acting antiviral drug regimens (targeting NS3/4A, NS5A, and NS5B HCV proteins) of shortened durations promises to improve sustained virological response rates [382, 384, 385].

HCV can cross the blood–brain barrier and is detectable in both the CSF and brain parenchyma [327]. In HIV-infected persons with HCV seropositivity, HCV RNA and proteins were found in brain tissue specimens, with HCV immunoreactivity localized to astroglia and perivascular macrophages, suggesting that HCV might undergo active replication and cause neural injury in conjunction with HIV [170]. HCV could also infect cerebral microvascular endothelial cells [386]. HCV seropositivity was found to associate with elevated levels of MCP1, TNF-alpha, and soluble TNF receptor II in the CSF [387].

The individual and combined effects of HIV and HCV on cognitive impairment have been described in several cohorts [388–393]. For instance, HCV seropositivity was associated with poorer neuropsychological performance after statistically adjusting for HIV seropositivity and methamphetamine dependence [387]. In a clinical study of adults with advanced HIV disease, HIV/HCV coinfected individuals were found to carry a higher risk of neurocognitive impairment, particularly in the functioning domains of learning and memory, compared to HIV mono-infected participants [394]. Still, not all clinical studies have reported greater neurocognitive impairment in HIV/HCV coinfected patients [327]. For example, in a CHARTER study [395], scores in all seven domains of neurocognitive functioning were not significantly different between groups of 160 HCV-seropositive and 707 HCV-seronegative individuals without serious comorbid neurological conditions that might impair neurocognitive performance. In the HCV-seropositive group without substantial HCV-associated liver damage, there was no significant association between neurocognitive performance and HCV RNA loads in blood.

8.10.6 Brain Aging

In populations with widespread use of cART, the dramatically improved survival due to cART and increase in newly diagnosed HIV infection in older adults lead to the growing number of older HIV-infected persons. Compared to HIV-infected younger adults, older patients usually show better adherence to cART and achieve systemic viral suppression, but may exhibit a slower immune recovery (as measured by CD4 T-cell count increases) [396]. Chronic comorbid conditions unrelated to HIV are more frequent in older HIV-infected adults [397], which may affect the clinical outcome of HIV infection. A substantial proportion of older HIV-infected patients are on chronic medications for comorbid conditions, such as hypertension, chronic airway disease, diabetes, arthritis, coronary artery disease, depression, renal disease, visual defects, and lipid disorders [398], leading to increases in the long-term pill burden and risk of drug–drug interactions when they concurrently receive cART [396].

A clinical study from the Hawaii Aging with HIV Cohort showed that the likelihood of having HAD among older adults was about three times that in younger adults after statistically adjusting for education, race, current substance dependence, cART status, plasma HIV-1 RNA loads, blood CD4 T-cell counts, and Beck

Depression Inventory scores [399]. In a study in Western Pennsylvania, the prevalence of neurocognitive impairment among HIV-infected patients over 50 years of age was higher than in younger individuals [400]. In a large European cohort in the cART era, older age at seroconversion was associated with higher risk of HAD, independently of current CD4 T-cell counts [20]. Nonetheless, the combined effects of age and HIV infection on cognition are not consistent, probably due to large individual differences in other comorbid factors among older HIV-infected adults [401]. In a longitudinal study of 146 participants (aged ≤40 years or ≥50 years) with normal cognition at baseline, Sheppard et al. [402] reported that HIV infection, independent of age, conferred a nearly five-fold risk of developing neurocognitive impairment 14 months later.

In the general population, a large proportion of elderly people are affected by neurocognitive decline in specific functioning domains not attributable to defined clinicopathological entities of dementia [403, 404]. The differential susceptibility to the development of age-related cognitive impairment may be driven by host genetic polymorphisms, epigenetic phenomena, and dissimilar life-long environmental exposure to stressors [405]. Mechanisms of brain aging have been proposed to involve cerebrovascular regulation, neuronal calcium homeostasis, synaptic plasticity, oxidative stress, neuroinflammation, and hypothalamic–pituitary–adrenal axis activity [404, 406].

In the CNS vasculature, age-related modifications in elastin and collagen compositions lead to the thickening of vascular basement membranes and reduction in distensibility of blood vessels, with progression to arteriolosclerosis [232]. Cerebral neocortical volume loss with increasing age is likely associated with a decrease in neuronal architectural complexity, rather than significant loss of neurons [404, 407]. During aging, there is a progressive deficiency in the handling and clearance of misfolded proteins, such as β-amyloid, phospho-Tau, and alpha-synuclein. Clearance mechanisms that contribute to β-amyloid elimination from the CNS include enzymatic degradation in glial cells, perivascular macrophages, neurons, and the extracellular space, transport across the blood–brain barrier, and perivascular interstitial fluid drainage along the basement membrane of capillaries and arteries [408–411].

The proportion of human brains involved by Tau pathology and cerebral β-amyloidosis in various stages increases with age [198]. Intraneuronal Tau lesions are observed initially during childhood in the noradrenergic locus ceruleus, serotoninergic dorsal raphe nucleus, and cholinergic nucleus basalis of Meynert in neurons that diffusely send axonal projections to the cerebral cortex, and then in the transentorhinal region. In contrast, extracellular diffuse β-amyloid plaques first appear in the fourth decade in the cerebral neocortex (basal temporal and orbitofrontal regions in particular) [198, 412–414]. Each of these lesions then expand to involve additional brain regions in a stereotypic spatial pattern of its own [198]. In the cerebral neocortex, the temporal progression of β-amyloid plaques and Tau lesions has been suggested to follow a stereotypic sequence: diffuse (predominantly nonfibrillar) β-amyloid plaques, Congo-red-positive (i.e. fibrillar) β-amyloid plaques, argyrophilic (silver-based histochemistry) phospho-Tau-immunoreactive

neuritic β-amyloid plaques and neuropil threads, and finally argyrophilic phospho-Tau-immunoreactive neurofibrillary tangles in neuronal soma [415]. It is the presence of argyrophilic phospho-Tau-immunoreactive pathology in the cerebral neocortex that is associated with neurocognitive impairment [416, 417].

Premature (accelerated) brain aging may occur in HIV-infected adults, especially in those with poor control of HIV replication [418], consequently promoting the development of neurocognitive impairment. A varying degree of systemic chronic inflammation inherent to HIV disease may accelerate aging process in the CNS. Even in individuals with systemic viral suppression, systemic chronic low-level inflammation [22] may trigger stress-induced premature cellular senescence of replication-competent glial and vascular cells in the CNS, creating a pro-inflammatory milieu (i.e. senescence-associated secretory phenotype) and consequently neuronal degeneration [419–421]. In addition, certain HIV proteins and comorbid factors may prematurely trigger or promote a cascade of metabolic disturbances that would otherwise occur only in the aging brain.

To study accelerated aging associated with HIV disease, Horvath and Levine [422] used an epigenetic biomarker of aging based on host DNA methylation levels. The epigenetic clock was defined as a prediction method of age, based on the linear combination of the DNA methylation levels of 353 cytosine–phosphate–guanine [CpG] dinucleotide sites in the human genome, assayed via the Infinium Human Methylation 450 K platform (Illumina, San Diego, California, USA). The HIV-infected group was found to have an increase in epigenetic age in both brain tissue (7.4 years) and blood (5.2 years), relative to the non-HIV control group. Interestingly, HIV infection seemed to accelerate the age of some brain regions (e.g. the occipital lobe and cerebellum) but not that of others (e.g. the frontal lobe). It remains to be determined as to how HIV infection leads to epigenetic age acceleration. In a subsequent analysis of DNA methylation data from the occipital cortex, Levine et al. [423] showed that individuals diagnosed with HAND within 1 year before death had greater accelerated aging relative to those HIV-infected persons with normal cognition.

Older HIV-infected adults may also be at increased risk of developing age-related cognitive impairment, mild cognitive impairment, and dementias commonly found in the general older population [424–426]. Sheppard et al. [427] studied 75 HIV-infected adults (aged ≥50 years) on cART with undetectable plasma HIV-1 RNA loads in comparison to 80 demographically similar non-HIV control participants. Mild cognitive impairment (according to the comprehensive diagnostic scheme described by Bondi et al. [428]) was diagnosed in 16% of HIV-infected persons compared to 2.5% of the non-HIV counterparts. Within the HIV-infected group, mild cognitive impairment had minimal overlap with ANI diagnosis and was significantly associated with older age, lower Karnofsky Scale of Performance scores, and mild difficulties performing instrumental activities of daily living. Further, the brains of certain older HIV-infected patients were found to have Alzheimer's disease neuropathological changes comparable to those seen in symptomatic Alzheimer's disease (personal observation).

8.10.7 Systemic Chronic Inflammation and Metabolic Disturbance

The pathogenesis of HAND may involve systemic chronic inflammation and metabolic disturbance that are related to the gut–liver–brain axis [22, 429]. In a study of HIV-infected individuals with advanced immune suppression by Ancuta et al. [430], high plasma levels of bacterial lipopolysaccharide (an indicator of microbial product translocation from the gut to the portal system and systemic circulation) were associated with HAD compared to normal cognition, independent of plasma HIV-1 RNA loads and blood CD4 T-cell counts. In a subsequent study by the same group [431], elevated plasma levels of soluble CD14 (a marker of monocyte activation) were associated with neurocognitive test scores indicating global impairment, especially in attention and learning domains, in individuals with advanced HIV disease; however, no difference in plasma-soluble CD14 levels was observed among HAND categories and normal cognition.

In a large community-based study by Towner et al. [432], HIV-infected individuals, compared to non-HIV control participants, had a greater overall risk of both hepatic dysfunction (as measured by serious clinical hepatic events or biochemical laboratory markers of dysfunction) and hepatic dysfunction-related death, even after statistically adjusting for known hepatic risk factors. In the HIV-infected group, clinical factors associated with increased risk included lower blood CD4 T-cell counts, higher plasma HIV-1 RNA levels, alcohol/drug abuse, HCV or hepatitis B virus coinfection, and diabetes. In a cross-sectional study by Matthews et al. [433], significant liver fibrosis based on transient elastography (FibroScan, Echosens, Paris, France) was observed in 7.7% of 221 untreated HIV-infected persons. Higher blood levels of HIV-1 RNA and alanine aminotransferase (a marker of liver injury) were clinical factors associated with higher transient elastography scores in multivariable analysis. The association with plasma HIV-1 RNA loads suggests that HIV itself may involve in liver inflammation.

In HIV disease, the intestinal mucosal barrier and gut-associated lymphoid tissue are compromised, leading to gut microbial translocation [429, 434–436]. The microbial products are delivered via the portal venous circulation into the liver, stimulating Kupffer cells (tissue macrophages) and hepatic stellate cells (pericytes) to become pro-inflammatory and pro-fibrotic, and contributing to hepatic dysfunction, impaired clearance of microbial products and metabolites, and dysregulated protein synthesis [22, 437]. In addition, HIV may directly infect Kupffer cells [438] and hepatic stellate cells [439].

The gut microbiota composition and stability are influenced by host genetic polymorphisms (particularly in those genes that are related to immunophenotypes), the use of antibiotics [440] and certain psychotropic drugs, such as selective serotonin reuptake inhibitors [441] and atypical antipsychotic olanzapine [442], stress, physical activity [443], and diet [444–446]. In HIV disease either untreated or treated with cART, the gut microbiota composition is altered [434, 435, 447–453]. These alterations may lead to modification in the levels or proportions of short-chain

fatty acids produced by colonic bacterial flora as byproducts of fermentation of dietary fiber, as well as elevation of ammonia production by urease-bearing bacteria in the large intestine.

Acetate, propionate, and butyrate are major short-chain fatty acids produced and absorbed in the proximal large intestine [454]. Short-chain fatty acids have multiple effects on metabolism, including being energy sources for colonic enterocytes, muscle, kidney, heart, and brain [455]. Lack of butyrate increases intestinal permeability to bacterial lipopolysaccharide, leading to systemic chronic low-level inflammation. The levels of short-chain fatty acids in the colon and blood circulation are important for immune regulation via chemoattractant G-protein coupled receptors [456]. Gut microbiota-derived short-chain fatty acids also regulate the maturation and innate immune function of microglia in the CNS [457–459].

Acetate in the blood circulation is derived from gut microbiota, as well as ethanol metabolism. Acetate is converted to acetyl-coenzyme A and undergoes subsequent oxidative metabolism in the Krebs cycle of most cells. In the CNS, nonetheless, acetate is metabolized mainly in astroglia (rather than neurons) because monocarboxylate transporter-2 (MCT2, the only MCT expressed by neurons) fails to recognize acetate to the same extent as MCT1 expressed by astroglia and endothelial cells [460, 461]. In a rat model of neuroinflammation [462], acetate supplementation attenuated lipopolysaccharide-induced neuroglial activation, suggesting that acetate is anti-inflammatory in the CNS.

The changes in the gut microbiota composition in HIV disease may lead to increased ammonia production by ammonia-producing bacteria using the enzyme urease, such as *Proteus mirabilis* [463]. Under physiological conditions, the small and large intestines are major sources of ammonia in the blood circulation via enzymatic breakdown of amino acids (mainly glutamine) and bacterial breakdown of urea and amino acids, respectively [464, 465]. The hepatic portal vein delivers ammonia to the liver, where ammonia is incorporated into urea via the (low affinity, high capacity) urea cycle within periportal hepatocytes or glutamine via the (high affinity, low capacity) glutamine synthetase reaction within perivenous hepatocytes [464, 466].

Hyperammonemia and hyperglutaminemia may lead to neuroinflammation and dysregulation of certain neurotransmitters, as evident in *minimal hepatic encephalopathy* in patients with chronic liver disease [467]. In the CNS, glutamine synthetase is expressed mainly in astroglia. One of the primary functions of astroglia is to protect neurons from ammonia toxicity and glutamate-mediated excitotoxicity by taking up ammonia (readily crossing the blood–brain barrier) and glutamate (at synapses) and converting them into glutamine in the glutamine-synthetase-dependent reaction [468]. HIV-1 Tat protein and methamphetamine can inhibit Wnt/β-catenin signaling in astroglia [337], thereby downregulating glutamine synthetase expression [339]. Ammonia toxicity in the CNS of HIV-infected methamphetamine users may be enhanced through this mechanism.

Ammonia itself could generate cellular oxidative stress and energy deficit through disturbances of the nitric oxide pathway, inhibition of the Krebs cycle, and opening of the mitochondrial permeability transition [469, 470]. Ammonia could

also mediate the inhibition of glutamate uptake into astroglia via the downregulation of glutamate transporters, which might lead to glutamate-mediated neuronal excitotoxicity [470]. Glutamine (both entering the brain from the systemic circulation and formed in situ in astroglia) could enter the mitochondria and be converted into glutamate and ammonia by the enzyme glutaminase [471], contributing to ammonia-induced mitochondrial dysfunction [472]. In agreement with this hypothesis, clinical studies of patients with urea cycle disorders showed that hyperglutaminemia and hyperammonemia occurred in parallel and both correlated with neurocognitive impairment [473, 474].

In sum, a conceptual model has emerged to explain the high prevalence of HAND, even in effectively treated HIV-infected adults [22]. That is, gut microbial translocation and alterations in the gut microbiota composition in HIV disease can lead to hepatic impairment, systemic chronic low-level inflammation, and systemic metabolic disturbance, which may induce indolent metabolic encephalopathy, neuroinflammation via the neurovascular unit, and chronic neurodegeneration [22, 429–431, 475, 476]. Also, it is reasonable to hypothesize that HIV-associated changes in the gut microbiota composition may cause a decrease in acetate levels and increases in ammonia and glutamine levels in the blood circulation and CNS, which in turn adversely affect astroglial functions at neuronal synapses. The innate immune function of microglia may also be disturbed by alterations in the levels of gut microbiota-derived short-chain fatty acids [457–459]. The disturbances of the gut–liver–brain axis could also be exacerbated by chronic alcohol use and HCV infection, comorbid factors common in HIV-infected adults [22, 477].

8.11 Conclusions

HAND is a common complication of HIV disease and profoundly affects the clinical outcome even in patients who have achieved systemic viral suppression with cART. HAND is a multifactorial syndrome attributed to the extent of HIV infection in the CNS and shaped by a variety of comorbid factors. Despite systemic viral suppression, low-level HIV replication may exist especially in the CNS due to variability in CNS penetration of cART regimens. A variety of comorbid factors that may be involved in the pathophysiology of HAND include HIV-1 genetic variants, host genetic polymorphisms, chronic cART-related neurotoxicity, substance use, HCV coinfection, and brain aging. These factors, together with certain HIV-1 proteins, may induce neuronal degeneration or alternatively trigger stress-induced premature cellular senescence of replication-competent glial and vascular cells, leading to neuroinflammation and neurodegeneration. In addition, gut microbial translocation in HIV disease may lead to chronic low-level systemic inflammation, hepatic dysfunction, systemic metabolic disturbance, and indolent metabolic encephalopathy. Accordingly, the pathophysiology of HAND in the cART era may vary from case to case, representing additive or synergistic effects of multiple contributing factors. In some cases, HAND is associated with robust CNS HIV replication as a primary

contributing factor, whereas in other cases, it is related to various combinations of small-effect comorbid conditions that interact with chronic low-level CNS HIV replication [92, 156].

It remains challenging to study the pathophysiology of HAND because of an inevitable selection bias toward end-stage AIDS cases coming to autopsy, which are not representative of the clinical profiles of virally suppressed patients living with HAND. To some extent, modern functional neuroimaging techniques (e.g. 3-Tesla MR spectroscopy and PET) [21, 478], as well as biomarker assays of the CSF, may elucidate some aspects of the living CNS pathology in relation to a waxing and waning pattern of HAND.

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Chapter 9
HIV-Related Peripheral Nervous System Illness

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Keywords HIV • Peripheral nervous system • Neuropathy • Neuropathic pain • Autonomic • Radiculopathy • Myopathy

Core Message

- Distal symmetric polyneuropathy (DSP) is the most prevalent disorder of the peripheral nervous system in HIV-infected individuals and it typically presents with uncomfortable sensory symptoms in the feet.
- As there is no disease-modifying treatment for HIV-DSP, treatment focuses on control of painful symptoms with agents such as antiepileptics and antidepressants.
- Autonomic neuropathy is also common in HIV, but it is typically unrecognized because its symptoms are often nonspecific and diagnosis requires specialized testing.
- Other forms of neuropathy experienced by HIV-infected individuals include focal involvement of an individual nerve (mononeuropathy) or nerves (mononeuropathy multiplex) and demyelinating neuropathies.
- Radiculopathy, the dysfunction of a nerve root, leads to pain in the distribution of that nerve root, which may be accompanied by motor and sensory dysfunction. Radiculopathies are likely to become more common in HIV as the population ages, due to their association with degenerative spine disease.
- Primary disorders of muscle are rare in HIV in the current treatment area, although nonspecific symptoms such as myalgia are common.

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9.1 Introduction

The widespread implementation of potent combination antiretroviral therapy (CART) has dramatically reduced HIV-associated morbidity and mortality, but it has not completely restored the health of the infected individual. Increased survival has allowed for the development of chronic complications of HIV infection, including those involving the peripheral nervous system (PNS). Disorders of peripheral nerves are prevalent in HIV-infected individuals, and these disorders may cause debilitating symptoms such as neuropathic pain and gait instability. Underlying etiologies include inflammatory, infectious, vasculitic, and neoplastic disorders of the PNS; however, the pathophysiology of many of these disorders remains incompletely understood. Management of HIV-related PNS disorders is complicated by challenges in diagnosis, limited pharmacological management options, and the medical and biopsychosocial complexity of the affected population. Therefore, the clear delineation of these disorders is critical to both patient care and the research efforts required to advance in this field. In this chapter, we discuss PNS disorders in HIV-infected individuals, which encompass a diverse group of localized, generalized, acute, and chronic clinical entities involving peripheral nerve (see Table 9.1) and muscle. We focus on HIV-1 in general without reference to particular subtypes and strains.

9.2 HIV-Associated Distal Symmetric Polyneuropathy (HIV-DSP)

9.2.1 Clinical Presentation, Epidemiology and Diagnosis

Distal symmetric polyneuropathy (DSP) is the most prevalent disorder of the PNS in HIV-infected individuals. HIV-DSP commonly presents with pain and other uncomfortable sensory phenomena in a distal symmetrical distribution, usually beginning in the feet and then, in more severe cases, progressing proximally to assume the classic "stocking and glove" distribution involving the legs and hands. The sensory symptoms of HIV-DSP can be either provoked or spontaneous and include: numbness, tightness, coldness, burning pain, pins and needles or electrical sensations (paresthesias), hypersensitivity to touch (hyperesthesia), and the perception of normally nonpainful stimuli as painful (allodynia). The clinical impact of neuropathic pain in HIV-DSP extends beyond the symptoms themselves, adversely affecting employment, quality of life, and mood. Estimates of the prevalence of HIV-DSP vary based on the population under study, the rigor of the diagnostic criteria applied, and whether asymptomatic individuals are included. When general HIV-infected populations are assessed using bedside diagnostic techniques, the prevalence of clinically relevant, symptomatic HIV-DSP is approximately 20% [1].

Table 9.1 Comparison of common peripheral neuropathic disorders in HIV-infected individuals

Neuropathic disorder	Prevalence in HIV	Risk factors	Clinical presentation	Diagnostic tools	Treatment options
HIV-DSP	Common	Older age DM[a] Lower nadir CD4 History of substance abuse	Distal, symmetric pain, and sensory dysfunction	NCS/EMG[b] Skin biopsy if NCS/EMG[b] normal	Foot care Symptomatic treatment of neuropathic pain (e.g. gabapentin)
Autonomic neuropathy	Common	Older age DM[a] HIV-DSP	Varied: orthostatic hypotension, dry eyes/mouth, N/V, diarrhea/constipation, urinary/sexual dysfunction, changes in body sweating/temperature/color	Autonomic reflex screen	Avoid agents with anticholinergic side effects Lifestyle modifications
Mononeuropathies	Common	HIV-DSP	Varied, depending upon the specific nerve that is involved	NCS/EMG[b]	Conservative (e.g. neutral wrist splint for carpal tunnel syndrome) Surgical decompression when indicated
Mononeuropathy multiplex	Rare	Severe immunodeficiency (CMV-related forms) Polyarteritis nodosa-like vasculitis	Asymmetric pain +/− sensory dysfunction +/− motor involvement	NCS/EMG[b] Lumbar puncture	If immune mediated: IVIg, plasmapheresis, or steroids If CMV-related: antiviral agents (e.g. ganciclovir)
Inflammatory demyelinating polyneuropathies	Rare	N/A	Symmetric, progressive ascending muscle weakness with loss of DTR's; sensory dysfunction may be less prominent in acute form	NCS/EMG[b] Lumbar puncture	IVIg Plasmapheresis Steroids (for CIDP)

(continued)

Table 9.1 (continued)

Neuropathic disorder	Prevalence in HIV	Risk factors	Clinical presentation	Diagnostic tools	Treatment options
Radiculopathy	Common	Older age Degenerative spine disease CMV infection	Pain at affected area of spine with radiation +/− sensory loss, decreased DTR's, weakness in nerve root distribution	MRI Lumbar puncture NCS/EMG[b]	If structural: conservative vs. surgical treatment In CMV-related polyradiculopathy: antiviral agents (e.g. ganciclovir)
ALS	Rare	Retroviral infections besides HIV (HTLV-1, HFV)	Progressive weakness and muscle atrophy with preservation of sensory function	NCS/EMG[b]	Initiation of CART
DILS	Rare	Untreated/uncontrolled HIV infection	Acute or subacute onset of painful, symmetric paresthesias of lower extremities	NCS/EMG[b] Nerve biopsy	Initiation of CART Steroids

[a]Diabetes mellitus
[b]Nerve conduction studies and electromyography

In studies including asymptomatic individuals, using more sensitive diagnostic testing, or examining sicker populations (e.g. older patients with long-standing HIV and multiple comorbid medical illnesses), the prevalence of HIV-DSP can exceed 50% [1–3].

In practice, HIV-DSP is often a clinical diagnosis, particularly in patients with typical symptoms who evidence the classic clinical signs of decreased or absent ankle reflexes, and decreased sensation at the toes to one or more modalities (vibratory, pinprick, temperature, or joint position sense). In such patients, additional diagnostic testing is usually unnecessary. However, good clinical practice dictates excluding other noninfectious causes of neuropathy, such as medication toxicity, alcoholism and other forms of substance abuse, diabetes mellitus, vitamin B12 deficiency, hypothyroidism, and monoclonal gammopathy [4]. With regard to medication toxicity, there are numerous commonly used medications, which may cause or exacerbate neuropathy (e.g. chemotherapeutic agents, fluoroquinolones, dapsone, isoniazid), and so it is prudent to review the medication list of any patient with HIV-DSP [5]. More specifically, certain antiretrovirals may cause neuropathy particularly the nucleoside analog reverse transcriptase inhibitors commonly known as "D-drugs" (ddI, ddC, and d4T) [6]. These agents, which are no longer recommended in the developed world but are still used in some developing nations, are posited to adversely impact peripheral nerve via mitochondrial toxicity [7].

Confirmatory tests for HIV-DSP are ordered when there is diagnostic uncertainty and may include nerve conduction studies and electromyography (NCS/EMG) and skin biopsy (see Fig. 9.1). NCS/EMG assesses the function of large nerve fibers such as those responsible for motor function and vibratory and joint position sense. NCS/EMG is useful for confirming the severity of HIV-DSP and for excluding other forms of neuropathy, which may require specific treatment such as mononeuropathies, and demyelinating neuropathies (described in later sections).

Occasionally, HIV-DSP preferentially involves small nerve fibers such as autonomic nerve fibers and those carrying temperature and pain sensation. In such small fiber neuropathies, patients may complain of significant neuropathic pain but display only subtle abnormalities on neurologic examination, for example, decreased temperature and sharp sensation in the feet. NCS/EMG is often normal in small fiber neuropathies, and so in these cases, skin biopsy is the diagnostic modality of choice, allowing visualization and quantification of epidermal nerve fiber density [8].

More extensive testing is often employed in the research setting where accurate and objective quantification of HIV-DSP is required. The Total Neuropathy Score (TNS) is a measure originally validated in diabetic and chemotherapy-induced neuropathy [9], which was subsequently modified and validated for use in HIV [10, 11]. The TNS integrates severity of reported sensory and/or motor symptoms, the neurologic exam, autonomic indices, and NCS to grade the overall severity of HIV-DSP. The Brief Peripheral Neuropathy Screen has also been used and incorporates neuropathic symptoms of the lower extremities and an exam of vibration sense and ankle reflexes [12].

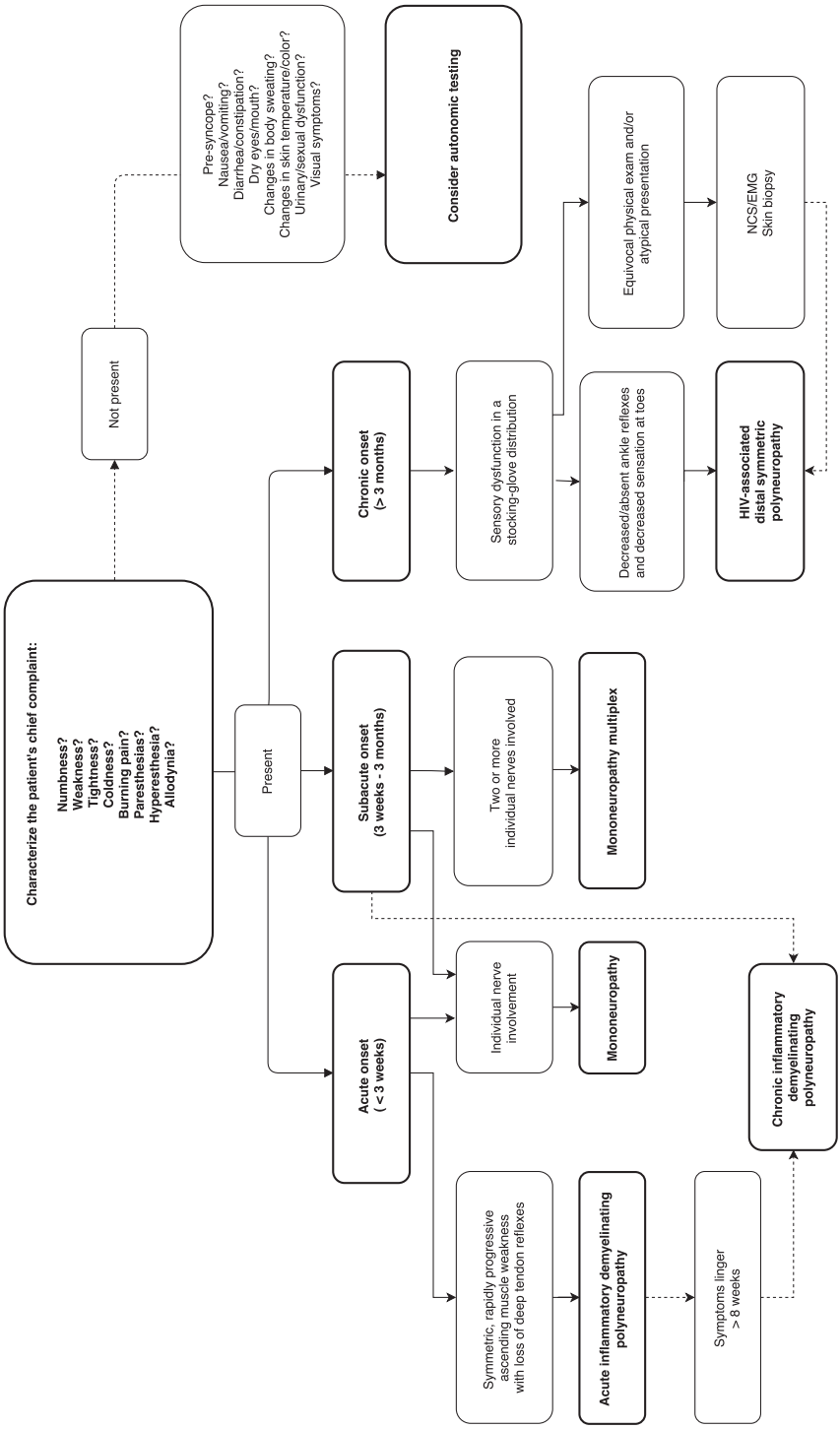

Fig. 9.1 Diagnostic algorithm distinguishing HIV-DSP from other peripheral nerve disorders in HIV

9.2.2 Risk Factors and Pathogenesis

Several studies have examined the risk factors for HIV-DSP and also for the development of neuropathic pain in HIV-infected individuals. Not surprisingly, one of the most consistent risk factors for HIV-DSP is advancing age, consistent with the observation that DSP is more common among older people in the general population [1, 3, 13, 14]. Additional risk factors include diabetes, lower nadir CD4 count, and history of substance abuse [14, 15]. Risk factors for neuropathic pain in the setting of HIV include female sex, current and past antiretroviral treatment, lack of virologic suppression, depressive symptoms, and lifetime history of opioid use disorder [16].

Pathologically, HIV-DSP is a length-dependent, axonal, predominantly sensory polyneuropathy. As such, the main pathology of HIV-DSP arises from the distal axon, although there are also inflammatory abnormalities observed at the level of the dorsal root ganglion. The pathogenesis of DSP is likely multifactorial in many cases, with HIV-specific mechanisms of nerve injury being compounded by non-HIV factors. HIV does not appear to infect nerve cells directly, rather the virus is brought to the nerve by HIV-infected immune cells (T cells and macrophages). Viral proteins, particularly gp120 and viral protein R (Vpr), then bind to chemokine receptors present on axons leading to activation of inflammatory signaling pathways and axonal damage [17, 18]. In addition to inflammatory mechanisms, HIV also negatively impacts the function of mitochondria, leading to axonal damage particularly in the more vulnerable distal axons [19]. The effects of aging, comorbid illness, and exposure to polypharmacy and substances of abuse add insult to injury and likely are collectively responsible for the continued high prevalence of HIV-DSP despite effective CART.

9.2.3 Management

Treatment of HIV-DSP is initiated by identifying and addressing any modifiable exacerbating factors, including eliminating neurotoxic medications when possible, and addressing any relevant comorbid medical issues such as diabetes mellitus, vitamin B12 deficiency, renal or liver impairment, or thyroid dysfunction. Currently, there are no neuroregenerative therapies for any form of neuropathy, including HIV-DSP, and so treatment is symptomatic. When considering initiation of treatment, it is important to establish the most bothersome symptoms to the patient. Loss of sensation is not specifically reversible, and so if this is the primary symptom, the patient should be counseled regarding proper foot care and pharmacologic agents should not be initiated. If the primary symptoms are neuropathic pain or paresthesias, then pharmacologic treatment may be offered based on patient preference. Patients should be made aware that currently available therapies are not disease-modifying, and so it is reasonable to forego treatment, particularly if minimizing

pill burden is a priority and symptoms are not too severe. If treatment is to be initiated, then it is important to establish realistic expectations. Neuropathic pain is difficult to treat, and it is unlikely that the painful symptoms will be entirely eliminated. Reducing painful symptoms to a tolerable level and improving function may be more realistic goals.

The pharmacological agents used to treat neuropathic pain and paresthesias due to HIV-DSP are the same as those used for other forms of neuropathy (e.g. postherpetic neuralgia and painful diabetic neuropathy) and include anticonvulsants such as gabapentin and pregabalin, antidepressants such as amitriptyline and duloxetine, topical agents such as the 8% capsaicin patch and topical lidocaine, and nonspecific analgesics including opioids. Clinical experience suggests that the use of such agents either alone or in combination can lead to a meaningful reduction of symptoms, albeit often after a prolonged period of trial and error, during which considerable caution must be exercised if opioids are to be used. However, most large, randomized, placebo-controlled clinical trials of agents that are effective in other types of neuropathic pain have failed to show the efficacy specifically in HIV-DSP, despite the fact that neuropathic pain in these conditions likely has common underlying mechanisms [20–22]. This may be due to unusually high placebo effects in these HIV-DSP studies and also to the unique biopsychosocial complexity of HIV-infected populations [20]. The lack of established efficacy of most pharmacologic agents has sparked interest in alternative treatments. Hypnosis, mindfulness-based stress reduction, and smoked cannabis have shown preliminary evidence of efficacy in HIV-DSP [23–25].

9.3 Autonomic Neuropathy

The autonomic nervous system innervates all major organ systems and is responsible for a wide array of bodily functions. Accordingly, the clinical manifestations of autonomic neuropathy – in both HIV-infected and HIV-uninfected patients – are varied and diverse, and often challenging to recognize and properly diagnose. Affected individuals may present with one or more of the following symptoms: presyncope or syncope due to orthostatic hypotension; nausea and/or vomiting especially with meals, diarrhea or constipation related to gastrointestinal dysmotility; dry eyes and mouth due to denervation of secretory organs; urinary or sexual dysfunction; changes in body sweating particularly decreased distal sweating due to denervation of sweat glands; changes in skin temperature or color, particularly coldness and bluish discoloration of the feet, which is variable and due to vasomotor instability; and visual symptoms such as light sensitivity and blurry vision due to loss of normal pupillary reactivity [26].

Unlike HIV-DSP, which has a typical presentation and can be diagnosed on the basis of history and physical examination, the clinical features of autonomic neuropathy are variable and its diagnosis requires specialized testing that is not typically available in community settings. Many different tests of autonomic function

have been described, and most often, several complementary tests are performed together to assess the different aspects of autonomic function. The Autonomic Reflex Screen (ARS) is a standardized and validated battery, which has the benefit of yielding a quantitative score, the Composite Autonomic Severity Score (CASS) [27, 28]. The ARS measures sweat output (quantitative sudomotor axon reflex test), and heart rate and blood pressure responses to standardized stimuli including deep rhythmic breathing, Valsalva maneuver, and tilt table testing.

Early studies employing differing forms of autonomic testing suggest that autonomic dysfunction is an important neurologic complication of HIV [29–37]. However, these studies were generally small, and autonomic characterization was performed in isolation, without additional clinical neurologic or neurophysiologic testing, and so it was unclear if the autonomic dysfunction was part of a larger neuro-AIDS syndrome. We studied 102 HIV-infected adults recruited from an HIV primary care clinic and found that autonomic neuropathy, diagnosed using standardized laboratory measures (ARS/CASS), was present in 61% of patients overall and in 90% of patients with severe HIV-DSP, suggesting that in many cases HIV-DSP and autonomic neuropathy occur together as part of a spectrum of HIV-associated neuropathies that may have common underlying mechanisms [2, 38]. The mechanisms by which HIV might lead to autonomic neuropathy have not been specifically elucidated; however, in the rhesus macaque model, simian immunodeficiency virus infection leads to a decrease in sympathetic innervation of lymph nodes suggesting that the virus may be neurotoxic to autonomic nerve fibers [39].

Similar to HIV-DSP, there are no neuroregenerative therapies available, which are capable of reversing autonomic neuropathy, and so treatment is symptomatic. The specific treatment required will vary greatly depending upon the particular organ system involved. Once autonomic neuropathy has been definitively diagnosed, a thorough investigation of the patient's medication list should be made to remove agents that may worsen symptoms, particularly agents with anticholinergic side effects such as certain antidepressants and antipsychotics [40]. In the specific case of patients with orthostatic hypotension, antihypertensives may need to be discontinued. After this initial step, the symptoms of chronic orthostatic hypotension can be ameliorated through lifestyle modifications such as maintaining hydration, avoiding overheating, arising slowly from supine to seated to standing, minimizing alcohol intake, salt supplementation, and waist-high compression stockings. Pharmacologic therapy with agents such as fludrocortisone, midodrine and droxidopa is rarely necessary.

9.4 Mononeuropathies

Mononeuropathies are common in the general population particularly among those with DSP, likely because diffuse nerve dysfunction increases the vulnerability of individual nerves to focal damage. Thus, due in part to the high prevalence of HIV-DSP, mononeuropathies are commonly encountered in the HIV clinic. The clinical

presentation varies depending upon the specific nerve that is involved. Median neuropathy at the wrist, or carpal tunnel syndrome, is the most common mononeuropathy. Clinical features include pain at the wrist, which may radiate into the hand and/or arm, and intermittent numbness and paresthesias primarily in the thumb, index, and middle fingers. Initial management is conservative with a splint designed to keep the wrist in a neutral position. Other common focal neuropathies of the limbs include ulnar neuropathy at the elbow, peroneal neuropathy at the fibular head and lateral femoral cutaneous neuropathy. NCS/EMG are often helpful in the diagnosis of these focal neuropathies. Acute hearing loss and diaphragmatic paralysis as a result of mononeuropathy have also been reported in HIV-infected patients.

Facial neuropathy, which typically presents as weakness of unilateral facial muscles, is more common in HIV-infected patients than in the general population [41]. In most cases, facial neuropathies are idiopathic (e.g. Bell palsy), in which case, according to guidelines derived from the general population, a brief course of oral steroids may hasten recovery [42]. Facial neuropathies may also occur as part of the acute retroviral syndrome [43], or be caused by Lyme disease [44] or reactivation of varicella zoster virus (VZV). In the case of VZV, vesicles in the ear, a condition referred to as Ramsay Hunt syndrome, accompany the facial weakness [45]. More rarely, facial neuropathy may be caused by syphilis, tuberculosis, and meningeal processes, for example, meningeal lymphomatosis [41, 46]. However, in these cases, the facial neuropathy is not usually an isolated finding.

9.5 Mononeuropathy Multiplex

Mononeuropathy multiplex (MM) is an infrequent complication of HIV that can occur both in early or advanced stages of HIV infection. As its name implies, MM is characterized by dysfunction of two or more individual peripheral nerves, nerve roots, and/or cranial nerves. The disorder is typically painful, and unlike HIV-DSP, it presents asymmetrically. In severe cases, where many nerves are affected, the patient's sensory and motor abnormalities may become confluent. MM disease presenting in an HIV-infected patient with relatively preserved immune function is most likely immune-mediated, and in some cases, it may be triggered by a polyarteritis nodosa-like vasculitis [47, 48]. MM in this context may be self-limited, with symptoms resolving without treatment over several months [49, 50]. However, if symptoms are severe or persistent, immunomodulatory treatment such as intravenous immunoglobulins (IVIg), plasmapheresis, or corticosteroids may be beneficial [51]. In contrast, MM in patients with severe immunodeficiency (e.g. CD4+ lymphocyte counts below 50) is classically caused by cytomegalovirus (CMV) infection of peripheral nerves [52, 53]. Evidence of CMV infection of other sites (e.g. eyes, gastrointestinal tract) should be sought, and a lumbar puncture should be performed to document the presence of CMV in the cerebrospinal fluid (CSF) by polymerase chain reaction (PCR). Treatment is with antiviral agents (e.g. ganciclovir). In hepatitis C coinfection, MM may also be a manifestation of cryoglobulinemia [54].

9.6 Inflammatory Demyelinating Polyneuropathies

Inflammatory demyelinating polyneuropathy (IDP) occurs in two forms: acute inflammatory demyelinating polyneuropathy (AIDP), also known as Guillain–Barre syndrome, and chronic inflammatory demyelinating polyneuropathy (CIDP). AIDP classically presents with symmetric, rapidly progressive ascending muscle weakness, with loss of deep tendon reflexes and relative sparing of sensory function. AIDP is by definition a monophasic illness, which typically reaches its nadir within 2 weeks of symptom onset. AIDP is uncommon in HIV but has been reported as part of the acute retroviral syndrome and at different stages of HIV disease severity [55, 56]. In contrast, CIDP has a more insidious onset and protracted course (>8 weeks), which may be steadily progressive, stepwise, or fluctuating. CIDP presents with relatively symmetric, proximal and distal weakness, and sensory impairment in the limbs. CIDP is considerably less common than HIV-DSP; however, there is sufficient phenotypic overlap that CIDP should be considered in the differential diagnosis of HIV-DSP, particularly in patients with significant motor weakness and/or deficits that do not follow a length-dependent distribution, for example, diffusely absent deep tendon reflexes as opposed to reflexes that are diminished at the ankles only.

The diagnosis of IDP rests on neurophysiologic evidence of demyelination on NCS/EMG including slow conduction velocities, increased distal motor latencies, increased F-wave latencies, and conduction block [55]. These findings should be reproducible across multiple nerves and not be confined to common entrapment sites. Lumbar puncture for CSF analysis is often performed as part of the diagnostic evaluation for AIDP and CIDP. In HIV-uninfected patients, the diagnosis of IDP is supported by the presence of elevated protein without associated leukocytosis. This finding carries somewhat less weight in HIV-infected patients in whom CSF protein elevation and a low-level lymphocytic leukocytosis sometimes occurs even in the absence of overt neurologic disease [56]. However, the CSF analysis can be useful for excluding other causes of neurologic symptoms, for example, CMV in the more severely immunocompromised patient.

Treatment options for AIDP and CIDP and response to these therapies are similar between HIV-infected and HIV-uninfected populations [57, 58]. A proportion of patients with AIDP may spontaneously recover; however, IVIg or plasmapheresis is typically offered in resource-rich environments, particularly if motor symptoms are prominent. Steroids are not indicated for the treatment of AIDP and may actually worsen symptoms. Steroids, IVIg, and plasmapheresis are all considered as first-line treatments for CIDP, though any medications with immunosuppressive side effects should be considered carefully in the HIV-infected population.

9.7 Radiculopathy

Radiculopathy refers to dysfunction of one or more nerve roots, which typically presents as local pain in the affected area of the spine radiating into the dermatomal distribution of the root. Depending on the severity of the radiculopathy, patients

may also display sensory loss, decreased deep tendon reflexes, and weakness in the affected dermatome/myotome. The most common radiculopathy encountered in clinical practice is lumbosacral radiculopathy, which is often referred to as "sciatica." Sciatica is caused by dysfunction of the L4, L5, and/or S1 nerve roots and is characterized by low back pain radiating into the buttock and posterior lower limb, which in more severe cases may be accompanied by a diminished ankle reflex, sensory loss in the lateral calf and sole of the foot, and foot weakness (e.g. foot drop). The most common cause of radiculopathy is degenerative spine disease, which leads to compression of nerve roots by a combination of degenerative disc disease, ligamentous hypertrophy, and osteophyte development. Recent epidemiologic work suggests that degenerative spine disease is highly prevalent in HIV-infected populations [59, 60], which may be related to the phenomenon of accelerated aging [61].

Although radiculopathy is most commonly due to structural degenerative etiologies, the clinician should be alert to other possible etiologies, such as focal infection or malignancy [59]. A syndrome of progressive lumbosacral polyradiculopathy due to CMV is well described in HIV-infected patients with advanced immunocompromise [62]. Symptoms are of a progressive (over days to weeks) cauda equina syndrome, with weakness and numbness of the lower extremities, frequently accompanied by sphincter dysfunction in the form of urinary retention/incontinence. Neurological examination reveals a flaccid paraparesis, ankle and knee hyporeflexia/areflexia, and sensory loss in the lower limbs including saddle anesthesia. Gadolinium-enhanced magnetic resonance imaging (MRI) may demonstrate enhancement of the lumbosacral roots of the cauda equina. Demonstrating CMV in the CSF via PCR confirms the diagnosis, though the presence of concurrent CMV infection elsewhere (i.e., retinitis) warrants empiric treatment in suspected cases. CSF also usually shows a marked polymorphonuclear pleocytosis and elevated protein levels [62]. Specific anti-CMV therapies include intravenous ganciclovir, foscarnet, cidofovir, as independent agents or in combination.

Although CMV is the most common etiological agent, similar clinical presentations have been described with neurosyphilis, lymphomatous meningitis, and neurolymphomatosis [63–65]. The differential diagnosis for lumbosacral polyradiculopathy in immunocompromised patients also includes other HIV-associated entities, including tuberculous meningitis, primary infection and/or reactivation of herpes simplex virus type 2 within the sacral dorsal root ganglia, and diffuse infiltrative lymphocytosis syndrome (DILS) (see Sect. 9.9) [66–69].

9.8 Amyotrophic Lateral Sclerosis in HIV

Amyotrophic lateral sclerosis (ALS) is a progressive, degenerative neurologic disorder of the upper and lower motor neurons, resulting in progressive weakness and muscle atrophy with preservation of sensory function. Diagnosis of ALS is made based on clinical neurologic examination and NCS/EMG findings as described in the El Escorial criteria [70]. Since 1985, there have been at least 29 case reports of

ALS-like disease in HIV-infected patients and a suggested ALS frequency of 3.5 patients per 1000 HIV-infected persons [71, 72]. Although ALS in an HIV-infected individual is clinically indistinguishable from ALS in a non-HIV-infected individual, some between-group differences have been reported. HIV-associated ALS has been reported to occur at a younger age [72], with a typical age of onset in the fifth decade of life (as opposed to the sixth or seventh in HIV-uninfected patients), although this may have been biased by the relative rarity of HIV in older adults at the time in which this data were collected. In addition, HIV-infected patients' progression of ALS-like symptoms has been reported to be accelerated [73], with reports of HIV-associated ALS progressing from onset of symptoms to severe handicap in weeks. Finally, some but not all cases of ALS in HIV have improved with initiation of CART [71, 72].

Although the relationship between HIV and ALS is unclear, there are some data to support a role for retroviruses in the pathogenesis of motor neuron diseases [74]. ALS-like syndromes have been reported in association with other retroviruses such as human T-lymphotropic virus-1 (HTLV-1) and human foamy virus (HFV) [71]. Also, there is an increase in serum reverse transcriptase activity in the blood and brain tissues of patients with ALS [75, 76]. Despite these findings, unlike viruses such as polio [77], there is no specific evidence of direct infection of motor neurons by exogenous retroviruses such as HIV and HTLV in humans, although recent work suggests a possible role for endogenous retrovirus in ALS pathogeneses [76]. It is as yet unclear how this finding fits into the other proposed etiologies of ALS, which include genetic factors, environmental exposures, excitotoxicity, immune mechanisms and others [78].

9.9 DILS in the Peripheral Nervous System

DILS is a rare multisystemic disorder usually seen in untreated or uncontrolled HIV infection. Initial pre-CART studies estimated that DILS affected 3–8% of the HIV-infected population, though its prevalence today is likely substantially lower. Its pathogenesis is rooted in the transient peripheral oligoclonal CD8+ T-cell expansion seen in early HIV infection. DILS occurs when these expanded CD8+ T-cell populations persist and infiltrate tissues and organs [79]. The most commonly involved sites in DILS are the salivary glands, with variable involvement of the lungs, PNS, kidneys, liver, and gastrointestinal tract.

DILS is associated with heterogeneous focal or multifocal neurological disorders including unilateral or bilateral facial nerve palsy, aseptic meningitis, and a painful sensorimotor polyneuropathy. Considering that a major feature of DILS is bilateral parotidomegaly, facial nerve palsy can be secondary to local compression or neuritis. The causal relationship between DILS and aseptic meningitis is less clear, though cases of DILS-associated aseptic meningitis have demonstrated CD8+ T cells and elevated protein on CSF analysis and diffuse dural and/or leptomeningeal enhancement on MRI [79].

DILS-associated polyneuropathy was described in a 12-patient case series in 1997 [80]. The disorder is characterized by the acute or subacute onset of painful paresthesias usually affecting the lower extremities symmetrically. Sensorimotor dysfunction follows the paresthesias, and the upper limbs can also become involved. Electrophysiology findings from these patients are consistent with axonal loss. Nerve biopsy demonstrated both angiocentric endoneurial and perineurial infiltrates and abundant HIV p24 protein in surrounding macrophages, suggesting that the DILS-associated lymphocytic infiltration is largely triggered by the presence of HIV antigens [80]. Correspondingly, patients suffering from DILS-associated polyneuropathy can improve with initiation of CART or steroid therapy [80].

9.10 Muscle Disorders

The majority of HIV-infected individuals complaining of myalgia – i.e., diffuse, deep, aching, cramping, or throbbing of muscles – do not have objective evidence of a primary muscle disorder. These patients can be identified by normal strength on neurological exam with normal creatine phosphokinase (CPK) levels. Further diagnostic testing is usually not necessary. The primary muscle disorders that occur in HIV-infected patients range in severity from asymptomatically elevated CPK levels to frank rhabdomyolysis. The former is relatively common, with 15% of HIV-infected patients experiencing transient CPK elevations and about 4% experiencing sustained CPK elevations [81]. These asymptomatic laboratory anomalies likely represent a mild HIV-associated myopathy and do not require treatment. A rare condition termed HIV-associated neuromuscular weakness syndrome has also been characterized as a rapidly progressive diffuse weakness associated with lactic acidosis, which occurs following exposure to nucleoside reverse transcriptase inhibitors [82]. The weakness may be due to toxic myopathy, neuropathy, or a combination of both. Zidovudine in particular has been associated with myopathy; however, this was mainly observed at high doses and is currently quite rare [83, 84].

HIV-associated myopathies, in contrast to myalgia and asymptomatic CK elevations, are rare. HIV-associated polymyositis is the best characterized of these symptomatic muscle disorders. Patients at any stage of HIV disease can be affected; they present with diffuse myalgias accompanied by slowly progressive, proximal, symmetric muscle weakness, usually manifesting as difficulty in climbing stairs or arising from a chair unassisted [85]. The physical exam should confirm objective evidence of proximal weakness. Further evidence supporting the diagnosis of HIV-associated polymyositis includes elevated serum CPK, myopathic findings on NCS/EMG, and muscle biopsy demonstrating an inflammatory myopathy.

Management begins with eliminating potentially myotoxic medications, such as statins. Pharmacotherapy is immunomodulatory, and the prognosis is generally favorable. In one case series of patients with HIV-associated polymyositis ($n = 13$), symptoms resolved completely in over half of those treated with steroids, and steroids were successfully discontinued after a mean of 9 months [86]. The remainder

of the patients also improved, albeit more slowly. A second case series ($n = 14$) from a different group showed similar steroid responsiveness [87]. Based on the treatment of polymyositis in HIV-uninfected individuals, other immunomodulatory treatments such as IVIG can also be considered.

9.11 Conclusion

Despite the successful treatment of HIV with CART, disorders of the PNS persist in the HIV-infected population. The most common of these, the HIV-associated neuropathies represent a spectrum of disease that can lead to debilitating symptoms of neuropathic pain, sensory loss, and autonomic dysfunction. Symptomatic control is difficult, and currently available therapies are suboptimal. Future research is needed to improve these therapies and to develop additional strategies targeting the underlying issue of axonal loss, for which there is currently no treatment. If successful, such efforts have the potential to greatly improve the quality of life of many people living with HIV.

Conflict of interest The authors report no conflicts of interest.

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Chapter 10
The Role of Early Life Programming in Vulnerability and Resilience in Relation to HIV

Luba Sominsky and Deborah Hodgson

Keywords Perinatal programming • Early life stress • Programming of the hypothalamic-pituitary-adrenal axis • Sympathetic reactivity to stress • HIV disease progression

Core Message

Despite significant advances in HIV/AIDS research, the disease still impacts millions of people worldwide. The psychosocial environment of the patient plays an important role in the disease progression. Psychological stress, mental health issues and lack of social support contribute to a poor prognosis, particularly in those patients with prior exposure to these risk factors. Early life stress is known to affect mental health and modulate neuroendocrine and immune function long term, influencing individual's vulnerability to adult stress and compromised health status. This increased susceptibility to the adverse effects of stress may in turn promote the rate of HIV disease progression. Understanding the possible interactions between early life experiences of an infected individual and their ability to cope with the diagnosis and health consequences of HIV infection may shed light on the underlying biological mechanisms contributing to the disease progression and, thus, to improve current therapeutic strategies.

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10.1 Introduction

The pathogenesis of human immunodeficiency virus (HIV)-1 is highly variable, and individual differences exist in the progression of the disease. Following a primary infection and spread of HIV-1 among $CD4^+$ cells, replication of the virus is controlled by the cytotoxic T-cell response [1] and HIV-1-specific antibodies [2, 3]. Other immune responses against HIV-1 include activation of natural killer (NK) cells that control viral replication [4] and enhance the cytotoxic T-cell response [5]. T helper (Th) 1 response is also essential in the control of the viral spread via production of cytokines, such as interleukin (IL)-2, IL-12 and interferon (IFN) γ that are essential for the development of cytotoxic T cells and promote cellular immunity [6]. These initial antiviral responses inhibit replication, without the depletion of the infected cells. After the acute antiviral response, which lasts between 2 and 4 weeks, the viral load set point is established. That is, the replication of the virus and its clearance reach an equilibrium [7]. Higher RNA levels of the virus in plasma typically predict more rapid progression to acquired immunodeficiency syndrome AIDS [8, 9]. After the set point is established, a clinical latency of the disease, when an infected individual remains asymptomatic, may last for years, with a median of 10 years, until the onset of clinical conditions defining AIDS begin [10], the most prominent of which is the decline in the absolute number of CD4+ T-cells below ~200/ul of peripheral blood [11]. Rapid decline in CD4+ T-cell counts is often followed by increased plasma viral load and a decline in HIV antibody production [1, 12]. Other immunological parameters affected by the progression of the disease include diminished NK cell levels and cytotoxicity [5], as well as a critical alteration in the cytokine balance and a shift towards Th2 immune responses, inhibiting the production of Th1 cytokines and cellular immunity [6]. Prior to the introduction of antiretroviral therapy, life expectancy after the diagnosis of AIDS has been on average up to 2 years [13]. The development of anti-retroviral therapies has substantially prolonged the life expectancy of the patients and dramatically decreased the incidence of AIDS [14]. However, even the most effective treatment cannot fully eradicate the virus, which will continue to replicate in lymphoid organs and the central nervous system. Over time, the virus undergoes mutation, and any treatment may be eventually rendered ineffective [15].

Although individual differences in the level of the HIV set point play an important role in the progression rate of the disease, its progression rate is not solely dependent on the established set point of the viral load. Other factors may influence the progression rate, such as the psychosocial environment and its impact on the immune status of the patient. HIV/AIDS diagnosis is undoubtedly a traumatic event. Like any other chronic illness, it causes a constant emotional and economic burden on the individual. But in addition to the expected stressful impact, HIV/AIDS diagnosis is often accompanied by a negative social stigma. The infected individual has now to deal not only with the illness but also with a potential judgemental attitude from their friends, family and even strangers. Even with the current increased awareness to the origins and transmission routes of the disease, HIV-infected

individuals are still facing a constant threat of social isolation, strongly negative attitudes, and even discrimination of basic civil rights [16–18]. With these issues in mind, the psychological quality of life of the patients may become substantially reduced.

There is a large and continually emerging literature demonstrating that the psychosocial environment can play a vital role in the health status of HIV-1 patients. Stressful events, lack of positive social support and the onset of mood disorders such as anxiety and depression may impair the already vulnerable immune status and impact upon HIV-1 disease progression, even in the presence of antiretroviral therapies [19, 20]. Herein, we will review the existing literature on several psychosocial factors that have been associated with the pathogenesis of HIV and discuss their potential biological mediators. We also aim to introduce a new model of HIV/AIDS disease progression, which takes into account preexisting conditions that may affect the progression rate and health consequences of the disease. These are early life factors that alter the internal milieu of an organism and may contribute to the interrelationship that exists among the immune system, mood and behaviour, and ultimately modulate disease progression.

10.2 Psychosocial Determinants of HIV Progression

10.2.1 Stressful Life Events and Coping Strategies

There is growing evidence demonstrating the deleterious impact of stress on health status in a variety of diseases [21, 22]. In the context of HIV, significantly increased depletion of $CD4^+$ T cells and the emergence of other AIDS-defining conditions were found in several longitudinal studies assessing the impact of stressful and traumatic life events among infected individuals, including loss of job, relationship breakup, financial difficulties and more [19, 23]. Other studies have shown that high levels of distress were associated with other immunological parameters in HIV-infected individuals, including decreased numbers of memory T helper cells and B cells [24], and impaired NK cell activity [25], which are indicative of decreased cellular immunity associated with accelerated HIV progression [6].

It appears that rather than exposure to the stressful event itself, it can be the subjective perception and coping strategies utilised which can produce the most profound detrimental impact on physiological responses [26]. A meta analytic review of 36 articles, investigating associations between the impact of the psychosocial environment on HIV/AIDS disease progression revealed that personality types or coping strategies and psychological distress were more strongly associated with accelerated HIV disease than the stressor per se. All of the health-related outcomes, such as CD4+ T-cell levels, the diagnosis of AIDS, the stage of the disease, and HIV/AIDS symptoms, were significantly affected by these adverse psychosocial factors [26]. Negative or pessimistic perceptions of the stressful event were found to

be correlated with accelerated decline in CD4+ cell levels [27], increased plasma viral load [28] and decreased NK cell cytotoxicity as well as decreased levels of cytotoxic T cells [29]. In contrary, positive proactive approach has been associated with slower disease progression [30]. Unrealistic optimism, however, reflecting denial and passive coping style, was found to be harmful and has been associated with decreased CD4+ T cell levels [31]. Another study indicated that direct and active coping approach to the HIV/AIDS-related stress is associated with better psychological adjustment [32].

10.2.2 Social Support and Relationships

Social support and meaningful relationships can mitigate the detrimental effects of stress by providing emotional, physical, or financial support [33]. As such, it has been shown to be important in the context of HIV disease, with a lack of positive social network generally being associated with an enhanced rate of HIV progression [34]. Several early studies have reported this phenomenon, demonstrating greater satisfaction with social support to be associated with slower progression to AIDS, as indicated by CD4+ lymphocyte counts [19, 23]. Perception of social support has been reported to be associated with greater adjustment and coping, as well as with greater self-perceived quality of life, among HIV-infected individuals [35], implicating the stress-buffering role of social support in coping with chronic illness.

There is, however, an important distinction between the impact of social support in the context of HIV infection, as opposed to other chronic illnesses (i.e. cancer). Provision of social support requires the infected individual to disclose their HIV status, which may ultimately lead to rejection and a potential loss of support, due to the negative reactions associated with the stigma of the disease [36]. This often results in a lower rate of disclosure of HIV infected over other diseases. Nevertheless, it appears that infected HIV/AIDS individuals who have chosen to disclose their HIV status to family and friends receive greater social support from these meaningful relationships and perceive less emotional distress [18]. The stage of the disease is likely to be a valuable indicator of the perceived benefit of social support, with larger social networks and greater support predicting longevity among individuals at a later stage of HIV disease [37].

Along with the beneficial impact of positive social factors on health status, there are possible detrimental effects of negative social environment. For instance, the nature of social relationships has been found to predict survival when support from some social networks may be associated with encouragement of sexual, drug-related and other HIV risk behaviours and therefore has a negative impact [38]. Other factors, such as sexual orientation, may play an important role in predicting the benefits of social relationships and support. Disclosure of homosexual identity was found to be associated with high CD4+ T lymphocyte counts only when those HIV-seropositive gay and bisexual men received high levels of social support [39]. Concealment of sexual identity, reflective of psychosocial inhibition and utilisation

of passive coping strategies, has been reported to have a negative impact on HIV-1 disease progression and increased risk of other infectious diseases [40–42]. Stability of social relationships is yet another important factor. Studies of rhesus macaques experimentally infected with the simian immunodeficiency virus (SIV) indicated a complex impact of social relationships on SIV pathogenesis, with a negative influence of random and unstable social interactions on the disease progression and survival, as opposed to stable social hierarchies [43].

10.2.3 Mental Illness and Cognitive Decline

Unsurprisingly, depression can become a common occurrence among HIV-1-positive individuals. Unfortunately, the development of major depression or depressed mood can impact negatively on the progression of HIV in patients. A number of studies have reported that depressive states in HIV patients correlate with increased risk of rapid disease progression [19, 44, 45]. Chronic depression is typically associated with accelerated HIV disease progression, including increasing morbidity and mortality [20], suggestive of the benefits of early detection of depression in these patients. Even with the use of highly active antiretroviral therapies (HAART), chronic depression was associated with rapid progression of HIV [46]. Although antidepressants have been shown to affect immune function in patients with depression [47], tricyclic and selective serotonin re-uptake inhibitors (SSRI) antidepressants do not appear to have an impact on CD4+ T-cell counts or plasma viral load [48, 49]. These drugs may however improve adherence to antiretroviral therapy [50].

Preclinical studies have produced recent support for the link between HIV-1 and depression. Increased activation of indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme in tryptophan metabolism, which results in metabolites such as serotonin and the excitotoxic NMDA receptor agonist quinolinic acid among others, has been implicated in inflammation-induced depression [51, 52], and is now considered to be a potential contributing factor to the onset of depression associated with HIV infection [53]. Depressive-like behaviour was reported in HIV-1 Tat protein-treated mice. Increased expression of IDO was found in the hippocampus of these animals, mediated via activation of the p38 mitogen-activated protein kinase (MAPK) inflammatory pathway [54]. Several studies have shown that MAPK signalling pathway can positively regulate replication of HIV-1 [55, 56]. Activation of IDO also inhibits T-cell proliferation via catabolism of tryptophan into its metabolite kynurenine. In the context of HIV, increased IDO-mediated tryptophan catabolism has been reported [57]. In rodents, inhibition of HIV-induced IDO activation has been shown to result in increased cytotoxic T cell response and depletion of infected cells [58]. In vitro studies have indicated that increased expression of IDO mRNA is present in peripheral blood mononuclear cells of HIV-infected patients. Inhibition of IDO in these cells resulted in increased proliferation of CD4+ lymphocytes [59]. Given the robust immunosuppressive activity of IDO pathways, further

investigation of therapeutic approaches aimed at controlling this mechanism in the context of HIV infection is required.

Despite the apparent lack of impact of the commonly used antidepressants on the biological indices of HIV disease progression [48, 49], there is evidence that psychological interventions, aimed at reducing psychological distress, may improve depression and anxiety symptoms as well as HIV-related health outcomes [46, 60]. These effects imply that not only serotonergic pathways, most prominently targeted by antidepressants, are affected in HIV-infected and depressed individuals, but other biobehavioural mediators of the psychosocial factors on HIV disease progression might be involved.

In addition to mental illness, individuals living with HIV tend to exhibit cognitive impairments. Before antiretroviral therapies became available, a majority of HIV-infected individuals with advanced infection suffered from HIV-associated dementia (HAD) or also known as Neuro-AIDS. HAD is a severe neurological impairment that includes motor dysfunction, loss of dexterity and coordination [61]. The discovery of HAART dramatically decreased the prevalence of HAD. However, although HAD incidence is now rare, the incidence of HIV-associated mild cognitive impairments remains common, leading to significant neurocognitive decline in otherwise virologically stable HIV-infected patients [62, 63]. With a significantly increased survival rate and extended life span for the HIV-infected individuals in the era of antiretroviral therapies, age is also a significant contributor to HIV-associated cognitive disturbances. The incidence of cognitive decline is three times greater in HIV-infected individuals over the age of 50 [64], further accelerative cognitive aging [65].

Despite significant advances in knowledge, the cellular and molecular mechanisms underlying the HIV-associated neurocognitive decline are not fully elucidated. However, several possible pathways have been proposed. First, it is evident that HIV itself can invade the brain, causing neurodegeneration, synaptic and dendritic damage, as well as pathological activation of astrocytes and microglia [66, 67]. HIV entry into the central nervous system (CNS) is mediated by infected lymphocytes and monocytes or through trafficking of cell-free virus (reviewed in [68]). HIV infection of the CNS leads to the loss of pericytes affecting endothelial integrity and inducing breakdown of the blood–brain barrier (BBB) [69, 70]. These processes further facilitate the ability of HIV cell-free virus to cross the BBB and enter the brain [71]. CNS infiltration initiates neuroinflammatory responses, leading to robust astrocytosis and microgliosis, even in the presence of antiretroviral therapy, primarily in the brain regions particularly associated with cognitive function, such as the hippocampus [72, 73]. In addition, activated astrocytes and microglia release proinflammatory cytokines and chemokines, contributing to neuronal damage and loss of myelin integrity [74]. Although the presence of HIV in the CNS is essential for the development of HIV-associated cognitive declines, it is still unknown whether increasing amounts of HIV in the CNS are positively correlated with the severity of cognitive decline [75–77]. Moreover, the effectiveness of CNS penetration by antiretroviral agents does not appear to be associated with improved neurocognitive outcomes in HIV-infected patients [78, 79]. Cognitive impairments,

psychological distress and depression in HIV patients further contribute to rapid HIV disease progression [45, 78]. More research is therefore imperative on effective preventative measures to treat CNS HIV infection and the associated neurocognitive decline and mental illness.

10.3 Physiological Systems Underlying Stress and Psychosocial Well-Being

The psychosocial factors discussed above have been demonstrated to impact on disease progression in HIV-1 patients. This is due to the underlying physiological systems involved in regulating, interpreting and processing stressful situations, social interactions and other psychosocial factors. The physiological signalling systems that mediate psychosocial processes have become the focus of several avenues of literature, not least of all because they are known to interact closely with the immune system. In particular, the neural–endocrine–immune relationship has seen great interest due to its capacity to modulate the antiviral response. The two major systems involved in stress responsiveness and regulation are the hypothalamic–pituitary–adrenal (HPA) axis and the autonomic nervous system (ANS). In this section, we will outline how these biological processes can impact HIV-1 status.

10.3.1 The Role of the HPA Axis in the Disease Progression

Exposure to physiological or psychological stress is manifested by activation of the HPA axis, indicated by the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) of the hypothalamus, stimulating CRH and AVP receptors in the anterior pituitary and the subsequent release of adrenocorticotropic hormone (ACTH), which in turn stimulates production and release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal cortex. Inhibition of the HPA axis responses to stress is induced via the binding of glucocorticoids to glucocorticoid and mineralocorticoid receptors (GRs/MRs) in various brain regions, although primarily in the hippocampus, hypothalamus and pituitary. This results in activation of a negative feedback loop and a return to homeostasis [80]. In this way, the HPA axis provides an effective stress-control mechanism by stimulating a relatively brief release of glucocorticoids. The HPA axis is also activated upon exposure to immunological stress. Immune activation leads to the initiation of the HPA axis response, which is primarily mediated by increased production of proinflammatory cytokines (such as tumour necrosis factor (TNF)-α, IL-1α and β, and IL-6) [81]. The resulting acute increase in glucocorticoid levels possesses adaptive anti-inflammatory properties alleviating the immune activation. However, if the exposure to stress is prolonged, or if the

adaptive physiological response fails, prolonged exposure to increased HPA axis activity initiates a complex neuroimmune response resulting in increased circulation of IL-6 and CRP, promoting CRH synthesis and further HPA axis activity. Prolonged escalations in glucocorticoid levels are, therefore, detrimental for normal physiological functioning. Glucocorticoids are known to exert immunoregulatory functions, via induction of a shift in immune responses from a cellular (Th1) towards a humoral (Th2) response [82]. As discussed above, the trend towards Th2 responses is also evident during the course of HIV infection and is associated with decline in antiviral activity and accelerated progression rate of the disease [6]. The HPA axis, therefore, represents one of the possible mechanisms by which changes in the psychosocial environment may intervene with the disease progression. Thus far, however, clinical studies have indicated only a correlative link between altered cortisol levels to the severity of the disease. Increased cortisol levels have been found to correspond with faster progression to AIDS [19]. On the other hand, low waking cortisol in patients at early stages of HIV infection has been shown to be associated with greater T cell immune activation, a known risk for rapid disease progression [83]. Because the progression of HIV disease itself can lead to changes in cortisol production [84], it is difficult to determine a causal link between the altered HPA axis activity and the disease progression. In vitro studies suggest that low glucocorticoid concentrations enhance HIV-1 replication by stimulating HIV-1 long terminal repeat [85], or arresting infected T cells in the G2 phase of cell cycles, when the expression of the viral genome is optimal [86]. However, further empirical evidence is required to establish this plausible hypothesis in vivo.

Interestingly, experimental studies of SIV infection revealed lower cortisol levels at baseline and in response to an acute stressor in animals assigned to unstable social conditions (43). This pattern of cortisol response corresponds with that found in individuals suffering from posttraumatic stress disorder (PTSD), which often present hypocorticolism in the presence of a stressful situation [87]. The blunted cortisol response in SIV-treated animals assigned to unstable social conditions was nevertheless associated with increased humoral immune responses and increased plasma SIV RNA levels [43]. Despite the inconsistency of these data with the hypothesis that increased release of glucocorticoids leads to a shift in T helper cells function towards Th2 immunity [82], data from PTSD research indicate an inverse relationship between cortisol levels and glucocorticoid receptor numbers on lymphocytes and enhanced negative feedback sensitivity of the HPA axis [88]. Because behavioural differences during the formation of the social groups predicted survival in SIV-infected animals even without alteration of the HPA axis activity, these data suggest that other biological pathways are involved [43]. Although subpopulations of PTSD patients have been demonstrated to exhibit a blunted cortisol profile, they are also known to exhibit prolonged ANS activation (adrenaline release), reflective of the inability to switch from the acute fight/flight mechanism of the ANS to the energy mobilisation mechanism of the HPA axis [89, 90]. The above studies describing blunted HPA function in correlation with increased HIV progression did not assess ANS indicators, which may also be a contributing factor. Therefore, given that increased noradrenergic responsiveness to stress has been previously reported

in PTSD patients in the absence of elevated HPA activity [91], another plausible mediator of the viral progression is activation of the ANS.

10.3.2 The Role of the ANS in the Disease Progression

The ANS and the HPA axis work in a coordinated fashion to modulate the stress response. ANS functioning is, however, regulated by different neurobiological pathways (reviewed in [92]). The ANS provides the most immediate response to stress via activation of its sympathetic and parasympathetic compartments, resulting in rapid physiological changes, mediated by innervation of end organs. The sympathetic nervous system (SNS), for instance, can rapidly induce changes in heart rate and blood pressure as well as the release of catecholamines [93]. Catecholamines are released from the adrenal medulla and sympathetic neurons in lymphoid organs in response to psychological or immune stress. Increased production of catecholamines can modulate immune function, by driving a Th2 shift [94]; inhibition of thymopoiesis [95] and by inhibiting the activity of NK cells, cytotoxic T cells and macrophages [96]. Activation of the parasympathetic division of the ANS via both the afferent and efferent vagal nerves, which is classically associated with the counterbalance of the sympathetic response, has been also noted to supress inflammatory signals [97, 98]. Increased release of acetylcholine, the major parasympathetic neurotransmitter, leads to deactivation of macrophages, inhibiting the production of proinflammatory cytokines, such as TNF-α, IL-1 and IL-18) but not anti-inflammatory cytokines, such as IL-10 [99]. Activation of the ANS has, therefore, an important role in the control of immune function, regulating the immune activity towards Th2 immunity and inhibiting a proinflammatory response [100]. In the context of HIV, there is a more consistent association between increased ANS activity and HIV-1 pathogenesis, than in the case of the HPA axis. Importantly, given that unlike the HPA axis, ANS activity does not change substantially after the onset of AIDS, more causal links can be drawn. Natural history studies have indicated that socially inhibited HIV patients with increased plasma viral load and poorer response to the HAART treatment demonstrated elevated ANS activity [101, 102]. Cognitive–behavioural stress management intervention in HIV-positive patients was found to decrease anxiety and self-perceived stress, along with a decrease in urine catecholamine levels and increase in the number of cytotoxic T cells, as compared with a group of HIV-positive individuals that did not receive a psychological intervention and exhibited high levels of ANS activity [103]. Another study that directly assessed ANS activity has found that HIV-seropositive men with high levels of ANS activity before initiation of active antiretroviral therapy demonstrated poorer adjustment to the therapy, with elevated plasma viral load and decreased CD4+ T cell levels in response to the treatment [101]. Animal studies have also revealed that SIV-treated rhesus macaques that were subjected to social stress had increased density of sympathetic innervation within the lymph node parenchyma. Exposure to stress in these animals also increased viral replication, which was attributed specifically to the

elevated density of catecholaminergic varicosities within the parenchymal tissue of the secondary lymphoid organs [104]. In vitro studies have shown that catecholamines can accelerate HIV-1 replication via several molecular pathways [105]. One such pathway is the cAMP/PKA signalling pathway, which mediates catecholamine response via activation of β-adrenergic receptors resulting in suppression of Th1 responses [106]. Activation of cAMP/PKA pathway has been shown to increase HIV-1 plasma viral load, whereas inhibitors of PKA were able to normalise HIV-1 biomarkers [105]. Catecholamines were also found to upregulate expression of viral CXCR4 and CCR5 co-receptors [107] and to enhance transcription of HIV-1 genes, which was abrogated by the blockade of β-adrenergic receptors and by inhibition of PKA activity [101, 105]. To date, however, no studies have assessed the impact of inhibition of ANS activity per se on HIV disease progression.

Despite the complexity of the current research on the biobehavioural determinants of HIV progression, it is clear that individual differences drive susceptibility to these factors and therefore may regulate the health consequences. The question that will be addressed below is whether these individual differences in susceptibility to the influences of psychosocial and physiological environments can be identified, and importantly, whether exposure to environmental changes early in life can predict predisposition to these alterations.

10.4 The Role of Perinatal Programming in Health and Disease

A large body of research has provided evidence for the reciprocal interactions of the neural, endocrine and immune systems. The establishment of these interactions begins during ontogeny, when the physiological systems are extremely sensitive to environmental impacts, a process that has been referred to as *developmental plasticity* [108]. During this sensitive period of development, the neuroendocrine systems exert not only a regulatory but also morphogenetic role [109–111]. The plasticity of physiological systems in perinatal development allows environmental factors to alter the functionality of an organism, providing for foetal adaptation to adverse conditions. However, any adversity experienced during this time may interfere with the development or formation of physiological interactions and negatively influence physiological functioning in later life.

Over the last two decades, a new field of research has emerged to investigate the ability of early life adversity to alter the normal course of development and predispose to pathologies in later life. The process by which the early life environment can have permanent effect on physiological systems has been described as *perinatal programming* [112]. This concept was originally explored by epidemiologist Professor David Barker. Barker proposed that disease states in adulthood may have their origins in the early developmental period [113]. His studies have demonstrated a link between being born at a low birth weight and greater risk of developing

coronary heart disease in adulthood [114]. His later studies have suggested that low birth weight is also associated with an increased risk of hypertension, stroke and type 2 diabetes [115], and led to the establishment of *Developmental Origins of Health and Disease* (DOHaD) hypothesis, which has associated the perinatal experience with disease susceptibility in later life [116]. Many lines of research have extended the concept of the DOHaD hypothesis, investigating how the different avenues of early growth and development may determine susceptibility to other health conditions such as osteoporosis [117], cancer [118] and even predict life expectancy [119]. Low birth weight has also been associated with psychopathological outcomes, including depression and suicide [120].

The DOHaD hypothesis incorporates the concept of perinatal programming, whereby changes in environment (i.e. nutrition) may alter the functionality of physiological systems and predispose to later health adversity. The process of perinatal programming can also be regarded as *phenotypic induction* [121], as the process of programming integrates involvement of genetic predisposition and determinacy in foetal development. Phenotypic induction allows for adaptive response to changes in environmental conditions, and health consequences are, therefore, dependent on the ability to develop an adequate response to such changes. While generally beneficial, under certain condition, this response may become maladaptive. In particular, when there is a mismatch between phenotypic adaptation during development and later life actual conditions. An extent to which the mismatch between environmental demands will have an impact on health outcomes is dependent on a more vulnerable or resilient genetic predisposition [122].

Many lines of research have addressed the impact of perinatal programming on a variety of physiological as well as psychological outcomes. Epidemiological and experimental evidence have indicated that discrepancies between the early and later life environments are not limited to nutritional factors but extend to other potential impacts, such as immune and hormonal statuses, as well as mental states. As such, the research has demonstrated how exposure to physiological or psychological stress factors during critical periods of fetal and neonatal development, which involve vigorous activation of the neuroendocrine and immune responses, disturb the internal milieu of the developing organism and are associated with an increased risk of pathologies and psychopathologies. This evidence will be reviewed in the following sections and incorporated in the discussion of plasticity of neural, endocrine and immune systems during early development.

The HPA axis and the ANS are the major stress response systems, controlled by the CNS which is known to exhibit an enormous degree of plasticity during perinatal development and therefore to be particularly sensitive to environmental stimuli. The critical development of most brain regions and systems occurs in-utero or in early neonatal life, so as the development of neuroendocrine control of stress responsivity. The HPA axis and the ANS are also involved in systemic response to immunological inputs, whereas the immune system, in turn, has an active role in the development of the neural and neuroendocrine responses to a variety of stress factors [123]. Even though variability in the developmental timeline exists among different species, the functional activity of the neural, endocrine and immune systems

is significantly lower in perinatal life than in adulthood. Therefore, the early life period is crucial for the definitive development of an organism and disturbances in normal ontogeny of any of the physiological systems may lead to long-term alterations in the functioning of other systems. The ability of environmental stimuli to produce robust programming effects is dependent on the developmental stage of an organism. The same environmental stressor may cause no lasting effects in mature organisms but can be detrimental if experienced during critical periods of development. The extent to which the trajectory of development of the HPA axis, the ANS and the immune system can be affected by environmental stimuli and how these alterations can be manifested in physiological and behavioural abnormalities is described below.

10.5 Early Life Plasticity of Neuroendocrine-Immune Interactions

10.5.1 Programming of the HPA Axis

Optimal exposure to glucocorticoids is important for normal brain development; however, increased glucocorticoids exposure may alter the developmental trajectory of brain maturation and function [124]. The HPA axis is known to be particularly sensitive to environmental influences early in life and exposure to stress during the critical period of HPA axis development may alter stress responsiveness long term [97]. Excess perinatal glucocorticoid exposure has been shown to down-regulate the expression of mineralocorticoid (MRs) and glucocorticoid receptors (GRs) [125, 126], resulting in increased release of glucocorticoids in response to later aversive stimuli [127, 128]. However, this profile of glucocorticoid release has been found to be largely dependent on the timing, strength, type and duration of exposure to not only the initial stressor but also a secondary stimulus. For instance, chronic stress has typically been shown to produce blunted glucocorticoid output, which can be maladaptive at times of stress due to the inability of the HPA axis to regulate its activity [129].

In rodents, brief periods of handling typically alter maternal behaviour, so that handled pups receive reduced maternal attention. This reduced maternal care has been shown to lead to decreased corticosterone levels and increased hippocampal GR expression in adulthood in those animals that were handled as neonates [130]. Increased hippocampal GR expression then leads to inhibition of CRH synthesis and reduced levels of ACTH and corticosterone in response to stress when compared with non-handled animals. Other animal studies have shown that low maternal care during the first week of life in rats affects methylation levels of the GR gene, resulting in hypermethylation within the exon 1_7 GR promoter and increased histone acetylation and transcription factor (NGFI-A) binding to the GR promoter in the hippocampus of adult offspring [126], which is typically associated with

reduced DNA binding and thereby reduced transcriptional activity [131]. These effects, however, can be reversed by cross-fostering the pups to dams that provide higher levels of maternal care [126, 132], suggesting a causal link between differences in maternal care and programming of gene expression. Thus, the sensitivity of the HPA axis is directly programmed by the degree of stress exposure during development.

Similarly, in non-human primates and in humans, parental care mediates and programs stress responsivity later in life [133]. Parental abuse and maltreatment tend to produce initial elevations in cortisol levels, followed by lower than normal cortisol release [134] and elevated ACTH response to psychological stressors [135]. In rhesus macaques, the deleterious effects of early life adversity appear to be so robust that even 3 years of normal social life could not reverse the decline in cortisol levels and abnormal behavioural patterns observed following exposure to maternal separation at birth [136]. Although maternal separation is an intense manipulation in infant monkeys, these findings provide convincing evidence for the long-term programming effects of the early adversity on brain development, particularly the HPA axis.

The lower cortisol secretion and suppressed ability to respond to stress associated with PTSD may be transmitted across generations through epigenetic programming, as has been shown in studies in offspring of Holocaust survivors, as well as other traumatic events, including children born to mothers who were pregnant on 9/11, reviewed in Yehuda and Bierer, 2008 [137]. These important observations may have potential implications for the consideration of factors influencing susceptibility and resilience to HIV disease progression, because severe life stress and PTSD have been linked with the severity of HIV disease [138, 139].

The variable and long-term outcomes of perinatal stress on adult stress responsiveness illustrate the complexity of HPA axis programming. The timing, the extent and the origin of stress exposure, all influence the long-term sensitivity and efficacy of the HPA axis in mediating an appropriate stress response. Ongoing research is still endeavouring to elucidate the critical determinants in programming of the HPA axis. In addition, there is an additional focus on the impact of early life events on the ANS and its subsequent role in stress regulation.

10.5.2 Programming of the ANS

While the majority of literature exploring the effects of early life stress focuses on programming of the HPA axis, the ANS is also susceptible to long-term functional alterations by exposure to a variety of stressors in early life. Similar to other physiological systems, although certain environmental exposures may program beneficial adaption to the environmental challenges, under some circumstances, these changes may prove maladaptive in adulthood and as such provide a basis for developmental origins of pathological states [140].

Due to the complexity of ANS structure and function, each compartment is likely to respond to different environmental factors, generating differing programming outcomes. For instance, thermoregulation, which is predominantly controlled by the SNS, is susceptible to environmental modifications in early life. Exposure to a cold environment during early development improves adaptation to subsequent exposure to cold. Conversely, exposure to elevated temperature enhances tolerance to heat later in life, in both animals and humans [140]. Another important developmental factor that has been extensively studied is maternal and neonatal nutrition, which contributes to the development of the ANS structure and function. In rodents, rearing in small litters results in a permanent increase in body weight and fat mass. Assessment of sympathetic function in these animals revealed that although no consistent differences were observed in adrenergic innervation of peripheral organs, sucrose-induced activation of cardiac sympathetic activity was diminished [141]. Neonatal handling in rat pups, which has been shown to induce a long-lasting reduction in the HPA responses to stress [130, 142], induces an increased autonomic response, as demonstrated by an increase in catecholamine concentrations in response to fasting in adulthood [143]. An acute neonatal immune challenge in rat pups has also been implicated in programming an increased autonomic arousal and anxiety-like behaviours long term [144]. Furthermore, exposure to various modalities of prenatal stress in rats has been shown to exaggerate stress responsivity in later life, as demonstrated by enhanced cardiovascular activity in response to restraint stress in adult offspring [145]. Maternal separation stress in rodents exacerbates responses to sympathetic stimulation, by inducing sensitisation of the renal and systemic sympathetic system and thus impairing blood pressure regulation in adulthood [146].

In children, early experience of neglect and the quality of relationship with their current caregivers has been shown to predict ANS reactivity. Children with a background of neglect and disordered attachment exhibited increased sympathetic reactivity, compared with those children with ordered/secure attachment [147], indicative of a potential reversal of the detrimental effects of early life adversity. Another encouraging evidence of plasticity in the HPA axis and ANS development, even after extreme psychosocial deprivation in early life, comes from the Bucharest Early Intervention Project. Children placed in foster care, after being raised in deprived institutional setting in Romanian orphanages, exhibited normalisation of their HPA axis and ANS responsiveness, compared with those who remained in institutional care [148]. Timing of placement in foster care, however, had a significant effect on the degree of reversal of the negative influences of psychosocial deprivation. Positive intervention effects of foster care on the HPA axis and the ANS were evident for children placed in foster care before 18 months and 2 years of age, respectively. These findings highlight a sensitive period during which the stress response systems, such as the HPA axis and the ANS, are most strongly influenced by environmental challenges [148].

Although a short window of developmental plasticity exists, the ANS and HPA axis hyper-reactivity is a typical consequence of childhood maltreatment and abuse,

contributing to adult psychopathology [149]. Prolonged activation of the sympathetic compartment of the ANS inhibits the activity of the innate immune system [150] and has, therefore, implications in the progression of immune-related diseases, including HIV [101]. Early life stress can also independently alter the immune system development, leading to lasting immune consequences.

10.5.3 Programming of the Immune System

The development of the immune system is dependent on the immune, autonomic and endocrine signals that it receives early in life [151–153]. The neonatal immune system is typically considered to be functionally immature, leading to an increased risk of infections during this period of life [154]. Inadequate exposure to immune stimuli in early life may disrupt the developmental trajectory of immune maturation and has been associated with a number of chronic immune-related diseases, including increased susceptibility to allergic and autoimmune diseases later in life [155].

Although the neonatal peripheral immune system generates a lower response when compared with that of the adult [156], the expression of many cytokines in the developing brain is significantly increased, even in the absence of an immune stimulus, coinciding with the appearance of "active" amoeboid microglial morphology. This increased central inflammatory activity in the developing brain stimulates neurogenesis, neuronal and glial cell migration, proliferation, differentiation, and synaptic maturation and pruning. The heightened central cytokine expression and "active" microglial morphology are likely to be indicative of the increased sensitivity of the developing brain to its environment. When adversity is experienced, this may lead to permanent alterations of major developmental processes and long-term programming of neuroimmune function (reviewed in [157]).

Microglia play important roles not only in the developing brain but also in the adult brain. Microglial activation significantly contributes to pathogenesis in neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [158], as well as to HIV-associated neurocognitive impairments [67]. Early life psychological, immune and nutritional stressors have been associated with lasting proinflammatory changes and microglial activation in the adult brain [159–161], suggesting that early life stress may contribute to the development of neurodegenerative disorders later in life. These detrimental effects of early life adversity on neuroinflammatory markers have, therefore, potential significant implications for HIV-associated cognitive impairments and Neuro-AIDS.

Reciprocal interactions between the neural, immune and endocrine systems are established during the perinatal period and together mediate organismal development. Therefore, not only may altered maturation of individual systems affect later life functioning, but disrupted interactions between these systems at critical periods of development may also lead to pathology. The effects of early life stress on the innate immunity of the offspring are generally inhibitory. In rodents, exposure

to prenatal psychological stress has been shown to decrease cytotoxicity of natural killer (NK) cells and resistance to experimentally induced tumours [162, 163]. Similar findings have been demonstrated in rats exposed to brief periods of maternal separation, following adult restraint stress [164]. These data suggest that perinatal stress may alter the ontogeny of the neonatal immune system leading to increased susceptibility to diseases and altered immune responsiveness to stressful stimuli.

In primates, prenatal stress and exposure to IL-1β in juvenility have led to a blunted inflammatory response with reduced plasma and cerebrospinal fluid levels of IL-6 and reduced febrile response to the IL-1β [165]. Exposure to an acute psychological stressor in pregnancy has also been shown to diminish cellular cytokine response to an in vitro stimulation with LPS [166]. Prenatal stress also impairs T cell-mediated antigen recognition in monkeys whose mothers were exposed to stress during gestation. These effects, however, were dependent on the timing of exposure to prenatal stress [167]. The suppressive effects of prenatal stress on the immune function in non-human primates appear to be similar to that observed in animals subjected to psychosocial stress in adulthood and SIV infection [43, 104], and may thus be potentially implicated in HIV disease progression.

Although a link between perinatal stress and immune function in the offspring has been investigated in animal models, so far limited information on this association exists in humans. Parental stress has been suggested to have implications for the development of allergic and atopic diseases in the children, influencing polarisation of T cell-mediated immunity towards Th2 cell dominance and contributing to the burden of childhood respiratory illness [168, 169]. On the other hand, additional epidemiological evidence has indicated a link between early life stress and an enhanced inflammatory profile. Specifically, patients with childhood abuse-related PTSD has been shown to display increased levels of inflammatory cytokines and decreased sensitivity of monocytes to glucocorticoids, indicative of increased inflammation [170]. Similarly, depressed adults with a history of childhood maltreatment were found to exhibit increased inflammation, as indicted by higher levels of C-reactive protein [171]. Stressful early life events have been also found to be associated with the reactivation of herpes virus, telomere shortening via increased T-cell proliferation and with immune dysregulation of the tumour environment in different types of cancers [151].

These diverse outcomes of early life stress on the immune function highlight the important gaps in the current knowledge regarding the specific influences of the timing and the extent of traumatic events, and how these factors mediate susceptibility to chronic diseases later in life. Therefore, the biological consequences of early life adversity require further investigation, particularly in human populations. The experience of early life trauma and its long-term programming effects on neuroendocrine and neuroimmune responses is inevitably associated with a variety of behavioural consequences and an increased incidence of mental health problems.

10.5.4 Behavioural Programming

The foetal and neonatal brain is characterised by an extensive network of developing neuronal connections and is especially vulnerable to the consequences of stress. Alterations of these developmental processes may affect cognitive function and behaviour, with the manifestation of these programming effects appearing later in life [172, 173]. Although data from human studies are able to provide correlational links, animal models of perinatal stress have been widely used to establish a causal relationship and to provide in-depth investigation of underlying mechanisms of behavioural programming. Multiple studies in rodents have demonstrated the long-term effect of early life psychological, physical and immune stressors on the emergence of anxiety-like and depressive behaviours, with the behavioural phenotype typically triggered in response to a secondary stressor in adulthood [174–176]. This unique phenotypic consequence of early life stress has received particular attention in a double-hit hypothesis of schizophrenia. Originally, the double-hit hypothesis of schizophrenia pointed out that a combination of genetic susceptibility together with a distinct developmental insult, as a 'first hit', can predispose an individual for a later traumatic event, a 'second hit', leading to the onset of psychopathology [177]. This hypothesis has since been extended to include infection and inflammatory insults as a 'first hit' that sets up this increased vulnerability [178–180] and is implicated in psychopathologies, including anxiety and depression [175, 181–183].

Epidemiological evidence has also indicated a link between maternal stress and increased risk for developing affective disorders. Specifically, it has been indicated that low birth weight and preterm birth, as a result of gestational stress, contribute to developmental impairments and motor dysfunction, common risk factors for schizophrenia [184]. Other research has reported maternal stress during pregnancy to influence developmental delays, emotional status and learning skills in childhood [185–187]. These findings in human populations are, however, confounded by the continuing influence of maternal anxiety on the quality of maternal care, which can affect development and behaviour [188]. Retrospective studies reported a higher incidence of schizophrenia in adults born to mothers exposed to severe stress during pregnancy, such as the stress of war, famine or a natural disaster [185, 189, 190]. Moreover, postnatal abuse, maltreatment and neglect have been linked to a greater risk of psychiatric disorders in later life, including PTSD [88], anxiety disorders [191], schizophrenia [190] and other mood disorders [192].

Cumulatively, both animal models and human studies of perinatal programming provide evidence that exposure to early life stress of various sources may alter the maturation of the HPA axis, the ANS and the immune system. These changes may in turn increase susceptibility to dysregulated stress responsiveness and pathological outcomes in adulthood, the nature and intensity of which depend on the genetic vulnerability of the individual, the severity of perinatal stress, the timing of exposure and the quality of the postnatal environment.

10.6 Perinatal Programming of Vulnerability or Resilience: Implications for HIV Disease Progression

Despite incredible advancements in HIV/AIDS research, the disease still affects millions of people worldwide. In 2015, there were 36.7 million people infected with HIV worldwide, with the rate of new infections falling only by 6% in the past 6 years. Continuous antiretroviral therapy is, currently, the most effective way to reduce the risk of disease progression, however, only half of those infected worldwide have currently access to these life-saving drugs [193]. Treatment interruptions are also common in the clinical practice, reducing treatment efficacy [194]. Importantly, the progression of HIV and the development of AIDS are not limited to physical and immunological symptoms. The disease is often accompanied by psychopathology and substance abuse, worsening disease outcome, even in the presence of antiretroviral therapy [101, 195].

Psychosocial stress is an inevitable component of every chronic disease, including HIV/AIDS. This relationship is influenced by the nature and the extent of stress, as well as by individual vulnerability to its effects. Here, we propose that this vulnerability may be at least in part affected by early life experiences of an individual that may eventually lead to more rapid disease progression in the context of HIV (Fig. 10.1). It appears that some outcomes of early life adversity are similar to those associated with poor prognosis of HIV infection, such as a blunted cortisol profile, overreactive sympathetic responses and diminished parasympathetic activity, as well as symptoms of PTSD, anxiety and depression [19, 46, 83, 102]. Therefore, these individuals may potentially be at a higher risk of adverse disease outcomes upon HIV infection.

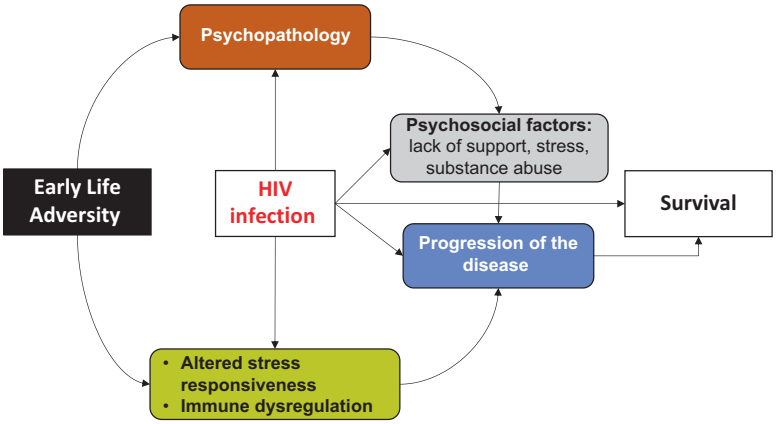

Fig. 10.1 Framework proposal: In the context of HIV infection, early life adversity may influence the progression of the disease and ultimately affect survival, by predisposing the individual to increased risk of psychopathology, blunted stress responsiveness and altered immune function. These parameters that are further exacerbated by HIV infection and may in turn accelerate the progression of the disease

HIV/AIDS research suffers from decline in investment in recent years. The newly established National Institute of Health (NIH) HIV/AIDS research priorities exclude behavioural and social science research activities [196]. Behavioural research is essential to optimise medication adherence and to improve the management of psychosocial disorders which can greatly impede HIV health outcomes. In combination with biomedical therapies, behavioural interventions have been shown to optimise the efficacy of treatment [197], to increase treatment adherence [198] and to increase knowledge of HIV and transmission risks among populations at risk [199]. Such activities are vital for the continuous improvement of HIV treatment and prevention strategies. More research is required into the potential for identifying biomarkers of early life adversity and altered stress responsiveness in HIV infected individuals, which may predict accelerated disease progression. Together with behavioural interventions, this approach may assist in early detection of reduced resilience and provide these individuals with timely and appropriate psychosocial support.

It is important to note that in this chapter we did not discuss the important issue of HIV infection in newborns and children. Although the rates of HIV infection among children have declined by 50% since 2010 [193], the challenges in the diagnosis and treatment of the disease at such young ages are still of utmost importance for healthcare providers and researchers. The interactions between the developing immune system and HIV are complex, leading to different HIV pathogenesis in children, characterised by more rapid disease progression, compared with adults (reviewed in [200]). While such differences in the disease progression and outcome are beyond the scope of the current chapter, further research into the particular susceptibility of the foetal to adolescent immunity to infectious diseases like HIV is essential to reduce pediatric HIV infection and improve the quality of care.

Conflict of interest The authors report no conflicts of interest.

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Chapter 11
Neurocognition in Viral Suppressed HIV-Infected Children

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Keywords Pediatrics • Children • Youth • HIV • Neurocognition • Neurodevelopment • Neuroinflammation • Perinatally acquired HIV (PHIV) • Neuroimaging • ART • Neurocognition in youth with PHIV • Neurodevelopment in children with PHIV • Neuroimaging in PHIV • Antiretroviral therapy associated neurotoxicity in PHIV • Neuroinflammation in PHIV

Core Message

Children and adolescents with perinatally acquired HIV (PHIV) infection remain at risk for subtle to severe neurocognitive deficits despite antiretroviral therapy (ART). Early ART initiation in infancy may mitigate global or selective deficits for some children; however, initiation of ART later in childhood does not appear to have similar protective benefits. The presence of neurocognitive deficits and/or the lack of neurocognitive improvement after ART initiation in older children may be due to damage associated with prior immunosuppression, intermittent periods of HIV replication and neuroinflammation during childhood and adolescence, and/or ART-associated neurotoxicities. The literature supports the need for early ART initiation, not only for survival benefit but also for optimizing neurodevelopmental and neurocognitive outcomes; however, prospective, longitudinal studies are still necessary to determine the long-term neurocognitive outcomes among children with early and deferred ART initiation as well as the functional impact of deficits and potential resilience.

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11.1 Introduction and Background

Antiretroviral therapy (ART) has transformed the spectrum of pediatric HIV infection. Children with perinatally acquired HIV (PHIV) who were unlikely to survive beyond childhood prior to the advent of ART are now approaching young adulthood. Progressive HIV encephalopathy (PHE) has dramatically declined since the introduction of ART [1] (Fig. 11.1) as has the incidence of severe neurocognitive impairments [2–4]. Yet mild or global neurocognitive deficits remain detectable in children and adolescents with PHIV receiving suppressive ART. As a result, youth with PHIV who have neurocognitive deficits are at risk for poor school performance, high-risk behaviors, diminished productivity as adults, reduced quality of life, and medication nonadherence [5–8]. As youth with PHIV survive into adulthood with families and careers of their own, it is crucial to optimize their neurocognitive and functional outcomes.

Children and adolescents with PHIV may experience neurocognitive deficits due to a variety of mechanisms that include (i) irreversible neuronal injury prior to ART initiation, (ii) detrimental effects of neuroinflammation that occur in response to HIV infection, (iii) ART-associated neurotoxicities, and (iv) ongoing viral replication in the central nervous system (CNS) [9–12]. The potential effects of lifelong or intermittent exposure to these processes, particularly during periods of rapid brain growth, are variable and unique to youth with PHIV. In addition, low socioeconomic and ethnic minority status, genetic and familial risk factors, poor nutrition, high social stress, and limited community or societal support may have distinctive and cumulative effects on neurocognitive outcomes [13–15], perhaps coincidentally affecting the developmental plasticity of neural systems [16–19]. Associated risks may appear or accelerate as youth age into

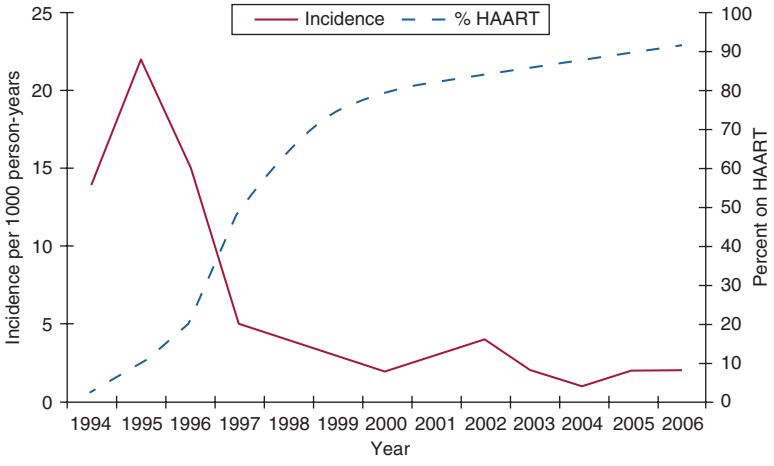

Fig. 11.1 HIV encephalopathy incidence in children and percentage on ART, from 1994 to 2006 (Reproduced from Patel et al. 2009 with permission from Wolters Kluwer [1])

adulthood when demands for autonomy increase and social support from caregivers and others vary or diminish significantly, placing youth at risk for nonadherence and disease progression.

Although there is a wealth of literature regarding HIV-associated neurocognitive disorders in adults [20], youth with PHIV require unique consideration, since HIV affects developing neural networks in children during sensitive periods of development, with potential for ongoing damage. HIV replication in the cerebrospinal fluid (CSF) is associated with neurocognitive impairment and PHE severity in children with PHIV, highlighting the importance of early viral suppression in the CSF [21, 22]. The purpose of this chapter is to summarize current understanding of the impact of ART and viral suppression on neurocognitive function in children and adolescents with PHIV. Although children and adolescents with PHIV residing in low- and middle-income countries are uniquely different from those in high-income countries in terms of predominant HIV subtypes, comorbidities, treatment availability and standards, and psychosocial factors, there is limited literature on this topic in pediatrics, and the majority of new PHIV infections currently occur in sub-Saharan Africa. Therefore, this chapter will consider studies conducted in the USA as well as internationally.

11.2 Neurodevelopmental and Neurocognitive Functioning

Early studies of children with PHIV infection revealed significant neurological and cognitive compromise prior to the availability and initiation of ART. The rate of PHE was as high as 76% of children with an AIDS diagnosis and 21–33% of all children with HIV infection [23, 24]. Investigations since the advent of ART have revealed a dramatic decrement in rates of PHE but not elimination of subtle to severe neurocognitive deficits among children and adolescents.

Results of investigations of the effect of ART or viral suppression on neurocognitive outcomes are complex and sometimes conflicting (Tables 11.1 and 11.2). Timing, duration, and efficacy of ART are critical to consider yet must be understood in the context of timing and severity of HIV infection, biogenetic vulnerabilities, social and cultural factors, and supportive interventions that are unique to each child [25]. In general, neurocognitive deficits vary in presentation and severity, and their etiologies are likely multifactorial and dynamic.

In retrospective studies of children and adolescents with PHIV, ART initiation prior to the diagnosis of an AIDS-defining illness including PHE had survival benefit but limited neurocognitive benefit [26–29]. Children with PHIV obtained lower neurocognitive scores than established norms and/or healthy controls [29, 30]. During the early years of ART availability for children, investigations of neurocognitive functioning were sometimes limited by absence of appropriate comparison groups, restricted time frames, and inadequate control for HIV disease severity in analyses, as well as other risk factors that are independently associated with neurocognition. A US-based retrospective study observed a marginally significant asso-

ciation between HIV viral load and neurocognitive scores and general stability in mean neurocognitive functioning over time among children with PHIV [31]. While the use of different neurocognitive assessment measures and absence of detail regarding inter-test reliability precluded firm conclusions, results suggested that older children initiating ART avoid neurocognitive decline but have ongoing unsalvageable neurocognitive deficits of varying severity.

Results of early studies of ART efficacy revealed limited improvement in neurodevelopmental and neurocognitive functioning. Among a cohort of children with PHIV, neurocognitive function was poorer relative to age-based norms [32]. Higher baseline HIV viral loads correlated with poor cognitive and fine motor scores among those receiving nucleoside reverse transcriptase inhibitor (NRTI) monotherapy yet, after 48 weeks of protease inhibitor (PI)-based combination therapy and favorable impact on viral suppression, positive neurocognitive effects were limited to vocabulary development only and baseline functioning remained significantly predictive of later outcomes. In a cohort of infants and children 3 years of age and younger, initiation of PI-based ART regimens was associated with positive but limited impact on neurodevelopmental trajectories in the context of declining performance of a comparison group of HIV-exposed but uninfected (HEU) children [33]. Since these investigations were conducted shortly after the introduction of protease inhibitors, it is possible that PHIV-infected participants received prior suboptimal antiretroviral treatment, if treated. In addition, neurodevelopmental outcomes are influenced by the interaction of genetic, health, treatment, and psychosocial factors that vary in their influence during the early years of life and were not fully accounted for in these investigations.

More recent studies conducted in low- and middle-income countries where ART is more homogenous suggest that ART maintains baseline cognitive functioning. A study of children with PHIV in Thailand, ages 6–12 years (median age 9.3 years), revealed low but generally stable neurocognitive functioning after 30 months of ART with viral suppression among the majority of children [15]. When compared to HEU children and HIV-unexposed uninfected (HUU) children, children with PHIV obtained lower scores than both groups of uninfected children and were at higher risk for poor cognitive outcomes. A subsequent study comparing neurocognitive functioning of children (median age 9 years) randomized to either early (CD4% >25% and no clinical criteria for ART initiation) versus deferred ART (CD4% <15% or CDC class C event) demonstrated no differences in global cognition, specific cognitive domains, or psychomotor scores between the early versus late ART groups at 3 years after study entry [34]. These studies highlight the likelihood that children with PHIV initiating ART at older ages are unlikely to experience significant neurocognitive improvement, regardless of timing of initiation during childhood.

While evidence suggests that ART does not eliminate cognitive differences between youth with PHIV and their HEU and HUU counterparts, particularly among school-age children with treatment initiation according to earlier clinical guidelines, some evidence suggests that ART may confer some, possibly short-term, neurodevelopmental benefit for young children. Results across studies, however, are

Table 11.1 Summary of studies of the impact of antiretroviral therapy and viral suppression on neurocognitive function in perinatally acquired HIV-infected children

Citation	Study design	Study population	Outcome(s) measured	ART/viral suppression	Findings[a]
Van Rie (2009) [35] *(Democratic Republic of Congo)*	Cohort	$N = 160$; 35 PHIV+, 35 HEU, 90 HUU Ages: 18–71 months; 44.8 months (median)	Age appropriate assessment at baseline, 6 and 12 months (BSID-II, Peabody Developmental Motor Scales 2nd edition, and SON)	ART as per WHO guidelines in 2004 71% initiated ART	Baseline scores: lowest for PHIV+, intermediate for HEU and highest for HUU One year scores: PHIV+ was lower than HUU but similar to HEU. PHIV+ children experienced the largest improvements in mean scores PHIV+ early vs. late presenters for care had similar baseline cognitive scores, but early presenters had greater improvement after 12 months (28.6 vs. 14.8, $p = 0.11$). For motor, late presenters performed worse at baseline (71.6 vs. 80.9, $p < 0.05$) and at both follow-up visits ($p < 0.05$ for both)
Puthanakit (2010) [15] *(Thailand)*	Cohort	$N = 121$; 39 PHIV+, 40 HEU, 42 HUU Ages: 6–12 years; 9.3 years (median)	WISC-III at enrollment and after 30 months	All PHIV+ children on ART with variable ART duration at enrollment 77% of PHIV+ had undetectable VL at 30 months follow-up	At baseline and after 30 months follow-up, PHIV+ children had lower scores compared to HEU and HUU and higher risk of poor cognitive outcome (OR: 6.20, $p < 0.01$) Among PHIV+ children, VL and duration of ART were not significant predictors of poor cognitive function (statistics not provided)
Laughton (2012) [40] *(South Africa)*	Cross-sectional sub-study of RCT with age-matched controls	$N = 152$; 115 PHIV+, 28 HEU, 34 HUU Ages: 11 months (median)	GMDS	All children on ART (early vs. deferred) In parent study >99% of children achieved viral suppression after 24 weeks of ART	PHIV+ children in the deferred arm scored lower than early ART, HEU and HUU PHIV+ children in the early ART arm had similar scores compared to HEU and HUU except in locomotor domain

(continued)

Table 11.1 (continued)

Citation	Study design	Study population	Outcome(s) measured	ART/viral suppression	Findings[a]
Lowick (2012) [37] *(South Africa)*	Cross-sectional	*N* = 60; 30 PHIV+, 30 healthy controls (HIV status unknown) Ages: 55–75 months; 63 months (median for PHIV+)	GMDS	All PHIV+ children had CD4% >15%, VL <50 copies/ml and had received ART for >1 year	PHIV+ children scored lower than controls in all domains
Puthanakit (2013) [34] *(Thailand)*	Cross-sectional sub-study of RCT with age-matched controls	*N* = 489; 170 PHIV+, 155 HEU, 164 HUU Ages: 9 years (median)	WISC-III or WPPSI-III (at week 144 for PHIV+)	All early arm on ART, 48% of deferred arm on ART 91% in early arm had viral suppression	No difference between early vs. deferred ART arms ($p > 0.10$) All PHIV+ mean scores were lower than controls
Whitehead (2014) [38] *(South Africa)*	Cohort	*N* = 56; 29 PHIV+, 29 HEU Ages: 4.08 months (mean)	BSID-III PHIV+: before, 3 and 6 months post-ART	All children on ART No VL data	PHIV+ scored lower at baseline in all domains Scores did not improve significantly 6-months post-ART ($p \geq 0.06$)
Brahmbhatt (2014) [36] *(Uganda)*	Cohort	*N* = 329; 116 PHIV+, 105 HEU, 108 HUU Ages: 45.8 months (mean)	MSEL for all children with a positive TQ10 screening test PHIV+: follow-up MSEL to assess for impact of ART initiation and duration	44% on ART No VL data	At baseline: PHIV+ vs. HUU: Impaired in FM (PRR = 2.39; CI, 1.15–4.95), VR (PRR = 5.86; CI,2.3–14.92), RL (PRR = 4.2; CI,1.83–9.64), EL (PRR = 2.27; CI,1.15–4.5) and ELC (PRR = 6.87; CI,2.54–18.58) PHIV+ vs. HEU: Impaired in VR (PRR = 2.86) ELC scores improved by 0.16 per 1 month duration of ART ($p = 0.02$)

Shanbhag (2005) [31] *(USA)*	Cohort	$N = 146$; all PHIV+ stratified by birth cohort (pre- vs. post-ART era) Ages: 21.5 months (median)	Varied depending on age. Not reported	97% of children born in post-ART era No VL data	VL log was marginally associated with neurocognitive scores; a 1-log unit decrease in VL log was associated with an increase in overall neurocognitive standard score of 0.88 points ($p = 0.06$) Increase in mean score in children born in the post-ART era versus those born before (87.2 vs. 82.3; $p = 0.001$)
Jeremy (2005) [32] *(USA)*	2 RCTs of PI-based vs. non-PI-based ART	$N = 489$; all PHIV+ Ages: 4 months-17 years; 6.8 years (median)	Age appropriate assessment at baseline and every 24–48 weeks (BSID-II, WISC-III, WIPPSI, WAIS-III)	All children on non-PI-based ART for ≥16 weeks 44% had viral suppression from week 24 to 48	Lower cognitive, vocabulary, and fine motor scores at baseline compared to population norms ($p < 0.001$) Higher baseline VL correlated with poorer cognitive and fine motor scores ($p < 0.05$) PI-based ART for 48 weeks associated with improved vocabulary scores only ($p = 0.043$) Viral suppression was not associated with improved scores
Martin (2006) [88] *(USA)*	Cross-sectional study	$N = 41$; all PHIV+ Ages: 11.2 years (mean)	WISC-III	All children on ART for ≥1 year 17% had viral suppression	Scores were not significantly related to VL ($p > 0.10$)
Hazra (2007) [89] *(USA)*	Cohort	$N = 12$; all PHIV+ Ages: 11.8 years (median)	WISC-III at entry, 24, 48, and 96 weeks after initiating study ART	All children initiated on study ART 10 children had viral suppression at week 48, 8 at week 96	No significant change in mean FSIQ between baseline and follow-ups (statistics not provided)

(continued)

Table 11.1 (continued)

Citation	Study design	Study population	Outcome(s) measured	ART/viral suppression	Findings[a]
Koekkoek (2008) [47] *(Netherlands)*	Cross-sectional study	*N* = 22; all PHIV+ Ages: 9.46 years (median)	SON 7 tests from the ANT program	20 children on ART 18 children had viral suppression	Only mean reaction time was associated with treatment duration ($r = 0.66$, $p = 0.01$)
Crowell (2015) [42] *(USA)*	Cohort	*N* = 396; all PHIV+ Ages: 9.6 years (mean) at time of WISC	WISC-III or WISC-IV	42% of children had viral suppression by age 5	FSIQ and PIQ/PRI were higher in PHIV+ children who achieved viral suppression by 4 and 5 years of age
Cohen (2015) [29] *(Netherlands)*	Cross-sectional study	*N* = 72; 35 PHIV+, 37 healthy age, gender, ethnicity, and SES matched controls Ages: PHIV+ 13.8 years (median), controls 12.1 years (median)	WISC-III or WAIS-III	83% virally suppressed at time of study	FSIQ, VIQ, and PIQ were higher in controls compared to PHIV+ children. CDC classification at HIV diagnosis was inversely associated with VIQ No association between VL, peak VL, or duration of detectable VL and cognitive function (IQ or VIQ)

ART antiretroviral therapy; *PHIV+* perinatally acquired; *HIV*-infected; *HEU HIV*-exposed, uninfected; *HUU HIV*-unexposed, uninfected; *BSID-II* Bayley Scales of Infant Development, second edition; *WHO* World Health Organization; *WISC-III* Weschler Intelligence Scale for Children, third edition; *WISC-IV* Weschler Intelligence Scale for Children, fourth edition; *VL* viral load; *RCT* randomized control trial; *GMDS* Griffiths Mental Development Scales; *WPPSI-III* Weschler Preschool and Primary Scale of Intelligence; *BSID-III* Bayley Scales of Infant Development, third edition; *MSEL* Mullen Scales of Early learning; *FM* fine motor; *VR* visual reception; *RL* receptive language; *EL* expressive language; *ELC* early learning composite; *PI* protease inhibitor; *WAIS-III* Weschler Adult Intelligence Scale, third edition; *FSIQ* full-scale intelligence quotient; *SON* Snijders-Oomen Nonverbal intelligence test; *ANT* Amsterdam Neuropsychological Tasks program; *PIQ* performance intelligence quotient; *PRI* perceptual reasoning index

[a]For studies reporting mean test scores, refer to Table 11.2.

Table 11.2 Mean neurocognitive testing scores for studies with control group comparison

Citation	Findings
Jeremy (2005) [32]	Baseline vs. 48 weeks Cognitive: 84 vs. 84.5 (NS) Vocabulary: 87.6 vs. 93.1 ($p = 0.043$) Baseline HIV viral load ≤50,000 copies/mL vs. >50,000 copies/mL: Cognitive: 84.5 vs. 80.6 ($p = 0.005$) Fine motor (z-scores): −0.08 vs. -1.29 ($p = 0.013$)
Van Rie (2009) [35]	PHIV+ vs. HEU vs. HUU (p-values for comparisons with PHIV+ group) Cognitive: Baseline: 65.8 vs. 74.8 ($p = 0.035$) vs. 84.6 ($p < 0.001$) 6 months: 75.8 vs. 74.9 ($p = 0.839$) vs. 87.3 ($p = 0.002$) 12 months: 84.3 vs. 87.6 (0.495) vs. 96.5 ($p = 0.006$) Motor: Baseline: 75.7 vs. 87.2 ($p < 0.001$) vs. 97.8 ($p < 0.001$) 6 months: 82.4 vs. 91 ($p = 0.003$) vs. 101 ($p < 0.001$) 12 months: 90.4 vs. 94 ($p = 0.178$) vs. 105 ($p < 0.001$)
Puthanakit (2010) [15]	PHIV+ vs. HEU vs. HUU (p-values for comparisons with HUU group) Baseline: FSIQ: 79 vs. 88 vs. 96 ($p < 0.01$) PIQ: 83 vs. 92 vs. 100 ($p < 0.01$) VIQ: 79 vs. 86 vs. 93 ($p < 0.01$) 30 months: FSIQ: 75 vs. 85 vs. 91 ($p < 0.01$) PIQ: 82 vs. 90 vs. 100 ($p < 0.01$) VIQ: 75 vs. 85 vs. 91 ($p < 0.01$)
Laughton (2012) [40]	Deferred vs. early vs. HEU vs. HUU (p-values comparing 4 groups) General quotient: 100.1 vs. 106.3 vs. 105.6 vs. 106.9 ($p = 0.14$) [post hoc deferred vs. early, $p = 0.02$] Locomotor quotient 88.9 vs. 97.7 vs. 105.3 vs. 101.6 ($p < 0.01$) Personal-social: 107.7 vs. 111.3 vs. 106.6 vs. 107.4 ($p = 0.46$) Performance: 95 vs. 100.3 vs. 99.8 vs. 102.7 ($p = 0.16$)
Lowick (2012) [37]	PHIV+ vs. controls General quotient: 70 vs. 78.0 ($p = 0.001$) Locomotor quotient: 75.7 vs. 82.7 ($p = 0.004$) Personal-social: 76.8 vs. 85.8 ($p = 0.001$) Hearing-speech: 60.6 vs. 66.9 ($p = 0.011$) Performance: 62.3 vs. 73.1 ($p = 0.014$) Practical reasoning: 68.2 vs. 75.4 ($p = 0.005$)
Puthanakit (2013) [34]	PHIV+ (early and deferred arms) vs. HEU vs. HUU (p-values for comparisons with PHIV+ group) FSIQ: 74–75 vs. 86 ($p < 0.001$) vs. 90 ($p < 0.001$) VIQ: 70 vs. 82 ($p < 0.001$) vs. 87 ($p < 0.001$) PIQ: 82–83 vs. 91 ($p < 0.05$) vs. 94 ($p < 0.001$) PS: 89–90 vs. 102 ($p < 0.001$) vs. 105 ($p < 0.001$)

(continued)

Table 11.2 (continued)

Citation	Findings
Whitehead (2014) [38]	PHIV+ vs. HEU Baseline: Cognitive composite: 86.11 vs. 93.97 ($p = 0.06$) Language composite: 87.85 vs. 100.26 ($p < 0.001$) Motor composite: 93.15 vs. 106.48 ($p = 0.004$) 6 months: Cognitive composite: 94.75 vs. 98.95 ($p = 0.18$) Language composite: 90.3 vs. 100.21 ($p = 0.004$) Motor composite: 93.7 vs. 105.58 ($p = 0.002$)
Crowell (2015) [42]	PHIV+ virally suppressed vs. unsuppressed Virally suppressed by age 4 FSIQ higher by 4.4 points ($p = 0.02$) PIQ/PRI higher by 4.6 points ($p = 0.01$) Virally suppressed by age 5 FSIQ higher by 3.9 at age 5 ($p = 0.03$) PIQ/PRI higher by 4.5 points ($p = 0.01$)
Cohen (2015) [29]	PHIV+ vs. controls FSIQ: 76 vs. 87.5 ($p = 0.002$) VIQ: 77.9 vs. 89.6 ($p = 0.003$) PIQ: 78.1 vs. 86.5 ($p = 0.03$)

PHIV+ perinatally acquired HIV-infected; *HEU* HIV-exposed, uninfected; *HUU* HIV-unexposed, uninfected; *FSIQ* full-scale intelligence quotient; *PIQ* performance intelligence quotient; *VIQ* verbal intelligence quotient; *PS* processing speed; *PRI* perceptual reasoning index

inconsistent. In a study of preschool-age children (median age 44.8 months) in the Democratic Republic of Congo, children with PHIV demonstrated the largest improvements in neurodevelopmental functioning after 1 year of ART compared to HEU and HUU controls [35]. In this study, children with PHIV scored lower than HEU and HUU children at baseline but had similar scores compared to HEU children after 1 year. Improvement in motor functioning was observed among children who presented for care prior to meeting ART eligibility criteria, suggesting that ART initiation prior to disease progression and in the preschool years is associated with positive neurodevelopmental change. In addition, development was accelerated for younger but not older children. Thus chronological age and status of HIV progression appear relevant. Results from a recent study in Uganda identify an association between general neurodevelopmental improvement and ART initiation and duration in a larger cohort of similarly aged children [36]. However, longitudinal studies of cohorts with early access to ART are necessary to determine if observed positive effects are temporary or sustained in childhood and adolescence.

Despite some encouraging results, positive impact of ART on neurodevelopmental and/or neurological functioning is not universally observed. A study of 60 preschool-age children with PHIV in South Africa examined neurodevelopmental outcomes among children who initiated ART at a median age of 24 months, with viral load <50 copies/ml at the time of enrollment, and median duration of 40 months on ART [37]. Despite viral suppression and ongoing ART, children with PHIV

scored lower than their healthy age-matched peers with unknown HIV status in all neurodevelopmental domains assessed. A study in South Africa of 56 infants, with either HIV infection or HIV exposure and average age of 4 months, examined neurodevelopmental outcomes before ART and 3 and 6 months after ART initiation [38]. Infants with PHIV had lower scores at baseline than HEU infants, with no significant neurodevelopmental improvement after 6 months of ART. The absence of information regarding viral load among this cohort leaves open the possibility that the infants with PHIV were not virologically suppressed and thus, without benefit from CNS viral control. Another possibility is that children with PHIV identified in infancy were clinically symptomatic, unless they were diagnosed via prevention of mother-to-child transmission, whereas those who remained well during their preschool years represent a cohort of survivors who have not yet experienced significant HIV-associated neurologic compromise or HIV-associated illness. In fact, among the infants with PHIV in this study, 74% experienced a prior illness and 44% were hospitalized before study enrollment. In contrast, HEU children in the control group had a 17% rate of prior illness and a 7% rate of prior hospitalizations. These observations suggest that the cohort with PHIV was relatively symptomatic at baseline and the absence of neurodevelopmental benefit after 6 months of ART may be related to high baseline disease severity and accompanying sequelae. The development of these children may be influenced also by greater psychosocial risk, if affected by parental illness and limited resources.

A meta-analysis of data from five US pediatric HIV/AIDS treatment trials, conducted during a period of rapidly evolving treatment approaches, found an association between higher HIV viral load 24 weeks post-ART initiation and cognitive decline (defined as >15 or 30 point decline in general intelligence) [39]. These associations were significant for children >1 year of age with only a weak association between viral load and cognitive decline in infants ≤1 year. The studies included in this meta-analysis were heterogeneous, and the ART strategies employed included NRTI and non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimens. Despite these limitations, the analyses suggest that HIV viral control has potential neurocognitive benefit but that its influence is related to age, disease severity at ART initiation, and efficacy of ART in the CNS.

To further address the question of whether early ART initiation during infancy confers neurodevelopmental benefit, infants enrolled in the CHER trial who were 3 months of age or younger with CD4% >25% were randomized to early ART (immediate) versus deferred ART. Infants with early treatment achieved higher scores across developmental domains than those with deferred treatment and were generally comparable to HEU and HUU controls, except in the locomotor domain [40]. These results suggest that early ART may reduce the negative neurodevelopmental impact of HIV viral activity prior to disease progression. It remains possible that improvements are selective and/or short term, given that study evaluations occurred at 11 months of age. A follow-up sub-study of this cohort evaluated a subgroup of children who developed neurological symptoms (mean age of 31.9 months), of whom 77% had been treated early, with a mean age at ART initiation of 8 weeks [41]. Although firm conclusions cannot be made in the presence of incomplete

reevaluation of the original cohort, a trend for association of white matter structural abnormalities in the brain of those with longer time on ART suggests that vulnerability occurs prior to ART initiation and/or develops in spite of early ART and suggests a potential role of ART toxicity with implications for ongoing neurodevelopment and later neurocognitive outcomes.

While some investigations suggest that early ART initiation may have favorable short-term effects on neurodevelopment, it remains unclear whether early ART and subsequent viral suppression provide long-term neurocognitive benefit. Analyses of data collected during two longitudinal observational studies of children with PHIV in the USA [42] specifically examined the association between age of viral suppression and school-age neurocognitive outcomes. Children with PHIV who achieved viral suppression by 4 or 5 years of age achieved significantly higher scores in global cognition (Wechsler III and IV FSIQ scores) and perceptual reasoning (Wechsler III Performance IQ and Wechsler IV Perceptual Reasoning Index scores) when assessed at an average age of 9.6 years. Viral suppression by 1, 2, or 3 years of age was not associated with school-age neurocognitive performance. However, this lack of association may have been related to small numbers in those age groups, given that effect sizes were similar across all age categories. Furthermore, the association between age of viral suppression and cognitive outcomes persisted only among those born after 1996, when use of combination ART became common.

11.2.1 Summary and Implications

There is no question that early ART confers survival benefit for infants with PHIV [43]. Recent evidence also indicates that early viral suppression, by age 1 year, is associated with reduced peripheral blood proviral reservoir size and longer duration of virologic control and immune health [44]. The data suggest that in infants with PHIV, early ART may provide mild neurodevelopmental benefit, sometimes limited to individual domains of development, and perhaps only to those without severe early CNS damage. It is unclear if this benefit is sustained and some data suggest that it is not. However, the absence of longitudinal data in most studies limits the ability to provide a definitive conclusion. It is important to recognize that during the early years of life, brain development is dominated by dynamic, progressive processes, with emphasis on growth that will be pruned and refined via maturation and experience throughout childhood [45]; insults during early developmental periods are unlikely benign. Additionally, the effects of socioeconomic and familial factors on neurodevelopment in this population are not fully understood but may certainly potentiate existing vulnerabilities.

For children with PHIV presenting for care later in childhood either well or with evidence of disease progression, data suggest that there is limited neurocognitive improvement with ART, which may be due to prior CNS damage or disruption that is unsalvageable. However, ART may prevent further decrement in function in this group or may limit deficits to selective aspects of neurocognitive functioning. Adult

literature suggests that the neurocognitive benefit conferred early in the course of ART for newly infected individuals stems from control of CNS virus, yet this benefit may be temporary as neurotoxicity of the antiretroviral drugs themselves, ongoing CNS inflammation, and other risk factors may cause additional or more severe CNS insults [46]. Certainly, this is of major concern for children and adolescents with PHIV who likely require lifelong ART from infancy or time of HIV diagnosis.

11.3 Selective Domains of Neuropsychological Function

As more children and adolescents with PHIV age into adolescence and young adulthood, examination of higher-order cognitive abilities has potential to identify subtle and significant forms of neuropsychological impairment during different stages of development and across variable socioeconomic and cultural contexts. Such examination may also inform our understanding of the impact of HIV, viral suppression, and ART on risk behaviors and requisite skills and functions for successful independence, employment, and management of health care. Executive functioning, which refers to neurocognitive processes important for behavioral and cognitive regulation, and other selective domains of neuropsychological functioning, including processing speed and memory, have been relevant among adults with HIV. However, discrepant outcomes among adults versus children and adolescents with HIV are not unexpected, given unique differences in timing of HIV infection, history of ART initiation, vulnerabilities inherent to each age group, and likely comorbidities. Executive functioning, memory, processing speed, and other abilities among youth with PHIV and their relationships with viral suppression and ART, as well as history of AIDS diagnosis and immune functioning, have been studied among cohorts of variable size, age, and HIV severity and with variable assessment strategies and tools. Results thus far are mixed.

In a cohort of 22 school-age children with median age of HIV diagnosis of 4.8 years and median age of ART initiation of 5.6 years, nonverbal reasoning skills were in the average range; however, performance on four of seven tasks that reflect executive function was significantly below average relative to norms [47]. Longer ART duration was associated with better executive function, including reaction time, accuracy, and attentional flexibility, supporting the efficacy of ART. A prospective longitudinal investigation of emerging executive functioning, designed to control for potential demographic and psychosocial confounders, examined a cohort of school-age children with PHIV, with and without a prior CDC class C diagnosis, and a comparison group of HEU children [48]. Analyses revealed no significant differences in functioning across executive functioning tasks, including abstraction, disinhibition, sustained visual and auditory attention, and others. Children on ART, regardless of disease severity, did not display executive functioning deficits relative to HEU children when differential psychosocial and environmental factors were considered. However, power to detect small differences in this cohort was limited

by the small number of children with CDC class C diagnoses. Additionally, the majority of children in this investigation were of ethnic minority backgrounds and normative samples were not necessarily representative of these groups. Thus, it remains possible that executive functioning is impacted by HIV, viral suppression, and/or ART, perhaps becoming evident as children age through adolescence and as sensitive and comprehensive measures of executive functioning are accessed.

To control for differences in environmental factors and potential biases in interpretation, school-age (6–16 years) children with and without PHIV residing in orphanages in Myanmar with identical nutritional support, caregiver status, education, and access to services were compared [49]. Children with PHIV were on ART with the majority receiving the same ART regimen. Children with PHIV performed significantly less adequately on 6 of 13 neuropsychological measures, including measures of psychomotor speed, visual motor integration, visuospatial skills, and executive function. However, no relationship was identified between these and either age of treatment initiation or duration of ART. Follow-up evaluations of a subgroup of the cohort revealed expected age-related growth in multiple domains for both groups. Of interest, children with PHIV demonstrated more consistent improvement and gains in more neuropsychological domains than children in the control group, suggesting differing trajectories of development and the possibility of greater than expected developmental growth among children with PHIV in the presence of adequate ART and consistent adherence.

A cross-sectional study of youth with PHIV and healthy controls matched on socioeconomic status revealed lower attention/working memory among youth with HIV, 83% of whom were virally suppressed at study entry [29]. However, there were no significant differences in executive functioning or immediate and delayed verbal memory between children with and without HIV. In contrast, among 40 children with PHIV adequately treated with ART and a control group of 72 children matched on age, gender, school type, and cognitive functioning, executive functioning was one of three discriminating domains, and those with more advanced CDC disease classification (AIDS) demonstrated lower performance overall and greater heterogeneity across tasks; effects were also observed in visual spatial memory and language skills [50].

Several cross-sectional prospective studies have also identified relationships between markers of disease severity and difficulties associated with neuropsychological functioning, including processing speed. In a prospective study of older school-age children, the majority with viral suppression, peak viral load >100,000 copies/mL, was associated with lower scores in global cognition and other cognitive domains including processing speed and working memory [51]. Higher risk of processing speed impairment was observed among those with AIDS diagnosis, including those without encephalopathy. A similar relationship between higher peak viral load and slower processing speed was observed in a prospective study of HIV disease severity and psychiatric and cognitive outcomes among 6–17-year-old youth [52] and has also been frequently detected in adults with HIV infection [53].

In an effort to determine if deficits in executive functioning are evident during later childhood and adolescence following periods of ART and viral suppression, a

large US-based prospective investigation among youth with PHIV and HEU youth examined the impact of indices of HIV disease severity and ART on multiple aspects of executive functioning, using measures specifically targeted to assess executive functioning [54]. Of interest, among youth with PHIV with and without AIDS diagnoses and HEU youth, no significant executive functioning differences in planning, cognitive flexibility, or divided attention were observed, although scores were below standardized means. In addition, caregivers reported fewer executive functioning problems for PHIV than HEU groups. However, youth with histories of HIV-associated encephalopathy and higher peak viral load self-endorsed more metacognitive (i.e., cognitive regulation) problems and also performed more slowly on a direct measure of executive functioning. In contrast to observations of youth with early severe HIV, behavioral regulation difficulties rather than cognitive regulation difficulties were reported by youth who were older at time of peak viral load. These results suggest that specific aspects of executive functioning may vary among youth with PHIV as a function at least in part of timing of most severe HIV disease.

The integrity of learning and memory functioning is critical for educational and functional competence and has been examined in a recent US-based longitudinal investigation of adolescent HEU youth and youth with PHIV, the majority with undetectable viral load at study entry [55]. Results of baseline evaluations revealed memory and learning abilities in the low-average to average range, with greatest difficulty in immediate recall on learning trials. Youth with history of AIDS diagnoses generally performed less adequately than others yet, when considering socioeconomic factors, significant differences between these youth and HEU youth were identified only in visual recognition memory. Thus risk for inefficient encoding and forgetting of visual designs may be a later outcome of early severe HIV disease. In contrast, youth who were older at the time of peak viral load achieved lower mean scores in design memory, design recognition, and verbal delayed memory. It is possible that CNS vulnerability may differ at older ages or that there is reduced plasticity at specific ages, resulting in variable difficulties in learning and aspects of neuropsychological functioning, dependent in part upon timing of HIV-associated complications.

11.3.1 Summary and Implications

Results of these and other investigations suggest that neuropsychological competency and deficits in selective neuropsychological domains differ among youth with variable disease severity and histories of viral suppression yet multiple additional factors likely influence outcomes. Brain development occurs over a prolonged period, and different tissue types, structures, and neural circuits have unique developmental trajectories with dynamic change throughout life and with both genetic and environmental influences [56]. Vulnerability and/or damage at different ages are likely to have unique effects. Longitudinal studies of large cohorts of children affected by HIV remain crucial to understand neuropsychological trajectories across

developmental stages and among those with variable HIV disease severity, timing of ART initiation, and timing and duration of viral suppression. Consideration of the unique impact of cultural, socioeconomic, educational, and family/societal factors is also crucial to advance knowledge and improve health care as well as prevention and intervention efforts.

11.4 Neuroimaging

To date, neurodevelopmental and neuropsychological evaluations have been the primary methods of characterizing HIV-associated neurocognitive deficits in children with PHIV. However, such evaluations have their limitations: (a) a comprehensive testing battery is time and labor intensive and sometimes available only in research settings; (b) assessment tools are often culturally specific and inappropriate for those with limited English-language exposure; and (c) neurocognitive testing does not necessarily provide information about the potential etiology of detected neurocognitive deficits. Cerebrospinal fluid (CSF) and neuroimaging are two additional modalities for characterization of brain phenomena. In the absence of clinical symptoms that trigger evaluation of CSF, it is rarely feasible to perform lumbar punctures in children and adolescents; thus, evaluation of CSF is rare in childhood and adolescence. There is significant literature on neuroimaging findings in adults with HIV-associated neurocognitive deficits; data in children and adolescents remain relatively limited, despite potential utility. Magnetic resonance spectroscopy (MRS), volumetric MRI, diffusion tensor imaging (DTI), and functional MRI (fMRI) are noninvasive and can potentially provide insight into the pathogenesis of HIV-associated neurocognitive deficits. They may also be of utility in surveillance of disease progression and efficacy of ART regimens.

MRS is the magnetic resonance modality most widely studied in HIV-infected adults and to a lesser extent in children with PHIV. The majority of studies have evaluated N-acetylaspartate (NAA), a marker of neuronal integrity; choline (Cho), a marker of cellular proliferation and inflammatory response; and myoinositol (MI), a marker of gliosis [57]. In general, these studies have found reductions in NAA and increases in Cho and MI in HIV-infected adults compared to controls, suggesting that HIV infection is associated with increased neuronal injury and inflammation (Table 11.3). Additionally, increases in Cho and MI and concomitant reductions in NAA appear correlated with the severity of neurocognitive impairment. To date, five pediatric studies of MRS have shown conflicting results and variable associations with cognitive functioning (Table 11.4) [58–63].

Diffusion tensor imaging (DTI) has become a more favored method for studying white matter integrity, and there are an increasing number of studies of adults with HIV using this neuroimaging modality. In general, studies of adults with HIV have shown increases in mean diffusivity (MD) and decreases in fractional anisotropy (FA) within white matter tracts, although subtle inter-study differences exist in the location of these changes [57]. White matter (WM) structure alterations have been

Table 11.3 Summary of neuroimaging findings in HIV+ adults and children

Marker	Description	Associated with
MRS		
NAA:Cr	Marker of neuronal injury	↓ in HIV+ adults compared to controls [90, 91]; ↓ in HIV+ adults on PI monotherapy vs. triple ART [92]; ↓ in HIV+ adults on NRTIs vs. no NRTIs [93]; ↓ in impaired HIV+ adults [91, 94, 95]
Cho:Cr	Marker of cellular proliferation and inflammatory response	↑ in HIV+ adults compared to controls [96]; ↑ in impaired HIV+ adults [95]; ↑ in HIV+ children compared to controls [61]
MI:Cr	Marker of gliosis	↑ in HIV+ adults compared to controls [90, 91, 95]; ↑ in HIV+ adults on PI monotherapy vs. triple ART [92]; ↑ in impaired HIV+ adults [94]; ↑ in HIV+ children compared to controls [58]
Lac:Cr	Marker of increased anaerobic glycolytic demands due to inflammation	↑ in impaired HIV+ adults [97]
Glu:Cr	Marker of neurotoxicity due to excess NMDA activation	↓ in impaired HIV+ adults [94]; ↓ in HIV+ adults compared to controls [98]; ↓ in adults with greater number of NRTIs and poorer cognitive performance [98]
DTI		
FA		↓ in HIV+ children compared to controls [65, 70, 71]
MD		↑ in HIV+ children compared to controls [65, 70, 71]

MRS magnetic resonance spectroscopy, *DTI* diffusion tensor imaging, *NAA* N-acetylaspartate, *Cho* choline, *MI* myoinositol, *Lac* lactate, *Glu* glutamate, *NMDA* N-methyl-D-aspartate, *FA* fractional anisotropy, *MD* mean diffusivity

associated with periods of immune deficiency, as reflected by CD4+ counts below 500 cells/μl among men with HIV, although these alterations were not associated with HIV-related cognitive deficits [64].

MRI studies of children and adolescents with PHIV are in varying stages of development, albeit often with relatively small sample sizes [65, 66]. Recent neuroimaging studies of large groups of normally developing children and adolescents [67, 68] as well as smaller studies of HEU youth permit comparison of neuroanatomical mechanisms of children with PHIV who have varying histories of HIV disease severity, ART exposure, and histories of viral suppression [69].

In a study comparing children and adolescents with and without PHIV, matched on age, sex, ethnicity, and socioeconomic status, MD, radial diffusivity (RD), and axial diffusivity were higher, and FA, cortical and total gray matter (GM) volume, and WM volume were lower among youth with PHIV infection, suggesting diminished integrity of WM. Peak viral load was associated with lower FA [70]. Furthermore, WM hyperintensities, which may represent myelin loss, vasculopathy, and/or gliosis, were prevalent in the well-controlled PHIV group (94% of those

Table 11.4 Summary of pediatric MRS studies

Study	Findings
Nagarajan et al. (2012) [62]	HIV+ had higher Scy/Cr and Scy/Cho in the right FL, higher Glu/Cr and higher Glu/Cho than controls
Prado et al. (2011) [61]	NAA/Cr not statistically different between HIV+ and controls HIV+ had higher Cho/Cr in FGM, FWM, left PWM, and overall than controls
Banakar et al. (2008) [58]	HIV+ had higher MI/Cr, MI/Cho, GABA/Cho than controls
Keller et al. (2004) [60]	HIV+ had decreased left FWM [Cho] compared to controls. Higher VL associated with decreased right BG [Cho] and higher midfrontal GM [Cho]. High NAA/Cho associated with spatial memory
Gabis et al. (2006) [63]	HIV+ only. Low NAA/Cho correlated with poor neurocognitive performance

Scy scyllo-inositol, *Glu* glutamate, *Cr* creatinine, *Cho* choline, *NAA* N-acetylaspartate, *FGM* frontal gray matter, *FWM* frontal white matter, *PWM* parietal white matter, *MI* myoinositol, *GABA* gamma-Aminobutyric acid, *VL* viral load, *BG* basal ganglia, *GM* gray matter

receiving ART had viral suppression) but were also observed in some youth in the comparison group. Among all participants, higher GM and WM volumes were associated with higher IQ and better processing speeds, and higher MD was associated with lower IQ. Although only the association between WM volume and IQ remained significant when restricting to the PHIV group, the coefficients for the other associations were similar. No associations between HIV subtypes and MRI outcomes were identified in this investigation, although HIV subtypes varied among participants as did other factors that could influence brain development, including the presence of malnutrition among immigrants from sub-Saharan Africa, and the possibility of other early risk factors among youth with PHIV living in foster or adoptive homes.

A study of adolescents with PHIV and typically developing youth also detected WM microstructure alterations among youth with PHIV, including reduced FA of the right inferior fronto-occipital and left uncinate tracts and elevated MD of the F minor [71]. Among PHIV youth, the majority of whom were receiving ART, peak viral load was associated with lower FA and higher MD. Furthermore, associations with working memory functioning of these youth were partially mediated by reductions in FA. Similar alterations have been observed in adults with HIV [72] and suggest impact of HIV on axonal organization and/or myelination. Among a subgroup of this cohort of youth with PHIV, resting state functional connectivity MRI (fMRI) was used to examine activity within and between brain networks during rest from specific tasks, as well as relationships between markers of HIV severity and cognitive functioning [73]. Both increases and decreases in BOLD signal correlations for within and between network default mode network (DMN) connectivity were identified. These alterations were related to peak HIV viral load, suggesting that HIV may be associated with reorganization of the DMN and its connectivity with other networks. Moreover, patterns of connectivity with the posterior cingulated cortex and medial prefrontal cortex, which varied as a function of peak HIV

viral load, were predictive of processing speed efficiency among these youth, the majority of whom were receiving ART at the time of imaging.

Longitudinal and task-based neuroimaging studies with larger cohorts of children and adolescents with PHIV at various stages of HIV disease progression and with known duration of ART and viral suppression as well as longitudinal studies of HEU and HIV-unaffected children and adolescents are clearly needed to confirm and extend these findings, particularly as youth age into adulthood. Such studies may reveal whether successful viral suppression and its timing are associated with normal brain development, stabilization, or improvement of indices of white matter injury, connectivity, and volume and may also identify their impact upon cognitive trajectories and functional outcomes. Ongoing surveillance of the neurobiological effects of early severe HIV CNS disease and intermittent or consistent periods of immune activation may guide decision-making regarding ART or other appropriate prevention/ intervention efforts.

11.5 ART-Associated Neurotoxicity

There is recent interest in tailoring ART regimens to include antiretrovirals with maximum CNS penetration in an attempt to optimize CNS viral control. However, this effort needs to be balanced against the increased potential for neurotoxicity associated with high CNS penetrating ART regimens [74, 75]. ARV-associated neurotoxicity is of particular concern in the pediatric population where there is rapid brain development and the potential for lifelong exposure to ARVs. NRTIs are known to be associated with mitochondrial toxicity to varying degrees and can result in lactic acidemia ranging from mild to life-threatening [76]. Efavirenz, a non-nucleoside reverse transcriptase inhibitor, is associated with multiple neuropsychiatric side effects, including dizziness, headaches, confusion, hallucinations, depersonalization, insomnia, and abnormal dreams [77]. Efavirenz metabolism is mediated by the hepatic cytochrome P450 CYP2B6, and some patients may harbor the CYP2B6-G516T polymorphism that is associated with increased plasma drug levels, which could in turn result in worse neuropsychiatric side effects [78]. While these side effects are typically short term, their potential to influence cognitive outcomes remains unclear.

11.6 Viral Escape

In adults, early initiation of ART after diagnosis results in maintenance of normal levels of brain metabolites with the exception of Cho/Cr, which is normalized after 6 months of ART [79]. This suggests that early ART mitigates neuronal injury by not only suppressing viral replication but also by reducing neuroinflammation. Unfortunately, the neuroprotective benefits of early ART are not necessarily

sustained over time, suggesting that there is either ongoing viral replication in the CNS despite peripherally suppressive ART, ongoing neuroinflammation, ARV-associated neurotoxicity, or some combination of the above. Indeed, HIV is often detected in the CSF in adult patients with undetectable HIV levels in the plasma who present with new or worsening neurologic symptoms [80–82]. Recent research reveals that genetic variants of HIV not found in blood sometimes evolve in the brain and become independent compartmentalized viral reservoirs, with potential to replicate in the CNS despite concurrent viral suppression in the periphery [83, 84]. This phenomenon, labeled "viral escape," is not well described in children with HIV due to the difficulties in performing lumbar punctures in children. However, the fact that cognitive deficits of varying severity across variable domains of function are reported in children and adolescents on ART, with undetectable plasma HIV viral loads, suggests that there is likely ongoing viral replication in the CNS despite effective systemic ART which may be associated with deficits observed upon neurocognitive evaluation [85].

11.7 Conclusions and Implications

In the era of ART, children and adolescents with PHIV remain at risk for subtle to severe neurocognitive deficits. Early ART initiation in infancy can mitigate global or selective deficits for some children, likely due to early viral control, ongoing viral suppression and potentially also due to reduction of the CNS HIV reservoir size. In contrast, children with PHIV who initiate ART later in childhood are less likely to demonstrate normal neurocognition across domains after ART initiation, not only due to damage associated with prior immunosuppression but also due to intermittent periods of HIV replication and neuroinflammation during childhood and adolescence. These findings support the need for early HIV diagnosis and early ART initiation during infancy. Prospective, cross-disciplinary longitudinal studies of appropriate samples of children and adolescents across the HIV illness spectrum are needed to determine the long-term global and domain-specific neurocognitive outcomes among children with early and deferred ART initiation as well as the functional impact of selective deficits and potential resilience. At the same time, the presence and effects of early and severe HIV disease, ART neurotoxicity, and neuroinflammation on brain structure and function in virally suppressed and unsuppressed youth should be more fully monitored, understood, and ameliorated if feasible, given the increasing availability of MRI brain imaging technologies and normative data on brain development in children and adolescents [45, 68]. Evaluation of markers of inflammation and neurodegeneration could also increase our understanding of HIV and ART effects.

In all investigations of youth with PHIV, variability conferred by differences in individual, family, and societal resources should be acknowledged. For example, risks associated with aging, substance use, psychiatric disorders, nonadherence, poverty, and stress may modify the spectrum of brain and cognitive alterations or

vulnerabilities experienced by those with longer lifespans in the presence of ART. Therefore, it is crucial to systematically evaluate the neurodevelopment and neurocognitive functioning of children with PHIV from infancy and during all developmental stages, with culturally appropriate neurodevelopmental and neuropsychological measures, including dynamic testing measures [86, 87], so that reliable estimates of change can be identified and strengths, delays, deficits, and risks can be understood and addressed in an individualized, socially sensitive manner. Throughout their lifespans, collaboration with children, parents, caregivers, and families is essential to ensure that children and adolescents with PHIV have full access to appropriate ART as well as to evidence-informed psychosocial, educational, rehabilitative, and structural support to optimize their health, learning, and functional outcomes as they age with chronic HIV disease.

Conflict of interest The authors report no conflicts of interest.

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Chapter 12
Psychiatric Comorbidities in HIV Infection

April D. Thames and Jacob D. Jones

Keywords Psychiatric comorbidities • Neuropsychiatric features • HIV-associated neurocognitive disorder • Mood disorders • Severe mental illness • Substance abuse

Core Message

1. Psychiatric comorbidities place individuals at risk for HIV infection and transmission.
2. Clinicians need to distinguish between pre-existing psychiatric disorders and CNS manifestations of HIV infection.
3. Treatment of comorbid psychiatric disorders is important for HIV-related outcomes.
4. Non-pharmacological interventions have shown promise for treatment of psychiatric disorders in HIV-infected persons

12.1 Introduction: HIV Psychiatry

The shift in public knowledge, concern, and attention to the HIV epidemic has resulted in a number of efforts toward prevention that include early screening, public distributions of condoms, and community needle exchange programs [1] https://www.cdc.gov/hiv/pdf/policies_nhas.pdf. For those recently diagnosed, there are a growing number of "linkages to, retention in, and re-engagement in HIV care" (LRC) programs [2]. For LRCs, entering and staying in care are pivotal in the care continuum, which begins with the diagnosis of HIV infection, entry into, and

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retention in HIV medical care, access, and adherence to antiretroviral therapy (ART) and viral load suppression.

Psychiatric comorbidities pose considerable challenges to the success of these intervention efforts. For example, risk reduction interventions assume that the individual has the capacity to make informed decisions about his or her behavior (e.g., engaging in risky sex). Interventions that use contingency management principles (i.e., the use of proximal awards for positive behavior change) assume that the patient has intact cognitive/neural reward systems. Among individuals with psychiatric illness, cognitive resources may be grossly limited and even further compromised in the context of HIV infection (see [3–7]).

In primary care settings, psychiatric disorders are often under-detected, misdiagnosed, undertreated, or treated improperly. Psychiatric disorders are more common in the HIV-seropositive population (~63%) as compared with the HIV-seronegative population (~30.5%) [8, 9]; therefore, the need for psychiatric presence in HIV care settings has become increasingly recognized [10]. Psychiatric comorbidities, particularly depression and substance abuse, are one of the most consistent barriers cited for nonadherence to antiretroviral therapy [11–17]. Findings also support that depression and recreational drug use contribute to continuous engagement in high-risk sexual behavior [18–20].

Early in the epidemic, a substantial majority of HIV-infected persons presenting for intake to an HIV primary care clinic suffered from depression, substance dependence, or another current psychiatric disorder; however, clinicians failed to identify most cases [21]. Similarly, population-based studies have found that individuals meeting the criteria for major depression are commonly undiagnosed and untreated [22, 23]. A more recent study examined attrition across four stages of care: (1) clinical recognition of depression, (2) treatment of depression, (3) adequacy of treatment, and (4) treatment response [24]. They estimated that among individuals with HIV, only 45% of individuals meeting the criteria for depression were clinically recognized; only 40% of recognized cases received treatment, only 40% of patients treated are treated according to standard guidelines, and only 70% of patients receiving adequate treatment achieve remission. This data suggested that among HIV individuals with depression, only 18% receive treatment, and only 5% achieve remission, which indicates that identifying and treating depression remain a problem. This is particularly concerning due to the fact that a majority (72%) of depressed HIV patients should achieve remission if properly identified and treated [25]. Toward this end, screening tools for depression (e.g., PHQ-9) have been evaluated for feasibility and show considerable promise for use within busy outpatient clinics [26, 27]. Nevertheless, more work is needed to fully understand the manifestations of psychiatric symptomatology and fluctuations throughout the course of the disease.

As such, it is foreseeable that psychiatric screenings and assessments will be a major component of HIV clinical care, if not at the forefront. Screening for mood disorders, anxiety, and psychosis in HIV-infected people is crucial because of the downstream impact of mental health on HIV-related health outcomes. Clinicians will need to be able to recognize psychiatric conditions that place individuals at risk for HIV or for worsening of HIV (if already diagnosed) versus those that occur secondary to infection or treatment. This is no easy task given that psychiatric

symptoms may manifest for a number of reasons including psychosocial stressors, medication side effects, neuroinflammation, and immune system complications (e.g., reduction in CD4 count, rise in viral load, or onset of brain infection – for a review, see [28, 29]). Once a psychiatric diagnosis is made, it is necessary to determine appropriate treatment – whether that is in the form of pharmacotherapy, behavioral or psychotherapy, or combined treatment.

12.1.1 History of HIV Psychiatry

Loewenstein and Sharfstein [30] were among the first to describe the clinical *sequelae* of psychiatric disorders in seven AIDS patients referred for consultation. These cases were identified as having organic neurologic disease, which included central nervous system (CNS) cytomegalovirus (CMV) infection, cryptococcal meningitis, and disseminated lymphoma. Subsequently, Hoffman [31] reported two AIDS patients with organic mental disorder, one with progressive dementia and one with severe delirium. Although the exact etiology was unknown, both patients shared similar clinical presentations [32]. Behaviorally, patients presented with fluctuations in sensorium, behavioral responses, speech, and cognitive processes, which did not correlate well with any objective factors such as normal EEG findings. These findings suggested that organic brain dysfunction should be considered despite normal EEG findings [31]. Following these observations, several studies described cases of clinical syndromes that included affective symptoms [33], delusions [34], and features of dementia [35] that were attributed to infection of the CNS.

12.2 Psychiatric Disorders Before Highly Active Antiretroviral Therapy (i.e., Pre-HAART)

Pre-HAART studies vary in the reported prevalence of comorbid psychiatric disorders, though most studies agree that the rates of psychiatric disorders are higher among individuals diagnosed with HIV/AIDS than in the general population. Differences in the reported rates of psychiatric disturbance may be attributed to the use of convenience samples as well as differences in clinical severity. Notably, some earlier reports used patients who had met the criteria for AIDS, whereas others used patients who were identified as HIV seropositive but had not yet advanced to AIDS. Studies generally reported a higher rate of mood disturbance among patients diagnosed with HIV and/or AIDS [36–38], and rates increased even further when factoring in the history of intravenous drug use [38]. In the same study, a diagnosis of depression continued to independently predicted serostatus after factoring in the history of intravenous drug use. An investigation of 60 patients referred to the

liaison psychiatric service over the course of 1 year found that the most common reason for referral was affective disorder (58%), adjustment reaction (15%), paranoid states (10%), personality changes (6%), and schizophrenia (3%) [39]. In this same study, 23 patients underwent CT scan, and of those, 17 showed brain abnormalities. Walkup and colleagues [40] found that 5.7% of persons listed on the New Jersey HIV-AIDS registry had received a diagnosis of schizophrenia, which is much higher than the national prevalence rate of 1.1%. They also found that 6.8% of persons on the registry had received a diagnosis of a major affective disorder, for a total of 12.5% with a serious mental illness.

In contrast to the aforementioned studies, Bialer and colleagues [41] did not report a higher rate of mood disturbance among AIDS patients in their large-scale investigation of patients who were referred for inpatient psychiatric consultation at Beth Israel Medical Center. Rather, they reported an increased prevalence of dementia among persons with AIDS in comparison with non-HIV patients, whereas substance use disorder and personality disorders were more likely to be found among HIV-seropositive patients in comparison with persons with AIDS. For many of the earlier studies, concerns have been raised that HIV-induced organic mood disorders were misdiagnosed as functional depression [36, 42–45], as data suggest that the two entities generally have different clinical manifestations. Early studies failed to find that depression was related to markers of neurologic compromise such as cognitive impairment [46, 47]. In fact, a common clinical impression was that individuals who seemed the most depressed and anxious would perform well on neurocognitive testing, whereas others who were calm and showed normal affect (possibly reflecting a lack of awareness) would show impairments in cognitive functioning [46]. Thus, features such as apathy, withdrawal, mental slowing, and avoidance of complex tasks associated with early HIV-induced organic mental disorders may be differentiated clinically from low self-esteem, irrational guilt, and other psychological features of depression. Collectively, these studies highlight the relationship between clinical severity and the prevalence of mood disturbance and the importance of identifying an organic etiology.

12.3 Prevalence of Psychiatric Disorders in the HAART Era

The advent of HAART has transformed HIV from a fatal disease to a manageable chronic illness. This was achieved through restoration of the immune system that allowed patients protection against opportunistic infections [48–51]. However, neurological deficits and cognitive disorders were still found to persist or progress in some patients with treatable infections [52]; therefore, the benefits of HAART on HIV-associated neurocognitive disorders were not as clear at the time [53].

The HIV Cost and Services Utilization Study (HCSUS; [54]) examined mental health and substance use in a large, nationally representative sample of adults receiving care for HIV in the USA. Study participants were administered a brief instrument that screened for mental health disorders and drug use during the previous 12 months.

Approximately 50% of the sample screened positive for a mental health disorder, primarily major depression (approximately 1/3 of the sample) and dysthymia (approximately 1/4 of the sample). These rates were similar to those reported in pre-HAART. Nearly 40% reported using an illicit drug other than marijuana, and 12% screened positive for drug dependence. Physicians from the Moore Clinic at Johns Hopkins Hospital [55] reported that more than half of the patients seeking HIV medical care have a major psychiatric disorder other than substance abuse or personality disorders, with approximately 20% with cognitive impairment [56].

Since these reports, there has been a demographic shift in the HIV population. Changing from a disease that once targeted homosexual, white men from middle-class communities, new infections are highest among people of color, in women, and in low-resourced communities. Although the majority of people currently living with HIV in the USA are men who have sex with men (MSM), heterosexual transmission accounted for 27% of all newly diagnosed AIDS cases in 2009 – which is approximately ten times the rate reported in 1985 [57]. In the USA, the rates of HIV among injecting drug users (IDU) has decreased significantly over time. In 2009, the Centers for Disease Control and Prevention estimates that about 9% of the 50,000 of annual new infections in 2011 were among IDUs. Women of color are a subgroup that is particularly affected.

HIV tends to be concentrated in highly vulnerable, marginalized, and stigmatized populations such as ethnic/racial minority populations. Racial/ethnic minorities are particularly vulnerable to aquiring HIV-infection, as this group is more likely to live in poverty, report histories of abuse and incarceration, and have social networks that place them at risk for both HIV and mental health problems. In 2010, African-Americans accounted for 50% of all AIDS cases diagnosed during the year, even though they accounted for only 12% of the population. Among African-American women, the figures are even more alarming – representing up to 72% of all new HIV cases in American women [57]. African-American/Black women accounted for two-thirds (64%) of new AIDS diagnoses among women in 2010; Latinas represented 17% and white women 15%. As such, cultural considerations in the diagnosis and treatment of psychiatric disorders are also critical for HIV care among these demographic subgroups.

12.4 Psychiatric Disorders and Risk for HIV Infection and HIV Transmission

As mentioned throughout this chapter, pre-existing psychiatric disorders are common in HIV infection and must be distinguished from those that occur secondary to infection. A number of psychiatric disorders, including substance use disorders, are associated with increased risk of HIV/AIDS and interfere with treatment [12]. Data from 645 South Africans who participated in a community-based study demonstrated that 33% reported depression, 17% reported alcohol abuse, and 15%

reported post-traumatic stress disorder. After adjusting for demographic characteristics, the presence of any of these three conditions was strongly associated with HIV risks, which included forced sex and transactional sex [58]. When identifying patients who are at risk for contracting HIV, clinicians should consider that patients with substance use disorders, patients with severe mental illness, and victims of sexual abuse/crimes have specific risks for becoming infected with HIV. Psychiatric disorders increase risk behaviors such as sexual contact with multiple partners, injecting drug use, and unsafe sex practices [59]. For example, in the Multicenter AIDS Cohort Study [60], the proportion of gay and bisexual men reporting the use of an illegal drug in the prior 6 months ranged from 92% to 55%, between 1985 and 1989. Among HIV-negative psychiatric patients, nonadherence ranges between 28–52% for major depressive disorder and 20–50% for bipolar disorder. A recent investigation found that loss of a loved one has consistently been associated with various health risks. Little is however known about its relation to STIs. A large population-based study of women from the Swedish Multi-Generation Register found that women who had experienced bereavement were at significantly higher risk for all of the STIs studied [61].

12.5 Comorbid Conditions

Comorbid mental illness among HIV-infected individuals in the USA is substantially higher than in the general population, with rates ranging between 5% and 23%, compared with a range of 0.3%–0.4% among the general population [62]. Mood disorders can result in the most adverse outcomes, as patients with mood disorders are at greater risk for suicide and failure to adhere to medications. Some studies suggest that depressive and anxiety comorbidities accelerate HIV disease progression [63–66], which may be mediated by such factors as changes in medication adherence [67] and lack of health-promoting behaviors [68]. A meta-analytic review of studies conducted in South Africa and other sub-Saharan African countries reported similar findings with respect to higher prevalence rates among those with HIV/AIDS, although the range in prevalence is large across studies (from 5% to 83%), which may result from differences in tools and populations studied [69]. In a study of an HIV-infected sub-Saharan population, [70] there was a substantial prevalence of both major depression (35%) and post-traumatic stress disorder (15%). In another investigation of individuals in South Africa, the prevalence of depression, PTSD, and alcohol dependence/abuse was 14%, 5%, and 7%, respectively [71]. Similar to US findings, studies of HIV+ individuals with late-stage disease found that common mental disorders such as depression tended to increase, while the use of HAART was shown in one study to improve general mental health [72–74] (Table 12.1).

Table 12.1 Clinical diagnoses and symptoms

Clinical disorder (DSM-V code) [75]	Description
Major depressive disorder (296.2×/.3×)	Feeling sad, empty or hopeless most of the day, guilt, fatigue, loss of motivation, sleep disturbance, and cognitive impairment. Apathy, irritability, demoralization, and neurovegetative symptoms may overlap or present as separate/independent features in HIV
Bipolar disorder (296.4×/5×)	Symptoms of a manic episode include elevated or irritable mood, increased activity, increased self-esteem, irritability, decreased need for sleep, and racing thoughts. Certain symptoms may overlap with HIV-related CNS disruption
Post-traumatic stress disorder (309.81)	Exposure to threat or violence, intrusive symptoms, avoidance, and negative thoughts or mood of the event
Substance-related and addictive disorders (305.00–312.31)	General features may include impaired control over substance, social/functional impairment, risky use of substances, tolerance, and withdrawals
Cluster B personality disorders (301.7–301.81)	Antisocial personality disorder, disregard and violation of rights of others; borderline personality disorder, instability in relationships, emotional lability, and impulsivity; histrionic personality disorder, emotional lability and attention-seeking behaviors; narcissistic personality disorder, grandiosity, persistent need for admiration, lack of empathy

12.5.1 Major Depressive Disorder (MDD)

A diagnosis of major depressive disorder is made when five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning: (1) depressed mood most of the day (nearly everyday), (2) markedly diminished interest or pleasure in activities, (3) significant weight loss when not dieting or weight gain or decrease or increase in appetite nearly everyday, (4) insomnia or hypersomnia, (5) psychomotor agitation or retardation, (6) fatigue or loss of energy nearly everyday, (7) feelings of worthlessness or excessive or inappropriate guilt, (8) diminished ability to think or concentrate or indecisiveness, and (9) recurrent thoughts of death, suicidal ideation, or suicide attempt or plan [75].

Studies have found significant relationships between depression and mortality in HIV-infected individuals [76–78], whereas others have not [79, 80]. For example, in the San Francisco Men's Health Study, a 9-year longitudinal study of HIV-seropositive men, higher levels of depression at the beginning of the study were associated with faster progression to AIDS [81]. In another study, involving 414 HIV-infected gay men studied over 5 years, baseline depression was associated with shorter time to death but not change in CD4+ count or progression to AIDS [78]. An analysis of 1809 gay men from the Multicenter AIDS Cohort Study found no relationship between depression measured with the Center for Epidemiological Studies Depression Scale (CESD) at study entry and progression of HIV infection during

8 years of follow-up [80]. Disease progression was defined as time to AIDS, death, or decline in CD4+ T lymphocytes. In later analyses of these data, the authors found that self-reported depressive symptoms appeared to rise during the 1.5 years before an AIDS diagnosis [82]. The authors interpret these findings as an indication that depression may increase toward the later stages of HIV infection and thus may be a manifestation of the disease process. While diagnosing depression uses standard criteria for all populations, certain symptoms may be more prevalent in individuals with HIV thereby obscuring clinical assessment of depression. Specifically, appetite and sleep disturbances are more frequently reported in HIV-seropositive patients as compared to HIV-seronegative patients [83]. Demoralization can be difficult to distinguish from major depression, but there are distinctive features between the two syndromes. Depression is characterized by persistent anhedonia, and demoralization is being often characterized by helplessness and linked to recent events or ongoing life circumstances [84–86]. Patients with major depression respond relatively well to antidepressant medications; those with demoralization tend to respond well to psychotherapy and not to medications [87, 88].

The diagnosis of comorbid MDD in HIV-infected persons may be difficult, because the neurovegetative symptoms of premorbid MDD (such as lack of energy, fatigue, anorexia, and sleep disturbances) may also be caused by the biological effects of HIV [89]. HIV infection stimulates increasing levels of proinflammatory cytokines such as interleukin-6, interleukin-1-beta, tumor necrosis factor-alpha, and interferon-gamma [90–92], which are associated with sickness behavior (fever, hypersomnia, anorexia, decreased motor activity, and loss of interest in the environment). In differentiating between pre-existing MDD and the neurovegetative symptoms of HIV, it may be useful to remove the somatic depression symptoms included in diagnostic instruments such as the Beck Depression Inventory-II (BDI-II) and the CESD. Indeed, studies have found that removing somatic subsets of depression symptoms improved the clinical utility of the BDI and CESD [83, 93].

Chronic stressors associated with HIV infection may exacerbate mood disorders. Studies have documented dysregulation of the HPA axis and blunted adrenocorticotropic hormone (ACTH) responses among HIV-infected individuals [78]; thus, it is plausible to expect that HIV status may increase vulnerability to depression HPA-axis dysregulation. Several studies have reported that HIV-seropositive individuals with a major depressive episode or self-reporting depressed mood on standard instruments may demonstrate reduced performance than non-depressed individuals in some cognitive domains (e.g., memory tasks) or report more cognitive complaints [94–96]. However, none of these studies reported an association with neurocognitive impairment. In contrast, others studies have suggested a possible link between increased depressive complaints and lower cognitive function [93, 97, 98]. A longitudinal study of 227 HIV-positive adult men who did not meet the criteria for a current major depressive episode at baseline reported no neurocognitive performance differences in association with lifetime or incident depression and suggested that neurocognitive impairment and major depression be considered two independent processes [99].

HIV-infected individuals who are at greatest risk for developing depression are those with a history of depression, homosexual men, women, or intravenous drug users (IVDUs [100]). Other risk factors associated with developing major depression in HIV-seropositive persons include social stigmatization, isolation, lack of social support, death of friends as a result of HIV/AIDS, and gender [101, 102]. In a longitudinal study of HIV-infected and HIV-uninfected men, lifetime prevalence of major depression or other psychiatric disorders did not differ between the groups. However, at 2-year follow-up, those with symptomatic HIV disease were significantly more likely to experience a major depressive episode than individuals who were either in the HIV asymptomatic or control group. However, HIV disease progression did not predict the presence of neurocognitive impairment [103].

Differential diagnosis in a patient with a complaint of depression includes (1) major depression and related mood; (2) demoralization and grief states related to the losses and stresses associated with HIV; (3) delirium, a waxing and waning mental state associated with global cerebral dysfunction and possibly disease acceleration; and (4) dementia, including AIDS dementia and other forms of subcortical damage.

12.5.2 Apathy and Irritability

Apathy (a loss of goal-directed behavior and motivation) and irritability are common neuropsychiatric features of HIV [104, 105]. Approximately 26% of HIV-seropositive individuals meet the criteria for clinically significant apathy, and multiple studies have shown both apathy and irritability to be more common/severe relative to HIV-seronegative controls [104–106]. The clinical importance of apathy has been debated [107, 108]. Nevertheless, within the HIV population, apathy is related to some meaningful outcomes, such as medication adherence [14], but only minimally related to other meaningful outcomes, such as quality of life [106]. We are unaware of any studies or clinical trials examining treatments of apathy in HIV. In general, treatment of apathy in other populations (such as Parkinson's disease or Alzheimer's disease) has not been promising, and findings have been inconsistent regarding the superiority of medications over placebo (for review, see Drijgers [109]). Future investigations of treatments for apathy may be particularly important as pathophysiology of apathy may dramatically differ from other common mood disorders, such as depression. In fact, Selective Serotonin Reuptake Inhibitors (SSRIs), a common treatment for depression, may be related to worsening of apathy [110]. Investigations into non-pharmacological treatments for apathy, such as behavioral activations, are also warranted.

Apathy and irritability are of particular interest in HIV because they are thought to reflect disruption of the central nervous system (CNS). In contrast other psychiatric symptoms (such as depression and anxiety) are less related to a neurologic insult and may reflect adjustment to social, medical, or financial stressors [104]. Although symptoms of apathy and depression may overlap, apathy has been

shown to be a unique construct, separate from depression [106, 111]. The high occurrence of apathy and irritability is thought to be related to disruption of frontal-subcortical networks important for initiation and monitoring of behavior [112]. Indeed, neuropsychological and neuroimaging studies have found support for apathy and irritability reflecting CNS disruption [113]. Frontal-subcortical networks are known to be an important mechanism for executive functioning. Among HIV-infected individuals, apathy and irritability, but not depression or anxiety, are associated with impairments in executive functioning [104, 105]. Studies using diffusion tensor imaging have shown that white matter tracts subserving the medial prefrontal cortex (an area important for motivation and goal-directed behavior) are less intact in HIV-infected individuals relative to HIV-uninfected controls [114–117]. White matter integrity in these regions was related to the severity of apathy in a study of HIV-infected individuals [114]. A similar study found the relationship between apathy and white matter integrity to be independent of depression and to be stronger among individuals with more severe HIV (lower CD4 counts). Such findings support that apathy may be a syndrome that arises from HIV-associated frontal-subcortical disruption.

12.5.3 Mania

HIV-infected patients with secondary mania, termed "HIV mania," may present as agitated, disruptive, sleepless, having high levels of energy, and being excessively talkative [89]. They have a high rate of psychotic symptoms such as auditory or visual hallucinations and paranoia. HIV mania is reported to be associated with irritability rather than euphoria. Unlike primary mania, cognitive deficits are usually present. Mania occurring in the early stages of HIV infection may represent bipolar disorder in its manic phase, whereas mania in persons with AIDS is secondary mania linked to the pathophysiology of HIV brain infection [118].

A first episode of HIV mania typically occurs in the context of a CNS disorder, such as CNS opportunistic infection (OI). The mechanisms are poorly understood; however, the HIV nef protein is reported to alter CNS dopamine metabolism leading to hyperactive, manic-like behaviors in animal models [119]. Reports of HIV mania have decreased coincidently with the widespread use of ART [120], but it remains a problem among untreated and undertreated persons [121]. The differential diagnosis of brain disorders underlying suspected HIV mania includes substance use (especially stimulants), alcohol withdrawal, metabolic abnormalities (e.g., hyperthyroidism), and CNS OI. Evaluations should include a neurologic and mental status examination, brain MRI scan with and without contrast, serology for syphilis, urine toxicology, and cerebrospinal fluid (CSF) examination (if medically safe), including tests for OI and a quantitative HIV CSF polymerase chain reaction (PCR) (viral load in CSF) [89]. HIV+ patients with comorbid bipolar disorder are less likely to be adherent to antiretroviral and psychiatric medications [122]. Therefore,

promoting adherence in this subgroup is critical in protecting against poor HIV outcomes.

12.5.4 Anxiety Disorders

Anxiety disorders include disorders that elicit fear, anxiety, and behavioral disturbances. Anxiety disorders include mild adjustment disorders, panic disorder, phobias, obsessive-compulsive disorder, post-traumatic stress disorder, acute stress disorder, and generalized anxiety disorder. The prevalence of anxiety disorders in the HIV populations ranges from about 25% to 40%. Manifestations of anxiety disorders, such as adjustment disorder, are more prevalent at diagnosis and during new treatment or acute illness [123]. It is important to recognize and treat anxiety disorders in the HIV-positive population. Increased rates of anxiety disorders are associated with treatment dropout, high-risk behaviors, and suicide. While the rates of anxiety disorders in the HIV population are generally similar to those of the general population, one of the more common anxiety disorders that disproportionately affect HIV patients is post-traumatic stress disorder. An assessment of an HIV-infected patient presenting with symptoms of anxiety disorder should first evaluate a patient's recent medication and substance use history (especially stimulants like cocaine and methamphetamine), previous psychiatric history, and sleep patterns. Because most anxiety disorders appear in adolescence or young adulthood, patients will often have a history of symptoms predating HIV [124].

12.5.5 Post-traumatic Stress Disorder

A diagnosis of post-traumatic stress disorder is made when the following criteria are met: (1) exposure to actual or threatened death, serious injury, or sexual violence; (2) presence of one (or more) intrusion symptoms associated with the event, (3) persistent avoidance of stimuli associated with the traumatic event(s), beginning after its occurrence; and (4) negative alterations in cognitions and mood associated with the traumatic event(s) [75]. Rates of PTSD have been found to range anywhere from 22% to 64% and tend to be higher among marginalized and stigmatized populations, such as ethnic/racial minorities, homosexuals, and low-income. Symptoms associated with PTSD include intrusive memories, avoidance of trauma reminders, numbness, hyper-arousal, and experiencing distorted or negative thoughts about oneself [75]. PTSD comorbidity among HIV+ individuals is prevalent among individuals with histories of early trauma and stress [125–127] and has been hypothesized to be linked to HIV-related neurobiological alterations which may render the system more vulnerable to stress (for review, see Neigh [128]). PTSD predicts worse HIV-related outcomes for both women [129, 130] and men [131]. While the research is correlational in nature, studies have suggested that individuals with

PTSD have a more negative course and progression of HIV/AIDS, continue to use drugs, and have altered immune levels. It has been found in some studies (although not all) that HIV-positive individuals who have been exposed to a traumatic event show a more rapid decrease in CD4+/CD8+ cell ratios as compared to HIV-positive individuals without a trauma history. A recent study of virologically suppressed HIV-infected patients found that those with PTSD had significantly higher total white blood cell counts, absolute neutrophil count, CD8%, and memory CD8%, lower naïve CD8%, and higher rate of high-sensitivity C-reactive protein than participants without PTSD [132]. In one study of HIV risk behaviors, PTSD was associated with increased HIV risk behavior, whereas depression was associated with increased condom use [58]. Elevated stress and emotional reactivity have been linked to increased sexual transmission risk behaviors [133, 134].

12.5.6 Substance Use Disorders

Substance use disorders are characterized by a cluster of cognitive, behavioral, and physiological symptoms indicating that the individual continues use despite significant consequences related to use. Drug use, especially the injection of drugs, has been associated with poor HIV-related outcomes. HIV-infected drug users have increased prevalence and frequency of medical, psychiatric, and substance use disorders that increase morbidity and mortality compared with age-matched HIV-infected non-users [135]. The number and range of these comorbid disorders complicate diagnosis and treatment, resulting in several challenges in the provision of comprehensive care. Injection drug use has been found to be highly prevalent among individuals with a psychiatric disorder, particularly mood disorders [136]. Studies of heroin users have documented elevated rates of major affective and anxiety disorders including PTSD [137, 138]. While injection drug use is a more direct route of HIV transmission, other substances such as alcohol can place individuals at risk for infection. For example, rates of injection drug use are high among alcoholics in treatment [139, 140].

Alcohol use disorders are common in people living with HIV/AIDS and in IDUs. Heavy alcohol use increases risk of HIV transmission to others [141], decreases retention in care [142], decreases treatment adherence [143, 144], increases HIV risk behaviors [145–147], and decreases likelihood of suppression of HIV [148, 149]. Earlier studies reported that individuals with a history of heavy alcohol use are more likely to report engaging in high-risk sexual behaviors, including multiple sex partners, unprotected intercourse, sex with high-risk partners (e.g., injection drug users, prostitutes), and the exchange of sex for money or drugs [139, 140]. According to McKirnan and Peterson [150], alcohol use may be used as an excuse for engaging in socially unacceptable behavior or to reduce conscious awareness of risk. This practice may be especially common among men who have sex with men. Treatment of alcohol abuse has the potential to positively affect HIV treatment outcomes and reduce HIV transmission.

Across studies conducted in South African and other sub-Saharan African countries, it was found that problem drinkers were more likely to be men, to engage in more risky sexual practices, and have a more frequent history of sexually transmitted infections (STIs) [151, 152]. Kalichman et al. [153] found that alcohol use in the context of a sexual encounter partially mediated the relationship between sensation seeking and HIV risk. In a study that compared a skills building and HIV risk reduction counseling session with didactic HIV-educational/control intervention, effects of increased condom use and decreased drinking prior to sex was found at 3-month but not 6-month follow-up, suggesting that the positive effects of behavioral risk reduction among problematic users may be time limited [154].

Contrary to popular belief, crack cocaine was not always considered a risk factor for HIV acquisition and transmission. In 1988, researchers in New York City suggested the adoption of crack smoking, in lieu of intravenous cocaine use, as a mechanism of AIDS risk reduction [155, 156]. However, a series of studies followed which indicated that when compared with intravenous drug users, crack smokers may be at equal or greater risk for HIV and other STD infections [157–160]. Methamphetamine (METH) use has been found to be associated with nonadherence [122, 161–163]. In a study of HIV+ individuals with and without a lifetime history of METH use and comorbid antisocial personality disorder (ASPD), major depressive disorder (MDD), and attention deficit disorder (ADD), results indicated that co-occurring ADD, ASPD, and MDD predicted ART nonadherence. Current METH use regardless of comorbidity was significantly associated with lower adherence [122].

Although the use of marijuana is more common among individuals with HIV relative to the general population, very few studies have examined the relationship between marijuana and HIV-related outcomes or risk. Specifically, 23–56% of individuals with HIV reported using marijuana in the last month of the survey period compared to 8.4% of the general population [164, 165] (Substance Abuse and Mental Health Services Administration, 2011). Individuals with HIV report they use marijuana to reduce anxiety/depression, to increase appetite and weight gain, and to treat pain [164]. In contrast, marijuana has been associated with negative outcomes such as cognitive impairment and lower adherence to ART, relative to non-marijuana-using HIV patients [166, 167]. Similarly, marijuana use has been found to be associated with unprotected sexual intercourse [168, 169] as well as STD infection [170]. However, these relationships tend to be moderated by such factors as HIV severity and amount of marijuana use. For example, Cristiani and colleagues [166] found that marijuana use was associated with cognitive impairment among symptomatic HIV participants but to a lesser extent among asymptomatic HIV participants. Regarding ART adherence, lower adherence, higher viral load, and more severe self-report symptoms of HIV/medication side effects were reported among individuals with HIV who met the criteria for cannabis dependence, but not casual/nondependent marijuana users. In addition, marijuana is also related to risk of contracting HIV, particularly for young adolescents [168].

12.5.7 Severe Mental Illness (SMI)

In a meta-analytic study of 52 studies, the majority of adults with SMI were sexually active, and many engaged in risk behaviors associated with HIV transmission (e.g., unprotected intercourse, multiple partners, injection drug use) [19]. HIV risk behaviors were correlated with factors from the following domains: psychiatric illness, substance use, childhood abuse, cognitive-behavioral factors, and social relationships. HIV prevention efforts targeting adults with SMI must occur on multiple levels (e.g., individual, group, community, structural/policy), address several domains of influence (e.g., psychiatric illness, trauma history, social relationships), and be integrated into existing services (e.g., psychotherapy, substance abuse treatment, housing programs) [19, 20]. While epidemiological evidence suggests that individuals with severe mental illness are more likely to live in risky environments that make them vulnerable to HIV [171, 172], the findings are mixed with regard to the link between HIV risk and psychotic-spectrum disorders, with many reporting small associations [173]. In a study by Carey et al. [173] that reviewed records of 889 patients with mental illness, it was found that only 11% reported HIV risk behavior. They found no direct association between psychiatric disorder and risky sex. In a follow-up study, 1558 records were reviewed and the numbers increased to approximately 23% [171]; however, risk behavior was less common among patients diagnosed with a schizophrenia-spectrum disorder. Similarly, in a study of 228 female and 202 male outpatients (66% mood disorder, 34% schizophrenia), it was found that risk behavior was more frequent among patients diagnosed with a mood disorder (compared to those diagnosed with schizophrenia) and/or with a substance use disorder (compared to those without a comorbid disorder).

12.5.8 Personality Disorders

Personality disorders (PD) are diagnosed if there is a pattern of inner experience and behavior that deviates markedly from the expectations of the individual's culture. The pattern is manifested in two (or more) of the following areas: (1) cognition, (2) affectivity, (3) interpersonal functioning, and (4) impulse control. The pattern must be inflexible and pervasive across many personal and social situations as well as lead to significant distress or impairment in social, occupational, or other areas of functioning [174]. Personality disorders that fall under the cluster B category of personality disorders (i.e., antisocial personality disorder (ASPD), borderline personality disorder (BPD), histrionic personality disorder, and narcissistic personality disorder) have been the most reliably associated with risk-related behaviors and outcomes in HIV. The literature supports a higher rate of PD in HIV-positive individuals, in particular BPD and ASPD. ASPD is characterized by a pattern of irresponsible, impulsive, and remorseless behaviors beginning in childhood or early adolescence and continuing into adulthood. ASPD is highly prevalent among drug

abusers [175–177]. ASPD participants reported higher rates of IVDU, frequency of needle sharing, and a number of equipment-sharing partners and lower rates of needle cleaning [178–181].

In a randomized HIV prevention study, Compton and colleagues [182] compared the effectiveness of standard HIV testing and counseling protocol to a four-session, peer-delivered, educational intervention for out-of-treatment cocaine users with and without ASPD and major depression. While all groups, regardless of assignment to standard vs. peer-delivered intervention or psychiatric status, improved significantly in outcomes of crack cocaine use, injection drug use, and number of IDU sex partners and overall number of sex partners, ASPD was associated with significantly less improvement in crack cocaine use and less improvement in having multiple sex partners and having IDU sex partners. Personality disorders are the most unreliably diagnosed out of all the psychiatric disorders, and it becomes more challenging when diagnosing BPD in the context of culture. Symptoms related to unstable self-image, impulsive substance abuse, and mood instability due to societal stigma and pressures may be the norm within certain cultures. For example, adolescents and young adults with identity problems may display transient behaviors that appear like BPD. Hence, clinicians should be cognizant to conduct thorough assessments when considering a PD diagnosis.

12.6 Psychiatric Assessment and Treatment

Central to conducting a psychiatric assessment of the HIV/AIDS patient is an adequate fund of knowledge about the pathophysiology and virology of HIV, epidemiology of HIV/AIDS, transmission of HIV, pathogenesis, staging of HIV disease, HIV treatment, and HIV effects on the central nervous system. The clinician should be prepared and well versed in a wide range of assessment tools and skills such as structured interviews, comprehensive diagnostic evaluations, and medical work-ups to rule out or consider new-onset symptoms [89]. Clinicians should also be aware of cultural and linguistic differences that may influence self-reporting of mental health problems, stigmas associated with both HIV and mental health problems, and social support networks. According to the guidelines set forth by the American Psychiatric Association Working Group on HIV/AIDS [183], (shown in Table 12.2), the clinician must be able to:

The development of a psychiatric treatment plan for patients with HIV infection requires a holistic approach accounting for the biopsychosocial context. Upon first diagnosis and throughout the course of the illness, there may be signs of bereavement and grief with various physical changes as well as the anticipatory loss of life [88]. The symptoms of bereavement (i.e., sadness, insomnia, poor appetite, and weight loss) overlap considerably with depression. Diagnostic decisions must follow a standard diagnostic practice, without minimizing or misattributing symptoms to the burden of having HIV. Depressed patients may also overstate their cognitive and functional limitations. A study by our group compared subjective complaints to

Table 12.2 American Psychiatric Association Working Group on HIV/AIDS

Practice guidelines for the treatment of patients with HIV/AIDS	
Establish and maintain a therapeutic alliance	Gather information of a patient's understanding of the illness and coping strategies. Discuss whether the treatment relationship should include family and/or significant others
Collaborate and coordinate care with other mental health and medical providers	Keep up-to-date with the evolving information about HIV by collaborating with infectious disease physicians, primary care, and other disciplines
Diagnose and treat all associated psychiatric disorders	Treatment should include active monitoring of substance abuse
Identify barriers in adhering to the overall treatment plan	Provide psychoeducation about adhering to treatment regimens. If necessary consider outreach efforts with public health services for adherence
Provide education about psychological, psychiatric, and neuropsychiatric disorders	To educate other clinicians and patients about the neuropsychiatric complications of HIV infection and to initiate and encourage treatment of current or emergent psychiatric disorders
Providing risk-reduction strategies to further minimize the spread of HIV	Assess the risk for HIV transmission from their HIV-infected patients to others and to provide risk-reduction counseling. Psychotherapy may help some individuals who are unaware of motivations that promote ongoing risk behavior. Risk assessment should be repeated when there are changes in the patient's clinical status or social situation, such as the onset of binge drug or alcohol use or new sexual relationships
Promote psychological and social/adaptive behavior	The clinician should consider the role of religion/spirituality, preparing the patient for issues related to HIV disability
Identify sources of social support	This could include significant others/family. The clinician should be able to educate family members about HIV disease

actual cognitive and functional performance and found that "over-reporters" – those who presented with complaints regarding cognitive function and performance on daily tasks (e.g., managing medications) but who performed normal on objective measures of function – exhibited higher levels of depressive symptoms compared to those whose self-reports matched their actual performance [184]. On the other hand, clinicians must caution not to quickly diagnosis a mood disorder without consideration of the context. It is common for patients to present with symptoms that mimic a major depressive episode or anxiety disorder shortly after learning about their HIV diagnosis. Usually these symptoms will subside over time as the patient begins to adjust to their illness.

The symptoms of major depression can be masked by symptoms that accompany other HIV-related illnesses. Overlapping symptoms include fatigue, sleep and appetite disturbance, general malaise, and feelings of illness. One study by Pugh and colleagues [185] found no difference in symptoms of fatigue, insomnia, or cognitive dysfunction between early-stage HIV-infected homosexual men and uninfected homosexual controls. When these symptoms do occur in early-stage HIV

infection, they are more likely to result from mood disturbance than HIV disease progression. Additionally, increased fatigue and insomnia at 6-month follow-up were highly correlated with worsening of depression but not CD4 count, change in CD4 count, or disease progression by CDC category.

12.6.1 Treatments

Extensive reviews of various types of pharmacological treatments for psychiatric disorders in HIV patients can be found elsewhere [186–188]. However, we would be remiss not to provide a brief overview of the various treatments for the more common comorbid psychiatric disorders. Treating psychiatric illness among individuals with HIV infection is frequently complicated by concurrent HAART regimens. The clinician must closely monitor drug-drug interaction and be alert to potential side effects and metabolizing profiles.

12.6.1.1 Antidepressants (Standard and Alternative)

As with treating depression among individuals without HIV, antidepressant medications are commonly used for treatment. In the early epidemic, tricyclic antidepressant (TCA) medications were often used [189, 190] with demonstrated efficacy. However, because of the high rates of negative side effects and concerns of lethality attributed to the use of TCAs, clinicians started to use SSRIs for treating depression [188]. SSRIs are now the most widely used antidepressant treatment for major depression among HIV-infected persons because of their more benign side effect profile and evidence of treatment efficacy [191]. In a study of a 6-week open-label trial with SSRIs, subjects who completed 6 weeks of SSRI treatment experienced significant reductions in both affective and somatic symptoms, which were initially attributed to HIV infection rather than depression [192]. A smaller trial showed no benefit of adding fluoxetine to structured group therapy versus group therapy alone; however, this study was limited by a small sample size of 20 individuals [193]. Another study compared fluoxetine and placebo in depressed patients receiving group psychotherapy [194]. Fluoxetine resulted in a significantly greater percentage of patients (64% vs. 23%) with a reduction in HAM-D score after 7 weeks. This study also showed that patients with mild to moderate depression may benefit from psychotherapy alone, but patients with severe depression did better with a combination of psychotherapy and fluoxetine. Overall, fluoxetine appears to have the most evidence for treating depression in HIV patients, with response rates between 50% and 75%. Limitations of many of the studies include exclusion of patients with active substance abuse and few studies with HIV-infected women.

Psychostimulants have also been used to treat depressive symptoms in patients with advanced HIV, especially when symptoms such as depressed mood, fatigue, and cognitive impairment are present. In a clinical trial's study of standard and

alternative antidepressants among HIV patients with depression, each treatment resulted in significant improvement after both 2 and 6 weeks of treatment according to the Hamilton Depression Rating Scale [195].

12.6.1.2 Anxiolytics

SSRIs are the first-line pharmacologic therapy for several anxiety disorders [196] including generalized anxiety disorders, panic disorders, social phobia, obsessive-compulsive disorder, and post-traumatic stress disorder in HIV. Specific agents have been approved by the Food and Drugs Administration for each of the major anxiety disorders: generalized anxiety disorder (paroxetine), social phobia (paroxetine), OCD (fluoxetine, sertraline, paroxetine, and fluvoxamine), and PTSD (sertraline). Buspirone has a low abuse potential. It is minimally sedating and has no known withdrawal effects [197]. Buspirone may, however, take up to 4 weeks before producing noticeable therapeutic effects. When a benzodiazepine is required, it is recommended that a low dose is prescribed and used only for short periods of time to minimize, respectively, the risk of abuse and adverse effects (confusion, sedation, cognitive impairment, disinhibition) and interactions with antiretroviral therapy [198].

12.6.1.3 Mood Stabilizers

Lithium, valproic acid, and carbamazepine are effective mood stabilizers in patients with bipolar affective illness. Lithium is the least likely to have specific drug interactions with antiretrovirals [187]. In HIV-infected patients, lithium has the potential to cause nausea, vomiting, diarrhea, tremor, thyroid dysfunction, and kidney problems at therapeutic doses [199]. el-Mallakh [200] described 14 cases and reported that AIDS-associated mania are responsive to lithium but that AIDS patients with associated neurologic and cognitive dysfunction may be more prone to neurocognitive side effects. Valproic acid can cause elevated transaminase levels and severe hepatitis, and it has been recommended that physicians observe liver enzymes periodically [187]. In a retrospective chart review of 11 HIV+ patients with an acute manic episode, Halman [201] found that neuroleptics and anticonvulsants were an effective alternative among those with poor tolerance of lithium. A number of factors must be taken into consideration when prescribing anticonvulsants to HIV+ individuals. Carbamazepine is metabolized via CYP3A4 and induces its own metabolism, increasing metabolism of protease inhibitors [202] and non-nucleoside reverse transcriptase inhibitors [203]. Such autoinduction and the potential for bone marrow suppression make its use complicated. There is clinical evidence of carbamazepine toxicity resulting from its use in combination with CYP3A4 inhibitors, such as ritonavir [204].

12.6.1.4 Antipsychotics

Antipsychotic drugs (also called neuroleptics) include both older "typical" drugs and the newer "atypical" (second-generation) medications that are FDA approved for the treatment of schizophrenia, bipolar disorder, and other psychotic disorders [204]. The typical neuroleptics (characterized by chlorpromazine and haloperidol) are specific dopamine receptor (D2) antagonists. Newer antipsychotics also interact with other receptor families, such as serotonin [187]. Newer antipsychotics are often preferred because of their efficacy in treating psychotic conditions and the decreased frequency of extrapyramidal adverse effects associated with their use. However, significant metabolic adverse effects (like hyperglycemia, weight gain, and hypercholesterolemia) often make newer neuroleptics less appealing. Clozapine, a very effective neuroleptic, has the potential to cause agranulocytosis, necessitating weekly blood count measurements for the first 6 months. In addition, this medicine can cause significant weight gain, orthostasis, sialorrhea, and seizures [199]. Molindone (20–180 mg/d), an atypical antipsychotic, was first reported to be beneficial for HIV-associated psychosis and agitation with minimal side effects [44]. Clozapine has been demonstrated to be effective and generally safe in treating HIV-associated psychosis (including negative symptoms) in patients with prior drug-induced Parkinsonism [205]. Risperidone (mean dose 3.3 mg/d) was reported to be effective in treating HIV-related psychotic and manic symptoms [206]. CYP inhibitors have the potential to increase the concentration of the antipsychotics, clozaril and pimozide. For this reason, these drugs have been contraindicated with antiretrovirals with CYP inhibition, such as ritonavir. In addition, the potential for toxic increases by CYP inhibitors exists in other antipsychotics, including chlorpromazine, haloperidol, olanzapine, and risperidone [207]. Antipsychotics do not generally significantly inhibit or induce P-450 enzymes and can safely be added to HAART regimens without causing toxicity or HAART failure [208]. Another concern with the administration of antipsychotics and antiretrovirals is the overlapping toxicities of metabolic disturbances, primarily with atypical antipsychotics. Metabolic disturbances are seen in 2–36% of patients treated with atypical antipsychotics [209]. In sum, patients with HIV infection are generally very sensitive to medication side effects as they often metabolize drugs more slowly and have compromised blood-brain barrier functioning [208]. Although most patients ultimately tolerate standard doses of most medications, it is advised to start at low doses and slowly increase over time.

12.6.1.5 Behavioral Interventions

For many HIV patients, psychotherapy and psychosocial interventions have been invaluable in the search for meaning during the course of living with HIV [210]. Psychotherapy can be an important intervention to address conditions that may interfere with a patient's acceptance of HIV illness or their ability to work

cooperatively with their healthcare team as well as addressing changes in role definitions and life trajectory as well as treatment adherence challenges.

Non-pharmacological interventions have been used to treat depression and increasing stress management for individuals with HIV. These interventions have consisted both cognitive (i.e., challenging and restructuring automatic negative thoughts) and behavioral approaches (i.e., progressive muscle relaxation), either in combination or separately. Reviews and meta-analyses found these approaches to be effective in terms of improving psychological symptoms (such as depression or anxiety). Additionally, these approaches were effective in improving psychosocial functioning, such as social support, medication adherence, quality of life, and decreasing engagement in risky sexual behaviors [211, 212]. However, there was only limited evidence that improvements in psychological functioning are generalized to improvements in markers' immunological functioning, such as stress hormones, CD4 counts, or T-cell counts.

Mindfulness-based interventions focus on increasing one's ability to purposely pay attention in the present moment, without judgment [213]. Mindfulness-based interventions have become a popular research topic within the last decade, but the research is still in its infancy, particularly as it relates to the HIV population. However, preliminary findings are optimistic, as the effect sizes have ranged from medium to small in terms of improvement in positive affect [214]. Perhaps even more impressive, mindfulness-based interventions were found to have a medium-to-large effect size in terms of healthier CD4+ cell counts, relative to HIV+ controls [214, 215]. Future studies are needed in examining the efficacy of mindfulness-based interventions in terms of other important outcomes, such as medical adherence, risk behaviors, and specific mood symptoms (rather than global affect).

In addition to therapies differing in terms of theoretical orientation, therapies can differ in the modality of administration. A past review found that 95% of intervention using behavioral techniques to improve stress management were delivered in a group format [216]. Although the use of group therapy is beneficial for delivering services to multiple individuals at once, one criticism was that group therapy may not be available/feasible for individuals living in rural or underserved populations. To address this concern, interventions delivered remotely, either over the phone or via the computer, may be of interest. Indeed two recent randomized controls trials found that a cognitive-behavioral therapy and a contingency management program delivered over the phone improved medication adherence among HIV+ individuals [217, 218]. More research focused on delivering psychological services to underserved populations is needed.

Limited research has been conducted in terms of examining other moderators that may be important for psychological therapies. In terms of dosage, one meta-analysis found that interventions consisting of ten or more sessions were more efficacious in improving depressive and anxiety symptoms, relative to interventions consisting of less than ten sessions [212]. A separate study is focused on the efficacy of stress-reduction interventions among different HIV subpopulations, including older adults, women, and individuals with a history of childhood sexual abuse [211].

Although very few intervention studies have focused on these subpopulations, the review has found preliminary evidence for decreased psychological distress and health risk behaviors following stress-reduction management.

12.7 Summary

Comorbid psychiatric features and disorders are important when working with and treating individuals diagnosed with HIV/AIDS. Thus, recognizing predictors of medication adherence among patients with dual psychiatric and substance use disorders is essential for identifying at risk individuals. The presence of pre-HIV psychiatric illness is the strongest predictor of psychiatric diagnosis after knowledge of seropositivity. As discussed throughout this chapter, psychiatric illness from the biological effects of HIV, CNS OI, prescribed medications, or substance abuse can arise. Optimum management of patients at high risk for HIV infection involves a wide range of psychiatric skills: comprehensive diagnostic evaluations, assessment of possible medical causes of new-onset symptoms, and initiation of specific treatment interventions. Early screening and assessment are essential for proper treatment for many persons infected and affected by HIV/AIDS which requires making important distinctions between overlapping symptoms in order to provide accurate diagnoses and treatments.

Conflict of interest The authors report no conflicts of interest.

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Chapter 13
Toward an Iterative Approach to Preventing HIV Risk Among Young MSM

J. Bryan Page

Keywords HIV • MSM • Risk • Youth • Mental health • Context • Continued risk behavior among young MSM • Serostatus and virus load as related to risk • Social context of risk • Iterative process of risk • Steps to intervention for preventing risk • Re-existing conditions and attitudes

Core Message
Because young MSM, especially young men of color, continue to have high rates of HIV+ seroconversion, research has focused on factors that might cause this ongoing trend. Factors that have received research attention in efforts to identify problem areas include (1) factors related to serostatus and virus load status and individuals' knowledge, attitudes, and beliefs about them, (2) factors related to mental health issues, (3) factors related to patterns of selecting sexual partners, and (4) factors related to community context. Most preventive interventions have focused on one or two of these factors. Future preventive interventions should combine all of them into an iterative model that relies on highly interactive learning. This kind of learning would take into account misconceptions about risk, the importance of mental health in avoiding risk, precautions necessary in selecting sexual partners, and the role of community environment in encouraging risk avoidance.

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13.1 Introduction

More than 30 years after the first realization that a virus could cause collapse of human immune systems, two factors have had a major impact on the willingness of people to risk infection by that virus: (1) the development of antiretroviral medications that arrest the process of destroying the immune system and (2) the coming of age of new cohorts of youths who have no memory of the original reactions to HIV as a menace to health. The first factor effectively removed the "death sentence" meaning from HIV seropositivity. The second presents a great challenge to those who would prevent young people from becoming infected and having to spend the rest of their lives dependent on the antiretroviral drugs mentioned in the first factor.

13.1.1 The Importance of Prevention

Ever since the first characterization of the human immunodeficiency virus (HIV) in the mid-1980s, prevention has received much attention from public health authorities. It had become clear by 1987 that the contact between human circulatory systems, via penetrative sex, parenteral drug use, and transfusion, were the principal factors in transmission of the HIV. Therefore, if people who engaged in these behaviors took the precautions of using condoms for sex, new disposable syringes for drug use, or testing blood and blood products before transfusion, they could avoid contracting HIV infection. The fact that behavioral strategies could affect prevention of HIV contagion, however, required public health authorities to convince people who engaged in behaviors involving risk of infection, to take the necessary precautions.

These relatively straightforward preventive strategies proved more difficult to promote than they first appeared to be. Condom use especially demanded constant attention and innovative campaign design, depending on the population of concern. In populations such as Haitian or African-American men, many of whom had never touched a condom, strategies that gave the individual an opportunity to apply a condom to a realistic dildo removed mystery of the unknown from the minds of prospective condom users. Needle/syringe (n/s) distribution required little ingenuity to attract users, but because infectious material could continue to circulate among the injecting drug user (IDU) population after initial use, n/s distribution schemes had to take measures to assure that the newly used n/s made its way back to the distribution center for disposal.

13.1.2 Prevention Declines

The Health Crisis Network of Miami, Florida, a public service organization focused on HIV/AIDS in the 1980s, dedicated its efforts to helping people living with AIDS and disseminating prevention messages to the community. Miami's public health department also did its part in furtherance of behavioral prevention, and these efforts

proved effective for a time. After the successes of antiretroviral medication in arresting disease progression among already infected people, preventive messages seemed to lose some of their life-or-death urgency. Furthermore, combined antiretroviral therapies reduced the risk of infection through sexual contact with infected individuals. As the sense of crisis-driven response to the AIDS pandemic shrank, efforts to prevent the spread of HIV became less energetic than they had been in the 1980s and 1990s. In fact, some agencies that had sounded the initial alarms for prevention in the 1980s had shut their doors by 2005, thinking their activities were no longer needed or relevant. Health Crisis Network, for example, maintained their address but changed their focus on health issues related to HIV infection in favor of a focus on mental health.

13.1.3 PREP as a Response to Rising Seroincidence Among Young MSM

The introduction of antiretroviral strategies for preventing HIV infection (pre-exposure prophylaxis or PREP) has broadened the array of messages to the public about the importance of HIV to the public health [1, 2]. This development, at least in part, was a response to the trend in populations at risk, especially young men who have sex with men (MSM), to be willing to take sexual risks, despite the pervasive warnings against sexual risk. In the 1990s, epidemiologists [3] reported that young MSM were unwilling to exercise the sexual caution that the previous cohort of MSM had strongly supported. The young men coming into the MSM social scene in the 1990s resented that the HIV epidemic had robbed them of the opportunity to express their sexual identity with the freedom enjoyed by the post-Stonewall cohort of MSM. Some rebelled against the rules of engagement in bars and other social settings, raising concern that the next cohort would experience a similar, decimating epidemic. Fortunately, the introduction of combined antiretroviral therapies (CART) began to make an impact on HIV progression and contagion by the mid-1990s, lowering the rates of both.

13.2 Marginalization and Risk

Notwithstanding these antecedents, it remains a concern that some populations of young MSM continue to generate high numbers of incident cases, especially populations of color [4–7]. In these populations, it is difficult to convince young people, who think of themselves as having nothing to live for, that they need to take up preventive measures in order to assure a long and comfortable life. In socioeconomic contexts where 30–60% of young men between the ages of 18 and 29 are unemployed or underemployed, the least of their worries is a virus that could infect them, eventually requiring treatment well into middle age.

In studies conducted among Haitian women in the early 1990s, we found similar attitudes in different structural circumstances [8]. The proximal concerns of these

women involved having the support of a man to assure that they and their children would have a roof over their heads and enough to eat. Whether or not the man used a condom in sex was a distal concern involving risk of an infection that might make them sick in 5 years and might kill them in 10 years (the perception of AIDS in 1991). As such, preventive behavior did not evoke the same intensity of action as the immediate needs of women and their children. One of the women expressed her situation succinctly: "I'd use a condom but my man won't. I'd leave my man, but he has a job. Do you have a job for me?" Her main concern lay with the daily provision of housing and food for her family, not a seemingly distal threat of HIV infection.

The attitudes of youth in marginalized circumstances parallel those of the Haitian women: I don't care when I die, as long as I survive tonight. Some are convinced that some other mode of death will catch them long before HIV sickens them. In the meantime, precautions against HIV spoil some of the fun of an active sexual life. Youths who face the crushing prospects of chronic unemployment and general lack of opportunities see little chance of having meaningful work, often opting to participate in illegal activity, such as armed robbery and drug dealing [9, 10]. Under the stultifying thrall of structurally limited options for employment that seldom offer advancement or eventual life comfort and satisfaction, it is not surprising that the brighter young people in these circumstances recognize the barriers that face them and opt for activities that will almost certainly land them in prison [9]. In the course of pursuing illicit options, risky sexual and/or drug using behavior become highly likely. In these circumstances, the concept of syndemic [6], where conditions of poverty exacerbate the impact of an epidemic, becomes highly relevant. Examination of incidence data from Europe [11] and elsewhere in the world [5, 12] has raised concern about young men and their still high rates of new infections. This concern has led researchers to seek explanations of the persistently high rates of HIV seroincidence among young men and to try to develop strategies for preventing further contagion.

13.3 Factors Identified in the Literature

The question, "what do I have to live for?" is especially applicable to preventing HIV risk among marginal populations of young men, but several other factors affecting risk have received attention in a variety of studies. These include factors involving knowledge or attitudes about HIV, such as:

1. Virus load measurements as they affect attitudes toward risky sex [13]
2. Degree of virus load control among HIV+ young men [14]
3. Disclosure of serostatus in sexual encounters [15]
4. Self-efficacy as a predictor of risky sex [16]
5. Developmental stages of young men who have recently entered the gay social scene [17]

Concomitant drug and alcohol use have also received attention: (1) the use of alcohol in sexual activity [18] and (2) the use of methamphetamine in sexual encounters [19].

Young men's approaches to relationships with sexual partners can also predict risk of HIV infection. For example, Bauermeister [20] proposed romanticism in relationship seeking as a protective factor in HIV risk. Age differentials in relationships, however, can result in elevated risk of HIV infection, according to Anema et al. [21]. Depression has demonstrable association with increased risk of HIV exposure among young men [22], and hypersexual behavior, according to Yeagley, Hickok, and Bauermeister [23], also exacerbates risk for young MSM. Consideration of race and diversity in sexual relationships appears in articles by Hernández-Romieu et al. [5] and Crosby et al. [24]. Cuervo and Whyte [25] comment generally on how the type of relationship affects risk among young MSM.

13.4 Kinds of Risk Factors

Four kinds of risk-related factors have emerged from the studies briefly described above: (1) psychological response to the possibility of risk, (2) mental health aspects of risk taking, (3) partnering patterns and risk, and (4) cultural context of risk. The first kind involves individuals' access to information about their own viral load and the potential complacency that the use of combined antiretroviral therapies (CART) might engender. The second kind of factor entails the mental health of actors in the MSM social context. The third kind of factor takes into account the patterns of sexual partner seeking in sociocultural context. The fourth kind of factor involves community context and peer influence. Table 13.1 arrays risk factors presented in the literature by the kinds posited here.

These kinds of factors are not mutually exclusive. They may operate to varying degrees in the behavior of an individual who responds to circumstances that bring them into play in the process of incurring or not incurring risk. For example, a seropositive young MSM takes his CART medications assiduously and obtains

Table 13.1 Kinds of factors the affect risk behavior and sources in the literature

Psychological response to risk	Mental health and risk	Partnering patterns and risk	Cultural context of risk
Beliefs about HAART efficacy and attitudes toward risk [13]	Depression and sexual risk behavior [22]	Romanticism as a protective factor [20]	Poverty in combination with other contextual factors increases risk [6]
Self-monitoring of virus load and risky sexual behavior [14]	Hypersexual behavior and risk [23]	Age difference enhances risk [21]	Alcohol use affects risk behavior [18]
Disclosure of serostatus and risk behavior [15]	Stigma, mental health, and race affect risk [7]	Race/ethnic preferences affect risk [5, 24, 27]	Methamphetamine use affects risk [19]
Self-efficacy and risk behavior [16]	Developmental stages of adolescence and risky behavior [17]	Types of relationships affect risk [25]	

virus load measurements regularly. He finds himself in the company of a very attractive potential partner at a social event that features an open bar. Although he normally does not drink, he celebrates his newfound acquaintance by having somewhat more alcohol than he would drink in social occasions (Long Island iced tea, a drink with high alcohol content, is the featured drink at the bar. He consumes four of these). After a night about which he remembers very little, he finds himself in bed next to his newfound acquaintance. Three of the four kinds of risk-related factors – known serostatus and control, community context, and partner patterning – have been part of this iterative experience, in which the individual has somewhat unexpectedly incurred risk. The individual imagined above responds in very understandable fashion to his circumstance. He has learned the discipline of controlling HIV infection and monitoring the medication's effect, but that discipline is relaxed in a moment of joy and celebration. His act may not even have consequences, thanks to the HIV's uncertain infectivity upon a single contaminated exposure [26]. The important point with regard to the interplay of factors related to risk of HIV infection is that the rate at which otherwise adherent people living with AIDS incur risk is affected in iterative, combinative fashion. Persons who know all about the nature of risk and have usually taken precautions to avoid it almost inevitably will find themselves breaking some rules in some situations. Preventive efforts that focus too exclusively on one or two risk-related factors may therefore be doomed to failure, just because they do not take into account how risk-related factors come together.

13.5 What Interventions Need to Do

According to some research on young MSM and their psychological responses to risk, those whose virus loads are most controlled and who are most aware of their own HIV loads tend to incur less risk to themselves or their partners than those who have uncontrolled loads and indicate low awareness of those facts [13, 14]. Those individuals who know their viral load is not controlled, the ones who need to be most careful about exposing themselves and their partners, are the least careful about avoiding that kind of exposure. Preventive interventions that aim at optimizing the effect of regular virus load monitoring do not need to combat complacency among HIV+ individuals who monitor virus load. Those individuals apparently are most careful about behaviors that might expose them and/or their partners to HIV. Nevertheless, those individuals who do not monitor their virus load and are not careful about risk of exposure may not be amenable to engaging in the self-monitoring behavior of the careful ones.

Little is known about how one group of HIV+ individuals chooses to monitor their personal virus loads, while another chooses not to do so. The factors that contribute to this differentiation may well involve factors studied elsewhere, such as acute intoxication, race/ethnic dynamics, syndemic processes, or age differential between partners. Again, the key concept in risk of HIV exposure is that risk behavior is a process.

Because all of the studies cited here have identified potentially important parts of the risk incurring (or risk avoiding) process, it would be useful to combine them into

an iterative model, identifying which risk-affecting factors fit into which parts of the risk process.

One category of factors affecting risky sexual behavior on the part of young MSM clearly precedes any sexual behavior – attitudes about what kind of partner the individual is seeking. These attitudes include age differential [21], choice of race/ethnic group [5, 7, 24, 26], and desire for idealized romantic relationship [20]. Age differential is associated with higher frequency of risky behavior [21]. Choice of race/ethnic group for selecting partners provides mixed reports on the nature of risky behavior, but, clearly, African-American MSM have proven more likely to seroconvert than their white non-Hispanic peers [5].

The second category of risk-affecting factors takes into consideration mental health. Bauermeister et al. [16] examined self-efficacy as a possible protective factor in predicting risky sexual behavior, finding the need to improve self-efficacy with casual sex partners. Hypersexual behavior [22] interacts with self-efficacy in predicting risky sexual behavior among young MSM. Lelutiu et al. [7] blended consideration of mental health, primarily in terms of stigma and bias, with race/ethnic group in their treatment of risk acceptance or avoidance. Of course, even moderate levels of depression, as shown by O'Cleirigh et al. [22], also predisposes in favor of risky sex. Regardless of their stress-mitigating conditions (e.g., stress buffers), MSM of color tend to incur more risk than white non-Hispanic MSM. In consideration of developmental stages of adolescence and transition to adulthood, Wong et al. [17] examined risk behaviors in adolescent MSM longitudinally. They noted that the context of these changes strongly influenced the rate of risky behavior among their respondent sample. If the young MSM spent time in contexts where older peers and peers drank heavily, they were more likely to drink and engage in risky sexual practices.

This finding is reminiscent of the differential association theory of drug use and delinquency forwarded in the 1950s through the 1970s [28, 29]. According to this interpretation of juvenile delinquency and uptake of illegal drug use, the key factor in predicting delinquent behavior and drug-related crime was the composition of the peer group. On strength of their findings, Wong et al. [17] also propose necessary attention to the relationship between time and context.

Two studies [18, 19] have focused on drug and alcohol use as factors in unprotected sexual behaviors among young MSM. Those risk-related behaviors constitute part of the cultural environment in which young MSM encounter risky circumstances. Therefore, drug and alcohol use, for purposes of preventive intervention, should be considered exacerbating factors in the larger cultural context of sexual risk encountered by young MSM.

Knowledge of HIV serostatus and attitudes about HIV risk based on the knowledge of virus load constitutes the third variety of risk factor that contributes to the risk process. Obviously, if the individual is already seropositive, he has already engaged in risky behavior. Nevertheless, knowledge of one's personal serostatus as well as knowledge of a partner's is clearly a component of the decision to take or not to take risks of exposure. Knowledge that one is HIV-seronegative may make an individual more careful when encountering circumstances of possible risk [15]. Not

Table 13.2 Ordering of attention to risk-related factors in intervention

Pre-existing conditions and attitudes	HIV+	Monitoring virus load	Divulging serostatus	Stigma	Depression
	HIV−	Beliefs about undetectable load	Asking about serostatus	Absence of Stigma	Depression
Partner pre-choice	Romantic or not?	Same race/ethnic or not?	Same age, older, or younger?	Types of relationships	Hypersexual behavior
In the moment	Acute effects of drugs (including alcohol)	Conditions of poverty as they affect risk	Developmental stage of adolescents	Response to scenarios – desirable partners	Rules of conduct

knowing one's personal serostatus, on the other hand, is associated with less caution in potential situations of risk. Similarly, knowledge that one has an undetectable virus load is associated with reduced risky behavior, whereas uncontrolled virus load is associated with more risk of further exposure [14]. These factors are the last considerations based on prior knowledge to precede the individual's risky or non-risky sexual behavior.

Four of the interventions reported in the literature on young MSM and risk of HIV infection [4, 30–32] suggested strategies to prevent HIV risk that involve interactive formats for learning of protective behaviors and take into account intrapsychic processes in the target group. These approaches, although they have not yet produced definitive findings, appear to be on the right track. Psychoeducational approaches (i.e., lectures and didactic films) tend not to work well with at-large study participants, and a young MSM audience would be no exception to this principle. The intervention reported by Hidalgo et al. [32] relied heavily on community cohesion, while the one reported by Mustanski et al. [30] relied on a form of social media. Parsons et al. [30] used a motivational interviewing strategy in their intervention.

In furtherance of the iterative approach to intervention for preventing HIV risk, the author suggests that each of the four kinds of factors identified in the literature on young MSM receive attention in the intervention studies to come. Because one group of researchers is already working with two varieties of intervention [30, 32], the combined approach appears eminently possible. Participants could learn in five sessions: a preventive approach to partnering, a preventive approach to mental health issues that affect partnering, a preventive approach to social context, and a preventive approach to serostatus and virus load considerations, with a summative session at the end. The ordering of the sessions would reflect the ordering of the attitudes and values that underlie each stage of the risk assessment process, supported by the latest data on how each is related to protective or risky behavior. Table 13.2 suggests the rough ordering of content, beginning with pre-existing attitudes and conditions (split into HIV+ and HIV-appropriate content), continuing with attitudes about partnering, and, finally, preparing to deal with specific circumstances for incurring or avoiding risk.

Conflict of interest The authors report no conflicts of interest.

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Chapter 14
NeuroAIDS in Drug Abusers: Associations with Oral Manifestations

Fariba S. Younai

Keywords Drug use • Cocaine • Methamphetaminhe • Cannabis • Maijuana • Oral health • Dental disease • Xerostomia • Oral ulceration • Dry mouth

Core Message
Both HIV infection and illicit drug use have significant oral health implications. While certain illicit compounds like cocaine, cannabis, and betel nut have the potential to directly cause oral soft tissue pathologies, most others including opioid agents and methamphetamine affect salivary flow and enhance the rate of dental decay and periodontal disease. Because immunologic deficiency caused by HIV infection leads to unique head and neck pathologic manifestations, among patients with HIV infection who are also users of illicit substances, special attention should be paid to recognizing the signs of oral diseases to help preserve their oral and general health.

Illicit drug use and dependence are major global health issues. It is estimated that 1 in 20 adults or a quarter of a billion people between the ages of 15 and 64 years used at least one drug in 2014, about 29 million are current problem drug users, and 0.2 million die each year from overdose or medical complications from heroin, cocaine, and other drugs [1]. Dependence on illicit drugs has major global and local economic and social impacts and contributes to crime, political instability, and the spread of communicable diseases such as HIV [1]. Cannabis remains the most widely used illicit substance globally. In 2014, some 3.8 percent of the global population, 183 million people, had used cannabis in the past year, a proportion that has remained stable since 1998 [1]. This was followed by opiates and cocaine at 0.4% or about 18 million people for each, amphetamine-type stimulants at 0.8% or 36

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million people, and "ecstasy" at 0.5% or 19 million users [1]. Some 10–13% of the drug users continue to be problem users afflicted with drug dependence and/or drug-use disorders, and among these, the prevalence of medical conditions such as HIV infection at approximately 14%, hepatitis C infection at almost 52%, and hepatitis B infection at 9% continues to add to the global burden of disease and death [1]. A recent systematic review of the published data on illicit drug use expanded on the information previously published from the Global Burden of Disease (GBD) study [2, 3] by demonstrating specific health outcomes associated with each major drug of abuse [4]. This analysis showed cannabis as a risk factor for schizophrenia (triggering an earlier onset of schizophrenia among those who would develop the disorder regardless); opioids, cocaine, and amphetamine dependence as risk factors for suicide; and injecting drug use (IDU) as a risk factor for HIV and hepatitis B and C viruses. Other studies have shown HIV prevalence to be almost 28 times higher among people who inject drugs [5]. In fact, in 2006, the HIV prevalence among IDUs in China, the United States, and Russia, the three leading countries for injecting drug use, was 12%, 16%, and 37%, respectively. In addition to contributing to higher infection rates, the use of many drugs of abuse by people receiving antiretroviral agents leads to both nonadherence and drug interactions that result in poorer virologic response to HIV treatments [6–9].

In the United States, the 2013 data provided by the National Survey on Drug Use and Health (NSDUH) has estimated 24.6 million individuals aged 12 and older or 9.4% of the US population are current illicit drug users (having used an illicit drug during the month prior to the survey interview) [10]. Among them, 2.2 million are aged 12 to 17 accounting for 8.8% of the US adolescents. According to this report, marijuana is the most commonly used illicit drug (7.5% of the population), followed by the nonmedical use of prescription-type drugs (2.5%), cocaine (0.6%), hallucinogens (0.5%), inhalants (0.2%), and heroin (0.1%), while nearly one quarter of adults (58.5 million) and 6.2% of the adolescents (1.6 million) are binge alcohol users. It is important to note that of the 22.7 million individuals 12 years or older who met the criteria for dependence and abuse for an illicit drug or alcohol,[1] only an estimated 2.5 million received treatment at a specialty facility for an illicit drug or alcohol problem [11]. The main barriers to receiving the appropriate treatment include not having appropriate health coverage, not being ready to stop, not knowing where to go for treatment, not having transportation and inconvenient hours [10].

Worldwide, a myriad of prescription drugs, over-the-counter preparations, and chemical compounds prepared from naturally occurring, semisynthetic, or synthetic compounds are used illicitly and are associated with significant medical consequences including conditions manifested in the head and neck region [12] (Table 14.1).

Naturally occurring opiates such as morphine and codeine, the semisynthetic opiates like heroin, and the synthetic opioids like methadone are all central nervous system (CNS) depressants and, because of their euphoric properties, are among the

[1] NSDUH defines dependence on and abuse of alcohol or illicit drugs using the criteria in the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), which include such symptoms as withdrawal, tolerance, the use in dangerous situations, trouble with the law, and interference with major obligations at work, school, or home during the past year.

Table 14.1 Categories of illicit drugs discussed in the chapter

Main drug categories	Class	Examples
CNS depressants	Naturally occurring Opiates	Morphine and codeine
	Semisynthetic opiates	Heroin, fentanyl, and oxycodone
	Synthetic opioids	Methadone
	Other depressants	Barbiturates, benzodiazepines, and GHB
CNS stimulants	Naturally occurring Stimulants	Coca, khat, and betel nut
		Cocaine and crack cocaine
	Synthetic stimulants	Methamphetamine, methcathinone, and methylenedioxy-methamphetamine
	Other stimulants	Cannabis

widely abused drugs; their repeated use, in addition to their serious physical and mental health complications, is associated with abnormalities in orofacial structures. Similarly, the nonmedical use of other CNS depressants like barbiturates, nonbarbiturate depressants, and benzodiazepines can have adverse consequences for the cognitive functions as well as the user's dentition. Naturally occurring central nervous system stimulants such as coca, khat, and betel nuts; products extracted from these plants like cocaine and crack cocaine; as well as fully synthetic forms like amphetamine and amphetamine-type compounds, illegally used for their strong hallucinogenic effects, can have significant health effects both systemically and also in the intra- and extraoral tissues. Cannabis, used for its sedative and hallucinogenic effects, is often mixed with tobacco for smoking purposes and for that has implications in the development of oral dysplasia and malignancy. Although HIV infection during its advanced stages and profound immunologic deficiency can be associated with a number of unique head and neck manifestations that have been well described over the past three decades, among illicit drug users, the intra- and extra-oral pathologies encountered are not specific to HIV infection. This chapter provides a comprehensive review of the oral health consequences of some of the most commonly used illicit substances and examines each drug of abuse for its harmful effects on the orofacial tissues.

14.1 CNS Depressants: Opiates

Opiates are the naturally occurring alkaloids derived from the opium poppy, *Papaver somniferum*. Members of this category of drugs include "opium," the coagulated juice of the opium poppy; "morphine," which is extracted from opium poppy straw; and "codeine," a methylated form of morphine [13]. There are many methods to abuse for these agents ranging from ingesting (raw opium and prescription codeine), chewing (raw opium), smoking (prepared opium) to injecting (morphine) [13]. Another member of this category is the semisynthetic drug "heroin" that is synthesized from morphine. It is illegally available in several forms from crude to different

grades of purified form and in these forms can be injected, inhaled, sniffed, snorted, or smoked [13]. Other semisynthetic opiates synthesized for medical use include oxycodone (OxyContin), hydrocodone (Vicodin), and buprenorphine (Suboxone) [13]. The final members of the opiate family are the fully synthetic opiate analogs referred to as opioids or opiate-like drugs that include methadone and fentanyl [12, 13]. Several types of fentanyl have been synthesized specifically for sale on the illicit market, and they can be either smoked or snorted and even injected [13]. Methadone, legally used to treat addiction to narcotics, is available in tablet, liquid suspension, and sterile solution forms and unfortunately has emerged as a drug of abuse in recent years [14].

Opiates and opioids are central nervous system depressants, and their main medical use is for their strong analgesic properties, but some are also prescribed as cough suppressants and in treatment of diarrhea [15]. Their nonmedical use is for their euphoric effect and also for reducing anxiety, boredom, or physical or emotional pain [12]. Based on the 2010 National Survey on Drug Use and Health, public health experts estimate that more than 35 million Americans age 12 and older used an opioid analgesic for nonmedical use some time in their life—an increase from about 30 million in 2002 [16]. Improper use of any opioid can result in serious side effects including overdose, respiratory depression, and death. In 2009, there were nearly 343,000 emergency department visits involving nonmedical use of opioid analgesics [17]; in 2008, nearly 36,500 Americans died from drug poisonings; and, of these, nearly 14,800 deaths involved opioid analgesics [18]. Despite these serious risks, ironically, some of the most severe health effects of injectable opioids like heroin is less related to the drug itself and more due to the unhygienic and needle-sharing practices which lead to the transmission of hepatitis viruses and HIV. One out of every ten new HIV infections is caused by injecting drug use, and currently 3 million injection drug users are living with HIV worldwide [19]. In fact, in parts of Eastern Europe and Central Asia, over 80% of all HIV infections is related to drug use [19].

The oral effects of opiate and opioid narcotics are both direct (causing pathological changes in the salivary, oral, and dental tissues) and indirect (lifestyle related). The most common self- reported and clinical finding among long-term users of narcotic drugs, including methadone, is high levels of dental decay and breakdown (Fig. 14.1).

The first reports of high rate of dental disease among opiate users were published in the 1940s [20]. Since then, both typical (interproximal and occlusal) and atypical (smooth surface and cervical) caries have been described among opioid and methadone users [21–30]. In one recent study of 41 heroin users in San Francisco, the decayed-missing-filled surfaces (DMFS) index[2] was determined to be very high and more skewed toward decayed surfaces [31, 32]. Although the xerostomic effect (inducing hyposecretion of saliva) of narcotic analgesics is a known property of these compounds and most likely a contributing factor in the development of tooth

[2] An index of past caries experience based on the number of decayed, missing, and filled surfaces of deciduous (indicated by lowercase letters) or permanent (indicated by capital letters) teeth.

decay among long-term users, other factors such as a taste preference for sweets [33–37], craving for carbohydrates [38, 39], poor self-care and oral hygiene practices [22, 30, 32, 39], and inadequate access to dental care [29, 40, 41] may all be influential in the high rates of tooth decay in this population. Long-term opioid use has also been reported to be associated with higher rates of periodontal disease including adult periodontitis [22, 42, 43] and necrotizing gingivitis [44] (Fig. 14.2).

Furthermore, opportunistic fungal and viral infections may also occur, [30, 44, 45] most likely due to an underlying immunological suppression reported to occur with drugs of abuse and independent of HIV infection [46–48]. In addition, there is at least one case report of tongue mucosa pigmented lesion attributed to a fixed-drug reaction to heroin pyrolysate vapors [49].

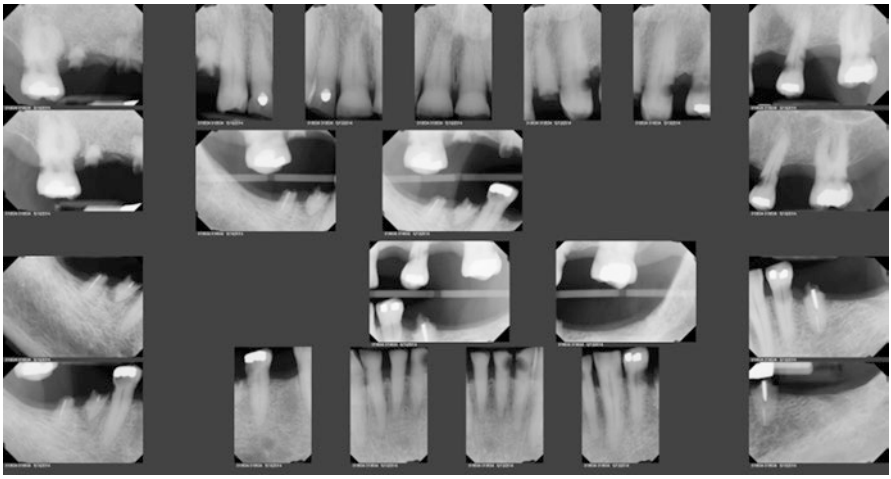

Fig. 14.1 Periapical radiographs of a patient with multiple interproximal and cervical tooth decay. Note teeth that have broken off because of advanced decay

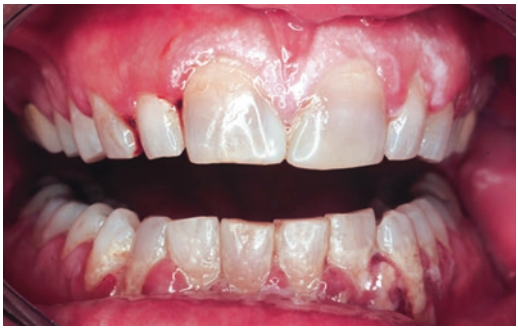

Fig. 14.2 Necrotizing gingivitis in an HIV-positive patient. Note areas of necrosis in the interdental papillae and the pseudomembrane in the gingivae of the maxillary central incisors

14.2 Other CNS Depressants

In this chapter, three members of this category are discussed: benzodiazepines, barbiturates, and *gamma* hydroxybutyric acid. They are available in a variety of forms, most as orally ingested tablets and suspensions and some as injectable solutions and even suppositories.

Benzodiazepines are a group of CNS depressants and among the most frequently prescribed medications worldwide as anxiolytic and sedative-hypnotic agents. About 2000 benzodiazepines have been synthesized by the pharmaceutical industry, and their illicit use is driven by diverting the legally synthesized preparations into illegal markets; they are mostly used for the enhancement of the "high" induced by other drugs or relief of side effects associated with overstimulation or withdrawal of other drugs [13].

Barbiturates are also CNS depressant compounds, but they have been largely replaced on both the licit and illicit markets by benzodiazepines. While they were formerly used as hypnotics/sedatives, their medical use today is limited to the treatment of seizure disorders as long-acting antiepileptics and as adjuncts to anesthesia as short-acting agents [13].

Sedatives/ hypnotics like benzodiazepines and barbiturates cause xerostomia, especially among long-term users, and tooth decay is the most important dental effect of chronic dry mouth [50].

Gamma hydroxybutyric (GHB) acid is a naturally occurring analog of gamma-aminobutyric acid (GABA) with CNS depressant properties. In medicine, it has been used as an adjunct in anesthesia and as an aid to alcohol/opiate withdrawal, but currently it is only legally available in the United States for the investigational treatment of narcolepsy [51]. GHB has become a major club drug over the past decade and has been implicated in a number of crime types, most notably in drug-facilitated sexual assault [52]. In its illicit use, it can quickly lead to systemic toxicity causing strong hallucinations and very erratic behaviors like self-mutilation; oral self-extractions have been reported in several case reports [53–57].

14.3 CNS Stimulants

Central nervous system stimulants include naturally occurring plants such as "coca," "khat," and "betel nuts"; products extracted from the leaf of the coca bush like "coca paste," "cocaine hydrochloride," and "crack cocaine"; and wholly synthetic substances such as "amphetamine," "amphetamine-type compounds," "MDA" (3,4-methylenedioxy-amphetamine), and "MDMA" (3,4-methylenedioxy-methamphetaime) [12].

For its euphoric effect, the leaves from the coca plant (*Erythroxylum coca*) can be chewed, brewed in form of tea, or transformed into coca paste that can be ingested or smoked [13].

Cocaine is the main alkaloid synthesized from coca leaves as a hydrochloride salt, and in this form it can be snorted. Crack cocaine is a smokeable form of cocaine made by processing cocaine with sodium bicarbonate into small rocks. Cocaine freebase is also obtained from cocaine hydrochloride by using a solvent or a process that converts it into its base form, which is no longer water soluble and can only be smoked.

The biological effect of cocaine is through blocking voltage-gated sodium channels and prevention of action potential; therefore, its medical use is in anesthesia as a topical anesthetic in eye and nasal surgery [13]. It is also a serotonin-norepinephrine-dopamine reuptake inhibitor and leads to an increase in these neurotransmitters, hence its euphoric properties [58]. The sympathomimetic effects of cocaine are responsible for significant cardiovascular effects including increased heart rate, coronary and systemic vasoconstriction, reduced myocardial perfusion and ischemia, and elevated systemic arterial pressure [59]. In the orofacial tissues, the use of cocaine is associated with a number of intra- and extraoral findings that seem to be related to its sympathomimetic effect. In the nose, chronic snorting leads to recurrent epistaxis, intranasal irritation and ulceration, and sinusitis and nasal septum perforation, referred to as cocaine-induced midline destructive lesions (CIMDL) [60–62]. CIMDL is reported to affect 4.5% of the users and mimic systemic conditions such as Wegener's granulomatosis by featuring positive antineutrophil cytoplasmic antibodies (ANCA), suggesting a complex inflammatory and autoimmune etiology [63–65]. The destruction of the sinonasal structures causes a loss of height of the nose and a broadening of its base, referred to as a saddle nose deformity. In the mouth, chronic use has a similar effect on the palate, causing palatal sialometaplasia or perforation [66–70].

Cocaine use has also been reported to be associated with oral leukoplakia, oral and gingival ulcers, and manifestations in the periodontal tissues including necrosis and recession. Finally, cocaine use can lead to bruxism and temporomandibular joint pain and dysfunction [71–78] (Fig. 14.3).

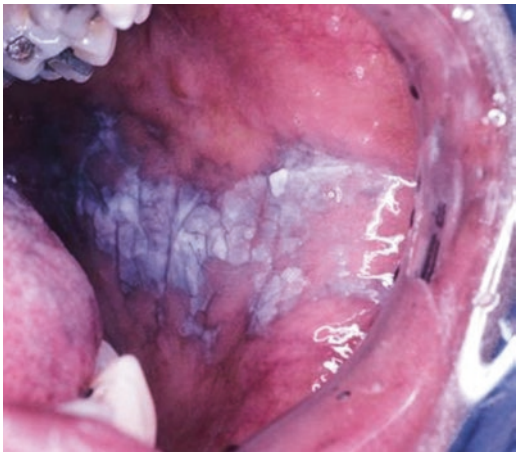

Fig. 14.3 An area of leukoplakia in the buccal mucosa of an HIV-positive woman with a habit of smoking cocaine-laced cigarettes

Khat (*Catha edulis*) is a flowering bush indigenous to East Africa and southern Arabia [79]. In North Yemen, khat is chewed on a daily basis and is restricted to men [80]. Leaves of the khat shrub are typically chewed and held in the cheek, like chewing tobacco, to release their stimulant chemicals. The main psychoactive ingredients in khat are cathinone and cathine [79]. These chemicals are structurally similar to amphetamine and result in similar stimulant effects in the brain and body, although they are less potent. Like other stimulants, cathinone and cathine stimulate the release of the stress hormone and neurotransmitter norepinephrine and raise the level of the neurotransmitter dopamine in brain circuits regulating pleasure and movement [79]. Oral leukoplakia, oral mucosal pigmentation, and oral dryness have been reported in individuals who chew khat [81].

Betel nut (areca nut), the seed of the areca palm (*Areca catechu*), grows in much of the tropical Pacific, Asia, and parts of east Africa [82]. The term "betel quid" (synonymous with "pan" or "paan") generally contains betel leaf, areca nut, and slaked lime and may contain tobacco along with other substances for flavoring (cardamom, saffron, cloves, aniseed, turmeric, mustard, or sweeteners) [82]. Arecoline is the primary active ingredient responsible for the central nervous system effects of the areca nut and has properties similar to nicotine [82].

One significant oral lesion seen among people who use betel quid regularly is submucous fibrosis (Figs. 14.4 and 14.5).

Lesions of submucous fibrosis start as blanched or marble-like pale mucosa and progress to fibrous bands in the buccal and labial mucosa which cause a restriction in opening the mouth [83]. It has been suggested that in a genetically proposed individual, areca alkaloids cause fibroblast proliferation, increased collagen synthesis, and inhibition of collagen phagocytosis [84]. Submucous fibrosis is considered a premalignant oral lesion [83]. Oral lichenoid reaction and excessive tooth abrasion and fracture have also been reported to occur with areca nut use [85].

The synthetic amphetamine and amphetamine-like compounds have been used medically for nasal decongestion and bronchial dilation, the promotion of weight loss, and also in the treatment of attention deficit disorder, narcolepsy, and depression [86, 87]. These include dextroamphetamine (Dexedrine), methamphetamine (Desoxyn), and methylphenidate (Ritalin) [87]. They are not only abused in their pharmaceutically available forms, but they are also illegally produced as methamphetamine, methcathinone ("bath salts"), and methylenedioxy-methamphetamine (MDMA, "ecstasy") [87]. Worldwide, common street names for methamphetamine are black beauties, chalk, crack meth, crystal meth, meth, ice, crystal, crank, glass, shabu, and yaba. "Speed" is a common street name for both methamphetamine (in the United States/North America) and amphetamine (in Europe) [13, 87]. Like cocaine, amphetamines result in an accumulation of the neurotransmitter dopamine. While in therapeutic doses they improve alertness, attention, and performance on various cognitive and motor tasks, when abused, their desired effect is increased alertness and energy, postponement of hunger and fatigue, exhilaration, and euphoria [13]. Globally, it is estimated that 36 million adults use amphetamines, including methamphetamine, amphetamine, and ethcathinone, and about 19 million use substances sold as "ecstasy" (MDMA) [1].

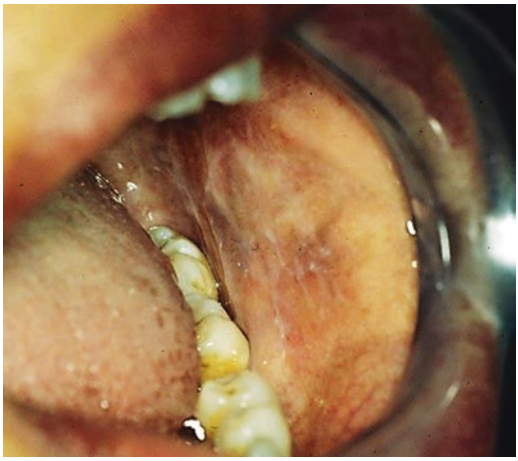

Fig. 14.4 Oral submucous fibrosis in the buccal mucosa of a man with betel nut chewing habit. Note the areas of tissue fibrosis

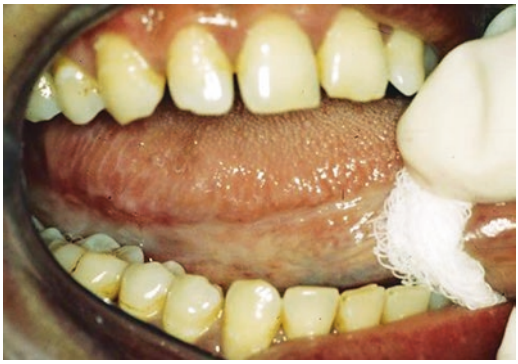

Fig. 14.5 Oral submucous fibrosis in the lateral and ventral aspects of the tongue of a man with betel nut chewing habit. Note the areas of tissue fibrosis

Methamphetamine was first synthesized in Japan in 1893, and its pharmaceutical form was heavily used during World War II by the British, German, American, and Japanese military personnel for its performance-enhancing properties; after the war, this drug penetrated the civilian market in Japan and led to its first epidemic in that country [88, 89]. In the United States, the epidemic gained a foothold, first, in Hawaii and California, and then it spread eastward with clandestine production by the "do-it-yourselfers" throughout the country, particularly in rural areas [90]. Methamphetamine is produced in a variety of forms that can be injected, orally ingested, sniffed/snorted, and smoked. Dependence on these drugs has significant neurologic, cardiovascular, and metabolic consequences including memory loss, aggression, hyperexcitability, paranoid and psychotic behavior, increased heart rate, hypertension, and malnutrition; in addition, it has been shown to contribute to increased transmission of infectious diseases, such as hepatitis and HIV [91]. Methamphetamine use has been reported to be associated with severe oral health problems such to the extent that the term "meth-mouth" started to gain popularity in

the late 1990s among the users and the professionals working in the field of addiction medicine and in the mid-2000s began appearing in the news media [92]. Since then, despite a paucity of evidence for methamphetamine-specific oral damage [32, 93], the term continues to be used, by both the dental professionals and the lay public, to convey an image of blackened, rotting, crumbling teeth and the associated soft tissue breakdown with gingival abscesses (Fig. 14.6).

It is more accurate to consider the specific oral conditions seen among methamphetamine users which include xerostomia and bad taste [94–96], rampant tooth decay [97–99], and bruxism and tooth wear [100, 101] (Figs. 14.7 and 14.8).

In one pilot study of 18 long-term methamphetamine users (average length of use of 8 years) in 1999, 50% of the participants reported severe dry mouth (22% had reduced salivary flow by clinical assessment) and 60% reported oral ulcer and irritation right after use [102] (Fig. 14.9).

Among the mechanisms suggested for the development of xerostomia are amphetamine-induced vasoconstriction in the vasculature of salivary glands [96] and stimulation of the inhibitory alpha-2 receptors in salivary secretory cells [103]. In one small pilot study of 28 subjects, no differences were observed in the rate of salivary flow between methamphetamine users and the control subjects, but there was a trend toward lower pH and decreased buffering capacity of saliva among the using group [104]. This reduction in salivary pH had previously been reported to be relatively small and among a small group of individuals who only smoked MDMA, raising doubt about the significance of its role in enamel erosion and the risk for tooth decay [105]. The pattern of dental caries among methamphetamine users is similar to that observed among patients with severe xerostomia (like those with a history of radiation to the head and neck region) and affects the cervical and the smooth tooth surfaces as well as the interproximal surfaces of the anterior teeth. Although the corrosive effects of the methamphetamine constituents such as anhydrous ammonia (found in fertilizers), red phosphorus (found on matchboxes), and lithium (found in batteries) on the tooth enamel have been suspected as etiologic factors in enamel erosion, the extent of tooth decay among some users and its rate of progression appear to be more related to lifestyle factors such as oral hygiene and the level of consumption of sugary food [106, 107]. Furthermore, a recent cohort study of adult methamphetamine users found the intravenous administration was associated with significantly more missing teeth in the cohort than the smoking

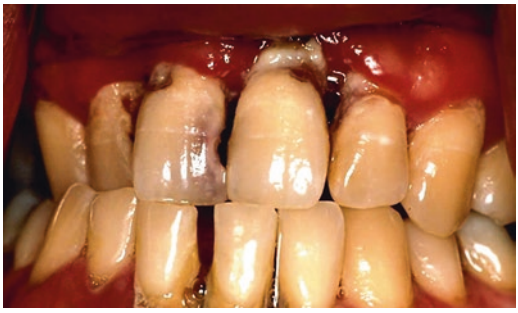

Fig. 14.6 Rampant decay, severe gingival recession, and purulence from a periodontal abscess in an HIV-positive man with a history of heavy crystal methamphetamine use

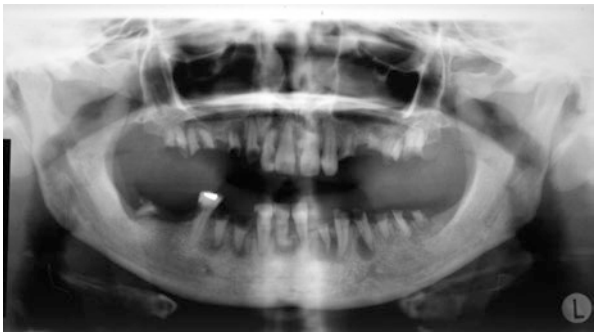

Fig. 14.7 The panoramic radiograph of an HIV-positive woman with a 4-year history of meth use. Note the number of dental abscesses

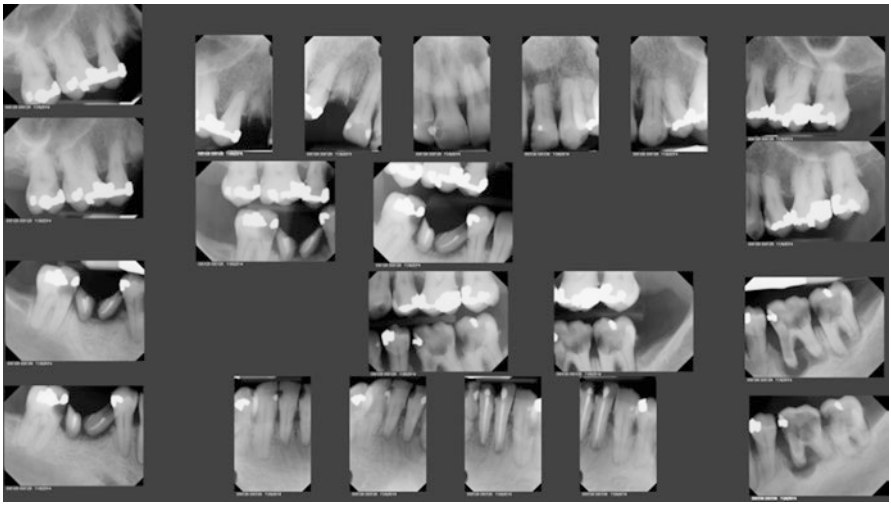

Fig. 14.8 The periapical radiograph of a person with a 10-year history of meth use (4–5 times daily). Note the level of tooth decay activity and broken teeth

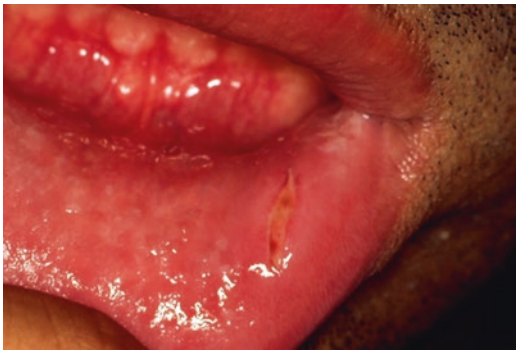

Fig. 14.9 An ulcer in the labial mucosa of an active crystal methamphetamine user

route [99]. Among other contributing factors are also forgoing dental care, grinding or clenching teeth, and the concomitant use of other substances such as alcohol and tobacco [106, 107]. In addition, increased neuromuscular activity in methamphetamine users may be responsible for bruxism, tooth grinding, and temporomandibular joint tenderness among methamphetamine users [100, 101, 108]. In one recent report of the osteonecrosis of the jaw (ONJ) in a chronic methamphetamine user, the authors suggest an etiologic correlation between ONJ and exposure to the toxic phosphorus vapor generated through heating and smoking this drug [109], a possibility that must be explored by future studies.

The oral effects of the designer amphetamine-like drugs, methylenedioxymethamphetamine (MDMA "ecstasy"), and methcathinone ("bath salts") are similar to those described for methamphetamine users. Ecstasy use is reported to cause significant dry mouth lasting up to 48 h after consumption, oral ulceration 1–2 days after use, and bruxism during use [110]. Bath salts have been reported to cause severe bruxism and grinding that can lead to tooth wear and fractures [40].

14.4 Cannabis

Cannabis remains the world's most widely used illicit substance with estimated annual prevalence ranging from 3.8 percent of the world adult population (183 million individuals aged 15–64) [1]. Cannabis preparations, marijuana, hashish, and hash oil, are obtained from the plant *Cannabis sativa* that contains over 60 types of cannabinoids [111]. Δ^9-Tetrahydrocannabinol (THC) is the plant's main psychoactive constituent, and several other cannabinoids such as Δ^8-THC, cannabinol, and cannabidiol have additive, synergistic, and antagonistic effects on THC [112]. THC is found in its highest concentration in the resin produced by the female flower heads, followed by the plant's flowers, leaves, stems, and seeds [111]. Marijuana is made from the plant's dry flowers and seeds and consists of 0.5–5% THC, while hashish, made from the resin produced by the flower heads, consists of 25% THC and hash oil, the liquid extracted from hashish, consists of 15–50% THC [111]. It is important to note that much of the cannabis used today comes from *Cannabis sativa* subspecies such as skunkweed and netherweed, developed through selective breeding techniques over the past 20 years, with THC contents in the magnitudes of 15 to 30 times higher than the old generations of cannabis used in the 1970s, the period of time when much of the research on the health effects of THC was performed [112]. In a 2001 review article, Ashton Gold is quoted from his paper in 1991: "This single fact has made obsolete much of what we once knew about the risks and consequences of marijuana use" [112, 113].

Some of the common street names for cannabis are bongo, buddha sticks, and ganja [12]. Cannabis can be smoked as hand-rolled cigarettes ("a joint") or from a variety of pipes including the water pump ("a bong"); hashish can be eaten when baked in cookies and cakes or be mixed with tobacco and smoked; hash oil is spread on the paper wrapping of cigarettes and smoked; yet, because of water insolubility,

cannabis is not suitable for intravenous use [111]. Cannabis exerts its mind-altering effects by interaction with endogenous neuronal receptor CB_1 located in the CNS, the cerebral cortex, the limbic areas, the basal ganglia, the cerebellum, the thalamus, and the brain stem; cannabis also has an endogenous ligand called anandamide with a high affinity for the CB_1 receptor [114–116]. Additionally, cannabis has immunosuppressive properties because of its interaction with another receptor, CB_2, found on immune cells such as macrophages in the spleen and also the B- and T-cell lymphocytes [117]. Medically, cannabis is used as an antiemetic, an appetite stimulant, and a pain reliever in the treatment of cancer and AIDS and in the management of glaucoma and neurologic conditions such as epilepsy, migraine, and bipolar disorder [116]. The nonmedical use of cannabis leads to a wide range of mood changes such as euphoria, relaxation, hallucination, confusion, and disorientation—effects that may be attributed to an additional interaction of THC on dopamine release from the nucleus accumbens and prefrontal cortex [112].

There is evidence for a potential relationship between cannabis and cancer development in the lungs and the oropharyngeal tissues. Other than nicotine and along with carbon monoxides and other bronchial irritants, the smoke from the herbal cannabis preparations contains the same carcinogens, the polycyclic aromatic hydrocarbons benzanthracenes and benzopyrenes, as the smoke from tobacco cigarettes [112]. The THC in cannabis has tumorigenic properties through promoting the transcription of P4501A1 (CYP1A1), an enzyme capable of converting the polycyclic aromatic hydrocarbons into carcinogens, as well as the formation of reactive oxygen species and also immunologic suppression [118]. On the other hand, laboratory studies have shown that THC may also possess protective antitumor properties by inducing apoptosis in several different human cancer-cell lines [119]. Taken together, the net effect of cannabis use on cancer development appears to be quite complex.

Compared to smoking tobacco cigarettes and because of the way it is inhaled, smoking cannabis exposes the person to a greater volume and a longer period of exposure to the smoke. In fact, smoking 3–4 cannabis cigarettes per day is associated with the same level of bronchial damage as 20 tobacco cigarettes per day [120]. Moreover, bronchial biopsies of marijuana smokers show more molecular abnormalities in Ki-67, EGFR, and p53 than nonsmokers [121], suggesting field cancerization effects on the bronchial epithelium and possibly the entire aerodigestive tract [119].

Studies of patients with head and neck cancers have shown marijuana use to be prevalent [122–124], and oral premalignant lesions such as leukoplakia and erythroplakia have also been reported among regular marijuana users [125]. One case-control study of 173 subjects with oral squamous carcinoma of the head and neck and 176 cancer-free controls showed the risk of cancer to be 2.6 times higher among marijuana users [126]. This positive association was refuted by a population-based case-control study of 407 individuals with head and neck cancer and 615 control subject where the use of marijuana did not show any association with oral cancer diagnosis or the molecular abnormalities in glutathione S-transferase genes (known to be involved in biotransformation of polycyclic aromatic hydrocarbons), while a

combined effect was observed with cigarette smoking and alcohol use [127]. In the latter study, however, the subjects were not long-term marijuana users. A pooled analysis of nine case-control studies from the United States and Latin America showed the association of marijuana use with head and neck cancer to differ by tumor site, with an increased risk for oropharyngeal cancer and a reduced risk for tongue cancer [128]. A recent review of 11 epidemiologic studies on all cancers confirmed the conflicting results among these studies for the effect of marijuana use and aerodigestive tract cancers [129]. It is clear that well-designed longitudinal studies of long-term cannabis users, separating the impact of smoking tobacco, alcohol use, and HPV infection, are necessary to show the true impact of cannabis use on oral cancer development at the population level.

Other oral health implications of cannabis use are xerostomia and poor oral hygiene [94, 117, 125, 130, 131] that may increase the risk of caries and periodontal disease. Other reported findings include multiple oral papillomas [132] and oral candidiasis [131, 133], both of which most likely related to the immunologic suppression effects of cannabis and also the hydrocarbons in cannabis acting as an energy source for candida growth [133].

Cases of gingival hyperplasia have also been reported with cannabis use [134] (Figs. 14.10 and 14.11).

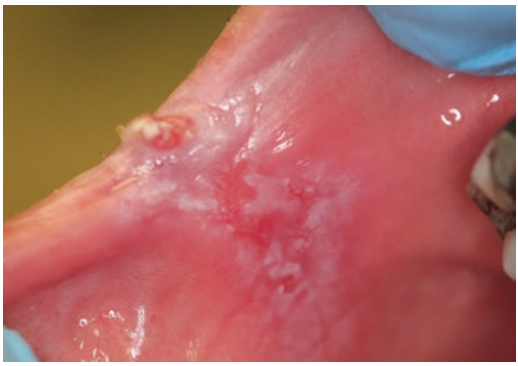

Fig. 14.10 Papillomatous lesion and leukoplakia in the inner aspect of the labial commissure in a man with a 30–40 per day cannabis cigarettes use

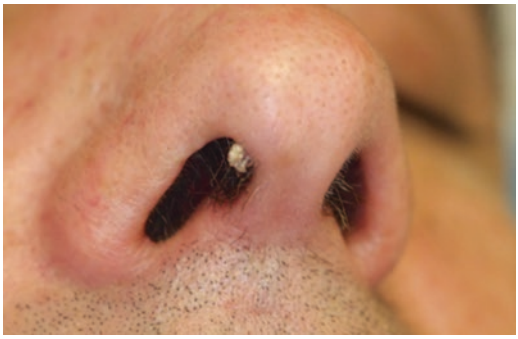

Fig. 14.11 Papillomatous lesion nasal mucosa in a man with a 30–40 per day cannabis cigarettes use

14.5 Summary

Illicit drug use and dependence are associated with significant systemic and oral sequelae. Oral presentations are mostly seen in the dentition with very high caries activity and/or periodontal disease. Dental breakdown in most instances is due to drug-induced xerostomia, but other factors such as diet and especially the high use of carbohydrates also play major roles. Other oral presentations of concern are pre-malignant and dysplastic lesions that occur with certain drugs such as cannabis. Patients who enter recovery programs for their drug dependence should not only be screened for HIV infection and other STDs, they should also receive an assessment by a dental professional for the diagnosis and treatment of drug-related dental and soft tissue pathologies and also instructions for appropriate oral hygiene practices and home care.

Conflict of interest The authors report no conflicts of interest.

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Chapter 15
Socioepidemiology of Injection Drug Users in Miami and HIV-1B Envelope (V1–V5) Genetic Diversity: A Preliminary Study

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Keywords Socioepidemiology • Relationship • Network • Risk locale • Risk behavior • Injection drug user (IDU) • Sexual risk • Psychiatric morbidities • HIV-1B infection • Envelope • ENV V1-V5 hypervariable domains • Nucleotide sequence • Cluster • Phylogenetic analysis (phylogeny) • Protein sequence • Molecular similarity • Glycosylation • Entropy

Core Message

Injection drug use is a major risk behavior associated with transmission of HIV-1B.

Sociocultural and socioepidemiological studies are required to characterize networks of injection drug users (IDUs).

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The possibility of links between subject socioepidemiology and viral sequence diversity, phylogenetic relationships, signatures, thermodynamics, and glycosylation patterns is studied.

This chapter addresses whether in risk locales where many people inject together, there are variations in probability of relatedness of HIV-1B env sequences.

15.1 Introduction

What became known as HIV-1 was discovered and first isolated in three different laboratories, namely of Drs. J. Levy, L. Montagnier, and R.C. Gallo [1–4]. The complex distribution of human immunodeficiency virus type 1B (HIV-1B) genotypic variants, or quasispecies, within infected individuals has been characterized extensively [5]. There are many potential routes of HIV-1B transmission, including parenteral (injection drug use, injection drug user (IDU), heterosexual and homosexual sexual transmission), blood transfusion, and perinatal (*prepartum* and *postpartum*). Upon transmission, some studies indicate that the major variant found in the donor is transmitted [6], while others report transmission of a minor variant [7–9]. For either alternative, the person newly infected through these routes has an HIV-1B sequence population that is initially homogeneous [7, 10, 11], irrespective of the heterogeneity of the donor's sequences. With time, the HIV-1B high mutation rate and selective pressures generate heterogeneous populations, quasispecies, or sequence variant clouds [8, 12–19].

Sequence heterogeneity in the envelope region occurs primarily in the hypervariable domains, designated V1–V5, which has attracted much attention for characterizing genotypic and phenotypic variants [20]. Because the env gene is particularly prone to mutation [21], it has proven invaluable in molecular epidemiological studies tracing the patterns of disease transmission and progression [9]. Several analytical approaches have been implemented using different variable domains of the HIV-1B ENV gene to investigate viral transmission: genetic distances, phylogenetic trees, and sequence signature patterns. Phylogenetic analysis has been used to establish the likelihood of HIV-1B transmission from infected health care workers to patients [11, 14]. Only recently, has such analysis been used to determine whether different transmission groups possess characteristic variants [22–24]. Risk group-associated variations in sequence signatures have been described between drug user, transfusion, and homo-

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sexual/hemophiliac DNA sequences within the V3 loop domain [25, 26]. Mother-infant transmissions reveal that the V3 sequences are much more heterogeneous in the mother than in the infant after birth. The amino acid sequence signatures correlated with linked vertical transmission between these pairs [27]. Earlier in the epidemic, two predominant genotypes of HIV-1B (Thai A and B) were known to exist in Thailand [28]; interestingly, they showed in studies of the V3 loop region that there had been independent introduction of the virus into two high-risk populations distinguished by mode of transmission (sexual activity vs intravenous drug use). Characterization of recombinant segments in the C2–V3 region demonstrated transmission between both individuals within a sexual risk couple [7]. In addition, dual transmission of HIV-1B and C subtypes was detected in a family, husband to wife to child [29].

Injection drug use is a primary risk behavior associated with transmission of HIV [30–52]. Many sociological and epidemiological characterizations of drug abuse and HIV infection networks include NIDA. There have been many detailed studies characterizing the IDU and sexual transmission of HIV-1B and other epidemiologically linked viruses including HCV. Detailed molecular studies identified and tracked virus strains in well-defined drug abuse networks as part of sociological and epidemiological studies.

One factor that complicates deducing HIV infection-related risk networks is the infectivity potential of virus strains. This involves inferring the probability that various virus strains infect new hosts after each single exposure due to use of contaminated drug injection needle-syringe, cotton, cooker, and washwater. The CDC reports that the average probability of HIV infection is 0.33 per 100 needlestick or cut exposures[1]. Consequently, the establishment of risk networks in which a single V1–V5 HIV-1B strain variant dominates is unlikely. There is also a lowered chance of transmission of any given strain, especially in circumstances of multiple IDUs injecting in the same risk locale. However, long-term intimate relations among socially characterized participants warrant a higher likelihood of exhibiting detectable clustering of HIV strains with increased sequence relatedness than diffusely distributed networks [53–57].

In this study, we characterized and defined the sociodynamics of four networks of seropositive IDUs. In addition, we characterized the viral sequence within specific hypervariable domains (V1–V5) of 37 HIV ENV genes derived from the IDUs.

15.2 Materials and Methods

15.2.1 Network Epidemiology

Epidemiological methods for assessment and outreach of individuals who inject drugs included several approaches and protocols. Four epidemiological networks linked by sexual interactions and/or IDU behavior were studied (Table 15.1).

[1] For comparison, the average risks for HCV and HBV infections are 1.8 per 100 and 6–30 per 100 needlestick or cutaneous cut exposures, respectively [53].

Table 15.1 Patient HIV-1B IDU demographics

Participant	Gender	Risk locale[a,b]	Risk behavior	Drugs used	Date of blood draw	HIV-1B DNA clones sequenced
1001	Female	Overtown	IDU and sex	Heroin and crack cocaine	8-7-1995 8-29-1996	3
1002	Male	Overtown	IDU and sex	Heroin and crack cocaine	8-7-1995 8-29-1996	5
1003	Female	Overtown	IDU and sex	Heroin and Dilaudid	8-21-1995 8-29-1996	2
1004	Male	Overtown	IDU and sex	Heroin	8-21-1995 8-30-1996	4 5
1005	Female	Overtown	IDU and sex	Heroin	9-20-1995 8-30-1996	4
1006	Male	Overtown	IDU and sex	Heroin and cocaine	10-13-1995 11-15-1996	8 2
1008	Female	Opa-Locka	IDU	Heroin and cocaine	2-8-1996	2
1011	Female	Opa-Locka	IDU	Heroin	3-7-1996	
1012	Male	Liberty City	IDU	Heroin	3-8-1996	
1013	Male	Liberty City	IDU	Heroin	3-5-1996	
1014	Male	Liberty City	IDU	Heroin	3-29-1996	
1015	Female	Opa-Locka	IDU	Heroin	4-9-1996	2
1017	Male	Liberty City	IDU	Heroin	11-15-1996	
1018	Male	Liberty City	IDU	Heroin	11-15-1996	
1019	Male	Liberty City	IDU	Heroin	11-15-1996	

This study was performed in Miami (Dade County, Florida, USA)

[a]Risk locale is the neighborhood where the participants carried out risk activities

[b]It should be noted that IDU risk activity involves the use of cottons, cookers, rinse waters, and injection needle-syringes. Paraphernalia used at shooting galleries have been shown to be HIV-1B contaminated [32, 42, 82, 83]

Fieldwork with questionnaires was used to investigate injection drug use in Miami. Factors including frequency of participation in shooting galleries, high-risk contacts, injection drug use, sex, gender, age, and mobility were characterized to elucidate the dynamic temporal and spatial relationships between risk and network members [30, 36, 37, 42–46, 48, 58, 59]. Confidentiality was strictly maintained throughout this work. The laboratory studies were all done devoid of any personal identifiers. The Internal Review Board (IRB) rules of the University of Miami were all maintained and strictly enforced. Human subject approvals were obtained at the time of these studies from the IRB at the University of Miami.

15.2.2 Specimens

Blood was obtained from 15 heterosexual HIV-positive subjects (Tables 15.1 and 15.2). Blood samples were obtained for individuals in networks 1, 2, and 3 at the same initial time, whereas those in network 4 were obtained 14 months later. Follow-up samples were subsequently obtained 12 months afterward for members of the first three networks. Blood was obtained from study individuals using standard EDTA-containing vacutainer tubes. Peripheral blood lymphocyte (PBMNC) pellets were produced using Ficoll–Hypaque density gradient centrifugation and were cryopreserved at −85 °C until needed [15–17, 19].

15.2.3 Polymerase Chain Reaction

Genomic DNA was extracted from the cryopreserved PBMC pellets using DNA isolation kits (United States Biochemicals, Cleveland, OH) and subsequently precipitated with ethanol and resuspended in Tris-EDTA (THE) buffer (pH 7.5).

Table 15.2 Patient networks (15 participants, 4 networks)

Network	Risk locale	Patient	Duration in network[a]	Additional information
1	Overtown	1001, 1002	4y IDU and sex	Recent couple
2	Overtown	1003, 1004, 1005, 1006	10y IDU	Couples with greater than 8 years of relations
		1003, 1004	13y IDU and sex	
		1005, 1006	8y IDU and sex	
3	Opa-Locka	1008, 1011	20y IDU	Female support group
		1015, 1011	20y IDU	
4	Liberty City	1017, 1018	20y IDU	Brothers
		1017, 1019	20y IDU	Brothers
		1012, 1013, 1014	15y IDU	Running partners

[a]*y* years, duration of risk activity

The V1–V5 region of the HIV-1B ENV gene was amplified using two rounds of PCR with a set of nested primers as previously described [19]. For the first PCR, we used approximately 1 mg genomic DNA in a 100 ul reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 20 nmoles (200 mM) each of dNTPs, 2.5 U AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA), and primers XPR1 (GGGATCAAAGCCTAAAGCCA, sense, nucleotide positions 6558–6577 in HXB2) and XPR7 (ACTTCTCCAATTGTCCCTCA, antisense, positions 7647–7666). Amplification consisted of a hot start (95 °C, 5′), then 30 cycles of 94 °C, 0′35″; 60 °C, 1′35″; and 72 °C, 2′35″, followed by a final extension (72 °C, 10′). For the nested PCR reaction, primers XPR2 (GAATTCACCCCACTCTGTGTTA, sense, positions 6590–6605) and XPR6 (AAGCTTCTCCTCCAGGTCTGA, antisense, positions 7625–7639) were used to amplify 1 ml of the first PCR reaction under the same conditions as above. The size of the PCR amplicon was determined by analysis on 1.2% agarose gel-1X TAE in 0.5-mg/ml ethidium bromide. Typically, a single PCR band was observed using only 10 ul of the nested PCR reaction.

Negative controls consisted of a water sample (instead of peripheral blood mononuclear cells), RT reaction mixtures without added reverse transcriptase, and reagent controls run in parallel with the tested samples [15–17, 19].

To minimize the possibility of carryover contamination, separate rooms (neither connected by airflow nor by air conditioning) were always used for the addition of reagents and sample DNA template vs performance of PCR and the handling of PCR products. Only one subject's sample was ever handled at any time during processing as well as subsequent amplification and cloning steps. In addition, sentinel tubes were evaluated weekly for contamination. Several subjects had blood samples redrawn after 12 months so that cloned sequences could be compared to verify phylogenetic relationships and to rule out sample mix-up and/or contamination [15–17, 19]. Sequence integrity was analyzed – Rodrigo and Learn [60] produced a review of several methods.

15.2.4 Molecular Cloning and DNA Sequencing

The PCR product was purified and concentrated using the High Pure PCR Product Purification Kit (Boehringer Mannheim, Mannheim, Germany) and quantified by fluorescence (Hoefer Instruments, San Francisco, CA). The amplified product (40 ng) was subcloned into pCR2.1 vector using the TA cloning kit (Invitrogen, San Diego, CA). An aliquot of the ligation reaction (110 ng) was then used to transform INVaF' cells. Plasmid DNA was isolated from positive clones, which were selected by kanamycin resistance and lacZa complementation (blue/white), using the Wizard Miniprep (Promega Corporation, Madison, WI). After digestion with EcoRI, the size of the cloned insert DNA was verified by agarose gel electrophoresis. Glycerol stocks of positive clones were prepared for long-term storage. A variety of sequencing primers both internal and external to the cloned fragment were used to sequence the primary and complementary strands (ACGT Inc., Northbrook, IL) [15–17, 19]. Clone designation: number designates subject ID; "L" designates blood, followed

by clone number; and then "D" or "R" designates DNA or RNA nucleic acid source, respectively. The addition of "+12", e.g., 1004 + 12L4D, designates follow-up samples, obtained 12 months after the initial samples.

15.2.5 Sequence Analysis

15.2.5.1 DNA and Protein Alignments

The env sequences of HIV-1B IDUs from two different risk locales such as Overtown and Opa-Locka, Dade County, Florida (Table 15.2), made a total of 37 nucleotide sequences, and their corresponding proteins were also used for sequence alignments. The alignment files were generated using Clustal X 1.83 [61] with default parameters. Both intra- and intermolecular sequence variations were analyzed using Clustal X. The sequence variations and consensus patterns (signatures) were displayed using Weblogo 2.8.2 http://weblogo.berkeley.edu/, [62].

15.2.5.2 Entropy Analysis

Shannon entropy [63] is a simple quantitative measure in uncertainty units in a dataset and was used to measure the variation in DNA and protein sequence alignments. Entropy calculation was done for each position of the input sequence set. It can be used as a measure of the relative variation in the regions of an aligned gene or protein. The online web server available at the Los Alamos National Laboratories (LANL) (Los Alamos, NM) (http://www.lanl.gov) and (https://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html) were used.

15.2.5.3 Neutrality Hypothesis

The null hypothesis assumes that in most protein-coding genes, the number of synonymous nucleotide substitutions per site (d_S) is equal to the number of nonsynonymous nucleotide substitutions per site (d_N), i.e., (H_0: $d_N = d_S$), and the alternate hypothesis is (H_1: $d_N \neq d_S$). The probability (P) of rejecting the null hypothesis of strict neutrality ($d_N = d_S$) is less than 0.05, considered significant at the 5% level. This may favor the alternative hypothesis. The variance of the difference was computed using the bootstrap method with 500 replicates [64]. Analyses were conducted using the Nei-Gojobori method [65] in MEGA5 [66]. All positions containing gaps and missing data were eliminated from the dataset. Tajima's test of neutrality [67] was conducted using MEGA5 to compare the number of segregating sites per site with the nucleotide diversity. An essential parameter in the theory of neutral evolution is "4Nu," where "N" is the effective population size and "u" is the mutation rate per site. The difference in the estimate obtained provides an indication of non-neutral evolution. Codon-based Z-test was also conducted using MEGA5 to

test whether positive selection is operating on a gene by comparing the relative abundance of synonymous and non-synonymous substitutions that have occurred in the gene sequences.

15.2.5.4 Estimates of Transition/Transversion Bias

Evolutionary analyses were conducted in MEGA5 [66]. Two statistical methods such as maximum likelihood (ML) and maximum composite likelihood (MCL) were used. Substitution pattern and rates were estimated for nucleotide sequences using Tamura-Nei model (+G) [68, 69]. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+*G*], parameter = 0.6115). All positions containing gaps and missing data were eliminated. The analysis involved 37 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding.

15.2.5.5 Disparity Index Test

A simple statistic (disparity index test) measures the homogeneity of substitution patterns between a pair of sequences [70]. It works by randomly comparing the nucleotide (or amino acid) frequencies of the two descendent sequences and using the number of observed differences between them. MEGA5 computed the disparity index per site, which is given by the total disparity index between two sequences divided by the number of positions compared, excluding gaps and missing data. It is more powerful than a chi-square test of the equality of base frequencies between sequences (http://www.megasoftware.net/manual.pdf). The test was performed to infer substitution pattern homogeneity on pairwise nucleotide sequence comparisons. Monte Carlo simulations with 500 replicates were computed. All positions containing gaps and missing data were eliminated.

15.2.5.6 Molecular Relatedness and Phylogenetic Analysis

Neighbor-joining (NJ) method was used to generate phylogenetic trees. MCL method assumed substitutions included transitions and transversions. Substitutions among lineages were treated as uniform rates, and the pattern among lineages was set homogeneous. All positions containing gaps and missing data were deleted. Codon positions included were 1st + 2nd + 3rd + Noncoding [69].

The generated sequence alignment files were imported from Clustal X to SplitsTree4 version 4.13.1, built 16 Apr 2013 [71], to compute splits as well as to infer variations among the sequences. The nucleotide dataset (37 sequences, each 1086 bp) had 578 constant sites, 277 non-parsimony-informative sites, and 196 gapped sites (no missing data). Whereas the translated proteins of the nucleotide dataset (37 sequences, each 367 residues) had 157 constant sites, 127 non-

parsimony-informative sites, and 71 gapped sites (no missing data). The distances were computed from characters (nucleotides) with default parameters, and then splits were generated from distances using neighbor-net [72] approach to produce a set of circular splits on the network as defined in split decomposition [73], and the amino acid distances used a neighbor-net variance approach performed using ordinary least squares.

15.2.5.7 Confidence in the Resulting Phylogenetic Trees

Confidence in the resulting phylogenetic trees was assessed using bootstrap analysis [64]. One thousand bootstrap replicates were generated to assess the reliability of each edge in the tree as well as the network.

15.2.5.8 Phi Test for Recombination

The characters were analyzed using Phi test [74] and found informative sites using window size of 100.

15.2.6 Nucleotide Sequence Accession Numbers

Sequences were submitted to GenBank and the Los Alamos National Laboratory HIV sequence database, and the accession numbers obtained are KT984127–KT984163 (Table 15.3).

15.3 Results

15.3.1 Socioepidemiology

The four IDU networks consisted of (i) a male/female dyad (subjects 1002/1001) with sexual relations and shared IDU habits for 4 years; (ii) two separate male/female dyads each of whom maintained sexual relationships for 13 years (subjects 1004/1003) and 8 years (subjects 1006/1005) and all of whom have shared IDU habits for 10 years; (iii) a female triad (subjects 1008, 1011, and 1015) with shared IDU habits for over 20 years; and (iv) a familial triad (subjects 1017, 1018, and 1019) with shared IDU habits. Shared IDU habits would include common use of needle-syringes, cookers, cottons, and rinse water. All individuals in these networks were located in Dade County, Florida. Subjects in networks 1 and 2 resided in Overtown (Dade County, Florida) that was a separate locale from those in network 3 who lived in Opa-Locka (Dade County, Florida) (Tables 15.1 and 15.2, Fig. 15.1).

Table 15.3 HIV-1B ENV clones (37 sequences)

Patients	Clone identification	NCBI accession numbers
1001	1001L6D	KT984127
	1001L7D	KT984128
	1001L8D	KT984129
1002	1002L1D	KT984130
	1002L2D	KT984131
	1002L3D	KT984132
	1002L5D	KT984133
	1002L7D	KT984134
1003	1003L4D	KT984135
	1003L8D	KT984136
1004	1004L1D	KT984137
	1004L2D	KT984138
	1004L4D	KT984139
	1004L5D	KT984140
	1004_12L1D	KT984142
	1004_12L4D	KT984143
	1004_12L8D	KT984144
	1004_12L9D	KT984145
	1004_12L11D	KT984141
1005	1005L1D	KT984146
	1005L2D	KT984147
	1005L3D	KT984148
	1005L4D	KT984149
1006	1006L1R	KT984152
	1006L2R	KT984153
	1006L3R	KT984154
	1006L4R	KT984155
	1006L5R	KT984156
	1006L6R	KT984157
	1006L7R	KT984158
	1006L8R	KT984159
	1006_L12R	KT984150
	1006_L13R	KT984151
1008	1008L4D	KT984160
	1008L5D	KT984161
1015	1015_12L6D	KT984163
	1015_12L12	KT984162

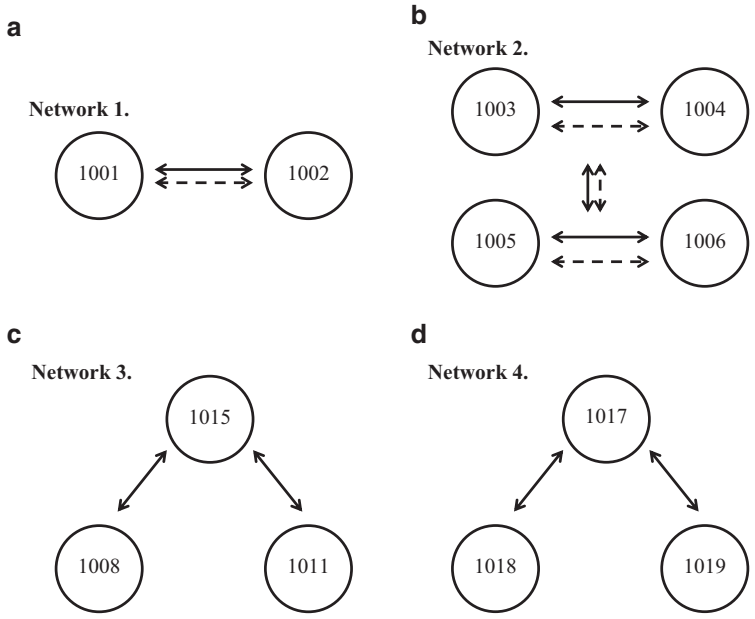

Fig. 15.1 (**a–d**) *Four socioepidemiological networks.* Refer to Tables 15.1 and 15.2 for additional information. *IDU* = injection drug use with sharing needle-syringes, cottons, cookers, and wash-waters. *Solid arrow* = IDU. *Dotted arrow* = sex

15.3.2 DNA and Protein Alignments

Nucleotide variations were analyzed based on sequence alignments and are presented in Fig. 15.2a. The transition/transversion bias was also estimated (Table 15.4). The protein translations of the variable regions (V1–V5) showed lesser variation in the V1 loop in comparison with the published global sequence variations of HIV-1 B isolates of blood and brain [75], whereas V2 loop is variable but not hypervariable. However, V3 is hypervariable especially between 182 and 190 and after the starting residues CTRP (Fig. 15.2b). The variable region (V4) is less variable compared to blood env proteins [75].

The overall amino acid variation in V1–V4 is lesser in comparison with blood isolates, and the variations are moderate in brain isolates. The differences in the variability of V1–V4 may be due to the accessibility of loops that may reflect mutations to escape from the immune system as well as in vivo variation in biological properties, such as tropism for macrophages or other cell types or ability to form syncytia [76, 77]. The analyzed dataset had a partial V5 start region. Hence, gp41 start, fusion peptide, and immunodominant regions were not available for further comparison.

It was observed that the residues in the start and end of the variable regions (V1–V4) are well conserved (Fig. 15.2b). There were no differences observed in the

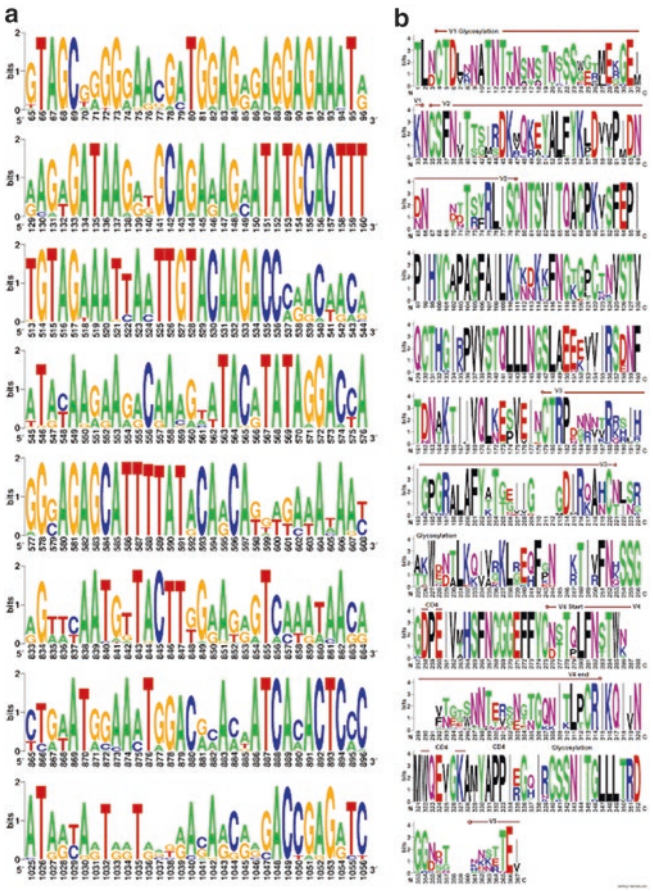

Fig. 15.2 (**a**) Env coding genes V1–V4 of HIV-1B IDU patients; (**b**) The translated env proteins show sequence conservations (*tall characters*), semi-conserved substitutions (*stacked with similar colored characters*), and variations (*stacked with different colors*). The annotation is based on the benchmark HXB2 sequence (accession K03455) provided in HIV Sequence Compendium [90]

Table 15.4 Maximum composite likelihood estimate of the pattern of nucleotide substitution

	A	T	C	G
A	–	*3.43*	*2.34*	**14.53**
T	*5.37*	–	**12.11**	*2.81*
C	*5.37*	**17.73**	–	*2.81*
G	**27.73**	*3.43*	*2.34*	–

Note: Each entry shows the probability of substitution (r) from one base (row) to another base (column [69]). For simplicity, the sum of *r* values is made equal to 100. Rates of different transitional substitutions are shown in **bold**, and those of transversional substitutions are shown in *italics*. The nucleotide frequencies are 38.48% (A), 24.57% (T/U), 20.17% (C), and 16.78% (G). The transition/transversion rate ratios are $k_1 = 5.165$ (purines) and $k_2 = 5.172$ (pyrimidines). The overall transition/transversion bias is $R = 2.532$, where $R = [A*G*k_1 + T*C*k_2]/[(A + G)*(T + C)]$

glycosylation patterns and CD4 regions of the analyzed dataset as in blood- and brain-derived Env sequences of HIV-1, as it recognizes the same CD4 receptor in all strains of HIV. The CD4 located between V3 and V4 is sequentially flanked by well-conserved residues from positions ranging between 254 and 274, except a single residue variation at 263. The most conserved residues throughout the sequences (V1–V4) are A, D, E, F, G, K, L, M, N, P, R, and W.

Intra- and inter-sample variations were compared using protein sequence alignment. The intra-clonal variations of patient (1005) shows a variation at the 93rd position for the clone 1005L2D (instead of "F" it is "S"). This variation occurs only in this clone. The clones (L4D and L5D) of the subject 1008, at position 120, "S" were found instead of "K," whereas in the following patients 1004 (L1D, L2D, 12L1D, and 12L11D) and 1006 (L5R, L6R, L7R, and L8R), it is "T," and the rest it is "K." In the subject (1004) at position 125, "T" is replaced with "Q" in L1D, L2D, 12L4D, and 12L9D, whereas in L4D, L5D, 12L1D, and 12L8D, it is replaced by "R." At position 155, the subjects 1001, 1002, and 1005 had "K," whereas the rest (1003, 1004, 1006, 1008, and 1015) it was replaced by "E," except two clones of 1004 (L4D and L5D), where "E" is replaced with "G." At position 186, the subject 1004 (all clones) had "Y," whereas the subjects 1001, 1002, and 1005 had "G," and in others 1003, 1006, 1008, and 1015, it is replaced by "N." At positions 200–210, there were some insertions and deletions only for 1004, and for all others it is absent. There are some sequence-specific signatures between 290 and 310; for 1001, 1002, and 1005, it is "FNGTWNNTERSNT"; for 1003 it is "NNNTWNSPNRLNS"; for 1008 it is "STSINANNTEGNE"; for 1004 it is "VTGESNNTVGNG" except for 12L1D and 12L11D ("GTEMSVENDT" and "FTRESNNTVGNGT"); for 1006 it is "VTEGSNNTEGN"; for 1015 it is "WSLNGTNTTNTNE." These unique subject-specific signatures can drive diversity at the molecular level.

15.3.3 Entropy Analysis

The sequence variations and conservations are analyzed using entropy plots for DNA (Fig. 15.3) and protein (Fig. 15.4). The entropy values >1 were observed at the following positions 23, 64, 70, 77, 200, 203, 336, 570, 601, 607, 608, 617, 721, 722, 744, 856, 869, 895–897, 900, 909, 1055, 1062, 1063, and 1070. The entropy values at positions 23, 200, and 203 were due to gapped alignment instead of variations. Similarly for the protein sequence alignment, the entropy values >1 were observed at following positions: 8 (N), 17 (N), 18 (S), 21 (I), 24 (W), 26 (R), 29 (K), 47 (M), 65, 70 (N), 71 (D), 123 (T), 184 (G), 186 (V), 187 (V), 189 (R), 190 (H), 203 (A), 205 (T), 206 (G), 226 (T), 228 (E), 236 (G), 239 (G), 243 (P), 253 (K), 263 (M), 286 (K), 292 (F), 294 (G), 295 (T), 296 (W), 300 (E), 301 (R), 302 (S), 304 (T), 337 (H), 356 (D), 357 (T), 361, 362 (N), 363 (K), and 364 (T). The entropy values at positions 65 and 361 were due to gapped alignment [63].

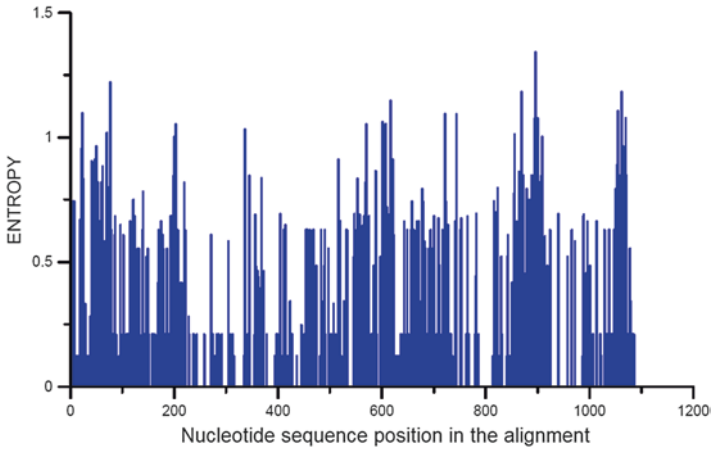

Fig. 15.3 Entropy plot of Env *nucleotide* sequence alignment. The plot is generated by comparing residue positions of the first sequence of the input (L6D_1001) with the rest

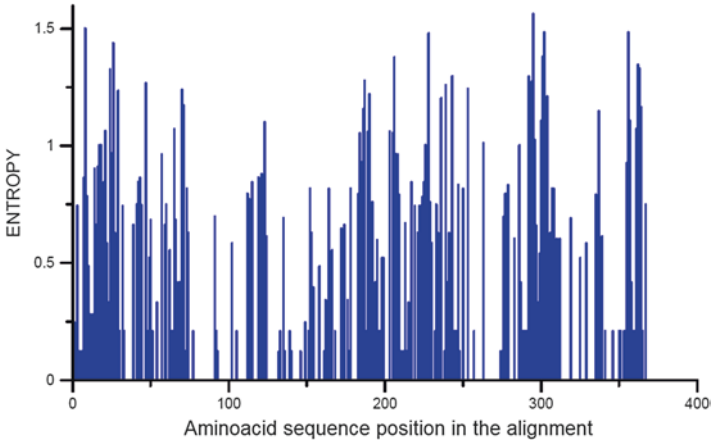

Fig. 15.4 Entropy plot of Env *protein* sequence alignment. The plot is generated by comparing residue positions of the first sequence of the input (L6D_1001) with the rest

15.3.4 Tajima's Neutrality Test

The analysis involved 37 nucleotide sequences (m), and the number of segregating sites (S) is 32, the nucleotide diversity ($\pi = 0.097$), and the Tajima's test statistic, $D = 0.593$. The positive test statistic reflects intermediate-frequency mutations, suggesting diversifying selection [78]. The HIV-1 sequences have not revealed evidence for natural selection in env [79]. Although the test assumes that nucleotides are equally mutable, it is not true for coding regions because the polymorphism is

not same for first, second, and third codon positions, and codon usage biases may further complicate the mutation pattern. The difference in the estimate obtained provides an indication of non-neutral evolution. Since Tajima's test is not very powerful and DNA polymorphisms are largely synonymous, it should be verified further with experimental work [80].

15.3.5 Codon-Based Z-Test of Selection

Only two random pair of clones (L2D 1005 vs L5D 1002) and (L2D 1004 vs L1D 1004) had probability (P)-value less than 0.05, i.e., 0.04 and 0.01, respectively, agreeing the null hypothesis. The rest of the sequence pairs suggest rejecting the null hypothesis of neutrality and preferring to accept the alternate hypothesis.

15.3.6 Estimates of Transition/Transversion Bias

There was a total of 890 transition/transversion positions in 37 nucleotide sequences. The estimated value of the shape parameter for the discrete gamma distribution is 0.6115. Mean evolutionary rates in these categories were 0.04, 0.21, 0.54, 1.16, and 3.05 substitutions per site. The nucleotide frequencies are $A = 38.48\%$, $T/U = 24.57\%$, $C = 16.78\%$, and $G = 20.17\%$. The maximum log likelihood for this computation was −3618.371. The ML-estimated transition/transversion bias (R) is 1.97, and MCL-estimated transition/transversion bias is $R = 2.532$ (Table 15.4).

15.3.7 Disparity Index Analysis

The substitution pattern between lineages was calculated by assuming that the sequences have evolved with the same evolutionary pattern of nucleotide substitution. The following sequences had disparity index (ID > 0) indicated evolutionary divergence between sequences based on composition bias. Therefore, we can reject the null hypothesis at the 5% level. The sequences of patients 1004 and 1006 (the clones are L1D 1004, L2D 1004, 12L1D 1004, 12L11D 1004, L5R 1006, L6R 1006, L3R 1006, L13R 1006, L4R 1006, L12R 1006, and L1R 1006.) had composition bias ($ID = 0.1$) in random comparison with patient 1005 (clone: L2D 1005), no bias with others. The sequences of patients 1001, 1002, and 1005 (their corresponding clones L8D 1001, L5D 1002, L3D 1002, L7D 1002, L2D 1002, L1D 1002, L7D 1001, L6D 1001, L4D 1005, L1D 1005, and L2D 1005) showed composition bias ($ID = 0.1$) with the patient 1005 (clone 12L6D 1015). A different clone of patient 1005 (12L12D 1015) showed composition bias ($ID = 0.1$) with the following sequences of patients 1001, 1002, and 1005 (their corresponding clones are L8D

1001, L5D 1002, L3D 1002, L7D 1002, L2D 1002, L1D 1002, L7D 1001, L6D 1001, L4D 1005, and L3D 1005). It is to be noted that for a couple of clonal sequences (L1D 1005 and L2D 1005) *ID* = 0.2, when compared with a patient clone 1015 (12L12D 1015). Thus, the test identified lineages and genes that are evolving with substantially different evolutionary processes as reflected in the atypical patterns of change [70].

15.3.8 Molecular Relatedness and Dendrogram

Dendrograms are suitable to display and infer evolutionary model assuming mutations and speciation events. A consensus tree is displayed after 1000 bootstrap replications (Fig. 15.5). However, it is well known that for some complex evolutionary scenarios involving gene loss, duplication, hybridization, horizontal gene transfer, or recombination, a dendrogram is not suitable for an appropriate representation of evolutionary events. Hence, the incompatible and ambiguous signals in the dataset (such as socioepidemiological data) were represented by split networks that provide only an "implicit" representation of evolutionary history [71]. The estimated proportion of invariant sites of nucleotides is 0.334 and for proteins is 0.427 [81].

As depicted in Fig. 15.1 (network 1), the IDU and sexual relationship between 1001 and 1002 agrees with the molecular connections as represented in the dendrogram with 92% confidence on the branch. The network 2 of the same figure is inconsistent with the molecular data, having 1003 and 1004 in two separate clusters with 100% confidence on the branch. Whereas the other subjects (1005 and 1006) were also distributed into two different clusters, 1005 is clustered along with 1001 at a confidence of 92%, and 1006 is entirely a unique cluster with 99% confidence. The network 3 is also in disagreement with the molecular data of 1015 and 1008. However, the sequence details of 1011 are required to confirm with 1015. (No molecular data is available to compare with network 4.)

The resulting nucleotide-based split network is showed in Fig. 15.6, and the protein-based split network is showed in Fig. 15.7. This pattern suggests that the dataset contains conflicting evolutionary signals (such as duration of IDUs, sexual relationship, hypervariable regions, recombination, risk locale, random genetic drift, etc.) and is consistent with the hypothesis of recombination events (refer, vi. Phi test) among the major lineages. Two of clones belonging to IDU patient (1004) appear isolated from the clusters, whereas few others clones of 1004 show sequence admixtures, preferably due to recombination events. Compared to all other subjects, 1004 cluster is more diverse and some clones converge. It is to be noted that split networks provide only an "implicit" picture of an evolutionary relationship and "nodes" in the "split network" do not represent ancestral species.

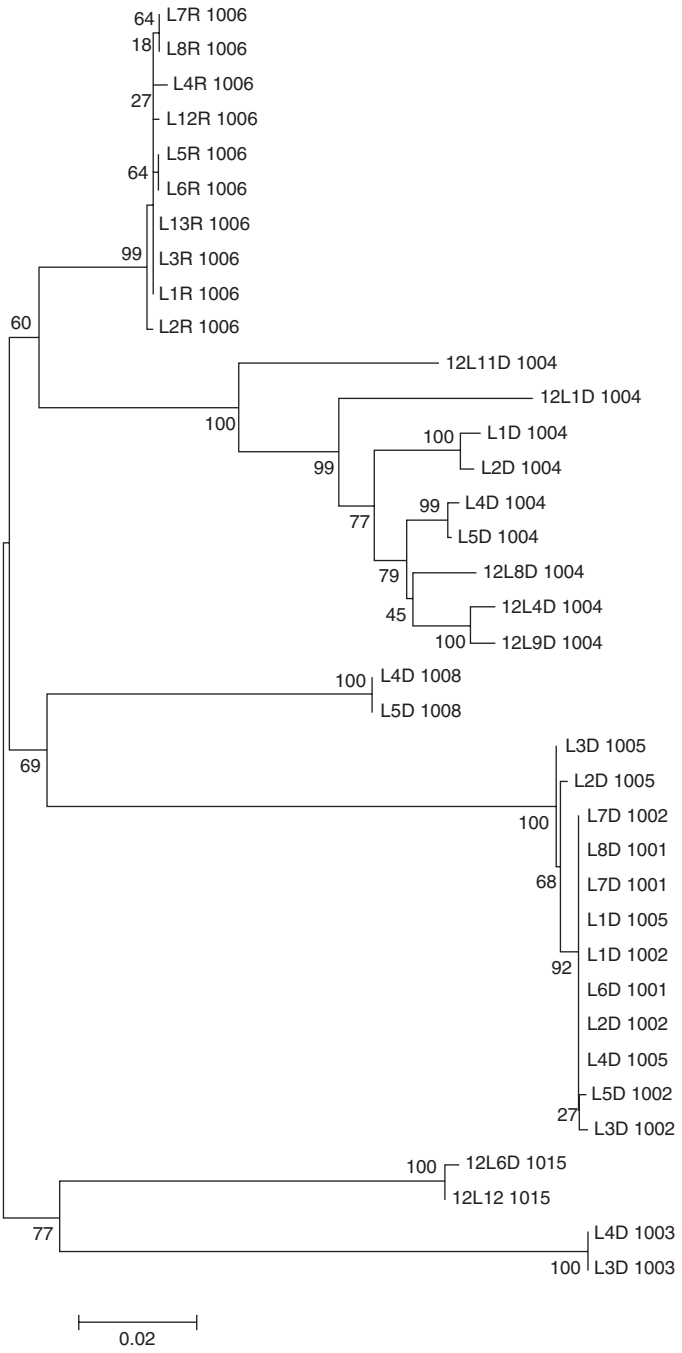

Fig. 15.5 Evolutionary relationships of HIV-1 B Env based on nucleotide alignment (37 sequences). The branching pattern was generated by the neighbor-joining (NJ) method, and the confidence of the clades was assessed by bootstrap values (n = 1000 replicates)

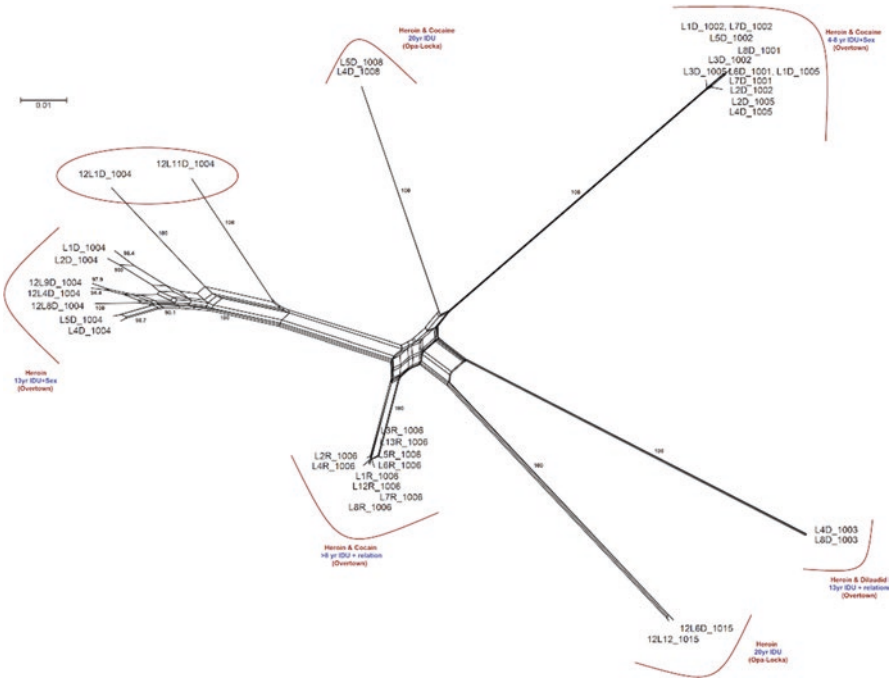

Fig. 15.6 A split decomposition network for 37 Env sequences of IDU HIV-1 B patients based on *nucleotide* sequence alignment, computed using the neighbor-net method with bootstrap values ($n = 1000$ replicates). The computed splits are displayed as a network with equal angle. Two of the IDU patient sequences appear isolated from the clusters and are encircled

15.3.9 Phi Test for Recombination

The nucleotide and amino acid characters were analyzed using Phi test [74]. The results are summarized in Table 15.5. Although the split network based on nucleotides and proteins had a similar tree topology (Figs. 15.6 and 15.7), only the network based on nucleotides showed significant evidence for recombination in the Phi test, which was not reflected in proteins.

15.4 Discussion

We address the question as to whether clustering observed in IDU networks reported in this chapter may reflect relatively concentrated shared co-injection behaviors. The dyads that we characterized injected with each other feasibly hundreds of times between 1982 and 1988, by which time all had seroconverted. The participants in the double dyad, additionally, had injected with each member of the group many

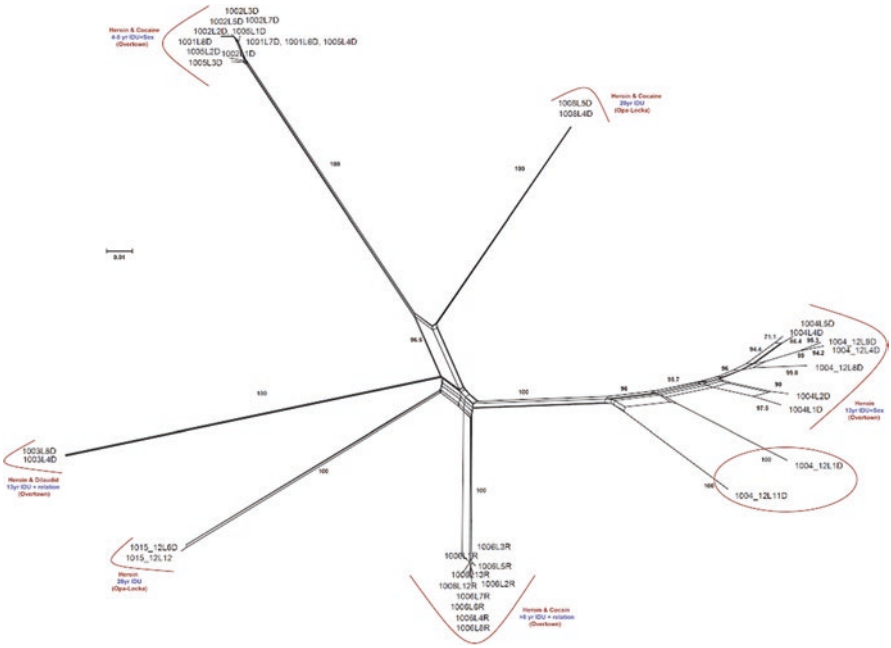

Fig. 15.7 A split decomposition network for 37 Env sequences of IDU HIV-1 B patients based on *protein* sequence alignment, computed using the neighbor-net method with bootstrap values ($n = 1000$ replicates). The computed splits are displayed as a network with equal angle. Two of the IDU patient sequences appear isolated from the clusters and are encircled

times. There is no evidence of direct interpersonal connections between participants 1005 and 1002 and 1001. It is relevant to note that the carrier of a founding HIV-1B infection need not be personally present when the next victim is exposed. That individual may no longer be on the premises; just his/her paraphernalia need be present for additional individuals to become HIV-1B infected with the strains at hand. These paraphernalia include contaminated needle-syringes, cottons, cookers, and washwaters, all generally used in the IDU venue. IDU paraphernalia have been extensively characterized in Miami-Dade County and shown to be contaminated with HIV-1B [42, 82, 83].

One observed condition in which this kind of exposure takes place is when a group of injectors arrives at a risk locale and uses needle-syringes and paraphernalia contaminated by the people who just finished ‘shooting up’ (injecting drugs). They use the contaminated available paraphernalia and syringes, not necessarily sharing them, but often not adequately cleansing them of prior contaminants. The primary hypothesis to be tested by sequencing ENV variable loops is, if we can expect sequence characteristics of strains of virus will reflect interpersonal risk behaviors characterized by sociocultural studies. In cases where people who run together (socialize) also inject together under private circumstances, we would expect clustering to reflect that fact. In cases where people who inject together do so in risk locales

Table 15.5 Phi test for recombination in the split network

Sl. No	Subject ID	Informative sites	*k*	Mean	Variance	Observed	*P*-value	Statistical significance
1.	1001–1006, 1008, and 1015 (genes)	360	33	0.899	6.703	0.080	1.190	Yes
2.	1003–1006 (genes)	286	27	0.692	7.459	0.056	7.143	Yes
3.	1003 and 1004 (genes)	192	18	0.053	1.160	0.039	1.695	Yes
4.	1005 and 1006 (genes)	116	11	–	–	–	–	No
5.	1008 and 1015 (genes)	131	13	–	–	–	–	No
6.	1001–1006, 1008, and 1015 (proteins)	168	46	0.090	2.008	0.090	0.497	No
7.	1001 and 1002 (proteins)	Non-informative	–	–	–	–	–	–
8.	1003 and 1004 (proteins)	105	30	0.029	7.989	0.030	0.595	No
9.	1005–1006 (proteins)	72	21	–	–	–	–	No
10.	1008 and 1015 (proteins)	81	23	–	–	–	–	No

where many inject, we would expect lower levels of clustering of virus strains. Additional study in more localities would aid in understanding the importance of shooting companions and shooting venues in the spread of HIV-1B (as well as additional viruses including HCV and HBV). Moreover, the extent to which individuals participate in out-of-network HIV-1B risk activity needs socioepidemiological comparisons and sequence relatedness characterization as well [42, 47, 48, 49, 84–89].

The full V1–V5 ENV domains are included in this study to characterize social effects and molecular changes. The structure and genomes of HIV strains are well known and on a firm basis for such studies [90]. For example, previous studies suggest that the overall tertiary conformation of the entire env protein may be important in tropism-determining activity. In particular, domains within the V1/V2 [91, 92] and/or V3 are critical determinants of macrophage cellular tropism [12, 25, 85, 91, 93–95]. A 94-amino acid domain, including the V3 loop, is involved with HIV-1B infection of macrophages being both necessary and sufficient for virus

entry [91, 93]. A mutation of residue 287 from a lysine to a glutamic acid converts a non-macrophagetropic isolate of HIV-1B to one capable of replicating in macrophages. In addition, the V3 domain is a 35–37-amino acid loop bounded by a pair of disulfide-bonded cysteine residues [96]. It forms two antiparallel beta turns and a short C-terminal alpha helix [97]. It is an epitope for neutralizing antibodies as well as cytotoxic T lymphocytes [98, 99].

Based on sequence analysis, specific signatures, transition/transversion bias, statistical test of neutrality, and molecular diversity as reflected in dendrograms, we conclude that there are genetic variations in V1–V4 region of HIV-1 B env. For example, disparity index confirmed composition bias in a couple of clones (12L1D_1004 and 12L11D_1004) belonging to 1004 with patient 1005 (clone: L2D 1005). This is very well reflected as isolated branches of split network. Similarly, the sequences of patients 1004 and 1006 had composition bias with patient 1005, and the sequences of patients 1001, 1002, and 1005 showed composition bias with the clone 12L6D of 1015 as reflected in the split network (Figs. 15.6 and 15.7), which confirms non-neutral evolution as indicated by Tajima's test. The unsystematic variations introduced by recombination may set an evidence for non-neutral evolution. It is also known that the natural selection is frequently masked by recombination and the natural selection over the env V1–V4 region had a minor role in driving diversity [76, 100, 101].

Finally, there is a non-intuitive and unexpected relationship between needle-syringe and paraphernalia sharing and psychiatric morbidities associated with IDU risk behavior. In the current volume of *Global Virology II*, Thames and Jones (Chap. 12) indicate that IDU needle-syringe and paraphernalia-sharing as well as reduced needle-syringe cleansing behaviors are associated with psychiatric comorbidities due to HIV-1 infection. These comorbidities include antisocial personality disorder (ASPD) and exhibit such as remorseless, impulsive, and irresponsible behaviors. Thames and Jones further report and discuss additional comorbidities associated with such behaviors including DSM-III axis II diagnosis as well as opioid and cocaine consumption. [102]. Additionally, marijuana use is also associated with such set of behaviors and conditions. [103].

15.5 Conclusions

The results described here may lead to new directions of understanding natural selection, random genetic drift, and recombination in the HIV-1B env protein as well as diversity during HIV-1 infections in a defined socioepidemiological context. Additional work is needed to characterize in detail the effects of differing risk activities including contemporaneously in the post-HAART, cART era as the world progresses into the next era of more advanced molecular and immunological therapies. Moreover, the contextual application of studies of IDU risk behaviors and molecular epidemiology should also include characterization of the associated psychiatric morbidities as well as the possible role of brain-related HIV-1 infections.

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Conflict of interest The authors report no conflicts of interest.

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Chapter 16
The Persistence of HIV-Associated Neurocognitive Disorder (HAND) in the Era of Combined Antiretroviral Therapy (cART)

Elyse J. Singer and Natasha M. Nemanim

Keywords Combined antiretroviral therapy • HIV–associated neurocognitive disorder • Dementia • Neurons • Macrophages • Viral persistence • Inflammation

Core Message
This chapter discusses four main issues: (1) the pathophysiology underlying human immunodeficiency virus (HIV) type 1 subtype B infection of the central nervous system (CNS) and its clinical manifestations, termed "HIV-associated neurocognitive disorders" or HAND; (2) how the clinical manifestations of HAND may persist despite reduction in viral replication with combined antiretroviral therapy (cART); (3) the reasons why current cART does not eradicate HIV in the CNS; and (4) why the clinical picture of HAND has been modified but HAND remains a serious and persistent problem.

16.1 Introduction: The HIV Epidemic and Its Neurological Consequences

Worldwide, approximately 34 million people are infected with human immunodeficiency virus (HIV) type 1 [1]. In 2014, the United States alone had more than 1.1 million people living with HIV infection, of which 12% were unaware of their HIV-seropositive (HIV+) status [2]. An estimated 40,000 new infections each year continue to expand the infected population [2]. In the United States, many HIV+

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individuals remain unlinked to care, do not receive combined antiretroviral therapy (cART), or fail to meet criteria for viral suppression [3]. The situation is far worse in countries with little or no public health infrastructure. Thus, HIV/AIDS and its consequences will likely remain a major problem for many years.

HAND/HAD Definition HIV targets the central nervous system (CNS) early and causes a broad spectrum of neurological disorders, including an aseptic meningitis, encephalitis, myelopathy, and peripheral neuropathy [4], in addition to the opportunistic infections (OI) and tumors related to immune suppression. The most important CNS complication of HIV is a unique syndrome that was identified early in the epidemic and has been termed "HIV encephalopathy," "AIDS dementia complex" (ADC), "HIV-associated dementia" (HAD), and more recently "HIV-associated neurocognitive disorder" (HAND) [5, 6]. The key feature of HAND is cognitive decline that may, or may not, be accompanied by clinically significant central motor and behavioral changes. The multiple changes in nomenclature over the course of the epidemic reflect changes in our understanding of the natural history of HIV in the CNS, as well as the ways in which cART has altered the HAND presentation and pathogenesis. The majority of information cited in this article comes from research performed in clade B-infected populations, unless specified otherwise.

16.2 HIV and the Pre-cART Era

Early in the epidemic, a progressive and potentially fatal dementia (termed ADC or AIDS encephalopathy and later HAD, the most severe form of HAND) was identified in persons with advanced, untreated HIV/AIDS [7]. In the pre-cART era, up to 15% of AIDS patients were estimated to develop such dementia before death [8]. Predictors of HAD included low CD4+ cell counts, high levels of viremia, anemia, and elevated beta-2 microglobulin (B2M) levels (an indicator of inflammation) [9, 10].

During the pre-cART era, the diagnosis of HAD was typically entertained in patients with advanced HIV/AIDS. These patients frequently suffered from comorbid medical conditions such as CNS OI (e.g., cytomegalovirus or CMV), CNS tumors, end-organ failure, sepsis, hypoxia, and delirium. All of these entities can confound the diagnosis of HAD in patients with altered cognition and behavioral changes. However, it was established that HIV infection alone is sufficient and capable of causing neurological deterioration even in the absence of comorbid conditions [11]. As more HIV+ patients were identified and prospectively studied in the preterminal phases of their disease, some individuals were noted to develop HAD relatively early in their course, as the sole or presenting sign of HIV/AIDS [12]. Investigators also reported that mild or subclinical forms of cognitive impairment, which did not meet the definition of a dementia, could be identified in a subset of HIV+ persons with relatively early systemic disease [13]. These clinical studies, as well as observations that HIV enters the CNS very early in infection [14, 15], even-

tually gave rise to the hypothesis that the pathological processes underlying HAD predate the clinical manifestations by months or years, similar to the course of other neurodegenerative conditions such as Alzheimer's disease.

16.3 Pre-cART Treatment of HIV and Subsequent Changes in HAND

In 1987, the Food and Drug Administration approved the first antiretroviral treatment for AIDS, zidovudine (AZT, Retrovir®). Two randomized, double-blind, placebo-controlled trials examined the effects of zidovudine monotherapy (administered at higher doses than are currently recommended) on neuropsychological test scores in patients with AIDS and AIDS-related complex [16] and in a smaller group of participants who had AIDS dementia complex [17]. Both studies showed improved test scores with treatment. However, the beneficial effects of monotherapy on the CNS were typically short-lived, in part due to the development of drug-resistant HIV.

In 1996, the introduction of cART (an antiretroviral regimen typically composed of at least three drugs) significantly slowed the evolution of drug-resistant virus and resulted in prolonged suppression of viral replication as measured by "undetectable" copy numbers of HIV RNA in plasma. The use of cART resulted in a significant improvement in immune function, as measured by rising CD4+ cells, fewer OIs, tumors, and constitutional symptoms, and a sharp drop in the overall morbidity and mortality of AIDS [18–20]. Investigators also reported an overall decline in new cases of HAD, the most severe form of HAND. An overall beneficial effect on cognition has been reported with virtually all cART regimens studied [21]. However, controversy remains over whether better neuroprotection is associated with cART regimens that include drugs with higher CNS penetration-effectiveness (CPE) or whether drugs with higher CPE should be used to treat individuals who manifest a persistent, resistant CNS infection with HIV detectable in CSF despite suppression in plasma, known as "CNS escape" [22, 23].

In contrast to the decline in cases of HAD, researchers have reported an increase in milder forms of cognitive impairment, indicating that the processes underlying HAD have been "attenuated" but not eradicated [24–26]. In 2007, the term HIV-associated neurocognitive disorders (HAND) was introduced as part of a new research nomenclature, the "Frascati criteria," for HIV [5]. This classification was intended to encompass the full spectrum of CNS disease by staging HAND as follows: a preclinical phase termed "asymptomatic neurocognitive impairment" (ANI), a "mild neurocognitive disorder" (MND), and an "HIV-associated dementia" (HAD). Key to the employment of this research-oriented classification system is the use of formal neuropsychological testing to assess the degree of cognitive dysfunction, the use of functional assessment to determine the impact on activities of daily living, and the ability to exclude other causes of cognitive impairment. Although this nomenclature represents an advance in respect to the previous classification systems, it does have several deficits as well. For example, it has been argued that

the Frascati criteria have the potential to overdiagnose HAND [27] and it may not be suitable in HIV+ children [28].

Using the Frascati criteria (or similar classifications), investigators have studied the prevalence of all forms of HAND in post-cART era research cohorts and have reported that the rate of milder neurocognitive impairment remains high. Depending on the study sample and methods used, impairment rates range from 18% of cART-treated HIV+ subjects who met criteria for viral suppression in an Australian cohort [29] to 37.5% of a cART-treated Thai cohort [30], 39% of an ACTG cohort (26% if more stringent standards were used) [31], and 52% of the participants in the CNS HIV Antiretroviral Therapy Effects Research (CHARTER) cohort (47% if severely confounded cases were excluded) [32]. In contrast, new cases of HAD, the most severe form of HAND, have declined sharply in cART-treated individuals [32]. The CHARTER investigators found that only 2.4% of their subjects were diagnosed with HAD versus approximately 33% who met criteria for ANI and 12% who met criteria for MND. The observation that HAND persists and even progresses despite cART is further supported by neuroimaging studies which demonstrate progressive brain abnormalities in cART-treated individuals [33, 34].

The persistence of even mild forms of cognitive impairment in HIV remains an important concern, for several reasons. First, the presence of ANI was reported to be predictive of symptomatic forms of cognitive impairment in the CHARTER and Multicenter AIDS Cohort Study (MACS) studies [35, 36]. In addition, even mild forms of impairment may impact life in an increasingly technological society where most adults are expected to drive in traffic, manage complex financial decisions, and use a computer. Mild forms of neurocognitive impairment can affect medication adherence, life span, driving, and other important activities of daily living [35, 37]. Even mild impairment in HIV+ patients may be a risk factor for mortality [38], which is in keeping with the effects of mild cognitive impairment caused by other forms of neurological disease [39]. Finally, the HIV+ cART-treated population is now expected to enjoy prolonged longevity [40], but the impact of mild HAND on the manifestations of age-related neurodegenerative conditions remains unknown.

16.4 HIV Neuropathology and Pathophysiology without cART Treatment

Brain autopsy series from the pre-cART era substantiated the hypothesis that up to 72% of HIV+ patients with the clinical manifestations of HAND during life (such as abnormal neuropsychological test performance) had findings consistent with HIVE at autopsy [41, 42]. These findings consisted of abnormalities such as perivascular infiltration of lymphocytes and macrophages, microglial nodules, multinucleated giant cells, astrogliosis, and white matter pallor or vacuolation [43], as well as the presence of HIV demonstrated by immunocytochemical and/or molecular techniques. These neuropathological findings reflect the activation of microglia

(resident macrophages of the brain), the recruitment of peripheral monocytes and macrophages into the CNS, and the proliferation of astrocytes following neuronal loss, as well as HIV replication [44].

Studies of HIV-infected brains localized productive HIV infection to CNS macrophages, microglia, and multinucleated giant cells [45]. Rarely if ever does HIV infect neurons or oligodendrocytes [45]. Astrocytes can be infected but infrequently produce replication-competent virus [46, 47]. However, neither the amount of HIV present nor the degree of neuronal loss has been reported to correspond well with a premortem history of HAD [48]. Subsequently, investigators reported that features such as the number of activated brain macrophages/microglia, synaptic degradation, and selective neuronal loss, as well as elevated levels of inflammatory mediators, were more likely to correspond to a premortem history of HAD [49–51]. In addition, some cases that showed HAD during life came to autopsy, and the classic histopathological findings of HIVE were sparse or lacking [43]. In the post-cART era, the number of autopsy cases where neurocognitive impairment was present during life, but the classical histopathological findings of HIVE were absent and/or minimal nondiagnostic abnormalities or neuroinflammation was present, has increased [52]. However, the CSF of living, cART-treated patients may show signs of continued immune activation despite years of cART treatment [53].

These observations, and others, led to the hypothesis that a complex spectrum of "indirect" mechanisms, which involve both HIV and the host response, such as inflammation and/or neurotoxicity, as well as impairment of repair mechanisms in the CNS, are the prime mechanisms underlying the pathogenesis of HAND [51, 54]. Unfortunately, little evidence has been presented to rank the relative importance of these respective mechanisms in generating HAND.

16.5 HAND in the Era of cART

The absence of demonstrated productive HIV infection in neurons and oligodendroglia, as well as the persistence and increase of the milder forms of HAND despite cART, has generated some of the most frequently discussed hypotheses as displayed in Table 16.1.

16.5.1 Overdiagnosis: Diagnostic Accuracy of Criteria for HAND

HAD was first identified as a unique entity in the pre-cART era, when it was known as "AIDS dementia complex (ADC)." The first ADC cases reported were in relatively young persons with advanced AIDS [43, 55]. The majority of such patients had a history of normal cognitive function. Their cognitive impairment was severe, progressive, and evident on bedside examination. In addition, many ADC patients

Table 16.1 Display of possible reasons for the persistence of HAND in the cART Era

Section-topic	Section-topic
5.1-Overdiagnosis	
5.2-Permanent damage to CNS pre-cART	
5.3-Persistence of the CNS viral reservoir	5.3.1-Inadequate penetration of the CNS compartment by cART 5.3.2-Unable to sterilize the CNS compartment
5.4-Neurotoxicity of some cART drugs	
5.5-Persistence of viral proteins, neuroinflammation, and neurotoxicity despite cART	5.5.1-Viral proteins 5.5.2 (A/B)-Persistent inflammation 5.5.3-Persistent neurotoxicity
5.6-Contribution of comorbid conditions	5.6.1-Hepatitis C virus (HCV) coinfection 5.6.2-Drug abuse 5.6.3-Cardiovascular risk factors

had neurological signs such as tremor, spasticity, and upper motor neuron-type weakness and had brain atrophy and/or white matter disease on neuroimaging [55].

Over time, HIV+ persons were identified earlier in the course of their disease, cART use became widespread, and the epidemiology of HIV changed. HIV is now increasingly under study among marginalized groups, such as minorities, drug users, and the poor. At the same time, the criteria for a diagnosis of HAND have become more focused on neuropsychological abnormalities (as demonstrated by standardized testing) and less on concomitant neurological signs. Consequently, some investigators have voiced concern that HAND may be overdiagnosed due to inappropriately stringent research criteria applied to HIV+ populations [27]. This may occur, for example, when there is a lack of appropriately matched controls and/or normative data for the group under study. For example, the diagnosis of neuropsychological test abnormalities in members of groups who have suffered from poor-quality education may be influenced by their lower reading ability [56, 57], even when such persons report the same number of years of education as do controls. Some studies that compare HIV+ and at-risk HIV groups also report a high base rate of problems in both groups which can affect cognition and which may contribute to poor neuropsychological performance [58].

The accurate assessment of cognition also requires culturally specific neuropsychological norms [59] that are derived from a well-matched control population, which may be difficult to obtain in many circumstances, such as in low-resource, international settings [60]. The Frascati criteria also require a thorough interrogation of comorbid conditions that may confound the diagnosis of HAND, which may also be difficult to obtain in resource-limited settings [60]. Finally, as the HIV+ cART-treated population ages, patients are more likely to have accumulated end-organ diseases that can compromise cognition [61]. In older HIV+ persons, an increased burden of comorbid conditions, such as lipid disorders, can be associated with poorer cognitive performance; this can make it difficult to assess the contribution of HIV to overall neuropsychological impairment [36, 62].

In summary, despite many studies, there is no single marker or constellation of markers that diagnose HAND; it remains a clinical diagnosis. In the continued absence of a diagnostic marker, it can be argued that there is a risk of overdiagnosis, particularly of the milder forms of HAND and especially in marginalized groups where good normative control data, baseline cognitive assessments, longitudinal assessment, and other resources for differential diagnosis are lacking. This could contribute to the apparent persistence of HAND despite cART.

16.5.2 Persistence of Early Damage to the Brain and Immune System Sustained Prior to Treatment May Not Be Reversed by cART

Prior to the cART era, certain biomarkers were associated with an increased risk of developing HAND. These included (but were not limited to) the presence of a low CD4+ count, high levels of plasma viremia, anemia, weight loss, advanced age, and elevated levels of beta-2 microglobulin [8, 10, 63]. Notably, most of these values were markers of advanced HIV disease, high levels of viral replication, and inflammation and can also be present in HIV+ persons whose cognition is deemed normal. In the post-cART era, predictors of HAND among cART-treated individuals have shifted to include a longer duration of known HIV infection and a low nadir (lowest ever) CD4+ count, with an increased risk in persons whose nadir was below 200 cells/mm^3 [64, 65]. In addition, a neuroimaging study of a large cohort of stable cART-treated HIV+ participants indicates that their brain volumes and neurometabolite levels remained persistently abnormal, and their corpus callosal widths (measure of white matter damage) were reduced, and that these findings correlated with a prior history of immune suppression [66, 67]. These observations suggested that individuals who do not receive cART early in the course of infection, and who suffer immune suppression, may be more susceptible to brain injury that is not reversed by cART.

Research on primary HIV infection (PHI) reinforces the notion that HIV enters the CNS very early [14] and can cause neuroinflammation [68]. In one study [68, 69] approximately half of individuals undergoing PHI developed clinical signs or symptoms of neurological disease. HIV was recovered from the CSF as early as 8 days after infection in one individual [68]. In general, the investigators found that persons with more neurologic signs/symptoms had higher plasma levels of HIV. The majority of neurologic findings during PHI were mild and resolved on cART, but a minority (9%) did not respond to cART. Evering et al. [70] reported that persons who were treated very early during PHI, and who never suffered severe immunosuppression, had a very low rate of subsequent cognitive problems. Another study compared HIV+ persons treated early after infection to HIV+ persons who entered treatment at a later time and with lower CD4+ and found a lower rate of cognitive impairment in patients treated with early cART [71].

In a contemporary study of cART-naïve clade C HIV+ individuals in India with CD4 under 200 cells/mm^3 [72], only a minority (15%) of cognitively impaired HIV+ individuals in the cohort showed global neuropsychological benefit from cART at 1 year follow-up, despite excellent adherence. Improvement was associated with lower CD4+ at baseline and better CD4+ and virological response (as measured by plasma viral load). However, there was no cognitive deterioration reported in treated HIV+ patients. In an African study of cART-naïve participants [73], overall cognition in the group was shown to improve with cART, with a predilection for improvement in more severely affected individuals. However, a number of subjects remained impaired, consonant with observations in other cART studies [74].

These studies, and others, suggest that at least some of the neurological benefits of cART may be time dependent and that persons who begin cART late (e.g., after a significant viral re established or after the immune system is severely damaged) may enjoy less benefit of treatment. They also suggest that some of the damage sustained before treatment may be permanent.

16.5.3 Persistence of the CNS Viral Reservoir Post-cART

Current cART drugs do not destroy the virus itself, but inhibit one or more steps in the viral life cycle (such as its entry into new cells, its ability to integrate into the host, and/or its ability to replicate [75]). They do not impair its ability to remain in a latent state. HIV latency refers to highly stable and transcriptionally silent integrated proviral DNA, which can produce infectious virus when the host cell is reactivated by antigen or during interruptions in cART [76]. Latency allows the virus to escape from the host's immune system. HIV reservoirs (a.k.a. sanctuary sites) are populations of infected cells that allow the persistence of replication-competent HIV in patients [76], despite optimal antiretroviral therapy. Reservoirs can be located within circulating cells such as resting memory T cells or in anatomical compartments, such as the CNS, gut, liver, lymphoid, and other tissues [76, 77]. Such reservoirs are often established early after HIV infection and may be resistant to cART penetration [78], thus allowing the continued propagation and evolution of virus [76]. Further, some cells such as macrophages, microglia, astrocytes, and pluripotent stem cells are long-lived and thus are ideally suited to harbor latent provirus [79, 80]. Exposure to antigens that stimulate cellular activation, or the withdrawal of cART, can activate latent virus in the CNS and elsewhere and initiate the resumption of viral transcription in reservoir cells. Replication within the CNS, a reservoir with known impediments to cART penetration [81], can lead to compartmentalization of HIV with the development of unique strains and patterns of drug resistance [22, 82].

As stated previously, HIV is believed to enter the CNS primarily via the transmigration of infected monocytes [83], e.g., CD14$^+$/CD16$^+$ monocytes [84], across the BBB, where these cells may then transform into long-lived perivascular macrophages [85].

This enhances their ability to support HIV replication to infect resident CNS cells, e.g., monocytes, macrophages, microglia, and astrocytes, and to sustain neuroinflammation [83, 85].

16.5.3.1 Failure to Sterilize the CNS Compartment

Failure of cART to clear the HIV reservoir in circulating monocytes [86] could facilitate the replenishment of virus in the CNS, as there is no evidence that cART halts transmigration across the BBB [84]. For example, cART-treated individuals with HAND have been reported to have high levels of HIV DNA in peripheral blood mononuclear cells [59], indicating that there may be an association between the size of viral reservoirs and CNS disease. Individuals with detectable HIV DNA in peripheral blood monocytes are also more likely to have cortical thinning on brain MRI, indicating more CNS damage and neuronal loss [87]. The number of copies of DNA in circulating monocytes from cART-treated person is associated with HAND, as well as increased cytokine production [88, 89].

Macrophages and microglial cells likely constitute the largest part of the HIV CNS reservoir. Macrophages are long-lived cells [90] that constitute a significant source of HIV within the CNS and other tissues [91, 92]. As stated above, some monocytes cross the blood-brain barrier and differentiate into perivascular macrophages in the CNS. In contrast, microglial cells are endogenous long-lived resident macrophages of the brain, express $CD14^+$ and $CD45^+$ antigens, and have a distinctive ramified morphology [93]. Chugh et al. have reported that HIV infection may actually increase the life span of microglial cells via a Tat-mediated mechanism [94]. Both macrophages and microglia can support productive HIV infection [93] and can harbor HIV, which is not cytopathic in these cells [91]. In addition, macrophages can protect HIV from the immune system [91], shed toxic viral proteins, express pro-inflammatory cytokines [95], and can infect the brain in an animal model without the presence of human T cells [92]. HIV can bud into the intracellular vacuoles of infected macrophages [96], where it can escape exposure to therapeutic concentrations of many cART drugs [97, 98]. In addition, HIV-infected macrophages can infect new cells by extracellular release of HIV and/or by direct contact with other cells (e.g., through so-called synaptic transmission or virological synapses) [99]. Eden et al. found evidence of persistent macrophage activation, such as elevated levels of CSF neopterin, in HIV+ persons treated with cART for over 4 years [53].

Astrocytes have also been demonstrated to be a part of the CNS reservoir [46]. Astrocytes are the largest cell population in the brain; they are essential to maintaining a healthy brain, normal cognitive function, neurotransmitter regulation, and CNS homeostasis [100]. They regulate the ionic environment, comprise a portion of the BBB, and influence neurons by calcium signaling, expression of neuropeptides, detecting and propagating signals, and regulating the uptake of glutamate (the major excitatory neurotransmitter in the brain) at neuronal synapses [100]. Failure to regulate synaptic levels of glutamate, the most abundant excitatory neurotransmitter in

the brain and a known toxin, is an important cause of neurotoxicity in many neurodegenerative conditions, including HIV infection [101].

HIV can infect astrocytes via a non-CD4+-dependent mechanism [47]. HIV-infected astrocytes can spread toxic signals to uninfected astrocytes by gap junction channels [102], can disrupt the integrity of the blood-brain barrier [103], can release pro-inflammatory cytokines [104], and can release toxic viral proteins that disrupt neurons [104]. Churchill et al., in an autopsy study of brains from persons known to have had HAD during life, found that up to 19% of astrocytes sampled were HIV infected [105]. However, even if a smaller fraction of astrocytes were HIV infected, they could potentially amplify and transmit toxic signals to many uninfected cells due to the enormous number of synaptic contacts of each astrocyte [102, 103]. Astrocytes are rarely observed to support productive HIV infection [106] but can be induced to do so when exposed to pro-inflammatory cytokines [107]. Even in the absence of productive infection, astrocytes can express neurotoxic viral proteins such as Nef [108], thus affecting many other cells that they contact.

Finally, neural stem/progenitor cells (NSPC, the undifferentiated, pluripotent precursors of neurons and macroglia (astrocytes and oligodendrocytes)) are a small portion of the CNS population but may have an important role in the viral reservoir and HAND pathogenesis. Evidence from postmortem pediatric brain tissues [109] and experimental models utilizing NSPC cell lines that express the cell surface marker CXCR4 [110] indicates that NSPC can be infected with HIV. In addition, such cell lines can produce proviral DNA and can release virus [110, 111]. While the clinical importance of these observations is still evolving, it appears possible that NSPC comprises part of the CNS reservoir and that HIV infection of NSPC may compromise the brain's ability to conduct neural repair.

16.5.3.2 Inadequate Penetration of CNS Compartment by cART and Failure to Clear the CNS Reservoir

Tissue reservoirs are compartments that are frequently surrounded by natural barriers, e.g., the blood-brain barrier (BBB), that can restrict the free influx of antiretroviral drugs [81]. The BBB is composed of both brain microvascular endothelial cells (joined by tight junctions) and the foot processes of astrocytes. It extends throughout the CNS, except for the area postrema/circumventricular organs, where the BBB may be incomplete [112]. The BBB has active mechanisms to take up nutrients and other materials, to defend against infection, and to "pump out" or exclude undesirable substances [81]. Nonetheless, some pathogens have adopted mechanisms to breech the BBB. HIV is thought to transmigrate via the infection of monocytic cells (e.g., CD14 + CD16+ monocytes). In addition, HIV infection can upregulate the production of soluble chemicals that increase BBB permeability and others that can function as chemoattractants [113], inducing more cells to transmigrate across the BBB. The BBB also contains efflux transporters such as P-glycoprotein, which can "pump" substances, including many cART drugs, out of the CNS [81, 114]. Thus, the brain may be "under medicated" despite the suppression of virus by cART.

The BBB can also inhibit the free exchange of genetic information between HIV in CNS tissue reservoirs and HIV in the peripheral circulation, inducing compartmentalization of the virus. This compartmentalized HIV can replicate independently and develops compartment-specific viral characteristics, genetic distinctions, viral loads, and drug resistance mutations [115–117]. HIV recovered from the brains of HAD patients is reported to be genetically distinct from that recovered from their lymphoid tissue [118]. Such virus is also more likely to be "macrophage trophic" [119], that is, it has evolved to infect, and replicate in, CNS macrophages. There are many case reports of HIV+ persons with excellent peripheral control of HIV replication on cART, who nonetheless developed symptomatic HAND due to the failure of their regimen to suppress HIV in the CNS. Such individuals may have very low or undetectable plasma HIV RNA, with high concurrent CSF HIV viral loads, a phenomena known as "CNS escape" [120]. CNS escape has been attributed to either a failure of the patient's cART regimen to adequately penetrate the CNS [121] or to a different drug sensitivity profile of HIV in the CNS versus the peripheral blood [122, 123].

Studies performed in simian immunodeficiency virus (SIV)-infected macaques (an animal model for HIV infection and encephalitis) demonstrate that early cART treatment of acute SIV can control CNS SIV replication and reduce severe CNS inflammation, but cART does not eradicate persistent SIV DNA from the brain [124]. The observed levels of SIV DNA were similar in the brains of cART-treated versus untreated macaques [124]. Further, cART treatment did not reduce latent viral DNA levels in chronically infected macaques. Macaque studies also indicate that SIV can escape from the CNS reservoir if cART is interrupted [125]. This work suggested that the brain is a significant reservoir of latent SIV despite treatment and, by extrapolation, is likely an important reservoir of HIV in humans [126]. One problem with these studies is that SIV-infected macaques are usually sacrificed as soon as they develop symptomatic SIV disease and are rarely studied after prolonged treatment with cART, unlike HIV+ humans and those with HAND.

Another animal model for the study of HIVE is the severe combined immunodeficiency (SCID) mouse [127]. This mouse can be inoculated with HIV-infected human macrophages and develops neurologic signs and neuropathological features similar to HIVE. Experiments in SCID mice using a CNS-penetrating cART regimen indicate that cART reduced the neuropathological signs of HIVE such as viral load, astrogliosis, mRNA for tumor necrosis factor (TNF, a pro-inflammatory cytokine), presence of mononuclear infiltrates, and increased markers of neuronal integrity, but cART did not eradicate the presence of HIV in the CNS, nor did it eliminate the cognitive abnormalities in the mice, despite a lack of apoptotic cell loss [127, 128].

Until recently, comparable studies of cART-treated HIV+ human brain and other tissues were not available. Lamers et al. published a study of 20 cART-treated HIV+ human patients from the National Neurological AIDS Bank (NNAB) cohort, who had prolonged peripheral virological suppression and were on cART until or very shortly prior to their demise [129]. Investigators recovered HIV DNA from 48/87 (55%) of brain tissues sampled from this cohort, as well as from many other tissues

and organs. This virus was also found to be replication competent [129], so it could contribute to viral persistence and rebound if cART was withdrawn.

The best way to approach the issue of cART penetration of the CNS in clinical care remains controversial. Letendre et al. [130] devised a major tool used in research known as the CNS penetration effectiveness (CPE) index. The CPE ranks various cART drugs by the ratio of their plasma/CSF concentration. However, CPE does not typically measure such drug ratios over time (as this would require multiple blood and CSF samples) [131], parenchymal drug concentrations (as this would require a brain biopsy), and unbound drug [131] and does not take into consideration individual genotypic patterns of drug transport [131] nor damage to the BBB [130, 131]. Thus, the usefulness of the CPE as a tool in clinical practice remains controversial. Some studies have reported that HIV+ individuals treated with a higher-penetration cART regimen had better cognition [130, 132] or found that they had better CSF viral suppression [133]. In contrast, other studies have found no neurocognitive benefit of a regimen with a higher CPE [134, 135]. A single controlled, randomized trial attempted to compare outcomes in HIV+ persons randomized to a high versus low CPE regimen but failed due to poor accrual and being underpowered [136]. One retrospective study suggested that persons treated with a cART regimen maximized for optimal penetration into monocytes, rather than the CNS, had better neurocognitive functioning [137].

In summary, CNS penetration of the various cART drugs is mostly measured in CSF and varies widely [138], and little information is available on the concentrations of drug reached within the cells of the CNS reservoir, e.g., macrophages, monocytes, and astrocytes. Previously, we discussed that HIV can be sequestered in macrophages and can also pass from cell to cell by synaptic transmission, thus evading cART. Thus, the failure of cART to sterilize the CNS is likely a major contributor to the persistence of HAND.

16.5.4 Putative Neurotoxicity of cART

The deleterious effects of cART on the peripheral nervous system (PNS) are well documented [139]. Nucleoside reverse transcriptase inhibitors (NRTIs) such as stavudine, didanosine, zalcitabine, zidovudine, lamivudine, abacavir, and emtricitabine are important components of many cART regimens. The dideoxynucleoside reverse transcriptase inhibitors (a.k.a. “d-drugs”) such as stavudine, didanosine, and zalcitabine are known to cause mitochondrial toxicity via inhibition of mitochondrial DNA polymerase **γ** which results in depletion of mitochondrial DNA in axons, and this clinically presents with a painful sensory neuropathy [140, 141]. Although many of these drugs have fallen out of favor in the United States, they are still used as first-line therapy in many low-income countries. In addition, some protease inhibitors are now suspected of causing peripheral neuropathy [142].

However, there was little interest in the potentially deleterious effects of cART on the CNS until an ACTG study reported that neuropsychological test scores of

immunologically stable HIV+ participants improved after cART interruption [143]. This was followed by several clinical studies indicating that high CPE regimens were associated with more HAND [135] and in vitro studies suggesting that efavirenz showed neuronal toxicity [144], prompting a serious examination of the long-term effects of cART.

Efavirenz, a non-nucleoside reverse transcriptase inhibitor (NNRTI), has good CNS penetration and is highly favored as a component of once-a-day combination regimens [145]. However, efavirenz is commonly associated with CNS adverse effects, such as mood instability, suicidality, and sleep disorders [146, 147]. This may be due to mitochondrial toxicity [148]. Efavirenz has also been reported to be associated with an increased risk of mild cognitive impairment in otherwise asymptomatic HIV+ patients [149]. Efavirenz, and particularly its metabolite 8-hydroxy-efavirenz, has also been associated with dendritic injury in neurons [150] in clinically relevant concentrations, associated with disturbance of neuronal calcium homeostasis and significant damage to dendritic spines in neuronal culture [149]. A genetic polymorphism has been identified that increases the plasma levels and neurotoxicity of efavirenz, so there may be a subgroup of persons who are especially susceptible [151]. Giunta et al. reported that in a murine model, lamivudine, zidovudine, abacavir, and indinavir increased the presence of beta-amyloid by impairing its phagocytosis by microglia [152], a finding also seen with efavirenz [153].

In addition to potential direct toxicity to CNS cells, some antiretroviral drugs have indirect adverse effects by altering elements of systemic metabolism. For example, some components of the cART regimen are associated with the development of diabetes [154], dyslipidemia [155], and atherosclerosis [156], conditions which have been associated with increased cognitive impairment in HIV-infected persons [157, 158].

The subject of cART neurotoxicity versus benefit is extremely complex and beyond the scope of this chapter. The reader is referred to several excellent articles on this subject for more information [144, 159, 160]. In summary, the beneficial effects of cART on the CNS, mediated by reducing HIV replication and inflammation, may be partly counterbalanced by neurotoxicity associated with long-term use, and this neurotoxicity may play a role in the persistence of HAND in the cART era.

16.5.5 Persistence of Indirect Mechanisms: Neurotoxic Viral Protein Secretion, Neuroinflammation, and Neurotoxic Metabolites During cART

As neurons and oligodendrocytes do not support productive HIV infection, the major mechanisms of damage to the CNS are deemed to involve indirect effects on primary CNS cells such as neurons and oligodendroglia [54, 95, 161]. While cART reduces the infection of new cells, low-level HIV replication in infected cells, as well as a persistent elevated inflammatory response, is not obliterated by cART [129, 162], and infected macrophages may escape cytotoxic lymphocytes [163].

Infected cells may also produce neurotoxic viral proteins, even from defective proviruses, while on long-term cART [164]. The slow production of HIV, viral proteins, and pro-inflammatory substances from chronically infected, "sanctuary" cells such as monocytes and macrophages may continue in cART-treated, virally suppressed persons for many years, perhaps resulting in a less severe form of HAND [91].

16.5.5.1 Viral Proteins

HIV-infected brain macrophages, microglia, and astrocytes can produce both replication-competent virions and individual viral proteins such as Gp120, Tat, Vpr, and Nef [165]. These proteins can be shed into the extracellular milieu where they bind to the cell membranes of immune cells and to neurons. Although these individual proteins are incapable of infecting new cells, they can trigger pathological mechanisms that cause cellular damage and death. In addition viral proteins expressed by HIV+ and/or activated monocytes and macrophages can stimulate nearby cells to become immunologically activated and to express damaging pro-inflammatory cytokines, glutamate, reactive oxygen species, and neurotoxic metabolites such as quinolinic acid and arachidonic acid into the CNS milieu, resulting in the dysfunction and/or death of neurons and oligodendrocytes [166].

There is strong evidence that at least four HIV proteins, such as Gp120 [167], Tat [168], Nef [169], and Vpr [170], are neurotoxic and play a role in the pathogenesis of HAND [95, 165]. These proteins can be synthesized in productively infected cells, e.g., CD4+ macrophages and microglia, and some (such as Nef) can be synthesized in astrocytes [171]. However, these proteins can also bind to cells that do not become productively infected, instigating a potentially lethal disruption of cellular metabolism and activating a cascade that can result in neuronal apoptosis despite the absence of neuronal infection [172]. In addition Tat can be transported through the nervous system within neurons, escaping immune surveillance [173]. However, investigators report that cART treatment does not block the production of Gp120, Tat, or Nef [174, 175]. Presumably, these proteins could constitute an ongoing source of neuroinflammation and neurotoxicity in a cART-treated individual. Viral proteins Gp120 [176], Tat [177], Nef [178], and Vpr [179] have also been reported to stimulate the synthesis of pro-inflammatory cytokines by microglia and macrophages.

16.5.5.2 Persistent Inflammation

Persistent Inflammation and Immune Functioning

Treatment with suppressive cART lowers viral replication and the degree of immune activation; however, immune activation does not normalize in many HIV+ individuals. Anzinger et al. reported that monocytes can remain activated in HIV+ individuals, despite lengthy treatment with suppressive cART [180]. These cells produce

inflammatory mediators that are associated with cell damage and many serious non-AIDS events [181] that cause morbidity and mortality in HIV+ persons. Like HAND in the era of cART, these serious non-AIDS events are correlated with a history of severe immunodeficiency [181]. A study that compared HIV+ persons who began cART late with a low CD4+ versus those who started with higher CD4+ [182] found that after 3 years of effective cART, the "late" group was still found to have lower CD4+ and elevated inflammatory markers for cardiovascular disease such as circulating insulin levels, elevated C-reactive protein (CRP), and higher levels of IL-6, a pro-inflammatory cytokine. Residual, low-level HIV viremia, coinfection with cytomegalovirus (CMV), and microbial translocation through the HIV-damaged gut are the major factors hypothesized to exacerbate persistent inflammation in cART-treated patients [180, 181].

Persistent Inflammation and HAND Pathogenesis

Persistent inflammation may also contribute to the pathogenesis of HAND despite cART. Astrocytosis, activated microglia, perivascular inflammation, and dendritic simplification persist in the post-cART brain [52]. Anthony et al. report that cART-treated brains had as much inflammation as those from the pre-cART era, particularly in the hippocampus [183]. Brain inflammation has also been reported in autopsied brain tissue of HIV-infected persons who had a history of neurocognitive impairment during life, in the absence of evidence of productive infection or HIV encephalitis [184]. Elevated levels of pro-inflammatory cytokines, such as IL-8, MCP-1, IP-10, and granulocyte colony-stimulating factor (G-CSF), persist in the CSF study of both cART-treated and untreated patients with and without HAND [185]. The use of cART had no effect on these cytokines, except that cART-treated persons had higher levels of IP-10.

16.5.5.3 Persistent Neurotoxicity

In addition to low-level replication of HIV in macrophages and the presence of persistent viral proteins, cytokines may also fuel neurotoxic pathways that contribute to HAND. Quinolinic acid (QUIN) is a neurotoxin and a by-product of the metabolism of the essential amino acid, tryptophan. The major metabolic pathway of tryptophan is the kynurenine pathway, and its rate-limiting step is an enzyme, indoleamine 2,3-dioxygenase (IDO). IDO activity is upregulated by inflammatory cytokines (such as gamma interferon, which is elevated in HIV infection). Increased activity of IDO results into shunting tryptophan away from the production of serotonin and toward the production of QUIN [186]. This process occurs in activated macrophages and, to a lesser extent, in microglia [186]. At high levels, QUIN inactivates glutamine synthetase, resulting in elevated levels of the neurotoxin glutamate. QUIN also acts as an agonist at the NMDA receptor, leading to cellular overactivation, excitotoxicity, increased cytosolic calcium, free radical

formation, oxidative damage, and cellular death in both neurons and astrocytes [187, 188]. The levels of CSF QUIN can be greatly elevated in AIDS and in HAND [189], and QUIN is considered an important mechanism in HAND neuropathogenesis [161, 190].

The use of cART lowers HIV replication and CSF QUIN levels [191]. However, a study of brain tissues in SIV-infected macaques found that cART did not restore normal kynurenine metabolism in the brain, although CSF QUIN levels are normalized [192]. While similar invasive studies have not been performed in HIV+ humans, it is likely that failure to normalize kynurenine metabolism and continued excessive production of QUIN may be an important mechanism in contributing to HAND in cART-treated humans.

In summary, the failure of cART to fully eradicate HIV from the viral reservoir results in the persistence of low levels of viral proteins, elevated inflammation, inflammatory cytokines, and neurotoxicity, which may account for the prevalence of milder forms of HAND in cART-treated persons.

16.5.6 Comorbid Conditions

HIV-infected persons are prone to many important comorbid conditions that may exacerbate the cognitive deficits associated with HAND or may confound the ability of the physician to make a HAND diagnosis. Some of these conditions are also independently associated with cognitive impairment.

16.5.6.1 Hepatitis C Coinfection

Hepatitis C virus (HCV) is, like HIV, a blood-borne pathogen which can be contracted by similar risk behaviors, such as unprotected sex and intravenous drug use [193, 194]. HCV coinfection occurs in 20–30% of HIV+ persons, depending on the population sampled [195]. HCV has also been shown to be a neuroinvasive virus, which can cause mild neurocognitive and behavioral impairment in up to 40% of HIV-negative, HCV-positive persons without advanced liver disease or hyperammonemia [196] [197]. As in HIV, some HCV proteins have been reported to be neurotoxic [198], and HCV is also an important contributor to immune activation [199]. While it is generally agreed that cognition and other neurologic functions may be affected in HCV+ persons as compared to normal controls [200], the contribution of HCV to cognitive dysfunction in HIV+/HCV+ coinfection is controversial, with some studies reporting no effect of HCV in HIV+ persons [201], whereas others have found an interactive effect.

Potentially, HCV could contribute to neurocognitive dysfunction in several ways, independent of the development of hepatic encephalopathy. An in vitro study demonstrating HCV infection of brain neuroepithelioma cells suggested that HCV could enter the CNS via receptors on brain capillary endothelial cells [202]. HCV has

been identified in brain astrocytes and microglia [203]. Some HCV proteins are deemed neurotoxic [198]. The brain can respond to HCV with microglial activation [204]. In addition, HCV stimulates an inflammatory response with upregulation of cytokines [205], and HIV+/HCV+-coinfected individuals are reported to have higher levels of systemic inflammation than monoinfected HIV+ persons [206].

16.5.6.2 Drug Abuse

A large percent of HIV+ individuals in the United States have a history of illicit substance use, and it can be difficult to distinguish the cognitive deficits caused by HIV from the effects of some drugs [207]. The precise mechanisms by which illicit drugs interact with the pathogenic mechanisms underlying HAND may vary according to the type of drug used, the amount of exposure, and whether the individual is adherent to (or even taking) cART. The interactions of drug abuse and HIV center around interactions with viral proteins, increased neuroinflammation (primarily affecting glial cells), dopaminergic dysfunction, and BBB breakdown [208, 209].

Opiates increase brain inflammation in HIV infection, primarily by interaction with macrophages and glial cells. In vitro, morphine has been reported to increase the secretion of IL-6, a pro-inflammatory cytokine, in human HIV-infected macrophages [210]. Of interest, in this experiment, morphine did not appear to increase viral replication, as had been reported in some other studies [211]. Opiates can bind to the CXCR4 receptor, in place of its natural ligand CXCL12. Under physiologic conditions, binding of CXCL12 to CXCR4 activates G protein-coupled receptors and pathways associated with neuronal survival; however, binding of opiates such as morphine to CXCR4 instead upregulates the protein ferritin heavy chain and impairs CXCR4, thus potentially decreasing neuronal survival [212]. Opiates may activate inflammatory pathways in HIV-affected astrocytes [213], resulting in increased cytokine production. The combination of opiates and HIV has been reported to increase abnormalities such as prevalence of microgliosis and multinucleated giant cells [214], production of TNF [215], toxicity of Tat in vitro [216–218], and synaptodendritic damage [219].

From a clinical perspective, persons who abuse opiates tend to have more neurocognitive abnormalities during life and more HIV encephalitis at death [214]. μ opioids such as heroin and morphine can act synergistically with HIV proteins such as Gp120 and Tat [220]. These observations form a basis for the notion that neurocognitive changes associated with prolonged opiate abuse may contribute to the manifestations of HAND in the post-cART era.

Independent of HIV infection, acute use of cocaine is associated with stroke [221], presumably due to hypertension and vasospasm. Cocaine has been reported to hasten progression to AIDS [222] and to increase HIV replication in human peripheral blood mononuclear cells. In vitro, cocaine increases HIV migration across a model of the BBB [223] and potentiates Gp120-induced neurotoxicity [224] and Tat neurotoxicity [225]. Cocaine also increases neuroinflammation by upregulating pro-inflammatory cytokines and chemokines, even in abstinent users [226].

Thus, there is ample reason why chronic cocaine use might contribute to the persistence of HAND despite cART.

Chronic methamphetamine use is toxic to neurons even in the absence of HIV and particularly targets dopaminergic neurons in the nigrostriatal pathways [227]. Methamphetamine use is also associated with neuropsychological impairment in HIV-negative/HCV-negative users [228]. Methamphetamine has been shown to increase the expression of pro-inflammatory cytokines such as TNF and IL-1B in response to lipopolysaccharide [229] and to cause neuroinflammation as evidenced by microglial activation [227], with neurodegenerative change by inducing BBB compromise, increased astrogliosis, excitotoxicity, and oxidative stress [101]. In HIV+ persons, methamphetamine use is associated with more rapid progression of HIV disease [230]. Methamphetamine enhances the neurotoxicity of the viral proteins Gp120 [231] and Tat [232]. Research from Chana et al. found that methamphetamine synergizes with HIV to damage inhibitory interneurons in the brain and that this degeneration correlated with premortem cognitive impairment [233]. Thus, there is ample evidence that methamphetamine use can cause neuroinflammation and neurodegeneration in the absence or presence of HIV and is a likely candidate to cause or exacerbate persistent neurocognitive impairment in cART-treated HIV+ persons.

16.5.6.3 Cardiovascular and Cerebrovascular Diseases

Becker et al. [157] found vascular risk factors to be an important driver of neuropsychological impairment in a post-cART, older sample of HIV+ men. HIV+ individuals are at higher risk of cardio- and cerebrovascular diseases, such as myocardial infarction and ischemic stroke, than comparable members of the general population [234, 235], and such disease tends to occur at younger ages [234]. In part, this occurs due to an increased prevalence of traditional risk factors such as smoking [236]. In part, this occurs due to the systemic, inflammatory nature of HIV [237] with or without treatment which increases the risk of metabolic disorders [237, 238], in addition to the propensity for specific drugs in the cART regimen to predispose to the development of lipid disorders and diabetes [239]. Further, systemic immune activation is not completely reversed by cART [237]. As in many other categories of illness that may contribute to persistent HAND in the era of cART, microbial translocation across the gut; coinfections with CMV, HCV, and other pathogens; the persistence of low-level HIV replication; and production of viral proteins, oxidative stress, and cytokine release are thought to play a role in the pathogenesis of cardiovascular disorders and to contribute to a persistence of HAND despite cART.

Summary

Due to the introduction of cART, HAND has now become a milder, more chronic, and more manageable manifestation of chronic HIV infection. However, until HIV can be eradicated from all body reservoirs including the CNS and until persistent

inflammation can be controlled, we can expect that neurological diseases will remain an important consequence of HIV infection. Until this can be accomplished, researchers will need to focus on instituting early treatment to reduce the size of the CNS reservoir, mitigating antiretroviral toxicity, insuring that adequate levels of cART penetrate the brain, and developing better means to assess the treatment of HIV in macrophage and other reservoir cells. Targeted treatments to reduce persistent CNS inflammation and neurotoxicity and their consequences will also be needed and should be started early in the course of infection, to reduce progressive neuronal loss.

Conflict of interest The authors report no conflicts of interest.

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Chapter 17
Antiretroviral Therapy: Brain Penetration

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Keywords HIV • Brain • Combined antiretroviral therapy • cART • HAND • CNS • BBB • Drug penetration

Core Message
In the era of combination antiretroviral therapy (cART), advanced neurologic complications of HIV infection are less common, than pre-cART. There is evidence that antiretroviral therapy prevents, delays, and may reverse neurocognitive complications of HIV infection. However, clinical trials leading to antiretroviral drug approval primarily measure HIV in the plasma as an indicator of therapy efficacy. In this chapter we assemble and present data on CNS exposure and penetration of antiretroviral drugs.

17.1 Introduction

Prior to the introduction of combination antiretroviral therapy (cART), neurologic complications of HIV infection were common, termed overall HIV-associated neurocognitive disorders (HAND). HAND ranges from asymptomatic neurocognitive impairment (ANI), to a mild neurocognitive disorder (MND), to full-blown HIV-associated dementia (HAD) and often exhibited as HIV encephalitis (HIVE) [1, 2]. The incidence of HAND decreased over time due to the use of cART. Especially, HAD is now less common, and it is rare in patients who are clinically stable on

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cART. The incidence of HAD per 1000 person-years reduced from 6.49 in 1997 to 0.66 during the years 2003–2006; this was associated with the introduction of highly active antiretroviral therapy (HAART) or cART, as mentioned [3]. HIV-1 invades the CNS early and can cause persistent infection and inflammation [4]. HAND shows decreased association with immune activation and has a more diffuse range of neuropsychological deficits that may overlap other brain diseases and, at times, with continued association with suppressed virus loads [4, 5]. Moreover, despite the advent of cART, an overall neurologic impairment is still prevalent, in some studies; for those patients on cART, it occurs greater than 80%, especially in its milder form. Reduction of HIV viral load in the CSF alone perhaps is not the only clinical indicator of treatment efficacy for HAND [4, 6–9]. For example, there was a lack of association between neurocognitive impairment with virologic and immunological factors that indicates ongoing neural injury regardless of the success of antiretroviral therapy based on these laboratory measures [8]. In addition to HIV load levels, several studies suggest other factors including immunological, aging, persistent HIV replication in the CNS including macrophages, evolution of highly neurovirulent HIV strains, and the long-term neurotoxicity of cART [9, 10].

In this chapter, we will discuss further the effects of antiretroviral therapy on HAND and the interaction between ARVs and the brain. Most data, when indicated, were studied in HIV-1 infection.

17.2 The CNS

17.2.1 The CNS Barriers

The CNS is surrounded by the BBB and the blood-cerebrospinal fluid barrier (BCB). These barriers prevent most molecules from entering the CNS and maintain a stable environment. The BBB inhibits the free diffusion of water-soluble molecules by complex tight junctions that interconnect endothelial cells within CNS microvessels. They lack intercellular pores and have low pinocytosis activity. The endothelial cells and pericytes are enclosed by basement membranes and are almost completely surrounded by astrocyte foot processes [11]. A functional BBB has numerous active transport systems, specifically expressed by brain capillary endothelial cells to ensure the transport of nutrients into the CNS and block potentially offending molecules from CNS entry. Moreover, BBB is the passage for HIV, viral products, infected cells, and activated immune cells to penetrate to the CNS [12]. On the other hand, the BCB is formed by *choroid plexus* epithelial cells and found at the apical tight junctions between the *choroid plexus* epithelial cells. The BCB inhibits paracellular diffusion of water-soluble molecules across this barrier. In addition, it has a secretory function and produces CSF. With CNS pathology, these barrier characteristics are disrupted, leading to edema and inflammation entry into the CNS [11]. Molecular diffusion and exchange can occur in both directions and additionally via the perivascular space as well as the CSF [13].

HIV is neurotropic, as indicated by neurocognitive impairment with HIV infection of the brain, on the one hand, and by decreasing neurocognitive impairment in patients with viral suppressions from cART, on the other hand. Moreover, HIV brain invasion results in neuronal loss, synaptic and dendritic damage, astrogliosis, microgliosis, and multinucleated giant cell formation [14, 15]. HIV infection of the CNS occurs by cell-free and HIV-infected cells that migrate from peripheral blood into the CNS. Cell-free HIV particles pass through the BBB using mannose-5-phosphate receptor and tight junction dissolution [16]. Moreover, HIV proteins and HIV infection activate T cells and monocytes resulting in immune cell trafficking across the BBB. Gp120 protein is a potent neurotoxin. Circulating gp120 increases BBB permeability by downregulating tight junction proteins [17]. Tat protein itself causes oxidative stress leading to compromised BBB integrity. Nef protein not only facilitates downregulation of CD4 and increases HIV replication; it also stimulates apoptosis and induces disruption of the BBB [18]. Lastly, viral protein R (Vpr) increases permeability of the BBB and recruits monocytes and macrophages into the CNS by dysregulating the astrocyte compartment [19].

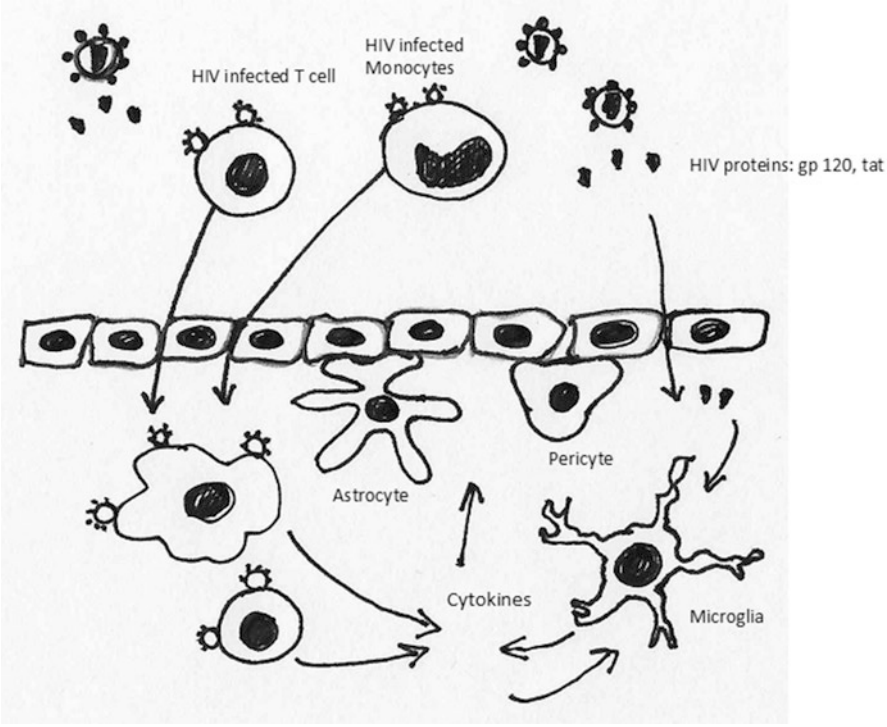

Fig. 17.1 Illustrates the mechanism of viral and cellular migration from peripheral blood into the brain. HIV, HIV proteins (gp120, tat), and HIV-infected cells can cross the BBB via transcytosis and infected microglia and astrocytes which then will be activated and release inflammatory cytokines, further activating microglia and astrocytes (Adapted from Hong and Banks [12])

Pericytes of the BBB endothelium secrete cytokines and increase HIV penetration of the BBB [16]. LPS stimulation, as a result of the increased gut microbial translocation [20], may facilitate HIV entry into the brain by inducing production and release of inflammatory mediators by brain endothelial cells. Additionally, cell-to-cell interaction facilitates HIV transmission from T lymphocytes to astrocytes. It was observed that virologic synapses formed by filopodial extensions binding of either cell type could be inhibited by anti-C-X-C chemokine receptor type 4 (CXCR4) antibody [18]. The interaction between HIV with the brain microvascular endothelial cells increases BBB leakiness, as a result, it increases brain-to-blood efflux of antiretroviral drugs [12, 18] (Fig. 17.1).

Penetrability of the ARVs across the BBB is facilitated by low molecular weight, high lipophilicity, degree of ionization, active transport pump, cerebral blood flow, and the degree of local inflammation [21, 22]. Many antiretroviral drugs, including protease inhibitors such as ritonavir and indinavir, are substrates for brain-to-blood transporters like P-glycoprotein (P-gp) [23]. Such transporters can pump the substrates out of the brain and prevent drugs from reaching therapeutic concentrations within the CNS [24].

17.2.2 The CNS as a Compartment

As mentioned above, the CNS is one of the compartments that HIV infects, and it provides sanctuary and allows independent replications. Occasionally, HIV escapes the CSF despite suppression in the plasma. It suggests that low-grade central nervous system infection may continue in treated patients and can cause further neurocognitive impairment [25, 26]. Different and inadequate penetration of antiretroviral agents can cause resistant mutations and distinct genetic profiles compared to HIV in plasma [27, 28].

17.3 cART

There seems to be an inverse correlation between concentration of antiretroviral agents in the CSF and the HIV CSF viral loads [22, 29]. Generally, the levels of ARVs in the CSF are low compared to plasma. There are questions if those ARVs in CSF are actually adequate to inhibit HIV replication. ARV concentration in the brain parenchyma is not uniform [30]. The ideal way to measure the ARV concentration in the CNS is the actual measurement of tissue concentration or the fluids (e.g., sinuses) from different parts of the brain. Such measurements are impractical in clinical settings. Most studies, however, use the ARV concentration in CSF as an indirect measurement of drug exposure, although it is unclear whether CSF concentrations accurately reflect parenchymal ARV concentrations [13].

Suppression of HIV replication requires a minimum drug concentration above the inhibitory concentrations (ICs). ICs are concentrations based on in vitro findings using HIV strains susceptible to the drug. For example, the concentration of the

drug necessary to inhibit 50% (IC50), 90% (IC90), or 95% (IC95) of viral replication is reported. For improved in vivo estimates, the corrected IC is derived by taking into account drug binding to plasma proteins, and the effective concentration (EC) is then calculated [31].

Frequently, IC50 of ARVs for wild-type HIV is often used as a reference and compared with half-maximal effective concentrations (EC50) to represent the plasma concentration [32]. However concentration of the drug necessary to inhibit 90% or 95% (IC90, IC95) offers greater accuracy. For example, for protease inhibitors, which are typically highly protein bound, the IC50 in CSF can be overestimated. It needs caution to compare IC50 values in plasma and in CSF [31, 33]. The measurement may vary depending on the methods to determine it, cell types, chronicity of infection, and HIV strains [34, 35]. Clinically, it also depends on the overall cART regimen, which may have additive or synergistic effects due to the use of multiple drugs. This rationale may be useful to treat CNS infection and to ensure that the cART regimen has adequate CNS drug levels. CNS escape, however, may occur as a result of inadequate treatment (suboptimal drug concentrations) of the CNS-compartmentalized HIV. Later, it may lead to the development of resistance mutations and additional neurologic complications [36–38]. Unfortunately, it is very difficult to compare drug levels and their IC values, because the results can vary with the protein concentrations as well as drug protein-binding ability [31]. Letendre et al. developed a quantification rank system of ARVs in CSF, which can be useful in selecting ARVs for patients with neurocognitive impairment [29, 39].

17.4 CNS Penetration and Effects of Current ARVs

17.4.1 Nucleoside/Nucleotide Reverse Transcriptase Inhibitors(NRTIs)

NRTIs are effective CSF concentrations because of their small molecular weights and low plasma protein-binding capacities; however, they have elevated hydrophilicity, which does not favor crossing the BBB. The optimum way to analyze the CNS concentration of NRTIs is to measure their intracellular triphosphate metabolites, which is not practical in the clinical setting [31]. Moreover, NRTIs are transported by organic anion transporter (OAT) at the *choroid plexus* [40]. Strazielle et al. studied delivery of ARVs and found that zidovudine (AZT) is the best among the NRTIs followed by stavudine, didanosine, and lamivudine [41]. Following is summary of the available data on CNS penetration of each NRTI.

17.4.1.1 Zidovudine (AZT)

AZT has the highest partition coefficient, which determines the ability of AZT to distribute, in the brain and CNS tissue [41]. It is the substrate for P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP)-4 and MRP-5 [42]. Since it

was approved, pharmacokinetics of AZT was studied extensively [43–45]. In the largest study, Burger et al. had 39 patient participants and studied CSF concentrations of AZT. The CSF/plasma ratio increased linearly over time without significant relationship between AZT dose and CSF levels of AZT. This suggests that CSF penetration of AZT is dose independent. This finding may be an explanation for the efficacy of AZT in the prevention and treatment of HIV-related neurological diseases despite in low doses. Moreover, Burger et al. demonstrated that AZT reached therapeutic levels in CSF and was able to decrease CSF HIV viral load. Thus, it improved neurocognitive dysfunction as a single agent [43].

It is also associated with improved neuropsychological functioning in children with progressive encephalopathy by reduction of HIV viral load in CSF and improves neurocognitive performance in children with HIV encephalopathy as found early in the HIV epidemic to the present [46–49]. In addition, ART improves neurocognitive outcomes in HIV-infected children when applied early in their disease process [47, 49].

17.4.1.2 Stavudine (d4T)

The main side effect of d4T is mitochondrial toxicity; this led to a drastic reduction of d4T use in current clinical practice. However, in the early HIV epidemic, the CSF concentration of d4T was studied extensively [50, 51]. In patients with long-term use of d4T, its concentration in CSF ranged from 0 to 109.9 ng/ml with the mean d4T concentration of 51.6 ng/ml that exceeds the EC50 of clinical isolates of HIV (230 nM, 52 ng/ml) [50]. Stavudine uses organic anion-transporting polypeptide (OATP)-like transporter for its uptake [52].

17.4.1.3 Didanosine (ddI)

Didanosine chemical structure contains hypoxanthine. It has less than 5% protein binding [53]. In HIV-infected patients, ddI reaches a negligible level in the CSF. In patients who had been chronically taking ddI, average CSF concentration at 4 h after administration was 0.16 mumol/l in the CSF and 0.70 mumol/l in plasma. CSF concentration primarily uses the OATP-2-like transporter [53]. In one study, it had median CSF/plasma ratios of 0 in five patients [54]. However, unlike AZT, the use of ddI monotherapy did not reduce HIV viral load in CSF [55].

17.4.1.4 Lamivudine (3TC)

3TC has optimum uptake from blood to *choroid plexus* using the dioxin-sensitive transporters and organic cation transporters [56]. The concentrations of 3TC were studied in combination with either AZT or d4T. All subjects had detectable HIV viral load in CSF. However, there was no correlation between plasma and cerebrospinal fluid HIV-1 RNA concentrations; after 12 weeks of treatment, none of the

subject had detectable CSF HIV viral load even when the subjects did not achieve complete plasma HIV viral load suppression. In this study, 3TC had the highest CSF concentration followed by d4T and AZT. Drug concentrations in plasma declined rapidly, while drug concentrations in CSF reduced more slowly. CSF to plasma concentration for d4T, AZT, and 3TC increased over time, and the time-dependent CSF to plasma drug/penetration ratios was highest for AZT followed by d4T and 3TC. And the 3TC level was well above the IC50 (range 66–80 ng/ml) [57].

17.4.1.5 Abacavir (ABC)

ABC has optimum CSF penetration with moderate plasma protein binding and lipophilicity. In animal model, it crosses BBB without influence of other drugs [58]. A human study of 54 subjects demonstrated its CSF/plasma ratio that is enhanced by dose escalation, and the CSF concentration is adequate to inhibit HIV replication. Subjects received ABC 300 mg twice daily as part of cART. The median CSF ABC concentration was 128 ng/mL (range 37–384 ng/ml). Predicted CSF trough concentrations exceeded the IC50 (70 ng/mL) for 85% of the dose interval. The CSF/plasma ABC ratio is approximately 31–44% [59]. However, its lacking in the active efflux mechanism and having P-gp as its major transporter limit CNS penetration [60].

There are no studies to demonstrate a virological or clinical effect in the CNS. Adding high-dose ABC for HAD patients on stable cART did not improve performance scores or reduce CSF HIV RNA levels more than placebo [61].

17.4.1.6 Tenofovir

According to the DHHS guideline, tenofovir has been the preferred agent in cART component (www.aidsinfo.nih.gov). The initial FDA-approved formulation, tenofovir disoproxil fumarate (TDF), demonstrated efficacy and persistency, with concerns of nephrotoxicity, osteopenia, and osteoporosis [62, 63]. To minimize such concerns, tenofovir alafenamide (TAF) was developed. Both TDF and TAF are prodrugs of tenofovir diphosphate. And clinically, TAF has been replacing TDF in the existing combination formulas [63].

CSF tenofovir concentration has been described to be very low due to its limited uptake by membrane transporters [64]. A study to determine tenofovir CSF penetration using random plasma and CSF samples from 183 subjects who were on tenofovir found median plasma, and CSF tenofovir concentrations were 96 ng/mL and 5.5 ng/mL, respectively. Thirty-four of 231 plasma (14.7%) and 9 of 77 CSF samples (11.7%) were below detection. CSF/plasma concentration ratio from paired samples was 0.057. Median CSF to wild-type 50% inhibitory concentration ratio was 0.48 (IQR 0.24–0.98). Moreover, 77% of CSF concentrations were below the tenofovir wild-type IC50. The tenofovir concentrations in the CSF are only 5% of plasma concentrations, which suggest limited transfer into the CSF and possibly active transport out of the CSF. Therefore, tenofovir may not effectively inhibit viral replication in the CSF [65].

17.4.2 Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

17.4.2.1 Efavirenz (EFV)

Despite its common CNS side effects, it has limited CNS penetration. One of the reasons is that EFV bounded extensively by plasma albumin left a small fraction of unbound EFV passively penetrated into CNS. The CNS EFV is not bound well by CSF protein; both unbounded EFV concentrations are similar resulting in distortion of CSF/plasma EFV ratio [66]. The first published study of the CNS penetration of EFV of ten patients showed a mean CSF concentration of 11.1 ng/mL (range 2.1–18.6 ng/mL) and a CSF/plasma ratio of 0.61% [67]. A pharmacokinetic study of EFV showed its CSF penetration of 0.44% of plasma concentration [31]. A recent larger study of 80 CSF samples reported a median CSF EFV concentration of 13.9 ng/mL with all except two samples that were above the IC50 (0.51 ng/mL). Its CSF concentration is only about 0.5–1% of plasma concentration. However, the CSF concentration offers the CSF concentration above IC95 of HIV wild type. This suggests the potent ability of EFV in inhibiting HIV in the CSF in such a low CSF concentration [68]. Its efflux mechanism uses P-gp expression and function. It also concentration-dependently inhibits MRP-1, MRP-2, and MRP-3 [69]. Moreover, EFV major haptic metabolite, 8-hydroxyefavirenz, neither has significant association with EFV plasma concentration nor association with CYP2B6 genotype; therefore, it reaches the 0.01 μM toxicity threshold [54, 70]. There are increasing evidences of EFV-related CNS toxicities. We describe cART especially EFV toxicities in Sect. 17.6 of this chapter.

17.4.2.2 Nevirapine (NVP)

NVP crosses BBB well, has stable CSF concentration, has highest CSF/plasma penetrability rate, and offers the highest penetrability rank in Latendre classification [39, 54, 71, 72]. It has median CSF/plasma ratio of 0.63 [54]. In a study of nine HIV-infected patients, the median CSF NVP concentration was 932 ng/mL (range 219–1837 ng/mL), which exceed the CSF IC50 by tenfolds [72, 73]. Therefore, it is suitable to use in patients with neurocognitive impairment.

17.4.2.3 Etravirine (ETV)

ETV has a CSF/plasma ratio of 4%. In one study, all 17 CSF concentrations exceeded the wild-type IC50 by a median of 13.6-fold [31]. In another study of 12 patients, the median ETV concentration in plasma was 611.5 ng/mL with the median CSF concentration of 7.24 ng/mL, which was above the IC(50) range (0.39–2.4 ng/mL). The median ETV CSF/plasma ratio was 0.01. All but one patient had undetectable CSF viral load. This study suggested that ETV use was associated with virus suppression in CSF and plasma and may help control HIV in the CNS [31, 72, 74].

17.4.2.4 Rilpivirine (RPV)

RPV CSF drug penetration has been limitedly studied. In a NVP to RPV switch study, CSF drug concentration was measured. The mean plasma RVP trough concentration was 29.7 ng/mL with the mean CSF RVP concentration of 0.8 ng/mL (95% CI: 0.7–1.0), resulting in a CSF/plasma ratio of 1.4 the protein. It was shown that switching NVP to RVP was safe with reassuring drug levels both in plasma and in CSF [75].

17.4.3 Protease Inhibitors (PIs)

Due to their lipophilicity, PIs are expected to have good CNS concentrations but have low CSF concentrations due to the efflux mechanism (all PIs are P-gp substrates) and high protein-binding capacity (except indinavir) [76]. Using ritonavir to boost PIs also increases CSF penetrability [29].

17.4.3.1 Ritonavir (RTV)

Currently, RTV is used exclusively to pharmacokinetic enhance (aka boost) other PIs rather than as a primary antiviral drug. It is a cytochrome P450 3A isoenzyme inhibitor and increases plasma areas under the curve (AUC), drug half-lives, and trough concentrations (lowest drug concentration at steady state) of others. It has large molecular weight, is highly protein bound, and is also a P-gp inhibitor. Therefore RTV can enhance CSF levels of other drugs both by increasing plasma concentrations and by inhibiting efflux [23].

A cross-sectional study of 28 subjects on saquinavir/RTV therapy was evaluated and resulted in a strong correlation between plasma and CSF HIV viral loads. Low CSF drug levels of both saquinavir (<2 ng/ml) and ritonavir (<25 ng/ml) with low CSF/plasma concentration ratio of <0.005 suggested that CSF ritonavir and saquinavir levels are consistent with the estimated known fraction of unbound drug in plasma (<2%), and suppression of plasma viremia can indicate low CSF HIV RNA levels. Likewise, CSF virologic breakthrough was the result of plasma virology failure [77].

17.4.3.2 Indinavir (IDV)

Currently, IDV is only rarely used because of its dosing frequency and renal toxicity; however, it offers the best CNS penetrations among the PIs because of its low protein-binding capacity [78, 79]. It is the only PI that has a CSF concentration that attains its IC95 (18–71 ng/mL) [78, 80, 81]. It is also the only PI that achieves therapeutic concentrations in CSF without RTV boosting (dosing 800 mg three times daily). Using it in a boosted fashion with RTV, there are even higher CSF concentrations with mean CSF IDV concentrations of 203 ng/mL. This is well above the IC95 (18–71 ng/mL) [79]. Using IDV showed clinically improved neurocognitive dysfunction [81].

17.4.3.3 Lopinavir (LPV)

The median CSF concentrations of LPV were 11.2–17.0 ng/mL based on three studies with the CSF/plasma LPV ratio that was approximately 0.2% in all of them [82–84]. All the CSF samples were above the median IC50 (1.9 ng/mL) for wild-type virus. RTV-boosted LPV (LPV/r) has CSF levels that exceed the IC50 [82]. It has been shown that LPV/r both monotherapy and as a component of cART reduces CSF HIV replication and immune activation [84]. However, a recent study with patients on effective cART randomized to LPV/r monotherapy (n1/429) or continued treatment (n1/431) had to be terminated prematurely because of the high rate of failures in the monotherapy arm. A total of four out of six patients with plasma virologic failure developed neurological symptoms, and all of them were on monotherapy. In five of the failing patients, all had elevated CSF HIV RNA levels (3.1–5.1 log10 copies/mL). In addition, 8 of 25 patients who consented to a lumbar puncture at study termination had detectable HIV RNA in the CSF. All these patients were on monotherapy at the time of study termination, whereas none of 15 patients in the continued treatment arm had detectable HIV RNA in their CSF. The use of LPV/r may benefit patients with neurocognitive disorders as part of cART, not as monotherapy [38].

17.4.3.4 Amprenavir (APV) and Fosamprenavir (FPV)

Boosted APV and, its prodrug, FPV (both in unboosted and boosted forms) reaches their IC50 rapidly after oral administration. FPV is almost completely hydrolyzed to APV prior to reaching systemic circulation. A study of 119 matched CSF-plasma pairs from 75 subjects found that APV concentrations were 5.6 ng/ml compared to the IC50 for wild-type HIV. The APV concentrations in CSF exceeded the median IC50 for wild-type HIV in more than 97% of CSF specimens with detectable APV by a median of 4.4-fold (IQR, 2.9–7.9). This showed that FPV may control HIV replication in the CNS as a cART component [85]. With a single dose of 630 mg of APV, only one of five CSF samples collected from healthy males had detectable CSF APV levels [86].

In a study of boosted FPV monotherapy (FPV 700/RTV 100 mg twice daily), 20 patients entered the study with 9 patients (45%) had therapeutic failure. Hence, this study ended prematurely and the use of boosted FPV monotherapy was discouraged. The CSF APV concentration was well above the IC50. Despite virologic failure, APV levels and undetectable HIV RNA levels in CSF were documented in all samples evaluated [87].

17.4.3.5 Nelfinavir (NFV)

NFV did not reach therapeutic CSF concentration and was below the detection limit [88]. However, in two studies, NFV was quantifiable in 9 of 15 samples and 8 of 18 samples, respectively. Some of the concentrations were in the range of

the IC50, but most of them were below it. When used as a single agent for 17 days (in three patients), NFV failed to suppress the CSF viral load [89, 90]. Another study measuring NFV concentration in both plasma and CSF in 6 study subjects, even though NFV was not detected in any of the CSF specimen, there was a significant reduction of HIV RNA PCR in CSF of patients who were treated with NFV-containing regimen. This finding demonstrated that reduction of CSF HIV RNA correlated to the reduction in plasma HV RNA [91].

17.4.3.6 Saquinavir (SQV)

SQV did not reach therapeutic CSF concentration and was below the detection limit even with ritonavir boosting [39, 77].

17.4.3.7 Atazanavir (ATV)

CSF RTV concentrations increase when used with ATV boosting. In a study of 68 patients on a treatment regimen with boosted ATV (ATV 300–400 mg/RTV 100 mg once daily), the median CSF ATV concentration was 10.3 ng/mL (range, 5–38 ng/mL) and the CSF/plasma ratio was 0.9% [92]. Fifty-four percent of the samples were below the IC50 with plasma (11 ng/mL) and 24% were close to the IC50 determined without human proteins (1.0 ng/mL). The authors concluded that ATV did not reach therapeutic CSF concentration and that it might not protect against HIV replication in the CSF. Moreover, it has highly variable CSF concentrations and more than 100-fold lower than the plasma concentration even with RTV boosting. Its concentration did not consistently exceed the IC50 for the wild-type virus [39, 92]. To evaluate the effect of monotherapy with boosted ATV on the CSF viral load, lumbar punctures were performed on 20 patients who had received this regimen as maintenance therapy for 24 weeks. Two patients (7%) failed this regimen. Excluding failing patients, individual measurements of HIV RNA in patients showed occasional viral "blips" in five patients. Samples with elevated HIV RNA greater than 500 copies/ml in CSF were all wild type. The mean ATV drug concentration ratio was 0.9%. This finding supports potential use of PI-based mono-maintenance therapies. However, their results in CSF caution against the uncontrolled use of PI-based monotherapies that can lead to CSF escape [36].

17.4.3.8 Darunavir (DRV)

DRV has detectable and stable levels in the CSF that exceed levels needed to inhibit HIV replication. In a study of 14 samples from eight treatment-experienced HIV-infected patients receiving 600 mg/100 mg of DRV/RTV twice daily plus optimized background therapy, the median CSF DRV concentration was 34.2 ng/mL (range 15.9–212 ng/mL); all of them had CSF DRV levels well above the IC50. The finding suggests DRV contributes to viral suppression in the CNS [93].

DRV/RTV monotherapy was investigated in 225 patients, and three patients developed virologic failure on DRV/RTV monotherapy, and none failed on DTV/RTV triple-drug therapy. No resistance to protease inhibitor emerged in patients with plasma viral load above 50 copies/ml. The patients failing on DRV/RTV monotherapy had no emergence of new DRV resistance mutations preserving future treatment options [37]. Another study of DRV/RTV monotherapy at 48 weeks HIV RNA was less than 50 copies/ml – 86.2 versus 87.8% in the monotherapy and triple therapy arms, respectively. One patient per arm showed at least one protease inhibitor mutation, and one patient in the triple therapy arm showed an NRTI mutation. In both studies, switching to DRV/r monotherapy showed noninferior efficacy versus triple antiretroviral therapy [94]. The addition of two NRTIs led to improvements of symptoms and reductions of CSF viral load.

17.4.3.9 Tipranavir (TPV)

There was no published data on the CSF concentration of TPV.

17.4.4 Integrase Inhibitors (Integrase Strand Transfer Inhibitors, INSTIs)

Although raltegravir, elvitegravir, and dolutegravir are the three agents in this class that are currently approved by the FDA, there are limited published data available for CSF penetration.

17.4.4.1 Raltegravir (RAL)

RAL is a substrate for P-gp in vitro [95]. A study of 18 subjects who took RAL-containing regimens demonstrated a median concentration in plasma that was 260.9 ng/ml, with a median CSF/plasma ratio of 0.058. RAL concentrations in CSF exceeded IC50 for wild-type HIV-1 (3.2 ng/ml) by a median of 4.5-fold. Its presence in CSF was sufficiently high concentrations to inhibit wild-type HIV in all subjects [96]. Another study showed that in patients who were on RAL-based cART regimen, the median CSF/plasma ratio was 0.20 and correlated with plasma and CSF trough concentration. Despite variability of RAL penetration into CSF, the concentrations were well above wild-type HIV IC50s in all patients and above IC95 in 28.6% of the patients [97]. Moreover, in another study, 50% of the CSF specimens, concentrations exceeded the IC95 levels reported to inhibit HIV-1 strains without resistance to INSTIs [93]. Based on its CNS penetrability, as a component of cART, RAL likely contributes to the control of HIV replication in the nervous system as well as being neuroprotective by suppression of HIV-infected inflammatory cytokine, IL-8, production [98].

17.4.4.2 Elvitegravir (EGV)

An ongoing CSF pharmacokinetic study to determine the CSF concentrations of EGV along with tenofovir and TAF (NCT 02251236) has completed subject recruitment; data are not yet available (https://clinicaltrials.gov/ct2/show/record/NCT02251236).

17.4.4.3 Dolutegravir (DGV)

In 12 treatment-naive subjects, using 50 mg doses of DGV in combination of 3TC and ABC, the median DGV concentration in CSF was 13.2 ng/ml, 2–6 h post dose after 16 weeks of treatment. However, clinical relevance of this information is not established (https://www.gsksource.com/pharma/content/dam/GlaxoSmithKline/US/en/Prescribing_Information/Tivicay/pdf/TIVICAY-PI-PIL.PDF).

17.4.5 Entry Inhibitors

17.4.5.1 Enfuvirtide

Enfuvirtide is entry inhibitor acting on the process of fusion. Its chemical structure suggests that it would not reach effective drug concentrations in the brain due to high molecular weight (4492 Dalton), high protein binding (92%), and lack of lipid solubility [31, 99]. Its CSF concentrations were below the lower limit of quantification (25 ng/mL) in 18 out of 18 CSF samples [100]. A report of a patient who developed virologic failure while on enfuvirtide-containing regimen, using genotypic analysis of CSF-derived HIV RNA, V38A mutation was detected in the CSF but not in plasma. This finding illustrated the selection of enfuvirtide-resistant virus in CSF, causing subsequent loss of viral suppression in plasma [101].

17.4.5.2 Maraviroc

To date, maraviroc is the only approved CCR5 coreceptor antagonist for the treatment of HIV-1 infection. It is also a substrate for P-gp [102]. In one study, maraviroc was detectable in all seven CSF samples with a median concentration of 3.6 ng/mL (range 1.8–12.2 ng/mL) [93] and 2.6 ng/mL (range, 0.5–7.2 ng/mL) in another study [103] with median CSF/plasma ratios of 3% and 2.2%, respectively. All CSF samples contained ≥3-fold maraviroc concentration above the median EC90 (0.57 ng/mL) [103] (Table 17.1).

Table 17.1 Characteristics of ARVs based on available pharmacokinetic and pharmacodynamic data

Drug class	Drug	Molecular weight (Da)	Protein binding (%)	Lipid solubility	Protein-free IC 50(ng/ml)
Nucleoside/ nucleotide reverse transcriptase inhibitors	Zidovudine	267	34–48	low	5.3
	Abacavir	286	50	Low	457.6
	Emtricitabine	247	<4	Low	70
	Didanosine	236	<5	Low	1180
	Lamivudine	229	16–36	Low	549.6
	Stavudine	224	Negligible	Low	112
	Tenofovir	288	<7	Low	201.6
Non-nucleoside reverse transcriptase inhibitors	Nevirapine	266	60	Intermediate	32
	Efavirenz	316	9.5–99.8	High	1.3
	Etravirine	435	99.9	High	0.9
Protease inhibitors	Indinavir	712	60	Intermediate	4.3
	Darunavir	548	95	High	0.4
	Fosamprenavir	586	90	Intermediate	4.3
	Lopinavir	629	97–99	Not available	3.1
	Atazanavir	705	86	Intermediate	5.3
	Nelfinavir	664	>98	High	11
	Ritonavir	721	98–99	Not available	Not available
	Saquinavir	671	98	Intermediate	3.6
	Tipranavir	603	>99.9	High	53
CCR-5 inhibitor	Maraviroc	514	76	High	Not available[a]
Fusion inhibitor	Enfuvirtide	4492	92	Not available	Not available[a]
Integrase inhibitor[b]	Raltegravir	594	83	Low	3.6
	Elvitegravir	448	98	Not available	54
	Dolutegravir	419	98	Not available	2.7

Adapted from Yilmaz et al. [31]

[a]Data of protein-free IC50 of maraviroc and enfuvirtide were not available in the same way they were generated for other drugs

[b]Data for newer integrase inhibitors are limited; additional data from recent publications were added in the table [104–107]

17.5 The CNS Penetration Effectiveness (CPE) Score

The CNS penetration effectiveness (CPE) score has been proposed as a method for estimating the combined CNS effectiveness of cART regimens. The study was done as part of the CHARTER (CNS HIV Antiretroviral Therapy Effects Research) study. Eight hundred and thirty-three HIV-positive individuals had enrolled, and 467 (71%) met eligibility criteria for CPE analysis by ARV drug use report and HIV viral load in both plasma and CSF measured. ARVs were classified into three categories based on chemical properties (molecular weight, protein binding, lipophilicity, charge at physiological pH), pharmacokinetic data (mainly CSF concentrations compared

with inhibitory concentrations for wild-type HIV-1), and pharmacodynamic data (effectiveness in CNS in clinical studies). For this initial version of the *CPE score*, individual ARV drugs were assigned a penetration rank of 0 (low), 0.5 (intermediate), or 1 (high) based on their chemical properties, concentrations in CSF, and/or effectiveness in the CNS in clinical studies. The CPE rank was calculated by summing the individual penetration ranks for each ARV in the regimen. The findings noted that the median CPE rank was 1.5. Lower CPE ranks correlated with higher CSF viral loads even after adjusting for the total number of ARV drugs, ARV drug adherence, plasma viral load, duration and type of the current regimen, and CD4 count [29]. In the revised 2010 version of this ranking system, individual antiretroviral drugs are assigned a penetration score of 1 (none), 2 (low), 3 (intermediate), or 4 (high) [39]. CPE rank has been shown to correlate with improvements in cognitive performance and with CSF viral loads in some studies [29, 108], while other studies have found no correlation with neurocognitive improvement, detectable CSF viral loads, or level of intrathecal immune activation [25, 81, 109]. This suggests that using a simple categorical scale still has limitations that might not be sufficient in judging CNS efficacy. Moreover, data for some drugs is very limited and do not take into account possible genotypic resistance. A study of 64 subjects focusing on the effects of CPE score on neuropsychological performance showed that CPE score is not related to cognitive outcomes [110]. In recent studies, there was evidence of worsening neurocognitive function in patients who were on a high CPE regimen. For example, in the HIV-CAUSAL collaborative, 51,938 patients were followed, and they were compared based on high (>9) vs. low (<8) CPE regimens (with regimen CPE scores range 4–16). The patients who were on high CPE score regimens had increased risk of HAD (hazard ratio 1.74), while there was a difference in the risk of developing *cryptococcal* meningitis, CNS toxoplasmosis, and progressive multifocal leukoencephalopathy [111]. Other studies suggested that high CPE regimens may be neurotoxic. Using NRTIs can cause mitochondrial toxicities by inhibiting mitochondrial DNA polymerase gamma. N-acetyl-aspartate (NAA) was used as a surrogate marker for neuronal integrity and mitochondrial function and can be measured by magnetic resonance spectroscopy (MRS). Robertson et al. used this technique in 18 patients and found that patients who were on d4T and ddI had depleted NAA levels [112]. Efavirenz is also found to be neurotoxic and damaged dendritic cells [113]. The antiretroviral drug score (ΣCPE) was investigated in a small study for its potential correlation with brain atrophy. The investigators used ventricular/brain ratio, calculated by lateral ventricular area divided by the brain area at the same level in T2 transversal MRI slices, as an index of overall brain atrophy. The ΣCPE scores were done in 2010 and 2008 version. ΣCPE 2010 version was found to be correlated with atrophy than the 2008 version [114]. Future revision as more information available perhaps helps to better correlate with clinical findings (Table 17.2).

This table shows the CNS penetration effectiveness (CPE) ranking of the currently available antiretroviral. The initial CPE rank proposed by Latendre et al. was based on physiochemical characteristics, CSF concentrations, and efficacy data [29]. However, the recent cross-sectional data that included CSF vs. plasma viral load studies from the CHARTER cohort led to a revised CPE ranking system. The new

Table 17.2 ARVs that penetrate the blood-brain barrier (BBB)

Drug class	Drug	CPE 2010 rank	Transporter	References
Nucleoside/nucleotide reverse transcriptase inhibitors	Zidovudine	4	P-gp, BCRP substrate, ENT, CNT, and OAT	[56, 115–117]
	Abacavir	3	P-gp	[69]
	Emtricitabine	3	MRP	[69]
	Didanosine	2	ENT and CNT	[118, 119]
	Lamivudine	2	OCT, MRP	[56, 69, 120]
	Stavudine	2	CNT	[56]
	Tenofovir	1	MRP	[69]
Non-nucleoside reverse transcriptase inhibitors	Nevirapine	4	P-gp, MRP	[69, 121]
	Delavirdine	3	P-gp, MRP	[69, 121]
	Efavirenz	3	P-gp, MRP	[69, 121]
	Etravirine	2	Unknown	
Protease inhibitors	Indinavir/ritonavir	4	P-gp	[122]
	Darunavir/ritonavir	3	MRP	[123, 124]
	Fosamprenavir/ritonavir	3	P-gp	[125]
	Indinavir	3	P-gp	[122]
	Lopinavir/ritonavir	3	MRP	[126]
	Atazanavir	2	P-gp	[127]
	Atazanavir/ritonavir	2	P-gp	[127]
	Fosamprenavir	2	P-gp	[125]
	Nelfinavir	1	P-gp, BCRP	[23, 128]
	Ritonavir	1	P-gp, BCRP	[128, 129]
	Saquinavir	1	P-gp, MRP, BCRP	[122, 126, 128]
	Saquinavir/ritonavir	1	P-gp, MRP, BCRP	[122, 126, 128]
	Tipranavir/ritonavir	1	P-gp	[130]
CCR-5 inhibitor	Maraviroc[a]	3	P-gp	[131]
Fusion inhibitor	Enfuvirtide[b]	1	Unknown	
Integrase inhibitor[c]	Raltegravir	3	Unknown	

[a]Limited CNS distribution of CSF concentrations is about 10% of the free plasma concentration [131].
[b]Negligible CSF penetration [131]
[c]Data for new integrase inhibitors, elvitegravir and dolutegravir, are not yet available

system reflects stronger associations with CSF viral load analysis by incorporating recent pharmacokinetic and pharmacodynamic data. A higher number estimates better penetration in the CNS [39].

Drug transporters involved in each antiretroviral distribution at the BBB are mentioned. These transporters are ATP-binding cassette (ABC) superfamily includ-

ing P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP). Solute-carrier superfamily (SLC) includes organic anion-transporting polypeptide (OATP), organic anion transporter (OAT), and organic cation transporter (OCT). Nucleoside transporters include equilibrative nucleoside transporter (ENT) and concentrative nucleoside transporter (CNT) (adapted from Shapshak et al. [3]).

Gao et al. proposed a method to predict drug BBB permeability that can apply to both small compounds and macromolecules by various mechanisms besides passive diffusion. The curated drugs with known BBB permeability dataset were extracted from SIDER database. They built classification models with support vector machine (SVM) algorithms using data mining software, evaluated the performance of the model independently from the dataset, and conducted de novo prediction for each drug in the SIDER database. This method uses clinical phenotypes including drug side effects and indications, using dataset of 213 drugs, which has three antiretrovirals including EFV, AZT, and RTV. In this model it was found that it was predicted of BBB permeability in AZT but not EFV and RTV. This dataset is found to have an accuracy of 85.5% and can potentially serve as a point to commence further CNS drug repositioning and combinatorial research [132].

17.6 Nanobiology and CNS ARV Drug Delivery

To date, available antiretroviral drugs are effective primarily in decreasing the viral load in the peripheral system but do not as yet eradicate virus in the CNS reservoir. The primary impediment is the BBB; drug delivery is still a challenge. HIV neurotherapeutics through nanocarrier-based delivery of the antiretroviral drugs through the BBB is a promising methodology against HIV cure by possibly eradicating persistent and latent HIV infection in the CNS. Delivery systems experimented include liposomes and magnetic liposomes, nanoART, cationic trans-activating transcriptor (TAT) nanoparticles, and polymer-based nanoparticles. All of these approaches created an improved ARV delivery to the CNS and are potentially applied to all ARV classes [133]. The global call of HIV eradication promotes research in nanocarriers and noninvasive strategies to deliver drugs through BBB such as site-specific release of ARVs, nanoformulations to eradicate HIV reservoirs, and diagnostic tools to detect and monitor HIV infection. Such developments will help to develop personalized nanomedicines toward HIV cure [134].

17.6.1 Liposomes

Liposome-based nanoformulation was benchmarked by nanoformulation of foscarnet, which was also employed with other ARVs [135, 136]. However, this delivery method seems to have shorter half-life by reticuloendothelial cell sequestration [137].

17.6.2 NanoART

NanoART was explored by Kuo and Chen by incorporating AZT and 3TC into polybutylcyanoacrylate and methylmethacrylate-sulfopropylmethacrylate nanoparticles. This formulation increases BBB permeability [138]. Additionally, they investigated the electromagnetic field-regulated transport of cationic solid lipid nanoparticles across human brain endothelial cells. Using this technique, the permeability of SQV across the brain endothelial cell monolayer was increased about 17- to 22-folds [139]. Later, this strategy was exploited further to deliver IDV nanoparticle loaded into bone marrow macrophages in mice with HIV-1 encephalitis. IDV was detected in the brain suggesting nanoparticles deliver IDV across BBB [140]. ATV and RTV crystalline nanoART and nanoART using monocyte-derived macrophages as Trojan horses (ATV, RTV, IDV, and EFV) are proven to increase ARV penetration through BBB [141, 142]. Additionally, magnetic nanoART was developed using a magnetically guided layer-by-layer technique coloads TDF and vorinostat. This formulation provides sustained drug release with acceptable BBB penetrations [143].

17.6.3 TAT-Nanoparticles

TAT-nanopeptides are the most commonly used cell-penetrating peptides. This demonstrated delivery of RTV to the brain by escaping the P-glycoprotein efflux without disruption of BBB integrity [133].

17.6.4 Actively Targeting Nanoparticles

Actively targeting nanoparticles have been studied in ARV drug deliveries. These include (1) PEGylated albumin nanoparticles encapsulating AZT [144]; (2) methylmethacrylate-sulfopropylmethacrylate nanoparticle functionalized with the bradykinin analogue, RMP-7, to increase permeation of D4T, SQV, and delavirdine (DLV) across the BBB [145]; and (3) brain-specific nano-NRTIs decorated with the peptide apolipoprotein E receptor, which provided low neurotoxicity and enhanced anti-HIV activities [146].

17.6.5 Polymer-Based Nanoparticles

Poly(dl-lactide-co-glycolide) (PLGA) nanoparticles were demonstrated to increase the peak concentrations of RTV, LPV, and EFV in mice brain [147]. Enfuvirtide conjugated with iron oxide nanoparticles coated with an amphiphilic polymer increases enfuvirtide penetrations across the BBB and increases its efflux into brain parenchyma [148].

17.7 cART and CNS Toxicities

Among antiretroviral drugs, EFVs have been well known to cause neurological and neuropsychiatric adverse reactions. Patients treated with EFVs present a wide range of symptoms including nightmares, dizziness, insomnia, nervousness, lack of concentration, as well as more severe symptoms including depression, suicidal ideation, or even psychosis. Moreover, EFVs have recently been shown to associate with mild/moderate neurocognitive impairment.

In fact, neuropsychiatric side effects are the most common cause of EFV discontinuation [149–151]. These side effects can occur as early as the first dose of therapy and likely to subside within the first month. However, some patients might experience them several months or years post-therapy, requiring switching to alternative agents [151–156]. Although the mechanisms of these adverse reactions were unclear, there has been increased evidence of mitochondrial function disturbances in the brain and the bioenergetic systems [70].

In addition, potential EFV neurotoxicity mechanisms include the following:

1. Upregulation of pro-inflammatory cytokines (IL-1β and TNF-α) in blood cells exposed to pro-inflammatory stimuli. In animal models, this is associated with anxious behaviors and impaired cognitive performance, causing spatial memory deficits and increased stress susceptibilities. Moreover, EFVs upregulate these pro-inflammatory cytokines [157, 158].
2. Increase in 5-HT levels occurring in parallel to a reduced activity of tryptophan 2,3-dioxygenase. Apostolova et al. found that EFV-exposed rats showed downregulation of serotonin via tryptophan 2,3-dioxygenase resulting in increased serotonin activities [158].
3. EFV acting as a partial agonist of the serotonin receptors 5-HT2C and, particularly, 5-HT2A [159].
4. EFVs significantly inhibiting creatinine kinase activities in the cerebellum, hippocampus, striatum, and cortex in a mouse model. EFVs affect mitochondrial function of the brain by depleting ADP that leads to cognitive impairment as well as increased seizure susceptibilities in EFV-treated mice [160, 161];
5. It was also shown that EFV reduced creatinine kinase activity in the mouse brain by a specific inhibition of complex IV (cytochrome c oxidase) of the electron transport chain in the cerebral cortex, striatum, and hippocampus, but not in the cerebellum [162].
6. EFV, not other NNRTIs, increased endothelial permeability by inducing reduction in, and disrupting localization of, a tight junction protein, claudin-5 [163].
7. EFV impacts mitochondrial function and neural bioenergetics. EFV was found to increase soluble amyloid beta, promote increased β-secretase-1 expression, and decrease clearance of the amyloid beta peptides, resulting in mitochondrial stress [164].

In addition, the impact of EFV on biogenetics especially in the neurons and glial cells has been shown in in vitro studies. It disrupts mitochondrial function by direct inhibition of complex I activity of the mitochondrial electron transport chain, lead-

ing to a decrease in total oxygen consumption, an increase in the production of reactive oxidase species, and a decrease in mitochondrial membrane potential [165, 166]. The action of EFV on the mitochondria of glial cells and neurons is similar. EFVs directly inhibit complex I resulting in reduction of mitochondrial respiration both in neurons and glial cells. However, the bioenergetic responses of reduction of mitochondrial respiration in glial and neurons are different. In glial cells, the increase in the AMP/ATP ratio induced by mitochondrial dysfunction causes the activation of AMPK (by phosphorylation forming P-AMPK), leading to upregulation of glycolysis (increased extracellular lactate) and consequently to increased intracellular levels of ATP. Glycolysis activation is not observed in neurons causing reduction in intracellular ATP [166–168].

Bioenergetic interference also plays a role in nitric oxide synthesis in glial cells. The decrease in respiration and the increase in glycolysis and mitochondrial reactive oxygen species generation were partially reversed when inducible nitric oxide synthase activity was inhibited in glial cells. Additionally, while EFV produced a decrease in complex I activity in both neurons and glial cells, a slight inhibition of complex IV activity was detected only in glial cells, which is consistent with an inhibitory action of nitric oxide on this mitochondrial complex. EFV-treated mice display inhibition of complex IV activity in different areas of the brain in EFV-treated mice, and no alteration of complex I activity was observed [158, 162].

Direct toxicities from EFV primary metabolite, 8-hydroxyefavirenz, have been observed by stimulation of glycolytic flux and decreased mitochondrial oxygen consumption. Moreover, increase in calcium into cells mediated by L-type voltage-operated calcium channels damages dendritic spines and induces apoptosis [113].

17.8 Conclusions

Using cART helps to alleviate and prevent HAND. However, the efficacy of each ARV compound in the CNS should be considered specifically as well as in concert with others in choosing cART regimens. Early diagnosis of neurocognitive impairment is needed to appropriately select cART regimen with good CSF penetrability rank that proffers neuroprotection.

Another consideration of adequate suppression of HIV replication in the brain is to prevent CNS escape. Compartmentalization of HIV infection is associated with genetic differences between plasma and CSF strains in terms of resistance. Efficacy of cART in the CNS sanctuary requires awareness on ARV penetrability, resistance mutations in CSF, factors as modification of BBB, drug interactions, additive or synergistic effects of cART components, and comorbidities. Unfortunately, technology and data are helpful when assessing individual agents, but more difficult evaluating each regimen. Nanotechnologies are the promising areas to develop efficient drug delivery to eradicate HIV CNS reservoirs. Further studies are needed to discover improved assays to measure cART regimen efficacy in viral compartments

and to find drugs that can assure a good balance between therapeutic effect and neurotoxicity, leading to HIV eradication.

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Conflict of interest The authors report no conflicts of interest.

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Chapter 18
Molecular Mechanisms of Cognitive Impairment in Patients with HIV Infection: Application of Bioinformatics and Data Mining

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Keywords Bioinformatics • Data-mining • Gene interactions • HIV • Infection • Molecular mechanisms • Protein interactions

Core Message

- AIDS patients often suffer from cognitive impairment, including distractibility, delirium, and dementia.
- Global brain atrophy was recognized from MRI images in HIV-associated neurocognitive disorders.
- A complex network of inflammatory molecules including cytokines, chemokines, growth factors, and excitatory compounds is associated with brain inflammation and damage in HIV-infected patients.
- Therefore, the role of each of those molecules should be studied within its network of interactions.
- To this end, genomics and proteomics could be applied to reach a deeper understanding of the molecular mechanisms underlying complex multifactorial disorders.
- Bioinformatics and data mining can become an added value in this context since they help clarify further the pathophysiology of complex diseases by analyzing complex networks of molecular interactions.

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- This approach could provide a starting point to plan targeted experimentation with simple but at the same time dominant tissue microarrays (TMAs) dedicated to HIV-associated neurological disease displaying only selected targets and hence simpler to analyze than larger ones.

18.1 Introduction

Research on the molecular mechanisms of cognitive impairment in patients with HIV infection commenced in the 1980s and is still very active. Since then, clinical studies reported that AIDS patients often suffer from cognitive impairment, including distractibility, delirium, and dementia. Antiretroviral therapy (ART) dramatically reduced the prevalence of HIV dementia, the most severe forms of HIV-associated neurological diseases, and a major AIDS-defining disease in the pre-ART period [1]. Despite these benefits, treated patients remain at high risk for chronic diseases affecting the circulatory system, gut, lung, and lipid, bone, and energy metabolism [1].

Autopsied specimens from brains revealed cortical atrophy, invasion of macrophages, nodules of reactive microglia, and giant cell formation, as well as loss of neurons in frontal, temporal, and parietal regions [2]. Global brain atrophy was recognized from MRI images in HIV-associated neurocognitive disorders [2].

Encephalitis is one of the most common pathologic correlates of cognitive impairment in patients with HIV infection. Cognitive impairment and abnormalities in cerebrospinal fluid (CSF) were observed by magnetic resonance imaging (MRI) in HIV-infected patients [3]. The virus can traverse the blood-brain barrier (BBB) through infected immune cells. Of note, infected immune cells can migrate easier into tissues than uninfected cells [3].

Many studies have shown that high HIV RNA is detected in the brain at the initial stage of the infection or shortly after seroconversion [4] rather than only in more advanced stages of infection. In fact, the presence of HIV proteins, HIV RNA, and HIV particles in the brain along with the central nervous system intrathecal production of anti-HIV antibodies is seen during the initial stages of infection [5, 6]. This supports the hypothesis that HIV may enter the brain from initial infection [7]. Mononuclear phagocytes, i.e., monocytes and blood-borne macrophages, are the primary carriers of HIV load in the brain. Moreover, in the central nervous system (CNS) of these patients, brain mononuclear phagocytes are reservoirs for persistent viral infection. Infected mononuclear phagocytes were found in the human brain. HIV-infected monocytes/macrophages release several viral proteins, some of which activate glial cells such as microglia and astrocytes to release chemokines, cytokines, and some soluble neurotoxic compounds. This stable latent HIV-1 reservoir, in particular in resting memory CD4+ T cells, provides a mechanism for lifelong persistence of infected cells that persist despite prolonged highly active antiretrovi-

ral therapy and present a major barrier to a cure of HIV-1 infection [8]. The memory CD4+ T cell compartment includes long-lasting central memory cells, developing to increase differentiation to short-lived effector memory and terminally differentiated T cells, promoting long-term viral persistence with stem cell-like properties of cellular immune memory [9]. These mechanisms, not entirely clarified, maintain HIV-1 persistence; their knowledge may help to induce HIV-1 eradication in conjunction with HAART treatments. Mononuclear phagocytes infected by HIV-1 regulate the production of chemokines and influence HIV-1 neuropathogenesis. HIV-1-infected and immune-activated mononuclear phagocytes (e.g., microglia) and astrocytes were shown to produce beta-chemokines, as shown in in vitro assays and infected brain tissue [10].

Neurotoxins, in conjunction with secreted HIV proteins, damage the synaptodendritic axis of neurons, resulting in neuronal dysfunction and cell loss via apoptosis [4]. Previous studies reported that HIV proteins abnormally activate the cyclin-dependent kinase CDK5 [5] and GSK3β [6] cascades. The deregulation of the GSK3β enzyme could contribute to HIV-induced neuronal apoptosis. In addition, HIV proteins might activate the CDK5 pathway and subsequently upset the various pathways that CDK5 regulates, including synapse formation, plasticity, and neurogenesis. Past studies also speculated that, in patients with cognitive impairment, the neurodegenerative process might correlate with an altered expression of neurotrophic factors such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and fibroblast growth factors (FGFs). FGFs exert their effects via receptor tyrosine kinases, leading to inactivation of GSK3β through phosphorylation of a serine residue. Other growth factors, such as insulin growth factor-1 (IGF-1), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), cause a similar inhibition of GSK3β activity by inducing phosphorylation [5].

CDK5 is a protein kinase with a postmitotic activity that phosphorylates cytoskeletal proteins (MAP1b, tau, NF, nestin, DCX), synaptic proteins (PSD95, synapsin, cadherin), and transcription factors (MEF2). Its activity is regulated primarily by the metabolism of the activating proteins p35 and p39. A recent investigation indicates that p25, a truncated form of p35, accumulates in neurons of patients with neurodegenerative diseases. Binding of p25 to CDK5 constitutively activates CDK5, changes its cellular location, and alters its substrate specificity. Expression of the p25/CDK5 complex results in abnormal phosphorylation of toxic substrates that induces cytoskeletal disruption, morphological degeneration, and apoptosis [10].

The role of Cdk5 was specifically explored in the development of medulloblastoma, a fast-growing pediatric brain tumor since this protein is expressed at high levels in patients with melanoma and brain, breast, and lung cancer, with poor clinical prognosis [11]. Moreover, cdk5 was found to regulate the expression of PD-L1, one primary controller of the cell cycle which is currently established as a therapeutic target [11].

18.2 3.0 Understanding the Molecular Pathology of Cognitive Impairment in Patients with HIV Infection: Current Concerns and the Need for New Investigation Tools

As described above, a complex network of inflammatory molecules including cytokines, chemokines, growth factors, and excitatory compounds is associated with brain inflammation and damage. Therefore, the role of each of those molecules should be studied within its network of interactions.

To this end, genomics and proteomics could be applied to reach a deeper understanding of the molecular mechanisms underlying complex multifactorial disorders.

In fact, gene and protein expression is not significant *per se* but only if inserted in a detailed cross talk of molecular pathways and gene/gene, gene/protein, and protein/protein interactions.

Many diseases, including HIV-correlated neurological disorders, are complex, polygenic diseases. Their etiology is therefore not attributed to the expression of a single gene or its encoded protein, but it is spread over several different contributing genes. A mere variation in expression of a single gene or of the encoded protein is not meaningful per se but only if put in a proper framework of interactions (i.e., physical interaction of different molecules, involvement in the same metabolic pathway, and co-expression in microarray studies) [12].

The analysis of the complex network of interactions between genes and proteins may allow the identification of potential molecular markers and targets [13]. This study requires the systematization and the analysis of a vast amount of information emerging from experimental evidence. However, a complete experimental analysis of all the molecules involved in a given process, including both genes/proteins and small molecules, appears a challenging task.

If we consider the gold standard for the analysis of expression of multiple genes, i.e., microarray technology, it appears that the greatest part of genes displayed on the arrays often is not directly involved in the cellular process under study. Commercial arrays with a lower number of genes – usually 150–200 – are available, but the genes displayed are generally once again chosen without a precise consideration of the particular target of the study [14]. The contribution of theoretical disciplines, such as bioinformatics and data mining, is therefore required to integrate this huge amount of data.

Bioinformatics can become an added value in this context [14]. This discipline is defined as the application of information technology to the field of molecular biology, via the development of original algorithms [15]. Another discipline playing an important role in the analysis of genomics/proteomics experiments is data mining, i.e., extracting patterns from data, thus developing new information from previous knowledge [16]. With these approaches, a further simplification of complex information emerging from genomics/proteomics experiments becomes possible. Appropriately combined with bioinformatics techniques and algorithms, data mining

may allow drawing a simpler but at the same time powerful picture of the complex amount of data.

18.3 4.0 Novel Approaches for the Analysis of the Molecular Events Underlying Neurodegeneration in HIV-Infected Patients

From the above, it appears that bioinformatics and data mining may be useful to clarify further the pathophysiology of complex diseases [15, 16]. In the context of HIV-related neurological disease, we (FC in Shapshak et al. 2006) provided a preliminary description of the molecular mechanisms underlying these processes using expression and gene annotation data [10]. This information has been stored in a user-friendly, online-available database that could enable scientists to retrieve biochemical and physiological information on neurodegeneration in HIV-infected patients.

Recently, another data-mining method, defined as the "leader gene approach," has been proposed (Fig. 18.1) [14, 17]. This algorithm is based on the systematic search for the genes involved in a given process; the interaction among these genes is then calculated, and genes are ranked according to the number and the confidence of all experimentally established interactions, as derived from free web-available databases, such as STRING (Search Tool for the Retrieval of Interacting Genes, Heidelberg, Germany). Genes in the highest rank are defined as "leader genes" since they can be preliminarily supposed to play a significant role in the analyzed process. These genes may become potential targets for a focused experimentation, which may be simpler than mass-scale molecular genomics and, at the same time, powerful [13, 14, 17]. The leader gene approach was applied to different cellular processes and pathological conditions. After a pilot analysis of human T lymphocyte cell cycle [14, 18], this approach was used to investigate complex processes such as human kidney transplant [19], osteogenesis [20], and the correlation between oral diseases such as periodontitis and lichen and systemic diseases [17, 21, 22]. Importantly, the role of the leader genes identified by ab initio analysis in the studied processes was confirmed experimentally [23–25].

As an illustrative study, let us consider the case of human *periodontitis*. A bioinformatics and data-mining ab initio analysis identified 61 genes involved or potentially involved in this condition, linked in a complex network of interactions (Fig. 18.2) [17]. Of these, only five were identified as leader genes since they presented the highest number and confidence of interaction with the other genes involved. Those genes were submitted to RT-PCR analysis, which allows to study only one gene at a time but is more precise than microarray analysis [25]. The experimental analysis confirmed the change in expression of leader genes in peri-

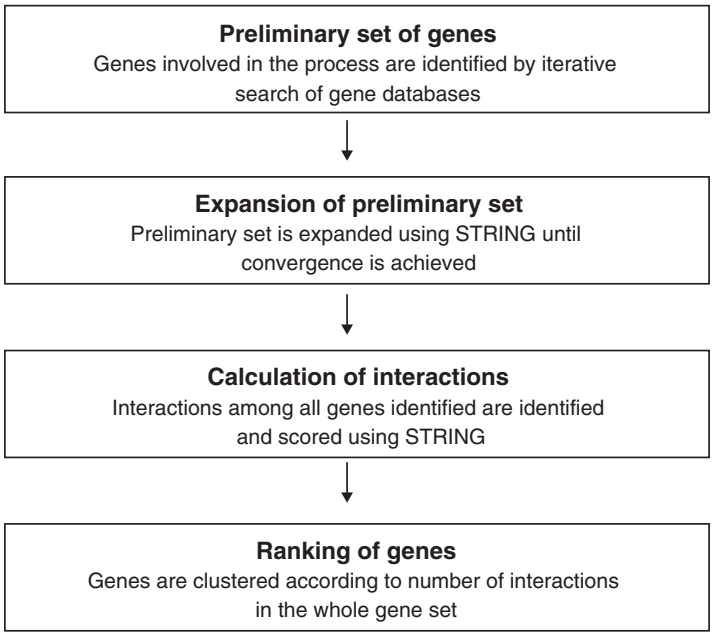

Fig. 18.1 Flowchart of the leader gene approach. A preliminarily set of genes with an established role in a specific process is identified by an iterative search of large-scale gene databases (PubMed, GeneBank, Geneatlas, Genecards), using several keyword-based searches and the official HGNC nomenclature. The preliminary set of genes is expanded using the STRING database (www.string-db.org), excluding text-mining-derived interactions, in order to identify genes linked to those playing an established role in the process under study and therefore potentially involved in it. Only interactions with a confidence score > 0.9, as given by STRING, are considered. Results are then filtered to discard false positives via a keyword-based query in PubMed, until no new genes are retrieved. The interactions between all the genes identified are mapped using STRING. This database gives a combined association score to each gene-gene interaction, representing its strength. The combined association scores referring to each single gene are then summed to obtain a weighted number of links (WNL). Genes are clustered (hierarchical and K-means algorithms) according to their WNL. The genes belonging to the highest class are defined as leader genes

odontal patients, as compared with controls; remarkably, the correlation of two ab initio-identified leader genes – GRB2 and CBL – with periodontitis was shown in that study for the first time. This approach was used to investigate, at a genomic level, the well-established correlation between periodontitis and diabetes [21]. Indeed, it was found that periodontitis and diabetes share four leader genes, and all leader genes are linked to a complex map of interactions (Fig. 18.3). On the other hand, periodontitis and another locoregional infection, sinusitis, share no leader genes, and no interactions were identified. Even with the limitations of any ab initio analysis, these theoretical results suggested the existence of some common genomic pathways between periodontitis and type 2 diabetes, despite the different pathogenesis of these diseases. In particular, the shared leader genes could be supposed to

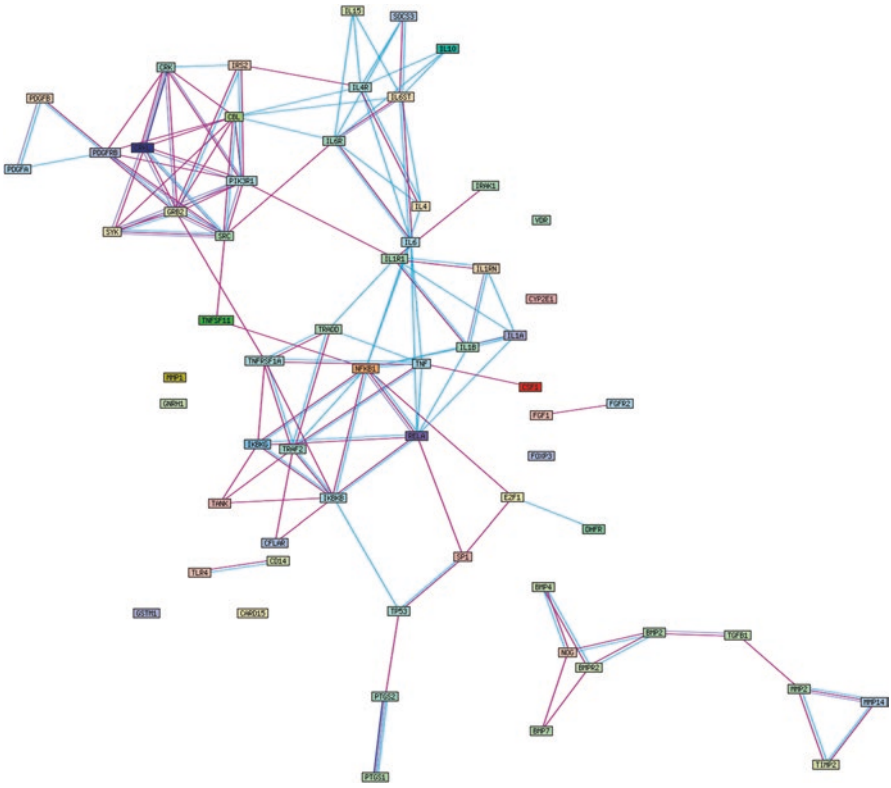

Fig. 18.2 Final map of interactions among genes involved in periodontitis, according to *STRING*. The lines which connect single genes represent physical interaction between proteins, confirmed by various experimental methods or the involvement in the same metabolic pathway. Leader genes are circled in *red* (From Covani et al. [17])

exert a major role in this relationship, which may be investigated further with targeted experimentation.

How can this approach apply to the study of HIV-related cognitive impairment? In recent years, genomic and proteomic studies in this field have been scant. However, the potential utility of tissue microarrays (TMAs) in the study of neuro-AIDS has been proposed [26]. In more details, TMAs constructed from autopsy brain material may allow screening large numbers of identical brain regions for evidence of microglial activation, astrocytic gliosis, and immunohistochemically demonstrable HIV. TMAs can also effectively show differential cell proliferation and hence subtle neocortical changes involving synapses and dendrites that may be early neuropathologic manifestations of HIV-associated cognitive decline.

In line of principle, the leader gene approach may fit well with such studies. Indeed, this data-mining approach could provide a starting point to plan targeted experimentation with direct and dominant TMAs displaying only selected targets

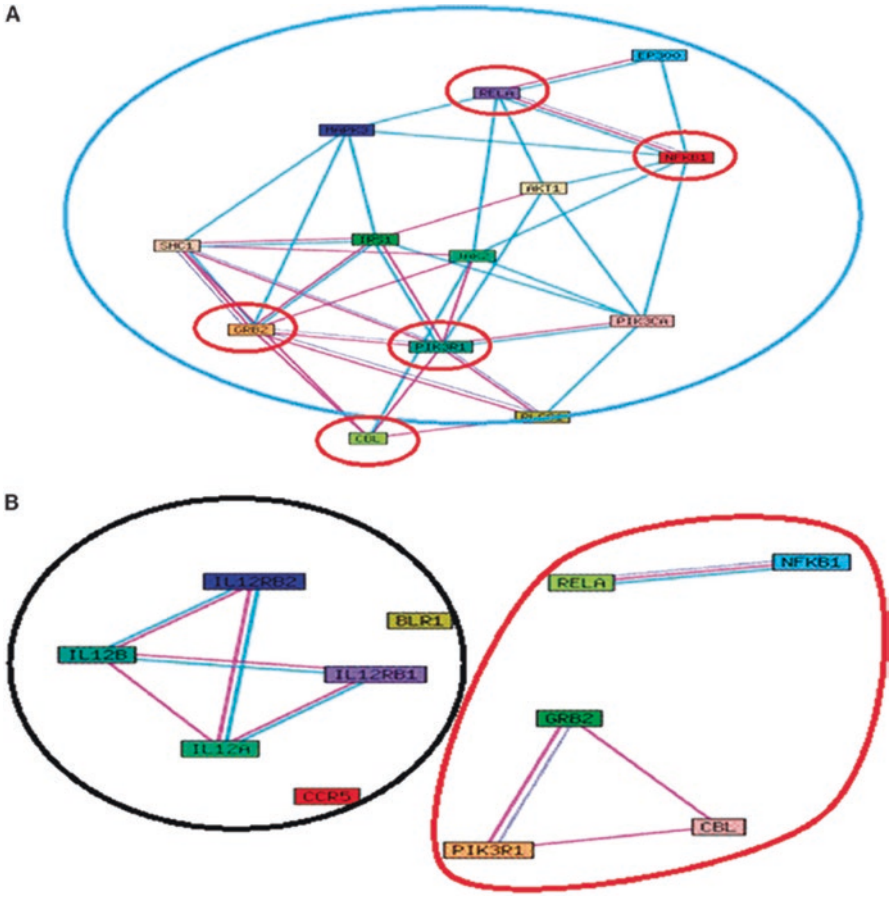

Fig. 18.3 Map of interactions, according to STRING software, among leader genes in human periodontitis and type 2 diabetes (**a**) (leader genes in periodontitis are circled in *red*; leader genes in diabetes are included in the *blue circle*) and leader genes in human periodontitis and sinusitis (**b**) (leader genes in periodontitis are circled in *red*; leader genes in sinusitis are circled in *black*) (From Covani et al. [21])

and hence simpler to analyze than larger ones. Within the current context of translational effectiveness, i.e., the use of the best available experimental data in specific clinical settings for patient-centered care, the cutting-edge concepts here presented suggest that bioinformatics and data mining may have a mounting role in the integration of translational research findings for evidence-based diagnosis and prognostic interventions for patients with neuro-AIDS.

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Conflict of interest The authors report no conflicts of interest.

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Chapter 19
Genetic, Epigenetic, and Transcriptomic Studies of NeuroAIDS

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Keywords Genetic • Epigenetic • Transcriptomic • NeuroAIDS • Mitochondria • Accelerating aging • HIV • HIV-associated neurocognitive disorders

Core Message

HIV-associated neurocognitive disorders (HAND) remain highly prevalent among those with HIV-1 infection. Risk for HAND appears to vary as a function of host genotype, in particular with regard to immune- and dopamine-related genes. Because HAND is a dynamic syndrome within individual cases, gene expression and epigenetic processes are also informative about HAND pathogenesis and potential treatment targets. Additional topics arising at the intersection of genomics and HAND include the iron dysregulation, HIV-associated central nervous system impairment in children, and leveraging epigenetic changes to study the effect of HIV on biological aging in the brain. Finally, several shortcomings of current HAND phenotypes are explored, as are promising alternatives.

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19.1 Introduction

Modern combination antiretroviral therapy (cART) has markedly improved the clinical outcomes of patients with human immunodeficiency virus-1 (HIV-1) infection. Severe neurological complications, such as HIV-associated dementia and HIV encephalitis, are now rare. However, milder forms of HIV-associated neurocognitive disorders (HAND) are increasingly prevalent. The reasons for this continue to be investigated and include chronic immune activation, interactive effects with biological aging, and antiretroviral drug toxicities, among others. In addition, some individuals appear to have inherent resistance not only to infection but also to HAND and other HIV-related complications. In this chapter, we review previous human genetic studies of HAND risk. In addition, because HAND is such a dynamic syndrome, we explore studies employing transcriptomic screening, as well as those investigating epigenetic processes associated with HAND. We also explore specific topics at the intersection of HAND and genomics. This includes the influence of iron dysregulation, mediated in part by genetic variants of iron-regulatory and mitochondrial-related genes; HIV-associated central nervous system (CNS) impairment in children, with a focus on host genetic variants that provide protection or added risk; the influence of aging on HAND risk and the genes that modify this risk; and research into a particularly exciting epigenetic measure of biological aging and its application to HAND. Finally, we address the difficulties of using current HAND phenotypes in genetic studies and offer some alternatives.

19.2 Genetic, Transcriptomic, and Epigenetic Studies of Hand in Humans

cART has resulted in a largely beneficial change in the course and clinical complications associated with HIV infection. In regard to complications involving the CNS, cART has resulted in a marked decrease in the frequency of severe conditions such as HIV-associated dementia (HAD) and underlying neuropathological conditions such as HIV encephalitis (HIVE) and microglial nodules [1–3]. However, in their place are milder forms of neurocognitive impairment, including asymptomatic neurocognitive disorder (ANI) and mild neurocognitive disorder (MND) [4]. The increasing prevalence of these milder forms is due to a variety of factors, including chronic immune activation, amplification of aging processes, and antiretroviral drug toxicities [5–10]. HIV-associated neurocognitive disorders (HAND), which include ANI, MND, and HAD, are diagnosed in 40–50% of unselected, chronically HIV-infected (or HIV+) individuals in the cART era who are able to complete neuropsychological testing [1, 11]. HAND is a public health concern, as it has adverse effects on medication adherence [12], activities of daily living [1, 13], employment, and overall quality of life [5].

The neuropathogenesis of HAND remains incompletely understood; it may overlap that of other common neurodegenerative diseases in which genetics has a role and with which HAND shares certain similarities [14–18]. Studies of genomic factors underlying symptoms and disease have led to helpful insights about HAND neuropathogenesis and identified potential treatment targets. In particular, recognition of the critical importance of neuroinflammation, reflected by elevated expression of inflammation or immune activation biomarkers in the brain, CSF, and plasma [19–21], the central role played by mononuclear phagocytes [16, 22, 23], and the possible role of dopaminergic dysfunction [24, 25], has provided a framework for studies of the role of host genomics in HAND.

However, while the application of genomic and other "omics" approaches coupled with bioinformatics and systems biology is promising, it faces a serious hurdle, the lack of a reliable phenotype for HAND. How can we, without a reliable neurocognitive, neuropathological, or neurophysiological biomarker for HAND, apply these methods in an effective manner? In this review, we present the current state of research involving human genetic, gene expression, and epigenetic data to understand HAND neuropathogenesis. We employ the term HAND to include all HIV-related neurocognitive deficits and their putative neuropathological causes. The benefits and limitations of these methods as applied to HAND are discussed. Finally, we propose potential solutions to overcome the primary obstacle in this research area, namely, a shift from behavioral to biological phenotypes and the application of systems biology as a path toward understanding the complexities of this disease process.

The purpose of this first section is to summarize the current state of understanding of host genomic, transcriptomic, and epigenetic factors that predispose individuals to HAND, with an emphasis on recent studies and reasonable conclusions that may be drawn from this rapidly growing volume of data. We then cover a number of special topics related to host genomic studies of HAND, including a focused examination of mitochondrial and iron-related genes, pediatric neuroAIDS, complement activation pathway polymorphisms, and the intersection of aging and genomics. We also discuss the acute challenge of omic studies of HAND due to lack of validated biomarkers and shifting definitions. We then discuss future directions for research in this field, suggested by the current state of knowledge, including innovative statistical methods, emerging genomic technologies, and therapeutic areas of promise.

19.2.1 Genetic Studies of HAND

19.2.1.1 Candidate-Gene Studies

The field of neurogenetics has long been interested in the role of genes in relation to psychiatric and neurologic characteristics and disease. However, in the case of HAND, there are no heritable neurocognitive deficits or neuropsychiatric symptoms

that would provide a foothold from which to explore genetic contributors of disease. Instead, the focus has been variants of genes involved in various biological processes that significantly impact risk of neurocognitive impairment, course of the disease, response to antiretroviral medications (ARVs), and also those associated with putative biomarkers of HAND. As such, genetic association studies in the realm of HAND serve both as a means to identify risk factors and to help delineate the neuropathogenesis. In this section, the focus is on studies of neurocognitive dysfunction; other HAND-related phenomena are covered later in the chapter. By and large, candidate-gene association studies have focused on immune-related genes and dopamine-related genes, as both the immune system and dopaminergic system are implicated in HAND pathogenesis. A comprehensive list of gene association studies is shown in Table 19.1.

19.2.1.2 Immune-Related Genes

There is a wide variety of immune factors that have been implicated in the chronic neuroinflammatory state leading to HAND, primarily involving cytokines, chemokines, and their cell surface receptors [71–73], as well as other immune factors such as human leukocyte antigen [58] and mannose-binding lectin-2 [50]. Genetic variants of these immune factors can affect HAND neuropathogenesis via numerous routes. For example, because HIV requires chemokine co-receptors to enter cells [74, 75], structural changes in receptors or expression levels of ligands can affect HIV replication [76] and disease progression [77, 78]. Chemokines also affect macrophage activation and chemotaxis of monocytes and other cells across the blood-brain barrier [79, 80], thereby leading to increased inflammation and viral entry into the CNS. Further, chemokines can affect neuronal signaling with subsequent disturbance of glial and neuronal functions [81, 82]. Several candidate-gene association studies have characterized how specific genetic variants of immune-related genes modify risk for HAND [17, 45, 47]. The most widely cited studies are discussed below. A comprehensive list that includes important study information such as sample description, phenotype, and results is provided in Table 19.1.

C-C chemokine receptor type 5 (*CCR5* gene). CCR5 is the most common HIV co-receptor, at least during the early course of infection. CCR5 mediates gp120 neurotoxicity [83]. A 32-base-pair deletion in the *CCR5* gene, resulting in the *CCR5-Δ-32* allele (rs333), leads to structural changes within the HIV co-receptor that confers high resistance to HIV infection among those who are homozygous at this locus [84, 85]. More recently, evidence for neurocognitive improvement among patients treated with CCR5 antagonists lends support for the potential role of variants of this gene in HAND risk [86]. Early genetic association studies suggested that this allele conferred protection against HIV-associated dementia. For example, Boven and colleagues [30] found that not a single case among their samples of European-American individuals diagnosed with HAD had a *CCR5-Δ-32* allele, which normally occurs in 10–20% of individuals with northern European ancestry. Although these findings were validated by another group shortly thereafter [31],

Table 19.1 Summary of candidate-gene and genome-wide association studies of HIV-related neurocognitive disorders

Study 1st author (year)	Sample description	Phenotype	Findings*
Dunlop et al. (1997) [26]	132 adult AIDS patients from Norway; majority were male Postmortem study of the basal ganglia, frontoparietal cortex, cerebral white matter, cerebellum, brain stem, thoracic spinal cord, and hippocampus Study compared the relationship of ApoE genotype with dementia and HIVE	HIV dementia was rated on a graded scale by a physician (no dementia, possible dementia, clinical dementia); however, the criteria were not specified. Determination of HIVE appeared to be based on presence of multinucleated cells, microglial noduli, and/or diffuse damage of white matter. Authors referred to a 1995 paper [27]	No association between *APOE* genotype (rs429358 and rs7412) and either HIV dementia or HIVE
Corder et al. (1998) [28]	44 HIV+ adults; majority were male and Caucasian Participants evaluated for neurological and other symptoms twice yearly for up to 10 visits	Dementia (described as predominantly mild). Criteria were not specified. Reported use of a battery of neuropsychological tests	*APOE ε4* allele carriers were twice as likely to be diagnosed with dementia during the study. The combination of the *ε4* allele and low CD4+ T cells increased risk for dementia over time. *ε4* allele carriers were also more likely to have peripheral neuropathy
Sato-Matsumura et al. (1998) [29]	44 AIDS patients with autopsy-verified HIVE or HIV leukoencephalopathy, 30 AIDS patients without these neuropathologies	HIVE and/or HIV leukoencephalopathy	*TNF-α* genotype (rs# unspecified) did not differ between the two groups
Boven et al. (1999) [30]	9 clinically demented AIDS patients; 8 non-demented AIDS patients; 6 HIV− control patients; age and ethnicity not provided; none of the AIDS patients were being treated with antiretroviral therapy Postmortem tissue specimens from the frontal cortex analyzed	AIDS Dementia Complex/HIV-associated dementia determined by a physician. Dementia cases had a score of 2 or higher on the Memorial Sloan-Kettering scale. Diagnoses were confirmed by postmortem neuropathological examination (methods not specified)	The *Δ32* deletion variant in the *CCR5* gene (rs333) was not found at the expected frequency among ADC patients. In vitro study showed heterozygosity of the *Δ32* deletion was associated with lower viral replication

(continued)

Table 19.1 (continued)

Study 1st author (year)	Sample description	Phenotype	Findings*
Van Rij et al. (1999) [31]	49 patients with AIDS Dementia Complex (ADC); 186 AIDS patients who died of AIDS with no ADC; age and ethnicity not provided; none received triple antiretroviral therapy	AIDS Dementia Complex – criteria were not specified	Lower frequency of the *Δ32* deletion variant in the *CCR5* gene (rs333) among ADC patients. *CCR2* 64I (rs1799864) genotype did not differ between ADC and non-ADC patients
Quasney et al. (2001) [32]	16 HIV+ adults with dementia, 45 HIV+ adults without dementia, and 231 healthy adult controls; 45–56% were Caucasian	HIV dementia per AAN criteria. The Memorial Sloan-Kettering criteria was used to classify severity [33]	*TNF-α-308* (rs1800629) A allele was significantly overrepresented among those with HIV dementia
Gonzalez et al. (2002) [34]	(1) 1151 HIV+ ethnically diverse adults (55% European-American, 36% African-American, 6% Hispanic-American, and 3% others). The majority was male (94%). Sample was followed prospectively with a median follow-up time of 5.9 years (2) 592 Argentinean children perinatally exposed to HIV (322 HIV+, 270 HIV-); drawn from a larger prospective study with a median follow-up time of 4.08 years	HIV-associated dementia. Criteria were derived from the Center for Disease Control [35] and included neuropsychological testing and neuroimaging	In Caucasian adults, homozygosity for the *MCP1 G* allele at rs1024611 was associated with 4.7-fold greater risk of HAD. Further analysis found this allele to be associated with greater transcriptional activity, enhanced protein production, increased serum MCP-1 levels, and increased monocyte infiltration of tissues

Singh et al. (2003) [36]	1049 HIV+ children; median age was 2.4 years; majority (59.7%) was non-Hispanic black Participants followed for up to 36 months	Neurological deterioration, a decline in neurocognitive test scores, or brain growth failure was considered evidence of disease progression between baseline and up to 36 months	Heterozygote carriers of the *Δ32* deletion in the *CCR5* gene (rs333) exhibited delayed disease progression, lower frequency of cognitive impairment at baseline, and lower frequency of either impairment at baseline or a decline in neurocognitive status (trend level) when compared to homozygous wild-type carriers. Among homozygotic carriers of the *Δ32* deletion, the most rapid disease progression was associated with A/A genotype at rs1799987 (*CCR5*) A/A genotype at rs1801157 on the *SDF1* gene was also associated with faster disease progression, including neurocognitive impairment over time. This was relatively uncommon, occurring in <2% of children studied. Modest or little effects were documented for rs1799864 (*CCR2*) or two SNPs on *CCR5* locally designated as 59,356 and 59,353
Cutler et al. (2004) [37]	10 HIV+ African-American males This was a postmortem study of brain tissue comparing sphingolipids and sterols in the medial frontal cortex, parietal cortex, and cerebellum of HIV dementia patients	HIV-associated dementia (determined by the presence of encephalitis in brain tissue and premortem Memorial Sloan-Kettering scale >1)	The *APOE ε4* allele was associated with dysregulated lipid and sterol metabolism, as well as elevations of sphingomyelin, ceramide, and cholesterol in the medial frontal cortex, parietal cortex, and cerebellum. The *ε4* allele was not related to astrocytes or activated microglia
Diaz-Arrastia et al. (2004) [38]	270 HIV+ persons who died from AIDS complications (unknown demographics); two separate cohorts assessed (one cohort whose members died during the monotherapy era, another whose members died during the dual therapy era)	HIVE as well as the presence of any of the following pathologies: microglial nodules, multinucleated giant cells, myelin pallor, and vacuolar myelopathy	No association between pathological findings and the *APOE ε4* allele (rs429358 and rs7412), *TNF-α*, *IL1B*2*, and *ILIRN*2*
Singh et al. (2004) [39]	121 HIV+ cognitively normal participants; the majority were Caucasian, non-Hispanic (68%) and male (88%) Prospective study with a median follow-up of 3.9 years and cognitive retesting every 6–12 months	Neurocognitive impairment, defined as a Clinical Rating score of 5 or higher and based on comprehensive neurocognitive testing [40]	At baseline, none of the alleles examined (*APOE Δ32* deletion, *MCP1-2518*, *CCR2*-V64I) were associated with neurocognitive impairment rates. In the longitudinal analysis, possession of one or two *CCR2*-64I alleles at rs1799864 was associated with earlier progression to neurocognitive impairment from study entry or from estimated time of seroconversion. Null findings: *Δ32* deletion at rs333 (*CCR5*) and rs1024611 (*MCP1–2518-G/A*)

(continued)

Table 19.1 (continued)

Study 1st author (year)	Sample description	Phenotype	Findings*
Valcour et al. (2004) [41]	182 HIV+ adults ($N = 85 < 40$ years, $N = 97 \geq 50$ years); sample was 54% Caucasian, 32% Asian or Pacific Islander, and 14% others	HAD (AAN, 1991), as determined via standardized neuropsychological testing, brain MRI, and serum tests	A significant association was observed between the *APOE ε4* allele (rs429358 and rs7412) and HAD among older (age ≥ 50 years) but not younger (<40 years) participants
Shiramizu et al. (2006) [42]	Repository CSF specimens from 27 prenatally infected with HIV	HIV-associated encephalitis	*MCP1 2578G* allele at rs1024611 was significantly more common in children with high HIV DNA in CSF. This allele was also associated with higher levels of supernatant *MCP1* in the CSF
Burt et al. (2008) [43]	1267 HIV+ adults; ethnically diverse (54% Caucasian); majority were male; they were followed prospectively with a median follow-up time of 5.9 years	HIV-associated dementia. Criteria not specified; however, the cohort appears to be the same as Gonzalez et al. (2002) [34], in which criteria were derived from the Center for Disease Control [35] and included neuropsychological testing and neuroimaging	*APOE ε4* allele was not associated with time to development of HAD, though it was associated with accelerated disease progression and time to death
Pomara et al. (2008) [44]	41 non-demented HIV+ adults, predominantly African-American (63%) and male (63%) This study assessed the effect of the *APOE ε4* allele on memory following acute lorazepam administration	Performance on verbal learning/ memory and psychomotor tests	The *APOE ε4* allele (rs429358 and rs7412) was associated with better immediate and delayed verbal recall at baseline assessment only
Pemberton et al. (2008) [45]	56 Caucasian HIV+ adults with HAD/ ADC, stage ≥1 and CD4 count <500 cells/ μL; other demographics unknown Data were also combined with genetic data from participants of other studies for a meta-analysis. This included HIV+ and HIV− controls	ADC/HAD, stage ≥1 (moderate to vegetative HAD) per [46]	HAD was more common among individuals who were homozygous for *A* allele at rs1800629 (*TNF-α-308*). When this data was combined with previously published data, possession of just one *A* allele was associated with HAD. No differences in allele frequencies were found for rs3783525 (*IL1A-889*), IL1B + 3953, and *IL12B* 3'UTR. *APOE* genotype (rs429358 and rs7412) did not differ between HAD patients and HIV+ controls in this study or when the data was combined with previously published studies

Levine et al. (2009) [47]	143 HIV+ adults (primarily Caucasian and African-American) who were either neurologically normal ($N = 117$) or who met criteria for HIV-associated dementia ($N = 26$) per established AAN criteria	HIV-associated dementia (HAD) per AAN criteria. Diagnosis was established via standardized neuropsychological testing and neuromedical exam	*TT* genotype at rs1130371 within the *CCL3* gene was associated with a twofold greater risk of HAD. Depression was associated with a fivefold greater risk of HAD. There was no association between HAD and any other of the other polymorphisms studied: rs1024611 (*MCP1*), rs1719130 (*CCL5*), rs17561 (*IL-1a*), rs1800872 (*IL-10*), rs1800629 (*TNF-α*), rs1801157 (*SDF1*)
Bousman et al. (2010) [48]	192 sexually active men with and without methamphetamine dependence (METH+/METH−) and/or HIV infection (HIV+/HIV−). Ethnicity was 71% Caucasian, 15% African-American, and 14% Hispanic	Executive functioning domain Deficit Score	There was a main effect of executive functioning but not of *COMT Val158Met* (rs4680) genotype on the total number of sexual partners. There was an interaction between rs4680 and executive functioning on total number of sexual partners and insertive anal sex (among *Met/Met* and *Val/Met* but not *Val/Val* carriers)
Joska et al. (2010) [49]	144 HIV+ young adults just entering care in South Africa, where Clade C is more common; the majority was female (74%)	HAND based on Frascati criteria (Antinori et al. 2007) using standardized neuropsychological testing	Null findings between *APOE* genotype and level of HAND severity. When comparing just HAD to non-HAD, the *ε4* allele (rs429358 and rs7412) was less common in HAD
Spector et al. (2010) [50]	201 Chinese HIV+ adults predominantly (93%) co-infected with hepatitis C	Global Deficit Score based on standardized neuropsychological testing. Considered both cross-sectional comparisons and rates of changes in neurocognitive status over the 12-month study period	A higher percentage of *APOE ε4* carriers (rs429358 and rs7412) were cognitively impaired at baseline. *MBL2* genotype (based on rs1800450, rs1800451, and rs5030737) was associated with neurocognitive changes over a 12-month period: 53% of those with *O/O* genotype declined whereas 23% of those with A/A genotype declined. No significant differences in baseline neurocognitive ability or change over time were observed for rs1799987 or rs333 (*CCR5*), *CCR2-180-G/A*, rs1801157 (*SDF1*), IL4-589-C/T, rs1024611 (*MCP1*), *CX3CR1-745-G/A*, and *-849-C/T* SNPs or for the *CCL3L1* copy number variant
Sun et al. (2010) [51]	44 HIV+ male adults; 11 HIV− adults; 62% Caucasian; all males. Ethnicity included 62% Caucasian and 22% African-American	Neuropsychological impairment (>1.5 SD below normative mean in two domains on a comprehensive test battery)	*APOE* genotype (rs429358 and rs7412) was not associated with neurocognitive outcomes

(continued)

Table 19.1 (continued)

Study 1st author (year)	Sample description	Phenotype	Findings*
Andres et al. (2011) [52]	48 HIV+; 39 HIV−; majority were male; largest ethnic groups were Caucasian (40%), Native or part-Native Hawaiians (28%), and Asians (13%)	Global Cognitive Score, based on the average of several domain *Z*-scores from a comprehensive neurocognitive test battery. The HIV Dementia Scale [53] was also examined as an outcome	Significant interactions were found between *APOE* genotype and HIV serostatus, with HIV+ ε4 allele carriers (rs429358 and rs7412) performing significantly worse than HIV-seronegative *ε4+* controls and seronegative *ε4-* controls on Global Cognitive Score and several specific cognitive domains. Among the HIV+ individuals, ε4 carriers performed worse than noncarriers on the HIV Dementia Scale
Chang et al. (2011) [54]	69 HIV+ adults of mixed ethnicity; predominantly male 70 HIV− adults of mixed ethnicity; predominantly male	Domain *Z*-scores and a Global *Z*-score were determined based on a comprehensive neurocognitive test battery	The *APOE ε4* allele (rs429358 and rs7412) was associated with poorer neurocognitive functioning (verbal fluency, executive function, learning, and memory) and smaller brain volume in HIV+ participants. This allele demonstrated a positive effect among HIV− individuals Further analysis by age group (older vs. younger), HIV serostatus, and *APOE* genotype indicated that the *ε4* allele had a deleterious impact on younger HIV+ individuals and a positive effect among younger HIV− individuals
Gupta et al. (2011) [55]	310 ethnically diverse males separated into four groups: 56 HIV−/methamphetamine nonusers, 77 HIV−/ methamphetamine users, 84 HIV+/ methamphetamine nonusers, 93 HIV+ methamphetamine users	Neurocognitive impairment, defined by a Deficit Score cutoff of ≥0.5. Based on a battery of neuropsychological tests	The C allele at rs6280 of the *DRD3* gene was associated with greater rates of neurocognitive impairment only among HIV+/ methamphetamine users
Singh et al. (2011) [56]	572 HIV+ children (206 progressors and 366 nonprogressors) enrolled in the Pediatric AIDS Clinical Trials Group study. Ages 3 months–18 years	Progression-free survival (defined as either time to first clinical HIV-related disease or death) and CNS-free survival (defined as time to deterioration in brain growth, psychological function, and/or neurological status)	The *B-27* allele was associated with complete protection against disease progression and CNS impairment over a median follow-up period of 26 months. The *Cw-2* allele protected against disease progression and the *A-24* allele was associated with more rapid CNS impairment. The HLA Class II *DQB1-2* allele was associated with a delayed disease progression and CNS impairment

Bol et al. (2012) [57]	86 HAD cases and 246 non-HAD AIDS patients as controls. All cases were from the Netherlands	Diagnosis of HAD was determined in various ways due to the retrospective nature of the data. This included DSM, AAN, and Frascati criteria	The *Δ32* deletion in the *CCR5* gene (rs333) was associated with HAD in cases that developed AIDS prior to 1991, but not after. *PREP1* genotype (rs2839619) differed between cases and controls irrespective of year of AIDS diagnosis. Null findings for: rs429358 and rs7412 (*APOE*), rs1130371 (*CCL3*), rs1799864 (*CCR2*), rs12483205 (*DYRK1A*), rs1024611 (*MCP1*), rs1046099 (*MOAP1*), rs12909130 (*PDE8A*), rs17519417 (*SPOCK3*), rs1800629 (*TNF-α*), and rs2905 (*UBR7*)
Schrier et al. (2012) [58]	Baseline sample of 203 HIV+ and 198 HIV- adults from a rural area of Anhui, China. HIV+ (N = 192) participants were reassessed at 12 months. HLA genotype data was analyzed for 178. 61% were male. Average amount of formal education was 5.5 years. 94% were HCV antibody positive	Global Deficit Score based on standardized neuropsychological testing. Considered both cross-sectional comparisons and rates of changes in neurocognitive status over the 12-month study period	*HLA-DR**04 was associated with a higher rate of baseline neurocognitive impairment, neurocognitive decline at 12 months, and HIV RNA in plasma. HLA Class I alleles (*B*27, 57, 58,A*03,33*) were associated with less impairment at baseline, at 12 months, and with less neurocognitive decline in the interval. The *HLA-DR*04* allele reduced the neuroprotective effect of the Class I alleles and, when present with the *APOE-ε4* in the same individual, had a synergistic negative effect on cognition
Brown et al. (2012) [59]	262 HIV+ individuals; 60% African-American	HIV dementia severity as determined by the Memorial Sloan-Kettering (MSK) classification	There were no differences in *CCL3L1* copy number in relation to HIV dementia severity
Levine et al. (2012) [60]	184 HIV+ adults (primarily Caucasian and African-American). All cases were diagnosed as neurologically normal or with mild cognitive/motor disorder or HIV-associated dementia per established AAN criteria or subsyndromic HIV-related neurocognitive impairment equivalent to asymptomatic neurocognitive impairment as per 2007 Frascati criteria	Neuropsychological domain T-scores (working memory, processing speed, learning, memory, motor). Scores were standardized based on entire NNTC cohort	Regression analysis found that *COMT Val158Met* (rs4680), *BDNF Val66Met* (rs6265), or *DAT1* (3′-UTR 40 bp) genotypes did not predict neurocognitive functioning after controlling for disease severity, depression, and demographic variables

(continued)

Table 19.1 (continued)

Study 1st author (year)	Sample description	Phenotype	Findings*
Soontornniyomkij et al. (2012) [335]	Brain tissue from an ethnically diverse sample of 160 HIV+ and 22 HIV− adult persons. The majority were male	HAND and Aβ plaques. HAND (mild cognitive/motor disorder or HAD per AAN criteria or subsyndromic impairment equivalent to asymptomatic neurocognitive impairment per 2007 Frascati criteria). Evaluation included comprehensive neurocognitive testing and neuromedical examination	*APOE-ε4* allele (rs429358 and rs7412) and older age (≥50) were independently associated with increased likelihood of cerebral Aβ plaque deposition. Although the *APOE-ε4* allele did not increase risk of HAND independently, ε4 carriers with Aβ plaque deposition had a higher risk of HAND
Morgan et al. (2013) [61]	466 HIV+ adults; 50% Caucasian; 78.8% were male	HAND based on 2007 Frascati criteria	This cross-sectional study found no effect of *APOE-ε4* allele (rs429358 and rs7412) on HAND or HAD in particular. No interaction between *APOE-ε4* allele and age, ethnicity, substance use disorders, duration of infection, or nadir CD4 on risk for HAND was observed
Panos et al. (2013) [62]	259 HIV+ ethnically diverse adults (55.2% Caucasian)	HAND (mild cognitive/motor disorder or HAD per AAN criteria or subsyndromic HAND equivalent to asymptomatic neurocognitive impairment as per 2007 Frascati criteria). Evaluation included comprehensive neurocognitive testing and neuromedical examination	94% of older *APOE-ε4* allele carriers had HAND compared to 56% noncarriers among older participants (age ≥ 50 years). No association between ε4 and HAND was found for younger (<50 years) participants Analysis by cognitive domain revealed the combination of advanced age, and the ε4 allele was associated with poorer executive functioning and information processing speed

Singh et al. (2013) [63]	1049 HIV+ symptomatic pediatric patients; predominantly of minority status (60% African-American, 26% Hispanic). Participants were enrolled prior to combined antiretroviral therapy availability Longitudinal study with median follow-up time of 18.6 months	CNS impairment defined as time from to deterioration in brain growth, psychological function, and/or neurological status. Neurocognitive decline was defined as the absence of any increase in raw scores or a decline in normalized scores by 2 SD for children <30 months of age or by 1 SD for older children. Deterioration in neurologic function was defined as the loss of previously documented motor skills, reflexes, or behavior	*APOBEC3G-H186R* (rs8177832) *G/G* genotype was associated with CNS impairment compared with the wild-type *A/A* or *A/G* genotype. Both additive and dominant models found the *APOBEC3G-F119F-C* allele (rs5757465) to protect against CNS impairment
Hoare et al. (2013) [64]	24 HIV+ individuals with at least one *APOE-ε4* allele were compared to 19 HIV+ without *ε4* allele. Participants were young, mostly females, and of Xhosa origin. This study was conducted in South Africa, where Clade C is more common; the majority was female (74%)	Group comparisons in neuropsychological functioning and diffuser tensor imaging were conducted	The *ε4* group had poorer immediate and delayed verbal memory and decreased fractional anisotropy in the corpus callosum
Morales et al. (2013) [65]	Cross-sectional study of 20 HIV+ and 16 HIV- women from the Hispanic-Latino Longitudinal Cohort of Women	AAN criteria (1996)	HIV+ women who were heterozygous at the rs4790084/rs1204828 loci in the *YWHAE* gene were three times more likely to exhibit reduced cognitive functioning, to have been diagnosed with HAND, and to express less YHWAE protein as compared to homozygotes. HIV+ women with HAND expressed 4.5 times less YWHAE in CSF compared to HIV+ neurocognitively normal women

(continued)

Table 19.1 (continued)

Study 1st author (year)	Sample description	Phenotype	Findings*
Levine et al. (2014) [66]	Longitudinal study of 952 individuals enrolled in the Multicenter AIDS Cohort Study. 952 individuals (914 Caucasian/non-Hispanic and 38 Caucasian/Hispanic). Roughly half were HIV+. All cases must have had 2+ neurocognitive evaluations between November 1985 and May 1995, a time frame chosen to avoid the possible confounding effects of HAART	Domain T-scores based on a comprehensive neurocognitive battery	No four-way interactions were found, indicating that HIV and stimulant use do not interact over time to affect neurocognitive functioning as a function of genotype. Numerous three-way interactions were found, but only rs1024611 (*CCL2*) and rs1719134 (*CCL3*) affected HIV+ individuals specifically. Specifically, for *CCL2* HIV+ cases with *TT* genotype improved at a faster rate than the HIV+ with *CC* or *CT* genotype. For *CCL3*, memory functioning declined in HIV+ individuals with *AA/AG* genotype for *CCL3*. Dopamine-related genetic variants generally affected HIV− individuals only
Chang et al. (2014) [67]	Cross-sectional study of 177 primarily of white or mixed race individuals. 80 were HIV+ and 97 HIV-. Mean age in the mid-40s. Most were men	Metabolite concentrations using MRS. Seven neurocognitive domain scores based on a comprehensive neurocognitive battery	Frontal white matter myoinositol was elevated in HIV+ participants across the age span. An age-dependent increase in HIV− participants was observed, most notably in those with the *APOE-ε4* allele. Only HIV− participants with the *APOE-ε4* allele showed elevated myoinositol in parietal cortex. All participants with *ApoE-ε4* had lower total creatine in basal ganglia. All HIV+ participants had poorer neurocognitive functioning, with *APOE-ε4* carriers having the poorest. In addition, higher myoinositol levels were associated with poorer motor function across all participants, slower speed of information processing in *APOE-ε4* participants, and poorer fluency in HIV+ participants with *APOE-ε4* genotypes
Sundermann et al. (2015) [68]	Two cross-sectional studies. A behavioral study involving 54 HIV+ and 33 HIV− women and an imaging study involving 23 HIV+ and 13 HIV− women	For the behavioral study, an N-back test for assessment of working memory. For the imaging study, the same test in conjunction with fMRI	HIV+ participants performed worse on the N-back test. An interaction between serostatus by *COMT* genotype was found, with *Val/Val* HIV+ women demonstrating poorer performance on the N-back test compared to HIV− women with the same genotype. Analogous findings resulted from the imaging study, with a serostatus X genotype interaction: HIV+ *Val/Val* carriers had greater prefrontal activation compared to HIV− *Val/Val* carriers. Conversely, HIV− *Met* carriers had greater prefrontal activation compared to HIV+ *Met*

Becker et al. (2015) [69]	Cross-sectional and longitudinal analysis of 2846 participants from the Multicenter AIDS Cohort Study who had *APOE* genotyping and neurocognitive data available for analysis. Age range 22–87, all male	Domain *T*-scores derived from a comprehensive neurocognitive battery. Overall cognitive functioning was characterized as follows: (1) within normal limits if one or fewer domains had *T*-scores 1 SD or more below the mean (i.e., $T \leq 40$), (2) mild impairment if two or more domains had *T*-scores ≤40, and (3) severe cognitive impairment if two or more domains had *T*-scores ≤30 or one domain had a *T*-score ≤ 25	*APOE* genotype was not significantly associated with time to death. The *APOE-ε4* allele was not related to incident cognitive impairment. No significant interactions between *APOE*, HIV status, and age on neurocognitive impairment were found
Villalba et al. (2015) [70]	Cross-sectional study of 267 HIV+ adults from urban areas of Miami-Dade County, Florida. Ages 18–60. All participants had history of alcohol use disorder	Three neurocognitive tests were used to characterize executive ability, cognitive flexibility, and visual memory	Significant associations between rs6277 (*DRD2*) and executive functioning and cognitive flexibility were found. When stratified by race and gender, significant results were seen in males and in African-Americans. The *VNTR* 7-allele (*DRD4*) was significantly associated with executive dysfunction

*When possible the Research SNP (rs) number is provided

more recent studies have not replicated this association [39, 50], possibly due to the changing pathogenesis of HAND. For example, Bol et al. [57] observed that the *CCR5-Δ-32* genotype was associated with HAD in individuals who developed AIDS prior to 1991, but not after, which was interpreted as reflecting the waning effect of this genotype on viral load set point. However, Singh and colleagues [36] found that children heterozygous for the *CCR5-Δ-32* allele had slower disease progression and less cognitive impairment than homozygous wild-type individuals. The phenotype in that study was neurocognitive functioning rather than HAND diagnosis.

Monocyte chemoattractant protein-1 (*MCP1* or *CCL2* genes). MCP-1 is a chemokine that recruits monocytes and other immune cells into the CNS and is therefore believed to be responsible in part for the neuroinflammatory response. In vitro HIV infection of human leukocytes results in increased transmigration across the BBB in response to MCP-1, which in turn increases expression of MCP-1 [87]. Levels of MCP-1 are elevated in the brain and CSF of patients with HIVE and HAD as compared to controls [19, 20], and MCP-1 in CSF is associated with pathologic magnetic resonance spectroscopy (MRS) indicators [71]. The HIV protein Nef induces MCP-1 expression in astrocytes with subsequent infiltration of infected monocytes into the brain [88]. The most commonly studied polymorphism in the context of HAND is SNP rs1024611, resulting in the *MCP1-2578* allele. This allele is associated with increased levels of MCP-1 in serum [89] and CSF [90] and has been linked to accelerated disease progression and a 4.5-fold increased risk of HAD [34]. However, this finding has not been consistently replicated [47, 50]. Also, while a recent study found evidence of a statistically significant rate of working memory ability change over time among carriers of this allele compared to noncarriers and HIV-uninfected individuals, the practical change was negligible [66]. Further, Thames et al. [91] reported that this polymorphism affects levels of inflammatory factors in CSF, which in turn affect neurocognition; however, no direct association between *MCP1* genotype and neurocognitive functioning was found in that study. Other polymorphisms outside this gene that affect the impact of MCP1 on neurocognitive functioning in HIV have been examined. For example, a recent study found a significant difference in *PREP1* allele distribution among HAD cases and non-HAD HIV+ controls [57]. Prep1 is a transcription factor with preferential binding to the promoter region of the *MCP1* gene. In addition, a polymorphism within the minor HIV co-receptor CCR2, the natural target receptor for MCP-1, may result in slower HIV disease progression [92]. Specifically, individuals heterozygous for the *CCR2-V64I* allele exhibited slower disease progression and developed AIDS 2–4 years later than those who were homozygous for the wild-type allele. Still another study found *CCR2-V64I* to be associated with slower progression to neurocognitive impairment [39].

Macrophage inflammatory protein 1-alpha (*CCL3* gene). CCL3, or MIP-1α, is a chemokine and natural ligand of the HIV co-receptor CCR5. MIP-1α expression is increased in the brains of those with HIVE and is released by both microglia and astrocytes [93]. SNP rs1130371 within the *CCL3* gene was previously associated with HIV disease progression [94] and was found to be associated with a twofold

greater risk for HAD [47] in the National NeuroAIDS Tissue Consortium (NNTC) cohort. An interactive effect was found between another SNP (rs1719134) and HIV status on changes in learning ability over time, such that HIV+ individuals demonstrate less improvement over multiple assessments as compared to their HIV-negative counterparts; however, the difference was negligible from a practical standpoint [66].

*HLA-DR. HLA-DR*04* genotype was identified as a predictor of HAND, low CD4+ T-cell responses to HIV, and low plasma HIV RNA levels in a US cohort. It was hypothesized that low CD4+ T-cell activation may lead to poor immune control of HIV in the CNS, predisposing to HAND, but it may also provide fewer targets (activated CD4+ T cells) for HIV replication. To assess the consistency of these HLA Class II associations in a new cohort and extend analysis to HLA Class I, HLA types, neurocognitive, and virologic status were examined in a cohort of former plasma donors in China [58]. In this study, 178 HIV+ individuals in Anhui, China, were HLA typed and underwent assessment of neurocognitive function (using locally standardized norms) and neuromedical, treatment, and virologic status at baseline and 12 months. *HLA-DR*04* was associated with a higher rate of baseline neurocognitive impairment ($p = 0.04$), neurocognitive decline ($p = 0.04$), and lower levels of HIV RNA in plasma ($p = 0.05$). HLA Class I alleles (*B*27,57,58,A*03,33*) that specify a CD8+ T-cell response to conserved HIV sequences were neuroprotective, associated with less impairment at baseline ($p = 0.04$) and at month 12 ($p = 0.01$) and less neurocognitive decline ($p = 0.02$) in this interval. Consistent with the theory that effective CD8+ T-cell responses require CD4+ T-cell support, the *HLA-DR*04* allele reduced the neuroprotective effect of the Class I alleles. The presence of *HLA-DR*04* and the Alzheimer's disease-associated allele *APOE-ε4* in the same individual had a synergistic negative effect on cognition ($p < 0.01$). Despite major background differences between US and Anhui, China, cohorts, *HLA-DR*04* predicted neurocognitive impairment and lower plasma HIV RNA levels in both populations. HLA Class I alleles associated with CD8+ T-cell control of HIV were associated with protection from HAND.

APOE-ε4 and MBL-2 genes [50]. For the above Chinese individuals, among 43/201 patients with the *APOE-ε4* allele, 58% were cognitively impaired, compared with 31% without the *APOE-ε4* allele ($p < 0.01$, odds ratio 3.09, 95% confidence interval 1.54–6.18). The mean global deficit score (GDS, a composite score derived from a battery of neurocognitive tests) for *APOE-ε4* carriers on antiretroviral drugs for 12 months was 0.88 [standard deviation (SD) = 0.55] compared with 0.63 (SD = 0.54) for *APOE-ε4* noncarriers [p = .05, 95% confidence interval (CI) -0.004 to 0.51]. For the *MBL-2* gene, 52% of patients with the *O/O* genotype experienced cognitive decline over 12 months, compared with 23% with *A/A* genotype [odds ratio (OR) 3.62, 95% CI 1.46–9.03, $p < 0.01$)]. The *APOE-ε4* allele was associated with increased risk for cognitive deficits, whereas the *MBL2-O/O* genotype was associated with increased risk for progressive cognitive decline in Chinese individuals infected with HIV through contaminated blood products.

19.2.1.3 Dopamine-Related Genes

In recent years, there have been numerous reports of polymorphisms within dopamine (DA)-related genes, resulting in measurable differences in neurophysiological and neurocognitive functioning in non-HIV cohorts. Among the most commonly examined are the catechol-O-methyltransferase (*COMT*) *val158met* allele [95–103], the dopamine transporter-1 (*DAT1*) 3'-UTR variable tandem repeat [104–109], and the brain-derived neurotrophic factor (*BDNF*) *val66met* allele [110–117]. While the effects of these variants on neurocognitive phenotypes have been small, it is conceivable that among HIV+ individuals, in whom DA functioning may already be compromised [107, 118–121], the effects will be additive or synergistic. Despite this plausible hypothesis, cross-sectional studies to date have not found reliable evidence that DA genotype modifies risk of HAND [60]. For example, Levine et al. [60], examining cross-sectional data from the NNTC, did not detect any interactive effect of disease severity (as measured by CD4+ T-cell count) and *COMT, DAT1,* or *BDNF* genotypes described above upon a number of neurocognitive domains in an exclusively HIV+ sample. Bousman et al. (2010) reported interactive effects of *COMT val158met* genotype (rs4680) and executive functioning on sexual risk taking in both HIV+ and HIV− individuals [122]. While no differences in executive functioning were noted between groups, they did find that among *Met* allele carriers, those individuals with greater deficits in executive functioning reported a greater number of sexual partners and other risky sexual practices. Very recently, Sundermann et al. [68] examined interactive effects of *COMT* rs4680 genotype and HIV on executive functioning and frontal cortex metabolism among two samples of women enrolled in the Women's Interagency HIV Study Consortium. While vastly underpowered for a genetic association study, they found that HIV+ *Val/Val* carriers performed significantly worse on working memory tests compared to uninfected *Val/Val* carriers and that HIV+ *Val/Val* carriers also showed greater prefrontal activation compared to uninfected *Val/Val* carriers during the task.

The additive or synergistic effects of DA-related alleles and stimulants such as methamphetamine and cocaine in HIV+ cohorts have also been examined. Gupta et al. [123] investigated the impact of a SNP (rs6280) within the dopamine receptor-3 gene (*DRD3*) upon neurocognitive functioning in four groups, stratified for HIV status and methamphetamine use. The biological connection between DRD3 and HAND is especially interesting, as macrophages are more likely to be infected by HIV in the presence of both methamphetamine and increased extracellular DA individually, and this process is mediated by DA receptors expressed on macrophages, including DRD3. As the authors hypothesized, only the HIV+ methamphetamine users were found to have genotype-related neurocognitive alterations.

Analyzing longitudinal neurocognitive data from the Multicenter AIDS Cohort Study (MACS), Levine et al. [66] examined the longitudinal interaction between HIV status, stimulant use, and DA-related genetic variants in a very large cohort ($N = 952$) that included both HIV+- and HIV-uninfected individuals. *COMT* genotype (rs4680) was found to influence the longitudinal neurocognitive functioning of uninfected individuals, but not HIV+ cases. Other DA-related genetic variants,

including those in genes for *BDNF* (rs6265), dopamine-β-hydroxylase *(DBH)* (rs1611115), dopamine receptor-2*/ANKK1 (DRD2)* (rs1800497), and *DRD3* (rs6280), did not affect the longitudinal neurocognitive functioning of HIV+ individuals.

19.2.1.4 Genome-Wide Association Studies

Genome-wide association studies (GWAS) have become increasingly affordable and a practical means to study disease pathogenesis. Several such studies have identified additional risk variants associated with HIV disease progression (including so-called rapid progressors), viral set point, and other disease-related phenotypes, as previously reviewed [124, 125]. GWAS have also proven valuable for the study of already relatively well-characterized neurologic diseases, such as Alzheimer's disease [126–129]. In the context of HAND, for which the cause remains poorly understood, GWAS also hold promise because of the potential to identify common genetic variants that contribute to neuropathogenesis. This potential to lead to improved mechanistic understanding of HAND and ultimately identification of pharmaceutical targets is tempered by the need for very large sample sizes to achieve the necessary power to detect those variants that influence phenotype. To date, only one GWAS focusing on HAND has been published [130]. The study sample consisted of 1287 Caucasian adults enrolled in the MACS, leaving it vastly underpowered by general standards. However, by leveraging a MACS protocol that includes serial neurocognitive testing and neuromedical examinations, several neurocognitive phenotypes were examined for their association with over 2.5 million SNPs. The phenotypes decline in processing speed or executive functioning over time, prevalent HAD, and prevalent neurocognitive impairment based on a comprehensive neuropsychological battery. Two SNPs within the *SLC8A1* and *NALCN* genes had p-values just below the strict GWAS threshold in association with change in processing speed over time. These genes, involved in sodium transport across cellular and intracellular membranes, support the role of mitochondrial dysfunction in HAD [131–133]. In the future, additional GWAS with larger samples will be possible by encouraging collaborative efforts across cohorts.

19.2.1.5 Summary

Targeted candidate-gene association studies are valuable for investigating HAND neuropathogenesis, in part because HAND is a syndrome that is many degrees separated from its molecular causes. However, a requisite for such studies is that the genes under investigation meet a standard of biological plausibility. Accordingly, genetic association studies have implicated a variety of immune-related genes for their role as risk or protective factors for HAND. However, very few of these associations have been replicated across studies. As discussed further below, there are several reasons for this, including lack of a reliable and consistently applied

phenotype for HAND (or more accurately a consistent definition or valid biomarker), focus on a narrow range of polymorphisms, and study design issues, such as failing to consider population stratification and admixture, Hardy-Weinberg equilibrium, and other factors. Going forward, investigators of genetic associations with HAND are encouraged to follow the strengthening the reporting of genetic association (STREGA) studies guidelines [134], which set standards for reporting and transparency of such studies. In particular, recruitment methods and statistical strategies must be especially rigorous. With regard to GWAS of HAND, collaborations across cohorts with the goal of increasing the statistical power to detect common variants contributing to neuropathogenesis will be necessary, and supplemental strategies to follow up GWAS analysis may also be useful for revealing associations that were undetected initially [135].

19.2.2 Transcriptomic Studies of HAND

Gene expression alterations have been widely studied in the context of neuroAIDS, including studies in human tissue and cells, animals, and in vitro models. For the purposes of this chapter, we limit our discussion to those studies that employed genome-wide microarrays (i.e., transcriptomic studies).

19.2.2.1 Brain-Based Gene Expression Studies

Most transcriptomic studies of neuroAIDS have taken advantage of genome-wide microarrays, allowing surveillance of virtually the entire transcriptome. The next-generation sequencing method, RNA-seq, is also available but has not to our knowledge been used for a published study of HAND. RNA-seq has several advantages, including increased coverage of the genome and the ability to assay miRNA, transfer RNA (tRNA), and other RNA in addition to mRNA. Regardless of the method, once expression data is generated, it must be evaluated using bioinformatics and systems biological methods that make it possible to discern biological networks associated with a chosen phenotype [136, 137]. There have been several transcriptomic studies of HAND or related phenotypes. Some focus on specific brain cells in vitro [136, 138–140], using methods such as laser capture microdissection. However, most transcriptomic studies to date have utilized brain tissue from HIV+ humans. Early studies focused on gene expression changes of frontal gray matter associated with HIVE and generally found altered regulation of genes involved in neuroimmune functioning; they also implicated neurodegenerative pathways based on dysregulation of genes involved in synaptodendritic functioning and integrity [141], toll-like receptors [142], and interferon response [143]. Findings from human microarray studies have been partially replicated in simian immunodeficiency virus (SIV) models, especially with regard to interferon-related and neuroinflammatory-related genes [144–146], providing some degree of validation. The overlap between animal and human brain transcriptomic studies was recently reviewed [147].

In perhaps a more relevant model to contemporary HAND, Gelman et al. [148] analyzed transcriptome data derived from multiple brain regions of HIV+ individuals diagnosed premortem with HAND alone or with both HAND and HIVE (as found postmortem). That analysis led to the discovery of different transcriptome profiles between the groups, implicating two distinct etiological pathways to HAND [148]. Specifically, HIVE with concomitant HAND was associated with high RNA viral load in brain tissue, upregulation of inflammatory pathways across all brain regions, and downregulation of neuronal transcripts in frontal neocortex. In contrast, HAND without HIVE was characterized by low brain viral RNA burden without evidence of increased inflammatory response and without downregulation of transcripts in frontal neocortical neurons. Only transcripts characteristically expressed by vascular- and perivascular-type cells were consistently dysregulated in HAND without HIVE. These data were recently reexamined by Levine et al. [149] using a systems biologic analysis method and weighted gene coexpression network analysis (WGCNA) [150]. While standard gene expression studies such as the study by Gelman et al. [148] utilize a group comparison approach, WGCNA enables a more systematic and global interpretation of gene expression data by examining correlations across all microarray probes, identifying biologically meaningful modules that are comprised of functionally related genes and/or correspond to cell types [151]. These modules can be examined for their association to clinical or biological variables of interest. Levine et al. [149] found a number of biologically meaningful gene expression modules that were correlated with a global neuropsychological functioning index and CNS penetration effectiveness (CPE). While the WGCNA largely validated the findings from Gelman et al., it also identified meta-networks composed of multiple gene ontology categories as well as oligodendrocyte and mitochondrial functioning. Levine et al. [149] also identified genes that were commonly associated with neurocognitive impairment in Alzheimer's disease and HIV (Table 19.2). Specifically, common gene networks dysregulated in both conditions included mitochondrial genes, whereas upregulation of various cancer-related genes was found. An earlier meta-analysis by Borjabad and Volsky (2012) compared global transcriptomes derived from frontal gray and/or frontal white matter from individuals with HIVE (regardless of HAND status) to those derived from various brain regions of individuals who had Alzheimer's disease, without consideration of NCI [152]. Both diseases (as well as multiple sclerosis) were associated with upregulation of a wide range of immune response genes, and HAND and AD also shared down-modulation of synaptic transmission and cell-cell signaling. However, there were several methodological differences between the studies, making it difficult to compare the results.

Transcriptomic studies have also been helpful in understanding the interaction of antiretroviral drug use and HAND. Borjabad et al. were the first to examine the relationship between cART use and global brain gene expression [153]. They found that the transcriptomes in cART-treated cases more closely resembled those of HIV-seronegative cases and had 83–93% fewer dysregulated genes, compared to untreated individuals if they were taking cART at the time of death. However, both cART-treated and untreated HIV+ brains were found to have approximately 100 dysregulated genes related to immune functioning, interferon response, cell cycle,

Table 19.2 Mechanisms and biomarkers of relevance to HAND

	Mechanisms of relevance to HAND	Biomarkers
Mitochondrial and iron	Basic metabolic processes: DNA, RNA, protein synthesis Maintenance of mitochondrial membrane potential Mitochondrial electron transport chain function Calcium homeostasis Lipid homeostasis Numerous other metabolic reactions Epigenetic modifications	Markers of oxidative and nitrosative damage to DNA, RNA, proteins, and lipids: 8-oxo-DG F_2-isoprostanes (specific) and neuroprostanes Isofurans, neurofurans Malondialdehyde (less specific) Protein carbonyls 8-nitroguanine (RNA, DNA) Oxysterols Aβ and α-synuclein aggregations
Iron transport	Mitochondrial biogenesis Iron-sulfur cluster biogenesis Neuronal repair/remyelination Myelination Monoamine neurotransmitter synthesis (dopamine, 5-HT) Cellular glutamate uptake/excitotoxicity Amyloid protein processing Calcium signaling Heme-oxygenase-1 deficiency Hypoxia-response pathways Macrophage-monocyte polarization/activation Macrophage-monocyte cytokine release HIV replication Endoplasmic reticulum (ER) stress	

and myelin pathways. Of note, gene expression in the HIV+ brains was not correlated with brain viral RNA, suggesting that even high CPE [154], which has been shown to reduce CSF viral load [8], may not reverse transcriptomic dysregulation. This finding is supported by a study by Levine et al. that showed no association between CPE and brain transcriptome utilizing both standard differential expression analysis and WGCNA [149]. These findings might help to explain the equivocal findings regarding CPE and HIV-related neurocognitive dysfunction to date [155–159].

19.2.2.2 Blood-Based Gene Expression Studies

Focus on peripheral (i.e., outside the CNS) mononuclear cells rather than brain tissue in studies of HAND necessitates different hypotheses and careful interpretation of results. By examining transcriptome changes in peripheral blood mononuclear cells (PBMCs), it is possible to identify biomarkers of HAND or anticipatory

cellular changes. These cells can be assayed easily, allowing for investigation of cellular events that occur considerably farther upstream to HAND onset. Due to their central role in HAND pathogenesis [160–166], monocytes have been the cells of choice for blood transcriptomic studies of HAND.

Using an in vitro model, Buckner et al. (2011) examined dynamic transcription changes in monocytes derived from healthy donors [167]. The cells were infected with HIV to produce a CD4 + CD16 + CD11b + Mac387+ monocyte subpopulation capable of crossing a laboratory model of the BBB. Gene expression analysis revealed upregulation of chemotactic- and metastasis-related genes but not inflammatory genes. Dynamic changes were also observed as the monocytes matured into macrophages, including an increase in the expression of enolase-2, followed by a decrease once the cell was fully differentiated. Osteopontin was also observed to have increased expression in the maturing monocytes.

Sun et al. (2010) reported the first study in which blood monocyte global transcription was associated with neurocognitive functioning in HIV+ individuals. More specifically, the authors examined whether or not monocyte gene expression and other peripheral factors (CD4+ T-cell numbers, *APOE* genotype, viral load, lipopolysaccharide, and soluble CD14 levels) were associated with neurocognitive functioning in a group of 44 HIV+ individuals on cART and 11 HIV-seronegative controls [51]. Monocyte gene expression, which showed a chronic inflammatory profile in the HIV+ participants with high viral load, was not correlated with neurocognitive impairment. The other blood markers were also not found to be associated with neurocognitive functioning. More recently, the same group of researchers focused their analysis on a neurophysiological phenotype rather than HAND [168] by examining whether peripheral immune activation and monocyte gene expression were associated with brain metabolite concentrations, as measured by MRS. Thirty-five HIV+ individuals on cART and 8 HIV-seronegative adults were examined. Among the HIV+ participants, an interferon-*alpha*-induced activation transcriptome phenotype was strongly correlated with N-acetyl aspartate in the frontal white matter. Notably, interferon-gamma inducible protein-10 (IP-10 or CXCL-10) was strongly correlated with plasma protein levels, and plasma CXCL-10 was inversely correlated with N-acetyl aspartate in the anterior cingulate cortex. This study is remarkable, as it is the first to connect transcription changes with neurophysiological changes in the context of neuroAIDS. As discussed below, we believe that this tactic holds the greatest promise for elucidating the neuropathogenesis of HAND.

Levine et al. [169] utilized the Illumina HT-12 v1 Expression BeadChip to analyze monocyte-derived transcriptome data from 86 HIV+ individuals enrolled in the MACS. Examining all HIV+ samples, the standard differential expression analysis identified a number of individual gene transcripts that were significantly correlated with global neurocognitive functioning. Of the 16 genes identified, many implicated oxidative stresses, including those encoding interleukin-6 receptor *(IL6R)*, casein kinase 1-alpha-1 *(CSNK1A1)*, hypoxia upregulated-1 *(HYOU1)*, low density lipoprotein receptor-related protein-12 *(LRP12)*, and Kelch-like ECH-associated protein-1 *(KEAP-1)* [170–185]. The *KEAP-1* findings are especially interesting, as they support a recently described role for *nuclear factor E2-related factor-2 (nrf-2)* in

HAND [186]. There has been some interest in recent years by neuroAIDS researchers of factors that modify the activity of nrf-2 (e.g., GSK3-β inhibitors [187] and curcumin [188]) or that are modified by it [189]. As such, members of this pathway deserve further investigation as potential pharmacological targets during early stages of HAND or even as potential prophylactic agents.

19.2.2.3 Summary

Genome-wide transcriptomic studies have implicated numerous genes and biological pathways in the neuropathogenesis of HAND. Some results of human studies have been replicated in simian and murine models. One limitation of previous studies is the use of homogenized brain tissue, which contains mRNA from numerous cell types [141, 145, 190, 191], thus making it difficult to determine cell-specific molecular processes. In addition, most studies describe gene expression from one brain region (e.g., frontal lobe), and those regional disease-related transcription changes may not reflect the disease-related transcription changes occurring in brain regions also commonly implicated in HAND (e.g., basal ganglia). Also, most in vivo studies utilizing brain tissue have sought to understand alterations in gene expression in brain tissue of humans or animals that expired in an advanced state of disease (i.e., HIVE or HAD). Therefore, it is unclear if the findings of those studies will generalize to contemporary HAND. In tandem with studies of brain tissue, there have been investigations of monocyte transcriptome, which may provide clues about the earlier stages of HAND pathogenesis. Finally, the interpretation of transcriptome data utilizing systems biological methods such as WGCNA [150] may point the way to novel therapeutic targets.

19.2.3 Epigenetic Studies of HAND

19.2.3.1 MicroRNA Studies

MicroRNAs (miRNAs) are small RNA molecules that modify transcription and translation via interactions with mRNA and which regulate a variety of cellular processes, including within the CNS. A small number of studies have examined the role of miRNA in HAND. The first study evaluated the impact of Tat upon expression of candidate miRNAs in primary cortical neurons in vitro [192]. Tat was found to upregulate mir-128a, which in turn inhibited expression of SNAP25, a presynaptic protein. A second study involved examination of postmortem caudate and hippocampal tissue of rhesus macaques with or without simian immunodeficiency virus encephalitis SIVE as well as caudate tissue from HIV-uninfected cases and humans with both HAND and HIVE [193]. Three miRNAs were found to be elevated in both SIVE and HIVE (miR-142-5p, miR-142-3p, and miR-21). miR-21, linked to oncogenesis, was significantly upregulated in both HIVE and SIVE.

miR-21 also induced stimulation of N-methyl-D-aspartate (NMDA) receptors, leading to electrophysiological abnormalities. Further, miR-21 was found to target the mRNA of myocyte enhancer factor 2C (MEF2C), a transcription factor crucial for neuronal function and a target of miR-21, ultimately reducing mRNA expression. In a third study, Noorbakhsh et al. conducted miRNA profiling in the frontal lobe white matter of four HIV-negative and four HIVE cases who were matched by age and sex [131]. Several miRNAs were found to be differentially expressed between the groups, using a standard twofold cutoff. Bioinformatics analysis revealed that most of the upregulated miRNAs targeted genes involved in immune response and inflammation, followed by nucleotide metabolism and cell cycle. A fourth study by Tatro et al. used both global mRNA and miRNA expression analysis in order to identify changes in miRNA expression in the frontal cortex of HIV+ individuals, determine whether miRNA expression profiles could differentiate HIV from HIV with concurrent major depressive disorder (MDD), and develop a method for integrating gene expression and miRNA expression data [194]. Their sample consisted of HIV-negative controls, HIV+, and HIV+ with concurrent MDD. miRNAs from three individuals within each group were pooled and used for the miRNA profiling, and mRNAs for three individuals from both HIV+ groups were used for non-pooled mRNA profiling. Importantly, neurocognitive functioning was not considered in this study, ages varied widely between groups, and one of the HIV+/MDD brains had pathology consistent with HIVE. With these caveats in mind, the HIV+/MDD group showed a greater number of downregulated miRNAs compared to the HIV+ group. Further, the miRNAs tended to cluster more tightly around the same chromosomal regions. After identifying mRNAs that were significantly differentiated in the HIV+/MDD group, and then identifying miRNAs that were dysregulated by at least a twofold change relative to the HIV-only group, the authors employed a target bias analysis to determine the relationship between miRNA dysregulation and target gene dysregulation. Using this method, they identified miRNAs belonging to four categories: (1) those with many dysregulated mRNA targets but of marginal statistical significance, (2) those with fewer dysregulated target genes but with high statistical significance, (3) those with numerous dysregulated gene targets that were of high statistical significance, and (4) those that did not have a significant number of dysregulated targets. The authors also identified a small number of genes with 3′-UTR miRNA target sequences. Those genes were considered to be "hubs" for miRNA activity, and the authors outlined their biological roles and association with neuropsychiatric illnesses.

A fifth study examined the impact of HIV viral protein R (Vpr) in a human neuronal cell line in order to investigate the mechanisms underlying the altered expression of cytokines and inflammatory proteins in CNS cells resulting from HIV infection. Both miRNA and gene expression assays of human neurons (primary cultures or cell lines) treated with recombinant Vpr proteins were used. Vpr was found to deregulate several miRNAs and their respective mRNAs [195]. As one potential mechanism for neuronal dysfunction, they found that expression of both miR-34a and one of its target genes (*CREB*) was dysregulated in the presence of Vpr. This study was the first to demonstrate a miRNA-dependent pathway through which Vpr damages neurons.

Most recently, Kadri et al. [196] sought to identify an epigenetic marker of HAND by screening over 750 miRNAs assayed from plasma in a group of 30 HIV+ adults who had been classified as neurocognitively impaired or normal based on a battery of cognitive tests. Utilizing a miRNA pairwise analysis to analyze the array data [197], the authors identified ten miRNA pairs that were differently expressed between impaired and unimpaired HIV+ cases and then validated their findings with qRT-PCR. The miRNA pairs that best differentiated impaired from non-impaired samples were miR-495-3p in combination with miRNA let-7b-5p, miR-151a-5p or miR-744-5p, and the pair miR-376a-3p/miR-16-532-3p. Sensitivity was further improved through the combination of two microRNA pairs: miR-495-3p/miR-744-5p and miR-376a-3p/miR-532-3p. Of note, none of these miRNA pairs were associated with other clinical characteristics. As the authors point out, it was also of interest that these miRNAs are all either enriched in brain tissue or have known neuronal functions.

19.2.3.2 Histone Modification Studies

Chromatin structure, and therefore gene expression, can be modified by the acetylation and deacetylation of histone proteins, a process that is mediated by histone deacetylases (HDACs) [198]. HDAC inhibitors have been shown to improve cognitive ability and may be candidates for treating a variety of neurologic diseases [199, 200]. We are aware of only one study examining histone modification in the context of HAND neuropathogenesis. Saiyed et al. examined the influence of Tat upon expression of HDAC2 in neuronal cells in vitro and the subsequent effect of HDAC2 modification on regulating genes involved in synaptic plasticity and neuronal function [201]. HDAC2 expression was negatively correlated with expression of *CREB* and CaMKIIa genes, which were reported to be involved in neuronal regulation.

19.2.3.3 DNA Methylation Studies

Genome-wide methylation platforms are now readily available (e.g., Illumina Infinium 450 K); however, very few studies have employed this method for the study of HAND. In addition to revealing information about cellular processes involved in HAND pathogenesis, whole-genome DNA methylation technology has been leveraged to create bioinformatics tools that can be used to study aging and HAND. DNA methylation levels are particularly promising biomarkers of aging, since chronological age profoundly affects them in most human tissues and cell types. The recently developed biomarker of aging (referred to as epigenetic clock [202]) was recently applied to the study of accelerated biological aging due to HIV in brain and peripheral blood mononuclear cells [203]. It was found that the brains of HIV+ adults exhibited age acceleration of 7.4 years compared to uninfected controls according to the epigenetic clock, whereas the age of PBMC was accelerated by 5.2 years. This

marker of aging may also be clinically relevant in HIV+ individuals. As recently shown by Levine et al. (2015), brain samples from individuals diagnosed with HAND within 1 year of death also showed an age acceleration of 3.5 years compared to samples from HIV+ neurocognitively normal individuals [204].

19.2.3.4 Summary

Epigenetic studies of HAND neuropathogenesis are relatively recent, with most studies focusing on miRNA pathways in infected tissue or cells. A variety of miRNAs have been implicated, lending validation to previously identified neuropathogenic mechanisms, such as increased caspase-6 and mitochondrial dysfunction. *CREB* has been implicated in both miRNA and histone studies. Improved neuropsychological performance was recently associated with global DNA demethylation, and a new biomarker of aging (the epigenetic clock) based on several hundred CpGs revealed that HIV infection accelerates aging in brain tissue and PBMC.

19.3 Special Topics

19.3.1 Roles for Iron and Mitochondrial Dysmetabolism in Neuro-HIV/AIDS

19.3.1.1 Overview

Accumulating data provides evidence of altered systemic iron metabolism in HIV infection, with sequestration of iron within reticuloendothelial cells and elevated circulating levels of the pro-inflammatory, master iron-regulatory hormone, hepcidin [205–207]. Furthermore, altered iron status (with or without anemia) has been associated with morbidity and mortality, even after accounting for potential disease-related confounding factors, such as CD4+ T-cell count [208–210]. Hepcidin levels increase, and plasma iron falls, as part of the inflammatory cascade triggered during acute HIV infection, and hepcidin subsequently remains high in untreated individuals. Hepcidin levels decline as the virus is suppressed with cART, but longitudinal studies have shown that they remain elevated compared to HIV-negative persons, even when the virus becomes undetectable. Intracellular HIV replication requires iron, and hepcidin levels measured early in HIV infection appear to predict subsequent plasma viral load set point [207, 211]. Studies of the role of hepcidin and iron transport in determining CSF HIV RNA concentrations in HIV+ persons are ongoing. By blocking gut iron absorption as well as the release of iron from cells of the monocyte-macrophage (M/M) lineage to erythroid and other metabolically active cells, hepcidin synthesis by the liver represents an important iron-withholding mechanism, leading also to anemia of chronic inflammation [212]. In addition, regional brain iron distribution may be abnormal in chronic HIV infection, based on brain imaging studies [213–215].

Over the past decade, considerable advances have taken place in understanding the roles of iron and mitochondrial metabolism in neurodegenerative disorders. It is increasingly recognized that these processes are interconnected and that iron and mitochondrial dysregulation may go hand in hand. Therefore, not to include a discussion of recent studies implicating iron and mitochondrial dysfunction in non-HIV-related inflammatory neurocognitive disorders would be to sidestep a burgeoning area of research with clear relevance to neuro-HIV/AIDS.

19.3.1.2 Insights from Studies of Iron in Non-HIV-Related Neurodegenerative Disorders

Disrupted iron homeostasis in the brain has been a long-recognized feature of both common and rare neurodegenerative disorders, including Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington disease, multiple sclerosis (MS), and the so-called "neurodegeneration with brain iron accumulation" (NBIA) disorders [216–221]. However, due to the ubiquitous nature of iron and its participation in so many fundamental metabolic processes, the independent contribution of iron to neurodegenerative disease pathogenesis has been difficult to discern from its possibly innocent bystander role. Substantial evidence linking iron homeostasis with mitochondrial function, lipid homeostasis, and energy metabolism implicates systemic and/or CNS iron dysregulation in neurocognitive disorders [222–224]. Iron accumulates in some areas of the brain with healthy aging, but the distribution and total amounts are abnormal in many neurodegenerative diseases, exceeding what is observed with normal aging. Inflammation is frequently also present in these disorders in areas of regional iron excess and neuronal cell death [225]. Iron redistribution has the potential to promote oxidative injury in areas of increased iron deposition as well as possible functional iron deficiency due to low bioavailable iron in other brain regions. Both eventualities may contribute to disease pathogenesis [226, 227].

Iron is a required cofactor for numerous essential metabolic enzymes and a critical component of cytochromes and flavoproteins of the mitochondrial electron transport chain [228–230]. Relevant to the CNS, iron is required for myelination, neuronal repair, and monoamine (dopamine and serotonin) neurotransmitter synthesis, an imbalance of which contributes to mood disturbances, oxidative stress within the brain, and excitatory neuronal loss [231–234]. Access to a steady supply of iron in non-oxidatively reactive forms is therefore essential for maintenance of cellular health and metabolism. However, iron is also a biological hazard due to its efficiency in catalyzing free radical reactions via Fenton chemistry. Most studies of the role of iron in CNS disorders have highlighted regional iron excess in the brain as the abnormality of interest and emphasized that iron accumulation is synonymous with oxidative stress, although there is relatively little direct evidence for this assertion [235, 236]. Mitochondria also contain high concentrations of iron in the presence of oxygen and superoxide radicals, yet they function normally under these conditions for extended periods. Both iron excess and iron deficiency can lead to

mitochondrial and cellular dysfunction and oxidative injury. Organisms have therefore evolved mechanisms to maintain tissue iron concentrations within a narrow physiologic range, tightly compartmentalize it within cells, and transport it in a soluble, nonreactive state, bound to a variety of large and small transporters and gatekeeper proteins [206]. Hence, iron-related mechanisms of neurocognitive impairment other than direct oxidative injury due to increased brain iron content also deserve consideration in evaluating the impact of iron.

As in the periphery, cells within the CNS tightly regulate iron homeostasis via iron-responsive expression of select proteins required for iron efflux, cellular import, transport, and storage. Recently, proteins directly implicated in pathogenesis of the most prevalent neurodegenerative diseases, such as amyloid-β precursor protein, tau, α-synuclein, prion protein, and huntingtin, have been linked to neuronal iron homeostatic control. This literature suggests that disrupted expression, processing, or location of these proteins may result in a failure of their cellular iron homeostatic roles and augment the common underlying susceptibility to neuronal oxidative damage that is triggered in neurodegenerative disease [237, 238]. Despite intensive research over the past two decades, mechanisms regulating brain iron transport and egress of iron from the brain are still only partially understood. Iron enters the brain mainly by transport across the BBB, a process expected to be tightly regulated under normal circumstances in order to buffer the brain from systemic iron fluctuations [239]. Hepcidin and the iron export protein ferroportin-1 (FPN-1, encoded by the *SLC40A1* gene) are linked to iron efflux and transport into the brain and, along with astrocytes, play key roles in iron release [238]; turnover of FPN-1 is controlled by hepcidin [240]. In vitro studies replicating the BBB, which is comprised of brain microvascular endothelial cells and underlying astrocytes, and studies in nonhuman primates, have recently suggested a model in which brain endothelial cells, far from being a passive conduit for iron, play an active role in regulating iron transport into the brain [241]. Additional in vitro studies of human brain microvascular endothelial cells suggest that expression of iron-transport proteins such as FPN-1, the copper ferroxidase ceruloplasmin (CP), transferrin (TF) and its receptor (TFR), and heavy chain ferritin are critical in regulating this process [242–244]. Other iron transporters such as iron-regulatory protein-2 (IRP-2) also play key roles in brain iron metabolism; *IRP-2* knockout mouse models show altered tissue iron distribution and mild behavioral and neurological impairments, despite showing no specific areas of neurodegeneration in the brain [245].

A substantial literature, albeit not entirely consistent, associates iron-regulatory gene variants with healthy aging and with altered incidence and age of onset of common neurodegenerative diseases like AD and PD [233, 246–255]. Polymorphisms in the iron-loading *HFE* gene (*H63D* SNP in particular) have been best studied in this regard, with in vitro studies implicating a multitude of mechanisms for the increased risk of AD associated with this SNP: increased iron accumulation, disruption of mitochondrial membrane potential, increased influx of intracellular Ca^{2+}, increased cellular glutamate uptake, increased secretion of pro-inflammatory MCP-1, increased endoplasmic reticulum stress, oxidative injury, β-amyloid (Aβ) peptide-mediated mitochondrial toxicity, and decreased activity of PIN1, which contributes

to tau phosphorylation seen in AD [222]. Recent studies of Friedreich's ataxia, NBIA disorders, and AD have also highlighted the interrelatedness of iron metabolism, mitochondrial function, autophagy (the cellular clearance of senescent or dysfunctional organelles), and lipid metabolism, processes which are critical to CNS and peripheral nervous system function [248, 256–258]. These findings demonstrate that *H63D-HFE* expression promotes processes that can influence pathways in neurons that ultimately lead to impaired cognition, such as lipid homeostasis, neurotransmission, and myelination. Iron, like other transitional metals, not only alters processing of β-amyloid, but soluble amyloid precursor protein has been shown to stimulate iron efflux from brain microvascular endothelial cells [243]. While changes in iron homeostasis may not be the primary triggering event that initiates the pathological cascade leading to HAND, disrupted iron transport may be an important factor in altering metabolism of structural proteins like β-amyloid, thereby promoting neuroinflammation and progression of these disorders (Table 19.2).

Disrupted iron metabolism has recently been confirmed in postmortem brain tissues from patients with AD [259], in their cerebrospinal fluid (CSF) [260], and using R2*-based magnetic resonance imaging of the brain in AD patients [261]. A meta-analysis of over 2500 studies of iron in AD suggested that the weight of evidence favors a role for iron dyshomeostasis in the serum and CSF, as well as in the brain in AD [262], but the literature is not entirely consistent in this regard [263, 264]. Very recent studies by Ayton et al. strongly implicate iron dysregulation in the association of the *APOE*-ε4 genetic variants with AD risk; and *APOE* genotype may modulate CSF ferritin levels, which in turn are associated with AD outcomes [260, 265]. Baseline CSF ferritin levels were inversely associated with cognitive performance during a 7-year follow-up in normal, mildly impaired and AD subjects and also predicted conversion from mild cognitive impairment to AD. CSF ferritin and CSF APOE levels were strongly correlated, and CSF levels of ferritin were elevated in individuals harboring the *APOE-ε4* allele. In PD, decreased brain iron levels have also been noted in individuals at postmortem [266]. Some studies even indicate abnormal systemic iron status in AD and PD, as well as in ALS [223, 267, 268]. Whether iron dysregulation in the brain in AD, PD, ALS, and other disorders is related to or induced by systemic iron dysregulation, or vice versa, remains unclear.

Taken together, these findings favor the concept that maldistribution of iron in the brain is not an epiphenomenon; rather that iron dysregulation is a likely pathogenic mechanism common to many types of neurocognitive disorders, including HAND. Furthermore, interactions between brain iron transport and systemic iron status are likely to be dynamic and complex: the current concept that increased regional brain iron leads to neurocognitive impairment via oxidative injury may be a gross oversimplification.

19.3.1.3 Links Between Iron Levels and HAND

Indications that HIV dysregulates systemic iron metabolism were first reported in the pre-cART era: HIV disease progression was found to be associated with altered iron metabolism in vitro, and increasing ferritin concentrations were linked to disease

progression [205, 269]. CSF ferritin was first measured in HIV+ individuals with AIDS Dementia Complex, individuals with CDC stage II HIV infection, and uninfected controls and found to be a possible marker of neurologic disease. Ferritin levels were detectable in CSF only in those with AIDS Dementia Complex, and it was speculated that the source of ferritin in CSF in these patients was activated macrophages involved in promoting neuroinflammation [270]. In HIV+ individuals, CSF ferritin levels were shown to be elevated during acute neurological episodes but were considered non-specific for HAD [271]. In another study, limited proteomic profiling of serum in a small study of HAD cases and non-demented HIV+ controls identified CP as a possible biomarker of HAD [272]. Interpretation of iron-related biomarkers remains somewhat challenging in HIV infection, due to elevation of these proteins in acute and sometimes chronic infection; CP is also an acute-phase molecule.

Neuroimaging studies have also provided evidence that brain iron deposition in HIV+ persons is abnormal; neuropathological evaluations have correlated T2 shortening (hypointensity) in magnetic resonance imaging (MRI) studies of the basal ganglia (putamen) with premature perivascular HIV-related iron deposition, but the significance of this observation was unclear [213]. A larger MRI study followed, showing significantly greater deposition of iron in the basal ganglia (globus pallidus and caudate nucleus) of HIV+ persons than in HIV-negative controls [214]. More recently, multi-contrast, high-field MRI has detected subtle structural defects in the brains of HIV+ individuals with mild neurocognitive disorder (MND) on ART, including loss of structural integrity (myelin and cellular macromolecules) and micro-edema in global white, cortical gray matter, thalamus, and basal ganglia. These subcortical changes were also found to significantly influence executive function in patients with MND, compared to those without MND, who were similar with regard to baseline demographic and HIV-related factors. Iron-sensitive imaging using susceptibility-weighted imaging (SWI) was performed in a subset of study participants. Although linear discriminant analysis incorporating T1, magnetized transfer ratio (MTR) and SWI data provided valuable information for distinguishing MND from non-MND individuals, T2* data which is most sensitive to brain iron content did not add appreciably to the model. Although there were longer T2* relaxation times in the caudate of non-MND patients vs. MND individuals, this was also observed in HIV+/non-MND subjects compared to controls and did not reach statistical significance; T1 relaxation times also suggested possibly lower iron content in HIV+ than in HIV− controls [215]. These findings clearly require further follow-up and study using iron-sensitive neuroimaging techniques, such as R2* MRI, functional MRI, susceptibility-weighted imaging (SWI), and the newer technique of quantitative susceptibility mapping [273].

19.3.1.4 Impact of Iron on HAND in the Setting of Substance Abuse/Dependence

Iron-storage and transport proteins may have multiple functions within the brain. Pitcher et al. [274] reported elevated levels of ferritin heavy chain (FHC) in cortical neurons from individuals with HAND premortem and in opiate abusers. Prior

studies showed that FHC levels increase in neurons in response to mu-opioid agonists. In neuroinflammation, the chemokine CXCL12 and its receptor CXCR4 perform many essential functions, and signaling through this receptor promotes neuronal survival and neuronal-glial communication [274]. CXCR4 is an HIV coreceptor which has been implicated in HIV neuropathogenesis, including elevated levels of excitotoxic mediators, synaptodendritic loss or "simplification," and release of inflammatory cytokines. Among HIV+ persons, opiate abuse, particularly intravenous opioids, may accelerate development of HAND. FHC regulates CXCR4 signaling by inhibiting its activation and downstream, pro-survival signaling pathways. FHC also functions to bind, oxidize, and sequester iron in nonreactive forms for stable storage within the cell, and its production is regulated in response to changing iron levels and the inflammatory milieu; of particular importance to HAND is the ability of inflammatory cytokines to regulate FHC levels. Pitcher and colleagues showed that opiate abuse may exacerbate NCI in HIV through FHC-dependent disruption of neuronal CXCL12-CXCR4 signaling. They determined that this signaling pathway increases dendritic spine density and that HIV+ persons with NCI had increased neuronal levels of FHC that correlated with reduced CXCR4 activation. These results were further confirmed in a SIV-infected nonhuman primate model with morphine administration. In vitro, transfection of a CXCR4-expressing human cell line with an iron-deficient FHC mutant resulted in increased FHC expression and dysregulated CXCR4 signaling, independent of iron binding. Furthermore, studies of neurons showed that FHC contributed to morphine-induced dendritic spine loss, suggesting that HIV (and SIV) infection independently dysregulate neuronal FHC, which in turn may actively contribute to neurocognitive decline in HIV infection among opioid abusers.

Synthesis of monoamine neurotransmitters requires iron; therefore, iron dysregulation may also impact the neurobehavioral outcomes of HIV infection, particularly in the setting of substance abuse [275, 276]. In animal models, iron deficiency has been shown to disrupt brain synthesis and metabolism of monoamine neurotransmitters and to contribute to memory deficits [277]. Specific stimulant drugs of abuse like methamphetamine and its metabolites are believed to contribute to cognitive impairment via increased dopaminergic and serotonin signaling in the brain, excitotoxicity to neurons, mitochondrial damage, and oxidative brain injury [234].

19.3.1.5 Potential Effects of Altered Iron Transport on HIV Replication and Neuroinflammation

HIV replication within the CNS is consistently associated with an increased risk of neurocognitive impairment, and much research has been aimed at reducing CSF viral load and viral reservoirs [278]. Increasing evidence has confirmed that elevated iron stores are positively associated with viral load and mortality in people infected with HIV, but interpretation of existing studies of HIV and iron must be interpreted with some caution, as most studies to date have been undertaken in

largely cART-naïve populations or those without access to modern combination antiretroviral regimens. Some work has suggested that *both* extremes of iron status are detrimental to HIV outcomes [209]. Insufficient attention has been given to the potential impact of functional iron deficiency to mitochondrial dysfunction, immune activation, and antiretroviral toxicity to mitochondria and neurons. Functional neuronal iron deficiency is suggested in some dementing disorders (e.g., AD) characterized by deposition of β-amyloid, which is often observed in HAND and exhibits altered metabolism in the presence of HIV.

Taken together, data from studies of HIV-infected individuals thus far point to independent effects of both HIV and aging on extracellular amyloid-β (specifically Aβ42) deposition [279]. Drakesmith et al. showed that HIV Nef downregulates the HFE expression on the surface of M/M, leading to iron accumulation and increased intracellular viral replication [280]. In addition, HIV infection in vitro alters cellular iron levels, which promotes viral replication, and several antiretroviral drugs alter the expression of iron-regulatory genes, increasing cellular iron content independent of HIV infection [281]. Over the past 15 years, research has also revealed close links between the iron-hepcidin-ferroportin axis and M/M-mediated inflammation, M/M polarization (M1 vs. M2 states) and activation, and regulation of innate immune responses [282]. The central role of M/M and microglia in mediating neuroinflammation and HAND has been consistently borne out by genetic, transcriptomic, and epigenetic studies to date, and it therefore begs further exploration of the role of cellular iron content in M/M polarization/activation in this phenotype. M/M with increased iron content produce more pro-inflammatory mediators and are more likely to have an activated M1 phenotype [283]. HIV replication within M/M in the CNS is associated with deficiency of heme-oxygenase-1, a neuroprotective enzyme produced by M/M, oxidative stress, and glutamate toxicity [189]. Recent ultrastructural studies have identified so-called dark microglia, which become abundant in areas of the brain affected by chronic stress, aging, depression, and AD pathology, and these cells play a role in remodeling of neuronal circuits, particularly at synapses [284]. These microglia exhibit signs of increased oxidative stress, with increased phagocytosis of synaptic elements as is seen in mouse models of HAND [285] and extensive engulfment of axonal terminals and dendritic spines. Hence, effects of iron in activating M/M or microglia, or in altering their polarization, may promote HAND.

19.3.1.6 Anemia and Erythrocyte Morphology as Predictors of HAND

Anemia, which is always associated with abnormal iron transport and occasionally with systemic iron deficiency, has been a consistently poor prognostic indicator in HIV infection in both retrospective and prospective studies [212]. Its relationship to dementia in HIV+ individuals was reported previously in the MACS and in another study [286, 287], and hemoglobin <12 mg/dl was one of the components of the Veterans Aging Cohort Study (VACS) index associated with neurocognitive impairment status in a cross-sectional analysis of the VACS, which was not designed to

evaluate anemia and therefore understandably did not include adjustment for comorbid conditions and ethnicity [288]. Until recently, its impact on milder forms of HAND prevalent in the cART era has therefore remained unclear. We conducted the first cART era prospective study in CHARTER which was designed to address the impact of anemia and erythrocyte indices in predicting milder forms of HAND. This study in >1200 individuals enrolled in the CHARTER study included a time-dependent analysis of anemia as a predictor of GDS-defined impairment and a cross-sectional analysis of erythrocyte indices in association with HAND defined by either Frascati criteria or GDS impairment. Anemia, defined as a hemoglobin <11.5 mg/dL in women and <13 mg/dL in men, was associated with incident GDS-defined HAND independent of numerous potential confounding factors, including zidovudine (ZDV) use and contributing comorbidities [adjusted hazard ratio (HR) 1.55, $p < 0.01$] [210]. Similarly, in a recently published longitudinal sub-study of CHARTER, current hematocrit emerged as an independent predictor of neurocognitive decline in multivariable analyses [289]. In addition, the Kallianpur et al. study determined that routinely available red blood cell indices such as the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were significantly and positively associated with the GDS-defined HAND, GDS as a continuous measure, and with HAND by Frascati criteria. These measures were also associated with milder forms of HAND (i.e., ANI and MND) in addition to HAD when these phenotypes were evaluated in separate models. Erythrocyte indices are often abnormal in HIV infection, commonly in association with ZDV use, protease inhibitors, or other cART, and they have also been shown to correlate with systemic mitochondrial dysfunction in human and animal studies [290, 291]. Erythrocyte indices may therefore indicate subclinical mitochondrial dysfunction and/or lipid dysmetabolism in HIV infection, which promotes HAND. Red cell membrane fatty acid composition was found to correlate with brain volumes [292]. To date, few studies have examined the value of evaluating and treating anemia in HIV infection, but in light of these findings, such studies would seem to be of value in potentially ameliorating HAND and preventing its progression [293]. Initiation of cART improves iron utilization, coincident with decreased immune activation. Fuchs et al. further demonstrated associations between anemia (low hemoglobin levels) and immune activation markers such as serum neopterin and β_2-microglobulin in HIV+ subjects; interestingly, the latter is also a protein important in regulation of HFE protein stability and expression in M/M [294, 295].

19.3.2 Iron-Related Genetics, RNA Expression, and Associated Biomarkers in HAND

19.3.2.1 Overview

Investigation of the role of altered iron transport in HIV infection on neurocognitive function is challenging due to the fact that many iron transporters are also acute-phase proteins and increased in acute inflammatory states. Until very recently, no

studies had been undertaken to measure iron and the levels of iron-transport proteins in CSF among HIV+ persons. CP and haptoglobin (HP), an iron-binding chaperone protein which serves as a ligand for the M/M scavenger receptor molecule CD163, were recently measured in CSF in CHARTER study participants [296]. Higher levels of CP were associated with an increased risk of GDS-defined HAND in multivariable regression analyses [adjusted odds ratio (OR) 1.8, $p < 0.05$], and higher levels of both CP and HP were associated with GDS in persons with only minimal comorbidities (ORs 2.4 and 2.1, respectively, both $p < 0.05$). In this subgroup, CSF CP and HP levels were also associated with GDS impairment and HAND in individuals with undetectable plasma HIV RNA (ORs 5.6 and 3.0, respectively, both $p < 0.01$). These associations were not merely due to inflammation, as only very weak correlations were observed between iron biomarkers and concurrently measured IL-6, CXCL-10, and TNF-α.

In neuroimaging studies of CHARTER participants who had minimal comorbidities, we identified five highly significant SNPs in iron-regulatory genes including *TFRC*, *CP*, and *SLC11A1*, which predicted alterations in brain imaging traits that are commonly associated with HAND. These traits included subcortical gray matter volume, frontal gray matter *N*-acetyl aspartate (a marker of neuronal integrity), abnormal white matter volume, and basal ganglia choline (a marker of neuroinflammation) [297]. Additional iron-related genes whose expression in monocytes has been associated with neurocognitive impairment also include: *NRF-2* (regulator of heme metabolism), the "hemoglobin complex" gene module, heme-carrier protein-1 (*HCP1)*, *CD163*, *IL6R* (required for hepcidin regulation of iron metabolism), and *BOLA2* (which binds iron-sulfur clusters in glutaredoxin and may play a role in sensing cellular iron status) [169].

In a collaboration with the NNTC, expression of transferrin receptor (*TFR*) messenger RNA in brain tissue (frontal neocortex) was evaluated for association with HAND; levels of *TFR* RNA were, surprisingly, unrelated to brain HIV burden but were significantly associated with all HAND (adjusted OR 5.2, $p < 0.05$) and for milder forms of HAND. Perhaps most interesting was the negative association seen between *TFR* RNA levels in the neocortex and specific domains of executive neurocognitive function, such as speed of information processing, and working memory [298]. Studies such as these support roles for altered iron regulation in HAND.

19.3.2.2 Iron and Vascular Disease in HIV Infection

HIV is associated with a significantly increased risk of atherosclerotic vascular disease, which may contribute significantly to HAND in older HIV+ individuals [6, 299]. Therefore, while elevated iron stores and resulting hepcidin-mediated degradation of the macrophage iron exporter FPN-1 may increase HIV replication within M/M and resulting immune activation, hepcidin may also increase development of foam cells and lipid peroxidation, destabilizing atherosclerotic plaques. Disorders of vascular remodeling such as pulmonary arterial hypertension (PAH) also occur with increased frequency in HIV+ individuals, and there is a well-described increase in prevalence of iron deficiency among persons suffering from PAH [300, 301].

Studies of the regulation of iron-sulfur clusters in PAH and HIV have revealed a role for epigenetic factors (hypoxia-related miRNAs or hypoxamirs) in the pathophysiology of vascular remodeling in PAH [302, 303]. Few studies of PAH in HIV infection have been performed [304]. The hypoxia-regulated response is modulated by iron-regulatory and RNA binding proteins [305]; studies in this area may help to elucidate potential common mechanisms linking iron-related chronic immune activation and vascular complications, which in turn promote neurocognitive decline.

19.3.2.3 Mitochondrial Genetics and Biomarkers in HAND

Evidence continues to accumulate supporting a role for abnormal mitochondrial metabolism in the pathogenesis of neurodegenerative disorders in non-HIV-infected persons, implicating defects in iron-sulfur cluster biogenesis. Iron-sulfur cluster dysfunction may contribute to cognitive decline and mitochondrial dysfunction with normal aging, as well as in specific neurocognitive diseases associated with premature aging like HAND, with brain iron accumulation and maldistribution in the basal ganglia [256]. Future studies will need to determine how iron-sulfur cluster defects may participate in the natural history of these disorders and whether targeted interventions can interrupt neuronal damage.

Until recently, few studies of mitochondrial genomic variation in HAND had been undertaken. The mitochondrial DNA (mtDNA) is a separate chromosome comprised of 37 genes that encode mitochondrial proteins; the remaining proteins required for mitochondrial function are encoded by nuclear genomic DNA. We performed a mtDNA haplogroup analysis in 1027 HIV+ participants from the CHARTER study, approximately two-thirds of whom were receiving cART and 46% of whom were diagnosed with HAND [306]. In this study, ancestry was genetically defined using principal components from genome-wide genetic data and categorized as European, African, or admixed Hispanic ancestry. Recent work in CHARTER has shown a consistently higher risk of HAND and, in longitudinal studies, a higher risk of neurocognitive decline over time, among self-reported Hispanic individuals. A reduced frequency of HAND among individuals of self-reported African ancestry in CHARTER has also been noted previously [210, 289]. The reasons for these differences have been widely debated. However, analyses within CHARTER have carefully accounted for differences in race/ethnicity and education, as well as for practice or learning effects in repeat neurocognitive assessments. The study by Hulgan et al. confirmed that individuals with genetically defined admixed Hispanic ancestry had a higher risk of neurocognitive impairment or HAND, as defined by the GDS, than did persons of European or African ancestry. The study also identified a subgroup of persons of admixed Hispanic ancestry with mitochondrial haplogroup B as having significantly reduced risk of GDS-defined impairment (adjusted OR, 0.16, $p < 0.01$) compared to other admixed Hispanic haplogroups. No other significant haplogroup associations were observed among CHARTER participants of European or African ancestry. Hence, mtDNA variation may constitute an ancestry-specific factor that influences risk of neurocognitive

impairment in HIV+ individuals [306]. Mitochondrial haplogroups have been shown in cytoplasmic hybrid (cybrid) studies to be associated with differing levels of systemic inflammation and ROS production [307, 308].

Samuels et al. recently evaluated a new measure of mtDNA copy number in PBMCs, estimated using genome-wide microarray data and comparisons of probe fluorescence intensities of mtDNA SNPs relative to all nuclear DNA SNPs, with neurocognitive impairment in CHARTER participants. Lower predicted mtDNA copy number per cell by this measure was associated with longer duration of cART, higher platelet count, and higher hemoglobin levels but surprisingly was not significantly associated with age. Adjusting for these factors as well as age, mtDNA copy number was inversely associated with neurocognitive performance (GDS impairment or HAND by Frascati criteria) in study participants (Samuels et al., *manuscript submitted for publication*) [309]. Higher mtDNA content may therefore indicate increased mtDNA replication in response to systemic mitochondrial dysfunction in HIV+ individuals, although further studies are needed to replicate these associations and clarify underlying mechanisms.

The role of cART in mitochondrial toxicity within the CNS in HIV infection has been extensively debated but remains understudied as a possible contributor to aging-related neurocognitive impairment [310]. Nevertheless, long-term systemic and brain mtDNA depletion and damage may occur after exposure to some nucleoside reverse-transcriptase inhibitors (NRTIs), and mitochondrial host genomics may interact with antiretrovirals in potentiating injury; this requires further study [311, 312]. Higher levels of oxidant damage to nuclear and mtDNA in the brains of HAND patients at autopsy suggest the possibility that mtDNA damage in these persons may promote chronic neuroinflammation and neuronal apoptosis during HIV infection [313]. These findings are supported by in vitro studies and studies in nonhuman primates [310], which show that cART can generate increased oxidative stress and lead to neuronal death. Measurement of biomarkers of oxidative damage to DNA and proteins (e.g., oxidatively modified DNA and protein carbonyls measured in CSF) may be helpful in clarifying these issues.

Extracellular (cell-free) mtDNA, which contains CpG motifs that act as immunogenic toll-like receptor-9 ligands and damage-associated mitochondrial patterns, has emerged as a biomarker of neuroinflammation and mitochondrial damage in HIV infection and HAND. Cell-free mtDNA is released during cellular injury and as part of the innate immune response to viral pathogens, and it may also relate to altered autophagy, the process by which cells under stress conditions recycle and dispose damaged organelles. Recent studies by Mehta et al. (Mehta et al., *manuscript submitted for publication)* evaluated relationships between mtDNA in CSF, neurocognitive impairment, and biomarkers of neuroinflammation and immune activation in HIV infection [314]. In a cross-sectional analysis of 28 HIV+ individuals, cell-free mtDNA levels measured in CSF by droplet digital PCR were strongly associated with CSF levels of CXCL-10 and with severity of neurocognitive impairment in impaired individuals, but not with neurocognitive impairment itself. In five individuals who participated in a longitudinal treatment interruption study, mtDNA levels rose in CSF preceding the onset of CSF pleocytosis and the rise in CSF HIV

RNA. In the first neuropathological study to evaluate mitochondrial injury in the brain in HIV infection, Var et al. compared levels of mtDNA (copies per cell) and the relative proportion of the mitochondrial common deletion (a 4977-bp deletion associated with mtDNA damage) in autopsy brain tissues from HIV+ persons with and without a history of methamphetamine (METH) use and in HIV-negative controls; all decedents in this study had undergone premortem neurocognitive characterization as NNTC participants [315]. Individuals with known AD were excluded from analyses. While no regional differences were seen in mtDNA copy number per cell in gray matter, higher mtDNA levels were seen in certain white matter regions (e.g., Brodmann's area), and a significantly higher abundance of the common mtDNA deletion mutation was also observed in these regions. Higher levels of mtDNA were observed in specific brain regions in HIV+ METH users compared to METH nonusers and HIV-negative controls, but mitochondrial injury, as evidenced by the abundance of the common deletion, was lower in the HIV+ METH users. MtDNA levels per cell were not associated with age in brain tissues, although the abundance of the common deletion was associated with age, as anticipated. In the HIV + METH+ group, a higher abundance of the "common deletion" was associated with lower GDS ($p < 0.01$); however, in the HIV + METH group, higher abundance of the "common deletion" was associated with higher GDS ($p < 0.01$). Finally, in both HIV+ groups, mtDNA injury was associated with HIV DNA levels in the brain, but not with mtDNA content. Nor were levels of extracellular (cell-free) mtDNA in CSF associated with GDS, inflammatory markers, or METH use.

19.3.2.4 Novel Iron-Modifying and Mitochondrial-Targeted Therapeutics

As noted by other investigators, there is a need to carefully balance trophic and toxic properties of iron in the CNS when designing and testing iron-modulating therapies [316]. The evidence suggests that treatment of HAND should be based on a combination of anti-inflammatory, regenerative, and neuroprotective strategies. Boelaert et al. [205] first discussed the possibility that iron chelators may have a role in treating the iron dysregulation of HIV infection. Iron chelators have shown considerable promise in AD and PD and need to be investigated in HAND. Future studies evaluating and treating even milder forms of anemia in HIV+ persons hold promise for reducing the impact of HIV on cognition. Studies of erythrocyte membrane and other properties of whole blood may provide a clue the pathophysiology of cognitive decline in this population [317].

Data linking brain iron redistribution and increased extracellular iron accumulation with dysregulation of calcium transport, abnormalities of NMDA receptors, and malfunction of voltage-operated calcium channels suggest that mitochondrial targeting of therapeutic agents could be a fruitful strategy for addressing HIV-induced iron-mitochondrial dysmetabolism in the brain [318].

Recent studies in nonhuman primate SIV models of neurologic disease have suggested that fluconazole and paroxetine are protective against HIV gp120- and Tat-mediated neurotoxicity. As evidenced by neurofilament light chain levels in CSF,

amyloid precursor protein accumulates in axons and calcium in the frontal cortex, although markers of neuroinflammation and plasma or CSF viral loads were not impacted. This study points out that neuroprotection is possible even in the face of viral replication and neuroinflammation [319]. Drugs such as paroxetine and deferoxamine (an iron chelator) may modulate iron levels in the brain and suppress iron-mediated Aβ accumulation in the CNS, with significant neuroprotective potential in HIV+ individuals [320]. A recently completed randomized, double-blind, placebo-controlled clinical trial showed particular promise for paroxetine [321]. Naturally occurring iron-containing molecules, including mitochondrial ferritin and (H)-ferritin, may also hold therapeutic promise in neurocognitive disorders like HAND [322]. Mitochondrial ferritin is a relatively recently identified iron-storage protein unique to mitochondria and important for proper iron partitioning between mitochondria and the cytosol. It is primarily expressed in the brain and structurally similar to (H)-ferritin. Mitochondrial ferritin is upregulated in AD and its overexpression attenuates β-amyloid neurotoxicity; its neuroprotective effects also include maintaining mitochondrial iron homeostasis and preventing dopaminergic cell death [322]. Chelation of extracellular iron released from activated macrophages and microglia may also be a way to address iron accumulation in certain brain regions and oxidative injury to the brain in chronic inflammatory disorders of the CNS, such as HAND [225, 323]. Neurons and glial cells may export iron via a glycophosphatidylinositol-anchored form of CP, and CP levels may promote iron deposition in certain parts of the brain. As in vitro and animal studies have suggested that excess iron in the brain can be chelated and that iron chelators hold promise in the treatment of neurological disorders like AD and PD, such interventions may have therapeutic benefit in HAND [323]. Ongoing studies addressing iron-mitochondrial dysregulation in the CNS promise new interventions to evaluate in clinical trials soon, providing hope for improving quality of life for this growing population of individuals surviving (and aging) with HIV infection.

19.3.3 Genetic Factors in CNS Impairment in HIV+ Children

19.3.3.1 Introduction

HIV infection has different effects on neurocognitive function in pediatric HIV disease as compared to adults. The natural history of HIV disease also differs between children and adults. Children generally do not have the same confounding factors such as drug abuse encountered in HIV+ adults. Since children's immune systems are immature, they are unable to fight HIV infection, and there is a risk of more rapid disease progression. Also, normal CD4+ T-lymphocyte counts are higher in young children than in adults, dependent on age; hence, these cell counts need to be interpreted differently from CD4 cell counts in adults. However, children also have higher potential for immune system reconstitution, as they have higher numbers of CD4+ T cells than adults.

HIV crosses the BBB and enters the brain early in infection. Compared to HIV infection in the CNS of adults, there have been fewer studies in children. Absence of coinfections or confounding factors in the CNS of children with HIV infection has allowed better understanding of the brain damage and lesions associated with primary HIV brain infection. In a comparison of primary HIV infection of the brain in children and adults, it was observed that children had more florid inflammation, more frequent multinucleated giant cell localization in the cerebral cortex, and more basophilic mineralization compared to adults. In contrast, adults had more perivascular brown pigment and more obvious white matter changes [324]. These neuropathological observations support the presence of more fulminant CNS disease in HIV-infected children, due to increased virulence of HIV in the immature CNS.

HIV+ infants may manifest early, catastrophic encephalopathy, with loss of brain growth, motor abnormalities, and cognitive dysfunction [325]. HIV-infected infants score lower than seroconverters on developmental measures, particularly language acquisition. Symptoms similar to adult HAD are occasionally seen in adolescents with advanced AIDS, including dementia, bradykinesia, and spasticity. The risk of HIV encephalopathy increases with very early age of infection and with high viral loads.

In a study of the French Perinatal Cohort of children born to HIV+ mothers and followed from birth with the French SEROCO Cohort of adults with a known date of infection, early encephalopathy in infants had a different pathophysiologic mechanism from that occurring in children, which showed similarities with mechanisms observed in adults [326]. Early encephalopathy was probably related to the occurrence of pathologic events during late fetal life.

Another study evaluated neuropsychological development, prevalence of neurological impairment, and neuroimaging in nine HIV-infected children for a period of 10 years using electroencephalography every 6 months and computed tomography/MRI once a year, which were very informative tools to follow the course of neuropsychological problems of HIV+ children [327].

Antiretroviral agents can improve or even reverse the course of neurological impairment in children due to various degrees of CNS drug penetration. Addition of the protease inhibitor ritonavir to nucleoside analogue therapy has been reported to delay disease progression and prolong life in adults with moderate to advanced HIV disease [328].

As part of the neurodevelopmental examination of children, the Clinical Adaptive Test/Clinical Linguistic and Auditory Milestone Scale (CAT/CLAMS) detected neurodevelopmental differences between HIV+ and uninfected children at 12 and 18 months of age [329].

In a study of HIV-related encephalopathy in 50 pediatric patients, born to HIV-seropositive mothers or infected by contaminated blood, 17 pediatric patients with HIV-related neurological impairment, 16 cases of encephalopathy, and 1 case of neurotoxoplasmosis were observed, demonstrating a high frequency of neurological impairment in HIV+ infants and children [330].

In a follow-up study of 784 HIV+ Argentinian children infected by vertical transmission, 311 developed neuroAIDS [331]. Also, antiretroviral treatment showed complete remission or noteworthy improvement of progressive and nonprogressive encephalopathy, conversion of the most severe cases of progressive encephalopathy (severe developmental delay, acquired microcephaly, spastic quadriparesis, and fatal progression) into a more moderate phenotype (less developmental delay, normal head growth, spastic paraparesis, and chronic evolution of the disease), and reversion of acquired microcephaly observed in the first years of the epidemic. Another study recently summarized the spectrum of neuro-HIV in children, the neurocognitive and behavioral sequelae, the effects of treatment on the primary neurologic effects of the disease, and the specific challenges of identifying and managing these problems in resource-limited contexts, such as those found on the African continent [332].

19.3.3.2 Effects of Host Genetic Variants on CNS Disease in HIV+ Children in the United States

Several studies in HIV+ children from two US cohorts are described here; 1053 children with symptomatic HIV infection from Pediatric AIDS Clinical Trial Group (PACTG) protocols P152 [333] and P300 [334] were studied. P152 and P300 were multicenter, prospective, randomized, double-blind, placebo-controlled protocols that assessed the efficacy of single or combination NRTI treatment regimens in symptomatic HIV+ children in the United States, prior to the availability of cART. Important eligibility criteria included an age range of 3 months to 18 years with symptomatic HIV infection for P152 [333], an age range of 42 days to 15 years with symptomatic HIV infection for P300 [334], and meeting the requirements that the original Centers for Disease Control (CDC) classification system had established to diagnose HIV infection in children at the time of these studies [335]. In these two protocols, CD4+ T-lymphocyte count and percentage and HIV RNA concentration were measured at entry, prior to initiation of therapy and baseline CD4+ T-cell count, and HIV RNA data were used as dependent variables in analyses to determine their associations with host genetic variants. Of the 1053 subjects, 1045 had baseline CD4+ T-cell counts, and 871 had baseline HIV RNA data.

The primary endpoints of the analyses were progression-free survival (PFS) and CNS impairment. PFS was defined as either time from study randomization to progression to first clinical HIV-related disease outcome or death, whichever occurred earlier. The disease outcomes included weight growth failure, ≥ 2 opportunistic infections, malignancy, CDC clinical disease category C, and/or abnormality of the CNS (e.g., neurological deterioration, decline in neurocognitive test scores, and/or brain growth failure). The CNS impairment endpoint, a subset of PFS, was defined as time from randomization to deterioration in brain growth, psychological function, and/or neurological status.

19.3.3.3 Chemokine and Chemokine Receptor Polymorphisms in CNS Disease in HIV+ Children

The prevalence of chemokine and chemokine receptor polymorphisms in symptomatic HIV+ children in the United States was determined first [336]. Furthermore, the genetic influence of *CCR5*, *CCR2*, and *SDF1* variants on HIV-related disease progression and neurological impairment in children with symptomatic HIV infection was studied [36]. Variants including *CCR2-V64I, CCR5-wt/Δ32, CCR5-59029-G/A, CCR5-59353-T/C, CCR5-59356-C/T,* and *SDF1-3'-G/A* were evaluated. Children with the *CCR5-wt/Δ32* genotype experienced significantly delayed disease progression, including less neurocognitive impairment.

The presence of genetic polymorphisms in the *CX3CR1* gene, a minor chemokine co-receptor of HIV, predicted HIV disease progression in children independently of CD4+ lymphocyte count and HIV RNA load [337]. Children with the *CX3CR1 I/I249* genotype experienced more rapid disease progression (*I/I249* vs. *V/V249*, HR 2.19 (95% CI, 1.30–3.68), $p < 0.01$; *I/I249* vs. *V/I249*, HR 1.77 [95% CI, 1.00–3.14], $p = 0.05$) and a trend toward more CNS impairment (*I/I249* vs. *V/V249*, HR 2.19 [95% CI, 1.00–4.78], $p < 0.05$; *I/I249* vs. *V/I249*, HR 2.02 [95% CI, 0.85–4.83], $p = 0.11$). Children with the *V249-T280* haplotype experienced significantly less disease progression (HR 0.42 [95% CI, 0.24–0.73]; $p < 0.01$) and CNS impairment (HR 0.39 [95% CI, 0.39–0.22]; $p < 0.01$). Of note, these effects remained significant after adjusting for CD4+ T-lymphocyte count and plasma HIV RNA load at baseline and in a longitudinal, multivariable analysis. *CX3CR1* genotypes and haplotypes impacted HIV disease progression independently of CD4+ T-lymphocyte count and plasma HIV RNA load, suggesting that the fundamental role of CX3CR1 in the alteration of disease progression might be the recruitment of immunomodulatory cells responsible for the control of HIV. MCP-1 is the ligand for CCR2, a minor co-receptor of HIV. The *MCP1 2518-G* allele was marginally associated with CNS impairment at study entry [338]. Alone or in combination with *CCR2-64I*, the *MCP1 2518-G* allele did not alter disease progression or subsequent CNS impairment. These findings differ from studies in adults and suggest that MCP-1-CCR2 protein interactions may play a different role in HIV immunopathogenesis in children. The interleukin *(IL)4 589-C/T* polymorphism has been reported to protect against HIV-related disease progression in white adults. The *IL4 589-T* allele was more prevalent in Hispanic and in black, non-Hispanic children, compared with white, non-Hispanic children. We found that the *IL4 589-C/T* polymorphism does not affect the risk of HIV-related disease progression or CNS impairment in children, and this result did not differ by race/ethnicity [339]. These findings suggest that the *IL4 589-C/T* polymorphism is not an important determinant of HIV disease progression in children.

19.3.3.4 Polymorphisms in Intracellular Antiviral APOBEC3G Gene Alter HIV Disease and CNS Impairment in Children

Apolipoprotein B mRNA-editing catalytic polypeptide 3G (APOBEC3G) protein is incorporated into nascent virus particles and mediates cytidine deamination (C-to-U) of first-strand reverse transcripts of HIV in target cells, resulting in *G*-to-*A* hypermutation of the coding strand and premature degradation. Genetic variants in the *APOBEC3G* gene were found to be associated with HIV-related disease progression and CNS impairment in children [63]. *APOBEC3G-H186R* homozygous *G/G* genotype was associated with more rapid HIV disease progression (HR 1.69; $p = 0.01$] and CNS impairment (HR, 2.00; $p = 0.02$) compared with the wild-type *A/A* or heterozygous *A/G* genotype in a recessive model. In both additive and dominant models, *APOBEC3G-F119F-C* allele was associated with protection against disease progression (HR [additive] 0.69, $p < 0.01$, and HR [dominant] 0.60, $p < 0.01$, respectively) and CNS impairment (HR [additive] 0.65, $p = 0.02$, and HR [dominant] 0.54, $p < 0.01$, respectively). These associations remained significant in multivariate analyses controlling for baseline characteristics or previously identified genetic variants known to alter HIV-related disease in this cohort of children.

19.3.3.5 An Age-Dependent Association of Mannose-Binding Lectin-2 Genetic Variants on HIV-Related Disease in Children

Mannose-binding lectin (MBL) is part of the lectin pathway of complement activation against various pathogens; however, its role in innate immune responses against HIV infection in children is unknown. The effects of mannose-binding lectin-2 (*MBL2*) alleles on HIV disease progression and CNS impairment in children [340] were determined. Children with the homozygous variant *MBL2-O/O* genotype were more likely to experience rapid disease progression and CNS impairment than those with the wild-type *AA* genotype. The effects were predominantly observed in children younger than 2 years. In unadjusted Cox proportional hazards models, children younger than 2 years with *MBL2-O/O* experienced more rapid disease progression (*O/O* vs. *AA*, HR 1.54; 95% CI, 1.07–2.22; $p = 0.02$; *O/O* vs. *A/O*, HR 2.28; 95% CI, 1.09–4.79; $p = 0.03$). Similarly, children with *MBL2-O/O* were more likely to experience rapid progression to CNS impairment (*O/O* vs. *A/A*, HR 1.69; 95% CI, 1.06–2.69; $p = 0.03$; *O/O* vs. *A/O*, HR 2.78; 95% CI, 1.07–7.21; $p = 0.03$). These effects remained significant after adjustment for CD4+ lymphocyte count, plasma HIV RNA, and other genotypes (*MBL2-H/L*, *MBL2-P/Q*, *MBL2-X/Y*, and *CCR5-wt/Δ32−59029-G/A*, *CX_3CR1-249-V/I*, *−280-T/M*, and *SDF-1-180-G/A*). *MBL2-O/O* genotypes, which result in lower expression of MBL, were associated with more rapid HIV disease progression, including CNS impairment, predominantly in children younger than 2 years.

19.3.3.6 HLA Alleles Are Associated with Altered Risk for Disease Progression and CNS Impairment of HIV+ Children

Five hundred seventy-two HIV+ children, identified as disease progressors or non-progressors, were selected from PACTG P152 and P300 through a case-cohort sampling scheme. Study endpoints were HIV-related disease PFS and time to CNS impairment. DNA was genotyped for *HLA* alleles using a Luminex 100 platform. Weighted Kaplan-Meier methods and Cox proportional hazards models were used to assess the effects of *HLA* alleles on study endpoints [56]. The presence of the *B-27* allele ($n = 20$) was associated with complete protection against disease progression and CNS impairment over the median follow-up of 26 months ($p < 0.0001$ for both). These findings held in multivariate analyses controlling for baseline covariates, including race, gender, age, HIV viral load, CD4+ T-lymphocyte count and percent, weight-for-age z score, and treatment, and for other genotypes known to affect HIV-related disease progression. Also, the presence of the *A-24* allele was associated with more rapid CNS impairment (HR 2.01; 95% CI, 1.04 to 3.88; $p = 0.04$). The *HLA Class II DQB1-2* allele was associated with a delayed disease progression (HR 0.66; 95% CI, 0.47–0.92; $p = 0.01$) and CNS impairment (HR 0.58; 95% CI, 0.36–0.93; $p = 0.02$) in children.

19.3.4 The Interaction of Age and Host Genomics on Hand

19.3.4.1 Overview

Although cART has been highly effective at preventing AIDS-related complications, treated patients are at significant risk for a number of diseases typically associated with aging, including cardiovascular disease, osteoporosis, cancer, cognitive impairment, and frailty [341–353]. Among the aging HIV+ population, it has become evident that the incidence of HIV-associated non-AIDS (HANA) conditions is increasing [354]. HANA conditions affect virtually every organ system and have as a common theme an association with advancing age and a pathogenesis likely based on chronic inflammation. Arguably, given its somewhat liberal inclusion criteria, the most common HANA condition is HAND. Early studies indicated that older individuals were at increased risk for HAND [355]. Additional evidence that HAND is related at least partially to accelerated aging includes studies of CSF metabolomics [356], in vitro analysis in astrocytes [357], and neurophysiological studies [358, 359].

In order to effectively investigate accelerated aging in HAND, one first needs to understand what is meant by normal aging and to find a way of measuring it. Telomere length, which relates to cellular senescence, has been the most popular method to date for studying biological aging. In the context of HAND, telomere length has not been consistently associated with neurocognitive impairment or other indicators of neuro-HIV/AIDS [360, 361], and reported associations have been

weak and difficult to interpret. For example, Malan-Müller et al. [361] found a positive correlation between telomere length and learning performance in HIV+ South African women and a negative correlation between telomere length and verbal fluency among HIV+ subjects who had also experienced psychological trauma. More recently, the utility of epigenomics as a tool for studying age acceleration has been demonstrated in HIV+ individuals. A recent study examining DNA methylation derived from HIV+ and HIV-uninfected brains indicates that infection accelerates aging by as much as 9.3 years [203], based on the recently developed epigenetic clock [202]. Similar accelerated aging has been found in PBMC, with an acceleration of 14 years in HIV+ adults compared to uninfected adults [362] and 3.6 years in individuals with detectable plasma viral loads as compared to those with undetectable plasma viral loads [203]. Whether or not these changes correlate with HAND remains to be seen. However, these findings are consistent with clinicopathological studies in which age-related pathology has been observed in relatively young HIV+ cases, including reduced Aβ-42 in CSF [363] and increased amyloid-β deposits in brain tissue [364]. These findings also suggest a role for *APOE* genotype in HAND. Despite these observations, the relationship between the *APOE*ε4 allele and HAND remains equivocal. Early studies appeared to show a robust relationship between this allele and risk for HAND. For example, Corder and colleagues [28] found that twice as many individuals carrying at least one *ε4* allele were given a diagnosis of HAD over the course of the 5-year study. Subsequent studies over the last decade, however, have yielded inconsistent findings. Potential mitigating factors include the deleterious influence of the *ε4* allele on disease progression and survival rates, methodological differences between studies in the operationalization of HAND, and differences between studies in terms of the inclusion of a (HIV) seronegative control sample [43, 365, 366]. Recent studies using such a control sample and objective measures of neurocognitive functioning suggest synergistic deleterious effects of the *APOE-ε4* allele and HIV on cognition [52, 54]. Within HIV+ samples, age has been found to be a modulating factor in some studies [41, 367] but not others [50, 54, 368]. In support of a modulating factor, Valcour and colleagues [41] observed older (age ≥ 50 years) *ε4* carriers to have higher rates of HAD compared to age-matched *ε4* noncarriers. This was not observed in their younger (<40-year-old) participants. Using more broadly defined HAND as the outcome variable, similar findings were recently documented by Panos et al. [367]. Of the published longitudinal studies of *APOE* genotype and HAND [28, 43, 50, 69], none employed a design aimed at measuring individual changes on objective neurocognitive measures over time while also accounting for mitigating factors such as disease severity. Such an approach may help clarify the nature of the relationship between *APOE* genotype and HAND. Most recently, Becker et al. [69] examined time to incident cognitive impairment in 1481 HIV+ individuals who were cognitively normal at their first neuropsychological evaluation. No association between *APOE-ε4* and time to develop neurocognitive impairment was found, nor did they observe interactions between *ε4*, HIV infection, age, and either death or neurocognitive impairment. However, that study was largely limited to younger (<65-year-old) individuals.

Looking beyond behavioral phenotypes, Diaz-Arrastia and colleagues [38] and Dunlop and colleagues [26] probed associations between *APOE-ε4* and pathological findings of HIVE or HAND; no associations were observed. However, while HIVE is thought to be a common pathological substrate for HAD, the two can occur independently of one another [369]. It should also be noted that these studies were conducted with patients who died in the pre-cART era. Given the deleterious relationship between the *APOE-ε4* allele and disease severity, individuals may have died before pathological effects emerged in the brain. *APOE-ε4* is associated with faster disease progression, possibly due to enhanced HIV fusion/cell entry [43]. More recently, Cutler and colleagues [37] found evidence for lipid metabolism derangements in the brain tissue of *ε4* carriers, although their sample also consisted of patients who died in the pre-cART era. Soontornniyomkij et al. [370] found that *APOE-ε4* and older age were independently associated with the increased likelihood of cerebral Aβ plaque deposition in HIV+ adults. While the Aβ plaques in HIV brains were immunohistologically different from those in brains of individuals who died with symptomatic AD, this study is among the first to provide a link between genotype and neuropathological findings in HIV.

19.3.4.2 Host Iron-Mitochondrial Interactions with Age and Age Acceleration in HAND

Despite being only a few percent of the total body mass, the brain is responsible for approximately half of the oxygen consumption and 20% of the mitochondrial oxygen consumption [371]. In the brain, a very high metabolic requirement for iron, coupled with the high susceptibility of brain tissue to iron-generated lipid peroxidative damage, requires particularly stringent regulation of iron availability and mitochondrial iron utilization in order to preserve structural integrity and energy metabolism. Mitochondrial dysfunction leads to neuromuscular degeneration, aging, energy depletion, and free radical production; defects in iron-sulfur cluster biosynthesis are important mitochondrial mediators of aging [372]. Iron-sulfur clusters are essential components of respiratory electron transport chain complexes as well as specific tricarboxylic acid cycle enzymes, including the iron-regulated cytoplasmic and mitochondrial isoforms of aconitase, succinate dehydrogenase, and DNA repair enzymes. Iron-sulfur cluster assembly and disassembly based on ambient iron levels in cells determines the binding of IRPs to their mRNA targets [373]. Disruption of iron homeostasis may therefore have significant impact on shifts in brain energy metabolism in the brain via effects on cytoplasmic and mitochondrial enzyme function [374].

Trace metals including but not limited to iron may also interact with proteins directly or indirectly involved in the pathogenesis of HAND. In non-HIV-related neurocognitive disorders (AD, prion diseases, and Lewy body dementia), there is accumulating evidence that misfolding of disease-associated proteins such as β-amyloid and α-synuclein is effected by interactions of these proteins with iron and that these proteins are also in part responsible for the iron dysregulation seen in disorders of aging [375].

Increased brain iron has been associated with age-related decline in motor strength as well as cognitive decline, and one study recently evaluated the association between iron content in the basal ganglia and hand grasp performance in older women [376]. Higher basal ganglia iron content assessed by T2* MRI was associated in this study with an increased number of errors committed during learned handgrasp tasks with the same and contralateral hands, suggesting a direct link between brain iron content and motor dysfunction.

Finally, it is significant to note that the effects of caloric restriction, long known to be neuroprotective and to slow the aging, have recently been shown in mouse models to include downregulation of hepcidin in the brain, which would be expected to limit aging-related iron accumulation in the brain parenchyma [377].

19.3.4.3 Aging, Host Iron-Mitochondrial Genomics, and HAND

Genomic, transcriptomic, and metabolomic studies have determined that HAND is associated with global mitochondrial dysfunction and a downregulation of mitochondrial protein expression, as have studies in animal models [149, 356, 378, 379]. These studies are consistent with the concept of age acceleration and increased oxidative stress in HIV infection. Changes in iron transport and mitochondrial function contribute to systemic oxidative stress. In HIV+ men, higher circulating iron levels are associated with increased levels of oxidative stress as measured by plasma F_2-isoprostane levels, a sensitive and relatively specific measure of in vivo oxidative injury. However women, in whom iron stores are significantly lower than in men, had higher F_2-isoprostane levels than men overall; the reasons for this finding are unclear [380].

The epigenetic clock, recently reported to show accelerated aging in HIV infection, also highlights the importance of nuclear-encoded mitochondrial genes [204, 381]. Whether mtDNA-encoded genes contribute to epigenetic aging is not known, but mtDNA methylation has recently been linked to neurodegenerative disease [382, 383]. Iron metabolism regulates mitochondrial biogenesis, and iron transport is itself regulated in part by mitochondrial function [384], so iron-related genes likely play a role in mediating age acceleration and neurocognitive impairment. Ongoing studies by our group are actively investigating interactive effects of nuclear and mtDNA variants in HAND.

Although studies in the modern cART era have generally not associated *APOE* alleles with HAND, *APOE* genotype interactions with age and HIV serostatus have been postulated [54, 69, 370, 385–387]. Increased influence of *APOE-ε4* genotypes in HAND among older HIV+ persons may therefore relate to iron dysregulation.

19.3.4.4 Iron-Sensitive Neuroimaging in Aging-Related NCI

Differentiating HAND from other neurocognitive disorders of aging such as AD and PD is increasingly challenging as the HIV+ population ages, and the use of neuroimaging techniques particularly sensitive to iron deposition in the brain may be of value, assuming that the characteristic patterns of iron deposition in these

disorders will differ from those in HAND. In addition, reliable estimates of brain tissue iron concentration are likely to be important in monitoring individuals with HIV infection for physiological age-related and pathological conditions such as HAND, AD, and vascular disorders. Several imaging techniques are helpful in this regard, including T2-weighted or T2*/R2* MRI, because they can detect differences derived from changes in signal due to the magnetic properties of the major tissue iron-storage proteins, ferritin, and hemosiderin; the magnetic properties of these iron-carrier proteins shorten the relaxation time of nearby water protons, leading to signal extinction in iron-rich areas. However these methods are not altogether specific for iron, due to the potential influence of water content of the surrounding tissue, other trace metals, and myelin density. More iron-specific imaging techniques under development are relaxometry, magnetic field correlation imaging, and phase-based multi-contrast imaging (covering susceptibility-weighted imaging and quantitative susceptibility mapping) [215, 388]. Diffusion tensor imaging is also reflective of white matter integrity and fractional anisotropy changes in HIV infection that appear to be impacted by iron [246]. In the future, monitoring changes in iron storage and content may serve as sensitive biomarker for diagnosis as well as treatment monitoring.

19.3.5 Alternative Phenotypes for the Study of Hand Genomics

One possible reason for the lack of replicability of findings in the genetic association studies described above is the use of different phenotypes across studies. Earlier studies primarily used HAD or similar diagnoses (e.g., AIDS Dementia Complex) as the phenotype [30, 34, 41, 45, 47]. Others have used composite measures of global neurocognitive functioning, usually derived from a comprehensive battery of neuropsychological tests [36, 39, 389], as the determination of neurocognitive impairment is more reliable than a formal diagnosis of HAND [390]. Perhaps the greatest shortcoming of these phenotypes is that they are influenced by a number of environmental, psychometric, and endogenous factors. This might be most problematic for detecting mild neurocognitive impairment characteristic of ANI or MND, which have a relatively low threshold to meet criteria [391, 392]. Furthermore, the numerous nongenetic contributors to variance in these measures (e.g., measurement error) make them less suitable targets for genetic analysis, especially when effect sizes for genetic variants are small. Finally, the use of global measures of neurocognitive function, which have become the norm in such studies, runs the risk of missing domain-specific associations with genes of interest. One partial solution is to utilize domain-specific composite scores (e.g., memory or processing speed) composed of measures with documented heritability. However, this strategy also increased Type I error rate, necessitating more strict corrections for multiple testing.

Unlike genomic studies, the majority of transcriptomic studies have focused on encephalitis (either SIVE or HIVE) as the disease phenotype. Prior to the development cART, HAD was common, and HIVE was considered to be its

neuropathological basis [19, 71, 87, 164, 286, 393–398]. In the current era of widespread cART use, however, the vast majority of HAND cases present with milder symptoms [399, 400], and upon autopsy examination, they do not have neuropathological findings consistent with HIVE as defined in the pre-cART era [401]. Increasingly, evidence suggests that for the vast majority of cART era HAND cases, HAND is the result of potentially reversible neurodegeneration driven by chronic neuroinflammation [164, 394, 396, 401]. The relevance of this concept to transcriptomic studies was best demonstrated recently in a study comparing transcriptome changes in individuals who had premortem HAND but no evidence of postmortem HIVE to those changes in individuals with premortem HAND, who also showed postmortem HIVE [148]. Despite the similar neurobehavioral phenotype, the resulting transcriptome profiles were highly distinct, as described above. This study underscored the need to evaluate currently relevant disease phenotypes.

Due to difficulties in using neuropsychological tests or diagnoses based primarily on such tests, as phenotypic measures of HAND, some investigators have explored alternative outcome measures. Among these are various neuroimaging indices. While beyond the scope of this review, a variety of MRI and MRS markers have been associated with HIV disease progression, neurocognitive impairment, and response to putative treatments [402–407]. Several studies have employed neuroimaging in conjunction with genotyping. A recent neuroimaging study of 177 (80 HIV+ and 97 HIV-) did not find interactions between *APOE* genotype and brain metabolite levels as measured via MRS [67], despite group differences in neurocognitive measures. Sundermann et al. [68] examined interactive effects of *COMT* rs4680 and HIV on executive functioning and frontal cortex metabolism using functional MRI in two samples of women enrolled in the Women's Interagency HIV Study Consortium. In the first sample, HIV+ participants who possessed *Val/Val* genotype performed significantly worse on the N-back tests than the uninfected controls with similar genotype, whereas those with a *Met* allele performed similarly to uninfected controls. In a second sample in which the N-back task was performed during fMRI, HIV+ *Val/Val* carriers showed greater prefrontal activation compared to uninfected *Val/Val* carriers during the task, suggesting that increased cortical activation was required by HIV+ *Val/Val* carriers in order to complete the task. Conversely, uninfected *Met* allele carriers demonstrated significantly greater activation as compared to HIV+ *Met* allele carriers; however, this activation occurred outside the prefrontal cortex and therefore may not be indicative of compensatory recruitment. Importantly, because the participants who generated the imaging data did not show the serostatus x genotype effects that the behavioral sample showed, it remains unclear if the altered metabolism in the prefrontal cortex is related to working memory deficits in HIV+ *Val/Val* carriers. It is important to point out that, as with candidate-gene studies of behavioral HAND phenotypes, these imaging studies are likely to be underpowered and difficult to validate. One potential solution is to pool imaging data across cohorts. One current endeavor with great potential of linking neuroimaging biomarkers of HAND with genetic variation is the ENIGMA-HIV consortium (http://enigma.ini.usc.edu/ongoing/enigma-HIV-working-group/), which ties together several hundred participants from a growing number of cohorts.

Another promising focus for alternative phenotypes is the histopathological changes in HIV+ brains that can be quantified via immunohistochemistry. Markers of dendritic simplification [408], or a combination of synaptic and dendritic markers, appear to have the strongest relationship with the neurocognitive deficit characteristic of HAND. For example, Moore et al. found that a combined histopathological phenotype consisting of synaptodendritic neurodegeneration, as measured by synaptophysin (SYP) and microtubule-associated protein-2 (MAP2), was associated with HAND across both subcortical and cortical brain regions [393]. Another histopathological candidate is β-amyloid deposition, which has been observed in the cortex in HIV+ individuals [409–412]. In addition to these markers of neurodegeneration and abnormal protein aggregation, markers of neuroinflammation have also been shown to be associated with neurocognitive impairment or HIV-related brain dysfunction [413–415]. Indeed, macrophage proliferation, microglial activation, astrocytes activation, and increased chemokine levels have all been found in CSF and brains from HIV+ individuals [19, 17, 87, 164, 394–398]. While these may all represent candidates for neuropathogenic processes underlying HAND, new methods are necessary to determine which ones are relevant. Toward that end, an innovative approach for simultaneously determining which histopathological markers are HAND relevant and which genetic susceptibility loci influence HAND is to examine the association between host genotype, histopathological findings, and behavioral (i.e., neurocognitive) outcomes. In this scenario, neuropathological changes (or neurophysiological changes, in the case of neuroimaging) are considered *intermediate phenotypes*. To put it more simply, if HAND is considered at its most basic level to be the end result of a sequence of physiological events that commences with HIV-induced cellular changes that are modified by genetic factors, then determining the extent to which known genetic susceptibility loci for HAND perturb candidate neuropathological and neurophysiological intermediate phenotypes may reveal which ones are most contributory. The advantage of this approach is that histopathological intermediate phenotypes, like neuroimaging phenotypes, are less prone to exogenous factors and have a stronger association with genetic susceptibility loci than neurobehavioral phenotypes. This approach has been successfully employed in genetic association studies of AD [416, 417], for example, delineating the relationship between *APOE* genotype and Alzheimer's-related cognitive impairment as a function of the sequential cascade of amyloid plaque formation and neurofibrillary tangle formation [416–419]. Relevant to HAND, to our knowledge, only three studies have examined the relationship between genetic susceptibility loci and neuropathological outcomes. Sato-Matsumura et al. [29], with a sample of 44 AIDS patients with autopsy-verified HIVE or HIV leukoencephalopathy and 30 AIDS patients without these neuropathologies, did not find an association between *TNF-α* genotype at rs1800629 and either of the neuropathological conditions. Diaz-Arrastia et al. [38] assessed for HIVE or vacuolar myelopathy in the brains of 270 HIV+ individuals who died with AIDS between 1989 and 1996. Neurocognitive functioning and HAND were not considered. They determined the presence of microglial nodules,

multinucleated giant cells, myelin pallor, and vacuolar myelopathy in the brains and/or spinal cords. None of the alleles examined were associated with the presence of these markers. More recently, Soontornniyomkij et al. found that *APOE-ε4* and older age were independently associated with an increased likelihood of cerebral amyloid-β plaque deposition in HIV+ adults [370]. While the amyloid-β plaques in HIV+ brains were immunohistologically different from those in brains of individuals who died with symptomatic AD, this study is the first to provide a link between genotype and neuropathological findings in HIV infection. However, neither study considered clinical manifestations of HAND in their design.

CSF and blood-based biomarkers of HAND have been difficult to identify and replicate. Still, studies relating host genomic factors with these markers have yielded interesting results. Morales et al. [65] examined differences in the frequency of *YWHAE* polymorphisms and protein levels between HIV+ and HIV− individuals who were neurocognitively characterized. The *YWHAE* gene product (also called 14-3-3ε protein) is considered a reliable biomarker for neurodegeneration and interacts with HIV. Drawing from 20 HIV+ and 16 seronegative, randomly selected samples from the Hispanic-Latino Longitudinal Cohort of Women, they found that HIV+ participants heterozygous at rs4790084/rs1204828 had a threefold higher risk of cognitive deficits, of HAND diagnosis, and they had less CSF 14-3-3ε protein expression as compared to homozygotes. Genotype did not affect neurocognitive function in the seronegative group. Furthermore, CSF 14-3-3ε protein levels were 4.5-fold lower in women with HAND as compared to HIV+, cognitively normal women. This latter finding was at odds with studies cited by the authors in which 14-3-3ε protein was not found in the CSF of healthy individuals and occurred almost exclusively in individuals with neurological illness (e.g., CJD and HAD). The authors explained these findings by suggesting that 14-3-3ε protein expression may be elevated in the early stages of neurological illness but depleted in advanced stages. This would not seem to be an adequate explanation for their findings, however, since the women in their study did not have advanced neurologic disease. Furthermore, the conclusion of a genetic association between *YWHAE* genotype and risk for HAND was based on an extremely small sample.

Thames et al. [91] examined interrelationship between *CCL2* (rs1024611) genotype, expression of inflammatory markers in CSF, HIV disease markers, and neurocognitive functioning in 145 HIV+ adults enrolled in the NNTC, hypothesizing that carriers of the *−2578-G* allele would have higher concentration of CCL2 and other inflammatory markers in CSF and worse neurocognitive function. That analysis revealed that while there was no difference in neurocognitive function between genotype groups, carriers of the *CCL2-2578-G* allele had higher levels of CCL2 in CSF, which was in turn associated with higher levels of other pro-inflammatory markers and poorer neurocognitive function. This study is particularly useful in demonstrating the importance of CSF markers as intermediate phenotypes of HAND in genomic studies (Table 19.3).

Table 19.3 Summary of genetic, epigenetic, and transcriptomic mechanisms of relevance to HAND

Genetic factors	**Mechanisms**
Immune-related genes (CCR5, CCR2, CX3CR1, MIP1alpha, RANTES, MCP-1, MBL2, HLA)	Chemokine receptors, chemokine ligands and for viral entry, immune response macrophage activation, monocyte chemotaxis across blood-brain barrier (BBB), viral epitope recognition and CD4 and CD8 cell lysis in brain, and neuroinflammation
Dopamine-related genes (DA, DAT-1, DRD2, DRD3, DBH, COMT, BDNF)	Impairment of dopamine receptor or dopamine expression, transport, or functioning
Intracellular antiviral genes (APOBEC3G)	Intracellular viral genome editing and deactivation
Genes affecting sodium transport across cellular and intracellular membranes (SLC8A1, NALCN)	Mitochondrial dysfunction
Epigenetic factors	**Mechanisms**
miRNA (miR-21, miR-34a, miR-495-3p, miR-151a-5p or miR-744-5p, miR-376a-3p/ miR-16-532-3p)	Interference in gene transcription, translation, and viral gene expression in brain
Histone modifications (HDACS)	Modification of brain gene expression, viral gene expression in brain affecting neuronal function
DNA Methylation (DNMTs)	Modification of global brain gene expression related to neurodegeneration, DA metabolism and transport, and oxidative phosphorylation
Brain/blood transcriptomic factors	**Mechanisms**
Brain gene expression in HAND and HIV encephalitis	Altered pathways of neuroimmune functioning, synaptodendritic functioning and integrity, myelin, neurodegeneration, neuroinflammation, neuropsychological functioning, mitochondrial functioning, cell cycle, cell-cell signaling
Peripheral blood gene expression	Peripheral blood-based markers of HAND: monocytes (BBB crossover), CD4, nadir CD4, CD8, viral load, oxidative stress, hypoxia, transcription factors

19.4 Summary

In this chapter we have summarized all genetic association and epigenetic studies of HAND of which we are aware, as well as gene expression studies employing whole-genome screening (i.e., transcriptomic studies). The reader will notice the general lack of replicability across genetic association studies and, to a lesser extent, across transcriptomic studies. This may be a function of inconsistent or variable neurocognitive phenotypes but may also reflect other aspects of study design (e.g., thresholds for statistical significance). A large portion of this chapter was dedicated to iron and mitochondrial dysregulation as it relates to genetic variants. This line of research is

particularly exciting as it may open doors to novel research efforts. We have also discussed the epigenetic clock as a tool for the study of HAND, but this tool may also be useful for the study of HIV disease progression in general. In sum, genetic, transcriptomic, and epigenetic studies continue to provide important information about HAND pathogenesis, lay foundations for novel and innovative methods, and uncover potential candidates for therapeutic drug development.

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Chapter 20
NeuroAIDS: A Review of Gene Expression in Neurons, Astrocytes, and Oligodendrocytes

Jeanie McGee and Alireza Minagar

Keywords NeuroAIDS • HIV-associated neurocognitive disorders • HAND • HIV-1 • Neurotropic virus • CNS • Diagnosis • Gene expression • Pathogenesis • Pathophysiology • HIV-associated neurocognitive disorders • HIV-associated neurocognitive impairment • HIV-associated dementia • CNS HIV infection • Pathogenesis mechanisms of HAND • HIV-1 infection of cultured human adult oligodendrocytes • Astrocyte expression in NeuroAIDS • Neuronal expression in NeuroAIDS • Oligodendrocyte expression in NeuroAIDS

Core Message

AIDS has been a constantly genetically evolving viral infection, which ultimately, annihilates the human immune system. A detailed assessment and evaluation of the genes, which are involved in pathogenesis of AIDS, in general and NeuroAIDS, in particular, enhance our fundamental knowledge of the pathogenesis and enable scientists to unravel the basic disease mechanisms of AIDS in order to develop more effective and highly focused treatments. The genetic infrastructure of HIV as well as its interactions with host genome governs every aspect of its infectivity, and such features can be utilized as potential means to counter the infection and halt its progression. Indeed, the neurogenesis of NeuroAIDS is fascinating and provides new views to study this devastating infection to all neuroscientists.

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20.1 Introduction

Human immunodeficiency virus (HIV) is a neurotropic virus and can affect the entire central and peripheral components of human nervous system as well as muscles, generating various clinical syndromes. HIV penetration through the blood-brain barrier (BBB) and into the central nervous system (CNS) milieu occurs shortly after the viral exposure and in the course of HIV seroconversion [1]. In fact, HIV RNA is detectable in the cerebrospinal fluid (CSF) of the affected individual as early as 8 days from the initial infection [2], and the brain atrophy occurs early in the course of infection. Such invasion of human body by HIV affects two main organs: the immune system and the CNS. Invasion of the CNS is associated with a series of significant neuropathological changes involving neurons, astrocytes, and oligodendrocytes as well as oligodendrocyte-myelin complex, which, in turn, play a significant role in development of a range of neurologic and psychiatric abnormalities (Table 20.1). Clinically, the neuropsychiatric abnormalities, which manifest with disease progression, are collectively recognized as HIV-associated neurocognitive disorders (HAND). Patients with HAND suffer from cognitive, behavioral, and motor disorders. HAND is relatively common among AIDS patients and engulfs a spectrum of abnormalities ranging from being clinically asymptomatic to mild neurocognitive decline to more severe dementia. In addition, with the common use of combination antiretroviral therapy (cART) (which was implemented as of 1996), the life expectancy of HIV-infected individuals and subsequently the prevalence of HAND have increased to 20–50% [3]. Interestingly, as AIDS patients live longer, they experience other compounding neurological complications (in addition to HAND) such as stroke at a young age, complication of infection with hepatitis C virus, and drug abuse-related complications.

Epidemiologically, HIV-1 is classified into three groups of M, O, and N and genetically is categorized into nine various subtypes, known as A to K. Among these, clades B and C make the largest part (86%) of circulating HIV-1 variants. Clade B is the principal form of infection in North America, Western Europe, and Australia, while clade C is predominant in Africa, Latin America, and Asia. The content of the current review reflects research specific to HIV-1 clade B (HIV-1B), the most dominant subtype in Europe, the Americas, Thailand, Japan, and Australia.

Neuropathogenesis of HIV infection of human CNS includes the initiation of a potent immune response with interactions among the immune cells—mainly the CD4 T lymphocytes, B lymphocytes, and monocyte/macrophages with HIV-1—and

Table 20.1 Common characteristics of HAND (HIV-acquired neurocognitive disorders) [2]

Forgetfulness
Confusion
Headaches
Changes in behavior
Impaired cognitive function
Weakness of arms or legs
Difficulty with movement
Trouble concentrating

the cerebral endothelial cells of the blood-brain barrier (BBB) as well as the resident cells of the CNS, neurons, glial cells, and oligodendrocytes. Within the CNS environment, HIV significantly infects perivascular macrophages/microglial cells as well as astrocytes. The HIV infective process within the brain, unlike periphery, which leads to destruction of CD4 T lymphocytes, leads to productive infection of macrophages and leads to development of syncytia (a hallmark neuropathologic feature of HIV infection of the brain). Apart from this, the HIV can stay dormant within the astrocytes, and identification of HIV-infected astrocytes can be difficult even utilizing immunostaining for HIV. The details of immune system dysfunction and its eventual failure in response to HIV infection and pathogenesis of AIDS, as a global infection of human body, are beyond the scope of this chapter. Instead, the authors will heavily focus on the role of gene expression in neuropathogenesis of HAND and the role of CNS native cells in its pathogenesis.

The core of this chapter is focused on an extensive review of literature concerning gene expression by the neurons, glial cells, and oligodendrocytes in the pathogenesis of HAND with emphasis on reviewing the genes whose expression is differentially expressed in patients with HAND.

20.2 Genetic Basis of HAND

In the past decade, scientific research has focused on exploring the genetic basis of neurologic and psychiatric manifestations of HAND. On the one hand, it is recognized that clinical traits of HAND do not have a genetically heritable basis, and this lack of hereditary factors in the pathogenesis of HAND makes its assessment even more complicated. On the other hand, utilization of specific novel techniques including genetic microarray expression and proteomic studies has made it possible to determine the expression of which genes and proteins are up- or downregulated during the pathogenesis of HAND. These studies are extensive and focus on several potential genes or proteins whose expression by postmortem brain tissue of patients with HAND, neurons, astrocytes, and oligodendrocytes are differentially changed. With today's advanced technology, postmortem studies of NeuroAIDS most often utilize laser microdissection (LMD) techniques to complete microarray profiling and analyses, which were employed in the basic research projects reviewed here. These studies included examinations of either the total brain tissue or specific cells such as oligodendrocytes and astrocytes.

In 2013, Levine and colleagues [4] utilized novel systems biology methods to assess existing transcriptome data derived from the brains of HIV+ patients in order to explore the pathogenic mechanisms of HAND. In addition, they compared the results with those collected from the brains of patients with Alzheimer's disease (AD) with the purpose of identifying common pathways of neuropathogenesis. This project was performed in the context of two studies. In the first study, the data collected from the three brain regions in 6 HIV seronegative and 15 seropositive patients were utilized, and the novel method of weighted gene co-expression network analysis (WGCNA) was applied in order to further explore transcriptome net-

works specific to HAND in patients with HIV encephalitis (HIVE) and those with HAND without HIVE. Next, standard expression analysis and WGCNA were used to identify networks associated with neurocognitive impairment (NCI), regardless of HIVE or HAND diagnosis. Lastly, the investigators assessed the association between the CNS penetration effectiveness (CPE) of antiretroviral regimens and the brain transcriptome. During the study, two correlating gene expressions with premortem neurocognitive functioning identified common gene networks associated with NCI in both HIV and AD. The results of this extensive study revealed that WGCNA largely substantiated findings from standard differential gene expression analyses. In addition, but also possible meta-networks composed of multiple gene ontology categories and oligodendrocyte dysfunction were recognized. Differential expression analysis recognized hub genes highly correlated with NCI, including genes implicated in gliosis, inflammation, and dopaminergic tone—which are some of the fundamental neuropathologic processes in HAND. Interestingly, further genetic analysis showed downregulation of genes involved in mitochondrial functioning. The authors concluded that WGCNA characterized dysregulated networks associated with NCI, including oligodendrocyte and mitochondrial functioning. The findings from the study pointed to the common gene networks dysregulated in relation to NCI in AD and HIV included mitochondrial genes, whereas upregulation of various cancer-related genes was found.

In a recent study by Yelamanchili and colleagues [5], the investigators studied the role of microRNAs (miRNAs) in regulating the neurodegenerative process of HIV-associated dementia in humans and monkeys. The authors found that the expression of miR-21, a particular miRNA with link to oncogenesis, was significantly increased in the brain of AIDS patients, and its expression was inducted in neurons. Such induction of miR-21 in neurons was executed by prolonged stimulation of NMDA receptor and caused neuronal dysfunction including decreased expression of myocyte enhancer factor 2C (MEF2C) (which serves as a transcription factor for neuronal function).

In another study with focus on an animal model for NeuroAIDS, Repunte-Canonigo et al. [6] published an original paper about alterations of gene expression in HIV-1 transgenic rats with NeuroAIDS and impaired working memory. The investigators utilized the gene set enrichment analysis (GSEA) algorithm in order to assess and report the results of gene expression profiling and alterations in their expression in the hippocampus cells of HIV-1 Tg rats. In this particular animal model of AIDS, several HIV-1 proteins are expressed under the control of viral LTR promoter in disease-relevant cells such as microglia and astrocytes.

They detected alterations in gene expression supportive of microgliosis and astrogliosis, which were indicative of the presence of inflammatory process and cellular proliferation. Among various genes whose expression was upregulated in the course of infection in HIV-1 Tg rats, they detected upregulation of the interferon-stimulated gene 15 (ISG-15). The ISG-15 was formerly demonstrated to be upregulated in the cerebrospinal fluid of HIV-seropositive patients, and it revealed correspondence with neuropsychology decline and neuropathology. The other gene whose expression was upregulated was prostaglandin D2 synthase (Ptgds), which was associated with acti-

vation of the immune system and development of astrogliosis and microgliosis. In addition, utilizing the GSEA-based pathway analysis, the researchers detected dysregulation of certain genes involved in neuronal tropism and neurodegenerative diseases. Some of these dysregulated genes were IGF, ErbB, and netrin signaling and the PI3K signal transduction pathway. While these gene expression alterations were captured, the infected HIV-1 Tg rats demonstrated decline of the working memory in spontaneous alternation behavior in the T-Maze—a sensitive paradigm to assess the activity of the prefrontal cortex and hippocampi. The investigators concluded that use of gene expression in order to identify which specific pathways are activated in the pathogenesis of HIV-1 infection can assist us to develop more focused treatments to enhance trophic support and decrease the inflammation.

Siangphoe and Archer [7] performed a meta-analysis of gene expression in the context of HAND in order to identify which gene is expressed differentially in patients with HAND versus those patients with HIV infection and without HAND and controls [7]. Their analysis focused on the publicly available gene expression information obtained from HIV postmortem brain tissue examinations. They utilized meta-analytic methods including combining P values and combining effect sizes with and without permutation method. They analyzed three studies, which included 48 postmortem brains from 25 patients with HAND, 7 patients with HIV encephalitis, 8 HIV-infected patients, and 8 controls. A total of 411 genes were differentially expressed in patients with HAND and HIVE when they were compared with the control subjects. Of the 411 differentially expressed genes, 94 genes were significantly expressed, and these genes were involved in a number of important pathogenic pathways such as immune system activity, interferon response, or antigen presentation. Of the 94 differentially expressed genes, again 66 genes were heavily upregulated with $\log_2$ intensities larger than twofold. Some of these strongly upregulated differentially expressed genes included PSBM8-AS1, APOL6, TRIM69, PSME1, CTSB, HLA-E, GPNMB, UBE2L6, PSME2, NET1, CAPG, B2M, PRL38, GBP1, and PLSCR1. They also detected that only BTN3A2 was expressed in HAND and HIV encephalitis patients when they were compared with those patients with HAND and without HIV encephalitis.

20.3 Astrocytes: Their Role in HAND and Gene Expression Studies

Astrocytes are the most common cell type within the CNS environment and perform a crucial role in homeostasis, activity, and survival of other cells. They play a supportive role as well as maintain function in preserving an intact blood-brain barrier (BBB). Commonly, and in the context of various neuropathologies, such as HIV-1 infection of the brain, astrocytes are targeted by immune mediators such as TNF-α, and the end result of such activity is astrogliosis—a commonly observed neuropathologic phenomenon resulting from viral infection invasions, including HAND, and discussed in more detail below. Astrocytes promote the neuropathogenesis of

HAND by altering the CNS environment and by releasing pro-inflammatory cytokines. Recent scientific observations indicate that apart from these mechanisms, astrocytic dysfunction leads to dysregulation of extracellular K+ homeostasis and intracellular calcium level as well as glutamate clearance.

Scientific experiments indicate that activation of the astrocytes translates into upregulation of the expression of several proteins by these cells, including cytokines, growth factors, and extracellular matrix proteins, as the astrocyte's defense response mechanism includes rapid activation of such cellular expression [8]. The human immunodeficiency virus (HIV) only infrequently infects and damages neurons directly but most commonly manifests through other indirect pathogenic mechanisms. However, astrocytes, under the effect of the activated immune system, also act as mediators of HIV-1-induced neuronal damage, and through this, they are believed to play a crucial role in the neuropathogenesis of HAND [9–11]. Indeed, during the pathogenesis of HAND, astrocytes are both activated and dysfunctional. Abnormalities of this astrocytic activity in the context of HAND consist of retraction of the foot processes from the blood-brain barrier (BBB) resulting from the astrocytic release of pro-inflammatory cytokines as the astrocytes begin to activate the central nervous system on a microscopic level. These overexpressed and active astrocytes, which have been exposed to the HIV protein resulting in Tat-mediated brain lesions, may demonstrate elevated TLR2 responsiveness to subsequent activation [12]. In addition, priming of astrocytes with Tat protein stimulates a decline in astrocyte expression of TLR9, which is necessary for suppression of viral infection, and stimulates increased glutamate expression, and as a result this increases neuronal excitotoxicity (39,7) [12–14]. Thus, structural changes in astrocyte morphology could result in impaired function [13, 14] causing neuronal dysfunction through excitotoxicity [15, 16], homeostatic imbalances [17], and damage to synapses as the abnormally functioning neurons attempt to send and receive messages [18].

Prominent neuropathologic features of HAND include the development of multinucleated giant cells, myelin pallor, and astrogliosis, as well as neuronal and glial cell loss [19]. A commonly occurring neuropathological alteration is astrogliosis, which refers to an abnormal increase in the number and size of astrocytes resulting from a defense response to the CNS damage induced by HIV-1. Astrogliosis is characterized by hypertrophy of the cells and their proliferation, as well as increased changes in molecular expression and elevated expression of glial fibrillary acidic protein (GFAP) [20]. HIV-1 infection of the astrocytes has been detected in the context of HIV-1 encephalopathy, and the infective process is limited only by both the virus entrance into the cells and post entry viral gene expression. In addition, the HIV-1 viral infection, the HIV-1 proteins (such as HIV-1 Tat), and the macrophages products in HIV-infected individuals can initiate astrogliosis. These findings, collectively, reveal astrogliosis as a salient feature of HIV-1 neuropathology and extenuate the potentially pivotal role of astrocyte dysfunction in the pathogenesis of HAND [19, 20].

GFAP, a type III intermediate filament protein present in the main processes and the soma of astrocytes, is involved in cell communication, cell migration, mitosis, and cytoskeletal alterations [21]. As a marker of astrogliosis, the expression of GFAP is upregulated in the pathogenesis of HIV-1 infection [17, 18]. Upregulation of GFAP expression has been attributed to multiplication of astrocytes through the

process of astrogliosis, and astrogliosis, in turn, has been connected to the productive infection of CNS and the cognitive disorders in patients with HAND. More interestingly, it has also been demonstrated that intracellular expression of HIV-1 Tat protein in astrocytes is associated with the development of astrocytosis recognized by elevated expression of GFAP, astrocyte dysfunction, and neuronal death [18]. Apart from these pathological alterations, it has also been shown that HIV-1-infected and HIV-1-activated astrocytes, along with activated microglia, participate in the process of neuronal loss as well as in the advancement of HAND-related dysfunctions by serving as inflammatory mediators for the infection [22].

During pathogenesis of HAND, the activated astrocytes show upregulation of expression of certain neurotoxic agents such as excitatory amino acids (glutamate) as well as TNF-α [15]. HIV-1 gp120 increases the expression of nuclear factor erythroid derived 2-related factor (Nrf2), which participates in regulation of antioxidant defensive mechanism [23]. Apart from this, HIV gp120 induces the expression of IL-6 [24], IL-8 [25], and CCL5 in astrocytes [26] via a nuclear-kappa β-dependent pathway.

Borjabad et al. [27] assessed the gene expression profile in HIV-1-infected glia and HIV-1-infected brain tissues. The investigators hypothesized that chronic exposure of versatile cells such as astrocytes to HIV-1 and viral pathogenic proteins leads to dysfunctional gene expression in astrocytes. They utilized functional genomics to assess such effects on astrocytes in vitro. In addition, they exposed cultured mouse astrocytes to HIV-1 and used microarray to evaluate their pattern of gene expression. The authors detected that multiple common gene regulatory alterations may be involved in responses of these cells to HIV-1 and its pathogenic proteins. In addition, they performed comparison between the transcriptional profiles of astrocytes and those obtained in analyses of the brain tissues of patients with HIV-1 dementia and macaques infected with simian immunodeficiency virus (SIV). Interestingly, several of the gene features of responses to HIV-1 in cultured astrocytes were also changed in HIV-1- or SIV-infected brains. This in-depth gene expression study further supported the significance of gene expression studies in exploring the pathogenic mechanisms of HAND.

Jayadev et al. [28] studied the astrocytic response to CNS HIV infection in patients with HIV-associated dementia. More specifically, they assessed the role of transcription p53 and its extent and cell type specificity in subcortical white matter of ten AIDS patients that had previously been shown to demonstrate white matter p53 accumulation. To determine if p53 activation functioned to alter gene expression in HIV-associated dementia, the investigators also immunolabeled cortical tissue sections for the p53 target genes Bax and p21 (WAF1). They looked at three different cell groups—microglia, astrocytes, and oligodendrocytes—and detected that they showed p53 activation in response to HIV infection. In addition, immunoreactivity for both Bax and p21(WAF1) in neurons and glia from patients demonstrating elevated p53 immunoreactivity was detected. The results of this study reveal the extensive elevated P53 expression in patients with HIV-associated dementia. The authors concluded that triggering of p53-mediated pathways within the astrocytes might contribute to the neurodegenerative process of HIV-associated dementia by suppressing astrocytic proliferation and promoting their dysfunction.

Atluri et al. [29], in a comprehensive study, assessed the differential expression profiles of 84 human synaptic plasticity genes in clade B and clade C infected primary human astrocytes, utilizing RT [2] Profile PCR Array human Synaptic Plasticity kit. The investigators searched for an increase of decrease in expression of these key human synaptic genes, and the results of their research indicated that among these 84 candidate genes, the differential expression of 31 and 21 genes was considerably increased and the expression of 5 genes was decreased in clade B and clade C infected cells, respectively, when they were compared to control astrocyte cells. Utilizing flow cytometry analysis, the investigators confirmed that expression of postsynaptic density and dendrite spine morphology regulator proteins (ARC, NMDAR1, and GRM1) in both clade B and clade C infected primary human astrocytes and SK-N-MC neuroblastoma cells were decreased as they were compared with uninfected and clade C infected cells . In addition, in clade B infected astrocytes, induction of apoptosis was prominently greater than in the clade C infected astrocytes. The authors concluded that based on the findings of their study, decreased expression of synaptic plasticity genes, lowered dendritic spine density, and induction of apoptosis in astrocytes may participate to severe neuropathogenesis of clade B infection.

20.4 Role of Oligodendrocyte/Myelin Complex in HAND

The oligodendrocytes, which produce CNS myelin, are the other less recognized and less researched cellular components of HIV infection of human CNS. One salient neuropathologic feature of the brain of AIDS patient is myelin pallor, which partly stems from HIV-induced vasculopathy and partially comes from damage to and loss of the oligodendrocyte/myelin complex. Potential transmission of HIV-1 strains from HIV-1-infected microglia to oligodendrocytes and HIV-1 infection of cultured human adult oligodendrocytes has been demonstrated previously [30]. Since oligodendrocytes do not express CD4 on their surface, many researchers do not recognize them as ideal targets for the HIV infection and raise the fundamental question as “by what mechanism(s) these cells become infected?” The studies on HIV infection of oligodendrocytes by HIV are contradictory, and certain scientific observations demonstrated the presence of HIV nucleic acid in these cells utilizing PCR [31, 32], while another study is facile to find indicators of HIV infection [33]. Despite these contradicting reports, oligodendrocytes should be regarded as another significant player in neuropathogenesis of HAND. Infection of these cells and their dysfunction comprises myelin production and presents as “myelin pallor” in histopathologic examinations. More than two decades earlier and utilizing immunocytochemical reactions specific for oligodendrocytes, Esiri and colleague [34] studied the destiny of oligodendrocytes in the brain tissue specimens obtained from HIV-1-infected patients and discovered that in individuals with HIV-1 infection, the myelin injury was initially associated with an increase in the number and hyperplasia of oligodendrocytes. In all cases, HIV-1 p24 antigen was detected. The authors included that during HIV-1

infection of the brain, the oligodendrocytes demonstrate a preliminary reactive hyperplasia in order to repair the myelin injury.

In another cross-sectional study which focused on the role of oligodendrocytes in development of HAND, Lackner et al. [35] explored the role of antibodies against myelin oligodendrocyte glycoprotein (MOG), in pathogenesis of HAND. Using ELISA, the investigators measured levels of CSF and serum levels of anti-MOG antibodies in 65 HIV-seropositive patients with HAND (*N = 14*), cerebral opportunistic infections (*N* = 25), primary HIV infection (*N* = 5), and asymptomatic patients *(N = 21*). They also included two other control groups, HIV-seronegative patients with viral or bacterial infection (*N = 18*) and patients with other neurological diseases (*N = 22*). The antibody measurements in patients with HAND were done prior and during antiretroviral therapy. The investigators detected significantly higher anti-MOG antibody levels in patient with HAND compared to those with other neurological diseases. In addition, they measured significantly higher CSF levels of anti-MOG antibodies in patients with HAND compared to those HIV-seropositive patients and the HIV patients with cerebral opportunistic infections compared to the asymptomatic patients and the patients with other neurological diseases. Based on the findings of this scientific study, CSF anti-MOG antibodies demonstrated high sensitivity and specificity for separating patients with active HAND from those with clinically asymptomatic HIV infection. In addition, those anti-MOG seropositive patients performed worse on HIV dementia scale and possessed higher CSF viral loads. The authors conclude that the perseverance of these antibodies (in the CSF and not in the plasma) despite viral clearance in HAND patients indicate ongoing neuroinflammation separate from the systemic immune cascade of AIDS. The findings of this scientific observation also point further to involvement of oligodendrocytes in pathogenesis of HAND. HIV-1-infected astrocytes release neurotoxic molecules such as glutamate and TNF-α [15]. In addition, viral gp120 increases the expression of Nrf2 and induces expression of IL-6 [24] (Shah 2001), IL-8 [25], and CCL5 in astrocytes [26]. The induction of IL-6, IL-8, and CCL5 by astrocytes by gp120 is medicated via a nuclear-kappa β-dependent pathway.

Utilizing gene expression studies, a number of published scientific observations have addressed the concept of gene expression and alterations of gene expression by oligodendrocytes in the context of HAND pathogenesis.

Chadhuri et al. [36] focused on the role of aberrant expression of microRNAs (miRNAs) in pathogenesis of HAND and upregulation of miRNAs in neurons and myeloid cells in the brain. The investigators assessed the expression of miRNAs in neuronal cells by comparing gene expression in stable clones of the human neuroblastoma cell line BE-M17 [2] expressing miR-142 versus controls. The investigators utilized microarray analysis for their assessment. The results indicated that miR-142 expression led to a reduction in monoamine oxidase (MAO) A mRNA. The results were validated by qRT-PCR. In addition to the mRNA, the MAOA protein level and enzyme activity had decreased. Based on their evaluation of the primary human neurons, they noticed that miR-142 expression led to a downregulation of MAOA protein level. Although MAOA is not a direct target of miR-142, SIRT1, a key transcriptional upregulator of MAOA, is the target, thus, miR-142 downregula-

tion of MAOA expression in indirect. MiR-142 induced decrease in MAOA expression, and activity may contribute to the changes in dopaminergic neurotransmission reported in HAND.

In another set of experiments, Zou and colleagues [37] focused on oligodendrocytes as potential targets for HIV-1 Tat protein. The investigators hypothesized that activation of NMDARs and consequent disruption of the cytoplasmic Ca2+ homeostasis may be the underlying mechanism of oligodendrocyte-myelin complex abnormality and dysregulated expression of the myelin sheath. Based on their observation, in culture environment, HIV-1 transactivator of transcription (Tat) protein caused concentration-dependent demise of immature oligodendrocytes, and the more mature oligodendrocytes demonstrated decreased myelin-like membranes. Additionally, Tat-induced [Ca(2+)] increases and Thr-287 autophosphorylation of Ca2+/calmodulin-dependent protein kinase II β (CaMKIIβ) in oligodendrocytes. Tat-induced disruption of Ca2+ homeostasis was lessened by the effect of NMDAR antagonist MK801 and also by the effect of AMPA/kainate receptor antagonist CNQX. Significantly, both of these antagonists blocked Tat-induced death of immature oligodendrocytes; however, only MK801 reversed Tat effects on myelin-like membranes. These results suggest that OLs can be direct targets of HIV proteins released from infected cells. The investigators concluded that effects of AMPARs and NMDARs were dissimilar and dependent on the stage of oligodendrocyte differentiation. A summary of all the gene expression-based studies is presented in Table 20.2.

Table 20.2 A summary of gene expression results from studies on HAND

Author/year	Examined tissue/cells	Results
Jayadev et al. 2007 [28]	Astrocytic response to CNS HIV infection in patients with HIV-associated dementia with focus on transcription p53 Three different cell groups—microglia, astrocytes, and oligodendrocytes were examined	Extensive p53 activation and expression in response to HIV infection in the three cell groups Immunoreactivity for both Bax and p21(WAF1) in neurons and glia from patients, demonstrating elevated p53 immunoreactivity
Borjabad et al. [27]	Using microarray, the authors studied gene expression profiles in HIV-1-infected glia and brain tissues Comparison of the transcriptional profiles of astrocytes with human astrocytes from HIV-1 patients was performed	Several of the gene features of responses to HIV-1 in cultured astrocytes were also changed in HIV-1- or SIV-infected brains
Yelamanchili et al. 2010 [5]	Role of microRNAs in pathogenesis of HIV-associated dementia in humans and monkeys	Significant elevation of expression of miR-21 in neurons along with decreased expression of monocyte enhancer factor 2C

(continued)

Table 20.2 (continued)

Author/year	Examined tissue/cells	Results
Atluri et al. 2013 [29]	Differential expression profiles of 84 human synaptic plasticity genes in clade B and clade C infected primary human astrocytes	Differential expression of 31 and 21 genes was considerably increased, and the expression of 5 genes was decreased in clade B and clade C infected cells Expression of postsynaptic density and dendrite spine morphology regulator proteins (ARC, NMDAR1, and GRM1) in both clade B and clade C infected primary human astrocytes and SK-N-MC neuroblastoma cells were decreased as compared with uninfected and clade C infected cells
Levine et al. 2013 [4]	The brain tissue from HAND (with and without HIV encephalitis) and Alzheimer's disease patients, utilizing weighted gene co-expression network analysis and standard expression analysis	Multiple meta-networks composed of multiple gene ontology categories and oligodendrocyte dysfunction were identified Hub genes related to gliosis, inflammation, and dopaminergic tone were identified
Repunte-Canonigo et al. 2014 [6]	Study of gene expression in HIV-1 transgenic rats with neuroAIDS and impaired working memory Gene expression profiling and alteration were done on hippocampus cells	Several HIV-1 proteins were expressed under the control of viral LTR promoter in microglia and astrocytes Upregulation of prostaglandin D2 synthase with dysregulation of genes involved in expression of IGF, ErbB, and netrin was detected
Chadhuri et al. [36]	Role of aberrant expression of microRNAs (miRNAs) in pathogenesis of HAND and upregulation of miRNAs in neurons and myeloid cells in the brain	miR-142 expression led to a reduction in monoamine oxidase (MAO) A mRNA
Siangphoe and Archer 2015 [7]	Meta-analysis of gene expression in the context of HAND with HIV postmortem brain tissue examinations	Of the 411 differentially expressed genes, 94 genes were significantly expressed Some of these strongly upregulated differentially expressed genes included PSBM8-AS1, APOL6, TRIM69, PSME1, CTSB, HLA-E, GPNMB, UBE2L6, PSME2, NET1, CAPG, B2M, PRL38, GBP1, and PLSCR1 Other findings: BTN3A2 was expressed in HAND and HIV encephalitis patients
Zou and colleagues 2015 [37]	Immature and mature oligodendrocytes were studied in the culture environment under the effect of HIV-1 Tat	Tat concentration-dependent demise of immature oligodendrocytes, with the more mature oligodendrocytes demonstrated decreased myelin-like membranes Tat-induced [Ca(2+)] increases and Thr-287 autophosphorylation of Ca2+/calmodulin-dependent protein kinase II β (CaMKIIβ) in oligodendrocytes

20.5 Conclusions

Utilization of modern molecular biology techniques with significant emphasis of gene, RNA, and protein expression has permanently and fundamentally altered the way we study NeuroAIDS, and the clinical subjective descriptions are being replaced by meticulous reports of the basic pathogenic mechanisms of NeuroAIDS and HAND. As a result, more effective and safer medications with narrower field of effect and specific targets are being developed, which in turn enhance the life span of AIDS patients.

Conflict of interest The authors report no conflicts of interest.

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Chapter 21
Neuronal Apoptotic Pathways in HIV-Associated Dementia, Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease

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Keywords Apoptosis • Dementia • HIV associated dementia • Alzheimer's disease • Parkinson's disease • Huntington's disease • Brain • Neurons • Genes • Signaling • Molecular biology • Gene therapy • Evidence-based • Translational healthcare

Core Message

HIV-associated dementia is analyzed in terms of neuronal apoptosis genes and signaling pathways. Contemporary advances in molecular biology and gene therapy are mentioned as well.

Alzheimer's disease, Parkinson's disease, and Huntington's disease are discussed for comparison.

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21.1 Introduction

Dementia is one of the most devastating healthcare problems in the twenty-first century. Dementia is defined as an acquired, progressive loss of several intellectual functions with no delirium. The cognitive impairment affects patients' behaviors and attitudes with persistent increase in dependency on others for activities of daily living and correspondingly increased expense of care. The number of people affected by dementia of all types is increasing annually with associated healthcare costs [1].

Human immunodeficiency virus-associated dementia (HAD) is one of the most important consequences of HIV infection. Despite antiretroviral therapy, the rate of HIV-associated neurocognitive disorders has not decreased and may be rising [2]. The pathogenesis of HAD has been under investigation for several decades, and activation of apoptotic pathways is among its proposed mechanisms [3].

This chapter reviews evidence regarding the role of apoptosis involvement in the pathogenesis of HAD. Furthermore, this chapter reviews evidence for the role of apoptosis in Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). This chapter briefly summarizes apoptosis pathways that may be common to the pathophysiology of these dementias.

21.2 Two Mechanisms of Neuronal Cell Death

There are primarily two different patterns of neuronal cell death: (1) apoptosis, intracellular or self-controlled programmed cell death, and (2) necrosis, extrinsic cell death. This classification is based on the historical difference in the signaling and molecular mechanisms that contribute to cell degradation and death. Classically, there are two pathways that trigger apoptosis: the intrinsic pathway involving factors released by mitochondria that activate an initiator caspase,[1] caspase-9 (CASP9), and the extrinsic pathway involving "death receptors" (i.e., tumor necrosis factor receptors, TNF-Rs, and Fas) that signal pathways for cell death [4]. Apoptosis can

[1] Caspases are a family of protease enzymes.

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occur in non-pathological as well as pathological conditions. In the non-pathological cases, it serves as a gene-directed programmed cell death to terminate aged, unneeded, or damaged cells [5]. This intrinsic process can be initiated by several environmental toxic factors, including viral infection.

Necrosis generally results from an insult that affects neurons with a reaction cascade, including spontaneous cellular and subcellular damage [6]. However, several additional types of necrosis have been identified recently in cell line cultures and in some diseases. These cell death mechanisms include necroptosis (programmed necrosis), feroptosis (implicated in liver and kidney diseases), oxytosis, parthanotos necrosis (implicated in PD), and cyclophilin D-mediated necrosis (implicated in MS) [6]. Molecular mechanisms and pathways in necroptosis may involve key signaling modules such as receptor-interacting kinase (RIP3) as well as its substrate, pseudokinase-mixed lineage kinase domain-like protein (MLKL) [7]. However, the involvement of necroptosis and these additional new pathways is not fully elucidated yet and so is not pursued further in this chapter.

21.3 Apoptosis and Apoptotic Genes in Neurodegenerative Diseases

21.3.1 Neuronal Apoptotic and Necrotic Pathways

The principal phenomenon in the patterns of neurodegeneration in HAD, AD, PD, and HD includes progressive neuronal death, which is generally accompanied by synaptic loss. This process is often associated with specific protein accumulations in the associated brain areas. The best-defined example of this phenomenon is in Alzheimer's dementia, in which death of cortical neurons is accompanied by aggregation of extracellular betaA41 amyloidal deposits that appear in parenchymal plaques and cerebral vessels.

Even though the presence of apoptotic reactions and markers has been supported by research utilizing animal models, tissue cultures, and studies of various neurodegenerative diseases, research involving the human postmortem brain has shown divergent results [5]. First, the detection of purely apoptotic neurons is extremely difficult since neuronal cell death in neurodegenerative diseases persists for numerous years, while neuronal cell death solely from apoptosis occurs within a few hours. Second, models, in which neurons only die from apoptosis, do not adequately explain synaptic loss, which is an essential component of the neurodegenerative phenomenon. Accordingly, apoptosis is not the only route of neuronal death, but other death pathways could participate as well. The multilevel overlapping interactions between the different death signaling pathways could terminate one reaction and promote another to produce the variable neurodegenerative presentations found. Such changes implicate major detrimental effects in the brain, including inducing neuroinflammatory reactions that might be associated with losses of interneuronal synaptic connectivity and initiation of related degenerative processes.

Table 21.1 Biochemical components in the two classical types of neuronal death

	Examples of apoptotic mechanisms	Examples of necrotic mechanisms
Basic description	Intrinsic cell programmed death (could be non-pathological)	Passive extrinsic pathological cell death
Triggers	Radiation, oxidative stress, viruses, toxins, loss of neurotrophic factors, etc.	Stroke, trauma, etc.
Examples of genes	*Apo-1/Fas*: death receptors *Apaf-1*: apoptotic protease-activating factor 1 *AIFs*: apoptosis-initiating factors, mitochondrial-released proteins in early stages of cell death *Caspase/calpain family proteins/ proteases* *Bcl-2 and p53 oncogene families:* Bax, Bid, Bad, Bak, and Bcl-Xs exacerbate apoptosis, and Bcl-2 and Bcl-xL prevent apoptotic reactions *Mitogen-activated protein kinase* pathways regulated by neurotrophins	Excitatory amino acids transmitters Neuronal membrane depolarization Calcium ions influx Increase in oxidative stress Production of ROS FFA increase and upregulation of IIA sPLA2 in reactive astrocytes Chromatin modification
Mechanism	Activation of the primary molecular participants will lead to proteolysis of cell structure that will initiate the death cascade	Activation of the primary molecular participants will lead to blebbing of the cells and eventually death associated with inflammatory phagocytosis
Special markers	*Postmitotic neuronal homeostasis regulators*: downstream-named effector caspases and their products represent the irreversible state of cell death	*Insult*: the main recognized hallmark

Interestingly in this regard, the death of CA1 hippocampal neurons that results from stroke and ischemia in rodents and in humans has been ascribed to either apoptotic or necrotic pathways [8–10]. Moreover, there appears to be a common early c-Jun N-terminal kinase (JNK) pathway that can lead to either apoptosis or necrosis in neurons [11]. In addition, neuronal necrosis may be regulated by a novel conserved chromatin-modifying cascade [12].

Table 21.1 shows a comparison of several biochemical components in the two classical types of neuronal death.

21.3.2 Apoptosis in HIV-Associated Dementia (HAD)

Human immunodeficiency virus type 1 (HIV-1) is a leading cause of progressive dementia. HAD includes the following clinical criteria: cognitive impairment, behavioral impairment, and progressively worsening motor defects. It is estimated that HIV-associated neurocognitive disorder (HAND) affects 25–60% of HIV-infected people worldwide [13]. Possibly, the various differing HIV-1 strains (clades and

mixed clades), the wide and disparate ranges in availability of diagnostic capabilities, and limitations of access to HIV-1 antiretroviral therapy (ART) may underlie the global variability in the rates of HAND criteria.

Although it is questionable as to the extent to which HIV-1 directly infects neurons, HIV infection damages neurons indirectly through the HIV-1-encoded proteins injuring the neuronal cells [14] and indirectly through the released neurotoxins from viral-infected macrophages and microglia mediating neuronal apoptosis. Apoptosis was initially detected in neurons in HIV encephalitis (HIVE) brain by CK Petito and colleagues [15] with extensive subsequent confirmatory observations [16, 17].

Additionally, HIV-infected glial cells are believed to be an important component of HAD neuronal apoptosis. The release of cytokines and chemokines, in the presence or absence of viral protein neurotoxins, can indicate the activation of the glial cells in HIV-positive patients. A clinical trial investigated the possibility of detecting the glial cell activation in vivo through tracing [11C]-R-PK11195 using positron emission tomography (PET) [18]. In this regard, the peripheral benzodiazepine receptor (PBR) is associated with the selective radioligand [11C]-R-PK11195 that is known to indicate the range of glial cell activity. The results indicate a glial cell activation role in HAD and support its use for early detection of neuropathology in HIV infection.

The presence of HIV-1 regulatory protein Vpr in the serum of HIV-positive individuals and in the cerebrospinal fluid of the acquired immunodeficiency syndrome (AIDS) patients showing neurological symptoms supports a role for this protein in HAD. Vpr has also been demonstrated in postmortem brain tissues of the AIDS patients with severe HAD symptoms. Consequently, the effect of added Vpr was examined in primary cultures of rat cortical and striatal neurons. Vpr induced apoptotic reactions involving caspase-3 activation, upon 3 h of extracellular exposure, and peaked after 3 h [19]. Additionally, added Vpr was associated with caspase-8 activation [17].

HIV-1 proteins gp120 and Tat can initiate neuronal cell apoptosis and excitotoxicity due to oxidative stress, perturbation of cellular calcium homeostasis, and by affecting neuronal mitochondria (Mattson et al. 2005). These effects occur through direct binding or indirect activation of cell surface receptors such as CXCR4 and N-methyl-D-aspartate (NMDA) receptor (NMDAR). This work supported a role of cell cycle protein regulation through chemokine receptors in HAD pathogenesis. The CDK/Rb/E2F-1 pathway, which participates in programmed neuronal cell death and differentiation, was studied in relation to the chemokine effect [20].

Cultures of differentiated rat neurons (cerebellum and hippocampus neurons where CXCR4 physiologically relevant) were used to indicate if CXCR4, the receptor for SDF-1 and X4-using gp120s, can regulate Rb and E2F-1 activity. The changes in the Rb levels in brain cells, nuclei, and cytoplasm led to loss of its function and initiation of apoptotic reaction. The CDK/Rb/E2F-1 pathway is affected by SDF-1 alpha and gp120. Added to CCR3- and CCR5-mediating roles in HIV-1-infected microglia, CXCR4 has an important role in the apoptotic steps involved in HAD [21].

The NF-KappaB pathway normally has a protective function for neurons. However, an inhibitory effect by HIV-1 infection of human macrophages was dem-

onstrated on NF-KappaB signaling pathways through HIV-1 Tat, which activates glycogen synthase kinase (GSK)-3beta in neurons. The activated GSK-3beta modifies the NF-KappaB subunit RelA at serine 468, controlling RelA interaction with histone deacetylase-3 corepressor molecules. Eliminating the effect of Tat through neutralization or inhibiting the GSK-3beta effect on the neurons led to termination of neuronal apoptosis [22].

Another effect of CNS HIV infection is astrocyte activation. A study published in 2006 focused on the role of ezrin (the actin-binding protein) immunoreactivity (IR) in activated astrocyte on *postmortem* autopsied brain tissues [23]. In this study, semiquantitative levels of ezrin-IR were evaluated as well as glial marker, GFAP, ferritin, and HLA-DR, and these were related to HIV encephalopathy clinical and morphological criteria. The results revealed a marked increase in GFAP, HLA-DR, and ferritin-IR levels in all HIV case neurons compared to neurons from normal controls. Ezrin-IR was specifically increased only in HIV cases accompanied by encephalopathy. HIV encephalopathy cases with high ezrin-IR levels were found to be associated with neuronal apoptosis (using the TUNEL assay). It was concluded that there was a significant correlation between the levels of ezrin-IR and GFAP-IR. Normal ezrin-IR combined with elevated GFAP-IR indicated astroglial activation and was not accompanied by neuronal apoptosis, whereas high ezrin-IR indicated neuronal damage and apoptotic reactions.

TNF-related apoptosis-inducing ligand (TRAIL), one of the TNF family of pro-inflammatory cytokines (also referred to as CD253 or TNFSF10), may also play a role in HAD-related neuronal cell death [24]. TRAIL is not normally present in the brain cells, but it can be induced because of the immune response of macrophage and microglia activation, which are major components in the HAD reactions. Additionally, TRAIL is induced in brain cells by beta-amyloid protein, which may be present in several brain diseases. Accordingly, TRAIL is suggested to play a significant role in several neurodegenerative diseases [25]. The mechanism of its apoptotic effect can be directly through TRAIL neuronal receptors or indirectly through the release of neurotoxins. Another investigation showed a different aspect of cytokine receptor activation, oxidative stress, and apoptosis, which included production of ceramide from membrane sphingomyelin and may have a role in HAD pathogenesis [26].

One of the earliest observations of stimulation of HIV replication due to abused drugs such as opiates and cocaine came from the Peterson laboratory [27, 28]. The involvement of apoptosis was implicated as well for opioid and cocaine in concert with HIV-1 [29]. Several subsequent studies examined the neurotoxic relationship between HIV and drug abuse. For example, ethanol, cocaine, amphetamine, and opiates appear to exacerbate the neurotoxic and apoptotic effects of HIV-1 proteins including gp120 [30, 31]. Chen's 2005 study elucidated the role of ethanol as a potentiating factor in neuronal HIV-1 gp120-induced apoptosis through the extrinsic pathway (death receptors) and the intrinsic pathway (NMDAR) [32]. Human gene expression profiles indicated apoptotic pathways that were associated with HIV-1 and ethanol-related neuronal damage. There was increased expression of

specific apoptotic functional genes, including TRAF5, which potentiates the HIV-1 gp120 apoptotic effect.

In related gene expression studies, exposure of cultured neurons to cocaine, gp120, and tat (separately and in combination) results in perturbation in expression of 35 genes including MAP-2 and KREMEN2. Changes in MAP-2 and KREMEN2 gene expression could disrupt wnt/β-catenin expression and function. If so, it was hypothesized that this could promote neuronal apoptosis associated with subsequent hyper-phosphorylation of MAP-tau and neurofibrillary tangle (NTF) formation [33]. In support of this pathway, it should be noted that siRNAs specific for wnt-1 rapidly induced apoptosis in other studies [34].

The addition of cocaine also increased gp120-induced apoptosis in rat brains, even at doses of cocaine that did not independently cause apoptosis. Nitric oxide (NO) production was associated with neuronal apoptosis. Inhibitors of NMDARs and iNOS decreased apoptosis in the presence of cocaine in conjunction with gp120 [35]. Other studies suggested that cocaine increases neuronal apoptosis by potentiating cathepsin B secretion from HIV-infected macrophages [36]. Furthermore, HIV-infected temporal cortex dissected from postmortem brain tissue showed increased numbers of dystrophic neurons containing elevated neuronal-NOS expression compared to HIV-negative controls [37].

Gp120 and cocaine exposure of rat primary neurons showed increased toxicity compared to either exposure alone. Apoptosis-promoting proteins caspase-3 and Bax in concert with intracellular oxygen species were associated with neuronal death. Signaling pathways implicated included nuclear factor (NF)-κB, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinases (MAPK), p38, and c-Jun N-terminal kinase (JNK). MAPK signal, mitochondrial, and oxidative stress pathways thus are components in HIV and cocaine neurotoxicity [38].

Briefly and germane, opiates may indirectly increase neuronal damage in HIV infection via their additional effects on autophagy due to HIV-infected microglia. HIV-1-infected microglia showed evidence of autophagosome formation without protein degradation by lysosomes; this is thought to be an essential mechanism to control viral replication and inflammatory responses in microglia. Exposure to morphine disrupted autophagosome formation in HIV-infected microglia [39].

21.3.3 Apoptosis in Alzheimer's Disease

Alzheimer's dementia is the most common form of dementia, accounting for 60–80% of all dementia cases. The gradual course of the disease and its tremendous impact on the patients as well as their families and the society at large are the main drives for the intense research efforts. Composed of deterioration of cognition and memory, AD is associated with the presence of plaques, neurofibrillary tangles, massive loss of neurons, and gliosis. In addition, there is a common characteristic of apoptotic cell death found in neurons, due to intensive oxidative stress. Additionally,

it has been proposed that the mixture of both apoptosis and necrosis may contribute to the neuronal degeneration found in AD [40–43].

Progressive neuronal cell death causes loss of brain volume and synapses with reduced connectivity (dendrite arborization) between neurons. The major characteristic, though not specific to Alzheimer's histopathology, is the extracellular β-amyloid protein (Aβ) and intracellular hyper-phosphorylated tau proteins accumulated as paired helical filaments (PHF) present in neurons as neurofibrillary tangles (NFT), in dendrites (neuropil threads) and around plaques (neuritic plaques). The amyloid cascade hypothesis has become the central position for modeling the general etiopathogenesis for AD. The clinical diagnosis of AD by the use of biomarkers is not confirmed until these pathological data are obtained through postmortem examination of the brain tissues, as much more research is needed to identify the most accurate biomarkers and to confirm their utility [44–46].

DNA microarray analysis of neurons undergoing apoptosis has been used to characterize the genomic control of apoptosis in cerebellar neurons as a model in vitro [47]. To understand the genomic control of apoptosis, Cavallaro and colleagues [47] used DNA microarray analysis of apoptotic cerebral granule cells (CGC) to investigate the signal transduction mechanisms involved in neuronal apoptosis. They utilized cerebellar neurons cultured in serum with elevated extracellular potassium concentrations, in vitro, and induced apoptosis by removing the serum and lowering the extracellular potassium from 25 to 5 mM. This caused rapid apoptosis in 50% of the granule cells within 24 h. This pattern of apoptosis mimics the natural process that takes place in the infant's third to fifth week of life in utero at which time, 20–30% of the Purkinje granule cells die. Microarray analysis of these cells through the precommitment period of apoptosis was used to identify the genome-wide expression profile of the basic genes controlling the molecular mechanisms in this Purkinje cell death apoptotic mechanism. The following Table 21.2 summarizes key genes categorized in functional clusters of co-regulated genes and pathways, illustrating the complexity of such apoptotic mechanisms.

Out of 8740 genes detected by the microarrays, 423 genes were markedly affected. Two hundred ninety-five genes of the 423 were categorized in relation to their final products into functional clusters.

Mitochondrial dysfunction is proposed as another pathway leading to AD. Nakamura and colleagues suggest that the Aβ protein may augment the level of mitochondrial fission in AD patients [48]. Bioenergetic impairment caused by mitochondrial impairment may eventually lead to synaptic damage and neuronal loss.

Research on inherited Alzheimer's disease (AD), familial AD (FAD), is associated with mutation in the amyloid precursor protein (APP) causing neuronal apoptosis and DNA synthesis [49]. The investigation of the mechanisms of producing the previous effects (DNA synthesis and apoptosis) indicates that abnormal activation of a signaling pathway regulated by APP, p21-activated kinase 3 (PAK3), and G-zero (G_o) protein is triggered by FAD interaction site mutations in the aberrant APP gene. The study estimated the time needed for the apoptotic cascade completion to be 16–24 h, suggesting loss of the neurons exposed to the offensive stimuli during that time. Surprisingly, that was not the case since only 30% of the exposed

Table 21.2 DNA microarray analysis of apoptotic cerebral granule cells was used to analyze genomic control of apoptosis

Signal transduction	Thyroid hormone (TH)	Apoptosis regulation	Cell to cell contact	Metabolism	Transcription or translation regulation
Eicosanoid metabolism: increased expression of GSH-dependent and GSH-independent prostaglandin (PG) D-synthases and PG F2 receptor negative regulator after apoptosis induction	Decreased TH after complete development of the brain is associated with apoptosis. The thyroxin deiodinase TD has three members that catalyze the intracellular TH. Decreased TD-1 and increased TD-III have been observed in apoptotic CGC	Differential expression of Bcl-X, caspase 3, caplain, and harakiri (neuronal death protein: DP5)	Increased MEGF1/ FAT2. Decreased major histocompatibility complex MHC as RT1-B alpha and 1b	Fatty acid metabolism: increased expression of eight enzymes: 2,4-dienoyl CoA reductase 1, acetyl-CoA acyl-transferase 2, carnitine palmitoyltransferase 1 alpha, 1 beta, and 2; delta3, delta2-enoyl-CoA isomerase; acyl-CoA thioesterase 1; fatty acid CoA-ligase; acyl-CoA dehydrogenase	Chromosomal structure: differential expression of HI-0, H2b, and H4 histones associated with chromatin structure. Increased expression of high mobility group box (HMBG)1 and 2 genes
G protein-coupled receptors: decreased expression of metabotropic glutamate receptor (mGluR) 7 leading to reduction of the survival efforts				Protein folding: increased expression of all genes associated with protein folding	Transcription factors: degreased expression of c-fos and increased expression of c-jun, jun D, c/ebp gamma, and early growth response d1 (Egr1 also called NGFI-A, Krox24, or zif/268)
Increased expression of cannabinoid receptor 1 reflecting an apoptotic activation in the neuronal cells				Protein modification: increased expression of ADP-ribosyltransferase and decreased expression of ADP-ribosylarginine hydrolase	Translation regulation: increased expression of eukaryotic translation initiation factor 2 alpha kinase 3

(continued)

Table 21.2 (continued)

Signal transduction	Thyroid hormone (TH)	Apoptosis regulation	Cell to cell contact	Metabolism	Transcription or translation regulation
As part of the apoptotic intracellular signaling: decreased expression of inositol 1,4,5-triphosphate receptor type 1, p38 mitogen-activated protein kinase (MARK), and two MARK phosphatase 1,5. Increased expression of endothelial nitric oxide synthase (NOS)-3 and RhoB				Protein-L-isoaspartate(D-aspartate) decreased expression of enzyme O-methyltransferase	
Differential expression of activin receptor 1 and 2b				Decreased expression of platelet activation factor, PAF	
Differential expression of proteins associated with ion homeostasis: three voltage-dependent Ca2+ channels and ten K+ channels. Potassium efflux has a role in enhancing apoptosis				Amino acid metabolism: increased expression of kynurinase	
Differential expression of ligand-gated ion channels: two ionotropic glutamate receptors, AMPA4 and NMDA2C				Glutathione metabolism: disturbed expression of glutathione S-transferase, glutathione reductase, and NADP-specific isocitrate dehydrogenase	

Increased expression of pentraxin proteins: neuronal pentraxin 1 (NPX1) and neuronal activity-regulated pentraxin (Narp or pentraxin 2)				Proteolysis: increased expression of presenilin 2 gene seen in familial AD and associated with b-amyloidosis	
Secreted peptides and their receptors: decreased expression of parathyroid hormone-related protein (PTHrP)					
Decreased cytokines A20, B13, D1, and growth-regulated oncogene (Gro also called KC)					
Increased expression of transforming growth factor beta-2 (TGFB2)					

As shown in this table, functional gene clusters included signal transduction, thyroid hormone (TH), apoptosis regulation, cell to cell contact, metabolism, and transcription or translation regulation [47]

neurons were affected. Thus, this suggests active compensatory mechanisms are induced to counteract the terminal apoptotic reactions, reflecting the progressive course of the disease and the anticipated intervals for each of its phases. The authors further suggest that FAD mutations in APP and interactions with PAK3 that result in activation of G_o confer cell cycle entry followed by apoptosis. Apoptosis in this model is inhibited or reversed by abrogation of cell cycle entry [49, 50].

In 2002, another study conducted by Uberti and colleagues examined skin fibroblasts cultures collected from eight randomly selected Alzheimer's disease (AD) patients and eight normal controls [51]. The fibroblasts were exposed to acute oxidative injury caused by hydrogen peroxide. The main target was to indicate the ability of the cells to arrest proliferation, to launch an apoptotic reaction, and to measure viability and DNA damage. The results indicated that AD patients' fibroblast resistance to oxidative agent was much higher than those of the controls with almost the same degree of DNA damage in both groups. This protective mechanism in AD patients could be explained by impairment of hydrogen peroxide-induced cell cycle arrest causing an accelerated reentry into the cell cycle and reduced induction of apoptosis. AD patients' fibroblasts showed excessive destruction in the hydrogen peroxide-activated, p53-dependent pathway including p21, GADD45, and bax. These observations suggest significant intracellular pathway modification in AD patients' peripheral cells [51].

The mechanisms of the studies by McPhie et al. and Uberti et al. could be related to each other. Furthermore, the induction of apoptotic inhibitors that decelerate neuronal cell death could be present in both studies. Moreover, the protective mechanisms extant in fibroblasts of the AD patients support a wider state of protection not simply limited to the nervous system.

Wang et al. in current rodent studies find that S-nitrosylation of neuronal proteins occurs. In a rodent model of Alzheimer's disease, they find that VGLUT1 and VAChT transporters are S-nitrosylated in hippocampal neurons, thus explaining the dysfunctional glutamatergic and acetylcholinergic neurotransmission systems in Alzheimer's disease [52].

Recent studies suggest that the loss of the synapses, rather than neuronal loss, is a direct cause of dementia. An alternate hypothesis is called "the attrition hypothesis." This hypothesis attempts to explain aspects of AD pathophysiology in terms of changes in cytoskeletal structure of neurons. The argument of this theory states that stimulation of a primary role of caspase-6 in AD, due to single or multiple insults, causes breakdown of the cytoskeletal structure of neurons. This subsequently negatively affects protein and organelle pathways and organization, leading to the clinical and pathological presentation of AD [53].

An additional novel approach to the problem of apoptosis induction in AD had been taken by the National Institute of Neurological Disorders and Stroke (NINDS) at NIH. They propose a mechanism related to early steps in AD that occur prior to the effects of Abeta that occur later in AD. They propose that early indicators of AD that are associated with olfactory dysfunction may comprise different components of the APP molecule itself at different early stages of AD. They propose that APP-induced apoptosis and neurodegeneration may occur independently of Abeta.

Early neuronal loss is due to APP activation of the intrinsic apoptosis pathway and thus emphasizes the involvement of mitochondrial regulatory factors and cellular stress [54].

It is thus possible that changes among several receptors for components of APP may be involved in the induction of AD.

21.3.4 Apoptosis in Parkinson's Disease

Parkinson's disease (PD) is a clinical disorder that can take many forms caused by dystrophy of the extrapyramidal motor neurons in the cortex as well as degeneration of *substantia nigra* (subcortical neurons) associated with low levels of dopamine (neurotransmitter) [1, 55, 56].

S-nitrosylation of proteins may be a factor contributing to neuronal cell death in PD. NMDAR hyperactivity could lead to an abnormally high level of Ca2+ influx and essentially lead to an elevated level of nitric oxide (NO) and reactive oxygen species (ROS). NO would then induce S-nitrosylation of various proteins, resulting in accumulation of misfolded proteins and Lewy bodies, leading to neuronal injury and cell death. Nakamura and Lipton found that a defect in mitochondrial fission and fusion is an additional factor for Parkinson's disease [48, 57].

As mentioned above, increased NOS expression and NO production are important mechanisms that occur in HAD and AD [35, 37, 52].

Aside from protein misfolding and mitochondrial fission and fusion, missense mutations in several codons in leucine-rich repeat kinase 2 (LRRK2) contributed to apoptosis in PD. An in vitro study conducted by Ho et al. suggested that this occurs via activation of the apoptotic extrinsic pathway [58].

α-Synuclein[2] was also found to play a role in the pathogenesis of PD. Point mutations in α-synuclein have been found in families with the autosomal dominant forms of PD. α-Synuclein is also a component in amyloid in patients with AD as well as in Lewy bodies in patients with PD. Two rodent models have been produced that mimic many components of PD by creating in vivo recombinant DNA. One model is based on the use of recombinant adeno-associated virus (rAAV) to transfer additional copies of the α-synuclein gene into the brain, and another model involves the production of α-synuclein fibrils. These models in the transgenic mice recapitulate PD characteristics including dopamine neuron death, motor defects, inflammation, striatal dopaminergic synapse loss, and formation of α-synuclein inclusions. The gene was overexpressed in the *substantia nigra*, and Parkinson's disease-like symptoms were produced. Moreover, extracellular α-synuclein oligomers have been shown to be toxic in cell cultures, leading to apoptosis in these exposed neurons [59, 60].

It should be noted that many of the animal models use several vectors for DNA transfer into the animals. For example, recombinant lentiviral vectors (rLVs) can

[2] α-Synuclein is a protein component of the protein aggregates that compose Lewy bodies.

transfer DNA into nondividing cells. Thus, rLVs have been used to transfer ciliary neurotrophic factor (CNF) and glial cell line-derived neurotrophic factor (GDNF) into animal models of PD and HD since GDNF and CNF are neuroprotective. In addition, rLVs have been used to transfer inhibitory RNA molecules into animal models for AD and amyotrophic lateral sclerosis (ALS) [61].

21.3.5 Apoptosis in Huntington's Disease

HD is an autosomal dominant genetic disorder, characterized by major neurological and psychiatric features including choreoathetosis, dementia, and caudate atrophy. The HD gene encodes the huntingtin (Htt) protein and is located on the short arm of chromosome 4. The disease is a result of increased triplet repetition of the DNA trinucleotide, cytosine, adenine, and guanine (CAG). The greater the number of CAG repeats, the more severe the disease. The expanded CAG repeats that occur in the Htt gene result in *striatum* and cerebral cortex bearing greatest neuropathology, post-mortem [1, 62].

Gabery and colleagues utilized recombinant serotype 5 adeno-associated viral vectors (rAAV5) that carried two types of an 853 amino acid Htt fragment. One protein had a mutation at amino acid 79 and another was wild type [63]. They over-expressed the Htt gene via an animal model to observe its phenotypical consequences. Like their experiments regarding α-synuclein , they utilized direct gene transfer to various neuron populations in the brain with AAV and LV vectors for gene introduction, resulting in in vivo recombinant DNA. The goal of such work is to verify a cause-effect relationship between Htt and HD symptoms.

Additional work showed that the mutant Htt triggers alpha thalassemia/mental retardation X-linked (ATRX) transcription, resulting in pericentromic heterochromatin condensation and HD pathogenesis [64]. In addition, research by Milnerwood et al. showed that an increase in extra-synaptic NMDAR signaling in *striatal* medium spiny neurons (MSNs) reduces phosphorylated cyclic AMP response element binding (pCREB) protein levels, leading to apoptosis [65]. NMDAR signaling that occurs in the synapse increases pCREB levels, promoting gene transcription and cell survival, but extra-synaptic NMDAR signaling that results in excessive Ca2+ influx is detrimental to the cell.

NO and ROS additionally cause neuronal damage in HD as mentioned above for HAD, AD, and PD. In HD, anomalous S-nitrosylation of dynamin-related protein1 (Drp1) because of NO synthesis results in neuronal dysfunction due to mHTT malfunction. Drp1 mediates mitochondrial fission and is a GTPase. Mitochondrial malfunction results in neuronal synaptic damage followed by neuronal collapse (S-nitrosylation of Drp1 similarly causes damage in AD.) [66]. In HD, mutant Htt (mHtt) is involved in NO toxic pathways. Htt is a substrate for autophagy, and NOS expression producing NO results in decreased mHtt autophagy in HD. mHtt protein is cleared at a faster rate when NOS is inhibited. The combined effects of mHtt and NO production result in increased neuronal apoptosis. Specifically, autophagosome

formation and function are inhibited via JNK1-Bcl-2 signaling pathways due to increased expression of nNOS, eNOS, or eNOS expression in HD [67] (Table 21.3).

21.3.6 MicroRNAs and Apoptosis in Brain Disease

Briefly, it should be noted that current work demonstrates that cellular microRNAs (miRNAs) can function to promote as well as counter apoptosis in cancer cells [68]. In neurodegenerative disorders, for most neurons, aberrant cell cycle reentry leads to apoptosis. A few examples are provided from this burgeoning field. In terms of neurodegenerative disorders, overall, miRNAs also play a role in regulation of apoptosis in neurons [69]. In an AD model, miRNA-34a prevents neuronal apoptosis in part by blocking cyclin D1 expression [70]. In a PD model, miRNA-7 prevents apoptosis by reducing Bax and Sirtuin 2 (Sirt2) expression. However, the hallmark PD-inducing molecule, 1-methyl-4-phenyl-pyridinium ion (MPP+), is toxic to dopaminergic neurons by inducing elevated levels of Bax and Sirt2 expression. However, miRNA-7 prevents the effects of MPP+ by inhibiting Bax and Sirt2 expression [71]. Neurons treated with HIV-1 Tat protein produced elevated levels of miRNAs, including high levels of miRNA-34a. This treatment resulted in apoptosis and decreased expression of miRNA-34a targeted genes such as CREB. This effect was abrogated by treatment with siRNA specific for miRNA-34a. Similar results were obtained using Tat transgenic mice [72].

Several pro-apoptotic genes are involved in the etiology of HD, and some of these are targets of miRNA-22. Primary cortical and striatal cultured neurons were exposed to mutated human huntingtin fragments (Htt171-82Q). In these cultures, miRNA-22 overproduction reduced expression of several pro-apoptotic genes including mitogen-activated protein kinase 14/p38 (MAPK14/p38), histone deacetylase 4 (HDAC4), tumor protein p53-inducible nuclear protein 1 (Tp53inp1), REST corepressor 1 (Rcor1), regulator of G-protein signaling 2 (Rgs2) mRNA, and reduced caspase activation. The experiments described support the development of miRNA-22 treatment as a possible therapeutic agent against HD and AD [73].

21.4 Conclusions

The evidence discussed in this chapter indicates that elucidation gene expression and apoptotic pathways are promising areas for research and have much to offer to understand mechanisms of dementia. The fact that the apoptosis can be counterbalanced by other inhibitor pathways and that the earlier stages of the apoptotic processes are reversible is critical for interventions. Moreover, these findings can be used to alter disease progression and possibly save and protect the affected neurons (while still in a reversible state).

Table 21.3 Apoptosis gene and pathway comparison among diseases

Apoptosis	Necrosis	HIV NeuroAIDS	Alzheimer's disease	Parkinson's disease	Huntington's disease
Casp9		+	+	+	+
TNFR, FAS		+	+	+	+
	Necroptosis	+	+	+	+
	Parthanotos			+	
	RIP3, MLKL		+	+	
JNK	JNK	+	+	+	+
Alpha-synuclein		+	+	+	
Beta-A41		+	+		
HIV-1 proteins		+			
gp120, tat, CXCR4, NMDA		+			
Chemokines and CDK/Rb/E2F-1		+			
HIV-1 proteins and CXCR4, CCR3, CCR5		+			
HIV-1 tat and NFkB, GSK-3beta		+			
Ezrin, ferritin, HLA-DR		+			
nNOS, iNOS		+	+	+	
gp120, cocaine, NMDAR, iNOS		+			
TNF, TRAIL		+			
ROS, oxidative stress		+	+	+	
Cytokine R, oxidative stress, ceramide		+			
gp120, tat, TRAF5, cocaine, amphetamine, opiates		+			
gp120, tat, cocaine, MAP-2, KREMEN, wnt/ beta-catenin		+			
MAP-tau, NFT		+			
Hyper-phosphorylation of tau, NFT		+	+		
Cathepsin B		+			
Casp3, Bax, ROS		+			

(continued)

Table 21.3 (continued)

Apoptosis	Necrosis	HIV NeuroAIDS	Alzheimer's disease	Parkinson's disease	Huntington's disease
Mitochondrial stress		+			
NFkB, ERK, MAPK, p38, JNK		+			
Signal transduction, cell to cell contact, regulation of transcription and translation, thyroid hormones			+		
Mitochondrial fission and fusion			+	+	
Synaptic damage		+	+		
APP, PAK3, Go protein			+		
APP-FAD			+		
ADD45, Bax			+		
Casp6			+		
LRRK2				+	
Htt					+
Htt, NO					+
JNK1, Bcl-2, nNOS, eNOS, iNOS, inhibition of autophagosome formation and function					+
NMDAR, pCREB					+
MAPK14, p38, HDAC4, Tp53inp1, Rcor1, Rgs2			+		+

Genes involved in each of the diseases HIV and NeuroAIDS, Alzheimer's disease, Parkinson's disease, and Huntington's disease based on the text in this chapter. Literature references and explanation of abbreviations are provided in the text
+ = associated with disease in vitro model or in vivo

In HAD, many studies demonstrated that HIV and drug abuse synergistically cause neurogeneration [30]. While the cornerstone of HAD prevention remains virologic control, these studies emphasize the importance of aggressively treating comorbidities, including drug abuse. In addition, the use of drugs in combination with HIV infection requires extensive study as many HIV-infected patients abuse

multiple drugs (polydrug abuse). The panoply of drugs used includes a wide range of compounds – alcohol, cocaine, marijuana, opioids, sedatives, and amphetamines. It should be noted that drugs abused at social clubs, "club-drugs," in the USA include hallucinogens, inhalants, ecstasy, ketamine, phencyclidine (PCP), rohypnol (Flunitrazepam), 3,4-methylenedioxymethamphetamine (MDMA), lysergic acid diethylamide (LSD), 4-bromo-2,5-dimethoxy-amphetamine (2-CB), and gamma-hydroxy-butyrate (GHB) [74, 75]. Ketamine often is taken in "trail mixes" of meth-amphetamine, cocaine, sildenafil citrate (Viagra), or heroin. The mixtures are injected additionally [76]. As a supplementary example, rohypnol, manufactured by a pharmaceutical corporation, is a rapid onset potent benzodiazepine used as a "date rape" drug in the social context. There is a wide availability of this drug in more than 60 countries in Europe and Latin America, and it is imported into the USA [77].

This is an area of active research [78, 79]. It would also be potentially helpful to study the affects of drugs of abuse in laboratory models of treated and partially treated HIV infection. Moreover, establishing models of HIV infection and drug abuse that closely resemble clinical and social situations would assist interpretation of epidemiological and clinical investigations [80].

The use of contemporary gene therapy methods, although at an early stage, is the goal of investigators in clinical and research laboratories to protect and regenerate neurons in the diseased brain, otherwise slated for apoptosis. In this regard, as an example, it should be noted that as of 2015, Dr. D. Kirik with the regional government in southern Sweden established a specialized hospital to implement and test gene therapy for brain diseases. In addition, advanced centers have been established at the Wallenberg Neuroscience Center, Department of Experimental Medical Science, Lund University, Lund, Sweden, and at the Center for Neurodegeneration and Experimental Therapeutics, Department of Neurology, University of Alabama at Birmingham, Birmingham, Alabama [60, 81, 82].

Pleiotropic effects and overlapping, redundant, and sometimes exclusive or opposing signaling pathways appear to operate under a variety of disease conditions and in several cell types. Thus, for example, when TNF binds to the TNF receptor (TNFR), Complex I is formed. Downstream, depending on a plethora of differing conditions, each of the three subsequent signaling complexes IIa, IIb, or IIc can be formed. Complex IIa is involved in classic apoptosis, Complex IIb is involved in RIPK 1-dependent apoptosis, and Complex IIc is involved in necroptosis [6].

NO produced by nNOS, iNOS, and eNOS is excitatory and implicated in widespread damage in neuropathogenesis including HAD, AD, PD, and HD [37, 67]. Overall, in these diseases, ROS degeneration of neurites occurs prior to neuronal death [83]. The precise conditions of excitatory induction vary among these diseases, but common signaling pathways are cognate to all. Producing methods to reduce excessive NO production remains a prime common goal in the defeat of these neuroinflammatory and neurodegenerative diseases.

Great strides are being made in research and clinical laboratories, worldwide characterizing complexities of cell death, and just a few are mentioned here. It should be noted that laboratory protocols are utilized to hypothesize, test, and implement improved therapies with having pinpointed the patient's underlying con-

ditions. Individual-focused, evidence-based, translational healthcare brings this perspective into the twenty-first century [84].

See the Appendix for a list of 113 select apoptosis-, dysregulation-, and dementia-related genes and their basic genetic information derived from NCBI and GeneCards, NIH, Bethesda, MD.

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Conflict of interest The authors report no conflicts of interest.

Appendix: Dementia-Associated Genes

This appendix summarizes information for 113 genes implicated in apoptosis, dysfunction, and dementias (NCBI and GeneCards, NIH, Bethesda, MD).

1. CASP9

   Official Symbol: CASP9; **Name:** caspase-9, apoptosis-related cysteine peptidase [*Homo sapiens*]
   Other Aliases: RP11-265F14.3, APAF-3, APAF3, CASPASE-9c, ICE-LAP6, MCH6
   Other Designations: ICE-like apoptotic protease 6; OTTHUMP00000044594; apoptotic protease MCH-6; apoptotic protease-activating factor 3; caspase-9; caspase-9, apoptosis-related cysteine protease; caspase-9c protein
   Chromosome: 1; **Location:** 1p36.3-p36.1
   GeneID: 842

2. LRP1

   Official Symbol: LRP1; **Name:** low-density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor) [*Homo sapiens*]
   Other Aliases: A2MR, APOER, APR, CD91, LRP, MGC88725, TGFBR5
   Other Designations: alpha-2-macroglobulin receptor; low-density lipoprotein-related protein 1; type V tgf-beta receptor
   Chromosome: 12; **Location:** 12q13-q14
   GeneID: 4035

3. CASP3

   Official Symbol: CASP3; **Name:** caspase-3, apoptosis-related cysteine peptidase [*Homo sapiens*]
   Other Aliases: CPP32, CPP32B, SCA-1

Other Designations: PARP cleavage protease; SREBP cleavage activity 1; Yama; apopain; caspase-3; caspase-3, apoptosis-related cysteine protease; cysteine protease CPP32; procaspase3
Chromosome: 4; **Location:** 4q34
GeneID: 836

4. RUNX1

Official Symbol: RUNX1; **Name:** runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene) [*Homo sapiens*]
Other Aliases: AML1, AML1-EVI-1, AMLCR1, CBFA2, EVI-1, PEBP2A2, PEBP2aB
Other Designations: AML1-EVI-1 fusion protein; acute myeloid leukemia 1 gene; aml1 oncogene; core-binding factor, runt domain, alpha subunit 2; runt-related transcription factor 1
Chromosome: 21; **Location:** 21q22.3
GeneID: 861

5. TNFSF10

Official Symbol: TNFSF10; **Name:** tumor necrosis factor (ligand) superfamily, member 10 [*Homo sapiens*]
Other Aliases: APO2L, Apo-2 L, TL2, TRAIL
Other Designations: Apo-2 ligand; TNF-related apoptosis-inducing ligand TRAIL
Chromosome: 3; **Location:** 3q26
GeneID: 8743

6. TP53

Official Symbol: TP53; **Name:** tumor protein p53 (Li-Fraumeni syndrome) [*Homo sapiens*]
Other Aliases: LFS1, TRP53, p53
Other Designations: p53 tumor suppressor; tumor protein p53
Chromosome: 17; **Location:** 17p13.1
GeneID: 7157

7. BCL2L11

Official Symbol: BCL2L11; **Name:** BCL2-like 11 (apoptosis facilitator) [*Homo sapiens*]
Other Aliases: BAM, BIM, BOD, BimEL, BimL
Other Designations: BCL2-like 11; bcl-2 interacting mediator of cell death; bcl-2 interacting protein Bim; bcl-2-related ovarian death agonist
Chromosome: 2; **Location:** 2q13
GeneID: 10,018

8. APP

   Official Symbol: APP; **Name:** amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer's disease) [*Homo sapiens*]
   Other Aliases: AAA, ABETA, ABPP, AD1, APPI, CTFgamma, CVAP, PN2
   Other Designations: A4 amyloid protein; amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer's disease); amyloid beta A4 protein; amyloid-beta protein; beta-amyloid peptide; cerebral vascular amyloid peptide; protease nexin-II
   Chromosome: 21; **Location:** 21q21.3
   GeneID: 351

9. FAS

   Official Symbol: FAS; **Name:** Fas (TNF receptor superfamily, member 6) [*Homo sapiens*]
   Other Aliases: ALPS1A, APO-1, APT1, Apo-1 Fas, CD95, FAS1, FASTM, TNFRSF6
   Other Designations: APO-1 cell surface antigen; CD95 antigen; Fas antigen; OTTHUMP00000059646; apoptosis antigen 1; tumor necrosis factor receptor superfamily, member 6
   Chromosome: 10; **Location:** 10q24.1
   GeneID: 355

10. TNF

    Official Symbol: TNF; **Name:** tumor necrosis factor (TNF superfamily, member 2) [*Homo sapiens*]
    Other Aliases: DASS-280D8.2, DIF, TNF-alpha, TNFA, TNFSF2
    Other Designations: APC1 protein; OTTHUMP00000037669; TNF superfamily, member 2; TNF, macrophage-derived; TNF, monocyte-derived; cachectin; tumor necrosis factor alpha
    Chromosome: 6; **Location:** 6p21.3
    GeneID: 7124

11. BCL2

    Official Symbol: BCL2; **Name:** B-cell CLL/lymphoma 2 [*Homo sapiens*]
    Other Aliases: Bcl-2
    Other Designations: B-cell lymphoma protein 2
    Chromosome: 18; **Location:** 18q21.3
    GeneID: 596

12. BAX

    Official Symbol: BAX; **Name:** BCL2-associated X protein [*Homo sapiens*]
    Other Aliases: Bax zeta
    Other Designations: apoptosis regulator BAX; bax protein
    Chromosome: 19; **Location:** 19q13.3-q13.4
    GeneID: 581

13. MAPK1

Official Symbol: MAPK1; **Name:** mitogen-activated protein kinase 1 [*Homo sapiens*]
Other Aliases: ERK, ERK2, ERT1, MAPK2, P42MAPK, PRKM1, PRKM2, p38, p40, p41, p41mapk
Other Designations: extracellular signal-regulated kinase 2; mitogen-activated protein kinase 2; protein tyrosine kinase ERK2
Chromosome: 22; **Location:** 22q11.21
GeneID: 5594

14. NFKB1

Official Symbol: NFKB1; **Name:** nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) [*Homo sapiens*]
Other Aliases: DKFZp686C01211, EBP-1, KBF1, MGC54151, NF-kappa-B, NFKB-p105, NFKB-p50
Other Designations: DNA binding factor KBF1; nuclear factor NF-kappa-B p50 subunit; nuclear factor kappa-B DNA binding subunit; nuclear factor kappa-B, subunit 1
Chromosome: 4; **Location:** 4q24
GeneID: 4790

15. BCL2L1

Official Symbol: BCL2L1; **Name:** BCL2-like 1 [*Homo sapiens*]
Other Aliases: BCL-XL/S, BCL2L, BCLX, Bcl-X, DKFZp781P2092, bcl-xL, bcl-xS
Chromosome: 20; **Location:** 20q11.21
GeneID: 598

16. IL6

Official Symbol: IL6; **Name:** interleukin 6 (interferon, beta 2) [*Homo sapiens*]
Other Aliases: BSF2, HGF, HSF, IFNB2, IL-6
Chromosome: 7; **Location:** 7p21
GeneID: 3569

17. IGF1

Official Symbol: IGF1; **Name:** insulin-like growth factor 1 (somatomedin C) [*Homo sapiens*]
Other Aliases: IGFI
Other Designations: somatomedin C
Chromosome: 12; **Location:** 12q22-q23
GeneID: 3479

18. ESR1

Official Symbol: ESR1; **Name:** estrogen receptor 1 [*Homo sapiens*]
Other Aliases: RP1-130E4.1, DKFZp686N23123, ER, ESR, ESRA, Era, NR3A1, major ORF
Other Designations: dJ443C4.1.1 (estrogen receptor 1); estrogen receptor 1 (alpha); estrogen receptor; steroid hormone receptor
Chromosome: 6; **Location:** 6q25.1
GeneID: 2099

19. PTGS2

Official Symbol: PTGS2; **Name:** prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) [*Homo sapiens*]
Other Aliases: COX-2, COX2, PGG/HS, PGHS-2, PHS-2, hCox-2
Other Designations: cyclooxygenase 2b; prostaglandin G/H synthase and cyclooxygenase; prostaglandin-endoperoxide synthase 2
Chromosome: 1; **Location:** 1q25.2-q25.3
GeneID: 5743

20. FASLG

Official Symbol: FASLG; **Name:** Fas ligand (TNF superfamily, member 6) [*Homo sapiens*]
Other Aliases: APT1LG1, CD178, CD95L, FASL, TNFSF6
Other Designations: CD95 ligand; apoptosis (APO-1) antigen ligand 1; fas ligand; tumor necrosis factor (ligand) superfamily, member 6
Chromosome: 1; **Location:** 1q23
GeneID: 356

21. AKT1

Official Symbol: AKT1; **Name:** v-akt murine thymoma viral oncogene homolog 1 [*Homo sapiens*]
Other Aliases: MGC99656, PKB, PRKBA, RAC, RAC-ALPHA
Other Designations: RAC-alpha serine/threonine-protein kinase; murine thymoma viral (v-akt) oncogene homolog-1; protein kinase B; rac protein kinase alpha
Chromosome: 14; **Location:** 14q32.32
GeneID: 207

22. PPARG

Official Symbol: PPARG; **Name:** peroxisome proliferative-activated receptor, gamma [*Homo sapiens*]
Other Aliases: HUMPPARG, NR1C3, PPARG1, PPARG2

Other Designations: PPAR gamma; peroxisome proliferative-activated receptor gamma; peroxisome proliferator-activated receptor gamma; peroxisome proliferator-activated receptor gamma 1; ppar gamma2
Chromosome: 3; **Location:** 3p25
GeneID: 5468

23. RB1

Official Symbol: RB1; **Name:** retinoblastoma 1 (including osteosarcoma) [*Homo sapiens*]
Other Aliases: OSRC, RB
Other Designations: retinoblastoma 1; retinoblastoma susceptibility protein; retinoblastoma susceptibility protein; retinoblastoma-1
Chromosome: 13; **Location:** 13q14.2
GeneID: 5925

24. IL1B

Official Symbol: IL1B; **Name:** interleukin 1, beta [*Homo sapiens*]
Other Aliases: IL-1, IL1-BETA, IL1F2
Other Designations: catabolin; preinterleukin 1 beta; pro-interleukin-1-beta
Chromosome: 2; **Location:** 2q14
GeneID: 3553

25. STAT3

Official Symbol: STAT3; **Name:** signal transducer and activator of transcription 3 (acute-phase response factor) [*Homo sapiens*]
Other Aliases: APRF, FLJ20882, MGC16063
Other Designations: DNA-binding protein APRF; acute-phase response factor; signal transducer and activator of transcription 3
Chromosome: 17; **Location:** 17q21.31
GeneID: 6774

26. CDKN1A

Official Symbol: CDKN1A; **Name:** cyclin-dependent kinase inhibitor 1A (p21, Cip1) [*Homo sapiens*]
Other Aliases: CAP20, CDKN1, CIP1, MDA-6, P21, SDI1, WAF1, p21CIP1
Other Designations: CDK-interaction protein 1; DNA synthesis inhibitor; cyclin-dependent kinase inhibitor 1A; melanoma differentiation-associated protein 6; wild-type p53-activated fragment 1
Chromosome: 6; **Location:** 6p21.2
GeneID: 1026

27. SNCA

Official Symbol: SNCA; **Name:** synuclein, alpha (non-A4 component of amyloid precursor) [*Homo sapiens*]
Other Aliases: MGC110988, NACP, PARK1, PARK4, PD1

Other Designations: alpha synuclein; alpha-synuclein; alpha-synuclein, isoform NACP140; non-A-beta component of AD amyloid; non-A4 component of amyloid precursor
Chromosome: 4; **Location:** 4q21
GeneID: 6622

28. GSK3B

Official Symbol: GSK3B; **Name:** glycogen synthase kinase 3 beta [*Homo sapiens*]
Chromosome: 3; **Location:** 3q13.3
GeneID: 2932

29. MAPK14

Official Symbol: MAPK14; **Name:** mitogen-activated protein kinase 14 [*Homo sapiens*]
Other Aliases: CSBP1, CSBP2, CSPB1, EXIP, Mxi2, PRKM14, PRKM15, RK, SAPK2A, p38, p38ALPHA
Other Designations: Csaids binding protein; MAP kinase Mxi2; MAX-interacting protein 2; cytokine suppressive anti-inflammatory drug binding protein; p38 MAP kinase; p38 mitogen-activated protein kinase; p38alpha Exip; stress-activated protein kinase 2A
Chromosome: 6; **Location:** 6p21.3-p21.2
GeneID: 1432

30. PTK2

Official Symbol: PTK2; **Name:** PTK2 protein tyrosine kinase 2 [*Homo sapiens*]
Other Aliases: FADK, FAK, FAK1, pp125FAK
Other Designations: focal adhesion kinase 1
Chromosome: 8; **Location:** 8q24-qter
GeneID: 5747

31. ESR2

Official Symbol: ESR2; **Name:** estrogen receptor 2 (ER beta) [*Homo sapiens*]
Other Aliases: 5p152, ER-BETA, ESR-BETA, ESRB, Erb, NR3A2
Other Designations: estrogen receptor 2; estrogen receptor beta
Chromosome: 14; **Location:** 14q23.2
GeneID: 2100

32. HD

Official Symbol: HD; **Name:** huntingtin (Huntington's disease) [*Homo sapiens*]
Other Aliases: IT15

Other Designations: huntingtin
Chromosome: 4; **Location:** 4p16.3
GeneID: 3064

33. CXCL12

Official Symbol: CXCL12; **Name:** chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) [*Homo sapiens*]
Other Aliases: PBSF, SCYB12, SDF-1a, SDF-1b, SDF1, SDF1A, SDF1B, TLSF-a, TLSF-b, TPAR1, chemokine
Other Designations: stromal cell-derived factor 1; stromal cell-derived factor 1 gamma; stromal cell-derived factor 1a
Chromosome: 10; **Location:** 10q11.1
GeneID: 6387

34. MAPT

Official Symbol: MAPT; **Name:** microtubule-associated protein tau [*Homo sapiens*]
Other Aliases: DDPAC, FLJ31424, FTDP-17, MAPTL, MSTD, MTBT1, MTBT2, PPND, TAU
Other Designations: G protein beta1/gamma2 subunit-interacting factor 1; microtubule-associated protein tau, isoform 4; tau protein
Chromosome: 17; **Location:** 17q21.1
GeneID: 4137

35. PSEN2

Official Symbol: PSEN2; **Name:** presenilin 2 (Alzheimer's disease 4) [*Homo sapiens*]
Other Aliases: AD3 L, AD4, PS2, STM2
Other Designations: Alzheimer's disease 3-like; presenilin 2
Chromosome: 1; **Location:** 1q31-q42
GeneID: 5664

36. MAP3K5

Official Symbol: MAP3K5; **Name:** mitogen-activated protein kinase kinase kinase 5 [*Homo sapiens*]
Other Aliases: RP3-325F22.4, ASK1, MAPKKK5, MEKK5
Other Designations: MAP/ERK kinase kinase 5; MAPK/ERK kinase kinase 5; apoptosis signal regulating kinase
Chromosome: 6; **Location:** 6q22.33
GeneID: 4217

37. IL2

Official Symbol: IL2; **Name:** interleukin 2 [*Homo sapiens*]
Other Aliases: IL-2, TCGF, lymphokine
Other Designations: T cell growth factor; aldesleukin; interleukin-2; involved in regulation of T-cell clonal expansion

Chromosome: 4; **Location:** 4q26-q27
GeneID: 3558

38. SOD1

Official Symbol: SOD1; **Name:** superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult)) [*Homo sapiens*]
Other Aliases: ALS, ALS1, IPOA, SOD, homodimer
Other Designations: Cu/Zn superoxide dismutase; Cu/Zn superoxide dismutase; SOD, soluble; indophenoloxidase A; superoxide dismutase (aa 120–154); superoxide dismutase 1, soluble; superoxide dismutase, cystolic
Chromosome: 21; **Location:** 21q22.11
GeneID: 6647

39. ALS2

Official Symbol: ALS2; **Name:** amyotrophic lateral sclerosis 2 (juvenile) [*Homo sapiens*]
Other Aliases: ALS2CR6, ALSJ, IAHSP, KIAA1563, MGC87187, PLSJ
Other Designations: alsin; amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 6
Chromosome: 2; **Location:** 2q33.1
GeneID: 57,679

40. MAP2K4

Official Symbol: MAP2K4; **Name:** mitogen-activated protein kinase kinase 4 [*Homo sapiens*]
Other Aliases: JNKK, JNKK1, MAPKK4, MEK4, MKK4, PRKMK4, SEK1, SERK1
Other Designations: JNK-activating kinase 1; JNK-activated kinase 1; MAP kinase kinase 4; MAPK/ERK kinase 4; SAPK/ERK kinase 1; c-Jun N-terminal kinase kinase 1; dual specificity mitogen-activated protein kinase kinase 4
Chromosome: 17; **Location:** 17p11.2
GeneID: 6416

41. EPO

Official Symbol: EPO; **Name:** erythropoietin [*Homo sapiens*]
Other Aliases: EP
Other Designations: epoetin
Chromosome: 7; **Location:** 7q22
GeneID: 2056

42. GZMB

Official Symbol: GZMB; **Name:** granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) [*Homo sapiens*]
Other Aliases: CCPI, CGL-1, CGL1, CSP-B, CSPB, CTLA1, CTSGL1, HLP, SECT

Other Designations: T-cell serine protease 1–3E; cathepsin G-like 1; cytotoxic serine protease B; fragmentin 2; granzyme B
Chromosome: 14; **Location:** 14q11.2
GeneID: 3002

43. TP53BP2

Official Symbol: TP53BP2; **Name:** tumor protein p53-binding protein, 2 [*Homo sapiens*]
Other Aliases: 53BP2, ASPP2, PPP1R13A
Other Designations: apoptosis-stimulating protein of p53, 2; tumor protein p53-binding protein, 2
Chromosome: 1; **Location:** 1q42.1
GeneID: 7159

44. AKT2

Official Symbol: AKT2; **Name:** v-akt murine thymoma viral oncogene homolog 2 [*Homo sapiens*]
Other Aliases: PKBBETA, PRKBB, RAC-BETA
Other Designations: Murine thymoma viral (v-akt) homolog-2; rac protein kinase beta
Chromosome: 19; **Location:** 19q13.1-q13.2
GeneID: 208

45. PRKCE

Official Symbol: PRKCE; **Name:** protein kinase C, epsilon [*Homo sapiens*]
Other Aliases: MGC125656, MGC125657, PKCE, nPKC-epsilon
Chromosome: 2; **Location:** 2p21
GeneID: 5581

46. CACNA1A

Official Symbol: CACNA1A; **Name:** calcium channel, voltage-dependent, P/Q type, alpha 1A subunit [*Homo sapiens*]
Other Aliases: APCA, CACNL1A4, CAV2.1, EA2, FHM, HPCA, MHP, MHP1, SCA6
Other Designations: brain calcium channel 1; calcium channel, L type, alpha-1 polypeptide, isoform 4; calcium channel, alpha 1A subunit
Chromosome: 19; **Location:** 19p13.2-p13.1
GeneID: 773

47. PARK2

Official Symbol: PARK2; **Name:** Parkinson's disease (autosomal recessive, juvenile) 2, parkin [*Homo sapiens*]
Other Aliases: AR-JP, PDJ, PRKN
Other Designations: E3 ubiquitin ligase; parkin
Chromosome: 6; **Location:** 6q25.2-q27
GeneID: 5071

48. PDCD8

Official Symbol: PDCD8; **Name:** programmed cell death 8 (apoptosis-inducing factor) [*Homo sapiens*]
Other Aliases: RP3-438D16.2, AIF, MGC111425
Other Designations: apoptosis-inducing factor; programmed cell death 8; programmed cell death 8, isoform 2; striatal apoptosis-inducing factor
Chromosome: X; **Location:** Xq25-q26
GeneID: 9131

49. GAPDH

Official Symbol: GAPDH; **Name:** glyceraldehyde-3-phosphate dehydrogenase [*Homo sapiens*]
Other Aliases: G3PD, GAPD, MGC88685
Chromosome: 12; **Location:** 12p13
GeneID: 2597

50. FLI1

Official Symbol: FLI1; **Name:** Friend leukemia virus integration 1 [*Homo sapiens*]
Other Aliases: EWSR2, SIC-1
Chromosome: 11; **Location:** 11q24.1-q24.3
GeneID: 2313

51. HSPB2

Official Symbol: HSPB2; **Name:** heat shock 27 kDa protein 2 [*Homo sapiens*]
Other Aliases: HSP27, Hs.78846, MGC133245, MKBP
Other Designations: heat shock 27kD protein 2
Chromosome: 11; **Location:** 11q22-q23
GeneID: 3316

52. F2R

Official Symbol: F2R; **Name:** coagulation factor II (thrombin) receptor [*Homo sapiens*]
Other Aliases: CF2R, HTR, PAR1, TR
Other Designations: coagulation factor II receptor; protease-activated receptor 1; thrombin receptor
Chromosome: 5; **Location:** 5q13
GeneID: 2149

53. NGFB

Official Symbol: NGFB; **Name:** nerve growth factor, beta polypeptide [*Homo sapiens*]
Other Aliases: Beta-NGF, HSAN5, NGF
Other Designations: beta-nerve growth factor; nerve growth factor, beta subunit

Chromosome: 1; **Location:** 1p13.1
GeneID: 4803

54. SMPD1

Official Symbol: SMPD1; **Name:** sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingomyelinase) [*Homo sapiens*]
Other Aliases: ASM, NPD
Other Designations: acid sphingomyelinase; sphingomyelin phosphodiesterase 1, acid lysosomal
Chromosome: 11; **Location:** 11p15.4-p15.1
GeneID: 6609

55. MET

Official Symbol: MET; **Name:** met proto-oncogene (hepatocyte growth factor receptor) [*Homo sapiens*]
Other Aliases: HGFR, RCCP2
Other Designations: Oncogene MET; met proto-oncogene
Chromosome: 7; **Location:** 7q31
GeneID: 4233

56. POMC

Official Symbol: POMC; **Name:** pro-opiomelanocortin (adrenocorticotropin/ beta-lipotropin/ alpha-melanocyte-stimulating hormone/ beta-melanocyte-stimulating hormone/ beta-endorphin) [*Homo sapiens*]
Other Aliases: ACTH, CLIP, LPH, MSH, NPP, POC
Other Designations: N-terminal peptide; adrenocorticotropic hormone; adrenocorticotropin; alpha-MSH; alpha-melanocyte-stimulating hormone; beta-LPH; beta-MSH; beta-endorphin; corticotropin; corticotropin-like intermediary peptide; corticotropin-lipotropin; gamma-LPH; gamma-MSH; lipotropin beta; lipotropin gamma; melanotropin alpha; melanotropin beta; melanotropin gamma; met-enkephalin; pro-ACTH-endorphin; pro-opiomelanocortin; pro-opiomelanocortin
Chromosome: 2; **Location:** 2p23.3
GeneID: 5443

57. GRID2

Official Symbol: GRID2; **Name:** glutamate receptor, ionotropic, delta 2 [*Homo sapiens*]
Other Aliases: MGC117022, MGC117023, MGC117024
Other Designations: GluR-delta-2
Chromosome: 4; **Location:** 4q22
GeneID: 2895

58. RASA1

Official Symbol: RASA1; **Name:** RAS p21 protein activator (GTPase-activating protein) 1 [*Homo sapiens*]
Other Aliases: CMAVM, DKFZp434N071, GAP, PKWS, RASA, RASGAP, p120GAP
Other Designations: GTPase-activating protein; RAS p21 protein activator 1; triphosphatase-activating protein
Chromosome: 5; **Location:** 5q13.3
GeneID: 5921

59. IL7

Official Symbol: IL7; **Name:** interleukin 7 [*Homo sapiens*]
Other Aliases: IL-7
Chromosome: 8; **Location:** 8q12-q13
GeneID: 3574

60. PINK1

Official Symbol: PINK1 **and Name:** PTEN-induced putative kinase 1 [*Homo sapiens*]
Other Aliases: BRPK, PARK6
Other Designations: protein kinase BRPK
Chromosome: 1; **Location:** 1p36
GeneID: 65,018

61. AKT3

Official Symbol: AKT3; **Name:** v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma) [*Homo sapiens*]
Other Aliases: DKFZP434N0250, PKBG, PRKBG, RAC-PK-gamma, RAC-gamma, STK-2
Other Designations: RAC-gamma serine/threonine protein kinase; protein kinase B gamma; serine threonine protein kinase, Akt-3; v-akt murine thymoma viral oncogene homolog 3
Chromosome: 1; **Location:** 1q43-q44
GeneID: 10,000

62. APP

Official Symbol: APP; **Name:** amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer's disease) [*Homo sapiens*]
Other Aliases: AAA, ABETA, ABPP, AD1, APPI, CTFgamma, CVAP, PN2
Other Designations: A4 amyloid protein; amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer's disease); amyloid beta A4 protein; amyloid-beta protein; beta-amyloid peptide; cerebral vascular amyloid peptide; protease nexin-II
Chromosome: 21; **Location:** 21q21.3
GeneID: 351

63. PSEN1

Official Symbol: PSEN1; **Name:** presenilin 1 (Alzheimer's disease 3) [*Homo sapiens*]
Other Aliases: AD3, FAD, PS1, S182
Other Designations: presenilin 1
Chromosome: 14; **Location:** 14q24.3
GeneID: 5663

64. NME2

Official Symbol: NME2; **Name:** non-metastatic cells 2, protein (NM23B) expressed in [*Homo sapiens*]
Other Aliases: MGC111212, NDPKB, NM23-H2, NM23B, puf
Other Designations: c-myc transcription factor; non-metastatic cells 2, protein (NM23) expressed in
Chromosome: 17; **Location:** 17q21.3
GeneID: 4831

65. PSEN2

Official Symbol: PSEN2; **Name:** presenilin 2 (Alzheimer's disease 4) [*Homo sapiens*]
Other Aliases: AD3 L, AD4, PS2, STM2
Other Designations: Alzheimer's disease 3-like; presenilin 2
Chromosome: 1; **Location:** 1q31-q42
GeneID: 5664

66. NME1

Official Symbol: NME1; **Name:** non-metastatic cells 1, protein (NM23A) expressed in [*Homo sapiens*]
Other Aliases: AWD, GAAD, NDPKA, NM23, NM23-H1
Chromosome: 17; **Location:** 17q21.3
GeneID: 4830

67. ACE

Official Symbol: ACE; **Name:** angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 [*Homo sapiens*]
Other Aliases: ACE1, CD143, DCP, DCP1, MGC26566
Other Designations: CD143 antigen; angiotensin I converting enzyme; angiotensin converting enzyme, somatic isoform; carboxycathepsin; dipeptidyl carboxypeptidase 1; kininase II; peptidase P; peptidyl-dipeptidase A; testicular ECA
Chromosome: 17; **Location:** 17q23
GeneID: 1636

68. NOS3

Official Symbol: NOS3; **Name:** nitric oxide synthase 3 (endothelial cell) [*Homo sapiens*]
Other Aliases: ECNOS, NOS III, eNOS
Other Designations: endothelial nitric oxidase synthase; endothelial nitric oxide synthase
Chromosome: 7; **Location:** 7q36
GeneID: 4846

69. ESR1

Official Symbol: ESR1; **Name:** estrogen receptor 1 [*Homo sapiens*]
Other Aliases: RP1-130E4.1, DKFZp686N23123, ER, ESR, ESRA, Era, NR3A1, major ORF
Other Designations: dJ443C4.1.1 (estrogen receptor 1); estrogen receptor 1 (alpha); estrogen receptor; steroid hormone receptor
Chromosome: 6; **Location:** 6q25.1
GeneID: 2099

70. TNF

Official Symbol: TNF; **Name:** tumor necrosis factor (TNF superfamily, member 2) [*Homo sapiens*]
Other Aliases: DASS-280D8.2, DIF, TNF-alpha, TNFA, TNFSF2
Other Designations: APC1 protein; OTTHUMP00000037669; TNF superfamily, member 2; TNF, macrophage-derived; TNF, monocyte-derived; cachectin; tumor necrosis factor alpha
Chromosome: 6; **Location:** 6p21.3
GeneID: 7124

71. FASLG

Official Symbol: FASLG; **Name:** Fas ligand (TNF superfamily, member 6) [*Homo sapiens*]
Other Aliases: APT1LG1, CD178, CD95L, FASL, TNFSF6
Other Designations: CD95 ligand; apoptosis (APO-1) antigen ligand 1; fas ligand; tumor necrosis factor (ligand) superfamily, member 6
Chromosome: 1; **Location:** 1q23
GeneID: 356

72. IL1B

Official Symbol: IL1B; **Name:** interleukin 1, beta [*Homo sapiens*]
Other Aliases: IL-1, IL1-BETA, IL1F2
Other Designations: catabolin; preinterleukin 1 beta; pro-interleukin-1-beta
Chromosome: 2; **Location:** 2q14
GeneID: 3553

73. FAS

Official Symbol: FAS; **Name:** Fas (TNF receptor superfamily, member 6) [*Homo sapiens*]
Other Aliases: ALPS1A, APO-1, APT1, Apo-1 Fas, CD95, FAS1, FASTM, TNFRSF6
Other Designations: APO-1 cell surface antigen; CD95 antigen; Fas antigen; OTTHUMP00000059646; apoptosis antigen 1; tumor necrosis factor receptor superfamily, member 6
Chromosome: 10; **Location:** 10q24.1
GeneID: 355

74. BACE1

Official Symbol: BACE1; **Name:** beta-site APP-cleaving enzyme 1 [*Homo sapiens*]
Other Aliases: ASP2, BACE, HSPC104, KIAA1149
Other Designations: APP beta-secretase; aspartyl protease 2; beta-secretase; beta-site amyloid beta A4 precursor protein-cleaving enzyme; beta-site amyloid precursor protein cleaving enzyme; memapsin-2; membrane-associated aspartic protease 2; transmembrane aspartic proteinase Asp2
Chromosome: 11; **Location:** 11q23.2-q23.3
GeneID: 23,621

75. E2F1

Official Symbol: E2F1; **Name:** E2F transcription factor 1 [*Homo sapiens*]
Other Aliases: E2F-1, RBBP3, RBP3
Other Designations: retinoblastoma-associated protein 1
Chromosome: 20; **Location:** 20q11.2
GeneID: 1869

76. IFNG

Official Symbol: IFNG; **Name:** interferon, gamma [*Homo sapiens*]
Other Aliases: IFG, IFI
Chromosome: 12; **Location:** 12q14
GeneID: 3458

77. MME

Official Symbol: MME; **Name:** membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10) [*Homo sapiens*]
Other Aliases: CALLA, CD10, MGC126681, MGC126707, NEP
Other Designations: membrane metallo-endopeptidase; neprilysin
Chromosome: 3; **Location:** 3q25.1-q25.2
GeneID: 4311

78. NRG1

Official Symbol: NRG1; **Name:** neuregulin 1 [*Homo sapiens*]
Other Aliases: ARIA, GGF, GGF2, HGL, HRG, HRG1, HRGA, NDF, SMDF
Other Designations: glial growth factor; heregulin, alpha (45kD, ERBB2 p185-activator); neu differentiation factor; sensory and motor neuron-derived factor
Chromosome: 8; **Location:** 8p21-p12
GeneID: 3084

79. CHRNA7

Official Symbol: CHRNA7; **Name:** cholinergic receptor, nicotinic, alpha polypeptide 7 [*Homo sapiens*]
Other Aliases: NACHRA7
Other Designations: a7 nicotinic acetylcholine receptor; alpha-7 neuronal nicotinic acetylcholine receptor; alpha-7 nicotinic cholinergic receptor subunit; alpha-7 nicotinic receptor subunit; neuronal acetylcholine receptor protein, alpha-7 chain precursor; neuronal nicotinic acetylcholine receptor alpha-7 subunit; nicotinic acetylcholine alpha-7 subunit; nicotinic acetylcholine receptor alpha-7 subunit precursor; nicotinic cholinergic receptor alpha-7
Chromosome: 15; **Location:** 15q14
GeneID: 1139

80. MAPT

Official Symbol: MAPT; **Name:** microtubule-associated protein tau [*Homo sapiens*]
Other Aliases: DDPAC, FLJ31424, FTDP-17, MAPTL, MSTD, MTBT1, MTBT2, PPND, TAU
Other Designations: G protein beta1/gamma2 subunit-interacting factor 1; microtubule-associated protein tau, isoform 4; tau protein
Chromosome: 17; **Location:** 17q21.1
GeneID: 4137

81. CTSD

Official Symbol: CTSD; **Name:** cathepsin D (lysosomal aspartyl peptidase) [*Homo sapiens*]
Other Aliases: CPSD, MGC2311
Other Designations: cathepsin D; cathepsin D (lysosomal aspartyl protease)
Chromosome: 11; **Location:** 11p15.5
GeneID: 1509

82. CD40

Official Symbol: CD40; **Name:** CD40 antigen (TNF receptor superfamily member 5) [*Homo sapiens*]
Other Aliases: Bp50, CDW40, MGC9013, TNFRSF5, p50

Other Designations: B cell surface antigen CD40; B cell-associated molecule; CD40 antigen; CD40 type II isoform; CD40L receptor; nerve growth factor receptor-related B-lymphocyte activation molecule; tumor necrosis factor receptor superfamily member 5; tumor necrosis factor receptor superfamily, member 5
Chromosome: 20; **Location:** 20q12-q13.2
GeneID: 958

83. MEOX2

Official Symbol: MEOX2; **Name:** mesenchyme homeo box 2 (growth arrest-specific homeo box) [*Homo sapiens*]
Other Aliases: GAX, MOX2
Other Designations: growth arrest-specific homeo box; mesenchyme homeo box 2
Chromosome: 7; **Location:** 7p22.1-p21.3
GeneID: 4223

84. GRIN2B

Official Symbol: GRIN2B; **Name:** glutamate receptor, ionotropic, N-methyl-D-aspartate 2B [*Homo sapiens*]
Other Aliases: NMDAR2B, NR2B, hNR3
Other Designations: N-methyl-D-aspartate receptor subunit 2B
Chromosome: 12; **Location:** 12p12
GeneID: 2904

85. OLR1

Official Symbol: OLR1; **Name:** oxidized low-density lipoprotein (lectin-like) receptor 1 [*Homo sapiens*]
Other Aliases: CLEC8A, LOX1, SCARE1
Other Designations: lectin-type oxidized LDL receptor 1; scavenger receptor class E, member 1
Chromosome: 12; **Location:** 12p13.2-p12.3
GeneID: 4973

86. Cdk5r1

Official Symbol: Cdk5r1; **Name:** cyclin-dependent kinase 5, regulatory subunit (p35) 1 [*Mus musculus*]
Other Aliases: RP23-252E4.5, Cdk5r, D11Bwg0379e, p35
Chromosome: 11; **Location:** 11 46.5 cM
GeneID: 12,569

87. APOC1

Official Symbol: APOC1; **Name:** apolipoprotein C-I [*Homo sapiens*]
Chromosome: 19; **Location:** 19q13.2
GeneID: 341

88. PRNP

Official Symbol: PRNP; **Name:** prion protein (p27–30) (Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, fatal familial insomnia) [*Homo sapiens*]
Other Aliases: ASCR, CJD, GSS, MGC26679, PRIP, PrP, PrP27–30, PrP33-35C, PrPc
Other Designations: CD230 antigen; major prion protein; prion protein; prion-related protein
Chromosome: 20; **Location:** 20pter-p12
GeneID: 5621

89. AGER

Official Symbol: AGER; **Name:** advanced glycosylation end product-specific receptor [*Homo sapiens*]
Other Aliases: DAQB-143 M3.1, MGC22357, RAGE
Other Designations: receptor for advanced glycosylation end products
Chromosome: 6; **Location:** 6p21.3
GeneID:

90. ABCA2

Official Symbol: ABCA2; **Name:** ATP-binding cassette, subfamily A (ABC1), member 2 [*Homo sapiens*]
Other Aliases: ABC2
Other Designations: ATP-binding cassette, subfamily A, member 2; OTTHUMP00000064733
Chromosome: 9; **Location:** 9q34
GeneID: 20

91. SNCB

Official Symbol: SNCB; **Name:** synuclein, beta [*Homo sapiens*]
Other Designations: beta-synuclein
Chromosome: 5; **Location:** 5q35
GeneID: 6620

92. CYP46A1

Official Symbol: CYP46A1; **Name:** cytochrome P450, family 46, subfamily A, polypeptide 1 [*Homo sapiens*]
Other Aliases: CP46, CYP46
Other Designations: cholesterol 24-hydroxylase; cytochrome P450, family 46; cytochrome P450, subfamily 46 (cholesterol 24-hydroxylase)
Chromosome: 14; **Location:** 14q32.1
GeneID: 10,858

93. APOM

Official Symbol: APOM; **Name:** apolipoprotein M [*Homo sapiens*]
Other Aliases: G3a, HSPC336, MGC22400, NG20
Other Designations: NG20-like protein; alternative name: G3a, NG20
Chromosome: 6; **Location:** 6p21.33
GeneID: 55,937

94. VSNL1

Official Symbol: VSNL1; **Name:** visinin-like 1 [*Homo sapiens*]
Other Aliases: HLP3, HPCAL3, HUVISL1, VILIP
Other Designations: hippocalcin-like protein 3
Chromosome: 2; **Location:** 2p24.3
GeneID: 7447

95. GRIN2A

Official Symbol: GRIN2A; **Name:** glutamate receptor, ionotropic, N-methyl-D-aspartate 2A [*Homo sapiens*]
Other Aliases: NMDAR2A, NR2A
Other Designations: N-methyl-D-aspartate receptor subunit 2A; NMDA receptor subtype 2A gene
Chromosome: 16; **Location**: 16p13.2
GeneID: 2903

96. LAMC1

Official Symbol: LAMC1**; Name:** laminin, gamma 1 (formerly LAMB2) [*Homo sapiens*]
Other Aliases: RP11-181 K3.1, LAMB2, MGC87297
Other Designations: formerly LAMB2; laminin, gamma 1
Chromosome: 1; **Location:** 1q31
GeneID: 3915

97. BCHE

Official Symbol: BCHE; **Name:** butyrylcholinesterase [*Homo sapiens*]
Other Aliases: CHE1, E1
Chromosome: 3; **Location:** 3q26.1-q26.2
GeneID: 590

98. APH1A

Official Symbol: APH1A; **Name:** anterior pharynx defective 1 homolog A (C. elegans) [*Homo sapiens*]
Other Aliases: 6530402N02Rik, APH-1A, CGI-78
Other Designations: anterior pharynx defective 1 homolog A
Chromosome: 1; **Location:** 1p36.13-q31.3
GeneID: 51,107

99. COL18A1

Official Symbol: COL18A1;**Name:** collagen, type XVIII, alpha 1 [*Homo sapiens*]
Other Aliases: KNO, MGC74745
Other Designations: alpha 1 type XVIII collagen; antiangiogenic agent; endostatin; multifunctional protein MFP
Chromosome: 21; **Location:** 21q22.3
GeneID: 80,781

100. GAL

Official Symbol: GAL; **Name:** galanin [*Homo sapiens*]
Other Aliases: GALN, GLNN, MGC40167
Other Designations: galanin-related peptide
Chromosome: 11; **Location:** 11q13.2
GeneID: 51,083

101. LAMA1

Official Symbol: LAMA1; **Name:** laminin, alpha 1 [*Homo sapiens*]
Other Aliases: LAMA
Chromosome: 18; **Location:** 18p11.31
GeneID: 284,217

102. APOD

Official Symbol: APOD; **Name:** apolipoprotein D [*Homo sapiens*]
Chromosome: 3; **Location:** 3q26.2-qter
GeneID: 347

103. GABBR1

Official Symbol: GABBR1; **Name:** gamma-aminobutyric acid (GABA) B receptor, 1 [*Homo sapiens*]
Other Aliases: GABAB(1e), GABABR1, GABBR1–3, GPRC3A, dJ271M21.1.1, dJ271M21.1.2, hGB1a
Other Designations: GABA-B receptor; GABAB, subunit 1c; gamma-aminobutyric acid (GABA) B receptor 1; seven-transmembrane helix receptor
Chromosome: 6; **Location:** 6p21.31
GeneID: 2550

104. NDRG2

Official Symbol: NDRG2; **Name:** NDRG family member 2 [*Homo sapiens*]
Other Aliases: DKFZp781G1938, KIAA1248, SYLD
Other Designations: N-myc downstream regulator 2; N-myc downstream-regulated gene 2; NDR1-related protein NDR2; cytoplasmic protein Ndr1; syld709613 protein

Chromosome: 14; **Location:** 14q11.2
GeneID: 57,447

105. CRH

Official Symbol: CRH; **Name:** corticotropin-releasing hormone [*Homo sapiens*]
Other Aliases: CRF
Chromosome: 8; **Location:** 8q13
GeneID: 1392

106. ADAM10

Official Symbol: ADAM10; **Name:** ADAM metallopeptidase domain 10 [*Homo sapiens*]
Other Aliases: HsT18717, MADM, kuz
Other Designations: a disintegrin and metalloprotease domain 10; a disintegrin and metalloproteinase domain 10; kuzbanian
Chromosome: 15; **Location:** 15q22
GeneID: 102

107. HTR2A

Official Symbol: HTR2A; **Name:** 5-hydroxytryptamine (serotonin) receptor 2A [*Homo sapiens*]
Other Aliases: RP11-147 L20.2, 5-HT2A, HTR2
Other Designations: 5-HT2 receptor
Chromosome: 13; **Location:** 13q14-q21
GeneID: 3356

108. SLC1A2

Official Symbol: SLC1A2; **Name:** solute carrier family 1 (glial high affinity glutamate transporter), member 2 [*Homo sapiens*]
Other Aliases: EAAT2, GLT-1
Other Designations: *H. sapiens* mRNA for glutamate transporter; dJ68D18.1.1 (solute carrier family 1 (glial high affinity glutamate transporter) member 2); dJ68D18.1.2 (solute carrier family 1 (glial high affinity glutamate transporter) member 2); excitatory amino acid transporter 2; glial high affinity glutamate transporter; glutamate/aspartate transporter II; solute carrier family 1, member 2
Chromosome: 11; **Location:** 11p13-p12
GeneID: 6506

109. SLC6A3

Official Symbol: SLC6A3; **Name:** solute carrier family 6 (neurotransmitter transporter, dopamine), member 3 [*Homo sapiens*]
Other Aliases: DAT, DAT1
Other Designations: dopamine transporter; variable number tandem repeat (VNTR)

Chromosome: 5; **Location:** 5p15.3
GeneID: 6531

110. SORL1

Official Symbol: SORL1; **Name:** sortilin-related receptor, containing L(DLR class) A repeats [*Homo sapiens*]
Other Aliases: LR11, LRP9, SORLA, SorLA-1, gp250
Other Designations: mosaic protein LR11; sortilin-related receptor containing LDLR class A repeats
Chromosome: 11; **Location:** 11q23.2-q24.2
GeneID: 6653

111. RNF41

Official Symbol: RNF41; **Name:** ring finger protein 41 [*Homo sapiens*]
Other Aliases: FLRF; NRDP1; SBBI03
Other Designations: This gene encodes an E3 ubiquitin ligase. The encoded protein plays a role in type 1 cytokine receptor signaling by controlling the balance between JAK2-associated cytokine receptor degradation and ectodomain shedding. Alternative splicing results in multiple transcript variants. Nrdp1 controls type 1 cytokine receptor degradation and ectodomain shedding. Nrdp1/FLRF mediates degradation of apoptotic inhibitor proteins (IAPs) and BRUCE (Qiu, 2004).
Chromosome: 12; **Location:** 12q13.2-q13.3
GeneID: 10,193

112. SYT1

Official Symbol: SYT1; **Name:** Synaptotagmin 1 [*Homo sapiens*]
Other Aliases: P65; SYT; SVP65
Other Designations: The synaptotagmins are integral membrane proteins of synaptic vesicles thought to serve as Ca(2+) sensors in the process of vesicular trafficking and exocytosis. Calcium binding to synaptotagmin-1 participates in triggering neurotransmitter release at the synapse. Mutant SYT1 involved in neurodevelopmental disorder.
Chromosome: 12; **Location:** 12cen-q21
GeneID: 6857

113. CDK5

Official Symbol: CDK5; **Name:** Cyclin-dependent kinase 5 [*Homo sapiens*]
Other Aliases: Protein Kinase CDK5 Splicing
Other Designations: CDKs are kinases that interact with cyclins and regulate cell division. However, the CDK5 gene encodes a proline-directed serine/threonine kinase. Unlike other members of the CDK family, this protein does not directly control cell cycle regulation. Instead the CDK5 protein, which is predominantly expressed at high levels in mammalian postmitotic CNS neurons, functions in diverse processes including synaptic plasticity

and neuronal migration through phosphorylation of proteins required for cytoskeletal organization, endocytosis and exocytosis, and apoptosis. In humans, an allelic variant of the gene that results in undetectable levels of the protein has been associated with lethal autosomal recessive lissencephaly-7. Alternative splicing results in multiple transcript variants. Dysregulation of CDK5 is associated with neurodegenerative disorders.

Chromosome: 7; **Location:** 7q36.1–2

GeneID: 1020

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Chapter 22
Clinical Relevance of Humanized Mice

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Keywords Humanized mice • Human immunodeficiency virus • Immunology • Infectious disease • Cancer • Immunotherapy • Vaccines • History of humanized mouse models • Humanized mice for the study of HIV • Expansion of chimeric mice into the development of treatment against bacterial and other viral diseases • Cancer immunotherapy and the utility of humanized mice • Assessment of the impact of substance abuse on HIV infection and human immunity • T cell selection, development, and egress

Core Message

Small animal models have been an instrumental tool in the study of human disease and immunity. The development of humanized mouse models to study the human immunodeficiency virus (HIV) has led, and continues to do so, to a better understanding of viral pathogenesis, development of effective therapies, and the testing of new ones. The evolution of these mouse models has been continuous enabling researchers to address further questions regarding HIV infection and pathogenesis. More importantly, with the increased levels of sophistication, humanized mouse

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models are now being used for the study of other infectious agents, chronic diseases such as cancer, immunotherapy approaches, as well as the development and function of the human immune system. While challenges remain, as these models become more advanced and used further, they will lend better understanding of human immunity and lead to the development of more effective therapeutic approaches against various diseases.

22.1 Introduction

Small animal models such as mice have been quite instrumental in addressing major immunological questions. Over the years, as science has evolved and new challenges posed, the need for more sophisticated chimeric mouse models was increasing. To this end, the past 30 years, a series of humanized mouse models was developed first to address the need for small animal models in HIV and, later as they improved, study other infectious and chronic diseases. In this chapter, we will present and discuss the various humanized mouse models and their various applications.

22.2 Humanized Mouse Models

22.2.1 hu-PBL

The hu-PBL mouse model was one of the early versions of humanized mice developed. This was a very basic model; human peripheral blood lymphocytes were transferred into mice with severe combined immunodeficiency (SCID) [1]. For the generation of these mice, CB17 *scid/scid* mice are implanted with human peripheral blood lymphocytes by a single intraperitoneal injection. Reconstitution of human immune cells is complete in approximately 2 weeks and can last up to 3 months. Cells can be recovered from various tissues such as the peritoneal cavity, spleen, blood, and thymus. The model was developed and used in a handful of studies to study HIV pathogenesis including the effects of cocaine use on virus replication [2–4]. However, the model was overshadowed by its contemporary, the SCID-hu.

22.2.2 SCID-hu

This humanized mouse model was also an early version but, unlike the hu-PBL, was extensively used for more than two decades studying T-cell development, HIV infection and pathogenesis, and gene therapy. The strength of this model lies on two

aspects of it; first, the major strength of this model is the generation of a human lymphopoietic organoid in an immunocompromised mouse. Second, it utilizes the strength, plasticity, and potential of human hematopoietic stem cells. Its main drawback is the reliance on fetal human tissue. This model of CB17 *scid/scid* mice is sublethally irradiated followed by transplantation of fetal thymus and autologous fetal liver pieces under the kidney capsule [5, 6]. Approximately a month later animals are ready for use as the thy/liv implant is established. These mice can be used for up to a year, after which the implant begins to shrink. The concept behind it is that the thymic piece keeps getting replenished with new progenitor cells from the liver piece leading to a continuous production of human T cells. The utilization of the hematopoietic stem cells has allowed researchers to engineer these mice through the genetic modification of human hematopoietic stem cells. Furthermore, while the early studies did not establish whether there was peripheral reconstitution, recent work has suggested that these mice can support T cell only for peripheral reconstitution [7]. However, to date, it is widely accepted that any studies using this mouse model are localized to the organoid and cannot be systemic. This major weakness, the lack of peripheral reconstitution, led to the exploration of the models outlined below.

22.2.3 Bone Marrow-Liver-Thymus (BLT) Model

The BLT humanized mouse model is a major evolution of the SCID-hu. The model addressed the major challenge posed by the SCID-hu, lack of peripheral reconstitution. First, changing the SCID mouse strain used for transplantation and then by adding one more transplantation in the protocol achieved this peripheral reconstitution. More specifically, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice have been shown to support xenografts and better immune reconstitution [8]. These mice lack not only mouse lymphoid cells but also mouse myeloid and NK cells. These properties allow for much improved reconstitution of human immune cells. These mice are transplanted with fetal liver and thymus tissues as in the SCID-hu. After the establishment of the graft (~1 month), the mice are irradiated and transplanted with autologous human hematopoietic stem cells. These progenitors will populate the mouse bone marrow as well as feed the thy/liv implant to generate the various immune lineages. Approximately 2 months later, these mice have fully reconstituted immune systems. Studies have shown that these mice have full reconstitution of all lineages [8–10]. More importantly, these lineages have largely followed the physiological process of maturation; thus, they are very functional immune cells [8–10]. The latter has made this small animal system as an excellent model to study not only HIV but also other human diseases and immunity. The major advantage of this model is the functional human immune system as well as the ability to genetically modify progenitor cells, allowing for immune engineering of these animals [8–10].

22.2.4 HSC Model

This model was developed alongside the BLT mouse model. Like the BLT, it takes advantage of the NSG as well as the BALB/c-Rag1$^{null}\gamma c^{null}$ and BALB/c-Rag2$^{null}\gamma c^{null}$ immunodeficient mouse strains [11–13]. However, this mouse model is distinct from the BLT as it utilizes neonatal mice for transplantations and does not require thymic transplantation, and the source of CD34 cells can be fetal liver or cord blood. More specifically, 1–4-day-old neonate mice are irradiated and injected intrahepatically with purified human hematopoietic stem cells. The source of stem cells as mentioned can be either fetal liver or cord blood. Approximately 2 months later, the mice are reconstituted with human immune cells of all lineages. This model yields more myeloid cells than the BLT; however, the T cells generated are not as mature as in the BLT. The latter can be attributed to the fact the HSC model relies on the murine thymus for differentiation as opposed to the human implant of the BLT.

22.3 HIV and Humanized Mice

The in vivo study of human immunology, infectious disease, and chronic conditions such as cancer was mainly carried out in murine models with some success, thus not underscoring the need for a humanized mouse model. However, the emergence of HIV posed new challenges. There was no murine equivalent of HIV; researchers could not utilize mice as small animal models to study the disease. The only alternative was the use on primates, but the costs were quite prohibitive. Thus, this drove the need for the development of a small animal model that can be used to examine HIV infection, pathogenesis, and treatment, a human/mouse chimera better known as humanized mouse.

The development evolution of these mouse models (as seen in the sections above) was gradual but quite valuable. The first humanized mouse model developed by the McCune group, the SCID-hu [5], addressed, as we will discuss below, a lot of the major questions in HIV infection and pathogenesis. However, due to the lack of peripheral reconstitution, studies were quite limited in scope. The development of the BLT and HSC mouse models allowed more expansive and clinically relevant studies taking the utility of humanized mice to an entirely new level. Here, we will discuss the studies carried out using all the humanized mouse models, the limitations, and implications for treatment.

Viral transmission and infection were studied in all animal models. In the SCID-hu, studies were mainly focused on viral tropism, depletion of CD4 T cells, as well as reconstitution of mice after the introduction of antiretroviral therapy [6, 14–27]. However, this was quite limited as infections were carried out by direct injection of the thy/liv. Thus, studying the routes of infection was not feasible. The

latter is quite important if these models were to be used to study chemoprophylaxis. However, with the advent of the BLT and HSC models, this became possible. Seminal studies by the Garcia and Akkina groups demonstrated, using the BLT and HSC mouse models, respectively, infection via the mucosal routes using both R5 and X4 tropic viruses [12, 28–34]. This was a major breakthrough as these models could now be used to (1) study HIV infection in the gut, a major site of HIV replication and an important reservoir, and (2) test various pre-exposure prophylaxis protocols such as antiviral gels.

Antiretroviral treatment (ART) has had a major impact in the control of HIV disease. Thanks to the development and evolution of antiretroviral drugs, HIV mortality has significantly decreased, and the quality of life for many HIV-infected individuals has vastly improved. All versions of humanized mice were used to test new treatments, examine new regimens, and assess drug efficacy [12, 25, 27–36]. Furthermore, with the development of the BLT and HSC models, ART studies begun to address even more relevant challenges in HIV disease. More specifically, while the use of ART has resulted in major advances in controlling the virus, the persistence of long-term viral reservoirs has prevented viral eradication. One running hypothesis is that ART drugs have varying half-lives and bioavailability in various tissues. Thus, ART studies using humanized mice have recently focused in addressing these very questions [33]. Data from these studies suggest that ART drugs are at high concentrations in tissues such as the mucosa more so than in blood [33].

In addition to ART, the use of neutralizing antibodies has been explored as a form of pre-exposure prophylaxis. Studies by the Baltimore group used an approach which they coined as vectored immunoprophylaxis. More specifically, they used an adeno-associated virus (AAV; serotype 8) vector to express the VRC07G54W antibody after injection into the muscle [37, 38]. The vector not only produced high levels of antibody but also protected humanized mice against infection. The Nussenzweig group also demonstrated similar results; however, they showed that having a broader repertoire of antibodies provided a more sustained protection [39–43].

One of the major strengths in using these small animal models to study HIV infection is the ability to assess viral latency and persistence. In all versions of humanized mice, research groups were able to establish HIV latency. The Zack group first demonstrated establishment of latency in humanized mice using the SCID-hu model [44, 45]. In these seminal studies, latently infected thymocytes were isolated, reactivated ex vivo, and targeted by immunotoxins. These studies were the first to demonstrate not only establishment of latency in vivo but also that lately infected cells can be reactivated and targeted for killing. The contemporary models, HSC and BLT, did recapitulate the establishment of latency [46–48]. As these models are more advanced, studies have also been focused on looking at reservoir sites, testing various reactivators, as well as examining approaches to purge and eliminate the reservoir [49, 50].

Gene therapy approaches to either protect from infection or provide treatment against the virus have been at the forefront of HIV research in the past few years.

The main focus has been to develop ways where the viral reservoir can be purged and then targeted for eradication. Here, humanized mice have been proven to be very useful because of the ease and flexibility in constructing them [9, 10]. The latter lies on the fact that for the making of these mice, one needs to use human hematopoietic stem cells. One of the key advantages of these cells is that they can give rise to various immune lineages and they can be genetically modified [9, 10]. Thus, one can generate humanized mice with an engineered immune system attacking HIV-infected cells or engineer the targets of the virus, CD4-expressing cells, to be resistant. Different groups have targeted the expression of CCR5, one of HIV co-receptors, using siRNA and have demonstrated that the cells are resistant to infection [9, 51, 52]. This approach is quite feasible, as it has been well demonstrated that abolishing expression of the receptor does not interfere with immune function and/or hematopoietic stem cell development. As a result of these studies, clinical studies are underway to test this approach. Other groups have explored a more proactive approach by engineering the immune system to attack HIV-infected cells. One of the major challenges of HIV as a chronic disease is immune escape and immune exhaustion. The generation of fresh, effective effectors can provide another tool in eradicating the virus. Hematopoietic stem cells have been genetically modified to express an HIV-specific T-cell receptor resulting in the generation of mature CD8 T cells that were able to kill HIV-infected cells [53, 54]. In addition, along the same lines, a CD4-zeta common antigen receptor (CAR) was introduced to stem cells to target gp120. The idea is that infected cells would be expressing the viral protein and thus be targeted by these CAR-expressing effector cells and killed [55]. Pilot studies have shown that this approach can also work in the efforts to clear the viral reservoir.

Overall, these gene therapy approaches while promising are technically quite challenging and costly. Thus, while potentially effective, the feasibility of such a therapy is still under investigation, especially since a number of studies have suggested that acute and/or chronic HIV infection can impact progenitor cells [56–58]. The latter do express the co-receptors for infection by HIV and have been shown to be latently infected [56–58].

Finally, neuroAIDS is an area of active investigation in the HIV pathogenesis field. As populations with HIV live longer thanks to ART, they are faced with new health challenges. The sustained inflammatory state due to baseline viremia has been linked to an increase on HIV-associated, non-AIDS diseases such as cancer, heart disease, and neurological problems [59]. Furthermore, long-term administration of ARTs is also believed to contribute to some of these ailments [60]. Thus, humanized mice can play a major role as they can be used as surrogates to test various interventions [36, 61–63]. Studies to date have demonstrated the presence of human immune cells in the brains of humanized mice, such as macrophages and microglia. However, as other cells of the brain are important in HIV infection such as astrocytes, there have been challenges in generating humanized mice to study neuroAIDS. To date, researchers have adoptively transferred neural progenitors to

try to get reconstitution with some success. This is definitely an area of HIV research that is in dire need of improved humanized mouse models.

22.4 Humanized Mice as a Model for Stimulant Abuse

Studies have linked HIV infection and substance abuse since the start of the epidemic [64–66]. However, the many studies outlined above have neglected this high-risk population. A number of in vitro studies have well demonstrated that stimulants such as cocaine and methamphetamine have major immunomodulatory properties and enhance HIV infection [64, 65, 67]. However, the use of humanized mice in this area of HIV is surprisingly quite limited. Considering the major challenges in recruiting and studying human subject cohort that use and misuse drugs, one would expect that humanized mice would be used more extensively to understand the role of controlled substances on HIV infection.

Studies by the Baldwin group used both the hu-PBL and SCID-hu mouse models to show that cocaine exposure resulted in increased HIV infection [4, 68]. In those early studies, it was demonstrated that cocaine increased CCR5 and CXCR4 expression, both HIV co-receptors. In addition, they showed that the σ1 receptor was key in the cocaine-mediated effects. We employed the BLT humanized mouse model to further examine the effects of acute cocaine use on HIV infection [69]. Using an acute exposure model, we found that 7-day cocaine exposure led to increased expression of CCR5 and inflammatory cytokines (IL-6 and IFN-γ). Following this exposure, the mice were infected and viral kinetics was monitored. We saw that mice pretreated with cocaine demonstrated faster viral kinetics and increased levels of viral mRNA expression in blood and the spleen. Furthermore, the cocaine-treated mice had blunted CTL responses. Thus, based on the acute cocaine exposure model, this stimulant not only promoted a higher inflammatory state to help the establishment of a productive infection but also blunted the immune system's effector arm to limit the virus and clear infected cells. These studies underscore the multifaceted impact of stimulants on HIV infection and pathogenesis. Moreover, this demonstrated the utility of the humanized mouse model in understanding and dealing with the effects of substance abuse on HIV infection.

22.5 Other Infectious Diseases

The peripheral reconstitution of human immune cells in the BLT and HSC chimeric mice has enable researchers to begin using these models for other human diseases. While the list below is not exhaustive and new studies may have come out already, it is indicative of the versatility and utility of these models going beyond the narrow confines of HIV infection.

22.5.1 Salmonella typhi

Researchers have used humanized mouse models to study human bacterial infections. Both the BLT and HSC models have been used to establish a humanized mouse model to study *S. typhi*. Three independent studies have successfully infected humanized mice with the bacterium intravenously [70–72]. In all studies, tissue pathology resembled that of the human condition. Moreover, following infection, expression of cytokines such as IL-6, IL12, IL-8, IL-10, TNF-α, IFN-γ, MCP-1, and IP-10 were upregulated at different points after infection. In one study, antibody production was detected in infected mice [71]. Finally, in the same study, the authors tested a number of mutants with the intent to develop and test vaccine approaches [71]. While these models have proven to be promising in studying the disease, the rapid progression of infection and subsequent death of animals do not permit for chronic disease studies yet. Nevertheless, the model can be useful for the development of protective interventions as well as early protective strategies.

22.5.2 CMV

Humanized mouse models have been used to study human CMV infection, pathogenesis, and antiviral drug efficacy. The earliest study utilized the thy/liv model in which they demonstrated that the Toledo CMV strain replicated very efficiently in this mouse model [73]. Infection lasted for about 9 months, and the site of viral replication was the thymic epithelium. The authors were able to suppress viral replication using ganciclovir. Since the FDA-approved drugs, ganciclovir (GCV), valganciclovir, foscarnet (PFA), cidofovir (CDV), and fomivirsen, have major limitations, the development of new therapeutic agents is of interest. Humanized mouse models can serve as a platform to assess efficacy and toxicity of new classes of drugs. Using the thy/liv model, Kern and colleagues [74] tested three benzimidazole nucleosides, 2-bromo-5,6-dichloro-(1-β-D-ribofuranosyl)benzimidazole (BDCRB), GW275175X (175X), and GW257406X (1263 W94, maribavir [MBV]) against GCV. Other studies using the CD34-NSG model established humanized mice as a system to examine the viral latency and reactivation in various cell types [75, 76]. The major gap in literature is immunological studies that can assess the effects of CMV in immune exhaustion [75].

22.5.3 EBV

Extensive studies have been carried out in the study of EBV using humanized mice [77]. Many aspects of the disease as well as the immune responses to the virus have been recapitulated using both the BLT and HSC models [77, 78]. One of the

malignancies developed in EBV is the appearance of B cell lymphoproliferative disease. Studies have been able to replicate the disease in humanized mice, and through the generation of EBV mutants, researchers have begun examining the mechanisms behind the development of this malignancy [79–82]. Furthermore, studies using humanized mice have potentially established a direct link between EBV and the development of rheumatoid arthritis [83]. This opens up significant opportunities in the development of treatment against the disease. Finally, work by several groups demonstrated using humanized mice the development of cell-mediated and humoral responses against the disease [8, 84–87]. Thus, they can be used as a model system for the development of vaccines and other immunotherapies against EBV. However, despite these advantages, most EBV-caused malignancies have not yet been recapitulated in this model [77]. Hopefully, later updated versions of these mice will make this possible.

22.5.4 Dengue Fever

Humanized mice have been used to study dengue fever [88–91]. Both the HSC-NSG and BLT humanized mouse models have been used to determine if they can be appropriate for understanding the disease. All studies, at varying levels, have demonstrated that humanized mice can be used to examine viral replication and disease progression. Regardless of the humanized mouse model, animals developed symptoms of erythema, fever, and loss of platelets. In both model systems, researchers have been successful to show the development of immune responses against the virus. Infected mice had elevated expression of various inflammatory cytokines including but not limited to IFN-α, IFN-γ, TNF-α, IP-10, MCP-1, and VEGF. Furthermore, in all studies, the mice developed humoral responses against the virus as demonstrated by the presence of IgM and IgG in the blood. Frias-Staheli and colleagues [91], using the BLT mouse model, were able to demonstrate effector T-cell immune responses based on T-cell maturation in the mice and ex vivo stimulation of effector T cells. One of the most fascinating studies though is the one by Cox and colleagues [89]. The majority of all studies using infectious agents and humanized mice use artificial routes of inoculation. However, in this study the authors used the natural route of transmission to infect mice and compared with inoculation. The author used *Aedes aegypti* to infect the mice. In their studies they compared against traditional inoculation protocols as well as the efficiency of bite number. Based on their results, infection by the natural method led to more severe symptoms and replication correlated with the number of bites the mice received. This has major implications in the study of the disease, as conventional inoculation routes may not reflect the true breadth and impact of the infection. Finally, the BLT model was also used to test antiviral therapy [91]. Thus, based on this, dengue fever can be studied using humanized mice. This is very exciting as these models escape the narrow confides of HIV infection. Moreover, it is clear the BLT model is more complete in understanding the disease.

22.6 Humanized Mice and Basic Immunology

The presence of a thymic organoid in humanized mice offers the opportunity to study T-cell development. Studies to date looking at T-cell development have focused exclusively on murine models alone. However, there are key differences between human and mouse T-cell development, and, thus, revisiting all human T-cell development in these systems is very important.

T cells are key players in adaptive immunity, specifically cell-mediated immunity. Briefly, T cells are comprised of several subsets, each of which is responsible for a specific task in the immune response. These tasks include cytotoxic activity (direct cell killing by CD8+ T cells or cytotoxic T lymphocytes (CTLs)), regulation of other immune cells (CD4 and CD8 regulatory T cells or CD4/CD8 Tregs), helper activity for CD4 T lymphocytes (Th1, Th2, and T follicular helper cells or Tfh), and protective immunity against extracellular bacteria and fungi (Th1, Th2, Th17, Th22, Tfh, and γδ T cells). Development of the various T-cell subsets begins in the thymus, but differentiation into the specific subsets occurs in the periphery after egress from the thymus [92]. In the thymus, T cells not only mature but also undergo training during which they are educated to target against antigens that are nonself and foreign. This is to ensure that our immune system does not turn against its own host.

The Uittenbogaart group examined thymocyte egress via the sphingosine-1-phosphate (S1P)/sphingosine-1-phosphate receptor 1 (S1PR1) pathway using both neonatal thymus and thy/liv tissues from humanized mice. Sphingosine-1-phosphate (S1P) is a signaling sphingolipid molecule, also known as a *lysosphingolipid*, with multiple tasks throughout the body [93]. S1P functions in a myriad of roles in humans, from regulation of cell death (specifically, suppression of apoptosis [94]) and survival [95, 96], immune responses [97] as well as autoimmune conditions and allergies [98–100], and B cell development [101]. Among its many roles, S1P is crucial for proper lymphocyte trafficking between the lymphoid organs and about the periphery [102, 103] as well as the trafficking of various other cell types such as smooth muscle and endothelial cells [104]. The S1P receptor family is known to include S1PR1–5; these receptors are present on various cell types [105]. In their studies (Resop et al. in press), Resop and colleagues demonstrated that a subset of human thymocytes, $CD3^{hi}CD27^{+}CD45RA^{+}CD62L^{+}$, was responsive to S1P. Gene expression analysis revealed that S1PR1 receptor was highly expressed in this subset of thymocytes. This observation was confirmed at the protein level via flow cytometry. Moreover, the authors showed that the receptor was functional, and they were able to recapitulate their observations using the thymic organoid from humanized mice. These studies make the humanized mouse model very relevant for the study of human egress. The latter can have major implications in the treatment of immunodeficiency and/or lymphoproliferative diseases.

22.7 Humanized Mice and Cancer Immunotherapy

Humanized mice were mainly developed to study HIV as there was no homologous small animal model to carry out such studies like in cancer. However, as cancer immunotherapy develops as an alternative to treat patients, researchers have seen the utility of humanized mice. The main goal of such approach is to establish a system of generating an effective, stable, and renewable T-cell population that specifically targets tumors. Our group developed tumor-specific T cells in vivo from genetically modified human hematopoietic stem cells (hHSC) through the use of a human/mouse chimera model [10, 106, 107]. Transduced hHSC expressing an HLA-A*0201-restricted melanoma-specific T-cell receptor were introduced into humanized mice, resulting in the generation of a sizable melanoma-specific naive CD8 T-cell population. Following tumor challenge, these transgenic CD8+ cells limited and cleared human melanoma tumors in vivo. The T cells also underwent proper thymic selection and established long-term bone marrow engraftment. Thus, this study was the first of its kind because it demonstrated not only that the immune system can be engineered from modified progenitors but also that the engineered immune cells were functional as shown by in vivo clearance of tumors. These studies have led to the establishment of clinical studies underway. Finally, these studies were carried out using in vivo positron emission tomography (PET) imaging to monitor migration of T cell as well as tumor clearance [10, 107]. As a result, these mice have also become a useful tool to test and evaluate new live imaging techniques that can lead to improved cancer diagnostic tools [107].

22.8 Conclusions

Humanized mouse models have evolved over time and become more sophisticated. As a consequence, the research community has been able to address more complex questions on HIV disease and pathogenesis. However, the more contemporary models can be used to study other human diseases. The latter is a major advance in the use of small animal models to study infectious and chronic conditions as well as human immunity. The use of purely mouse models led to major discoveries in these fields. Yet, their relevance, due to difference among species, always undermined their true impact in the development of effective therapies. Finally, humanized mice are not of course the perfect models. The presence of graft versus host disease, the limited presence of immune cells in tissues such as the brain, and the difficult task in obtaining fetal tissues to generate these models are still challenges. However, these models will continue to evolve, minimizing present disadvantages.

Model	Name of mice	Implanting method	Reconstitution	Used HIV	Strengths	Disadvantages
hu-PBL	CB17 *scid/scid* mice (scid = severe combined immunodeficiency)	Single intraperitoneal injection (human peripheral blood lymphocytes)	2 weeks	HIV pathogenesis (the effects of cocaine use on virus replication)	Fast and peripheral reconstitution	Overshadowed by SCID-hu Short term reconstitution
SCID-hu	CB17 *scid/scid* mice	Sublethal irradiation followed by transplantation of fetal thymus and autologous fetal liver pieces under kidney capsule	1 month	T-cell development, HIV infection, HIV pathogenesis, gene therapy	Generation of a human lymphopoietic organoid in an immunocompromised mouse Utilize the strength, plasticity, and potential of human hematopoietic stem cells	Reliance on fetal human tissue Lack of peripheral reconstitution (T cell only reconstitution) Studying the routes of infection (chemoprophylaxis) was not possible
BLT mode (bone marrow-liver- thymus)	NOD. Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/Szl (NSG)	Transplantation with fetal liver and thymus tissues (as in SCID-hu) ➔ Irradiation and transplant with autologous human hematopoietic stem cells	2 months	HIV and also other human diseases and immunity	Address lack of peripheral reconstitution Different SCID mouse strains that support xenografts and better immune reconstitution Full reconstitution of all lineages All lineages largely follow the physiological process of maturation ➔ functional immune cells Able to genetically modify progenitor cells ➔ allow immune engineering of animals	Reliance on fetal human tissue

HSC model	NSG and BALB/c-Rag1$^{null}\gamma c^{null}$, BALB/c-Rag2^{null} γc^{null}	Irradiation and intrahepatic injection with purified human hematopoietic stem cells into 1–4 day old neonate mice	2 months	Study HIV infection in the gut, a major site for HIV infection	Different SCID mouse strains that support xenografts and better immune reconstitution Utilize neonatal mice for transplantation ➔ does not require thymus transplantation and source of CD34 cells can be fetal liver or cord blood Yield more myeloid cells than the BLT model	T cells generated are not as mature as in BLT because this model relies on murine thymus for differentiation as opposed to human implant of BLT

Humanized mice and their applications

Diseases	hu-PBL	SCID-hu	BLT	HSC
HIV	Viral transmission Test new treatments, examine new regimens, and assess drug efficacy Assess viral latency and persistence	Viral transmission Viral tropism, depletion of CD4 T cells, and reconstitution of mice after the introduction of antiretroviral therapy (ART) Test new treatments, examine new regimens, and assess drug efficacy Assess viral latency and persistence (Zack group) [44, 45] Gene therapy [9, 10, 51–58] NeuroAIDS [36, 59–63] and testing various interventions	Viral transmission Can be used to study chemoprophylaxis and the routes of infection HIV infection in the gut, a major site for HIV replication and an important reservoir Test various pre-exposure prophylaxis protocols such as antiretroviral gels Test new treatments, examine new regimens, and assess drug efficacy Study viral reservoirs and viral eradication and whether ART drugs have varying half-lives and bioavailability [33] Study the use of neutralizing antibodies as a form of pre-exposure prophylaxis ➔ coined vectored immunoprophylaxis [37, 38] Assess viral latency and persistence [46–48], in addition to looking at reservoir sites, testing various reactivators and examining approaches to purge and eliminating the reservoir Gene therapy [9, 10, 51–58] NeuroAIDS [36, 59–63] and testing various intervention methods	
Stimulant abuse	Used to show that cocaine exposure resulted in increased HIV infection [4, 68] (Baldwin) BLT further used to examine the effects of acute cocaine use on HIV infection [69]		The use of humanized mice in this area of HIV and stimulant abuse is surprisingly quite limited	
Salmonella typhi	*BLT and HSC:* Three independent studies have successfully infected humanized mice with *S. typhi* [70–72] Used to study expression of cytokines at different points after infection, antibody production, and different mutants with the intent to develop and test vaccine approaches [71] Useful for development of protective interventions and protective strategies Limitation: rapid progression of infection and subsequent death of animals result in inability to study chronic diseases			
CMV	*BLT and HSC:* Used to study human CMV infection, pathogenesis, and antiviral drug efficacy and toxicity [73, 74] Used to examine the viral latency and reactivation in various cell types [75, 76]			

(continued

(continued)

Diseases	hu-PBL	SCID-hu	BLT	HSC
EBV	*BLT and HSC:* Used to examine many aspects of the disease as well as the immune responses to the virus, including malignancies and their mechanisms [77–82] Establish a direct link between EBV and the development of rheumatoid arthritis [83] Demonstrate the development of cell-mediated and humoral responses against EBV [8, 84–87] Can be used as a model system for development of vaccines and other immunotherapies against EBV Limitation: EBV-caused malignancies have not been recapitulated [77]			
Dengue fever	*BLT and HSC:* Used to examine viral replication and disease progression Used to show development of immune responses against virus [88–91] Used to test antiviral therapy [91]			
Basic immunology	Thy/liv tissues were used to examine thymocyte egress (Uittenbogaart group) [93–105] Shown to be very relevant for the study of human egress and thus have major implications in the treatment of immunodeficiency and/or lymphoproliferative diseases			
Cancer immunotherapy	Used to establish a system of generating an effective, stable, and renewable T-cell population that specifically targets tumors [10, 106, 107] Used to demonstrate that the immune system can be engineered from modified progenitors and that the engineered immune cells were functional as shown by in vivo clearance of tumors Used as a useful tool to test and evaluate new live imaging techniques that can lead to improved cancer diagnostic tools [107]			

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Conflict of interest The authors report no conflicts of interest.

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Chapter 23
HIV and Stroke

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Keywords Human immunodeficiency virus • Cardiovascular disease • Cerebrovascular disease • Stroke • Ischemic stroke • Risk factors • Antiretroviral therapy

Core Message
Approximately 795,000 Americans suffer from a new or recurring stroke every year, claiming more than 130,000 lives per year. While there has been an overall decrease in the number of stroke hospitalizations in the United States, the number of stroke hospitalizations in the HIV-infected population continues to increase. Stroke has often been reported as a complication of AIDS; however, limited data exist that address the mechanism of the risk of HIV-/AIDS-associated stroke. HIV treatment, specifically HARRT (PI and NNRTI), has been linked with metabolic syndrome and accelerated atherosclerosis, which may lead to an increased stroke risk.

23.1 Introduction

Stroke is the fifth leading cause of death and of preventable disability and a major source of healthcare costs in the United States [1]. In 2015, Americans paid approximately $34 billion in stroke-related medical costs that include hospital services,

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medications, and time off from work. Approximately 795,000 Americans suffer from a new or recurring stroke every year, an average of one stroke every 40 s. Stroke claims more than 130,000 lives per year, affecting more African-American than Caucasians. Stroke mortality remains higher in the southeastern region defined as the "stroke belt," including the following eight states: Alabama, Arkansas, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Tennessee [2]. The increased incidence of stroke in these regions is very complex, but some of the risk factors include consumption of fried and high-fat foods, tobacco smoking, and less access to healthcare in many of these states. As the US population continues to grow, the prevalence of stroke in the general population is predicted to increase. By 2030, it is estimated that nearly 4% of the US population will have suffered a stroke. Individuals over the age of 65 have an increased risk of stroke, and this population is expected to grow substantially over the next two decades, resulting in an increase prevalence of stroke as a whole [3].

Stroke, cerebrovascular accident (CVA), or "brain attack" is defined as a sudden onset of neurological deficits of a vascular origin lasting for greater than or equal to 24 h and confirmed on brain scan (CT or MRI) [4]. Stroke occurs when an area of the brain does not receive adequate blood flow due to a blocked or ruptured blood vessel, resulting in deprivation of oxygen and nutrients, as well as an accumulation of toxic metabolites. The decrease in blood supply can lead to severe and lasting damage to the neural tissue. Blockages can be caused by a blood clot or rupture of a blood vessel and can result in the loss of function in the affected area of the brain. Damage to the neural tissue may manifest in sensory, motor, and speech deficits. Signs and symptoms of a stroke include the onset of one-sided weakness or numbness and difficulty with vision, speech, thought, or coordination [1]. Sometimes these symptoms will improve within 24 h, often within minutes, with no evidence of stroke seen on a brain scan and is referred to as a "mini-stroke" or transient ischemic attack (TIA). A TIA is caused by a temporary clot. The individuals affected by "mini-strokes" are at an even higher risk for subsequent strokes [1].

Strokes can be classified into two categories: ischemic or lack of blood supply to the brain and hemorrhagic or bleeding in and around the brain. The ischemic strokes are 87% more common and are similar in mechanism to a heart attack, in that ischemic strokes are caused by the hardening of the blood vessels (*atherosclerosis*) or a blood clot (*thrombus*). A hemorrhagic stroke results from a ruptured blood vessel in the brain, increasing pressure, and swelling. Strokes can be treatable if diagnosed within a few hours of symptom onset, although frequently they go untreated because of a missed diagnosis. In fact, patients who arrive to the emergency room within 3 h of symptom onset are less disabled 3 months post stroke than those who received delay of care or no care at all [5].

Over 1100 primary and comprehensive stroke centers, healthcare systems that have been awarded a Certificate of Distinction by the Joint Commission, exist within the United States. These centers make exceptional efforts to foster better patient outcomes related to stroke care. However, many telemedicine network programs exist between these primary and comprehensive stroke centers and underserviced healthcare systems, offering multidisciplinary care and state-of-the-art approaches to care for patients affected by stroke [5].

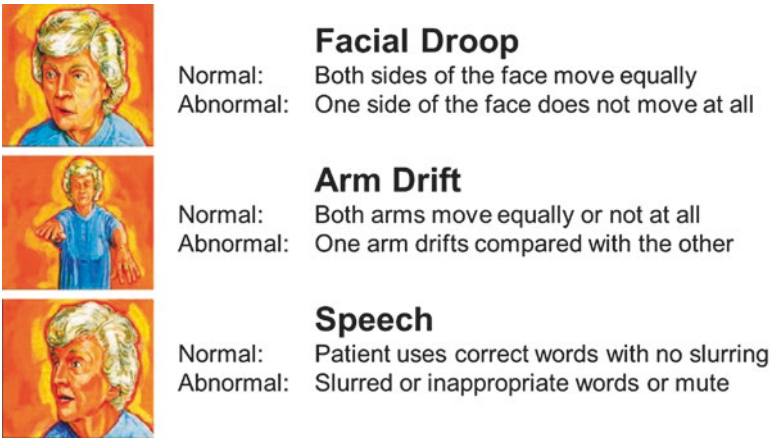

Fig. 23.1 FAST – a simple means of screening for stroke. F, facial droop; A, arm/limb weakness; S, speech difficulty; and T, time to dial 911

The American Heart and Stroke Associations recommend using FAST (facial droop, arm/limb weakness, speech difficulty, time to dial 911) as a simple screening tool used to quickly recognize a stroke and improve outcomes; see Fig. 23.1. Abnormal facial droop is present when one side of the face does not move at all or moves unequally compared to the opposite side. When one arm drifts either slightly or considerably compared to the other, this is abnormal and can be an early sign of a stroke. An individual showing early signs of a stroke may also have slurred speech, use inappropriate words, or be unable to speak at all. When these abnormalities are present, it is important to call 911 as soon as possible to request transport to the nearest hospital equipped for stroke treatment and care [5].

23.2 Stroke Treatment

Current treatments of ischemic stroke revolve around salvaging the penumbra or area that is functionally impaired, due to a lack of oxygen, but structurally sound. When stroke patients reach a hospital emergency department (ER) within 3 h, they are subjected to brain scans (CT or MRI) and can be treated with a clot-busting medication called tissue plasminogen activator (tPA), the gold standard treatment for ischemic strokes. tPA works by dissolving the blood vessel blockage and restoring blood flow to the stroke-affected part of the brain. If administered within a 3-h window from stroke symptom onset, tPA may significantly improve the chances of stroke recovery. The National Institute of Neurological Disorders and Stroke (NINDS) [6] study suggests that patients who received tPA in accordance with a strict protocol were at least 30% more likely to recover within 3 months of the stroke without any significant disability. In some medical centers with interventional capabilities, endovascular catheter-based clot retrieval devices can be used

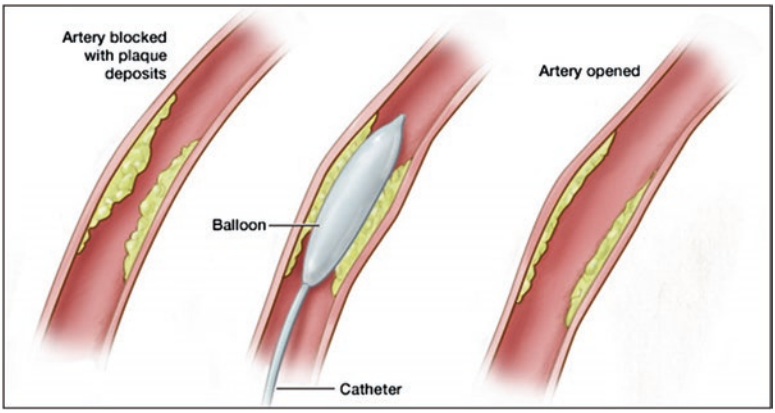

Fig. 23.2 Balloon angioplasty, where a balloon catheter is inserted to the site of occlusion and inflated to increase blood flow

(up to 8 h from stroke symptom onset), and further improvement of the stroke deficits may occur [6]. When arteries (e.g., common carotid artery) show signs of plaque buildup or blockages, one of several surgical procedures may be necessary such as (1) carotid endarterectomy, a surgery to remove plaque buildup from the carotid arteries; (2) balloon angioplasty, a procedure where a balloon catheter is inserted to the site of occlusion and inflated there to increase blood flow, see Fig. 23.2; or (3) stent placement where an artificial tubular support piece is inserted at the occluded area to dilate the stenotic blood vessel, see Fig. 23.3.

Endovascular procedures may also be used to treat certain hemorrhagic strokes similar to the procedure used for treating ischemic strokes. This less-invasive procedure involves the use of a catheter introduced through a major artery in the leg or arm that is guided to the cause of the hemorrhage and then deposits a mechanical agent, such as a coil, to prevent rupture or further bleeding; see Fig. 23.4. For strokes caused by a bleed within the brain or by an abnormal tangle of blood vessels or arteriovenous malformation (AVM), surgical treatment may be a consideration to stop the bleeding. If the bleed is caused by a ruptured aneurysm, swelling of the vessel that causes a rupture, a metal clip can be placed surgically at the base of the aneurysm to secure it [5].

23.3 Stroke Prevention

23.3.1 Guidelines for the Primary Prevention of Stroke

According to the American Heart and Stroke Associations, 80% of strokes are preventable. The current standards for prevention strategies for ischemic stroke include blood thinners, cholesterol-lowering medications, lifestyle changes, and treatment of risk factors such as high blood pressure [5].

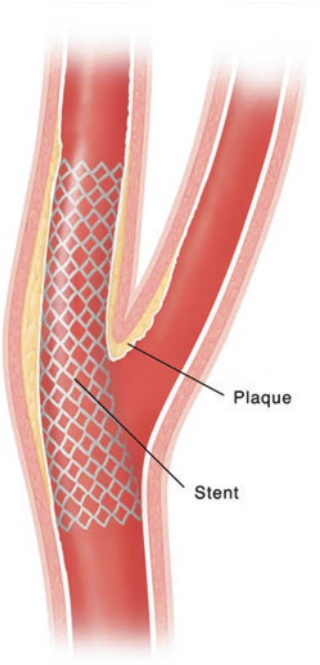

Fig. 23.3 Stent placement, where an artificial tubular support piece is inserted at the occluded area to dilate the stenotic blood vessel

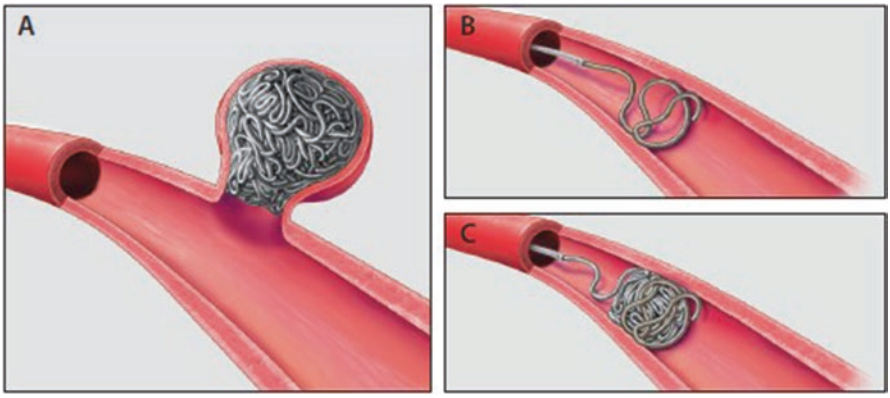

Fig. 23.4 Ruby coil filling in an aneurysm (**a**). Illustration of the peripheral occlusion device system (**b**, **c**)

The 2014 American Heart Association/American Stroke Association Guidelines for the Primary Prevention of Stroke suggests that modifiable risk factors that include high blood pressure, high cholesterol, diabetes, physical inactivity, diet, nutrition, obesity, and cigarette smoking can be crucial in the primary prevention of stroke. High blood pressure is the most important modifiable stroke risk factor because the treatment of high blood pressure is one of the most effective strategies

for preventing both ischemic and hemorrhagic strokes. Patients who have high blood pressure should be treated with antihypertensive drugs with a target blood pressure of 140/90 mmHg. Patients with high cholesterol are estimated to have a high 10-year risk for cardiovascular events and should seek treatment with a statin for primary prevention of stroke. Individuals affected by diabetes should seek a comprehensive program that includes the control of high blood pressure and glycemic control to reduce microvascular complications and the risk of stroke. Capable adults should take part in moderate to vigorous aerobic physical activity for at least 40 min 3–4 days a week. Additionally, epidemiological studies and randomized trials have determined that diets low in sodium, high in potassium, and rich in fruits and vegetables may help reduce the risk of stroke. Such diets included the Mediterranean- and Dietary Approaches to Stop Hypertension (DASH)-style diets. These diet styles focus on overall well-being and on eating more plant-based foods such as fruits, vegetables, nuts, and legumes. It also prefers the use of olive oil instead of butter, spices instead of salt for seasoning, and replacing red meat with lean sources such as fish and poultry. In addition to dietary changes, overweight (BMI = 25–29 kg/m^2) and obese (BMI > 30 kg/m^2) individuals should aim for weight reduction in order to lower blood pressure and thus reduce the risk of stroke. Finally, cigarette smoking is known to increase the risk of ischemic stroke and subarachnoid hemorrhages. Studies have shown a reduction in stroke risk due to smoking cessation and community-wide smoking bans. To assist active smokers in quitting, both drug therapy (nicotine replacement) and counseling are recommended [7].

23.3.2 Guidelines for the Secondary Prevention of Stroke

Individuals who have suffered an ischemic stroke or TIA are at high risk for a recurrent cardiovascular event. On average, individuals having had a previous stroke or TIA are at 3–4% increased risk for a recurrent ischemic stroke within the first year. The estimated risk for individuals is dependent upon many additional factors including comorbidities and adherence to preventive therapies. Due to the increased risk of recurrent ischemic events, the American Heart Association and American Stroke Association have prepared the Guidelines for the Prevention of Stroke in Patients with Stroke and Transient Ischemic Attack for prevention of future ischemic events among survivors of strokes or TIAs [8].

Most imperative in the prevention of secondary stroke is the treatment of high blood pressure, defined as systolic blood pressure greater than or equal to 140 mmHg or a diastolic pressure greater than or equal to 90 mmHg. The prevalence of high blood pressure among patients with a recent ischemic stroke is approximately 70%; therefore, patients with documented blood pressures greater than or equal to 140 mmHg systolic or greater than or equal to 90 mmHg in the days following an initial stroke or TIA should be started on a blood pressure therapy regimen. For patients who have an initial stroke or TIA assumed to be of ath-

erosclerotic origin and are documented to have a LDL-C level greater than or equal to 100 mg/dL, a statin therapy with lipid-lowering effects is recommended in an effort to reduce the risk of recurrent events. All patients, after an initial stroke or TIA, should also be screened for diabetes by testing fasting plasma glucose, hemoglobin A1c, or an oral glucose tolerance test. However, hemoglobin A1c may be more accurate in the immediate time period following the event. Pre-existing American Diabetic Association guidelines for glycemic control is recommended for patients with stroke or TIA who may also be prediabetic or diabetic. Nutritional assessments should also be conducted on these patients to test for signs of overnutrition or undernutrition, and patients should be referred for individualized nutritional counseling as necessary. Patients with stroke or TIA should reduce their sodium intake to approximately 2.4 g per day or less than 1.5 g per day to further reduce blood pressure. More evidently, these patients should also follow diets like the Mediterranean and DASH diets that are low in sodium, high in potassium, and rich in fruit and vegetables to reduce their recurrent stroke risk. Capable adults should also make an effort to participate in moderate to vigorous aerobic physical activity at least three to four times a week. These sessions should last on average 40 min and should be sufficient to break a sweat or noticeably raise the heart rate (e.g., walking briskly, exercise bicycle). Healthcare providers should also strongly advise those patients who have had a stroke or TIA and have smoked in the last year to quit and avoid secondhand tobacco smoke. In an effort to help patient quit smoking, providers can offer counseling, nicotine replacement products, and oral smoking cessation medications [8].

23.4 Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is a blood-borne virus typically transmitted via sexual intercourse, shared intravenous drug paraphernalia, and mother-to-child transmission, which can occur during the birth process or during breastfeeding. HIV disease is caused by infections with HIV-1 or HIV-2, which are retroviruses in the *Retroviridae* family, *Lentivirus* genus. HIV causes immunosuppression via a decreased CD4 T-cell count which leads to opportunistic infections, neoplasms, vasculopathies, and AIDS. HIV cells may activate the vascular endothelium, increasing its permeability and allowing for leukocyte invasion and thus chronic inflammation. This inflammatory process is what links HIV infection to accelerated atherosclerosis [9]. Chemokines are released from damaged endothelium, as a signal for chemotaxis, leading to further aggregation of leukocytes and monocytes. Prolonged exposure to HIV-infected cells leads to increased reactive oxygen/nitrogen species (ROSs/RNSs) causing cellular damage through free radical formation. In addition, exposure of subendothelial collagen of the blood vessels to thrombotic factors in the blood leads to a prothrombotic state, which may ultimately lead to a thrombotic occlusion of the cerebrovasculature causing an ischemic stroke. According to the World Health Organization, HIV infection average survival is estimated at 10 years

due to the progression to AIDS resulting in a plethora of associated life-threatening diseases including tuberculosis, varicella zoster virus, and syphilis, all of which have been linked to increase stroke risk [10].

23.5 Stroke and Human Immunodeficiency Virus

HIV infection is strongly associated with stroke in the young (15–44 years of age) [11–14]. While there has been an overall decrease in the number of stroke hospitalizations in the United States, the number of stroke hospitalizations in the HIV-infected population increased by 61% from 1997 to 2006 [15]. Stroke has often been reported as a complication of AIDS; however, limited data exist that address the mechanism of the risk of HIV-/AIDS-associated stroke. The younger patients most often do not have comorbidities typically associated with stroke such as diabetes mellitus (DM), hypertension (HTN), obesity, atrial fibrillation (AF), or hypercholesterolemia (HCL). This increase rate of stroke in the young appears to correlate with increasing rates of contracting HIV in young populations [16].

23.5.1 HIV Mechanisms of Ischemic Stroke

Several possible mechanisms have been hypothesized to account for stroke in association with AIDS, including covert HIV-induced vasculopathies [17, 18]. There is also clinical and histopathological evidence suggesting that HIV infection may cause a variety of inflammatory vascular diseases [19]. Cerebral vasculitis during HIV infection and AIDS has been found in postmortem examinations [20–22]. Several case-control studies, using single-photon emission computerized tomography (SPECT), have revealed baseline cerebral hypoperfusion. These studies have been reviewed by Tucker et al. [23]. Recent MRI case-control studies have revealed cortical and subcortical gray matter hypoperfusion in asymptomatic HIV subjects [24, 25]. Other studies have evaluated regional cerebral glucose metabolism using FDG PET and noted a hypermetabolic state in the deep subcortical gray matter including the basal ganglia [26–28]. Although not demonstrated in the same patients, such a resting imbalance between blood flow and metabolism could produce a state that renders the brain more susceptible to minor degrees of subsequent ischemia. A similar imbalance between resting blood flow and oxygen metabolism leading to increased oxygen extraction fraction (OEF) is associated with a marked increased risk of stroke in patients with symptomatic carotid artery occlusion [29]. Only a single study has measured CBF and glucose metabolism with FDG in the same HIV-infected patients and, contrary to the previous understanding, found no abnormality in either [30]. A recent MRI study of HIV subjects, most of them on antiretroviral therapy, found decreased cerebral blood flow (CBF), and a related uncoupling between CBF and cerebral metabolic rate of oxygen (CMRO2) changes

during neuronal activation, suggesting an increase in metabolic activity due to HIV-mediated inflammation [31]. MRI has been used to measure whole brain and regional OEF and gray matter CBF in treatment of naïve asymptomatic HIV-infected subjects and controls to gain insight into the pathophysiology of HIV-/AIDS-related ischemic stroke.

HIV-1 can lead to increase carotid intima thickness, increased wall stiffness, and decreased compliance. These are associated with accelerated atherosclerosis and thus cardiovascular disease and ischemic stroke. Because HIV-1 can also cause hypertension, the number one risk factor for ischemic stroke, due to increased arterial wall stiffness caused by the virus. Pro-inflammatory cytokines associated with HIV-1 infection can penetrate the vascular endothelium, causing atherosclerosis, thus damaging the blood-brain barrier, creating the potential for thrombosis [32, 33].

Smoking is often associated with HIV patients and is a risk factor for cardiovascular disease and ischemic stroke. Smoking frequency is approximately three times greater in HIV+ patients than in the same HIV- patients [34]. Smoking causes increase in TAGs, drop in HDLs, and increased chemotaxis leading to increased narrowing of arteries [35]. Thousands of toxic compounds create oxidative stress via ROS and RNS and vascular inflammation, degrading endothelial cells and compromising the integrity of the blood-brain barrier. HIV has also been associated with periodontal disease, a known risk factor for cardiovascular disease and ischemic stroke [36].

HIV is known to cause a variety of other conditions which may have an effect on vasculature and thus cerebrovascular accidents. However, it is important to consider that the relationship between HIV and stroke might not be a causal relationship, rather they may simply be correlated with one another (Table 23.1).

23.6 Epidemiology

Epidemiological data on stroke in HIV-infected patients may vary depending on the type of population studied (e.g., industrialized countries versus sub-Saharan Africa) and the date in which the study took place (pre- versus post-highly active antiretroviral therapy implementation). Thus, determining whether HIV is an independent risk factor for stroke is difficult. Retrospective case-control studies have indicated that HIV infection, and specifically the diagnosis of AIDS, appears to be associated with an increased risk of stroke in industrialized countries such as the United States [12, 14, 16]. Epidemiological studies have also suggested that HIV-associated stroke affects a younger population with a risk factor profile that differs from the HIV-negative young stroke population in that hypertension, diabetes, hyperlipidemia, and smoking are not significant risk factors [37]. A limitation of many of the existing studies is that they fail to distinguish between strokes associated with medical conditions known to be associated with HIV infection including lymphoma, opportunistic infections, antiretroviral therapy, and substance abuse and strokes resulting from an undetermined HIV-related process [13, 38].

Table 23.1 Causes and mechanisms of stroke in HIV-infected patients

Direct effect of HIV
HIV-related vasculitis/vasculopathy cardiac mechanisms
Cardiac mechanisms
Cardiac embolism
Endocarditis
Infection
No infection
Cardiomyopathy (low left ventricular ejection fraction)
With dilated left ventricle
Without dilated left ventricle
Valvular disease (particularly myxoid valvular degeneration)
Opportunistic infectious/neoplastic mechanisms
Opportunistic vasculitis/vasculopathy
Opportunistic infections (with or without meningitis)
Tuberculosis
Cytomegalovirus
Varicella zoster infection
Syphilis
Toxoplasmosis
Cryptococcosis
Other opportunistic infections
Opportunistic tumors
Lymphoma
Prothrombotic mechanisms
Coagulation diathesis
Protein S deficiency
Antiphospholipid antibodies
Others
Drug-related mechanisms
Intravenous drug use
Cocaine
Heroin
Cardiovascular risk-associated mechanisms
Atherosclerosis (may be accelerated by HAART)
Dyslipidemia, insulin resistance
Endothelial dysfunction

23.6.1 Pre-HAART/cART and Stroke Era

Currently, the treatment of HIV and AIDS generally includes the use of multiple antiretroviral drugs in an attempt to control the HIV infection. There are several classes of antiretroviral agents that act at different stages of the HIV life cycle. The use of multiple drugs that act on different viral targets is known as highly active antiretroviral therapy (HAART). The use of HAART results in decreasing a patient's total HIV burden, maintaining functions of the immune system and preventing opportunistic infections that can often lead to death. The pre-HAART era lasted

until 1996 when an effective anti-HIV treatment – protease inhibitors (PIs) and non-nucleotide reverse transcriptase inhibitors (NNRTIs) or HAART – was utilized. However, some third-world countries, especially those in the sub-Saharan Africa and the Caribbean basin, are still considered to be within the pre-HAART era because this treatment is still not available. In the current HAART era, more deaths associated with HIV and AIDS were more accurately attributed to associated illnesses and opportunistic infections, i.e., HIV and AIDS alone. Furthermore, in a study of South African patients, considered to be a part of the existing pre-HAART era, incidence of cryptogenic stroke, or unknown etiology, was 91% among young stroke patients with HIV as compared to 36% in young stroke patients without [13]. HIV patients in the pre-HAART era also had decreased cerebral blood flow as measured by carotid Doppler as compared to non-HIV patients of the same age, likely due to the pro-atherogenic nature of HIV [18]. When T and CD4 lymphocytes become activated, they can cause inflammation in conjunction with C-reactive protein. HIV infection alters the endothelium similar to an atherosclerotic plaque, resulting in increased von Willebrand factor circulation and thus platelet adhesion. Hyperactivated macrophages associated with HIV infection also causes apoptosis of endothelial cells causing greater injury and inflammatory cell recruitment (propagation of signal cascade). Activated macrophages play a key role in the atherosclerotic process, having been shown to migrate toward atherosclerotic plaque. HIV replication is thought to be responsible for enhancing the activation of macrophages which express tissue factors found in plasma. This process may encourage a procoagulation state and lipid expansion, in turn increasing plaque vulnerability and activation of T-cells. The hyperactivation of T-cells can lead to a pro-inflammatory response, resulting in endothelial dysfunction [39]. HIV virus causes a distinct vasculopathy along the length of the vessel with an abundant proliferation of smooth muscle cells and elastic fibers, leading to protrusions which may play a role in the onset of coronary artery disease in AIDS.

23.6.2 Post-HAART and Stroke Era

With the availability of HAART in mid- to late 1990s, patients with HIV that progressed to AIDS had fewer opportunistic infections and associated deaths. However, there have been increasing reports of strokes, mostly of ischemic origin, in children and young adults without related comorbidities [14]. After beginning HAART therapy, risk of stroke seems to go down, but this trend reverses as the patient ages. This is likely association rather than causation as HAART provides HIV patients with an extended life span and which allows them time develop traditional risk factors (hypertension, diabetes, etc.), in turn increasing their stroke risk [40].

HAART and protease inhibitors in particular (PIs) are thought to be associated with dyslipidemia, redistribution of body fat to more dangerous parts of the body, and insulin resistance. PIs can cause an increase in fatty acid release from storage or a decrease in uptake from the blood, resulting in fat redistribution. Some studies

indicate that NRTIs however are not associated with increased inflammation and atherosclerosis; other studies indicate that NRTIs such as abacavir may increase the risk of accelerated atherosclerosis. It is however not completely clear whether PIs or non-nucleoside reverse transcriptase inhibitors (NNRTIs) are more closely related to these comorbidities [41].

Although HAART increases CD4 counts and decreases HIV RNA/protein levels [42], it has been associated with the development of metabolic syndrome, a cluster of conditions that increases the risk of cardiovascular disease and stroke via mechanisms similar to HIV infection [43]. Thus, the link between cardiovascular disease and HAART may be the inflammatory marker increase post-HAART administration, such as interleukin-6 (IL-6). IL-6 causes inflammation that contributes to the pathogenicity of HAART treatment. In general, more people are living longer with HIV because of treatment but are dying of other issues from complications that normally plague the elderly. Overall, it is unclear if the cardiovascular disease, stroke, and other causes of death are related to HAART treatment or simply to longevity of the patients that are treated [42].

23.6.3 Protease Inhibitors

Protease inhibitors (PIs) are a class of antiviral drugs that are widely used to treat HIV and AIDS. HAART was created by combining PIs and two NRTIs. This combination treatment method was groundbreaking but caused many adverse side effects specifically gastrointestinal problems such as nausea, diarrhea, and elevated lipid levels. Today, there are PIs that are more tolerable for patients, but even as the backbone of HAART treatment, PIs continue to have adverse effects, including lipodystrophy and premature carotid atherosclerosis leading to increased risk of cardiovascular incidents [9]. Furthermore, PIs also block CD4+ T-cell apoptosis and thus may also block damaged cell apoptosis leading to cellular damage propagation. HIV PIs may inhibit apoptosis via three different mechanisms: (1) altered expression of apoptotic regulatory proteins, (2) mitochondrial stabilization, and (3) blocking cysteine protease, calpains, which activate the HIV aspartyl protease. These mechanisms affect all apoptotic cycles to some extent and may explain neurodegenerative diseases [44].

23.6.4 NNRTIs and NRTIs

Non-nucleotide reverse transcriptase inhibitors bind to and change the conformation of the HIV-1 reverse transcriptase enzyme. Typically, combinations of NNRTIs are given to prevent HIV from obtaining mutations, which is a frequent occurrence [45].

NRTIs are converted to their active metabolite form in vivo at which point they compete with deoxynucleotide triphosphates for a position in the growing viral DNA. When they are incorporated, they terminate further reverse transcription by the HIV reverse transcriptase [46].

23.7 Treatment of Comorbidities

Patients with HIV infections and those on HAART regimens are often diagnosed with comorbidities typically associated with stroke risk (diabetes – insulin resistance, hypertension, arterial stenosis, atherosclerosis, etc.). Treatment of these comorbidities in HIV patients remains similar as in patients that are HIV negative. Prior studies have shown that patients on HAART regimen and drugs such as statins, aspirin, or insulin do not experience any adverse interactions between drugs, making combined therapy safe [47].

23.8 HIV and Opportunistic Infections

23.8.1 HIV and TB

HIV infection reduces cell-mediated immunity and thus makes one susceptible to *Mycobacterium tuberculosis* infection (MTB) or reactivation of latent MTB infection. In fact 1/3 of the 38.6 million people living with HIV are coinfected with TB [48]. Risk of TB contraction in HIV patients can occur at any stage of the HIV infection, and risk also increases with decreasing CD4 counts [49]. For this reason, TB infection is rampant in areas in which HIV infection is common, making it difficult to eradicate or control TB incidences [50].

Additionally, multidrug-resistant MTB strains are emerging because of the inefficiency of isoniazid therapy when a concomitant HIV infection is present. Most of these cases of TB are seronegative, and thus clinicians must rely primarily on physical presentation of disease symptoms [51].

The decreased cell-mediated immunity makes typical radiographic evidence of TB, i.e., cavitary upper lobe lesions, less likely. Thus, in HIV immunosuppression, military TB and systemic effects are more commonly used as diagnostic features. Treatment is similar to HIV treatment, but outcomes vary based on when TB treatment is started [52].

The increased incidence of tuberculosis infections in HIV patients is concerning as it is known to increase the risk of stroke. This risk has been primarily attributed to the neurological complications of TB: meningoencephalitis. This is an infection of the meninges and brain matter which leads to an immune-complex deposition within the small blood vessels in the brain leading to an inflammatory response.

This response leads to hyperplasia of the intima or the internal layer of blood vessels ultimately causing decreased blood flow to those areas of cerebral tissue, causing cerebral infarction.

23.8.2 HIV and Toxoplasmosis

Toxoplasma gondii is an obligate intracellular protozoan infection that is controlled via cell-mediated immunity. Transmission generally occurs via oocysts in cat feces or by undercooked lamb or pork. The parasites can then cross the blood-brain barrier via several mechanisms and invade the endothelial cells that line blood vessels and cause an inflammatory response in the cerebral vasculature [53]. *Toxoplasma gondii* is typically asymptomatic in immunocompetent patients, but when CD4 cell counts dip below 100microL, encephalitis ensues. Toxoplasmosis is found in AIDS patients due to a reactivation of a latent infection. Typical presentation includes ring-enhancing brain lesions on imaging, headache, altered mental status, and fever. Focal neurological symptoms include motor weakness, speech disturbances as well as possible central nervous system abnormalities, cerebellar dysfunction, and movement disorders. Ocular and pulmonary symptoms may also present as extracerebral manifestations.

Diagnosis and confirmation of *Toxoplasma gondii* can be determined by visualization of the parasite in the serum. Treatment of HIV infections can lead to a recovery of cell-mediated immunity, which may also assist in defending against *T. gondii* infection [54].

23.8.3 HIV and Cryptococcus neoformans

Cryptococcus neoformans spores are found ubiquitously in soil which we all inhale regularly and defend against, but CD4+ counts under 100 are associated with complications. Presentation of infection with *C. neoformans* includes ring-enhancing lesions on neuroimaging and nonspecific meningitis-like symptoms including headache, fever, papilledema, photophobia, altered mental status, and stiff neck [55]. Stroke risk in patients with active *C. neoformans* infections is also associated with small vessel vasculitis, similar to those found in tuberculosis infections.

23.8.4 Polyomavirus (JC Virus)

Polyomavirus is a progressive multifocal leukoencephalopathy (PML), which can lead to gait abnormalities, hemiparesis, and visual field changes. Presentation includes non-ring-enhancing lesions of the white matter due to the demyelinating

nature of this disease affecting the oligodendrocytes of the CNS. The conditions may improve with HAART as CD4 counts increase over 200 [56]. Symptoms of PML include gait disturbances, ataxia, and hemiparesis, which are commonly confused with stroke. Thus, HIV patients with JC virus infection need to be treated to prevent masking of a cerebrovascular accident by the neurological complications of the JC virus disease process [57].

23.8.5 HIV and HSV/VZV

Many people live with the varicella zoster virus (VZV), which can be reactivated and cause shingles later in life, particularly when one is in an immunocompromised state. Such a state often occurs when HIV is left untreated. Herpes simplex virus infections, which often remain undiagnosed in many HIV patients, can result in cranial nerve palsies, cerebral vasculitis, TIAs, and meningoencephalitis [58]. Both VZV and HSV can potentially cause encephalitis due to invasion of the endothelial cells by virions. This leads to an inflammatory reaction in the blood vessels in the brain and stroke via secondary thrombosis of the vasculature [59].

23.8.6 HIV and Cytomegalovirus

Cytomegalovirus, CMV, is a common virus that can infect almost anyone. Most people never know they have CMV because it rarely causes symptoms. However, for those with weakened immune systems like HIV patients, CMV can be a cause for concern. Presentation of symptoms may include fever, pneumonia, diarrhea, ulcers in the digestive tract, hepatitis, encephalitis, behavioral changes, seizures, coma, and visual impairment and blindness [60].

23.8.7 HIV and **Treponema pallidum**

Syphilis is caused by a thin, tightly coiled spirochete, *Treponema pallidum*. HIV has not yet been found to predispose one to a syphilis infection, but these often are coinfections.

Neurosyphilis can manifest with abnormal gait; numbness in the toes, feet, or legs; cognitive issues; depression; irritability; headache; seizures; incontinence; tremors; and visual disturbances. Neurosyphilis can be passed from mother to child in first trimester leading to bony malformations and CN VII palsy deafness [61]. Stroke in patients with neurosyphilis is often a result of vasculitides and decreased cerebral perfusion.

23.8.8 HIV and Candida albicans

Candida species are a part of the normal flora of the mouth and gut but, in an immunocompromised state, may progress to disseminated candidiasis and causes meningoencephalitis. Other opportunistic infections include blastomycosis, mucormycosis, coccidiomycosis, and aspergillosis. Much of these come from endemic areas such as the Southwestern United States for coccidiomycosis and Ohio River Valley for blastomycosis [62].

23.9 Future of HIV Treatment

Due to the lack of a clear mechanism, prevention of stroke in HIV-infected patients is similar to prevention of stroke in patients without HIV, with only a few additional considerations. As in patients without HIV, the first steps in stroke prevention are the assessment of stroke risk and the treatment of conventional risk factors. Additionally, the treatment of HIV can be considered as an important part of stroke prevention in these patients due to the evidence that HIV may lead to vasculopathy. It is important to note that treatment using HAART may produce or worsen vascular disease risk factors, and this treatment method requires particular choices of medications and monitoring of side effects and adjustments of medications, specifically in patients with a history of stroke of other vascular diseases.

23.9.1 Assessment of Risk

As with all patients, primary prevention of vascular disease and secondary stroke prevention rely heavily on the estimate of overall vascular risk. Antiplatelet therapy (aspirin, clopidogrel, aspirin/extended-release dipyridamole, etc.) or anticoagulant therapy (rivaroxaban, dabigatran, warfarin, heparin, etc.) should be considered for patients who are ≥10% risk of myocardial infarction or coronary death over 10 years [63]. Patients who have already experienced a cardiac event are candidates for maximal medical management for secondary prevention, which included statins [64]. Additional risk factors for coronary disease are diabetes mellitus, occlusive atherosclerotic disease, and symptomatic carotid artery disease [65]. It is important to consider stroke among the relevant outcomes, especially in minority groups who are at an increased risk of stroke relative to coronary events. Furthermore, these minority groups are also likely to be overrepresented among HIV-infected patients [66].

23.9.2 Treatment of Conventional Risk Factors

There is little evidence for secondary prevention of stroke in HIV patients, but current guidelines regarding the use of antiplatelets, statins, and blood pressure-lowering therapies should be used in HIV-infected patients as they are used in patients without HIV for the management of risk factors [67, 68]. Determining the cause of any previous ischemic stroke is essential to treat these patients effectively [69].

Because smoking is highly prevalent among HIV-infected patients, smoking cessation and abstinence should be aggressively pursued to include the use of pharmacological therapies [66]. Obesity is also common among patients with HIV; thus, encouraging weight loss and aerobic exercise for a duration of 30–60 min at least five times a week is important. Patients may also benefit from seeking guidance from a nutritionist when struggling with weight loss [70].

23.9.3 Treatment of HIV

As HIV has become more reasonably associated with an increased risk of stroke, treatment of HIV may also be considered part of a strategy to reduce one's stroke risk. Current guidelines for the treatment of HIV-infected patients include the presence of a high risk of vascular disease as a reason to initiate HAART, even among patients with CD4 counts greater than 350 mm^3 [67, 71]. However, the risk of medication-induced metabolic abnormalities, stroke risk factors in themselves, must be weighed against the vascular protective benefits of HAART.

23.9.4 HAART in the Setting of Vascular Disease

Primarily, the guide in choosing the appropriate antiretroviral therapy should be the efficacy of the agent in the individual patient against the virus. Treatment of complication or side effects can then be managed as needed. Data suggests that the use of statins and fibrates helps to manage lipid levels among patients with HIV receiving HAART, rather than changing to a different antiretroviral [72]. When considering initial therapy options, it is important to remember that there are advantages to avoid or choose specific antiretroviral and lipid-lowering agents. For example, PI atazanavir appears less likely to be associated with high cholesterol and insulin resistance and should be considered in patients who are known to be at a high cardiovascular risk [73]. Abacavir has been associated with increased cardiovascular risk and should be avoided in those patients already at high risk [74]. PIs are also known to downregulate the cytochrome P450 enzymes involved in the metabolism of specific statins and may increase the level of that statin in patients. It is reasonable to consider the use of pravastatin, atorvastatin, or rosuvastatin in preference to lovastatin

or simvastatin [74]. Insulin resistance and diabetes mellitus are also common side effects of HAART. HIV-infected patients on HAART should be counseled to adhere to a diet that contains 50–60% carbohydrates, 10–20% protein, less than 30% fat, less than 100 mg cholesterol, and less than 10% of total calories from saturated fat. Furthermore, these patients should be advised to exercise regularly. When necessary, medications to control diabetes should be prescribed to maintain a goal, i.e., hemoglobin A1C less than 6.5% and a fasting blood sugar of 73–110 mg/dl [70].

23.10 Conclusions

HIV infection may be associated with an increased risk of stroke; however, the mechanism by which HIV infection may lead to an increase risk of stroke still needs to be determined. HIV treatment, specifically HARRT (PI and NNRTI), has been linked with metabolic syndrome and accelerated atherosclerosis, which may lead to an increased stroke risk. These associations need to be confirmed by population-based studies, which could generate immense changes for stroke prevention in HIV-infected patients.

Conflict of interest The authors report no conflicts of interest.

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Chapter 24
Hepatitis C and HIV Neurological Implications

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Keywords Hepatitis C • Neurology • Cerebrospinal fluid (CSF) • Cerebrovascular disease • Human immunodeficiency virus (HIV) • Intravenous drug use (IDU) • Mental health • Stroke • Interferon-alpha

Core Message

Hepatitis C virus (HCV) disease is becoming more prevalent globally. Extrahepatic manifestations of hepatitis C are increasingly being recognized as important factors driving timely treatment of HCV infection. Such manifestations include neurocognitive as well as neuropsychiatric conditions. Chronic HCV infection is associated with increased rates of acute and chronic cerebrovascular disease as well as increased rates of atherosclerosis. There is also an increased prevalence of emotional distress, anxiety, and depression. The incidence of human immunodeficiency virus (HIV) and HCV coinfection is increasing in both the United States and worldwide, and the level of understanding about the neurological manifestations of HIV infection has become more sophisticated. Some of the common neurological impacts of untreated HCV infections are slower reaction times, slowing of motor performance, depression, and speed of information processing. HCV and HIV coinfection augments neurological manifestations. Prevention and treatment of HCV as well as HIV infection have been associated with improved outcomes.

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24.1 HCV Epidemiology

The Global Burden of Disease Study last published in 2016 by the World Health Organization estimated that, globally, hepatitis C virus (HCV) infection prevalence is currently 2.5% (upper estimate 5.9%) of the global population, accounting for nearly 130 million individuals infected globally, with highest concentrations in the Southeast Asia and East Asia, with more than 50 million infected in each area, spanning nearly nine genotypes [1–3]. Hepatitis-C-related liver cancer has increased by 21.3% from 2005 to 2015 worldwide [2]. HCV genotype 1 remains as the most prevalent genotype worldwide, whereas genotype 2 is significantly concentrated in Central and West Africa; genotype 4 is prevalent in the Middle East and North Africa [4]. Intravenous drug use as well as iatrogenic healthcare exposures, such as the reuse of vaccination needles, continues to be the leading cause of transmission worldwide [5, 6]. For example, a national campaign to treat *schistosomiasis* in Egypt in the 1970s led to the reuse of needles among the entire villages, leading to the rapid spread of HCV within the population [7]. Further, suboptimal infection control standards in hemodialysis units have led to an increased risk of nosocomial HCV transmission in various countries, including Italy, Japan, and Spain [8]. In the United States, the estimated HCV infection prevalence ranges from 3.4 to 6.0 million, although the traditional National Health and Nutrition Examination Survey (NHANES) surveys exclude incarcerated and homeless groups [9]. These individuals carry an elevated HCV prevalence, with additional sources of potential underestimation, suggesting that the true prevalence is higher [10]. The latest figures from the Centers for Disease Control and Prevention as of 2017 reported 2194 cases of acute HCV infection in the United States from 40 states in 2014, with an incidence of 0.7 cases per 100,000 population [11]. HCV infection remains the leading indication for liver transplantation in the United States, often potentiated with alcohol use disorder [12] (Fig. 24.1).

Traditionally, individuals at an increased risk for HCV infection include current or former injection drug users, chronic hemodialysis individuals, healthcare workers, recipients of blood or organs from an HCV-positive donor, persons with human immunodeficiency virus (HIV) infection, recipients of blood products or solid organ transplants predating July 1992, and recipients of clotting factor concentrates admixed prior to 1987 [6, 13]. The Veterans Health Administration estimates a higher prevalence of HCV among US veterans (8.4%) as compared to the general nonveteran US population (1.3%) [14], due to higher incidence of war injury-related blood transfusions in the field before universal screening and concentration of those born in the 1945–1965 cohort within the VA care system [15].

More recently in the Northeast United States in 2007 and in the Midwest United States in 2014, increasing intravenous drug use practices using heroin and crushed oxycodone were reported among young Caucasians who shared injection needles and equipment. These practices accounted for a new wave of HCV infections among the younger generations in both the United States and Canada [10, 16–19].

Percutaneous accidental transmission is secondary to intravenous drug use as a cause of transmission within the United States, with 2% of affected individuals becoming infected through occupational health exposure [5, 6]. In addition, an estimated 17% of HCV infections within the United States were attributed to receiving

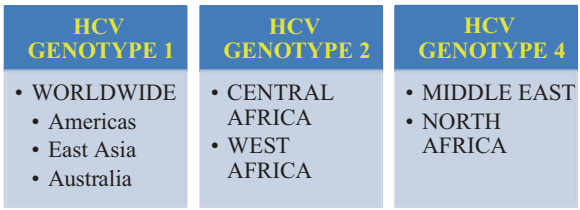

Fig. 24.1 Most common prevalence of HCV genotypes based on geographical location

blood transfusions prior to blood supply screening in 1992; currently HCV transmission through blood transfusion in the United States is nearly not nonexistent due to excellent screening procedures [13].

Transplantation of HCV-positive donor tissue had been a prior risk factor for HCV infection in times past. In one study of 716 organ donors between 1986 and 1990, 13 (1.8%) were positive for HCV antibodies; their organs were transplanted into 29 recipients, with a subsequent development of HCV infection in 14 (48%) organ recipients [20, 21]. However, in 2017, routine donor screening has exponentially decreased the risk of HCV transmission via this mode as organ donors are prescreened. Other routes of transmission are uncommon due to stringent public health licensing regulations and include tattooing and unsafe or unlicensed acupuncture practices. Additionally, transmission of HCV to healthcare workers has been reported, especially with hollow-bore needle-stick injuries with unexpected percutaneous exposure. The risk of HCV transmission through needle-stick injury is approximately 3%, intermediate to that of HIV (0.3–5%) and hepatitis B virus (33–37%). With appropriate infection control standards, however, risk among healthcare workers approximates that of the general population [22, 23] (Fig. 24.2).

Risk of HCV transmission is directly linked to the degree of HCV viremia, with greater viremia being more infectious. Specifically, cases of acute HCV transmission has been observed in men who have sex with men (MSM), [5] in commercial sex workers, in those in which one sexual partner continues to inject drugs, and in sexual practices that involve mucosal tearing [24]. However, the sexual transmission of HCV is very low to nearly nonexistent among couples in monogamous heterosexual and homosexual relationships that are without other high-risk features [25].

Transmission of HCV from mother to infant, "vertical transmission" or "mother-to-child transmission," occurs and is at higher risk in mothers with uncontrolled HIV infection, i.e., unsuppressed HIV viral load [26]. HCV RNA can be found in breast milk of affected mothers, proportional to the peripheral level of viremia [27–29].

24.2 HCV and Neurological Disorders

24.2.1 Overview

As HCV infection is a systemic disease, it causes extrahepatic inflammation through immune-mediated mechanisms and metabolic derangements, and there are inflammatory markers [30]. Inflammation in particular is generated through different pathways including cytokine release and oxidative stress, and these mechanisms also

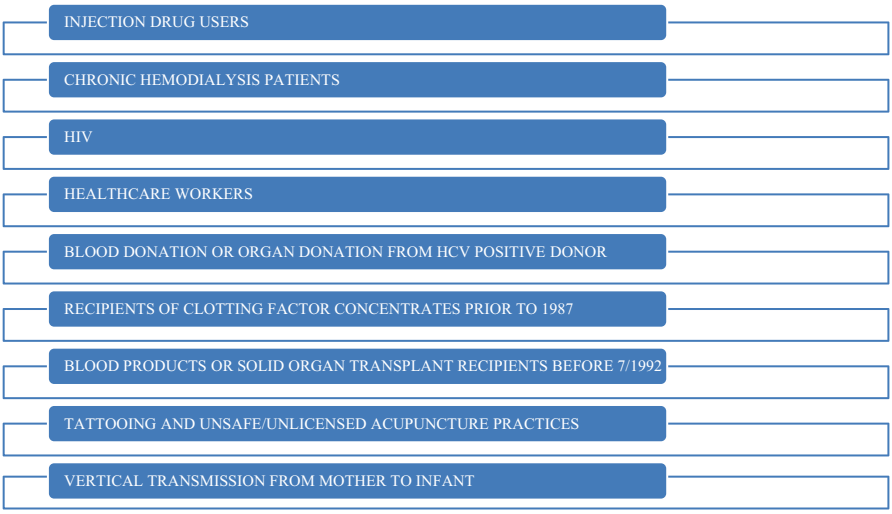

Fig. 24.2 Sources of infection for individuals with hepatitis C

affect nervous system function. It has also been linked to insulin resistance and diabetes type 2 and compromises of ocular, intestinal, renal, thyroid, and cardiac function. Neurological and psychiatric disorders are particularly observed in chronic HCV infection accounting for about 50% of individuals [31]. Generally, those with HCV infection are known to have lower quality of life as evidenced by a lower score on the commonly used depression symptoms' questionnaire, called the "short form 36" (SF36), which evaluates physical functioning, bodily pain, social functioning, and mental health, among other features. Frequently HCV patients present more physical symptoms, especially those who previously used intravenous drugs [32].

24.2.2 Pathogenic Implications

HCV infection-related neurological disorders are multifactorial and include a combination of adaptive host immune responses, production of autoantibodies, immune complexes, cryoglobulins, and in rare cases direct neurological invasion [33]. HCV RNA has been isolated postmortem in the brain cells of infected individuals, and entry and replication of HCV in astrocytes, microglial cells, and brain endothelial cells suggest independence and pathogenicity of the virus in the brain, previously believed to occur only in the liver [31]. However, not every cell in the nervous system is permissive to replication of the virus. For example, epineurial macrophages have not shown replication of the virus in biopsy specimens of individuals with peripheral neuropathy [33].

The exact pathophysiological mechanisms of cognitive impairment, neuropsychiatric disorders, and sleep disturbance to this date can be explained by

inflammatory cytokines, antibodies, immune-mediated complexes, iatrogenic factors, and confounders of substance use and mental health comorbidities [30]. Studies also report defective central serotoninergic and dopaminergic neurotransmission in some HCV individuals with neuropsychiatric symptoms, which also might suggest a role for HCV infection in inducing dysfunction via such selective systems or pathways. Moreover, cytokine release and cytotoxic effects can affect both the central nervous system (CNS) and neuroendocrine system.

Though no longer used in HCV infection therapy in the United States, interferon alpha (IFN-α) itself is well known to cause depression and neurological derangements. IFN-α has been correlated with depletion of platelet serotonin, an effect also expected within the CNS, due to the effectiveness of antidepressant drugs via their inhibitory action on serotonin reuptake [30, 31]. Some individuals who were treated with IFN-α also manifested systemic long-lasting neuropathy past the end of treatment [34]. However, since 2014, IFN-α has no longer been used for HCV infection therapy in the United States as is now replaced by all oral direct-acting antivirals (DAAs), though these drugs are still used in many parts of the developing world [35] (Fig. 24.3).

24.2.3 Neurocognitive Disorders

Chronic HCV infection is thought to cause chronic inflammation that leads to cerebrovascular events; cognitive impairment; CNS inflammatory disorders, including myelitis and encephalomyelitis; and peripheral neuropathy [30, 31].

24.2.3.1 Cerebrovascular Events

Chronic HCV infection has been associated with higher rates of acute and chronic, cerebrovascular events than seen in the general population. Small studies have shown an association between HCV infection and stroke. Higher risk of stroke in HCV individuals and higher prevalence of HCV infection in stroke patients are seen when compared to non-HCV individuals by gender and age. Also, younger age at the time of stroke and worse prognosis with higher level of HCV RNA in serum have been correlated [30]. Mixed cryoglobulinemia has been shown to play an important role in many HCV individuals with stroke [31].

HCV infection has an association with atherosclerosis, mediated by inflammation, formation and rupture of plaques, and thromboembolism. Atherosclerosis is influenced by both local and systemic inflammation, as well as glucose and lipid metabolic aberrations affected directly by the viral infection [30]. HCV core protein, HCV viral load, and HCV-related steatosis are all considered independent risk factors and predictors for stroke. Some studies mention up to five- to sixfold higher risk for stroke in HCV patients than HCV-negative patients [30]. Further, HCV-induced cryoglobulinemia involves the cerebral vasculature, particularly arterioles

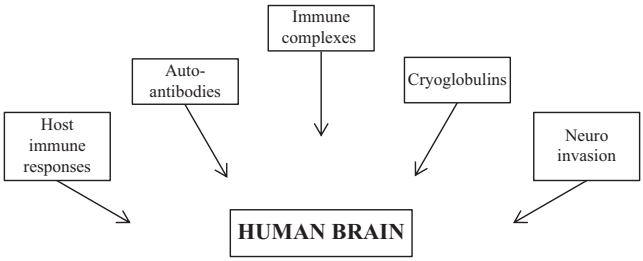

Fig. 24.3 HCV infection-related neurological disorders: pathogenic implications

and small vessels, through immune complex development and deposition in vessel walls.

24.2.3.2 Cognitive Impairment

Cognitive impairment is commonly seen in individuals with chronic HCV infection with 33% presenting at least some degree of mild cognitive impairment. This typically develops independently of HCV genotype or even in the absence of structural or signal abnormalities in neuroimaging [33]. Cognitive impairment can appear in the absence of significant liver disease; however, there is a correlation between impaired individuals and actively replicating virus when compared to individuals that have achieved virus clearance. The positive effects of successful antiviral hepatitis therapy on neurocognitive impairment also suggest an evident correlation [33, 36].

Cognitive impairment might be related, as some studies suggest, to metabolic changes. For example, increased level of choline or creatine has been noted in the *basal ganglia* of HCV-positive subjects. In addition, N-acetyl-aspartate level, which is a marker of ongoing repair, is decreased in the *frontoparietal* white matter of some individuals [33]. Microstructural changes and metabolic alterations, studied by magnetic resonance imaging, magnetic resonance spectroscopy [36], and positron emission tomography (PET) signals, were also seen in different areas of the brain including the *striatum*, the *external capsule*, and the *fronto-occipital fasciculus*. HCV core antigen was found in astrocytes and microglial cells. Even in HIV-coinfected individuals, the presence of HCV RNA has been detected in the *frontal cortex*, *basal ganglia*, and *subcortical white matter*. Mild cognitive impairment seen in HCV infection alone is like the type seen in HIV-/HCV-coinfected individuals; the underlying pathogenesis has been postulated common to both diseases.

Manifestations of cognitive impairment occur in a variety of forms. Executive function can be directly affected, impaired abstract thinking, verbal response, reasoning, and mental flexibility. Individuals also present with lack of concentration, poor ability to maintain attention, impaired working memory speed, decreased psychomotor speed, verbal learning, and auditory attention [30, 37]. Some studies

have shown that dementia is increased by HCV infection. However, additional research is needed to understand the mechanisms and to buttress the strength of this association [30].

HCV-positive individuals, who are specifically more vulnerable to these changes and present greater impairment, are commonly the individuals that have an initially lower cognitive reserve prior to acquiring the virus. Conversely, individuals endowed with greater cognitive reserve and level of education may be spared with more serious impairment. Research in this area is still limited and inconclusive [30].

When assessing the extent of the cognitive impairment in an HCV-positive individual, it is important to consider the role of lifestyle factors like alcohol and substance abuse, as well as medications like IFN-α, prior to social problems and other comorbidities.

24.2.3.3 CNS Inflammatory Disorders

HCV infection can cause encephalitic and meningeal inflammation. There are different clinical presentations: *encephalomyelitis*, urethral or anal sphincter dysfunction, spastic quadriparesis, and sensory loss like lower extremity weakness have been reported to be associated with demyelination [30]. Transverse myelitis and acute disseminated *encephalomyelitis* (ADEM) have been described in association with HCV infection and were thought to be mediated by a high titer of IgM [34]. Manifestations include motor, sensorial, and autonomic dysfunctions, with cerebral and cerebellar white matter compromise [38].

24.2.3.4 Peripheral Neuropathy

In contrast to the central nervous system, studies have not proven HCV entry and replication in peripheral nerves. Peripheral neuropathy has been mainly related to the occurrence of mixed cryoglobulinemia, seen in individuals with HCV infection [31]. Approximately 80% of the cases present this correlation. HCV neuropathy is thought to be related to immune-related inflammation due to viral replication and not direct nerve invasion or in situ viral replication within the peripheral nerve [39]. For similar reasons, demyelinating and transverse *myelitis* has been associated in a case series with HCV infection [40, 41]. Mixed cryoglobulinemias specifically affect peripheral nerves due to necrosis and ischemia seen in small vessels, more specifically vasculitis-like changes [42]. Individuals with HCV infection and mixed cryoglobulinemia can also present with peripheral neuropathy, but the number of reported cases is lower, and there is less severity of the manifestations when present. The mechanisms of pathogenesis are also related to immune-mediated deposits in vessels and axons eventually causing ischemia of the latter [30, 31].

Small- and large-fiber neuropathies have been described with a variety of manifestations such as burning and tingling or numbness and paresthesias; additionally, restless legs syndrome has also been reported. Other forms of neuropathy have

been described as well such as mononeuritis multiplex, polyneuropathies, and autonomic dysfunction [30].

24.2.4 Neuropsychiatric Disorders

HCV-infected individuals are known to have a higher prevalence of emotional distress, anxiety, and depression, also described are "brain fog," "somatization," "compulsiveness," and "insecurity" [30, 37]. In addition, aggression, phobia, psychosis, and sexual dysfunction have also been observed. About 30% of individuals with HCV infection suffer from depression, sometimes secondary to a sense of poor quality of life and lowered self-esteem of being infected with HCV [33]. However, some studies have suggested that depression also may be a direct result and intrinsic to the infection itself. Related to these manifestations are also problems with intimacy and family relationships, social marginalization due to the diagnosis, inappropriate coping strategies, stigma, and anger, all of which in turn trigger secondary psychiatric disturbances [33]. In Taiwan, a preliminary study showed a significant incidence rate difference of dementia from 56.0 vs. 47.7 cases per 10,000 person years for those with HCV infection in a study of nearly 59,000 matched controls [43].

Fatigue, or the lack of energy after rest, is one of the most commonly reported symptoms of infection with HCV [36], and such debilitating fatigue can contribute to cognitive impairment as well as other psychosocial disorders. Fatigue prevalence ranges from 20% to 80% and is not related to the severity of liver disease or viral load [36]. Thirty HCV-monoinfected individuals with mild to severe fatigue were compared to controls and found to have higher levels of anxiety and depression and decrease of N-acetyl-aspartate/creatine ratio in the cerebral cortex. EEG slowing also occurred in up to 25%, which was correlated with the degree of fatigue reported symptomatically [44].

Headaches, joint pain, and mood alterations can be present in addition to fatigue. Multiple factors can influence the development of fatigue, and the infection itself may not be the only cause in these individuals. Nonetheless, improvement in fatigue seen after successful therapy may indicate a direct role for HCV infection. Unfortunately, even HCV infection therapy with IFN-α alone can cause fatigue and significant depression necessitating interruption of therapy [33].

It is worth noting that neuropsychiatric symptoms are usually underestimated in routine examinations. But psychiatric screening, psychometric tools, and confirmatory studies are recommended for accurate diagnosis.

24.2.5 Sleep Disorders

There is a dearth of data found in the medical literature about HCV infection and its relationship to sleep disturbances. However, in multiple studies, individuals with HCV infection have been consistently dissatisfied with sleep quality and have

exhibited altered sleep patterns, increased nocturnal activity, and poorer sleep efficiency regardless of their viral load when compared to controls [33].

On one hand, HCV-positive individuals with disrupted sleep noted negative effects including altered mood, quality of life, and lack of energy. On the other hand, other studies have shown no alterations in 24-h activity level despite individuals reporting problems with sleep, and no association between fatigue and nocturnal activity has been reported. In cirrhotic individuals, however, there is reduced 24-h activity level and poor sleep. Also hepatic encephalopathy, delayed sleep phase syndrome, pronounced daytime sleepiness, and frequent awakenings were seen [33]. In conclusion, sleep disruption negatively impacts those infected with HCV (Fig. 24.4).

24.3 Neurocognitive and Neuropsychiatric Disorders in HIV- and HCV-Coinfected Individuals

24.3.1 Introduction

An estimated 25% of the approximately 1.2 million people infected with HIV-1 in the United States also have HCV coinfection [45]. HIV and HCV infections share common transmission risk factors, and hence both are commonly seen in those with high-risk behaviors, such as sharing of needles to inject drugs. Chronic HCV infection is increasingly becoming a major cause of morbidity and mortality among HIV-infected individuals [46]. Untreated HCV infection not only results in the deleterious effects on the liver but also affects other end organs especially the skin, eyes, heart, kidney, and brain. Considerable research substantiates the CNS involvement of chronic HIV infection, but much less is known regarding CNS manifestations of chronic HCV infection, and considerably less is known about such manifestations in those coinfected with HIV and HCV (Fig. 24.5).

Studies of autopsy brain tissue revealed that both positive- and negative-strand HCV RNA were found on the frontal cortex and subcortical white matter. However, since six of the twelve patients were also coinfected with HIV, one would need more information about HIV status to determine the significance of this observation on HCV presence in brain tissue as well as a larger study of subjects and controls [47] (Fig. 24.6).

24.3.2 Effects of HIV/HCV Coinfection on the CNS

HCV infection can be asymptomatic and spontaneously clears in 75% of cases naturally without antiviral treatment. Complications are rarely seen in this setting [33]. However, with HIV infection, HCV infection is less likely to be cleared and more likely to progress to systemic symptoms including hepatocellular carcinoma. HIV infection does not clear but causes chronic inflammation in the body and even with successful antiretroviral therapy establishes latent viral reservoirs [48].

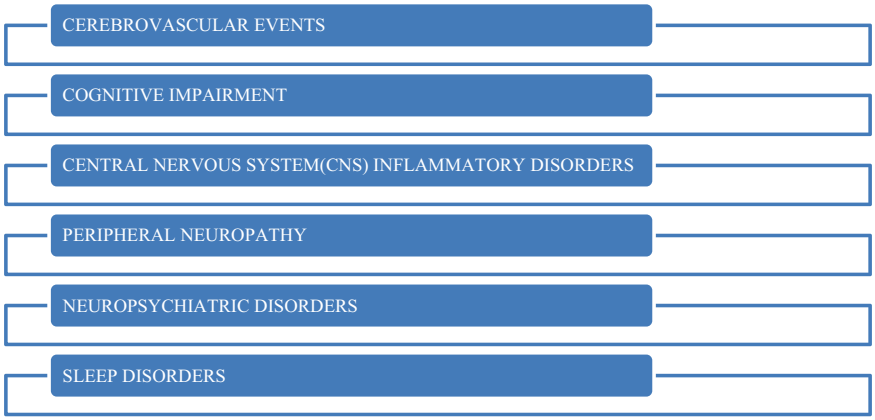

Fig. 24.4 Types of neurological disorders associated with hepatitis C infection

The exact mechanism by which these two viruses interact with each other to promote neurological disease is currently not very well understood but thought to be due to synergies of immune suppression and inflammation. One of the first studies to examine the effects of coinfection with HIV and HCV reported that coinfected individuals were much more likely to exhibit overall neurocognitive impairment than were individuals infected with either HIV or HCV alone [49]. This study suggested a possible synergistic effect of both infections on the CNS. Another study showed that coinfected individuals had much slower reaction times based on in vivo magnetic resonance spectroscopy and neurophysiological studies than did individuals infected with either virus alone [50].

There have been other studies indicating the effect of HIV and HCV coinfection in specific brain areas, especially frontal-subcortical regions. More recent studies suggest that in individuals optimally treated for HIV infection, there is no clinically significant neurocognitive dysfunction nor peripheral neuropathy [51].

A series of neurocognitive evaluations among 118 HIV-positive adults showed higher cognitive impairment among those with both HIV and HCV coinfection as compared to HIV infection alone (63% vs. 43%), especially in the area of learning and memory [52]. This finding is supported by a poorer score on the International HIV Dementia Scale (IHDS), a standarized dementia assessment tool, among those individuals with HCV and HIV coinfection in contrast to those with HIV infection alone. Those individuals controlled on antiretroviral therapy showed similar scores on formal neurocognitive testing and memory [53]. Some conclude that on consistent antiretroviral therapy and HIV virologic suppression, one may be able to halt any effect of HCV coinfection on the decline in neurocognitive function [54].

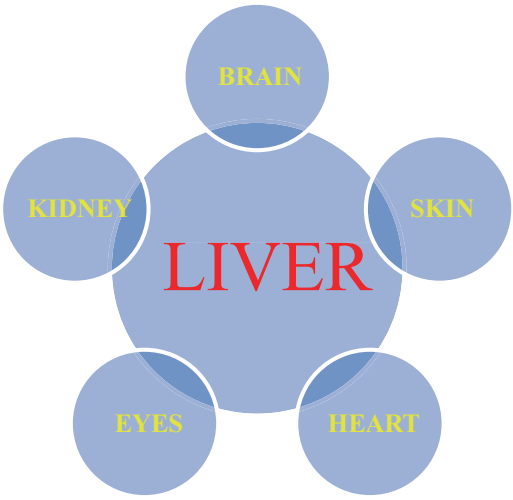

Fig. 24.5 Organs affected by HCV

24.3.3 Pathophysiology of CNS Manifestations in HIV/HCV Coinfection

HIV and HCV can be found in cerebrospinal fluid (CSF) as well as in brain tissue which supports their combined role in affecting neurological function in the host [55]. Both viruses tend to replicate in the same cells such as macrophages which can likely contribute to the synergy of these two viruses. There is also an upregulation of HIV replication in brain tissue in coinfected individuals [55].

24.3.3.1 Microglial Cell Activation

Microglial cell activation has been proposed as a promoter of neurodegeneration and inflammation in HIV-related CNS disorders and chronic HCV infection-associated cognitive decline. [56] A specific protein named PK11195, a high-affinity ligand for protein receptors in activated microglial cells, has been used as a tracer to study microglial cell activity in the brain. Many studies have demonstrated that there is an increased binding of PK11195 protein in individuals with severe HIV encephalopathy as well as in chronic HCV infection but not found in acute HCV infection. This difference may be because chronic inflammation in the CNS due to persistent viremia may result in more neuronal damage and subsequent clinical sequelae. Microglial cell activation is a late feature in individuals with chronic HCV infection but not found in acute HCV infections. Hence, there is substantial benefit in treating and eliminating HCV in the acute phase.

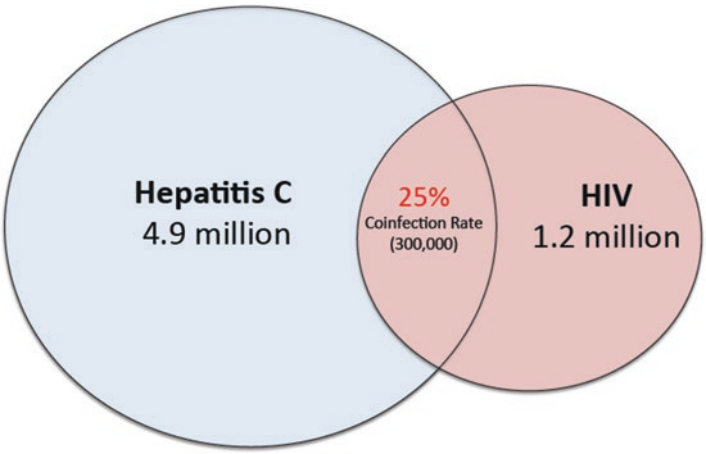

Fig. 24.6 Estimates of HIV and HCV coinfection prevalence in the United States (2015)

24.3.3.2 Changes in Cerebral Metabolites

In addition to microglial cell activation, there is a reduction in inflammatory cerebral metabolites in the basal ganglia which are infected with both HIV and HCV. Studies have shown an increase in myoinositol in the frontal white matter and choline in the basal ganglia in both HIV- and HCV-monoinfected and HIV- and HCV-coinfected individuals. One study conducted by Aronow et al. analyzed cryoglobulin levels in blood and glutamine levels in CSF in coinfected individuals and found that there was a greater cognitive-motor impairment in coinfected individuals compared to monoinfected individuals [57].

24.3.3.3 Immunologic Phenomenon in HIV- and HCV-Infected Individuals

Extrahepatic manifestations related to chronic HCV infection include neurological involvement, and most of it is related to either immunologic or rheumatologic mechanisms. The brain is a permissive site for viral replication as evidenced by an analysis that detected replicative intermediate forms of HCV RNA/viral proteins and HIV viral proteins within the CNS [58]. Neurological dysfunction has also been associated with circulating inflammatory cytokines that affect brain tissue. There is a significant aberrant type of interferon response that triggers immune activation in coinfected individuals [59]. This immune activation is largely responsible for accelerated neuronal injury resulting in various cognitive and psychiatric dysfunctions seen in both HIV- and HCV-monoinfected and HIV- and HCV-coinfected individuals.

24.3.3.4 HCV Core Protein Effect on HIV-Related Proteins in the CNS

Both HCV and HIV have been associated with replication in brain tissue. Proteins from both viruses can affect neuronal structures and be in turn responsible for neurological effects in a host. HCV-encoded RNA and HCV core and nonstructural 3 (NS3) proteins have been detected in human microglia, as well as in astrocytes, in HCV-infected individuals. This HCV core protein exposure in brain tissue induces pro-inflammatory cytokines such as interleukins and tumor necrosis factor. HCV core protein has been found to both modulate neuronal membrane current and cause neuroimmune activation (by several pathways) which in turn can lead to neurotoxicity. This neuroimmune activation is potentiated by HIV-1 Vpr protein in neuronal cells. Together, HCV core protein and HIV-1 Vpr protein can cause gliosis as well as a reduction in neuronal counts resulting in progressive neurological dysfunction (Fig. 24.7).

24.3.4 Neuropsychological Aspects in Coinfected Individuals

24.3.4.1 Neurocognitive Disorders

HIV-/HCV-coinfected individuals have been shown to have a higher rate of acquired immunodeficiency syndrome (AIDS) dementia complex than monoinfected individuals with HIV. One study conducted by Hilsabeck et al. showed that approximately 80% of coinfected individuals had neurocognitive impairment such as psychomotor slowing compared to monoinfected individuals [60]. However, the study did not show any differences in other measures of cognitive function between coinfected and monoinfected individuals such as global cognitive function.

One study conducted by Clifford et al. revealed that HCV-/HIV-coinfected individuals have significantly poorer performance on digit symbol testing and more depressive symptoms than HIV-monoinfected individuals [46]. Digit symbol testing is used to test motor persistence, sustained attention, response speed, and visual-motor coordination [61]. Deficits in digital symbol testing reflect deterioration in motor performance rather than cognitive status as such. Digital symbol testing is impaired in both HIV- and HCV-monoinfected individuals, but when combined, both viruses have a synergistic effect on motor function. In addition, a study conducted by Clifford et al. also showed an association indicating higher prevalence of depression in coinfected individuals [51] (Fig. 24.8).

24.3.4.2 Neuropsychiatric Disorders

There are marginal differences in the coinfected group with respect to poorer sleep [46, 51, 54]. There was no statistical difference noted between coinfected and monoinfected individuals with respect to anxiety. Both HIV- and HCV-infected

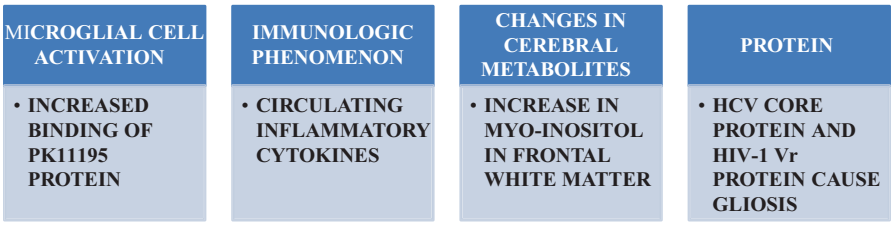

Fig. 24.7 Mechanisms of neurocognitive disorders in HIV-/HCV-coinfected patients

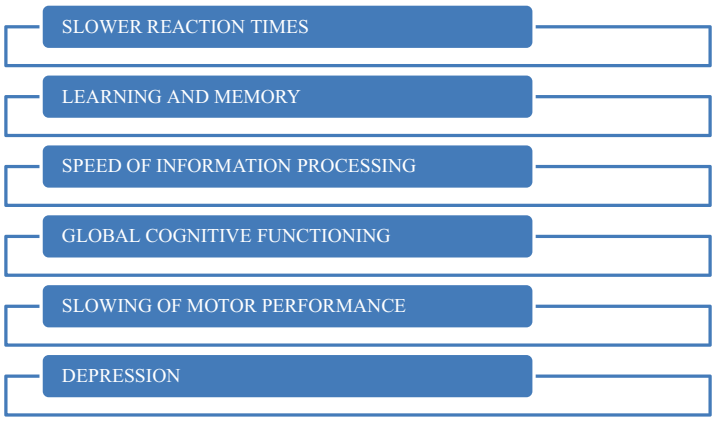

Fig. 24.8 HIV/HCV coinfection effects on neurocognitive disorders

individuals by themselves show affective disorders, but in general, depression is more common in HCV-infected individuals compared with HIV-infected ones. Grassi et al. conducted a study in injection drug users in which 83% of individuals with HIV infection and who use injection drugs were coinfected with HCV [62]. This study revealed that coinfected individuals had greater number of physical symptoms than those with HIV or HCV infection alone. Coinfected individuals are also more likely to have a history of cocaine dependence, opiate abuse, as well as substance-induced depression [63].

24.3.5 Other Factors That Confound the Effect of HIV and HCV on Neurological Disorders

24.3.5.1 Substance Use Disorders

Many HIV-/HCV-coinfected individuals have a history of injection drug use. Such injected substances such as cocaine, heroin, and opiates can themselves intrinsically cause psychiatric dysfunctions which can confound the impact of HIV and HCV

coinfection on neuropsychiatric involvement. Substance use is noted to have a higher prevalence among those with underlying undiagnosed or mistreated psychiatric conditions that often are self-treated with such substance use, leading in turn to higher acquisitions to blood-borne pathogens such as HIV, hepatitis B virus, or HCV [64].

24.3.5.2 Decompensated Liver Cirrhosis

With dysfunctional hepatocytes, the metabolites associated with hepatic decompensation have an impact on neurological performance. Hepatic encephalopathy is one such example where high levels of the ammonia metabolite can cause neurocognitive as well as neuropsychiatric dysfunction. This can confound the impact of the virus itself in the CNS as a whole [64].

24.3.5.3 Staging and Therapeutic Status of HIV

With the onset of antiretroviral therapy (ART), studies have shown that there is a demonstrable improvement in cognitive performance in HIV-infected individuals. HIV-infected individuals who are not on effective ART with high viral load are known to have worse neurological dysfunction. Also, sometimes specific drugs in the ART regimen can intrinsically affect neurocognitive and neuropsychiatric function [65].

24.4 Conclusion

Substantial evidence shows that there is an augmented neurological dysfunction regarding neurocognitive as well as neuropsychiatric functions due to HCV coinfection, which is potentiated in HIV/HCV coinfection. Well-controlled HIV infection with compliance of antiretroviral therapy will mitigate the hepatic and extrahepatic inflammatory effects of HCV infection and will minimize neurocognitive dysfunction. Aggressive measures to prevent, screen, treat, and cure HCV infection are ways to prevent progression of neurological and CNS sequelae definitively.

Conflict of interest The authors report no conflicts of interest.

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Chapter 25
Central Nervous System Reactivation of Chagas Disease in Immunocompromised Patients with HIV/AIDS

Silvana Maria Eloi-Santos, Unai Tupinambás, Helena Duani, and Jose Eymard Homem Pittella

Keywords Chagas disease reactivation • Trypanosoma cruzi • HIV/AIDS • Central nervous system

Core Message

Although endemic in the Latin-American countries, Chagas disease is now present in all continents in the world. In the coinfection HIV/Chagas disease, *T. cruzi* behaves, potentially, as an opportunist microorganism, and the central nervous system (CNS) is most commonly affected during reactivation. The reactivation is defined by the presence of trypomastigotes in the peripheral blood or in biological fluids, through direct microscopy and/or through the presence of inflammatory infiltrate similar to the acute phase of the disease, and in the presence of numerous amastigotes. Mortality is high and etiological treatment is recommended.

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25.1 Chagas Disease and Human Immunodeficiency Virus (HIV)

Chagas disease (CD) is an anthropozoonosis caused by *Trypanosoma cruzi* (*T. cruzi*) and can appear as an opportunistic infection in patients infected with HIV and in other forms of immunosuppression, especially in endemic tropical countries, as well as in non-endemic regions, such as North America and Europe, where this diagnosis is even more challenging [1]. The reactivation of the infection most commonly appears as meningoencephalitis [2]. In some cases, this may represent the first manifestation of AIDS and has thus been considered a defining condition of AIDS by the World Health Organization (WHO) since 2005 [3]. The reactivation of CD in AIDS patients is almost always associated with high parasitemia, and the treatment must be implemented in its early stages [4]. The frequency of the disease's reactivation, clinical and laboratory characteristics, specific treatment, impact of antiretroviral therapy, and incidence and mortality rates are issues that still need to be clarified in a systematic manner in patients who are coinfected with *T. cruzi*/HIV.

25.2 Epidemiology

CD, also known as American trypanosomiasis, is endemic throughout much of Mexico and Central and South America, affecting an estimated 6–8 million people. CD is estimated to cause approximately 12,000 deaths per year. During the last century, CD may have caused more deaths and illnesses than all other tropical diseases combined. Globally, there are 28,000 new cases per year, and 8000 newborns have become infected vertically [5]. Although the disease occurs mainly in Latin America, in recent decades, the migration of infected people has led to a dissemination from endemic countries to North America and Europe. In the USA, more than 300,000 people are believed to be infected with *T. cruzi*, and CD is considered one of the most neglected parasitic infections. These represent a group of parasitic diseases that have been targeted by the Center for Disease Control (CDC) to receive public health actions. In Europe, several studies have estimated a total of more than 100,000 Chagas patients [6].

The most important mode of transmission in Latin America is the transmission by infected triatomine bugs (reduviid) and less commonly by transfusion, by organ transplant, from mother to infant, and, in rare instances, by ingestion of contaminated food or drink.

In the vector-borne transmission, the hematophagous triatomine vectors defecate immediately after feeding on a person, and the protozoa present in the feces of infected bugs enters the human body through the bite wound or through the intact conjunctiva or other mucous membrane.

This vector-borne transmission is under control in Uruguay, Chile, and in most of Argentina and Brazil, as well as in other countries of Central America, due to the widespread campaigns to combat the vector [7].

The transmission of CD via blood transfusion is a recognized risk. In non-endemic countries, transmission via blood donation should be considered the main source of potential infections. In the USA, screening tests have recently been approved by the Food and Drug Administration. Currently, an estimated 90% of the US blood supply is screened. Most donors will only be tested once. If the result of their test is negative for CD, they will not be tested when they donate again [8]. Blood transfusions or their donors with a risk for CD are screened in France and Spain. In Italy and the UK, people who are native from endemic countries are excluded from blood donation. In the other European countries, there is no common proceeding for the screening of transfusions or screening at all [9].

CD can also be transmitted to an organ transplant recipient. In the USA, as well as in other regions where CD is not endemic, control strategies are focused on preventing transmission from blood transfusion and organ transplants. Nevertheless, other forms of transmission, such as vertical, still persist.

In HIV/AIDS patients, a major risk can be found in the reactivation of a chronic infection, in most cases acquired in early childhood. The reactivation of CD in an AIDS patient was described for the first time in the literature in 1988, through the finding of antibodies against *T. cruzi* in the cerebrospinal fluid (CSF) of an HIV patient [10]. Subsequently, other cases were diagnosed elsewhere, especially in Brazil and Argentina [11–18]. In a prospective study, the reactivation of CD in patients coinfected with *T. cruzi*/HIV was estimated to occur in approximately 20% of the patients [12, 16]. In fact, the real reactivation rate is not fully known, and different descriptions of this event appear in the literature [15]. However, it is recognized that the reactivation of CD in an HIV patient may well be even greater than that associated with other forms of immunosuppression [11].

The reactivation of the chronic latent Chagas' infection can occur in HIV patients in 80–90% of the cases as a uni- or multifocal necro-hemorrhagic meningoencephalitis [19]. The other 30–50% of the patients can reactivate the disease in the form of myocarditis and present cardiac insufficiency or arrhythmias [16]. In a systematic review of the reactivation of CD in AIDS patients reported in the literature between 1998 and 2010, the study identified 120 cases (41.2%) out of 291 cases of *T. cruzi*/HIV coinfection, of which 74.2% affected the central nervous system (CNS) and 16.7% presented myocarditis. Others included the duodenum (3.3%), skin (2.5%) and uterine cervix, peritoneum, eye, pericardium, and stomach (0.8% each) [16]. The evolution to death was reported in 86 (29.6%) of the 291 patients coinfected with *T. cruzi*/HIV, given that in 63 (73.3%) of those that evolved to death, a reactivation of the disease occurred [18].

The epidemiological profile of the patients coinfected with *T. cruzi*/HIV includes adult, male, and native of or coming from endemic regions, with serological diagnosis and with an indeterminate chronic form of the disease. However, Almeida et al. in a population study reported that the prevalence of coinfected patients in Brazil was 1.3% and that the coinfected group consisted mainly of white women with an average age of 43 years [18].

The real frequency of the reactivation rates of the disease and mortality are issues that still require better clarification. Most likely, the number of coinfected individuals is still underestimated in the literature.

25.3 Biology

T. cruzi is a flagellate protozoan of the Mastigophora class, Kinetoplastida order, Trypanosomatidae family, and *Trypanosoma* genus. The *Trypanosoma cruzi* species is a heterogeneous population comprised of diverse strains circulating in nature.

The *T. cruzi* life cycle has existed in nature for millions of years. Some accidental human cases may even have occurred at the time when humans still lived in caves, as evidenced by human infections found in mummies from 4000 to 9000 years ago [19, 20].

T. cruzi is capable of infecting difference cell types, including macrophages, smooth and striated muscle cells, as well as fibroblasts [21]. However, some parasite populations present a preferential tropism for phagocytic cells of the spleen, liver, and bone marrow, called macrophagotropic strains, or for smooth, skeletal, and cardiac muscles, called myotropic strains. Other types of tropism have also been described, as in the case of neurotropism [22].

25.4 Clinical Manifestations

The clinical manifestation of CD in immunocompetent individuals can be divided into two stages: acute and chronic. The acute stage is usually observed in children and begins shortly after the infection, with a duration of 1–2 months, and is, at times, asymptomatic. The symptomatic acute stage is characterized by fever, anasarca, hepatosplenomegaly, and lymphadenomegaly. At the bite site of the reduviid bug, a red, indurated inflammatory nodule called chagoma can be present for weeks afterward, and it is characterized by the presence of intracellular amastigotes. If the trypomastigotes are transferred to the conjunctival sac by rubbing the eye after scratching at a freshly infected bite wound, the patient can develop a unilateral painless periorbital swelling, called Romaña's sign, the classic sign of acute Chagas disease. It may be associated with lymphadenitis or even pre-septal cellulitis [23].

A small percentage of patients develop myocarditis and meningoencephalitis during the acute infection. Excluding the rare, severe forms of the involvement of the heart and the CNS, the acute phase presents a low mortality rate, which has been reduced in recent decades with the implementation of early specific treatment. In the majority of patients, a spontaneous resolution of the symptoms occurs in 4–8 weeks, after which time the patients evolve to the chronic stage of the disease, given that there is no record of the spontaneous cure of the infection [24, 25].

The chronic stage of CD is long lasting, and in immunocompetent individuals, it is characterized by low parasitemia and high levels of antibodies from the IgG class. While asymptomatic and without signs of cardiac or digestive damage, the clinical form is referred to as "indeterminate." One or two decades later, 10–30% of the infected patients will evolve into the cardiac or digestive form of the disease. Cardiomyopathy develops insidiously and varies widely in its manifestations, ranging from asymptomatic electrocardiographic abnormalities to congestive cardiac insufficiency, arrhythmias, thromboembolic phenomena, dizziness, and

Table 25.1 Classification of Chagas cardiomyopathy (Brazilian Consensus on Chagas Disease 2015) [25]

Stage	Electrocardiogram	Echocardiogram	Heart failure
A	Abnormal	Normal	Absent
B1	Abnormal	Abnormal. Left ventricular ejection fraction >45%	Absent
B2	Abnormal	Abnormal. Left ventricular ejection fraction <45%	Absent
C	Abnormal	Abnormal	Present but treatable
D	Abnormal	Abnormal	Refractory

syncope, which are the typical findings in the chronic cardiac form (Table 25.1) [25]. The gastrointestinal form can appear as megaesophagus, which presents similar symptoms to idiopathic achalasia, with retrosternal pain, dysphagia, odynophagia, cough, and regurgitation, or as a megacolon, in which the patient presents abdominal pain and chronic constipation. In the more advanced stage, it can evolve into fecaloma, intestinal obstruction, perforation, and sepsis [25].

25.5 Reactivation of the CD in HIV Patients

Since the 1980s, with the beginning of the use of immunosuppressants and of organ transplants, as well as with the emergence of HIV, clinical conditions appeared for the reactivation of the pre-existing chronic infection caused by *T. cruzi*, with severe repercussions, especially in the CNS. The reactivation of CD is defined by the identification of *T. cruzi* trypomastigotes in the peripheral blood or in biological fluids, through direct microscopy and/or through the presence of inflammatory infiltrate similar to the acute phase of the disease, and in the presence of numerous amastigotes. The great majority of patients coinfected by *T. cruzi*/HIV contracted the disease through the (triatomine) vector in childhood and were later infected by HIV when adults [26]. Thus, nearly all of the coinfected patients suffered a reactivation of the disease and not an acute infection [14]. What factors are associated with reactivation is still relatively unknown, given that not all of the patients with a T CD4+ lymphocyte count of below 200 cells/mm^3 undergo reactivation, but it is known that the risk of reactivation increases with a decline in the T CD4+ lymphocyte count [16].

25.5.1 Reactivation in the CNS

25.5.1.1 Clinical Picture and Laboratory and Neuroimaging Exams

CNS is the most commonly affected in the reactivation of CD, occurring in nearly 75% of the cases [17, 27, 28]. Expanding mass lesions are commonly viewed in AIDS patients. The classic manifestations are those of acute meningoencephalitis,

with fever, headache, meningism, convulsions, altered mental state, vomiting, and focal neurological deficits. The CSF exam shows low to moderate numbers of cells, in general lower than 100 cells/ml, with a predominance of lymphocytes, an increase in proteins, and a reduction in glucose. The most relevant finding is the presence, in the CSF, of trypomastigotes in the direct, fresh exam. The prior centrifugation of the CSF increases the already high sensitivity of this method. Imaging methods, such as computed tomography, can reveal single or multiple hypodense lesions with contrast enhancement. Magnetic resonance imaging demonstrates one or more heterogeneous expansive lesions with a mass effect, hypointense in T1 and hyperintense in T2, and FLAIR, with an irregular or ring enhancement after the administration of gadolinium. The lesions predominantly affect the white matter. Though the infection of the CNS by *Toxoplasma gondii* in AIDS patients affects the basal ganglia and even more commonly, the thalamus, the CNS lesions in CD are erroneously attributed to toxoplasmosis and other opportunistic infections. This lack of specificity of the neuroimaging findings and the broad spectrum of differential diagnoses of the CNS expansive lesions in AIDS patients shows the difficulty of reaching a diagnosis, which, at times, demands the use of a brain biopsy to establish the precise diagnosis and the appropriate early treatment (see item 25.7).

25.5.1.2 Neuropathology

Three aspects differentiate the involvement of the CNS in immunocompromised chagasic patients from the acute nervous form observed in immunocompetent patients: encephalitis in multiple foci tends to take on a necrotizing aspect; numerous amastigotes are always present; and many patients present a pseudotumoral form (cerebral chagoma), characterized by the presence of single or multiple necrotic-hemorrhagic nodular lesions, usually located in the white matter of the cerebral lobes and, less frequently, in the brainstem and cerebellum [29, 30]. The lesions are poorly defined, softened, and measured several centimeters in diameter; the largest lesions are usually located in the cerebral hemispheres [31]. Histologically, there are necrosis, hemorrhage, microglial nodules, and infiltration of macrophages, lymphocytes, plasma cells, and, to a lesser degree, neutrophils, in the brain parenchyma and perivascular spaces. The amastigotes are abundant in astrocytes and macrophages and can be identified by conventional histology (Fig. 25.1), immunohistochemistry (Fig. 25.2), hybridization in situ, and PCR. There is no evidence of parasitism in neurons and oligodendrocytes. An inflammatory infiltrate of lymphocytes and macrophages is commonly observed in the leptomeninges, representing an extension of the encephalitic necrotic-inflammatory process. The pseudotumoral form was first described by Queiroz in 1973, in a patient with a clinical diagnosis of fungoid mycosis, the most common type of T-cell primary cutaneous lymphoma [32]. Although there is the possibility of the appearance of other opportunistic infections in patients coinfected with *T. cruzi*/HIV, the association of cerebral chagoma

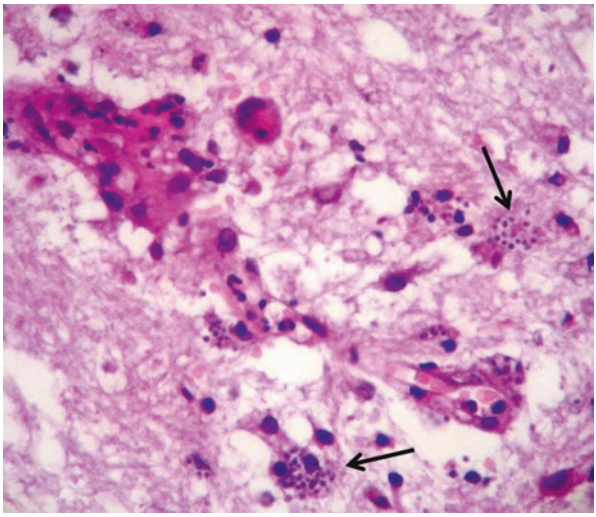

Fig. 25.1 Chagas disease. Reactivated acute form. Chagasic encephalitis. Necrosis of the nervous tissue. Amastigote nests surrounding small vessels (*arrows*). Hematoxylin and Eosin, ×450

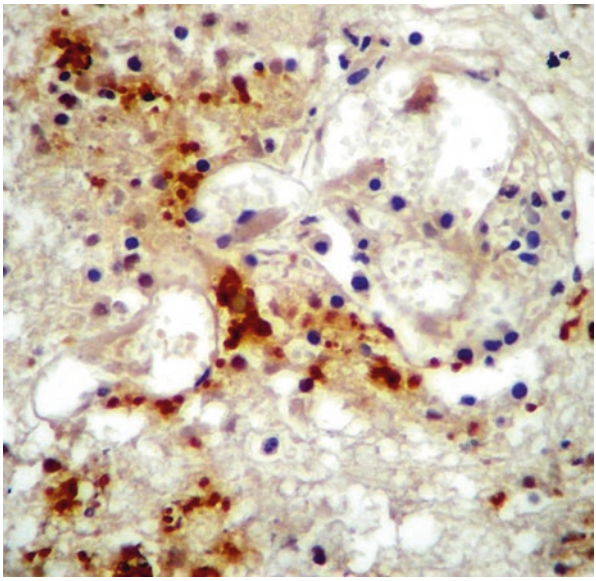

Fig. 25.2 Chagas disease. Reactivated acute form. Chagasic encephalitis. Numerous amastigotes. Immunohistochemistry of *Trypanosoma cruzi*, Peroxidase-antiperoxidase, hematoxylin counterstaining, ×450

with neurotoxoplasmosis has only been reported in two patients [31, 33]. One of these patients also presented a cerebral infection caused by cytomegalovirus and the herpes virus. In the few patients with a longer survival time, submitted to the tripanosomicide and anti-retroviral treatment, regression/healing of the lesions was observed in the neuroimaging exams.

25.5.2 Other Manifestations

The heart is the second organ most associated with the reactivation of CD in AIDS in about 25–45% of the cases [1]. The clinical manifestations generally include pericardial stroke, new arrhythmias, acute decompensated heart failure, or a quick progression of a pre-exiting chronic myocardiopathy.

Patients in general present a high level of immunosuppression, mostly with an advanced disease caused by HIV and a T CD4+ lymphocyte count of below 200 cells/mm^3 [4, 8, 12, 18, 34]. Myocarditis as a single form of the manifestation of CD in HIV patients is quite uncommon. This is usually associated with the involvement of the CNS, and its diagnosis is performed clinically or by autopsy [1, 31]. Findings from autopsy studies suggest that a significant proportion of the CD cases in patients with HIV/AIDS have a heart disease that is clinically silent, despite the presence of inflammation and amastigote nests in the myocardium [31]. In the largest Brazilian cohort, reactivation in the heart was as frequent as in the CNS [16].

Cutaneous lesions are frequent and are most commonly seen as erythematous nodules or plaques [35]. Less common manifestations include peritoneal invasion and esophageal, gastric, or intestinal forms. In a systematic review, Almeida et al. showed that the cases reported in the literature with involvement in other sites other than the CNS or the heart corresponded to approximately 9.2%, with the duodenum and the skin being the most common locations [18].

Limited data suggest that the vertical transmission rate of *T. cruzi* is greater in coinfected women when compared to immunocompetent pregnant women, even if there is no reactivation of the disease [36]. Children who are coinfected with *T.cruzi*/HIV have a greater risk of presenting severe, especially neurological, symptoms [37, 38].

25.6 Diagnosis

The diagnosis of the reactivation of CD in HIV patients requires a high suspicion index. Serological tests are required to diagnose CD, but these are insufficient to confirm reactivation and, in some cases, can be negative, despite pathological evidence of reactivation [39–41].

In immunocompetent individuals, high levels of parasitemia are characteristics of the acute stage of the disease [42]. During the chronic stage, parasitemia is quite low and cannot be detected by a direct microscopic exam. Indirect methods, such as xenodiagnosis, hemoculture, and molecular methods, are necessary to detect the parasite [42].

During the reactivation, the high level of parasitemia is quite common, and the parasites can easily be found in many body fluids: blood, CSF, ascitic fluid, etc.

The parasitological or histopathological studies are the final confirmation tests. The most useful parasitological tests are the direct exam of the peripheral blood and of the CSF, ideally performed after centrifugation [2, 39, 42]. These methods allow direct visualization of the flagellate and elongated forms of the trypomastigote.

Blood concentration techniques, such as capillary centrifugation, can improve sensitivity [37]. In centrifuged blood, *T. cruzi* trypomastigotes are found just above the buffy coat. Centrifugation and microscopic examination of the CSF can also be applied for patients with suspected CNS CD. Parasites may also be observed in lymph nodes, bone marrow, skin lesions, or pericardial fluid. Hemocultures are somewhat more sensitive than direct methods but take 2–8 weeks to identify parasites.

Conventional PCR is not useful in diagnosing reactivation, given that the method can yield a positive result in chronic *T. cruzi* infection when reactivation is absent [43]. However, quantitative PCR assays (real-time PCR) performed on serial blood specimens that show rising parasite numbers over time provide the earliest and most sensitive indicators of reactivation. Few published reports on PCR of the CSF can be found in the literature, but high sensitivity would be expected when diagnosing reactivation in the CNS [44].

The identification of trypomastigotes in the CNS is the major criteria for the definitive diagnosis of chagasic encephalitis. In a series of cases from Argentina, the parasite was detected in the CSF of 11 of 13 patients [11]. The importance of the brain biopsy to diagnose the pseudotumoral form of chagasic encephalitis can be exemplified by the identification of *T. cruzi* amastigotes in all of the brain tissue samples in which this procedure was carried out [45].

The dynamic of *T. cruzi* parasitemia in patients coinfected with HIV can play an important role in the reactivation process. The presence of parasitemia is more often detected in patients coinfected with HIV without reactivation than in HIV-negative patients. Patients with AIDS also present a higher level of parasitemia than patients with a less advanced form of HIV [46]. Some studies in HIV-positive patients show that the high level of parasitemia is a predictor of the reactivation of the disease [12, 47].

In the largest Brazilian cohort, 2–11 patients with reactivation diagnoses performed by microscopic parasitemia proved to be asymptomatic [16]. The authors hypothesized that the high levels of parasitemia quantified by more sensitive methods can, in some cases, detect an intermediate stage before clinical reactivation and preemptive treatment can be justified. However, no systematic data exists that can confirm this hypothesis. Despite the existence of techniques for the early diagnosis of the reactivation of CD, in general, the diagnosis is only performed during the later stages of reactivation, when the patient already presents the symptoms; therefore, many patients die even after beginning treatment. The T CD4+ lymphocyte count is the main prognostic factor, given that 80% of the reactivations occur in patients with CD4 < 200 cells/mm^3 [43].

25.7 Treatment

Treatment of CD with benznidazole or nifurtimox focuses on killing the parasite. Both medicines are nearly 100% effective in curing the disease if given soon after infection at the onset of the acute stage, including the cases of congenital transmission. However, the longer a person has been infected, the more the efficacy of both diminishes.

Both benznidazole and nifurtimox are limited in their capacity to induce parasitological cure, especially in chronically infected patients. Moreover, properly structured trials have not established whether or not the treatment of chronically infected individuals with either benznidazole or nifurtimox actually improves the outcome. Thus, the use of these drugs in such patients continues to be controversial [25].

Benznidazole (5–8 mg/kg/day for 30–60 days) is the first choice and most commonly recommended treatment. Nifurtimox (8–10 mg/kg/day, administered for 90–120 days) is an alternative, although less clinical experience exists regarding the use of this drug [48]. The duration of therapy with either of these agents has not been studied in patients who are coinfected with HIV [49].

In HIV-infected patients, it is necessary to optimize anti-retroviral therapy [44].

For HIV-infected pregnant women with a symptomatic reactivation of *T. cruzi* infection, anti-retroviral therapy should be applied as a first-line treatment. Although benznidazole and nifurtimox should not be taken during pregnancy, the literature does give two uneventful accounts of the treatment of CD with benznidazole during pregnancy [50].

Before the advent of anti-retroviral therapy, the prognosis of the reactivation of CD in HIV patients was quite grim. The average survival rate was about 10 days, and death occurred within 6 –8 months after the diagnosis [39]. The prognosis after the introduction of anti-retroviral therapy has been greatly improved, with a survival time that reaches 3–5 years [51]. The case reported with the longest survival time was 5 years [41]. In these few cases of longer survival times, neuroimaging exams show regression and/or healing of the lesions [41, 50–53]. Factors related to a better prognosis include early diagnosis and trypanosomicide treatment, adequate anti-retroviral therapy, and secondary prophylaxis [54, 55].

Conflict of interest The authors report no conflicts of interest.

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Chapter 26
Central Nervous System Tuberculosis

Beata Casanas, Douglas Holt, and Kelly Kynaston

Keywords Central nervous system tuberculosis • Disseminated tuberculosis • Extrapulmonary tuberculosis • Tuberculoma • Tuberculoma • Tuberculosis • Mycobacterium tuberculosis • Central nervous system tuberculosis • Extrapulmonary tuberculosis • Disseminated tuberculosis • Basilar meningitis • Paradoxical worsening • Hydrocephalus • SIADH

Core Message

- The relationship between human immunodeficiency virus and tuberculosis constitutes a well-recognized syndemic relationship due to the ability of each entity to affect the cellular immune response in a way that potentiates the spread and progression of the other.
- Index of suspicion and the ability to recognize the clinical syndrome of central nervous system tuberculosis is important as laboratory testing can be difficult to obtain and takes time for the results to become known.
- Managing therapy in this population can be difficult due to compliance, drug interactions, toxicities, immune reconstitution, paradoxical worsening, and emerging resistance.
- Without appropriate and effective therapy, CNS TB rapidly progresses to severe neurologic *sequelae* and death.

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26.1 Introduction

It is estimated that a third of the world's population has active tuberculosis [1], defined by the 2009 CDC case criteria as either a positive skin test or positive interferon gamma release assay plus signs and symptoms compatible with tuberculosis or isolation of *Mycobacterium tuberculosis* from a clinical specimen. In the USA, in 2014, the Center for Disease Control's national surveillance system reported 9412 incident cases [2]. Central nervous system tuberculosis (CNS TB) accounts for 1% of those [3]; the majority of whom were also HIV positive. Because of its rarity in the USA, CNS TB poses a significant challenge to physicians who may not consider it in their differential diagnosis during an encounter with a patient exhibiting neurological symptoms. While more common outside of the USA, the relative scarcity of diagnostic and therapeutic measures in developing nations means the approach to this syndrome requires strong clinical acumen. Understanding the relationship between the disease entities, their clinical presentation, and the management of central nervous system tuberculosis are paramount to stemming the tide of the two most common causes of worldwide mortality.

26.2 The Relationship Between HIV and TB

The relationship between these two microorganisms is unique due to the ability of either disease to reactivate and accelerate the progression of the other. This section will explore the impact this phenomenon has had on the reemergence of TB, the impact it continues to have on particularly susceptible populations, as well as the immunologic mechanisms through which it takes place.

26.2.1 Historical Context

With the twentieth century advent of modern pharmacotherapeutics, there was a steady decline in the rate of tuberculosis cases until 1985. At that time, a sudden surge in TB morbidity was noted. This trend was first reported in Dade County (Florida) where it was observed in immigrants [4, 5]. It was followed, in relatively rapid succession, by demographic studies in Newark (New Jersey), San Francisco (California), and the state of Connecticut. In these cases, it was linked to injection drug abuse and homosexuality [6, 7]. The common denominator seemed to be the emergence of the HIV/AIDS epidemic. In a morbidity and mortality weekly report from 1987, in response to the 1986 demographic data, health departments around the nation were urged to match their AIDS and tuberculosis registries [8]. Once done in New York, researchers concluded that "human immunodeficiency virus infection (HIV) is causing a resurgence of TB in NYC" [9].

26.2.2 Epidemiology

This epidemiological relationship still holds 30 years later, though with the development of highly active antiretroviral therapy and improved public health measures in industrial societies, it still most profoundly impacts developing nations. Statistics from the World Health Organization rank South Africa, India, Nigeria, Mozambique, and Zimbabwe among the highest incident HIV-TB coinfected cases in 2014 [10]. When accounting for immigration patterns into the USA, the Center for Disease Control lists immigrants from China, India, Mexico, the Philippines, and Vietnam to be at highest risk [2]. Repeatedly, studies published in developed nations list "foreign-born" status as a risk factor for both HIV and TB infections [3].

In addition to foreign-born status, other risk factors for morbidity seen among coinfection patients have been identified. This includes malnourishment which has a negative effect on the immune system making malnourished people more susceptible to disease [11]. It also includes crowded living conditions (residents of nursing homes, prisons, homeless shelters) which increases the chance for transmission [3]. Finally, also at risk are injection drug users and excessive alcohol users who may be not only engaging in high-risk behavior that could transmit disease but also tend to be noncompliant with medications [12]. Medication compliance is extremely important in all populations, and prognosis can usually be tracked by CD4 count; however, HIV/TB coinfected persons have been shown to generally have a higher mortality than those with either infection alone, regardless of CD4 count [13], suggesting a basis for synergism or other factors in the pathogenesis of coinfection.

26.2.3 The Important Role of the Cellular Immune Response

Mycobacterium tuberculosis is an intracellular pathogen; as such, it is inaccessible to antibodies for most of its lifecycle. T lymphocytes, notably CD4 and CD8 cells, play a predominant role in adaptive immunity against tuberculosis [14] which, when the immune system is intact, leads to the formation of granulomas. Histopathologically, granulomas are aggregates of macrophages, dendritic cells, B cells, and T cells. *Mycobacteria tuberculosis* bacteria may inhabit these T cells; in an HIV positive patient, especially one with uncontrolled disease, the T cells possibly also contain HIV virus. Whether a TB-infected cell acquires HIV or an HIV-infected cell acquires TB, the result is the accelerated replication of both diseases, leading to more rapid clinical progression and death. This has been explained by four main immunologic mechanisms.

First, it has been shown that active TB infection increases the HIV viral load. When an HIV-infected T cell gets exposed to tuberculosis, it activates a complicated cascade of molecular signals involving the T-cell antigen receptor (TCR). Downstream, TCR activation increases the expression of HIV coreceptor CCR5 resulting in increased susceptibility of CD4 T cells to HIV infection. Simultaneously, TCR activation stimulates the nuclear factor of activated T cells-c (NFATc) and NF-κB to promote the reverse transcription of HIV [14]. Therefore, *M. tuberculosis*-

infected macrophages are activated to produce HIV that is then available to infect adjacent macrophages and CD4 T cells in the granuloma itself.

Conversely, it has been shown that an active HIV infection accelerates the clinical progression of tuberculosis. In animal studies, where CD4, CD8, or both cells have been selectively depleted, the control of mycobacterial replication is poor [14]. Additionally, HIV is well known to cause T-cell depletion from the peripheral blood, mucosa, and lymphoid tissue either due to direct infection or due to the induction of apoptosis in neighboring cells. This suggests that CD4 T-cell deficiency in the HIV-infected patient is a direct cause of their increased susceptibility to TB.

In addition to quantitatively depleting the CD4 cell population, HIV infection also impairs the qualitative function of the remaining CD4 T cells. This is most clearly elucidated in the role of interferon-gamma (IFN-γ). In one study, CD4 cells from HIV-infected patients, who had undetectable HIV in plasma after treatment with ART for a minimum of 1 year, exhibited defective IFN-γ secretion in response to stimulation with M. tuberculosis purified protein derivative. The defective IFN-γ responses observed were more severe in chronically infected, ART-treated patients than in a group with primary HIV infection, suggesting that chronicity of infection causes a persistent defect that is not restored with effective HIV treatment [14]. Therefore, qualitative defect in the function of CD4 cells of HIV-infected individuals leads to more disseminated TB infections.

This disseminated infection is also noted to be more active, which has been linked to the presence of the HIV Nef protein. The HIV Nef protein downregulates the surface expression of the costimulatory molecules CD80 and CD86 on antigen-presenting cells which results in an impaired activation of naïve CD4 T cells. By disrupting antigen presentation and by undermining the essential molecular interactions critical for CD4 cell recognition and stimulation, HIV has evolved the ability to evade the host immune system. It should be noted that the impairment of host CD4 T-cell recognition of non-HIV antigen-bearing target cells, such as those infected with *M. tuberculosis*, is compromised as well [14]. This suggests that HIV-infected patients will have a greater likelihood of active rather than latent TB infection.

Recent studies have shown that the aforementioned molecular mechanisms can be extrapolated to the clinical setting. In a study of lung cells via an analysis of bronchialalveolar lavage (BAL) fluid, it was found that *M. tuberculosis* antigen-specific CD4 T cells were significantly less frequent in BAL fluid from HIV-infected subjects than in BAL fluid from subjects without HIV. Another study has utilized intracellular cytokine staining to quantitate the frequency of polyfunctional (IFN-γ-, TNF-, and/or IL-2-producing) mycobacterial antigen-specific CD4 T cells in sputum. It also revealed a significant reduction in the amount of polyfunctional CD4 T cells in BAL fluid of HIV-infected versus HIV-negative subjects [14]. This is a clinical confirmation that local adaptive immune responses are reduced in HIV-infected individuals. It follows that compromised local lung adaptive immune responses contributes to mycobacterial disease progression.

In summary, there are four primary immunologic mechanisms by which HIV and TB act synergistically. First, TB increases the HIV viral load. Second, HIV

increases susceptibility to acquisition of TB infection. Third, HIV accelerates the progression of TB. And, finally, HIV promotes the atypical presentations of extrapulmonary TB.

26.3 CNS TB Provides the Setting that Illustrates this Synergistic Relationship

According to the mechanism described above, as HIV-related immunosuppression increases, the clinical pattern of tuberculosis changes [15]. As it is more likely to be disseminated, it becomes more difficult to diagnose. This section will review the clinical presentation of CNS TB.

26.3.1 Pathophysiology

TB is acquired via inhalation of droplet nuclei which contain the *Mycobacterium tuberculosis* bacilli. Once in the alveoli, they trigger the immune system to activate and form a granuloma to contain the infection. However, before the granuloma is fully formed, the bacilli are filtered through lymph nodes and can enter the bloodstream causing a low-level bacteremia. This allows TB bacilli to disseminate to distant sites in the body; they can deposit anywhere, but they favor sites that are well oxygenated, such as the brain. A similar interplay of host immune factors and TB virulence factors as that described for the lungs takes place in the brain as well. When small tuberculous foci begin to develop in the brain, spinal cord, or meninges, it is the inauguration CNS TB [16]. As in the lungs, the CD4 quantitative and qualitative defects seen in HIV coinfection will predispose to a more aggressive disease. Several forms of CNS TB may ultimately occur.

26.3.2 Clinical Syndromes

As the pathophysiology suggests, CNS TB can be detected at different stages of the disease and thus its manifestations can vary.

26.3.2.1 Meningitis

If the tuberculous foci in the CNS rupture into the subarachnoid space, they cause a dense gelatinous exudate to form. This is composed of erythrocytes, macrophages, neutrophils, and lymphocytes. Though viscous, this envelops the meninges and causes irritation. This is the most common form of CNS TB [3].

26.3.2.2 Hydrocephalus

The gelatinous exudate has a predilection for the interpeduncular fossa as well as suprasellar regions of the brain. However, it may extend to the prepontine cistern and spinal cord; if it extends to the level of the tentorial opening, it leads to hydrocephalus [3].

26.3.2.3 Tuberculoma/Brain Abscess

If the tuberculous foci enlarge in the brain without rupturing, they can form a mass-like lesion [16]. Histopathologically, this is characterized by an inflammatory reaction consisting of epithelioid and giant cells mixed with lymphocytes around an area of caseating necrosis. It is called a tuberculoma if the central area of necrosis contains clear, straw-colored fluid. It is considered a tuberculous brain abscess if the central area of necrosis contains pus and viable bacilli [15]. These manifestations occur in 4–8% of patients without HIV and 20% of patients with HIV [17].

26.3.2.4 Vasculopathy

If *Mycobacterium tuberculosis* gets deposited in the cerebral vasculature, proliferation there (as well as the subsequent recruitment of inflammatory response) can cause luminal thrombosis or vessel rupture and intracerebral hemorrhage. This commonly occurs in territories of the middle cerebral artery perforating vessels [17].

26.3.2.5 Encephalitis

Encephalitis is characterized by brain edema and extensive demyelination. Histopathology in this scenario is characterized by microvascular necrosis and perivascular macrophage reaction and demyelination with focal glial nodules in the white matter [17]. This is the rarest manifestation, common in the younger population and alcoholics.

26.3.3 Diagnosis

Diagnosis of CNS TB can be difficult because the spectrum of symptoms which are indicative of one of the above presentations can be heterogeneous depending on the stage of progression, the exact location of cerebral insult, and the degree of immunosuppression. Thus, a high index of suspicion becomes very important in making the diagnosis. The clinician must be cognizant of a wide spectrum of clinical symptoms ranging from asymptomatic to focal deficits to global encephalopathy. If a patient does manifest symptoms, they tend to be broad spectrum and can include headache, fever, vomiting, confusion, and malaise. While the classic symptoms of tuberculosis – fevers, night sweats, and weight loss – are possible [18], the

differential for this "classic triad" is broad in the HIV population including lymphoma, histoplasmosis, coccidioidomycosis, MAI, toxoplasmosis, cryptococcus, or noninfectious cerebral vascular accident [20].

A few physical exam findings are characteristic of CNS TB but they should not be considered pathognomonic or universally present. Tuberculous meningitis can have classic meningismus or cranial nerve palsies. Special attention should be paid to cranial nerve VI as it is the most commonly affected [17]. Hydrocephalus can also present with visual symptoms including complete visual loss due to compression of the optic chasm. Ophthalmic exam may reveal choroid tubercles (yellow lesions with indistinct borders) or papilledema. Tuberculomas and abscesses will manifest with various different focal deficits depending on their location. Intracranial hemorrhage, too, can present with signs of mass effect or seizures. While working up seizures in this setting, however, it is important to consider hyponatremia as syndrome of inappropriate antidiuretic hormone (SIADH) does occur with CNS TB [1].

One clue on the physical exam maybe is the signs and symptoms of tuberculosis in extrameningeal locations. This will help to support the diagnosis and provide an easier location from which to obtain a culture. Indeed, demonstration of *Mycobacterium tuberculosis* in culture remains the gold standard for diagnosis [19]. There are several reasons why this may be challenging in the CNS. One, tuberculous lesions may be difficult to obtain depending on their location in the brain or the availability of neurosurgeons in a resource poor setting. Two, there is a paucity of tubercle bacilli circulating in the cerebral spinal fluid (CSF), and good lumbar puncture samples are positive only about 10–60% of the time. Three, once medical therapy has been initiated, the yield of the culture falls even lower [18]. Finally, *Mycobacterium tuberculosis* is a slow-growing pathogen, and the time it takes for the cultures to become positive may lead to progression of disease if not treated empirically before the results are known. Nonetheless, it remains a good practice to obtain CSF where feasible as sensitivity testing should be ordered.

CSF fluid analysis can provide clues to the diagnosis long before the culture becomes positive. On cytology, CSF classically demonstrates a lymphocytic pleocytosis, with moderately elevated protein levels and low glucose [1]. In some cases, neutrophils are seen and it is thought that they are indicative of either an early infection or a partially treated infection.

Several molecular-based testing techniques are available for pulmonary tuberculosis. While they may be off label, they can be ordered on the CSF, and the results can be extrapolated if the tests are understood and their limitations recognized. For example, antibodies can be detected in the CSF, but they cannot differentiate acute from previous infection. Nucleic acid amplification testing by polymerase chain reaction is also available. This has a sensitivity of 56% and specificity of 98% and can be used, in theory, up to 1 month after treatment has been begun [3]. Adenosine deaminase is a marker of cell-mediated immunity that is associated with lymphocytic proliferation. Its sensitivity varies from 44 to 100% and can be positive in lymphomas, malaria, brucellosis, pyogenic meningitis, and cryptococcal meningitis [18]. Tuberculotearic acid is a fatty acid component of the cell wall. While its sensitivity and specificity is good, it can only be in CSF using gas chromatography, so it is expensive and limited to research settings [3].

Tuberculin skin testing (TST) can also be ordered to help support the diagnosis. However, it is not specific enough to differentiate between active, latent, or treated disease. Also, its performance varies according to age, vaccination with bacillus Calmette–Guerin (BCG), nutritional status, HIV infection, and technique administration [18].

Interferon gamma release assays were developed as tests that are more specific and less technique dependent than the TST. They can be used on patients with a history of BCG vaccination [18]. Three assays are commercially available, all of which detect the release of the cytokine gamma interferon from T lymphocytes after stimulation with *M. tuberculosis*-specific antigens. The QuantiFERON tests use anticoagulated whole blood and measure the release of gamma interferon by ELISA. The T-spot uses isolated blood mononuclear cells and determines the number of gamma interferon-secreting cells by ELISPOT. Each test uses positive and negative controls; indeterminate results are reported if the negative control has a high signal or if the positive control has a low signal. An indeterminate result is common in patients with advanced immunodeficiency; because of this, the test is FDA approved in HIV-positive patients for the diagnosis of only latent TB [12].

As with the symptoms presented earlier, there are no pathognomonic findings on imaging for CNS TB, though computed tomography (CT) and magnetic resonance imaging (MRI) remain important tools. Findings that suggest CNS TB are varied. They include enhancement of the basal cisterns or choroid plexus suggestive of meningitis. Ventriculomegaly is suggestive of hydrocephalus. Lobulated masses with irregular walls, central nidus of calcification, and ring enhancement after contrast is suggestive of tuberculoma. Small hypodensities at the corticomedullary junction that also demonstrate ring-enhancing post contrast may be indicative of miliary TB. Vascular infarcts are common and attention should be paid to the lenticulostriate or thalamoperforating arteries. Infarcts in this vascular distribution will cause ischemic lesions in the internal capsule and basal ganglia. Brainstem lesions; enhancement of cranial nerves II, III, and VII; and dural venous sinus thrombosis have also been reported with CNS TB [3].

A final diagnostic strategy that can be utilized in case of suspicion of CNS TB may be to empirically administer treatment and assess for the likelihood of diagnosis by the appropriateness of response.

26.4 Management

This section will explore the principles of management of CNS TB including choosing pharmacotherapy, the expected clinical response (which oftentimes may manifest as worsening), and strategies for mitigating clinical deterioration.

26.4.1 Pharmacotherapy

For the most part, the principles of TB pharmacotherapy in HIV-infected patients are the same as those for HIV-negative patients. This includes a treatment phase with a combination regimen as well as a continuation phase with a two-drug regimen.

Specifically, the 2016 ATS/CDC/IDSA guidelines recommended a 2-month course of isoniazid, rifampin (or rifabutin), pyrazinamide, and ethambutol followed by a continuation phase with isoniazid and rifampin (or rifabutin) if the infection is caused by susceptible strains. Although an optimal duration of therapy is not explicitly defined, it is typically continued for 7–10 months and monitored by changes in cerebrospinal fluid cell count, glucose, and protein, especially early in the course of therapy [20].

It is important, when treating infection in the central nervous system, to understand which medications have the ability to cross the blood–brain barrier. Isoniazid and pyrazinamide pass easily across the BBB so that, in the case of CNS TB, isoniazid remains the backbone of therapy. Rifabutin has good penetration [15], while rifampin and ethambutol have less penetration. The aminoglycosides, second-line agents for TB, penetrate only when there is inflammation of the meninges. Flouroquinolones penetrate but at much lower concentrations than in the serum. The only other second-line agents that are able to penetrate are ethionamide and cycloserine [21]. Rarely, therefore, in drug-resistant CNS TB, has intrathecal administration been reported [22].

26.4.2 Interactions

Choosing therapy for the coinfected patient may be more challenging in terms of drug interactions with antiretrovirals; when possible, regimens should be chosen that minimize those interactions. A few drug interactions are of primary concern. First, as potent inducers of the cytochrome P450 system, rifampin and rifabutin have significant interactions with protease inhibitors. The combination of rifampin and protease inhibitors (PI) greatly accelerates the metabolism of the protease inhibitors resulting in negligible concentrations of PI in the serum. Furthermore, it induces activity of the efflux pump P-glycoprotein which further exacerbates this problem, decreasing the area under the curve (AUC) of protease inhibitors by 90%. The ideal alternative to bypass this interaction would be to keep rifampin and avoid the protease inhibitors altogether. However, if a PI-based medication must be chosen, then rifabutin can be used as an alternative to rifampin as it is a less potent inducer of CYP3A4 [12]. It does need to be dose- reduced to avoid toxicities [13].

Another noted drug interaction is also between rifampin and the integrase inhibitors. Of note, the 2015 DHHS Guidelines recommended using integrase inhibitors such as dolutegravir, raltegravir, or elvitegravir as part of the first-line therapy [23]. Rifapentine is not recommended to be coadministered with dolutegravir or elvitegravir. Rifampin, however, can be given with either raltegravir or dolutegravir so long as dosages are adjusted. The dose of raltegravir needs to be increased to 800 mg twice daily when it is coadministered with rifampin. The dose of dolutegravir should be increased to 50 mg twice daily when coadministered with rifampin [20]. An alternative strategy could be to utilize rifabutin instead of rifampin if an integrase inhibitor is needed as no dosage adjustments will be necessary.

Rifampin also has recognized drug interactions with the non-nucleoside inhibitors, namely, it is noted to decrease the AUC of nevirapine by 50%. If an alternative is needed, efavirenz and rifampin could be used together. While rifampin does

decrease the AUC of efavirenz by 22%, this was not shown to be clinically significant and the US guidelines do not recommend routine dose adjustment, though it could be considered for patients weighing over 60 kg [24].

A final interaction should be noted between rifampin and the CCR5 inhibitors. Rifampin reduces maraviroc by 78% [24], though this is less significant as maraviroc would not be a first-line therapy in the USA. If needed, it could be given with rifabutin (no dose adjustment) or with rifampin (at increased maraviroc doses).

26.4.3 Paradoxical Worsening

HIV patients that receive a diagnosis of TB and do not receive highly active antiretroviral therapy (HAART) therapy have a mortality approaching 91% [15]. Therefore, the recommendation that patients receive treatment for both diseases is very compelling. What remains less clear, however, is the timing, although recent data from South Africa supports earlier rather than later therapy. The SAPIT trial was a randomized controlled trial designed to answer this question. It randomized 642 TB-HIV coinfected patients to one of three arms. The first received ART 4 weeks after the start of TB treatment. The second arm received ART after 2 months of TB therapy. And the third arm received ART after 6 months. Researchers concluded that the earliest therapy reduced mortality by 56%, and this was true among all CD4 counts [25]. These results have been largely replicated in the CAMELIA trial so the recommendation for earlier therapy, regardless of CD4 count stands [26].

The reason that the timing remains controversial is that the use of HAART in patients with active infections of the CNS leads to a paradoxical immune reconstitution inflammatory syndrome (IRIS) that can be more serious than IRIS of other organ systems. In general, IRIS is a clinical deterioration 4–8 weeks after the initiation of HAART due to the sudden surge in the ability of the immune system to respond to opportunistic pathogens that were left unchecked when the immune system was depressed. It sets off a cascade of inflammatory cytokines that recruits inflammation to any remaining mycobacterium antigens. When this happens in the CNS, it exacerbates neurological symptoms [12]. These symptoms should be managed with steroids which modify cytokine production by CNS microglial cells. Specifically, the 2016 ATS/CDC/IDSA guidelines recommend dexamethasone or prednisolone tapered over 6–8 weeks. This use of adjunctive corticosteroids has been shown to have a mortality benefit [20].

There is no laboratory test for IRIS; it remains a clinical diagnosis which means that understanding of the timelines involved in defervescence after the initiation of therapy is important as is understanding of the differential diagnosis of acute clinical worsening. The international network for the study of HIV-associated IRIS has made criteria for more objectively identifying TB-IRIS, provided that the diagnosis of TB was made and that there was an initial positive response to TB therapy both prior to the initiation of ART therapy. Major criteria include new or enlarging lymph nodes, cold abscesses, or other focal tissue involvement; new or worsening radio-

logical features of TB; new or worsening CNS TB; or new or worsening serositis. Minor clinical criteria include new or worsening constitutional symptoms (fever, night sweats, weight loss); new or worsening respiratory symptoms; new or worsening abdominal pain with peritonitis, hepatomegaly, splenomegaly, or abdominal adenopathy. To meet criteria for diagnosis, a patient must have one major or two minor criteria. Also, they must occur within 3 months and alternate explanations for the symptoms must be ruled out [27] as management strategies for these other entities is entirely different. Importantly, IRIS due to other opportunistic organisms requires therapy for those other organisms.

A patient may also experience worsening clinical status after the initiation of HAART due to adverse effects of therapy, especially as therapies for both TB and HIV have overlapping toxicities. Peripheral neuropathy is a concern with isoniazid, stavudine, and didanosine but can be mitigated by the coadministration of B6 [13]. Hepatotoxicity is a concern among isoniazid, rifabutin, pyrazinamide, protease inhibitors, and nonnucleoside reverse transcriptase inhibitors. Concomitant hepatitis B or C may contribute to that risk. Drug-induced hepatotoxicity becomes a concern when the liver function tests (LFT) are 5x above normal. In this situation, pyrazinamide should be stopped but isoniazid, rifampin, and ethambutol may be continued with daily LFT monitoring. If the labs continue to rise or if serum albumin falls, then isoniazid and rifampin should be changed to streptomycin and a fluoroquinolone. Rifampin and isoniazid can be rechallenged once the LFTs return to normal; the recurrence rate is expected to be about 10% [12].

Finally, a patient may experience worsening clinical status after the initiation of HAART due to the unmasking of antimycobacterial treatment failure, which occurs in about 10% of coinfected patients [28]. If multidrug-resistant TB is suspected, then resistance testing should be obtained. Mycobacterium tuberculosis is naturally resistant to many antibiotics because it has hydrolytic and drug-modifying enzymes. These include periplasmic beta-lactamases, aminoglycoside acetyltransferases, and drug efflux systems. Moreover, it has a highly hydrophobic cell wall which makes antibiotic penetration more difficult [29]. Resistance is usually conferred via random chromosomal mutations [29]. Acquired rifamyacin resistance is also of concern in the coinfected population and has been correlated with advanced HIV disease (CD4 less than 100), intermittent anti-TB therapy, and low drug levels of both rifamycin and isoniazid [24]. The principal drivers of resistance include patient noncompliance as well as prescription patterns that are inappropriate for the clinical setting. Inappropriate therapy is defined as the use of a single drug, inappropriate combinations of drugs, short treatment periods, and low absorption of administered drugs as all these conditions may lead to exposure of MTB to sublethal concentrations which will favor the selection of resistant bacilli. When resistance is suspected, the addition of a single drug is inappropriate as this mimics monotherapy [29]. Second- and third-line TB regimens include aminoglycosides though it is important to note they can be nephrotoxic with tenofovir or increase the peripheral neuropathy found with stavudine and didanosine. Cycloserine may have increased psychiatric disturbances when used with efavirenz [13] (Tables 26.1 and 26.2).

Table 26.1 Adapted from Sterling TR, Pham PA. HIV Infection – Related tuberculosis: Clinical manifestations and treatment. Clinical infectious disease 2010 (50): S227 [24]

HIV infection treatment	TB treatment	Interaction	Recommendation
PIs, unboosted (no ritonavir)			
Atazanavir, indinavir, nelfinavir, and saquinavir	Rifampin	Rifampin reduces C_{max} AUC and trough by >80%	Do not coadminister
Atazanavir, indinavir, and nelfinavir	Rifabutin	Increased concentrations of rifabutin with variable effects on PI exposure	Rifabutin and unboosted PIs may be coadministered (with dose adjustment), but alternative regimens are preferred because of limited safety and efficacy data
PIs, boosted (with ritonavir)			
Lopinavir, fosamprenavir, atazanavir, indinavir, darunavir, and tipranavir	Rifampin	Rifampin reduces C_{max} AUC, and trough significantly; double dosing of PI is toxic and may not overcome interaction	Do not use
	Rifabutin	Modest decreases in PI exposure; ritonavir increases rifabutin exposure, potentially resulting in toxicity	Usual PI administered with ritonavir, rifabutin dosage of 150 mg every other day
Nonnucleoside reverse-transcriptase inhibitors			
Efavirenz	Rifampin	Rifampin reduces efavirenz exposure by −20%	Administer both drugs at usual doses; some recommend increasing efavirenz dose to 800 mg
	Rifabutin	Efavirenz increases rifabutin clearance by 30–40%	Rifabutin dosage should be increased to 450–600 mg daily or 600 mg 3 times weekly
Nevirapine	Rifampin	Rifampin reduces nevirapine AUC by 37–58% and C_{min} by 37–68%	Avoid combination if possible because of higher rate of virological failure; use of full-dose nevirapine (200 mg twice daily) with rifampin may be effective
Eiravaine	Rifabutin	Minimal interactions	May be coadministered safely at usual doses
		Significant interaction of rifampin on etravirine exposure	Do not coadminister

	Rifabutin	Modest bidirectional interaction with reductions in exposure to both agents	May be coadministered with rifabutin dosage of 300 mg daily; do not coadminister with DRV/r or SQV/r in regimen because of interaction between etravirine and DRV/r or SQV/r
Integrase inhibitors			
Raltegravir	Rifampin	Rifampin reduces C_{max} AUC, and trough levels by 60–70%; doubling raltegravir dosage to 800 twice daily improves C_{max} and AUC but does not affect reduction in trough concentration	Do not coadmineter; consider rifabutin with raltegravir coadmirastration
	Rilabutin	Rifabutin reduces raltegravir trough by 20%, but raltegravir AUC is not affected	Administer rifabutin (300 mg daily) with raltegravir (400 mg twice daily)
Coreceptor inhibitors			
Maraviroc	Rifampin	Rifampin reduces maraviroc exposure by >60%	Do not coadminister or increase maraviroc dosage to 600 mg twice daily
	Rifabutin	Modest impact of rifabutin on maraviroc exposure likely	Administer maraviroc (300 mg twice daily) and rifabutin (300 mg daily)
Fusion inhibitors			
Enfuvritide	Rifampin and rifabutin	No interactions	No dose adjustments necessary
Nucleoside analogues			
Zidovudine	Rifampin	Rifampin reduces zidovudine AUC by 47%, but effect on intracellular concentrations unknown	Clinical significance unknown

Table 26.2 Adapted from World Health Organization. Treatment of tuberculosis: guidelines 4th edition. 2010: 61 [30]

Side effects	Drug(s) probably responsible	Management
Major		*Stop responsible drug(s) and refer to clinician urgently*
Skin rash with or without itching	Streptomycin, isoniazid, rifampicin, pyrazinamide	Stop anti-TB drugs
Deafness (no wax on otoscopy)	Streptomycin	Stop streptomycin
Dizziness (vertigo and nystagmus)	Streptomycin	Stop streptomycin
Jaundice (other causes excluded), hepatitis	Isoniazid, pyrazinamide, rifampicin	Stop anti-TB drugs
Confusion (suspect drug-induced acute liver failure if there is jaundice)	Most anti-TB drugs	Stop anti-TB drugs
Visual impairment (other causes excluded)	Ethambutol	Stop ethambutol
Shock, purpura, acute renal failure	Rifampicin	Stop rifampicin
Decreased urine output	Streptomycin	Stop streptomycin
Minor		*Continue anti-TB drugs, check drug doses*
Anorexia, nausea, abdominal pain	Pyrazinamide, rifampicin, isoniazid	Give drugs with small meals or just before bedtime, and advise patient to swallow pills slowly with small sips of water. If symptoms persist or worsen, or there is protracted vomiting or any sign of bleeding, consider the side effect to be major and refer to clinician urgently
Joint pains	Pyrazinamide	Aspirin or nonsteroidal anti-inflammatory drug or paracetamol
Burning, numbness, or tingling sensation in the hands or feet	Isoniazid	Pyridoxine 50–75 mg daily (*3*)
Drowsiness	Isoniazid	Reassurance. Give drugs before bedtime
Orange/red urine	Rifampicin	Reassurance. Patients should be told when starting treatment that this may happen and is normal
Flu syndrome (fever, chills, malaise, headache, bone pain)	Intermittent dosing of rifampicin	Change from intermittent to daily rifampicin administration (*3*)

26.5 Conclusion

This chapter reviewed the pathophysiology, diagnosis, and treatment of central nervous system tuberculosis in the unique setting of the HIV coinfected patient. These two clinical entities are a well-recognized syndemic, individually causing a depression of the cellular immune response that potentiates the spread and progression of the other. Understanding of this complex interplay between the two diseases is critical for both recognition as well as management of CNS TB as both rely heavily on clinical reasoning over laboratory testing. Without appropriate and effective therapy, CNS TB rapidly progresses to severe neurologic sequelae and death. Further research would be beneficial in mitigating these devastating effects. Of crucial necessity, are new medications for MDR strains, biomarkers for IRIS, more rapid diagnostics that are less dependent on T-cell count, and hopefully ultimately a vaccine to prevent the development of disease entirely. This would have significant impact around the world, but especially in areas of high endemicity and limited resources where HIV and TB are the number one and number two causes of death.

Conflict of interest The authors report no conflicts of interest.

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Chapter 27
Neurosyphilis and HIV Infection

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Core Message

- HIV and syphilis have a complex interaction at a molecular level that leads to a higher rate of neurosyphilis, among HIV-coinfected patients than those who are HIV negative.
- Neurosyphilis rates are likely to be high globally among HIV-infected patients since there is a high rate of syphilis-HIV coinfection given shared demographic and route of transmission.
- In HIV patients with ophthalmologic and/or otologic complaints, there should always be a high index of suspicion for neurosyphilis.
- Diagnostic workup for neurosyphilis in HIV-coinfected patients is similar to non-HIV patients although a negative finding should not preclude additional modality of testing in the setting of clinical suspicion.
- Intravenous penicillin remains the treatment of choice for neurosyphilis.

27.1 Background

HIV and syphilis remain diseases of major public health importance globally. According to the 2008 World Health Organization (WHO) estimate, there was a 36.7 million prevalence of HIV as well as 2.1 million new cases diagnosed that year. Interestingly, there were also an almost equal number of syphilis prevalence and 11

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million new sexually transmitted cases worldwide [1]. Since the presence of genital ulcers increases the likelihood of both the acquisition and transmission of HIV, syphilis rates are much higher among HIV-positive patients [2]. There has been a worldwide increase in the incidence of syphilis parallel to the rise of HIV. In some parts of Africa, rates of HIV-syphilis coinfection are reported to be 2–8% [3, 4], and rates of neurosyphilis are estimated to have increased correspondingly. Similar increases in syphilis and neurosyphilis rates have been reported in Asia and Europe [5–8]. The incidence of neurosyphilis is difficult to quantify partly because of incomplete reporting even in the industrialized nations such as the USA; however neurosyphilis rates mirror the rates of syphilis in general and thus likely to be on the rise as well. HIV and syphilis coinfection is estimated to account for 25% of primary and secondary syphilis cases reported in the USA [9, 10]. Moreover, as indicated, there is a complex demographic, epidemiologic, biological, and clinical interplay between HIV and syphilis [11]. For instance, the pro-inflammatory lipoprotein component of *Treponema pallidum* has been shown to induce the major HIV co-receptor CCR5 [12, 13]. Thus not only is there an increased influx of inflammatory cells to syphilitic lesions, but also there is an increased susceptibility of these cells being infected by the HIV virus. HIV is also known to alter the natural history of syphilis in such a way that HIV-positive patients who contract syphilis tend to have a persistent chancre (primary lesion) and more extensive ulceration of genital and cutaneous lesions [11, 13, 14]. It is worth noting that the presence of *T. pallidum* in the cerebrospinal fluid (CSF) has been shown less than 24 h postinoculation in animal studies [15]. Neurosyphilis, a central nervous system (CNS) invasion by *T. pallidum*, has been shown to develop early in the course of syphilis [16, 17], and it is estimated that CNS invasion occurs in 15–30% of people who contract syphilis [18, 19]. The clinical syndromes of neurosyphilis range from asymptomatic to major and at times irreversible neurologic deficits [20]. It is generally agreed that neurosyphilis occurs early in the course of the disease in HIV-coinfected patients and presents in the form of acute syphilitic meningitis, and neuro-ophthalmologic involvement is increasingly recognized as a common presentation [21–23]. Penicillin treatment failure rates of as high as 5% have been reported in HIV-positive patients although this has been held in doubt since it could represent either true treatment failure versus equivocal interpretation of follow-up serologic tests which may have persisted in spite of successful prior treatment. Although there is discordant expert opinion on the subject, there may be a subtle difference between HIV-positive and HIV-negative patients in regard to interpretation of diagnostic tests as well as approach to therapeutic and posttreatment follow-up strategies, for instance, initial CSF serology for syphilis could be negative, and posttreatment decline of CSF serology may take a longer course in HIV patients [24–26].

27.2 Brief Review of *Treponema pallidum* and HIV Biology Pertaining to Neurosyphilis

T. pallidum, subspecies pallidum, is the causative agent of syphilis. It is an obligatory intracellular gram-negative bacterium with one of the smallest genomes of all bacteria. Its outer membrane is mainly composed of lipopolysaccharides and has an

unusually low density of peptide residues [27, 28]. This property may explain the lower sensitivity of some of the available serologic tests for syphilis and the ability of *Treponemes* to persist in several types of tissues despite a vigorous immune response [28, 29, 30]. One such immune response induced by *T. pallidum* is overexpression of the chemokine ligand CCR5 on inflammatory cells particularly in CD4 lymphocytes and dendritic and microglial cells of the CNS. The HIV virus glycoprotein (gp120) in turn mimics chemokines that bind to CCR5 which is known to be a major co-receptor for HIV binding and cellular internalization. Thus the complex interplay between *T. pallidum* and the HIV virus has a molecular basis as well as epidemiologic and clinical dimensions [13, 31].

27.3 Pathology/Pathogenesis of Neurosyphilis in HIV

Once contracted, *T. pallidum* attaches to host epithelial tissues. The expression of adhesin peptides that bind to fibronectin, laminin, and collagen residues found in the extracellular matrix, plasma, and epithelial cell surfaces of the host has been shown to mediate this process of binding and is believed to be one of the determinants of virulence of T. strains [32–34]. This adherence to cellular structures at the portal of entry, local replication, subsequent inflammation with tissue destruction, and ulceration results the characteristic syphilitic chancre in the first stage of (primary) syphilis [27, 30]. Recent studies have also revealed that activation of vascular endothelial cells and the induction of intercellular adhesion molecule (ICAM-1) expression by treponemal infection play an important role in the pathogenesis of syphilis [35, 36]. The early involvement of arterioles by syphilis and the ensuing arteriolar inflammation (the endarteritis, which is sometimes referred to as "luetic vasculitis") assures that syphilis obtains entry into the bloodstream and access to almost all tissues including the CNS. Thus it is important to note that from its onset, syphilis establishes itself as a disseminated disease even at the early primary stage and in the absence of other systemic clinical symptoms. Symptomatic early syphilis was considered in the past to have been a rare presentation of neurosyphilis. However the consequence of early dissemination and intense inflammation of the meninges leading to *acute syphilitic meningitis* is one of the primary manifestations of neurosyphilis in HIV-coinfected patients [37]. If left untreated, meningeal inflammation involves surrounding vascular structures with lymphocytic infiltration leading to a gradual destruction of neurons and generalized atrophy of the involved cortex, usually of the frontal and temporal lobes of the brain along with dilatation of the lateral ventricles; this process characterizes a different neurosyphilis pathology known as *parenchymatous neurosyphilis (also known as general paresis)*. This feature of neurosyphilis at times also affects the cerebellum and basal ganglia. The predilection of *T. pallidum* for arterioles of the CNS, ensuing obliterative endarteritis, thrombosis with micro infarctions, and ischemic necrosis results in a distinct clinical entity which results in stroke-like symptoms and is generally termed *meningovascular syphilis* [38, 39]. Degeneration of the posterior column of the spinal cord is seen in up to 10% of neurosyphilis and leads to one of the most well-known

syphilis syndromes called *tabes dorsalis*, and it can occur up to two decades after the primary infection [23, 40]. Formation of *syphilitic gumma* may be another presentation, which had been rare even before the advent of antibiotics. Gumma is characterized by a central area of coagulative necrosis, surrounded by epithelioid giant cells and a perimeter of fibrotic tissue. Anatomically it can occur anywhere in the brain or the spinal cord and has recently been reported in the optic nerve in HIV-coinfected patients [41].

27.4 Clinical Features of Neurosyphilis in HIV-Coinfected Patient

Neurosyphilis generally presents as an early and/or late manifestation of CNS invasion by *Treponema pallidum*. Early neurosyphilis in the form of *acute syphilitic meningitis* is an increasingly recognized manifestation of syphilis in the HIV-infected patient. In addition to the classic symptoms and signs of meningitis such as headache, neck stiffness pain, mental status change, and stiff neck, cranial nerve neuropathies have also been reported. Cranial nerves II and VIII are most commonly affected, and manifestations include visual loss and impairment of hearing and balance. Facial nerve involvement and palsies have also been reported [42–44]. While visual symptoms may be due to optic nerve involvement, another presentation that is an increasingly recognized presentation of neurosyphilis in HIV-coinfected patients is *ocular syphilis*. The prevalence of ocular involvement in HIV syphilis coinfected patients is estimated to be about 10% [45, 46]. Some experts consider ocular syphilis to be an extension of neurosyphilis, although a distinct form of the disease presenting as *acute pan-uveitis* is the more typical manifestation in HIV-infected patients [47, 48]. Ocular involvement is more likely to be bilateral in HIV-infected patients, and in addition to uveitis, inflammation may involve any or all parts of the eye, thus causing keratitis, scleritis, chorioretinitis, or even syphilitic gummas of the optic nerve [49, 50]. *Ear involvement (otosyphilis)* presents as hearing loss, tinnitus, and dizziness and is considered as part of the neurologic manifestation of neurosyphilis with or without meningitis.

Meningovascular syphilis results from endarteritis of the meningeal vasculature and the resulting thrombosis and infarction which lead to symptoms of a cerebrovascular event. Hemiparesis, hemiplegia, impairment of speech, and seizures are the most common clinical presentations. Symptoms can start suddenly as an acute stroke, or they may take a more insidious course [22, 44, 51, 52]. Meningovascular syphilis has also been reported to involve the spinal cord in the form of acute or subacute transverse myelitis and may occur 5–10 years after the initial infection. Clinically patients present with paraplegia, sphincter dysfunction, and sensory levels [38, 53]. Another manifestation of neurosyphilis is *general paresis* (sometimes referred to as paretic neurosyphilis) which is a neuropsychiatric degenerative disorder leading to progressive cognitive decline, tremors, myoclonus,

hyperreflexia, seizures, and impairment of speech, memory, mood, and sleep that can eventually lead to frank psychosis. Hence it is sometimes referred to as "general paralysis of the insane" [46, 51, 53, 54]. *Tabes dorsalis* is one of the late manifestations of neurosyphilis occurring 15–20 years after the primary infection. Loss of dorsal column function such as position and vibration sense, gait ataxia, urinary dysfunction, paresthesia, and periods of intense neuropathic pain (tabetic crisis) characterizes its clinical presentation [44, 55]. Even though tabes dorsalis is considered to be rare in the modern era, it has been reported in recent years in an HIV-positive patient [56, 57].

27.5 Diagnosis of Neurosyphilis in the HIV-Coinfected Patient

Although most experts generally agree that serologic tests for syphilis have similar utility in HIV-positive patients as in their use in the general population, it is important to keep in mind that delayed seroreactivity and higher rates of false-negative results have been reported in HIV-coinfected patients [58]. In the setting of suggestive clinical presentation and negative serologic tests, alternative testing such as dark field examination, PCR testing for *Treponema pallidum*, or examination of a biopsy specimen should be obtained [59]. Neurosyphilis should be considered in the differential diagnosis of neurologic signs and symptoms in both persons with HIV and those considered to be high risk for HIV [58, 60, 61].

Serologic tests for syphilis are generally divided into treponemal and non-treponemal tests.

27.5.1 Non-treponemal Serologic Tests

The two most common non-treponemal serologic tests are RPR (rapid plasma regain) and VDRL (venereal disease research laboratory) test which both detect and measure IgM and IgG antibodies formed in response to phospholipid residues of damaged host cells or treponemal surface components. RPR is a simplified form of the VDRL test and is used for screening as well as monitoring of syphilis activity. These tests lack specificity and could have a false-positive rate of up to 2% in the general population [62]. Several non-treponemal conditions including malaria, tuberculosis, hepatitis, rheumatic heart disease, autoimmune disorders, Lyme disease, and IV drug use could lead to a "positive" non-treponemal serology. This is particularly important from a global perspective since hepatitis, rheumatic heart disease, malaria, and tuberculosis affect a large proportion of the world population, and the specificity of the non-treponemal tests may be even lower in these subpopulations. Non-treponemal tests also have reduced sensitivity in both early

syphilis and, according to some reports, also in HIV-coinfected patients. The prozone phenomenon, which is a disproportionately high level of antibodies interfering with the testing procedure and resulting in false-negative results, is also another aspect of the non-treponemal test that should be considered when performed in the HIV-coinfected patient [63, 64].

27.5.2 Treponemal Tests

Treponemal tests use antigens derived from the specific *T. pallidum* subspecies or an antigen produced using recombinant techniques. It is generally performed as a confirmatory test to verify a reactive non-treponemal test. These tests detect total IgG and IgM levels. Even though they are used as a screening test in Europe, in the USA their use for screening is limited to occasion when clinical suspicion is high in the presence of a negative non-treponemal test [62, 65].

FTA-ABS (Fluorescent treponemal antibody absorption) is an indirect fluorescent antibody test with samples pretreated for nonspecific antibodies, thus tend to be highly sensitive and specific for *T. pallidum*.

TP-PA (*Treponema pallidum* particle agglutination) test is a commonly used treponemal test. A positive TP-PA with a non-treponemal test such as RPR or VDRL indicates current or past infection. It has high sensitivity and is also used in monitoring response to therapy [66, 67].

Enzyme Immunoassay (EIA) For specific IgM and IgG anti-*T. pallidum*, immunoblot tests have equal or greater sensitivity and specificity as FTA-ABS and TP-PA tests; however its high sensitivity may be misleading for those patients who may have had a successful treatment for syphilis in the past but continue to have circulating antibodies. This issue is applicable to HIV-coinfected patients. Thus the CDC recommends that if EIA is used as a screening test, a follow-up RPR should be done, and if it is positive, a second confirmatory test such as FTA-ABS and TP-PA test should be done for a more definitive diagnosis. This may limit the utility of EIA especially in HIV-coinfected patients [62, 68].

Western Blot Molecular characterization of antibody response used to resolve inconclusive serologic tests. It is a highly sensitive and specific test but not suitable for routine use as it is not widely available; it may have more utility in the future [69, 70].

Point of Care tests for syphilis Latex agglutination or immunochromatographic tests are commercially available that can be used at the point of care using whole blood, serum or plasma. They are not approved for use in the US. but WHO has evaluated a number of them showing sensitivity and specificity in the range of 85–98% [62, 71, 72]. These tests are easy to perform, and require very limited technical skill and do not require refrigeration or automation; thus it could be of significant utility in the developing world once their validity is ascertained in the future.

27.5.3 *Direct Detection of* T. pallidum

Dark Field Microscopy It requires fluid from a lesion or exudate. It may be particularly useful in HIV where the primary lesions could persist longer. However it is operator dependent, and false-negative rates are high.

Direct Fluorescent Antibody Test for *T. pallidum* It uses fluorescein isothiocyanate-labeled antibody specific to pathogenic treponemes and therefore is suitable for the examination of specimens from oral and rectal lesions. Although its sensitivity is high, it doesn't differentiate venereal from non-venereal endemic syphilis such as pinta and yaws.

Direct Test for *T. pallidum* **in Tissue Sections** Nucleic acid amplification test: it is a PCR-based test that holds promise as a test of choice for neurosyphilis and to monitor response to therapy. It is not widely available but can be done at select laboratories.

There is some discordance among experts on whether neurosyphilis takes a different course in HIV-coinfected patients and whether diagnostic (and therapeutic) approaches need to be different. However it is generally agreed that the diagnosis of syphilis in an HIV-infected patient should raise the index of suspicion for the likelihood of neurosyphilis, bearing in mind that neurosyphilis could be asymptomatic especially in the early phase. It is also generally agreed that among HIV-syphilis coinfected patients, a lower CD4 count alone increases the likelihood of neurosyphilis. The odds of having neurosyphilis is reported to be up to 3× higher when CD4 < 350 and/or serum RPR titer is 1:32 or higher. Understandably, one recommendation based on the literature uses this cutoff to recommend that CSF analysis be performed in HIV patients manifesting these CD4 and/or RPR levels [46, 73, 74]. It is also generally agreed that presentation with ophthalmologic complaints in an HIV-positive patient should raise suspicion for neurosyphilis. CSF analysis is also recommended when the duration of untreated syphilis is over 2 years or when the duration is unknown.

CSF specimens should be examined for total protein, leukocyte counts, and VDRL. It is important to note that the CSF protein level and the cell count could be normal in neurosyphilis, especially in cases of parenchymatous neurosyphilis such as patients with general paresis and tabes dorsalis. On the other hand, high number of mononuclear cells could alone be observed in the CSF of HIV-positive patients [51, 75]. The CSF VDRL test has high specificity (99–100%) but low sensitivity for neurosyphilis [67, 76]. Thus, while a reactive CSF VDRL test is an indicator of neurosyphilis, a nonreactive test cannot be used to exclude neurosyphilis. In contrast, the FTA-ABS CSF test has high sensitivity; thus, when a negative FTA-Abs CSF test result is obtained, there is a high probability that neurosyphilis can be excluded [35].

CSF RPR is believed to be equally valid, although the quantitative titers from the two tests (RPR and VDRL) cannot be compared. When serologic tests do not correspond with clinical findings that are suggestive of early syphilis, presumptive

treatment is recommended, and use of other tests (e.g., PCR, Western blot and/or biopsy if feasible) should be considered.

CSF PCR for the presence of *T. pallidum* to help establish a diagnosis of neurosyphilitic is currently considered of little value since results to date have shown low sensitivity and specificity [19, 63].

27.5.4 Imaging in Neurosyphilis

Imaging studies have a role (though limited) as diagnostic tools, since some of the neurosyphilitic pathologies do lead to abnormal imaging signals. Typical findings include lacunar infarctions in the middle cerebral artery distribution and intracerebral gummas which show as space-occupying lesions and generalized atrophy which is seen in general paresis and syphilitic myelitis of the spinal cord which on MRI can appear as diffuse white matter T2 hyperintense signals . Orbital MRI may show nerve sheath enlargement in ocular syphilis [58]. Cranial nerve involvement may be seen on MRI as well, but in cases of chorioretinitis, cerebral angiography may have a limited role since its use as a diagnostic tool is probably limited. Vascular ectasias and irregular stenosis in large and small vessels may be seen as well [65].

When available single-photon emission computed tomography (SPECT) and positron emission tomography (PET) could be useful in cases of negative MRI which clinical suspicion warrants it. Increased cerebral blood flow consistent with increased inflammation may also be used to gauge posttreatment follow-up [68].

27.5.5 Electrophysiologic Testing

Several reports of complex partial seizures and subclinical status epilepticus have been reported as manifestations of neurosyphilis. Epileptiform discharges even in the absence of focal imaging abnormality on CT or MRI have been reported. Nerve conduction abnormalities can be demonstrated in tabes dorsali.

27.6 Treatment of Neurosyphilis

Below are the recommendations for the treatment of neurosyphilis from the WHO, European guidelines, and the CDC (Tables 27.1 and 27.2).

The WHO recommends that penicillin serum levels be raised to 0.018 mg/L and be maintained for 7–10 days to be effective therapy for neurosyphilis. The above-recommended regimens have been shown to achieve this drug level. Some authorities recommend adding benzathine benzylpenicillin, 2.4 million units by intramuscular injection, in three consecutive weekly doses after completing the

Table 27.1 Recommendations for the treatment of neurosyphilis

WHO	Aqueous benzylpenicillin, 12–24 million IU by intravenous injection, administered daily in doses of 2–4 million IU, every 4 h for 14 days
Europe ECDC	Benzylpenicillin 18–24 million units IV daily, as 3–4 million units every 4 h during 10–14 day
CDC	Aqueous crystalline penicillin G 18–24 million units per day, administered as 3–4 million units IV every 4 h or continuous infusion, for 10–14 days

Table 27.2 Alternative recommendations for the treatment of neurosyphilis (if IV infusion is not possible)

WHO	Procaine benzylpenicillin, 1.2 million IU by intramuscular injection, once daily, and probenecid, 500 mg orally, four times daily, both for 10–14 day
Europe	Procaine penicillin 1.2–2.4 million units IM daily AND probenecid 500 mg four times daily, both during 10–14 days or ceftriaxone 1–2 g IV daily during 10–14 days
CDC	Procaine penicillin G 2.4 million units IM once daily plus probenecid 500 mg orally four times a day, both for 10–14 days

above IV/IM regimen; however this weekly dose doesn't achieve the recommended drug level, and this protocol is not commonly followed in the USA or Europe.

WHO also recommends the option of using doxycycline or tetracycline for penicillin-allergic nonpregnant patients, doxycycline, 200 mg orally, twice daily for 30 days, or tetracycline, 500 mg orally, four times daily for 30 days. This alternative to penicillin for the treatment of neurosyphilis has not been evaluated in clinical trials.

27.7 Follow-up After Treatment for Neurosyphilis

After treatment of Neurosyphilis, the tests that were utilized for the initial diagnosis should be repeated, and a fourfold decline in titer is required to signify serologic response. Such a decline may not be achieved by up to 15% of patients. Some authorities recommend that these patients should be retreated with 2.4 million units of procaine penicillin weekly ×3 weeks. It is important to note that a treponemal test may remain positive for life following effective treatment; in such patients serial serology can be monitored, and an increase of ≥2 dilution steps (fourfold) could be assumed to suggest a reinfection, whereas a stable titer when present may represent a "serologic scar". If CSF leukocytosis was present initially, a CSF cell count should be repeated every 6 months until the cell count is normal. The CSF leukocyte count is a more sensitive measure of the effectiveness of therapy; if the CSF cell count has not decreased after 6 months or if the CSF cell count or protein fails to revert to normal after 2 years, retreatment should be considered [77, 78].

Conflict of interest The authors report no conflicts of interest.

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Chapter 28
Orthopoxviruses and Human Disease

Robert Castro and Beata Casanas

Keywords Orthopoxviruses • Smallpox • Variola virus • Monkeypox • Cowpox • Vaccinia virus • Bovine vaccinia • Human monkeypox • Human cowpox • Vaccinia virus associated disease

Core Message
Orthopoxviruses have played a role in human disease for thousands of years. There are four commonly known species of *Orthopoxvirus* that cause human disease with variola virus (smallpox) being the most well known. Since the eradication of naturally occurring smallpox, the other human pathogenic orthopoxviruses have come to the forefront including monkeypox virus, cowpox virus, and vaccinia virus. Over the past few years, novel pathogenic orthopoxviruses have been described whose existence indicates that orthopoxviruses may continue to play a role, however limited, on future human disease.

28.1 Introduction

The *Orthopoxvirus* genus belongs to the family of *Poxviridae* and subfamily *Chordopoxviridae*. Orthopoxviruses are large complex double-stranded DNA viruses that replicate in the cell cytoplasm. The virions are described as brick shaped in electron micrographs and measure about 300 by 250 by 200 nm [1]. Within this genus, the species that are of main interest in human disease include variola virus (smallpox),

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Table 28.1 Human pathogenic orthopoxviruses

Virus	Disease	Clinical manifestation
Variola virus	Smallpox Variola major Variola minor	Ordinary-type smallpox: fever, headache, backache followed by diffuse pustular rash
Monkeypox virus	Human monkeypox	Same as smallpox but considered less severe due to lower pox lesion burden and mortality. Poor human-to-human transmission
Cowpox virus	Human cowpox	Localized painful pustular lesion with associated systemic constitutional symptoms. Systemic disease is possible in immunocompromised patients
Vaccinia virus	Smallpox vaccine-related disease Bovine vaccine	Bovine vaccinia: usually localized pustular lesions with intermittent period of systemic constitutional symptoms
Novel orthopoxviruses: 2013_13 NY_v014 2012_037	Unnamed	Localized painful pustular lesions with associated constitutional symptoms

monkeypox virus, cowpox virus, and vaccinia virus, though novel pathogenic orthopoxviruses have emerged over the past few years [2, 3] (Table 28.1). With the exception of variola virus, infections by these species are zoonotic. Variola virus holds the distinction of being the only microbiological pathogen, which has been eradicated purposely. The successful eradication program started in 1967, tracked the last naturally acquired infection in 1977, and concluded with the World Health Organization (WHO) General Assembly declaring that naturally acquired smallpox had been eradicated in 1980 [4]. It is considered among the greatest public health achievements in history.

28.2 Epidemiology

Variola virus is thought to have emerged as a human pathogen about 3000–4000 years ago in East Africa [5]. Smallpox is unique among orthopoxviruses in that it has no animal reservoir and humans are its strict host. It is estimated that in the twentieth century alone, approximately 400 million people around the world suffered from smallpox [6]. The incidence had been highest during the winter and early spring, because aerosolized orthopoxviruses survive longer at lower temperatures and low levels of humidity [1].

The monkeypox virus was first isolated from captive primates in Denmark (1959) with the first identified human monkeypox infection occurring in the Democratic Republic of the Congo (DRC) in 1970 [7]. The virus is currently endemic in the DRC and occurs sporadically in neighboring countries (Republic of the Congo, Central African Republic, Sudan) within the Congo Basin [8, 9]. Human monkeypox is a zoonotic infection and has been the foremost orthopoxvirus affecting human populations since smallpox eradication with its emergence in the DRC

receiving significant interest [10–12]. Two monkeypox virus clades have been identified, the Congo Basin clade and the West African clade, with the former considered to be more pathogenic [13]. In 2003, the first outbreak of human monkeypox in the Western Hemisphere occurred in the United States (USA) when there were 72 confirmed or suspected cases of human monkeypox [14, 15]. The cases were traced back to purchased prairie dog pets that had been in contact with infected Gambian giant-pouched rats [14, 15]. The monkeypox virus that was responsible for the outbreak was derived from the West African clade, which correlates with the milder clinical presentation seen in the US cases.

Human cowpox occurs via direct animal contact, usually with domestic cats since they tend to hunt rodents (wood mice and bank voles), which are the reservoir hosts [6, 16]. The virus is currently distributed in Europe, the former USSR, and adjacent areas of Northern and Central Asia [17]. Travelers who have hands-on contact with affected bovines, felines, rodents, or captive exotics (zoo animals) may be at risk for cutaneous infection [8].

Vaccinia virus-associated disease can present as a complication of smallpox vaccination because it is an active component of the vaccine or it can occur as a zoonotic infection. Vaccination complications include inadvertent self-inoculation, generalized vaccinia, eczema vaccinatum, progressive vaccinia, and post-vaccinial encephalitis. Vaccinia virus is a cause of zoonotic infections in Asia and South America, particularly in Brazil where it causes a disease called bovine vaccinia [18, 19]. Bovine vaccinia is a disease that affects milking cows and the dairy workers who have direct contact with them [19]. There have been reports of possible non-zoonotic transmission that possibly involved an environmental route or human-to-human transmission [20].

In recent years, novel pathogenic orthopoxviruses have been described in the literature. In 2013, in the country of Georgia, two cattle herdsmen developed multiple painful pruritic lesions on their hands with associated fevers and lymphadenopathy after reporting contact with sick cattle [2]. Real-time polymerase chain reaction (RT-PCR) was positive for a non-variola virus orthopoxvirus, and follow-up DNA sequence analysis revealed a novel orthopoxvirus species. More recently, in September 2015, there was a case of a male patient in Alaska who was diagnosed with a previously unknown orthopoxvirus when a papulovesicular lesion was sampled from his back [3]. In 2015 there were cases published that reported novel poxviruses in both immunocompetent (2012_037) and immunosuppressed patients (2013_013 and NY_v014), which presented with similar skin manifestations and likely zoonotic transmission [21, 22].

28.3 Pathogenesis

Orthopoxvirus infections in general present either as a localized, benign skin infection or as a systemic infection. In systemic orthopoxvirus infection, the virus enters the respiratory tract, invades the mucous membranes, and then spreads to the local lymph nodes [1]. There is initial viremia with a subsequent incubation period of

4–14 days during which time the virus multiplies in the reticuloendothelial system [1]. Another brief period of viremia precedes the prodromal phase. During the prodromal phase, the mucous membranes of the mouth and pharynx are infected. The virus then invades the capillary epithelium of the dermal layer in the skin, leading to the development of the lesions. The migration of infected macrophages to lymph nodes after the initial infection elicits the production of cytotoxic T cells and B cells. Neutralizing antibodies appear during the first week of illness. Hemagglutination-inhibition antibodies are detectable by day 16 of the infection and complement-fixation antibodies by day 18 [1]. Neutralizing antibodies remain present for many years, whereas levels of hemagglutination-inhibition and complement-fixation antibodies begin to decrease after 1 year [1].

28.4 Clinical Manifestations

28.4.1 Smallpox

There are two clinical forms of smallpox: *variola major* with an associated mortality of about 30% and *variola minor* with an associated mortality of 1% [23, 24]. *Variola major* is further subclassified based on the presentation of the smallpox rash or the absence of the rash. The rash can be described as ordinary (discrete, confluent, semi-confluent), modified, flat, hemorrhagic (early, late), and, in the case of no rash, *variola sine eruptione*. In the case of ordinary discrete, the most common presentation, the rash involves diffuse discrete lesions throughout the body. An ordinary confluent presentation indicates that the rash is confluent on the face and forearms with semi-confluent indicating that the rash is confluent on the face but discrete everywhere else. A modified presentation usually occurs in previously vaccinated people with a less severe prodromal period, more superficial lesions, and faster evolution of the skin lesions. A flat presentation indicates that the lesions are confluent or semi-confluent but appear flat. Hemorrhagic smallpox, as implied, involves widespread hemorrhage into the skin. Flat and hemorrhagic smallpox are usually fatal; the flat type alone has a reported fatality rate of about 97% among unvaccinated patients [1, 20].

In the case of ordinary-type smallpox (90% of cases), including ordinary discrete, ordinary semi-confluent, and ordinary confluent, the incubation period is 7–17 days with a mean of 10–12 days [25]. The incubation period is followed by a prodromal phase that lasts about 2–3 days and is characterized by abrupt onset of fever, severe headache, vomiting, and backache. The fever then subsides with the development of an exanthema over the tongue, mouth, and oropharynx. The diffuse rash then begins 1 day later as small reddish macules, which progress to papules then vesicles and then pustules. The pustules appear by day 7 on the onset of the rash and last another 5–8 days prior to crusting and scabbing over. The total time from the onset of the rash to the time that the lesions crust is about 2 weeks. The lesions start on the face and extremities but then develop over the rest of the body

with all the lesions in general being in the same stage of development. The person remains contagious until all of the scabs have fallen off. When the scabs fall off, they leave pitted scarring ("pockmarks"), as seen in 65–80% of survivors of severe smallpox [1, 23].

Other clinical manifestations of smallpox include panophthalmitis and blindness from viral keratitis (1%), arthritis in children (2%), and encephalitis (1%) [1]. Severe cases can also present with complications including pneumonia or bacteremia [1].

28.4.2 Monkeypox

The clinical manifestations of human monkeypox are similar to those of ordinary or modified smallpox except that it is associated with pronounced submandibular, postauricular, cervical, and inguinal lymphadenopathy [7]. The lymphadenopathy usually presents prior to or concomitant with the development of the rash, though there have been cases in which the rash was not present [10, 26]. Human monkeypox is less severe than smallpox as reflected in its mortality rate of 10% and decreased severity of rash. In one study [6], 58% of smallpox patients and 11% of human monkeypox cases from the Congo Basin presented with more than 100 pock lesions. The most common long-term *sequela* of those who survive the infection is the typical pitted scarring [10].

28.4.3 Human Cowpox

In immunocompetent persons, human cowpox most often is self-limited and characterized by localized painful pustular lesions or at times ulcerations with central hemorrhagic necrosis [8, 27]. Generalized infections can be seen in eczematous or immunocompromised patients including those with HIV [28, 29]. The lesions may last for weeks and occur at the site of inoculation with the possibility of multiple lesions if there are multiple sites including self-inoculation. The lesions undergo similar phases of development, which, previously mentioned, characterizes all orthopoxviruses. The lesions progress through macular, papular, vesicular, and pustular phases. Fever and other constitutional symptoms may appear when the lesion first appears.

28.4.4 Bovine Vaccinia

In the case of bovine vaccinia, infection is also characterized by the development of skin lesions at the site of inoculation [19]. Lesions progress from nodular swellings to edematous papules to umbilicated pustules to painful necrotic ulcers. It takes

about 12 days from the time of the onset of the skin lesions to the development of the necrotic ulcers [19]. Constitutional symptoms are also usually present for about 2–5 days sometime after the onset of symptoms [19]. The illness usually lasts about 4 weeks [19].

28.5 Diagnosis

Multiple diagnostic tests are available which can help with the diagnosis of an orthopoxvirus infection when used in conjunction with the patient's clinical and epidemiological history. The diagnostic tests for orthopoxviruses include viral culture, electron microscopy, immunohistochemistry, real-time polymerase chain reaction (RT-PCR), and anti-orthopoxvirus serology [10]. RT-PCR may be the most useful to help differentiate among species of orthopoxviruses since there is cross-reactivity among species, which the other tests cannot distinguish. More recently, the use of multiplex RT-PCR can also be used to rapidly differentiate orthopoxvirus species among themselves but can also detect other viruses which may be included in the diagnostic differential such as varicella virus [30]. Orthopoxviruses, when sampled from lesion exudate or crust specimen, are grown in viral culture which produces characteristic "pocks" on chorioallantoic membranes of chicken embryos [6]. Further characterization must be done for specific viral identification [7]. Viral culture is not assayed from blood samples due to patients not being consistently viremic during the duration of the entire illness. Electron microscopy helps identify the presence of an orthopoxvirus by showing the image of a brick-shaped particle [10]. Immunohistochemistry and anti-orthopoxvirus serology help identify the presence of orthopoxvirus-specific antigens and antibodies, respectively, but do not differentiate among species due to cross-reactivity.

In the case of smallpox, given the public health ramifications if a single case was confirmed, the Centers for Disease Control and Prevention (CDC) has a number of available Internet resources for practitioners who are suspecting a case of smallpox. According to the CDC, testing to rule out smallpox should be limited to cases that fit the clinical case definition in order to lower the risk of obtaining false-positive test results. The CDC clinical case definition of smallpox is an illness with acute onset of fever, greater or equal to 101 °F, followed by a rash characterized by firm, deep-seated vesicles or pustules in the same stage of development without other apparent cause [23]. Multiple other illnesses, particularly varicella, may present like smallpox, so differentiating between those illnesses and smallpox is fundamental. The CDC provides an algorithm that can be used to stratify risk for patients into low, moderate, or high risk of smallpox using clinical criteria, which then helps with diagnostic testing decision-making [31]. Confirmed cases, according to the CDC, should meet both clinical case definition and laboratory criteria. The laboratory criteria for diagnostic confirmation includes polymerase chain reaction (PCR), identification of variola DNA in a clinical specimen, or isolation of variola virus from a clinical specimen with variola PCR confirmation [23]. Any case of suspected smallpox should be reported immediately to the appropriate local health authorities.

28.6 Treatment

Although treatment of patients with orthopoxvirus infections is usually supportive and focused on containment, there are antiviral therapeutics that have shown promise including cidofovir, CMX001, and ST-246 [1, 10]. Cidofovir and CMX001 have proven activity against orthopoxviruses in vitro and animal studies; however, there is no data available on the effectiveness of these antivirals in treating human cases of smallpox [15, 23]. CMX001 is a modified cidofovir compound that lacks the extent of nephrotoxicity seen with cidofovir [10]. It is a cidofovir prodrug synthesized by covalently coupling cidofovir to the hexadecyl propanediol alkoxyalkanol (HDP-CDV) [6]. It was designed to mimic lysophosphatidylcholine and its natural pathway, which involves absorption through the small intestine [6]. It has an 88% bioavailability and is distributed to tissues via plasma or lymph without significant concentration in the kidney [6]. ST-246 has also been shown to be effective in animal studies, but there is no data on its effectiveness in treating human cases of smallpox [16, 32]. ST-246 works by targeting the F13 L protein and impedes the envelopment of virions as well as the egress of enveloped viruses from the infected cell [10, 33].

28.7 Conclusions

Orthopoxviruses have played a role in human disease for several thousand years. The eradication of naturally occurring smallpox in 1980 is recognized as a triumph over a human pathogen that has plagued humanity since the beginning of history. Since the completion of the smallpox eradication program, more attention has been given to other human pathogenic orthopoxviruses, including monkeypox virus, cowpox virus, and vaccinia virus. The endemic areas and outbreaks related to these viruses have been well documented and publicized. Their continued role in human disease is a reminder that there is still an ongoing fight with orthopoxviruses. Novel pathogenic orthopoxviruses are being identified globally over the past few years. Thus, it appears that despite the eradication of naturally occurring variola virus, we will continue to be affected by orthopoxviruses.

Conflict of interest The authors report no conflicts of interest.

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Chapter 29
Opportunistic Neurologic Infections in Patients Infected with Human Immunodeficiency Virus

Kelly Kynaston, Minh Ho, Robert Castro, and Jose A. Montero

Keywords Opportunistic infections • Encephalitis • Meningitis • Toxoplasmosis • Progressive multifocal leukoencephalitis • Herpes simplex • Varicella zoster • Cytomegalovirus • Cryptococcosis • Histoplasmosis • Coccidioidomycosis • Penicilliosis • Paracoccidioidomycosis

Core Message
Opportunistic neurologic infections in patients with HIV are among the most clinically devastating. Reactivation during times of profound immunosuppression is a hallmark of these infections that include parasitic, viral, and fungal pathogens. While CNS disease attributable to the direct neuronal effects of HIV and tuberculous meningitis is discussed elsewhere in this book, this chapter delves into the other opportunistic infections still commonly seen affecting the CNS of those infected with HIV.

29.1 Introduction

Infections of the central nervous system (CNS) in individuals with human immunodeficiency virus (HIV) remain a significant cause of morbidity and mortality despite the availability of highly active retroviral therapy (ART). While the rates of these CNS opportunistic infections have declined, these AIDS-defining infections still pose a threat to many infected with HIV, often with devastating clinical outcomes. Pathogens of various phylogenies including parasites, viruses, and fungi are all capable of posing threats to the nervous system. We focus on the most commonly encountered opportunistic neurologic infections in the HIV infected herein.

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29.2 *Toxoplasma gondii* Encephalitis

29.2.1 Epidemiology

Toxoplasma encephalitis (TE) is caused by the protozoan *Toxoplasma gondii*. Toxoplasma encephalitis is almost exclusively caused by reactivation of a latent infection. Primary infection is only rarely associated with acute cerebral or disseminated disease.

Toxoplasma gondii is carried by more than 60 million men, women, and children in the United States. [1]. In the United States, it is estimated that 22.5% of the population 12 years and older have been infected with Toxoplasma [2]. Seroprevalence of anti-toxoplasma antibody varies on geographic location with lower rates in the United States compared to certain European, Latin American, and African countries. HIV-infected patients whose CD4 count is <50 cells/uL are at greatest risk for disease with those with a CD4 >200 cells/mm^3 developing disease only rarely [3].

Primary infection occurs after eating undercooked meat containing tissue cysts or ingesting oocytes that have been shed in cat feces and sporulated in the environment [2]. In the United States, eating raw shellfish has been identified as a novel risk factor [4]. The organism is not transmitted through person-to-person contact. Up to 50% of individuals with documented primary infection do not have an identifiable risk factor [5].

29.2.2 Clinical Manifestations

Toxoplasmosis of the CNS produces brain abscesses, which are often found in the cerebral cortex (near the gray-white junction) and deep gray nuclei, less often in the cerebellum and brainstem, and rarely in the spinal cord [6]. The lesions lead to edema which may result in mass effect on surrounding structures leading to focal and non-focal neurological deficits.

The clinical findings tend to be subacute. The most common clinical presentation of *T. gondii* infection is focal encephalitis with headache, confusion, lethargy, and fever. Focal neurological findings include hemiparesis, cranial nerve palsies, ataxia, and sensory deficits. Without treatment, the disease can progress to seizures, stupor, and coma [7].

29.2.3 Diagnosis

Definitive diagnosis requires a compatible clinical syndrome; identification of one or more mass lesions by CT, MRI, or other radiographic testing; and detection of the organism in a clinical sample. MRI is generally preferred to CT due to its better sensitivity in detecting multiple lesions with 90% of these lesions displaying ring enhancement after administration of contrast [8].

The differential diagnosis of focal neurological disease in a patient with AIDS often includes primary CNS lymphoma, mycobacterial infection, cryptococcosis, Chagas disease, pyogenic brain abscess, and progressive multifocal leukoencephalopathy (PML). PML can at times be differentiated from toxoplasmosis by imaging studies because PML lesions usually involve white matter rather than gray matter, are non-contrast enhancing, and produce no mass effect [9]. Imaging studies in general are not specific as similar findings are found in other conditions.

Most clinicians initially rely on a presumptive diagnosis, which can be established as an objective response, documented by clinical and radiographic improvement after approximately 2 weeks of specific anti-*T. gondii* therapy in the absence of a likely alternative diagnosis. A brain biopsy with hematoxylin and eosin stains can be used for detection of *T. gondii*. Sensitivity is increased if immunoperoxidase staining is used and if experienced laboratories process the specimens. Brain biopsy is usually reserved for patients who fail to respond to specific therapy, though a recent retrospective cohort analysis showed that neuroradiologic diagnosis was correct in only 50% of cases when compared to histologic diagnosis from biopsy [10]. Medical treatment was also changed in 69% of the patients in the analysis after they had a biopsy performed [10].

29.2.4 Treatment

Guidelines for the treatment, primary prevention, and secondary prevention of *toxoplasma gondii* encephalitis have been published by the US Centers for Disease Control and Prevention, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America [11]. The initial therapy of choice for toxoplasma encephalitis consists of the combination of pyrimethamine plus sulfadiazine plus leucovorin (Table 29.1). Leucovorin reduces the likelihood of development of hematologic toxicities with pyrimethamine therapy. Pyrimethamine plus clindamycin plus leucovorin is the preferred alternative regimen for patients who cannot tolerate sulfadiazine or who do not respond to first-line therapy. This last combination does not prevent pneumocystis jiroveci pneumonia, so additional prophylaxis will be required. If pyrimethamine is unavailable, then trimethoprim/sulfamethoxazole (TMP-SMX) should be used in place of pyrimethamine-sulfadiazine or pyrimethamine-clindamycin. If a patient has a history of a sulfa allergy, then sulfa desensitization should be attempted. There are no well-studied options for patients who cannot take an oral regimen. Most will use parenteral TMP-SMX or oral pyrimethamine plus parenteral clindamycin as initial treatment in severely ill patients who require parenteral therapy.

Clinical response to acute therapy occurs in 90% of patients with toxoplasma encephalitis within 14 days of initiation of appropriate therapy [12]. Acute therapy should be continued for at least 6 weeks with a longer course if the disease is extensive or if the response is incomplete. Once acute treatment is complete, patients should be continued on chronic maintenance therapy.

Table 29.1 Toxoplasmosis treatment

Preferred regimen	Alternative regimens
Initial regimen (minimum 6 weeks)	*Initial regimen (minimum 6 weeks)*
Pyrimethamine 200 mg PO once followed by pyrimethamine 50–75 mg PO daily + sulfadiazine 1000–1500 mg PO every 6 h + leucovorin 10–25 mg PO daily	(Pyrimethamine + leucovorin) + clindamycin mg IV or PO every 6 h TMP-SMX (TMP 5 mg/kg/dose and SMX 25 mg/kg/dose) IV or PO BID Atovaquone 1500 mg PO BID (with or without sulfadiazine or pyrimethamine + leucovorin)
Chronic maintenance therapy[a]	*Chronic maintenance therapy*[a]
Pyrimethamine 25–50 mg PO daily + sulfadiazine 2000–4000 mg PO daily (in 2–4 divided doses) + leucovorin 10–25 mg PO daily	Clindamycin 600 mg PO every 8 h + (pyrimethamine 25–50 mg + leucovorin 10–25 mg) PO daily TMP-SMX 1 DS PO BID Atovaquone 750–1500 mg PO BID (with or without sulfadiazine or pyrimethamine + leucovorin)

Adapted from Guidelines for the prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: recommendations from the Centers for Disease Control and Prevention, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America [11]

TMP-SMX trimethoprim-sulfamethoxazole

[a]Stop maintenance therapy once no signs or symptoms of infection and CD4 > 200 for > 6 months in response to antiretroviral therapy

The use of adjunctive corticosteroids may be clinically indicated when there is mass effect in the CNS due to local lesions or associated edema [12]. If steroids are used in the setting of presumptive diagnosis by clinical response, there should be awareness that radiographic findings and clinical symptoms of primary CNS lymphoma may also improve in the setting of steroids. Corticosteroids should be discontinued when medically feasible due to their potential to cause immunosuppression and that they may contribute to the development of other opportunistic infections. Anticonvulsants should be administered in patients who have a history of seizures, but current guidelines recommend that they should not be administered prophylactically to all patients [11]. If administered, they should be continued at least for the duration of acute treatment.

Timing to initiate ART is not defined in a patient with toxoplasma encephalitis. Many clinicians however would initiate ART within 2–3 weeks after the diagnosis of toxoplasmosis, based on the lower incidence of AIDS progression and death [13].

Patients should be regularly monitored for treatment response and treatment-related adverse effects. A brain biopsy should be considered in patients who show clinical and radiographic deterioration during the first week of treatment despite adequate therapy or who do not show clinical improvement within 10–14 days of therapy [12]. If the patient already has biopsy-proven disease and shows clinical decline or no clinical improvement in the setting of therapy, then consideration should be made on switching to an alternative regimen.

29.2.5 Prevention

HIV-infected patients should be tested for IgG antibody for toxoplasma soon after diagnosis to detect for latent infection [14]. For those that are seronegative, they should be advised on how to prevent exposure. They should be cautioned to avoid eating raw or undercooked meat (including lamb, beef, pork, or venison) and not to eat raw shellfish (including oysters, clams, and mussels). Handwashing after contact with raw meat and after gardening or other contact with soil should be advised. Also, fruits and vegetables should be washed well before eating them raw. If the patient is seronegative, they should not handle stray cats. If they have a cat, they should not be the ones changing the litter box daily nor should they feed their cats raw or undercooked meats.

Patients who are toxoplasma IgG positive with a CD4 count <100 cells/mm^3 should receive primary prophylaxis [15]. If a toxoplasma seronegative patient is receiving pneumocystis jiroveci prophylaxis which does not cover toxoplasma and their CD4 count declines to <100 cells/mm^3, they should have their toxoplasma serology retested [11]. Prophylaxis should then be started if they have become seropositive. The preferred regimen for prophylaxis is TMP-SMX 1 DS tablet PO daily (Table 29.2). Alternative regimens include but are not limited to TMP-SMX 1 DS tablet PO TIW, TMP-SMX SS tablet PO daily, and dapsone 50 mg PO daily plus (pyrimethamine 50 mg plus leucovorin 25 mg) PO weekly. Primary prophylaxis may be discontinued if the patient's CD4 count is >200 cells/mm^3 for >3 months in response to ART [16, 17]. Primary prophylaxis should be restarted if the patient's CD4 count drops <100 to 200 cells/mm^3.

Patients who have completed a treatment course for toxoplasmosis should be given chronic maintenance therapy for secondary prevention until immune reconstitution occurs as a consequence of ART. The preferred regimen is pyrimethamine plus sulfadiazine plus leucovorin. Besides being highly effective as suppressive therapy for patients with TE, it also provides protection against *Pneumocystis*

Table 29.2 Toxoplasmosis OI primary prophylaxis

Indication and discontinuation	Preferred regimen	Alternative regimens
Begin if CD4 count < 100 cells / mm^3 and toxoplasma IgG positive Stop if CD4 count > 200 for > 3 months in response to antiretroviral therapy; Reinstate if CD4 falls < 200	TMP-SMX 1 DS PO daily	TMP-SMX 1 DS PO 3 times weekly TMP-SMX 1 SS PO daily Dapsone 50 mg PO daily + (pyrimethamine 50 mg + leucovorin 25 mg) PO weekly (Dapsone 200 mg + pyrimethamine 75 mg + leucovorin 25 mg) PO weekly Atovaquone 1500 mg PO daily

Adapted from Guidelines for the prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: recommendations from the Centers for Disease Control and Prevention, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America [11]
TMP-SMX trimethoprim-sulfamethoxazole

jiroveci. An alternative regimen of pyrimethamine plus clindamycin is commonly used as a suppressive therapy for patients who cannot tolerate sulfa drugs. A disadvantage of this regimen is that it does not provide pneumocystis coverage, so additional prophylaxis will be required. Discontinuation of chronic maintenance therapy should be considered in patients that have successfully completed initial therapy, have no signs or symptoms of active TE, and have an increase in their CD4 counts to >200 cells/mm^3 after ART that is sustained for more than 6 months [17]. Secondary prophylaxis should be restarted if the CD4 count decreases to <200 cells/mm^3.

29.3 Progressive Multifocal Leukoencephalopathy (PML)

29.3.1 Epidemiology

Progressive multifocal leukoencephalopathy (PML) is an opportunistic infection of the CNS caused by the polyomavirus JC virus (JCV) and characterized by focal demyelination.

The virus is ubiquitous and has worldwide distribution, with a seroprevalence of 39–69% [18–20]. Primary infection usually occurs in childhood, is asymptomatic, and establishes a chronic asymptomatic carrier state in most individuals. Outside of HIV infection, PML is rare and occurs in the setting of other immunocompromising diseases or therapies such as immunomodulatory humanized antibodies. Before the advent of ART, PML developed in 5% of patients with AIDS [21]. With the use of ART, the incidence of PML has substantially decreased. Unlike other opportunistic infections which are almost wholly prevented when CD4 counts are maintained above 100–200 cells/mm^3, PML can still appear in such patients and in those on ART.

29.3.2 Clinical Manifestations

The JC virus preferentially infects oligodendrocytes which leads to demyelination being its principal pathologic effect. The lesions consist of patches of irregular, ill-defined destruction of the white matter ranging in size from millimeters to extensive involvement of an entire lobe of the brain [6]. These lesions lead to neurological deficits that correlate with the different parts of the brain that are involved.

PML presents with usually insidious focal neurological deficits. Areas more commonly involved include the occipital lobes (hemianopsia), frontal and parietal lobes (aphasia, hemiparesis, and hemisensory deficits), and cerebellar peduncles and deep white matter (dysmetria and ataxia). Spinal cord involvement is rare. Headache and fever are not characteristic, but seizures develop in nearly 20% of PML patients and are associated with lesions immediately adjacent to the cortex [22].

29.3.3 Diagnosis

A presumptive diagnosis can be made by the combination of clinical and neuroimaging findings. MRI of the brain is recommended to evaluate for distinct white matter lesions in the areas of the brain corresponding to the clinical deficits. The lesions are hyperintense on T2-weighted and fluid-attenuated inversion recovery sequences and hypointense on T1-weighted sequences [23].

Confirming the diagnosis starts with testing the CSF by PCR for the presence of JC virus DNA. A positive result can be considered diagnostic in the appropriate clinical context (subacute onset of neurological abnormalities and suggestive imaging findings). In cases where the diagnosis is not clear, a brain biopsy may sometimes be required to establish the diagnosis. The use of serology is not considered useful due to the high anti-JC virus seroprevalence in the general population [18–20].

29.3.4 Treatment

There is no effective specific antiviral therapy for JC virus infection or PML. Treatment mainly involves initiation of ART to reverse the immunosuppression. If the patient is ART naïve, they should be started on ART immediately. Approximately half of treatment-naïve patients who are initiated on ART experience a remission in which disease progression stops [24–30]. Some may experience improvement in neurological deficits, but the deficits often persist and may progress. A higher CD4 count has been shown to be predictive of survival, and a high plasma HIV RNA level has been associated with worse prognosis [24, 26, 28–30]. Anecdotal reports of targeted treatments for PML have not been confirmed by controlled studies.

There are no clear guidelines for the timing of follow-up assessments. Clinical progress should guide decision making, but in general repeat neuroimaging can be obtained 6–8 weeks after ART is initiated to evaluate for radiographic signs of progression or of immune response. PML-immune reconstitution inflammatory syndrome (PML-IRIS) which includes unmasking of cryptic PML and paradoxically worsening in a patient with an established PML diagnosis has been observed [23]. There has been suggested benefit of the use of corticosteroids in the setting of PML-IRIS associated with contrast enhancement, edema, mass effect, and clinical deterioration. However, this is currently discouraged by published guidelines [11].

PML remission can take several weeks after initiation of ART, and treatment failure may be considered if there is clinical worsening and continued detection of CSF JC virus without substantial decrease within 3 months [11]. However, no clear guidance exists for timing of follow-up assessment and therefore should be reasonably guided by clinical progress.

29.3.5 Prevention

There is no way to prevent initial exposure to the virus. For HIV patients who are at risk for disease, the main preventive measure is treatment with an effective ART regimen that suppresses viremia and maintains CD4 cell counts [11].

29.4 Herpes Simplex Virus (HSV)

29.4.1 Introduction/Epidemiology

Herpes comes from the Greek word meaning "to creep" and is a name given to a family of viruses consisting of herpes simplex viruses 1 and herpes simplex viruses 2 (HSV-1 and HSV-2). Together they are etiologic agents of an array of common human illnesses. HSV-1 is primarily associated with orofacial infections and encephalitis, while HSV-2 is primarily associated with genital infections. After initial or recurrent infection with either HSV-1 or HSV-2, the virus can invade the CNS and establish latency via trigeminal nerve or olfactory tract. Once established, it can reactivate and cause encephalitis. HSV encephalitis is thought to be the most common cause of sporadic fatal encephalitis and is usually focal. If left untreated, mortality rate can be as high as 70%. In those treated promptly, only 2–5% return normal neurological functions. HSV-1 is the predominant cause of encephalitis; however, those infected with HSV-2 often have worse outcome. HSV meningitis on the other hand is predominantly caused by HSV-2. HSV myelitis has also been associated in patients with AIDS [31, 32]. These viruses have no seasonal variations and are distributed worldwide. There are no known animal reservoirs and humans are believed to be the only natural reservoir. Demographic factors such as economic and race affect seroprevalence of HSV-1. In developing countries, one third of children at 5 years of age will have seroconverted and over 70% will have seroconverted by adolescence. This is in contrast to children from developed countries where only 20% by 5 years of age will seroconvert. This number remains stable until the prevalence increases to 40–60% at age 20–40 years. In general, HSV-1 infection occurs more frequently and earlier than infection with HSV-2 with over 90% of adults possessing antibodies to HSV-1 by their 5th decades. Since HSV-2 infections are usually sexually transmitted, its seroprevalence rises with increasing age from 20–30% at age 15–29 years to 35–60% by age 60 years. Factors affecting rate of transmission for HSV-2 include female sex, African-American race, single, higher number of sexual partners, and higher rates in cities than suburbs. In general, HSV-2 seroconversion is highest among female prostitutes (75%) and male homosexuals (83%) [33].

29.4.2 Clinical Manifestations

HSV infections occur when it comes in contact with mucosal surface or abraded skin. It is usually transmitted via different routes and can affect different areas of the body. The most common areas of the body are skin and mucosal membranes, irrespective of virus type. Incubation period for HSV-1 or HSV-2 is about 4 days and ranges from 2 to 12 days. Virus excretion can occur for up to 23 days with an average of around 7–10 days in primary HSV-1 infection of the oropharynx and HSV-2 infection of the genital tract. Orolabial lesions (e.g., cold sores, fever blisters) are the most common manifestations of HSV-1 infection. Preceding symptoms are pain, burning, tingling, or itching; they are followed by 3–5 vesicles appearing along the vermillion border of the lip. Within 8–10 days, lesions are typically completely healed. Genital herpes presents themselves as macules and papules. These then turn into vesicles, pustules, and ulcers.

Aseptic meningitis, transverse myelitis, and sacral radiculopathy can occur as complications of genital HSV infection. These complications appear more frequently in women than in men. HSV meningitis is often preceded by genital lesions in 85% of cases. These lesions often appear about 1 week prior to CNS symptoms with a range between 3 and 12 days. CNS symptoms include meningismus, fever, vomiting, and photophobia. Benign recurrent lymphocytic meningitis, previously known as Mollaret's meningitis, is characterized by recurrent meningitis episodes. These episodes can last for 3–7 days and often resolve without neurological sequelae.

HSV encephalitis, on the other hand, presents with more focal neurologic findings that are often less than 1 week in duration and is associated with altered mental status. Often focal cranial nerve deficits, hemiparesis, dysphasia, aphasia, ataxia, or focal seizures may follow. HSV encephalitis is often preceded by clinical or serologic evidence of mucocutaneous HSV-1 infection before the onset of symptoms [34]. Behavioral syndromes such as hypomania, Klüver-Bucy syndrome (KBS), and amnesia have been documented. Hypomania can present as elevated mood, excessive animation, decreased need for sleep, inflated self-esteem, and hypersexuality. KBS manifests as a loss of normal anger and fear response. These behavioral syndromes are believed to be due to HSV's affinity to the temporal lobes and limbic structures. HSV myelitis often presents as a transverse myelopathy of the cervicothoracic spinal cord and shows a non-ascending pattern [35].

29.4.3 Diagnosis

Diagnosis is made by virus isolation in cell culture or PCR detection of HSV DNA. Serological diagnosis is often only useful for establishing a history of past infections. Sensitivity of viral isolation is higher in vesicular mucocutaneous lesions than ulcerative lesions. DNA detection is the preferred diagnostic method due to its increased sensitivity by three to four times more than viral isolation. In CNS

infection, PCR detection of HSV DNA is preferred. CSF studies are often helpful in providing clues. In HSV aseptic meningitis and encephalitis, the CSF is usually clear with cell counts ranging from 10 to 1000 cells/mm^3. It is predominantly lymphocytic in the course of disease. If CSF is obtained early in disease course, polymorphonuclear response can be seen. CSF glucose level is often more than 50% of blood glucose and CSF protein is slightly elevated. If there is suspicion for HSV encephalitis, magnetic resonance imaging is the neuroimaging technique of choice, and gadolinium-enhanced lesions are often seen in the temporal lobes. EEG changes such as focal δ activity and periodic lateralized epileptiform discharges (PLEDs) over temporal lobes unilaterally or bilaterally were once considered classic for herpes simplex infection. However, this can now be seen in another condition such as brain tumors, acute strokes, or other acute encephalitides [36].

29.4.4 Treatment

Treatment for HSV mucocutaneous infections in HIV individuals is similar to those without HIV and can be done via episodic therapy when symptomatic lesions are present or with daily suppressive therapy for preventing recurrences. Orolabial and genital HSV lesions can be treated with oral acyclovir, valacyclovir, or famciclovir for 5–10 days. IV acyclovir can be considered for treatment of severe mucocutaneous HSV lesions; de-escalation to oral regimen should occur after lesions have regressed.

In patients with suspected HSV meningitis, encephalitis, or transverse myelitis, treatment should begin with IV acyclovir at a dose of 10 mg/kg every 8 h for 14–21 days (Table 29.3). Despite improved availability of treatment, sequelae such as severe neurological deficits, seizures, and neuropsychological dysfunction exist. In a retrospective review done in France of non-HIV patients, a 6-month morbidity

Table 29.3 Treatment of selected viral CNS opportunistic infections

CNS viral infections	Preferred regimen	Alternative regimen
PML	HIV antiretroviral therapy	
HSV encephalitis	Acyclovir 10 mg/kg IV every 8 h × 14–21 days	Foscarnet 90 mg/kg IV every 12 h
VZV encephalitis	Acyclovir 10 mg/kg IV every 8 h × 14 days	Ganciclovir 5 mg/kg IV every 12 h
CMV	Ganciclovir 5 mg/kg IV every 12 h + foscarnet 90 mg/kg IV every 12 h x 21 days	Ganciclovir 5 mg/kg IV every 12 h × 14–21 days

Adapted from Guidelines for the prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: recommendations from the Centers for Disease Control and Prevention, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America [11]

PML progressive multifocal leukoencephalopathy, *HSV* Herpes simplex virus, *VZV* Varicella zoster virus, *CMV* cytomegalovirus

assessment revealed only 14% of patients completely recovered, while 23% had mild impairment [37]. Other published studies showed a 6-month fatality rate to vary from 19% to 28% in those treated with acyclovir [38–40]. A major determinant of poor prognosis is a delay in initiation of acyclovir therapy [37].

29.4.5 Prevention

Persons with HIV infections have high rate of HSV-1 and HSV-2 infections. As a result, prevention of acquisition of HSV is important in the uninfected. Serodiscordant couples should use latex condoms consistently and should avoid sexual contacts when their partners have overt herpetic lesions. Nevertheless, most sexual transmission of HSV occurs during asymptomatic viral shedding. Oral suppressive therapy has been shown to reduce HSV-2 transmission to susceptible heterosexual partners by 48% in persons without HIV [41]. However, in HIV-1/HSV-2 seropositive persons not on antiretroviral therapy, suppressive acyclovir did not prevent HSV-2 transmission [42]. As a result, current guidelines recommend against prophylaxis antiviral drugs to prevent primary HSV infection, and suppressive anti-HSV therapy is not recommended to prevent HSV-2 transmission in those HIV persons not on antiretroviral therapy [11].

In those with HSV disease, suppressive therapy with oral acyclovir, valacyclovir, or famciclovir is effective in preventing recurrences. It is preferred for person with severe or frequent HSV recurrences or those who want to minimize frequency of recurrences. In cases of recurrent benign lymphocytic meningitis, the role of antiviral therapy for the prevention of HSV-2-related recurrent meningitis remains unclear. In cases of herpes encephalitis, no clear role for suppressive therapy post-treatment has been established. Study in neonates revealed that oral acyclovir suppression for 6 months after initial treatment with IV course of acyclovir showed improved neurodevelopmental outcomes [43]. However, a double-blind, randomized, controlled trial using 500 mg of valacyclovir twice daily after initial treatment for HSV meningitis did not decrease recurrent rates of meningitis. The author of this study did note that the dosage may have been too low to penetrate the central nervous system effectively [44].

29.5 Varicella Zoster Virus (VZV)

29.5.1 Introduction/Epidemiology

VZV is a human virus belonging to *Herpesviridae* family. Humans are the only known reservoir. VZV causes two distinct clinical syndromes: primary infection and reactivation. Primary infection with VZV results in varicella, also known as chicken pox. After this initial infection, the virus becomes latent in the dorsal root

ganglion. Reactivation from its latent stage results in herpes zoster. Chicken pox is a disease of childhood and is rarely seen in adults. Primary infection with varicella in the HIV-infected adults is unusual, as more than 95% of HIV-infected US adults [45], 91% of HIV-infected Zaire adults, and over 95% of HIV-infected UK adults [46] are VZV seropositive. Herpes zoster can affect all ages with lifetime risk between 15% and 20%, with higher incidence in person older than 60 years and immunocompromised individuals. Herpes zoster can occur at any CD4 T lymphocyte (CD4) cell count, but its incidence is higher in individuals with CD4 count <200 cells/mm^{3}and in patients shortly after initiation of antiretroviral medications.

29.5.2 Clinical Manifestations

Varicella appears as erythematous macules that progress to papules with an edematous base. These papules subsequently become vesicles, with each vesicle initially with appearance of "a dewdrop on a rose petal." These same vesicles evolve into pustules and umbilicate with subsequent crusting over appearance within the next 8–12 h. Lesions will vary by stages although lesions in all states can be present. New vesicles may continue to develop in the next 48–96 h. These vesicles are often accompanied by pruritus, fever, headache, malaise, and anorexia. Herpes zoster presents as an erythematous maculopapular rash in a dermatomal distribution. It is often preceded by prodromal pain. The rash changes to clear vesicles and is accompanied by pain, often at severe intensity. Thoracic dermatomes are the most common sites occurring in 40–50% of cases. Cranial dermatomes occur in 20–25%, followed by cervical 15–20%, lumbar 15%, and sacral 5% dermatomes.

Central nervous system infections associated with VZV in HIV patients are often preceded by varicella or herpes zosters. In VZV encephalitis, symptoms usually develop 3–8 days after onset of chicken pox and 1–2 weeks after development of zoster [47]. Zoster with ophthalmic distribution has a higher incidence of encephalitis. It is also associated with higher incidence of stroke, which is attributed to VZV direct infection of cerebral arteries. Cerebellar symptoms such as ataxia, tremors, and dizziness can also be presenting symptoms. Other VZV-associated CNS infections include vasculitis, multifocal leukoencephalitis, ventriculitis, myelitis and myeloradiculitis, optic neuritis, cranial nerve palsies, focal brain stem lesions, and aseptic meningitis [48].

29.5.3 Diagnosis

Diagnosis is made via confirmation of virus isolation in cell culture or PCR detection of VZV DNA. However, both varicella and herpes zoster have distinct cutaneous and mucocutaneous appearance, and diagnosis can often be made clinically. However, in severely immunocompromised patients, atypical varicella presentation

may be hard to differentiate from disseminated herpes zoster. Serologies are often helpful in this instance to establish past infections. DNA detection, viral culture, or direct fluorescent antigen testing of these atypical skin lesions will help to differentiate from other exanthems. In CNS infection, PCR detection of VZV DNA is preferred for diagnosis. CSF studies are often nonspecific with mononuclear pleocytosis and elevated protein concentration.

29.5.4 Treatment

Treatment for uncomplicated varicella may include oral valacyclovir or famciclovir for 5–7 days. However, no prospective studies for treatment of varicella in HIV-infected adults have been reported. IV acyclovir for up to 10 days can be considered in severe varicella. Treatment for uncomplicated herpes zoster may include oral valacyclovir, famciclovir, or acyclovir for 7–10 days. IV acyclovir can be considered in severe cases with subsequent switch to oral and treatment duration of 10–14 days' course. No study or clinical trials to date have determined the best regimen; however, due to its efficacy in herpes virus encephalitis, expert opinions recommend initiation with acyclovir [49]. In patients with suspected VZV encephalitis, treatment should begin with IV acyclovir at a dose of 10 mg/kg every 8 h for 14–21 days (Table 29.3). Outcome data of HIV-infected patients with VZV encephalopathy is lacking. However, in non-HIV-infected patients from a French study, VZV encephalopathy case fatality rate is as high as 15% [50].

29.5.5 Prevention

HIV-infected person without history of varicella or shingles, seronegative for VZV, or without history of vaccination against VZV should avoid exposure to individuals with varicella or herpes zoster. Household contacts of HIV-infected individuals without evidence of immunity should consider being vaccinated with VZV vaccine. HIV-infected person who is susceptible to VZV should promptly receive VariZIG, a purified human immune globulin that contains high levels of anti-varicella antibodies, within 10 days after exposure to varicella or herpes zoster. The use of acyclovir or valacyclovir 7–10 days after exposure may be considered but have not been studied in HIV-infected population. An attenuated live varicella zoster virus vaccine under the trade name Zostavax, for prevention of herpes zoster, can be considered in HIV-infected individuals age 60 years or older. However, it is contraindicated in person with CD4 cell counts <200 cells/mm^3.

29.6 Cytomegalovirus (CMV)

29.6.1 Introduction/Epidemiology

Cytomegalovirus, a betaherpesvirus, is the largest virus to infect humans. Infection is common in all human population and seropositivity reaches 60–70% in the US cities [51]. In HIV-infected homosexual men, more than 90% are coinfected with CMV [52]. Clinical manifestations can vary from no disease in adult healthy hosts to fatal infections in immunocompromised hosts. In patients with AIDS, it is the most common viral pathogen and often causing retinitis, encephalitis, colitis, and pneumonitis. Similar to other herpesviruses, CMV establishes latency in the host after initial infection, and its activation often occurs after immunosuppression or after the use of chemotherapeutic agents. Both primary and secondary infection can occur. Primary infection occurs in patients who are seronegative and have never been infected with CMV. Secondary infection represents activation of latent infection as individuals with prior infection are not protected against future infection. Usually in primary infection, virus replicates to a higher level and thus disease is more severe. Most clinical disease occurs in seropositive individuals. End-organ disease typically occurs in those with CD4 T lymphocyte cell (CD4) counts <50 cells/mm^3, who are either not receiving or have failed to respond to antiretroviral therapy (ART). Other risk factors include previous opportunistic infections (OIs), high level of CMV viremia, and high plasma HIV RNA levels (>100,000 copies/mL). Prior to antiretroviral therapy, 21–44% of patients with AIDS acquired CMV disease [53]. CMV infection in the CNS has been found in several autopsy studies with incidence between 20% and 30% of pathology consistent with CMV infection [54]. Yet, clinical diagnosis of CMV encephalitis is reported to account for fewer than 2% of neurological disorders [55].

29.6.2 Clinical Manifestations

CMV central nervous system disease includes dementia, ventriculoencephalitis, and polyradiculomyelopathies [56]. Polyradiculomyelopathy caused by CMV causes a Guillain-Barre-like syndrome characterized as ascending weakness in the lower extremities and is accompanied by loss of deep tendon reflexes. Subsequently, this leads to loss of bowel and bladder control. It initially begins as low back pain with radicular or perianal radiation. Then progressive flaccid paralysis often occurs over the next 6 weeks. Spastic myelopathy has been documented and is associated with sacral paresthesia. CMV encephalitis, on the other hand, often presents with lethargy, confusion, and fever. Those with ventriculoencephalitis have a more acute course with more focal neurologic findings often involving cranial nerve palsies or nystagmus [55].

29.6.3 Diagnosis

Diagnosis of CMV usually is based on laboratory confirmation and often cannot be made on clinical grounds alone. Laboratory confirmation consists of demonstration of viral components (viral antigens or viral DNA) from body fluids. Prior to advent of PCR and direct antigen testing, growth of virus would be required and this was often done in human fibroblast cultures (MRC-5 cells). This step was often laborious and time consuming, often requiring several weeks for growth to occur. Then direct detection of antigens against the CMV matrix protein pp65 became available. This technique allowed direct measurement of the amount of virus present. A PCR assay is also available for detection of CMV. It uses primers in gene that encodes CMV immediate early antigen [57] or in the CMV DNA polymerase [58]. This detection of CMV DNA especially in the blood of patients with AIDS has allowed provider to predict subsequent development of clinical disease. This technique has also shown that a high number of CMV DNA copies/mL of plasma were correlated with CMV disease activity in AIDS patients [59]. Due to high sensitivity of PCR assay for CMV DNA, this allows providers to detect small amounts of CMV DNA in body fluids such as cerebrospinal fluid. As a result, it has been used to aid in the diagnosis of patients with CMV encephalitis or CMV polyradiculopathy syndrome [60, 61]. CSF PCR for CMV in immunocompromised patient has an estimated 82–100% sensitivity and 86–100% specificity [49]. Lumbar puncture for CSF in patients with polyradiculopathy and meningoencephalitis often reveals polymorphonuclear cells, with mildly elevated protein and low CSF glucose levels. Median CD4 count values are typically around 150–650 cells/mm^3 [62, 63]. Magnetic resonance imaging is often the modality of choice in patient suspected with CMV infection of the CNS. Radiographic findings in those with polyradiculopathy may include increased enhancement of pial lining of the cord, conus, cauda equina, and lumbosacral nerve on T1-weighted images. Lesions can also present as areas of increased T2-weighted signals [64, 65]. As for those with CMV ventriculoencephalitis, most cases show nonspecific findings, often associated with increased signal intensities in white matter and enhancement in areas such as periventricular subependymal lining along the lateral ventricles, septum pellucidum, corpus callosum, and fornices [66]. Diffuse encephalitis may present as widespread hyperintense lesions in the hippocampus, cerebellum, and brain stems on T2-weighted images [55].

29.6.4 Treatment

In general, there have been a paucity of controlled, randomized, double-blinded studies assessing efficacy of antiviral agents in patients with CMV infection of the CNS in AIDS patients. This is mostly due to limited number of patients with an early diagnosis of symptomatic CMV infection. Reported observations have suggested ganciclovir along with foscarnet may benefit those with CMV meningoencephalitis

in patients with AIDS [67]. Most recent guideline for treatment of CMV diseases in patients with AIDS has also suggested therapy with either IV ganciclovir or IV foscarnet or a combination of the two drugs, with preference to combination drugs if patient has previously received prior anti-CMV therapy [68] (Table 29.3). Cidofovir is currently not recommended due to its poor ability to penetrate the blood-brain barrier. Duration of treatment remains unclear, although a combination of ganciclovir and foscarnet for 3 weeks, followed by maintenance therapy, has been recommended as it has led to improvement or stabilization in 74% of 31 patients with CMV encephalitis or myelitis in HIV-infected patients [69]. Currently the role of oral valganciclovir in the treatment of CMV-related CNS infection is not clear. In combination with starting treatment, attempt to improve the profound suppression of cell-mediated immunity should be undertaken such as optimizing ART.

Since treatment of CMV infection of the CNS often requires weeks of IV therapies followed by weeks of maintenance therapy, providers undoubtedly will come across many of the side effects associated with treatment. Ganciclovir is an acyclic nucleoside analogue of acyclovir and acts via inhibition of viral DNA synthesis. Valganciclovir is the pro-drug form of ganciclovir and can be given orally. Adverse effects of ganciclovir/valganciclovir include anemia, neutropenia, thrombocytopenia, nausea, diarrhea, and renal dysfunction. Neutropenia associated with ganciclovir often occurs in about 40% of patients. Ganciclovir may also interact with antiretroviral drugs such as didanosine and zidovudine by increasing their concentration. When used concurrently with zidovudine, hematological toxicity of ganciclovir may be increased [70]. Foscarnet is a pyrophosphate analogue and it acts by reversibly inhibiting the DNA synthesis of CMV. Its main adverse effect is kidney damage, resulting in a mostly reversible decrease in the glomerular filtration rate. This occurs up to 50% of patients treated with foscarnet. Due to its toxic effects in the kidney, other adverse effects include hypocalcemia and hypokalemia. Other adverse effects may include anemia, epileptic seizures, headaches, nausea, and emesis. Cidofovir is a nucleoside analogue of deoxycytidine, and it acts by selective competitive inhibition of viral DNA polymerase. Its major adverse effects include asymptomatic proteinuria, elevation of serum creatinine, and neutropenia [71].

Antiviral drug resistance should be considered when active replication persists despite several weeks of therapy. The molecular basis for resistance mutations involve the UL97 protein kinase and UL54 DNA polymerase. Mutations in UL97 and UL54 may lead to resistance against ganciclovir [72]. UL54 mutation leads to resistance against cidofovir and foscarnet [73].

29.6.5 Prevention

Maintenance therapy may be considered in patients with AIDS and CNS infection post-appropriate treatment duration [69]. If there is no relapse of CNS disease, current guideline does not recommend maintenance therapy to prevent a second recurrence [11]. Prevention is aimed at not allowing the profound suppression of cell-mediated immunity to occur.

29.7 Cryptococcosis

29.7.1 Epidemiology

Central nervous system infection due to *Cryptococcus* is caused by two species: *C. neoformans* and *C. gattii*. *C. neoformans*, with its widespread distribution worldwide, is far more common. *C. gattii* is currently found primarily in Australia and the Pacific Northwest of North America and is thus less common. In the pre-ART era, 5–8% of HIV patients were diagnosed with disseminated disease.

Currently, the overall incidence is about one million per year and is accountable for 600,000 deaths [74]. The demographics have changed with the availability of HIV medications, and in countries with ART widely available, it is mostly diagnosed concurrently with a new diagnosis of HIV seropositivity. Infection is more likely when patients present with CD4 counts under 100 cells/mm^3.

29.7.2 Clinical Manifestations

CNS infection with *Cryptococcus* usually presents as subacute meningitis or meningoencephalitis. Common symptoms include fever, malaise, and headache. Depending on the degree of immune system depression from the HIV virus, the classic symptoms of meningitis (neck stiffness and photophobia) manifest in only 1/4 to 1/3 of patients. Lethargy, altered mentation, personality changes, and memory loss can also occur and are usually a manifestation of increased intracranial pressure.

When diagnosed in a patient infected with HIV, *Cryptococcus* may be disseminated. Other systemic manifestations, when coupled with CNS symptoms, can be a clue of disseminated disease. A characteristic skin lesion with central umbilication can be noted in a minority of patients. On rare occasion, it can cause pneumonia, usually in a lobar and less commonly a nodular pattern, which can result in acute respiratory failure [75].

29.7.3 Diagnosis

Cryptococcal disease can be diagnosed through culture, cerebrospinal fluid (CSF) microscopy, or detection of cryptococcal antigen. Fifty-five percent of blood cultures and 95% of CSF cultures should be positive after 7 days [11]. While it can occasionally be detected on a routine CSF gram stain, India ink prep (which will demonstrate an encapsulated yeast) is more reliable. Prior to definitive diagnosis via culture, several clues on CSF analysis are characteristic and may be diagnostic of disease. CSF fluid analysis typically demonstrates mildly elevated levels of protein, low to normal levels of glucose, and a lymphocytic pleocytosis (depending on CD4 count and that

patient's ability to mount an inflammatory response). Opening pressure may be another clue with 60–80% of patients demonstrating pressures of over 25 cm H_2O. Finally, serum cryptococcal antigen may be present weeks to months before CNS symptoms even start; therefore, a positive cryptococcal antigen in the serum should prompt a lumbar puncture to evaluate for the presence of CNS disease [76].

29.7.4 Treatment

Treatment for cryptococcal CNS disease is prescribed in three phases (Table 29.4). The first phase is called induction and consists of IV amphotericin B given along with 100 mg/kg PO of flucytosine in four divided doses. Historically, 0.7 mg/kg/day of amphotericin B deoxycholate has been used. However, several recent studies have shown liposomal amphotericin B to have similar efficacy with less nephrotoxicity. Its recommended dose is 3–4 mg/kg/daily. The induction phase should be given for at least 2 weeks.

During this time, it is important to monitor serum Cr and a 50% dose reduction of flucytosine should be ordered for every 50% decline in creatinine clearance. If available, serum levels of flucytosine should also be ordered for monitoring. Ideally, levels should be between 25 and 100 mg/L. If patients cannot tolerate standard therapy, due to renal function or otherwise, then the less efficacious second-line therapies may also be used. In order of preference, they are amphotericin B deoxycholate + fluconazole 800 mg daily, followed by lipid formulation amphotericin B + fluconazole 800 mg daily followed by fluconazole 1200 mg PO daily alone [77–79].

Successful induction therapy is defined as substantial clinical improvement and a negative CSF culture after repeat lumbar puncture [11]. This usually occurs after about 2 weeks of induction therapy, after which consolidation therapy can begin. This usually consists of 8 weeks of fluconazole 400 mg PO daily. The final phase is known as the maintenance phase and consists of fluconazole 200 mg PO daily for at least 1 year (and when CD4 count is over 100 cells/mm^3). Though the optimal timing for initiation of ART remains controversial, current recommendations state that antiretroviral therapy can be begun in either the consolidative or maintenance phases, with recognition that earlier timing leads to greater likelihood of IRIS (defined as worsening symptoms despite microbiologic improvement) [80].

Elevated ICP is very common in cryptococcal meningitis and is the most common manifestation of IRIS in the setting of cryptococcosis. Measures to decrease ICP should be undertaken for all patients who experience confusion, blurred vision, papilledema, or lower extremity clonus. This usually consists of the removal of 20–30 mL of CSF fluid until the opening pressure is halved, with repeated procedures daily until signs and symptoms are improved. CSF shunting should be considered in patients who cannot tolerate repeated lumbar punctures or in patients whose elevated intracranial pressures persist despite numerous lumbar punctures. Corticosteroids, mannitol, and acetazolamide are not recommended interventions in this situation.

Table 29.4 Treatment of selected fungal CNS opportunistic infections

CNS fungal infections	Preferred regimen	Alternative regimen
Cryptococcus		
Induction (at least 2 weeks)	Liposomal ampho B 3–4 mg/kg IV daily + flucytosine 25 mg/kg PO QID or Ampho B 0.7–1.0 mg/kg IV daily + flucytosine 25 mg/kg PO QID	Ampho B lipid complex 5 mg/kg IV daily + flucytosine 25 mg/kg PO QID, liposomal ampho B 3–4 mg/kg IV daily + fluconazole 800 mg IV/PO daily, ampho B 0.7–1.0 mg/kg IV daily + fluconazole 800 mg IV/PO daily, liposomal ampho B 3–4 mg/kg IV daily, ampho B 0.7–10 mg/kg IV daily, fluconazole 400–800 mg IV/PO daily + flucytosine 25 mg/kg QID, or fluconazole 1200 mg IV/PO daily
Consolidation (min 8 weeks)	Fluconazole 400 mg IV/PO daily	Itraconazole 200 mg PO BID
Maintenance	Fluconazole 200 mg PO daily	
Histoplasmosis	Liposomal ampho B 5 mg/kg IV daily × 4–6 weeks followed by itraconazole 200 mg PO BID–TID ×12 months at least	
Coccidioidomycosis	Fluconazole 400–800 mg IV/PO daily, followed by chronic maintenance therapy with fluconazole 400 mg PO daily or itraconazole 200 mg PO BID	Itraconazole 200 mg PO BID, voriconazole 200–400 mg PO BID, posaconazole 200–400 mg PO BID followed by chronic maintenance with either itraconazole 200 mg PO BID, voriconazole 200 mg PO BID, or posaconazole 200 mg PO BID
Penicilliosis	Liposomal ampho B 3–5 mg/kg IV daily ×2 weeks, followed by itraconazole 200 mg PO BID ×10 weeks, followed by itraconazole 200 mg PO daily	Voriconazole 6 mg/kg PO BID ×1 day, then 4 mg/kg PO BID × at least 3 days, then 200 mg PO BID × 12 weeks, followed by itraconazole 200 mg PO daily
Paracoccidioidomycosis	Ampho B 1 mg/kg IV daily until improved, then itraconazole 200 mg PO daily until CD4> 200 for >18–24 months on HIV therapy	Voriconazole 200–400 mg PO BID

Summarized from [11, 111]

Treatment failure is defined as either persistently positive cultures after 2 weeks of appropriate therapy or as initial positive response with relapse in symptoms and cultures after about 4 weeks of treatment. Rarely, resistance to fluconazole is encountered and so resistance testing is recommended should this situation occur. Resistance is defined as strains with MIC greater than or equal to 16 micrograms/mL. If found to be resistant to fluconazole, second-line therapy is liposomal amphotericin B 5 mg/kg/day, as it is better tolerated and exhibits higher efficacy than conventional amphotericin B [81]. Third line is high-dose fluconazole plus flucytosine [79].

29.7.5 Prevention

Cryptococcus is ubiquitous in the environment and exposure cannot be avoided. However, prudent screening in select populations can help to prevent this exposure from manifesting as overt symptomatic disease [82]. Due to the relative infrequency of disease, lack of survival benefit, the possibility of drug interactions, potential for resistance, and cost, routine prophylaxis for patients with isolated cryptococcal antigenemia is not routinely recommended [11]. However, in select few settings around the world, prophylactic fluconazole or itraconazole can be used. This is limited to locations with high prevalence of the disease and in resource-limited settings where a delay in ART therapy cannot be avoided [83].

29.8 Histoplasmosis

29.8.1 Epidemiology

CNS disease can rarely be caused by *Histoplasma capsulatum*. This dimorphic fungus is common in the Central and South Central United States, Puerto Rico, and Latin America. In these areas, incidence is almost 5% in the HIV population, especially when their CD4 count falls below 150 cells per microliter [84]. There is also an African version known as *H. capsulatum var. duboisii* [85].

29.8.2 Clinical Manifestations

Symptoms of histoplasmosis are nonspecific and include fever, fatigue, weight loss, and hepatosplenomegaly. Cough, chest pain, and dyspnea can occur as pneumonia is present in 50% of patients [86]. Histoplasmosis infection of the central nervous system usually manifests as meningitis; however, mass lesions and cerebritis can also occur. Symptoms may range from asymptomatic to more localized complaints including headache, altered sensorium, cranial nerve deficits, seizures, ataxia, and meningismus [85].

29.8.3 Diagnosis

Diagnosis of histoplasmosis meningitis can be difficult. CSF findings include elevated protein, low glucose, and a lymphocytic pleocytosis, but fungal stains and cultures are usually negative. A positive histoplasma antigen or antibody in the CSF is diagnostic, but often a presumptive diagnosis must be made based on clinical suspicion. Clues that would heighten clinical suspicion include history of residence or travel in an endemic area, CNS symptoms not explained by another cause, and symptoms of disseminated histoplasma infection. Certain tests suggestive of disseminated infection include detection of histoplasmosis antigen in the blood or urine [87]; peripheral blood smear showing histoplasma being engulfed by white blood cells; positive cultures from the blood, bone marrow, or respiratory secretions; or histopathological exam demonstrating a 2–4 micrometer budding yeast.

29.8.4 Treatment

Recommended therapy for CNS histoplasmosis is 5 mg/kg/day IV liposomal amphotericin B for 4–6 weeks [88] followed by maintenance therapy with itraconazole 200 mg PO Q8–12 h for at least a year [89] (Table 29.4). Due to interactions between itraconazole, the protease inhibitors, and efavirenz, monitoring of itraconazole levels in the serum is recommended. The target level is between 1 and 10 μg/mL. Fluconazole may be an alternative if a patient is intolerant of Itraconazole but is not recommended first line.

IRIS is uncommon in the HIV patient infected with histoplasma, and therefore antiretroviral therapy should be started concurrently with antifungal therapy [90].

29.8.5 Prevention

Like *Cryptococcus*, HIV patients that live in an endemic area cannot avoid exposure. However, patients infected with HIV and with CD4 counts below 150 cells/mm^3 should avoid activities that stir up dust and dirt. If a patient cannot avoid exposure, due to occupation or otherwise, then prophylaxis with itraconazole can be offered. At least one randomized controlled trial showed reduction in the frequency of histoplasmosis, though not mortality, with itraconazole prophylaxis at dosages of 200 mg PO daily and discontinued when CD4 rises back above 150 for 6 months [91].

29.9 Coccidioidomycosis

29.9.1 Epidemiology

Coccidioidomycosis can be caused by both *Coccidioides immitis* and *Coccidioides posadasii*, which are soil-dwelling fungi. These are endemic in the Southwestern United States; the San Joaquin Valley in California, Mexico; as well as Central and South America. Risk of developing disseminated or severe CNS disease is increased in certain ethnic groups including Filipinos and Africans [92]. It also increases with decline in CD4 count, with those patients below 250 cells/mm^3 at highest risk [93].

29.9.2 Clinical Manifestation

Meningitis is the most serious form of disseminated coccidioidal infection. It manifests as headache, vomiting, and altered mental status. Less frequently, hydrocephalus or vasculitis can occur [92].

29.9.3 Diagnosis

When diagnosis is suspected and lumbar puncture is obtained, CSF fluid analysis typically demonstrates low glucose, high protein, and lymphocytic pleocytosis. Coccidioides can be cultured in the CSF of patients in about one third of cases. The lab must be notified beforehand to take precautions. Coccidioides complement fixation can also detect IgG in the CSF but is less reliable at lower CD4 counts [94].

29.9.4 Treatment

Preferred therapy for coccidioidal meningitis is fluconazole IV or PO at 400–800 mg daily [95] (Table 29.4). Successful reports of treatment with voriconazole, itraconazole, or posaconazole have been described but are not considered preferred [96–99]. Intrathecal amphotericin can also be used if there is a contraindication to triazoles [11]. Occasionally, despite successful therapy, CNS shunting may be required for the development of hydrocephalus. ART should be started as soon as possible and continued even in the event of the development of IRIS.

Response to therapy can be monitored via downtrending serial titers of the complement fixation antibody. It is recommended that this be ordered every 12 weeks. If they unexpectedly rise, this should be a cause for reassessment [11]. A common reason for therapy failure has to do with complex interaction of the triazoles with ART, and dosage adjustments should be made accordingly.

As relapses have been reported in up to 80% of patients with coccidioidal disease of the CNS who have had triazole therapy discontinued, secondary prophylaxis is recommended indefinitely regardless of CD4 count [92]. Again, fluconazole 400 mg PO daily is recommended first line. Itraconazole, posaconazole, and voriconazole are considered alternatives.

The only exception to the above description of first-line therapy is in pregnancy. Because *Coccidioides* is more likely to disseminate if acquired in the second or third trimester [100] and because azoles can cause limb deformities if used after the first trimester, the risks and benefits of intrathecal amphotericin versus triazoles should be discussed. Amphotericin B lipid formulations are the preferred initial therapy in pregnant patients [11].

29.9.5 Prevention

Coccidioidomycosis, like histoplasmosis, is present in dirt and dust; patients living in endemic areas are hard pressed to avoid it completely, but they can take precautions. No benefit has been shown with oral prophylaxis at any CD4 count and is therefore not recommended [101]. Yearly serologic testing, however, is reasonable for HIV patients who live in endemic areas, and preemptive therapy with fluconazole 400 mg given oral daily is recommended for those patients under 250 CD4 cells/mm^3 who present with new seroconversion [11].

29.10 Penicilliosis

29.10.1 Epidemiology

The dimorphic fungus *Penicillium marneffei* can cause central nervous system disease in HIV patients. It is endemic to Northern Thailand, Vietnam, Southern China, and India. Most cases are seen in patients with CD4 counts under 100 cells/mm^3 [102].

29.10.2 Clinical Manifestations

The most common clinical manifestations are generalized and include fever, anemia, weight loss, and skin lesions [103, 104]. However, symptoms of CNS disease include altered mental status with confusion, agitation, or depressed consciousness [105].

29.10.3 Diagnosis

Diagnosis of penicilliosis is based on isolation of *Penicillium marneffei* in culture [103, 104], either a white colony of yeast at 37°C or a deep red mold at 25°C. Alternatively, intracellular and extracellular basophilic, spherical, oval, and elliptical yeastlike organisms with central clearing can be demonstrated via wright staining of biopsy material or a peripheral blood smear.

29.10.4 Treatment

Treatment for penicilliosis, like the other CNS fungal diseases, follows three phases (Table 29.4). First, 3–5 mg/kg of body weight per day of IV liposomal amphotericin B is recommended for 2 weeks. Then, 400 mg/day of oral itraconazole is recommended for 10 weeks [106]. Finally, secondary prophylaxis should be continued until CD4 counts are more than 100 cells/mm^3 for 6 months [107].

During treatment with amphotericin, monitoring of serum creatinine is recommended. During treatment with itraconazole, serum levels should be obtained to ensure adequate therapeutic dosing despite potential interactions with ART.

Initiation of ART should be started as soon as possible after the diagnosis of HIV has been made. However, there are no studies regarding the optimal timing of ART in this subset of HIV patients, but current guidelines recommend a 2-week delay for the initiation of antifungal therapy so long as the CD4 count is above 50 [11]. While unmasking IRIS has been reported with penicilliosis [108], paradoxical IRIS has not been.

29.10.5 Prevention

Primary prophylaxis is recommended for all HIV-infected individuals with CD4 counts under 100 cells/mm^3 who reside in endemic areas [102]. Itraconazole 200 mg PO daily is the preferred drug, but fluconazole 400 mg PO weekly could be an alternative. Though there are no studies supporting a recommended timeline for discontinuation, consensus of expert opinion suggests that once the CD4 count is above 100 cells/mm^3 for greater than 6 months, primary prophylaxis can be safely discontinued [11].

29.11 Paracoccidioidomycosis

29.11.1 Epidemiology

Central nervous system infection with the *Paracoccidioides brasiliensis* or *Paracoccidioides lutzii* can infrequently occur in HIV patients, especially when CD4 counts drop below 200 cells/mm^3 [109–111]. This dimorphic fungus is found in the subtropical forests of Latin America, from Mexico to Argentina, and is especially prevalent in agricultural areas of Brazil [112].

29.11.2 Clinical Manifestations

Isolated CNS disease is uncommon; instead, paracoccidioidomycosis usually affects the CNS as a disseminated process in an immunosuppressed patient. As such, fever, asthenia, malaise, and weight loss are nonspecific presenting symptoms along with lymphadenopathy, liver and splenic hypertrophy, and skin lesions – usually multiple, small, flat, crusting lesions with no inflammation in the surrounding tissues. More specific CNS symptoms include those that manifest from the formation of cerebral and meningeal lesions [112, 113].

29.11.3 Diagnosis

Diagnosis of paracoccidioidomycosis is usually made by direct visualization of the fungi in clinical specimens, especially skin scrapings and sputum, in conjunction with the right clinical setting. It is characteristically a large yeast with translucent walls and multiple buds. Other immune-based diagnostic tests are available and antibodies can be detected in the CSF; however, they may be unreliable in immunocompromised individuals. Therefore, antigen detection may be preferred in HIV patients [114].

29.11.4 Treatment

Treatment for CNS paracoccidioidomycosis is usually IV amphotericin B deoxycholate at 1 mg/kg/day until the patient recovers enough to be treated by the PO route (Table 29.4). Maintenance therapy is usually itraconazole 200 mg/day thereafter until CD4 count recovers above 200 cells/mm^3 or for 18–24 total months [111, 112]. Voriconazole can also be used, though drug interactions may limit its use in patients who are taking non-nucleoside reverse transcriptase

inhibitors or ritonavir. ART should be started concurrently though IRIS has been described; it can be treated with corticosteroids [109].

29.11.5 Prevention

Though treatment of milder forms of the disease can be undertaken with trimethoprim-sulfamethoxazole, in one clinical series, 40% of AIDS patients diagnosed with paracoccidioidomycosis had received trimethoprim-sulfamethoxazole for treatment of pneumocystis [111]. There are no studies on primary prophylaxis, so it is not recommended. Secondary prophylaxis, or rather maintenance therapy, with itraconazole is usually continued until CD4 counts rise above 200 cells/mm^3 [111].

Conflict of interest The authors report no conflicts of interest.

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Chapter 30
Zika Virus and HIV/AIDS

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Keywords Zika virus • HIV • AIDS • Retrovirus • Flavivirus • Arbovirus • Mosquito • Fear • Education • Epidemiology • Injection drug abuse • Needle and syringe sharing • Paraphernalia • Risk vector pathways • Multidisciplinary • Vaccine • Chemotherapy • Public health • Global warming • NIH • CDC

Core Message

The new Zika epidemic has many similarities and parallels with the HIV/AIDS pandemic that commenced more than 30 years ago.

Ignoring the rapidly emerging prevalence of the ZikaV globally has the potential to follow the trends experienced during the HIV/AIDS epidemic on a global scale.

The emergence of the ZikaV pandemic has occurred globally and its spread is well documented. Insufficiently addressing the potential global impact could be extremely dangerous.

The unprecedented upsurge of this epidemic stresses the importance for continual monitoring and the incorporation of sustainable public health measures as soon as possible.

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30.1 Introduction

Viral infections are a continued and expanding threat to global public health and are under continual epidemiological and medical scrutiny. Viral infections cause approximately 11 million deaths, annually, of which 1 million were due to HIV/AIDS, for example, in 2010 [1]. These fatalities are heightened with the periodic epidemics and pandemics of various strains of influenza, Ebola, continued HIV and HCV spread, TB, and many other viruses. These emerging and periodically re-emerging epidemic diseases pose an ongoing threat to global health security. At the end of 2016, the World Health Organization (WHO) Department of Pandemic and Epidemic Diseases (PED) was monitoring worldwide viral outbreaks of avian influenza, coronaviruses (MERS-CoV, SARS), Ebola, Hendra, influenza (seasonal, pandemic), Nipah, Rift Valley fever, viral hemorrhagic fevers (including Ebola, Marburg, Lassa, Crimean-Congo hemorrhagic fever), yellow fever, and ZikaV [2]. The global spread of ZikaV has evolved into a global pandemic since its original emergence in the Americas, since 2015; ZikaV has evolved into a highly virulent and pathological virus for children and adults, becoming a current unparalleled pandemic. The spread of viral diseases is generally associated with fear that can hinder public health efforts [3]. This fear response has been evident in the public's reaction to the Zika pandemic. This and many other factors closely mirror the setting that has existed with HIV.

30.2 Virus Descriptions

30.2.1 HIV Virus and AIDS

Human immunodeficiency virus (HIV) can lead to acquired immunodeficiency syndrome (AIDS) if not treated. People are unable to rid themselves of HIV completely, even with treatment, unlike some other viruses. Consequently, once infected with HIV, the infection is for life, as reservoirs of infection remain in various organs [4, 5].

HIV is a retrovirus, lentivirus, which has a lipid bilayer envelope. HIV binds to CD4 T-cell and macrophage receptors and infects these and other cell types. HIV contains an RNA genome that is reverse transcribed into proviral DNA, which is integrated into the host cell's genome [6].

Due to its extreme nucleic acid sequence heterogeneity, HIV consists of several different strains, swarms, clades, and recombinant forms. Individuals may harbor different strains, simultaneously [7–9, 36]. HIV is classified into two overall groups with numeric designations, HIV-1 and HIV-2. HIV-1 is most common and is found globally. HIV-2 is primarily found in Western Africa [10]. Due to its extreme ability to mutate, HIV can escape from immune surveillance and continue infection and proliferation [11].

Figure 30.1: A representative phylogenetic tree for HIV

By infecting blood cells such as CD4 T cells and macrophages, HIV damages the immune system and increases the likelihood of acquiring opportunistic infections [12, 13]. If left untreated, it can take approximately 5–10 years for AIDS to develop

Fig. 30.1 Phylogenetic tree of HIV

[10, 14]. AIDS is considered when the number of CD4 cells falls below 200 cells per cubic millimeter of blood [15]. Without treatment, once diagnosed with AIDS, people typically survive about 3 more years. Life expectancy falls even further due to opportunistic infection, without treatment [7].

30.2.2 Zika Virus

Zikavirus (ZikaV) is a flavivirus, which is in the arbovirus group that includes two additional virus subgroups – alphaviruses and bunyaviruses. ZikaV had been considered, originally a relatively mild or benign virus. However, the other related viruses have been known for several years to be associated with several severe and often fatal diseases, including central nervous system disorders, viral meningitis, encephalitis, and many other serious health outcomes. Arboviruses are often in the forefront of discussions regarding public health and their global prominence is a major public health concern. In addition to ZikaV, these viruses include dengue (DEN), Japanese encephalitis (JE), West Nile virus (WNV), chikungunya fever (CHIK), hemorrhagic fevers such as Crimean-Congo hemorrhagic fever (CCHF), and Kyasanur forest disease virus (KFDV) [16–19]. However, since 2015, in the Americas, ZikaV evolved into a highly virulent and pathological virus for children, nearly seven decades after its discovery. ZikaV has further evolved into strains that are more virulent and lethal for adults as well [19, 20]. In fact, this process of evolving toward increased pathogenicity (pathogenization) appears to be ongoing. Globally, there is an increased association of Guillain–Barré syndrome (GBS) with ZikaV infection as well [21–23].

Figure 30.2: A phylogenetic tree for arboviruses with ZikaV highlighted

An individual infected with ZikaV will often manifest only mild symptoms or none. The most common symptoms are fever, rash, joint pain, and conjunctivitis. Occasionally, there can be related muscle pain or headaches. ZikaV manifests itself very much like the common flu, and consequently most people do not seek medical attention when infected. Moreover, like the flu, it can last from several days up to a few weeks. An increased association with Guillain–Barré syndrome, an uncommon disease of the nervous system, has also caused concerns. Death results rarely from ZikaV infection. Be that as it may, at the end of 2016, the most distressing effect of ZikaV infection has been for mothers during pregnancy because ZikaV was associated with a brain birth defect – microcephaly. Additional complications for infants born of ZikaV-infected mothers include defects of the eyes, ears, and growth [16].

30.3 Modes of Transmission

30.3.1 HIV/AIDS Routes

HIV can be transmitted through several risk factor routes, which disputes the initial assumption in the 1980s that this epidemic was only experienced in limited demographics and/or risk groups.

Fig. 30.2 Phylogenetic tree of ZikaV

The following are categorical routes for HIV infection and spread.

- High risk sexual contact [24]
  - Anal sex
  - Vaginal sex
  - Oral sex
  - Multiple sexual partners
  - Lack of condom use
  - Deep kissing
- Needle/syringe sharing [24–29]
- Injecting drug user (IDU) paraphernalia including cottons, cookers, and wash waters [24, 26–29]
- Transmission via mother/prenatal [24]
  - Pregnancy
  - Breast feeding
- Medical procedures [24]
  - Blood transfusion
  - Organ transplantation
- Occupational exposure [24]
  - Needle/syringe stab
- Miscellaneous [24]
  - Bites
  - Wound contact

30.3.2 Zika Virus Vectors

Recent reviews summarized the status of clinical syndromes involving ZikaV. Issues discussed include ZikaV associated microcephaly, Guillain–Barré syndrome, potential Dengue virus–ZikaV interactions, and the prospects of a future medical establishment response by developing vaccines and other antiviral agents [19, 30].

The following are several risk factors involving ZikaV.

- Mosquito bites

ZikaV is transmitted to people primarily through the bite of an infected *Aedes* species mosquito. These mosquitoes typically lay eggs in standing water, for example, in tires, buckets, bowls, animal dishes, flowerpots, vases, and leaves that trap water in trees. Further, they become infected when they feed on people already infected with ZikaV and then spread the virus to other people through bites [30].

- Mother/Prenatal

The ZikaV can be transmitted from the mother to an infant near the time of delivery, if the mother contracted the virus during her pregnancy. No cases of ZikaV have surfaced through mothers' breastfeeding. Thus, mothers are continually encouraged to breastfeed even in areas where this virus is prevalent [30].

- Sexual Contact

A man can spread ZikaV to his sexual partners. It is unknown yet whether women can transmit the virus. Moreover, the virus is present in semen longer than blood, stressing the importance of condom use and other such means that prevent the unnecessary transmittal of the ZikaV [30]. In addition, the issue of viral reservoirs continues as has been found with HIV infection.

- Blood Transfusions
  - Public health services are considering the risk of transmission of ZikaV during blood transfusions. Although no confirmed cases have occurred, reports have indicated potential cases in Brazil and historical records indicated that blood donors have tested positive for the virus after they had given blood [30]. Several news sources have reported that donated blood has tested positive for the presence of ZikaV [31]
  - ZikaV vaccines are on the way from NIH and Walter Reed [30].

30.3.3 A Comparison of Zika Virus and HIV/AIDS Modes of Transmission

Although many modes of transmission for the HIV/AIDS virus are known, graphically presenting these modes will assist in highlighting current and potential mechanisms that can affect the spread of both the HIV/AIDS and ZikaV.

Figure 30.3: Modes of transmission for Zika and HIV

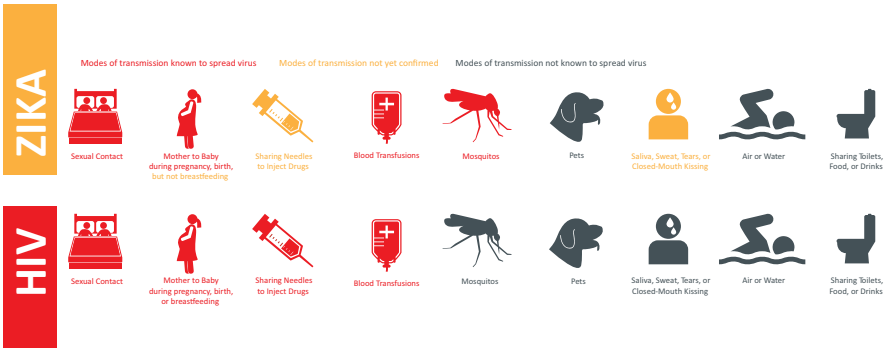

Fig. 30.3 A comparison of the modes of transmission between HIV and Zika

30.4 Pathways

30.4.1 HIV Pathway

Figure 30.4: HIV pathway [32, 33]

30.4.2 AIDS Pathway

Figure 30.5: HIV->AIDS pathway [34, 35]

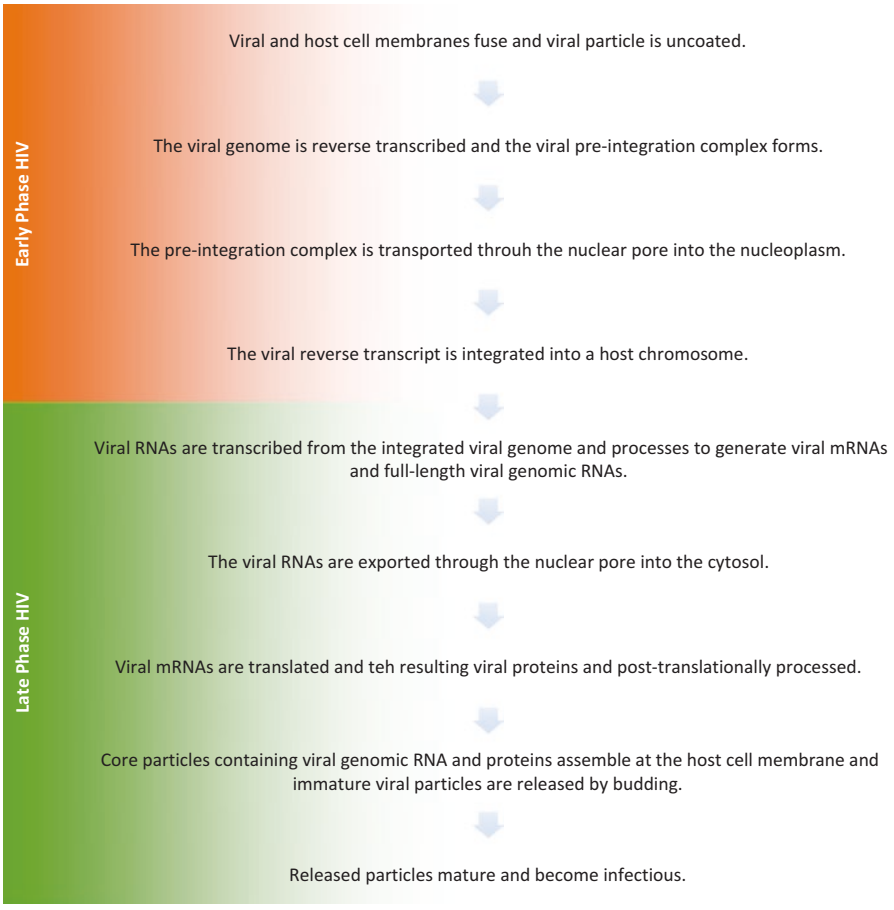

Fig. 30.4 HIV pathway [32, 33]

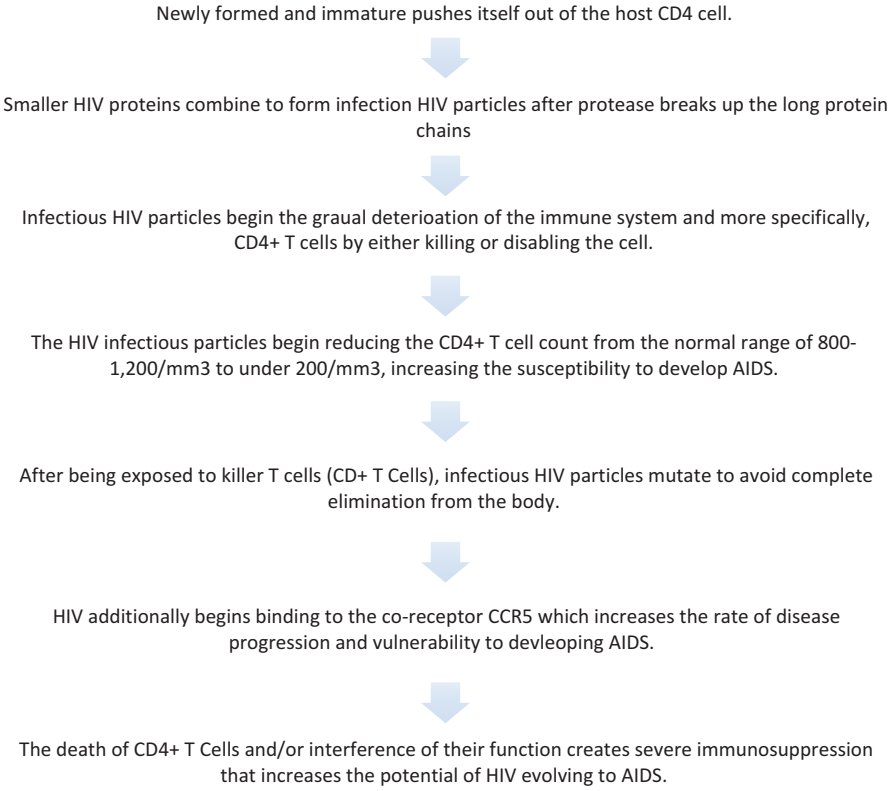

Fig. 30.5 HIV -> AIDS pathway [34, 35]

30.4.3 Zika Virus Pathway

Figure 30.6: Zika virus pathway [36]

30.5 HIV/AIDS Epidemic

The HIV/AIDS epidemic forever changed the health landscape of the United States as well as globally. A lack of understanding of epidemiology in general and of this virus-caused disease created a sense of fear and apprehension for the public.

The first reported case in 1981 at the University of Miami School of Medicine Grand Rounds involved a young Caucasian man of north European descent diagnosed with *Kaposi's* sarcoma. The physician reporting this case noted how unusual and unique the symptoms were as *Kaposi's* sarcoma was unknown in the patient's

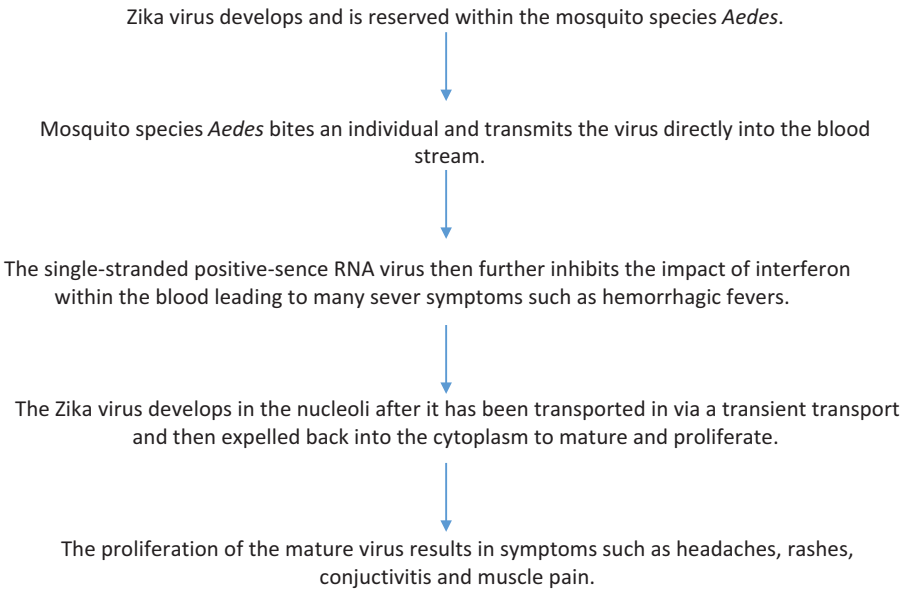

Fig. 30.6 Zika pathway [36]

age group or among those of north European decent. In addition, a second unusual case was also reported. This time, the young man had been diagnosed with *Pneumocystis carinii pneumonia* (PCP) – a disease that usually occurred among the very old and those living in nursing homes. These cases were puzzling and many questions arose including where the patients lived, traveled, what acute or chronic diseases they had, and with whom they interacted. It was further reported there was little in the patients' background information that indicated any major acute or chronic diseases that would suggest they would be susceptible to these illnesses [37].

Figure 30.7: Map of HIV spread [2]

The public misconceptions and misinterpretations of the HIV/AIDS epidemic did not help the scientific attack on this disease; it resulted in a limited embracing of the scientific approach by communities to solve medical issues. Early on, after this virus was discovered, immunologists speculated that HIV infection could perhaps be managed through a vaccine approach [38]. Because of further molecular research, it was ascertained that HIV underwent a high mutation rate making the development of vaccines highly difficult [24]. This fundamental molecular understanding of the mutation rate, gene structure, and evolution of HIV and the AIDS epidemic at the county, state, and national levels is still at the forefront of current concern as well.

Figure 30.8: Multidisciplinary risk framework

The HIV-associated novel agglomeration of signs and symptoms was frequently referred to as (1) Gay-Related Immune Deficiency (GRID) and (2) Gay Disease, which indicated a widespread misunderstanding and misconception of HIV epidemiology. The HIV/AIDS epidemic was thought to be primarily transmitted through

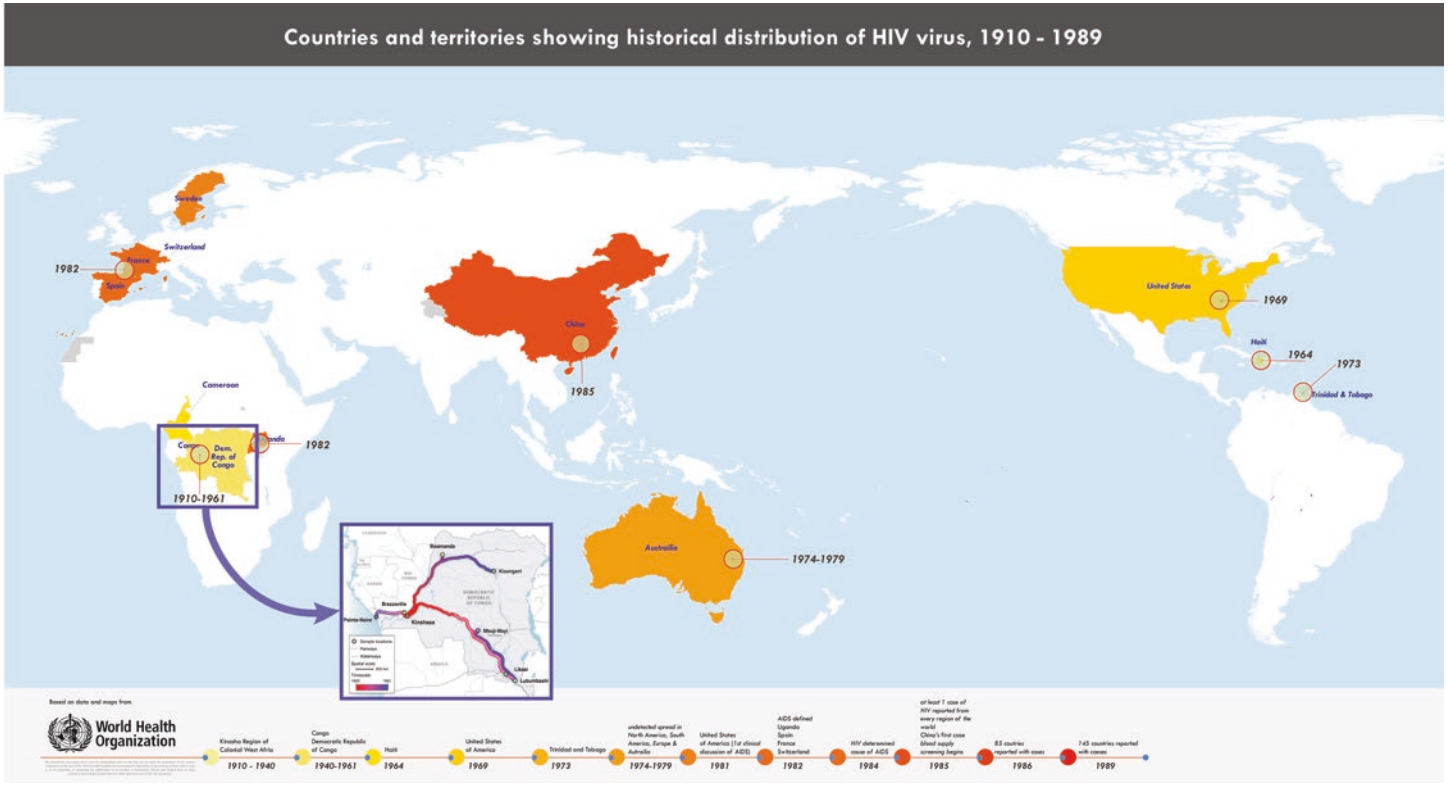

Fig. 30.7 Proliferation of HIV [2]

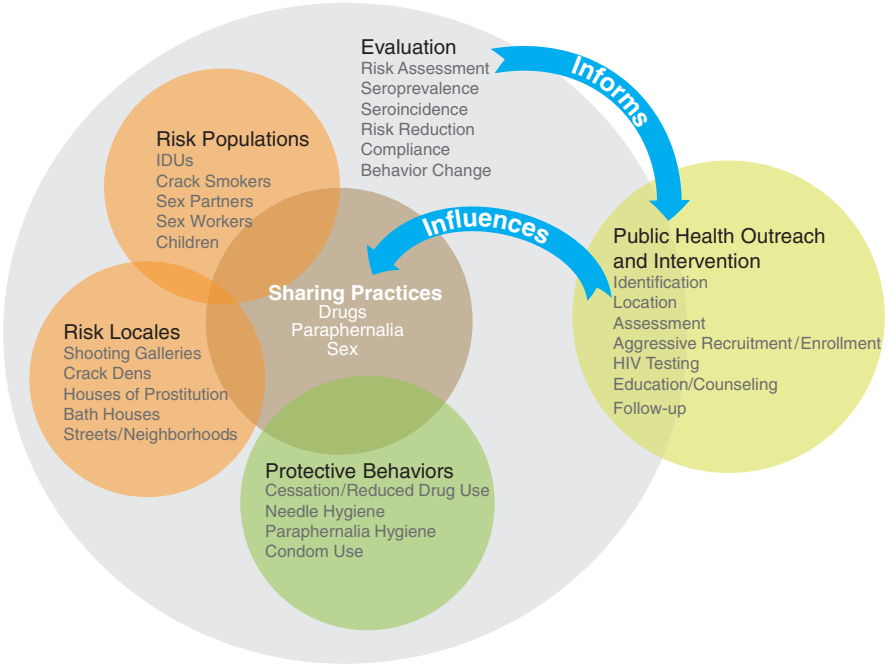

Fig. 30.8 Multidisciplinary risk framework. This chart was developed to graphically display risk populations, HIV risk, evaluation methods, and various other components that directly impact the HIV/AIDS epidemic

Caucasian and/or homosexual males. Moreover, the lifestyles within the United States during this disease's initial phase were not supportive of practices that could help contravene the emerging epidemic. It was a period of substantially increased urbanization, rapidly increased human trafficking and prostitution, venereal diseases, and drug abuse, including the use of hypodermic needle/syringes [39, 40]. Additionally, the sexual "revolution" that occurred during the 1960s and the gay liberation movement in the 1970s enhanced the severity of this new epidemic [38]. These specific manifestations combined with a dearth of scientific knowledge regarding HIV/AIDS further delayed foundation of the most rapid and preventable approaches against acquiring and transmitting HIV.

30.6 Zika Virus Epidemic

On April 18, 1947, the first documented case of the ZikaV emerged in the Zika forest of Uganda [31]. This information only surfaced as the monkey, Rhesus 766 was being observed and studied for research on yellow fever, which is an arbovirus related to the ZikaV [24]. Within a year, the virus was isolated from *Aedes*

africanus mosquitoes and serological studies commenced, examining the impact of this virus [41].

Since its discovery, ZikaV global spread was methodically tracked as follows: 1947 – Zika forest, Uganda, Nigeria, east Africa; 1954 – India; 1978 – Indonesia, Malaysia; 1999 – Ivory Coast; 2001 – Sabah, Malaysia; 2007 – Micronesia, Yap Islands, Easter Islands, Nepal, New World, Argentina, Hawaii, Scandinavia, and Saudi Arabia; 2008 – Southeastern Asia and Australia; 2008 – Senegal, Egypt, Pakistan, North Vietnam, Indonesia, the Philippines, and Borneo/Java, USA; 2012 – Singapore, Australia, Tahiti, and Germany. Tracking ZikaV highlights the ability of an initially, supposedly innocuous virus to spread globally [19, 41, 42].

In 2011, ZikaV was first detected in the United States (Colorado), where it was spread by sexual risk, having been brought to the United States by an infected health care worker, who had been in Indonesia [43].

Later, additional cases of ZikaV infection were reported within the United States and its territories, including Puerto Rico, US Virgin Islands, and American Samoa – 274 cases had occurred as of March 2016 [44]. As of November 16, 2016, every state and territory (other than Guam and Alaska) had reported cases of Zika totaling 36,323. Locally acquired cases were restricted to South Florida, Samoa, US Virgin Islands and Puerto Rico. Puerto Rico accounted for 86% of these cases [44, 45]. It is interesting to note that as of February 2016, according to WHO, there was no ZikaV detected yet in Sierra Leone (a medium risk country); however, by September 2016, ZikaV was detected in another medium risk area, Cape Verde islands, off the west African coast. (There are 195 countries in the world [46].) As of the latest report, March 10, 2017, ZikaV has spread to 184 countries, 13 countries report person-to-person spread, 31 countries report CNS malformations including microcephaly, and 23 countries report GBS – all specifically related to ZikaV infection. The spread of ZikaV, therefore, has rapidly expanded its domain. Thus, local and global public health services, in concert, continue to monitor ZikaV infection [21–23, 47, 48].

Figure 30.9: The map indicates the proliferation of the ZikaV [2]

Figure 30.10: ZikaV in the United States, November 16, 2016 [17]

30.7 Urbanization and Globalization

Several factors probably contributed to the global expansion of ZikaV (as well as many other viruses), including increased urbanization, economic expansion, swelled human populations and migrations, international travel, wars, and global warming. The rapid expansion of the global landscape as well as major climate changes (global warming) augmented the vector of transmission, the *Aedes aegyptus* and *Aedes albopictus* mosquitoes (including in Florida). Moreover, increased temperatures not only increase the transmission vectors but also additional health outcomes that can negatively heighten the effects of ZikaV infection. Moreover, there has not been a corresponding increase in quality and quantity of sanitation and healthcare,

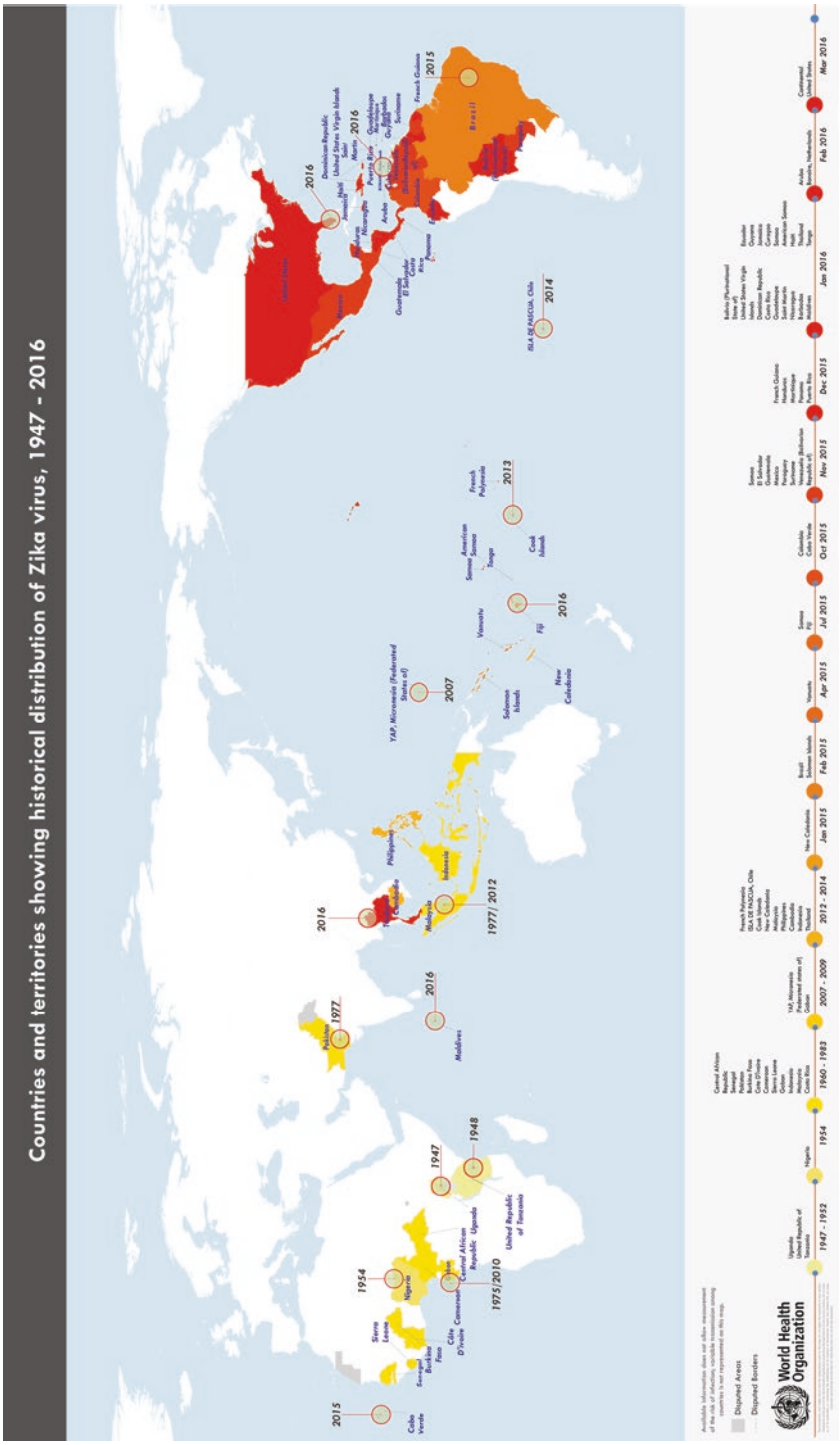

Fig. 30.9 Proliferation of ZikaV [2]

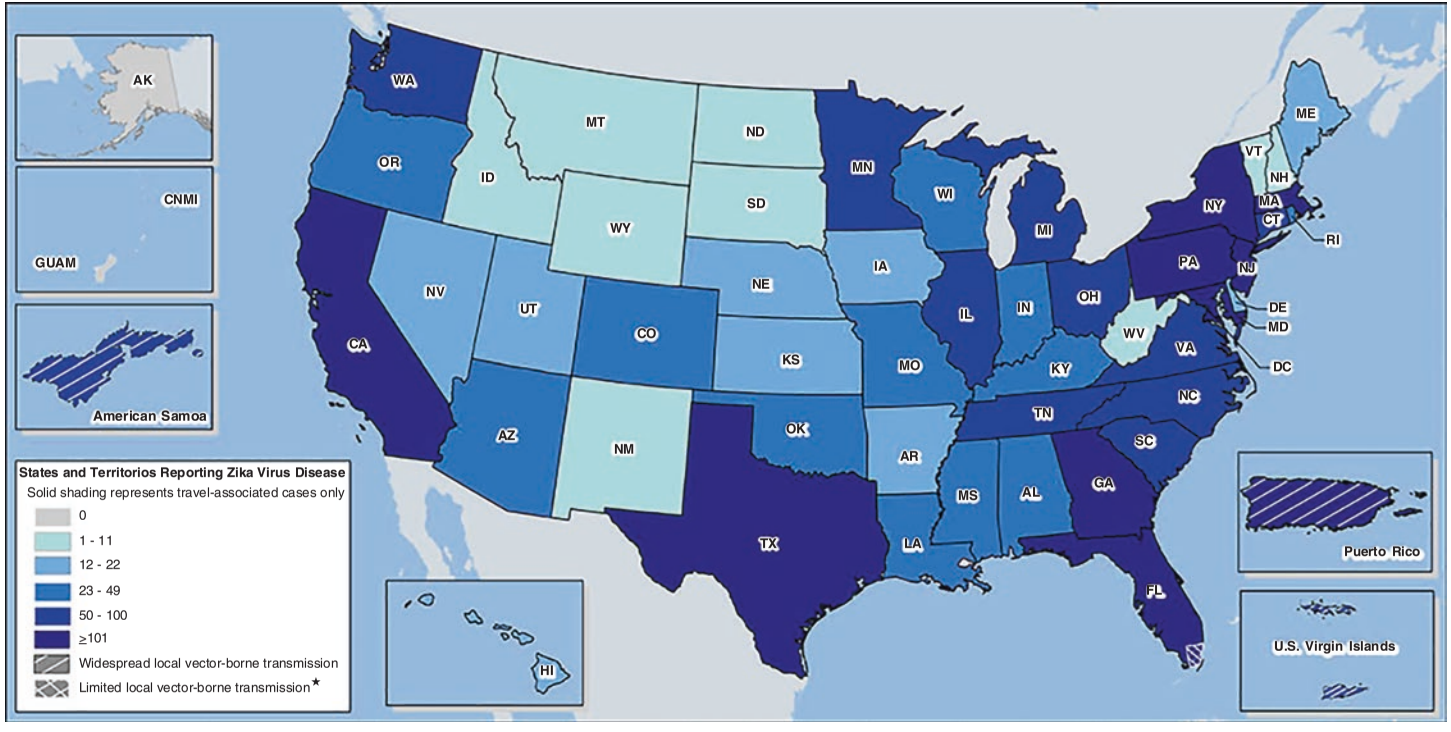

Fig. 30.10 ZikaV in the United States, November 16, 2016 [17]

which further contributes to the problem. Sanitary infrastructure and practices are necessary to address the rapid spread of the epidemic; however, pleas for funding of such efforts are underway [19, 20, 49].

30.8 Zika Virus and HIV/AIDS

The expansion of the ZikaV pandemic has been disturbingly like the HIV/AIDS epidemic. Initially, the HIV/AIDS epidemic was difficult to diagnose, the modes of transmission were not understood and the risk groups were unknown, thus, in retrospect, making the proliferation of this disease all but certain. The recent emergence of ZikaV mirrors similar delays in public health action and understanding that initially accelerated the spread of HIV/AIDS globally. Without a comprehensive and collaborative approach, ZikaV will have a similar damaging effect, globally, as the HIV/AIDS epidemic has had during the past decades.

The ZikaV pandemic reinforced that there is global human domination, urban crowding, expanded international travel, disruption of environmental equilibria, and other human behaviors combined with human-caused perturbations in ecologic balance that have promoted several latent infectious agents to emerge so unexpectedly [19, 44]. It is urgent to approach this epidemic through a multidisciplinary and collaborative approach to minimize the spread of the ZikaV [44]. The implementation of a global framework is needed in which collaborative efforts drive the research and clinical efforts for containing and eliminating this new epidemic and to anticipate other epidemics to come [44, 45].

It should be noted that the current damage caused by the ZikaV pandemic was predicted as likely to occur in a 2015 publication [19]. The reasons provided at that time are as follows: global warming and mosquito spread; war, pestilence, and malnutrition; relatedness of clinical symptomatic and immune relatedness to other diseases and their viruses; the proximity of ZikaV molecular and viral properties to all the other related viruses that are well-known to be pathogenic; viral superinfection issues; and lack of support from global economic and social structures.

Further, the impact of the ZikaV on pregnancy and newborn infants is different compared with HIV/AIDS. Both viruses can have deleterious effects on mothers and newborn children and have the potential to produce major health challenges and negative birth outcomes. Unlike HIV/AIDS for which manageable chronic interventions have been developed over the decades for both adults and children growing up HIV positive, ZikaV causes additional outcomes including intrauterine growth constraints, eye defects, congenital brain abnormalities, and microcephaly risks for infants, and GBS risk for adults [50–52]. The immediacy and relationship between ZikaV and pregnancy highlights the viruses' virulence and capability to cause major health challenges for future generations if it is not immediately controlled (Table 30.1).

Table 30.1 Comparison of HIV and ZikaV

HIV	ZikaV
Retrovirus	Arbovirus closely related to dengue, chikungunya, and West Nile viruses
Effects children and adults alike, yet with treatment children are living to adulthood	Highly virulent and pathologically disastrous for children
Most people are asymptomatic for long durations (years) before displaying disease and contracting other rare diseases (*Kaposi's* sarcoma)	Symptoms are rash and/or fever for 2–7 days but many are asymptomatic
Primary transmission is via sexual contact. Effective HIV prevention targets safe sex	Primary transmission is via mosquitoes, *Aedes aegypti* and *Aedes albopictus*
Most eventually require long-term expensive medical care for duration of life. Death from AIDS still occurring globally	Four-fifths of those with disease never need or receive medical care
Transmission still occurring in the United States yet threats continue in Africa and India	Local transmission just beginning in the United States though disease is now worldwide
No vaccine but many regimens of medication treatment in use	No vaccine or medication currently (4–2017)
Originated in Africa, Kinshasa, in the early 1900s, years before adverse health seen because of infection	Originated in Africa, Zika Forest in 1950s, years before adverse health seen because of infection

30.9 Conclusions

During the initial phases of the HIV/AIDS epidemic, an accurate diagnosis could not be made and neither were there clinical and/or therapeutic treatments available that were efficient and effective. It is similarly very difficult to discern a "pure" ZikaV diagnosis as it is closely related to dengue and other arbovirus diseases and many of the clinical tests cannot readily distinguish among the viruses [44]. A modern highly diagnostic technique in widespread use for neuroAIDS is brain imaging. Neuroimaging has also become important in ZikaV infection-related microcephaly, GBS, and acute disseminated encephalomyelitis (ADEM) [53, 54].

From the viewpoint of evolution and at the molecular level, HIV and ZikaV are widely divergent although they entered the human framework near each other in tropical Africa. For example, their mutation rates are divergent. The mutation rate of HIV is $4.1 \pm 1.7 \times 10^{-3}$ mutations per base in a viral genome of 9749 bp. That means that there are about 39.97 mutations per genome for HIV-1. This is the most rapid mutation rate of any living organism on planet Earth. However, in comparison, the mutation rate of ZikaV, in vivo, is 12–25 bases a year, in a viral genome of 10,272 bp. However, comparisons of HIV-1 and ZikaV mutation rates are made difficult because their mutation rates are not measured or reported under identical laboratory or epidemiological conditions [55, 56].

The risk groups, lifestyles, and poor accessibility to preventive measures and healthcare enhanced the spread of the HIV/AIDS epidemic and this trend appears to

be paralleled by ZikaV. The different modes of transmission coupled with the rapid spread of ZikaV stresses the importance of being alert, pre-emptive, and proactive rather than reactive to prevent this virus from continuing its pandemic trajectories and trails.

Conflict of interest The authors report no conflicts of interest.

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Chapter 31
Global Convergent Translational Science for Neuro-involvement in Ebola Viral Disease: Lessons Learned from Neuro-AIDS

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Keywords Human immunodeficiency virus (HIV) • Acquired immune deficiency syndrome (AIDS) • Neuro-AIDS; Highly active antiretroviral therapy (HAART) • Immune reconstitution inflammatory syndrome (IRIS) • Ebola virus disease (EVD) • Neuro-EVD; Hypothalamus-pituitary-adrenal (HPA) axis • Cell-mediated immune (CMI) surveillance • Blood-brain barrier (BBB) • Comparative effectiveness research (CER) • Bibliome • Analytic framework • Level and quality of evidence • Best evidence base (BEB) • Tele-health; Comparative individual patient effectiveness research (CIPER) • Publication bias

Core Message

Translational research in HIV/AIDS over the past decades has yielded a new in-depth understanding of the pathological processes in the neuroinvolvement of HIV/AIDS (neuro-AIDS). Comparative effectiveness research of these findings in observational studies, clinical trials, systematic reviews, and meta-analyses has established the best evidence base in support of novel interventions for patients with HIV/AIDS, and neuro-AIDS specifically, in a translational effectiveness modality. This new paradigm is informative in this new age of Ebola and Zika.

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31.1 Introduction: EVD – Parallels and Differences with HIV/AIDS

The Ebola virus was first discovered along the Ebola River across northern Zaire, now the Democratic Republic of the Congo, in the mid-1970s. In 1998, that specific new viral genus family was named the "Ebola-like viruses" (Vertebrate Virus Subcommittee proposal to the International Committee on Taxonomy of Viruses). By 2002, its name was simplified to Ebola virus (EBOV, ebolavirus).

The genus EBOV belongs to the negative strand RNA family of Filoviridae, order *Mononegavirales* (genus: *Ebolavirus,* Group V, sRNA virus). Flaviviruses are 40–65 nm in size, enveloped with an icosahedral nucleocapsid, negative-sense single-stranded RNA viruses. EBOV are transmitted to humans through an infected mosquito of the genus *Aedes* (i.e., *A. albopictus*, *A. aegypti*), and possibly through contact with infected body fluids (e.g., sweat, perspiration) [1].

The human immunodeficiency virus (HIV) is a lentivirus, a subgroup of the retroviruses (Group VI ssRNA virus, Family: Retroviridae, genus: *Lentivirus*). HIV is transmitted via exchange and mixing of body fluids. HIV infects cells in the human immune system that express the cluster of differentiation number 4 (CD4). These cells predominantly constitute lymphocytes (CD4+ T cells) and myeloid (CD4+dim monocytes/macrophages and dendritic cells). HIV infection is associated with a progressive weakening of cell-mediated immune (CMI) surveillance, and pathologies of increasing severity associated with the acquired immune deficiency syndrome (AIDS). HIV-infected lymphoid and myeloid cells can immigrate in the central nervous system through the blood-brain barrier (BBB), which triggers the onset of neuro-AIDS [2–4].

Both EBOV and HIV replicate in a host cell through the process of reverse transcription and are thus termed retroviruses. Retroviruses are single-stranded RNA viruses with a DNA intermediate. Once inside the host cell cytoplasm, retroviruses use their reverse transcriptase enzyme to produce complementary DNA from their RNA genome in the provirion stage of the infection cycle. The retroviral DNA is incorporated into the host cell genome by the viral integrase enzyme. The viral stage of retrovirus infection involves the viral DNA being processed by the infected host cell as its own, replicating it, transcribing it, and translating along with the cell's own genes. Viral proteins are thus produced that assemble into new copies of the virus.

In the case of EBOV, the negative-sense viral RNA is complementary to mRNA and thus must be converted to positive-sense RNA by an RNA polymerase before translation. Indeed, the purified RNA of a negative-sense virus is not infectious by itself as it needs to be transcribed into positive-sense RNA. Each virion can be transcribed to several positive-sense infectious RNAs. RNA viruses, in general, and negative sense RNA viruses, in particular, have higher mutation rates compared to DNA viruses because viral RNA polymerases lack the proofreading ability of DNA polymerases [1]. Both HIV and EBOV have high mutation rates due to their retro-

virus nature, but EBOV has a higher mutation rate than HIV because it is a negative-sense RNA virus.

The grave difficulties caused by the high rate of mutations of HIV in our concerted efforts to combat HIV/AIDS and neuro-AIDS in recent decades, together with the fact that EBOV is likely to show an even greater mutation rate than HIV depict the serious challenge clinicians will increasingly face in controlling Ebola virus disease (EVD).

EBOV infects dendritic cells and other myeloid cells, it interferes with and disables the interferon system, and it disrupts the host antiviral cell-mediated immune (CMI) surveillance response. Furthermore, EBOV induces alterations in the blood clotting pathway, contributes to a significant increase in proinflammatory cytokines, including interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α. EBOV raises nitric oxide and induces tissue damage by oxidation. The glycoprotein from EBOV forms trimeric complexes, which bind the virus to the endothelial cell lining and the interior surface of blood vessels, and damage the lining of blood vessels. A dimeric complex of the glycoprotein is also formed that interferes with the signaling of neutrophils, thus inhibiting early steps of neutrophil activation and impairing neutrophil-initiated antiviral CMI surveillance. Neutrophils, infected with EBOV, become transport vehicles for the EVD-causing virus to the lymph nodes, liver, lungs, spleen, and other organs, and presumably the central nervous system. Together these events converge into EVD, which is manifested as serious hemorrhage, hypotension, drop in blood pressure, and catastrophic organ failure, shock, and death [5, 6].

If intervention is commenced promptly and sustained with efficacy, EVD can be avoided or curbed, EBOV controlled, and patients led to survive in an asymptomatic state. As for HIV, however, because of the life cycle of retroviruses, an individual infected with EBOV remains EBOV seropositive asymptomatic for weeks, months, and years. As for HIV, it is suspected that EBOV can find niches (e.g., mesenteric lymph nodes) where it can hide, thus giving the – fallacious – impression that the patient is cured and free of that virus. In fact, because retroviral DNA inserts into the genome of the host, as noted above, once infected with HIV or with EBOV, patients always will remain HIV or EBOV seropositive.

It is possible and even probable that concerted investigative effort will soon establish that diverse virus families lead to alterations in HPA-CMI regulation, similar to what has been observed in HIV/AIDS and neuro-AIDS [2], including important elevations in members of the IL-17 cytokine family systemically and centrally. Central elevation of IL-17 [7] in a variety of pathologies could be observed at certain stages of EVD, such as those observed in neuro-AIDS [7], before the onset of critical and terminal pathology.

The remainder of this chapter explores similarities and differences between EBOV and HIV infection, particularly in the context of neuro-AIDS and putative neuro-EVD from a translational science perspective.

31.2 Central-Peripheral Manifestations in HIV/AIDS and EVD

31.2.1 IRIS in HIV/AIDS and Putative Corresponding IRIS in EVD

Over one-third of the patients seropositive for HIV with signs of AIDS and under treatment with highly active antiretroviral therapy (HAART) interventions develop the immune reconstitution inflammatory syndrome (IRIS) [3, 4, 8]. It is not clear what variables determine whether a patient with HIV/AIDS will develop HAART-related IRIS, but the best evidence base, thus far indicates that HIV/AIDS patients with low CD4 cell count, and HIV/AIDS patients whose CD4 count recovery shows a sharp slope, suggesting a particularly fast "immune reconstitution," are at a greater risk of developing IRIS [4, 8]. Still, even taking into account these two significant predictors, the medical establishment is currently at a loss to explain the variables that may determine the rather important odds for HAART-related IRIS [3].

It is perhaps inappropriate to use the term "inflammatory syndrome" to describe a state of chaotic inflammation where regulatory feedback loops are dysfunctional and to refer to "immune reconstitution" as simplistically as recovered CD4 cell numbers. Be that as it may, the term IRIS is the favored medical nomenclature.

Among the most striking features of IRIS consequential to blunted CMI surveillance is an aggressive resurgence of HIV/AIDS-related cancers, including Kaposi sarcoma, Hodgkin and non-Hodgkin's lymphoma, and a reoccurrence of oral manifestations of HIV/AIDS (e.g., oral Kaposi, hairy leukoplakia, a variety of oral ulcers). The latter observation may be a bit surprising because oral ulcers, such as recurrent aphthous stomatitis (RAS), were traditionally not among the oral manifestations in HIV/AIDS before the onset of HAART treatment interventions [2–4, 8].

CMI surveillance mechanisms are finely regulated cellular and physiologic processes that involve more than just CD4 cell number. There is a vast literature that describes the role of neuroendocrine modulation of cellular immunity. There also is ample evidence to support the intertwined biological effects of psychological and physiological factors on health and disease, including HIV/AIDS and oral biology and medicine [9, 10]: thence derived the fields of scientific endeavors now referred to as "psychobiology," "psychoneuroendocrinology," "biological psychiatry," "psychoimmunology," and "psychoneuroimmunology."

It is possible and even probable that the exacerbation of IRIS in HIV/AIDS patients with incipient IRIS can be significantly curbed by interventions directed to relieving anxiety. Patients with IRIS often develop anxiety in observing the recurrence of symptoms that ought to have been controlled by HAART interventions. Anxiety has profound psychoneuroimmunological sequelae [11–15]. As a corollary, it is logical to expect that anxiety is a significant predictor of IRIS and that controlling and reducing anxiety may control and reduce the emergence of IRIS [3, 16]. We predict that interventions directed at reducing anxiety, such as mindfulness meditation [17–19], delivered either contemporaneously with HAART, or immediately at

the earliest onset of IRIS symptoms, will be shown to curb or prevent full-blown IRIS within this decade.

CMI, in general, and inflammation, in particular, reflects a complex immunophysiological process that requires and involves a plethora of cytokines, and noncytokine immune factors. Together these elements are involved in a delicately balanced orchestra of effects and countereffects, activation, and regulatory feedback loops that finely regulate all aspects of CMI surveillance, including the myriad of events in the immune-inflammation microenvironment in viral infections, including HIV, EBOV, as well as dengue [20] and other related viruses such as Zika [2].

In brief, an inflammatory response is broadly composed of two phases: the first is triggered and sustained by myeloid CD45+CD14+ cells (i.e., white blood cells that express the common leukocyte antigen, CD45, and the lipopolysaccharide receptor, CD14). A significant number of CD45+CD14+ cells also express CD4, albeit weakly (i.e., CD4+dim). These immune cells produce IL-6, IL-1β, and TNF-α in addition to prostaglandins and other noncytokine factors that favor inflammatory processes. This first phase of inflammation is a rather sharp and short-lived response. By contrast, the second phase of inflammation is triggered by lymphoid CD45+CD14− cells (many of which are CD4+high) that engender a TH17 response (i.e., predominant cytokines: IL-17 and IL-23). The TH17 cytokine family is finely regulated by IL-9 and other TH9 cytokines representatives. The second phase of inflammation is a sustained event, such as that observed in chronic inflammatory diseases (e.g., inflammatory bowel disease). Both phases of inflammation are subject to fine regulation brought up in part by regulatory T cells (CD4+CD25+FoxP3+) [1, 10, 20].

A more detailed perspective on IRIS in patients with HIV/AIDS reveals the necessity to distinguish carefully which subpopulation of CD4+ cell is fast recovering in order to modulate the prognostic course with greater effectiveness. Moreover, when our diagnostic and prognostic modalities for EVD will be such that we will successfully treat EBOV patients, we will conceivably face clinical situations similar to IRIS in EBOV seropositive EVD patients. It will behoove us to consider the pathological signs and symptoms of this subpopulation of surviving EBOV seropositive patients from the perspective of the more detailed considerations about immune inflammation in viral CMI surveillance derived from HIV/AIDS research.

31.2.2 Osteoimmunology

Physiology is a delicately intertwined network: case in point, bone metabolism is intimately linked to cellular immune regulation – thence the emergence of the new field of osteoimmunology [21]. From a translational science viewpoint, the best evidence base supports the concept that osteoimmunology cannot be separated from psychoneuroimmunology. Thus, we proposed the novel domain of psychoneuroendocrinology-osteoimmunology in the HIV/AIDS field [22]. Here, we suggest that future research will soon establish significant psychoneuroendocrino-osteoimmune modulations in EBOV/EVD as well.

In brief, the immune and skeletal systems have major interactions. Osteoclasts are important cellular mediators of skeletal homeostasis, derived from hematopoietic precursors that also give rise to immune cells. Numerous cytokines, originally shown to regulate CMI function, also regulate bone cells and influence skeletal health. Products of bone cells appear critical for the engraftment of marrow in bone, the normal development of the hematopoietic and immune systems, and can also provide niche for long-term memory B and T cells [23].

In the pathogenesis of bone destruction associated with rheumatoid arthritis, for example, the synovium is the site of active interplay between immune and bone cells. The interaction between T cells and osteoclasts is critical at this juncture. T cells that produce IL-17, the predominant driver of the sustained process of inflammation outlined above, induce the expression of receptor activator of nuclear factor-κB (NF-κB) ligand (RANK-L) in synovial cells. This factor, together with inflammatory cytokines, stimulates the differentiation and activation of bone-resorbing osteoclasts. Other pathways that involve transcription factors, signaling molecules, and membrane receptors are also involved in the bone resorption process [24].

Interferons (IFNs) play crucial roles in the regulation of a wide variety of innate and adaptive immune responses. Type I interferons (IFN-α/β) are central to the host defense against pathogens such as viruses, whereas type II interferon (IFN-gamma) mainly contributes to the T-cell-mediated regulation of the immune responses. Studies of bone destruction associated with rheumatoid arthritis have highlighted the importance of the interaction between the immune and skeletal systems. IFN-γ interferes with RANK-L. In addition, RANK-L induces the IFN gene in osteoclast precursor cells, and this induction constitutes a critical aspect of the negative feedback regulation mechanisms of RANK-L signaling to suppress excessive osteoclastogenesis. Furthermore, a novel function of signal transducer and activator of transcription 1 (Stat1), the essential transcription factor for both type I and type II IFN responses, was revealed in the regulation of osteoblast differentiation. Collectively, these studies unveil novel aspects of the IFN system and indicate the operation of the intricate signaling network among IFN and other cytokine systems in bone remodeling, which might offer a molecular basis for the treatment of bone diseases [25]. Taken together, it is possible and even probable that translational research will demonstrate that EBOV infection causes significant osteoimmune pathologies.

It is clear that HIV infection and HAART therapy are clinically relevant risk factors for decreased bone mineral density, osteopenia, and osteoporosis [26, 27]. It is, therefore, possible and even probable that emerging data will soon establish a similar etiopathological role for EBOV for osteoporosis and other bone diseases.

31.2.3 HPA Axis and HPA-CMI Regulation

Extensive evidence now supports the important role for psychoneuroendocrine modulation and specifically of the hypothalamus-pituitary-adrenal (HPA) axis, in the regulation of CMI surveillance in normal healthy adults [2, 9, 10, 28, 29], as

well as certain patient populations, including patients with HIV/AIDS [30–32], and to some extent patients with autism spectrum disorder [33]. It is reasonable to expect that such alterations in immunophysiological regulation will be observed in Ebola and in Zika pathologies.

In a typical protocol, the integrity of HPA-CMI regulation can be tested by dynamic or static challenges of HPA responses, and monitoring CMI products outcomes in peripheral blood and other body fluids. Case in point, Ebola survivors or EBOV seropositive patients could be entered in a protocol and be randomly sampled from the population for an experimental design constructed to test the HPA response to external stressor (e.g., cold press, mental arithmetic, Trier stress test [34]), as well as internal static and dynamic challenges of the HPA axis.

The Trier Social Stress test induces replicable psychosocial stress in human subjects by means of rigorously standardized tasks, such as public speaking and mental arithmetic [9, 34]. The test yields significant elevations in serum and salivary cortisol about 10 minutes following the conclusion of protocol in most normal health subjects [34–36]. Males show a more vigorous elevation in cortisol across body fluids compared to pre-menopausal females, in part because of the differential confounding effect of female hormones at different stages of the menstrual cycle [34–36]. Stress-related free salivary cortisol and total plasma cortisol net increases do not differ as a function of the time of day (morning of afternoon) when the Trier test is administered, despite the fact that morning, compared to afternoon prestress salivary and plasma cortisol levels differ significantly due to diurnal variations [34–36]. The test has not been used with HIV/AIDS patients or with asymptomatic survivor Ebola patients.

In a dynamic test of HPA, subjects are typically challenged by intravenous administration of either ACTH (synthetic ACTH, sACTH 1–24, Cortrosyn Organon Inc., 0.5 μm/kg) or CRH (ovine CRF, oCRF, 1 μg/kg) at 11:00 PM. Injections must be closely monitored by the medical staff particularly in the case of oCRF, and discontinued immediately if the subjects show signs of dizziness, fainting, nausea, vomiting. Minor side effects may include flushing, slight fever, slight unease, and do not preclude continuation of the protocol. The static approach tests the functionality of the HPA axis by temporarily arresting the glucocorticoid feedback mechanism. Participants are administered 1–5 mg of metyrapone after a meal during midafternoon to early evening. Metyrapone acts as a glucocorticoid synthesis inhibitor, thus preventing the metabolism of plasma cortisol. Alternatively, subjects are administered a 5 mg bonus of the synthetic glucocorticoid, dexamethasone at 11:00 PM. Dexamethasone competitively loads the cytosolic glucocorticoid receptor, blocks translocation of the charged receptor to the nuclear compartment, and blocks glucocorticoid-mediated genomic modulatory events. Minor side effects, or none at all, are associated with either metyrapone or dexamethasone administration [2, 29, 32].

Typically, subjects subjected to a dynamic HPA test spend the night at the clinical research center (CRC) for continued observation. It is not necessary but recommended that subjects undergoing static HPA tests also spend the night at the CRC and be monitored, particularly with respect to the time of administration of metyrapone or dexamethasone, food intake before blood draws, and related confounding variables.

For the purpose of individual patient data outcomes research (*vide infra*) sampling, following admission into the study – optimally a cluster randomized stepped wedge blinded controlled trial (CRSWBCT) [37, 38] – and signing informed consent, participants should provide three baseline samples of peripheral venous blood (5 cc, in EDTA vacutainers) between 3:00 PM and 6:00 PM, when the circadian variations of the HPA axis and of CMI measures in plasma, serum, saliva, and other body fluids are relatively flat [2, 9, 29, 39, 40]. Salivary samples are best obtained by presenting the subjects with a cut half lemon, which triggers salivation, in EDTA- and protease inhibitors-coated tubes. Optimally, the baseline protocols should be structured around 5 cc collections at 4:30 PM, 5:00 PM, and 5:30 PM. Blood and saliva samples are centrifuged (1500 g [3000 RPM], 15 min, 4° C), and supernatants (plasma or saliva free of cell debris) labeled and stored frozen at −80°C until batch assay for HPA (e.g., ACTH, CORT) or CMI product (e.g., proinflammatory cytokines: IL-1β, TNF-α, IL-6; or TH1, TH2, TH17, and TH9 cytokines, neopterin [41], or related soluble products of immune activation [42]). Peripheral blood mononuclear cells are obtained immediately on the fresh samples (although uncoagulated blood can be preserved in EDTA vacutainers with continuous gently shaking for up to 24 h, as our studies have shown [43]), and processed either for the identification of lymphocyte subpopulations by dual- or tricolor fluorescence flow cytometry, followed by Komolgorov-Smirnov analysis [2, 28–30], or functional responses in vitro function, following nonspecific (i.e., mitogens such as concavalin-A [5 μg/ml, 72 h] for lymphoid cells, or lipopolysaccharides, LPS for myeloid cells [5 μg/ml, 72 h]) or specific activators (e.g., via the CD3 pathway with CD28 co-stimulation). Functional responses can be recorded as ^{3}H-thymidine incorporation, cell cycle traversal, expression of markers of immune activation (e.g., transferrin receptor, CD71), or production of certain cytokines, including TH1, TH2, and TH17 [42, 44, 45]. Glucoroticoid resistance can also be monitored by obtaining the number of cytosolic glucocorticoid receptors in circulating lymphocytes at baseline and every sample collection thereafter [28, 30].

In the morning, before coffee or breakfast, subjects provide another 5 cc venous blood and 5 cc saliva samples. After a light breakfast, clinical subjects are free to go about their morning with the instruction not to eat after 11:00 AM. By noon, subjects provide another 5 cc venous blood and 5 cc saliva samples and are free to have lunch and to go about their day. Subjects are not to eat after 4:00 PM and to return to the CRC by 5:00 PM for the final blood and saliva sample collection. As a routine, following each sample collection, the subjects are given the State-Trait Anxiety Inventory (STAI) [46] to monitor their level of stress and anxiety. Alternatively, subjects can be administered the Stroop test for executive functions [47], although the Stroop test is not, *per se*, a tool to measure anxiety. Rather, performance on the Stroop is impacted by anxiety: the Stroop test measures the patients' difference in selective attention capacity and skills as well as their processing speed ability and provides a useful neuropsychological glimpse on the patients' psychocognitive executive processing function, which can aid in the diagnosis of certain neurological abnormalities [48].

The approach outlined above rests on the rationale that the HPA axis is a sophisticated psychoneuroendocrine system that is activated by and responds to a variety of stressors, including challenges to cellular immunity. CMI in turn regulates HPA responses. Stressors can be physical, mental, or immunological in nature and induce a finely coordinated HPA-CMI response. Upon activation of higher brain processes to a potentially threatening stressor, the hypothalamus releases CRH, which in turn stimulates the production of ACTH from the pro-opio-melanocortin gene expressed by the anterior pituitary gland. The release of ACTH into the systemic circulation stimulates the adrenal cortex to synthesize and produce glucocorticoids, namely, cortisol (humans) and cortisone (rodents, fish). These glucocorticoids find their way to cells throughout the body and bind as ligands to cytosolic glucocorticoid receptors, in which this signal translocates to the nucleus of the cell where it can regulate transcription of glucocorticoid-related genes. These regulatory events, when localized in cells of the immune system, lead to a finely regulated downsizing of CMI responses. This finely homeostatic physiologic balance can be seriously altered by psychoemotional stress, such as the perception of lack of fit between one's perceptions of skills vs. environmental demands, and one's objective skills and environmental demands, namely the person–environment fit [49, 50], as well as by pathologies of the central nervous system, from major depression to anorexia nervosa, and putatively autism spectrum disorder, as noted above. It is now evident that certain viral infections of the central nervous system, including HIV that leads to neuro-AIDS, also significantly impair HPA regulation, and its ability to modulate CMI responses, as we discussed earlier.

Taken together, and based on our current understanding of the similarities in virological properties between HIV and EBOV, and for that matter the virus that causes Zika as well, it is reasonable to expect that, as we develop, test, and standardize more effective treatment interventions for Ebola (and Zika), we will significantly increase the number of patients who survive the acute eruption of the disease. The number of asymptomatic patients seropositive for EBOV will swell, even if that virus, like HIV, will tend to hide, as noted above, in certain privileged compartments (e.g., testicles, lymph nodes, brain parenchyma). That situation, which superficially will be attractive to the few because it will suggest to some that Ebola was "cured," should actually be as alarming in EBOV patients as it was revealed to be in HIV patients, in light of the points discussed earlier.

That is to say, one important lesson that we can learn from the HIV pandemic and the consequential important emergence of neuro-AIDS, with the associated significant neuroendocrine immunopathology that includes, as we outlined, impaired HPA-CMI interactions, is that a similar pattern of neuro-Ebola (and possibly neuro-Zika – case in point, we already know much of the direct pathology Zika inflicts on neurons and glias), with associated impaired HPA-CMI interactions are to be anticipated.

Impaired HPA-CMI interactions imply, evidently, impaired regulation of viral CMI surveillance [6]. In brief, during the invasion of a viral pathogen such as HIV or EBOV, the body's natural innate immune response, from immune cells of infected tissues, elicits the production of TH1 cytokines and other inflammatory mediators

(i.e., TNF-α, IL-1β, IL-6) [17]. HIV infection, and presumably EBOV as well, is an immune stressor, which activates the HPA axis and subsequent glucocorticoid production. The secreted glucocorticoids prevent the potential damaging of excessive immune response by reducing inflammation of affected tissues, a process mediated by TH2 cytokines and other anti-inflammatory mediators and cytokines that counter TH1 responses, such as IL-10. The immunosuppressive character of glucocorticoids has been shown to prevent the reduction of CD4 lymphocytes caused by HIV-mediated immune activation, thereby increasing CD4 lymphocytes and their therapeutic effects and to inhibit HIV viral replication [19, 20]. Reciprocally, diminished secretion of ACTH to CRH and consequential low serum cortisol suggested an imbalance of cytokine production needed to suppress the consequential immune response from HIV-infected tissues, an observation confirmed by an experimental study that reported lowered HPA response (i.e., blunted ACTH and GC response to the cold press or test) in patients with HIV/AIDS, as noted [6]. The intertwined nature of HPA-activating CMI responses and immunosuppressive HPA activation is a critical and timely key aspect of homeostasis, which is seriously hampered and deregulated in certain patients with viral infection, including HIV and presumably EBOV as well as the Zika virus as research will show within this decade.

Proof of principle is given in our recent discussion of neuroendocrine-immune interactions in Ebola [1] and dengue [20]. Both Zika and dengue are members of the genus *Flavivirus*, which also includes the West Nile virus, the tick-borne encephalitis virus, the chikungunya virus, the yellow fever virus, and contribute to the incidence of and morbidity of encephalitis, among other pathologies.

31.2.4 BBB: Viral Ports of Entry

The blood-brain barrier (BBB) consists of a fine filtering mechanism of the capillaries that carry blood to the brain and spinal cord tissue and that serves to block the passage of certain substances and cells. BBB separates the brain parenchyma from the circulatory system and thus protects the central nervous system from potentially harmful chemicals and infectious agents, while regulating transport of essential molecules, peptides, transmitters, and hormones maintaining a stable balance of central-systemic exchange. BBB is made by specialized endothelial cells that line the brain capillaries and work in concert to transduce signals from the vascular system and brain. Both the structure and function of the BBB is dependent upon the complex interplay between the different cell types, which include endothelial cells, astrocytes, and pericytes, and the extracellular matrix of the brain and blood flow in the capillaries [51–53]. Three sites have been identified that are devoid of a physical barrier via tight junctions that include the brain endothelium that forms the BBB, the arachnoid epithelium that forms the middle layer of the meninges, and the choroid plexus epithelium that secretes the cerebrospinal fluid CSF [54].

At the cellular level, the BBB proper consists of specialized endothelial cells that are connected through tight junctions and adherence junctions, which separate

and protect the CNS from the circulation and restrict and prevent blood-borne molecules and peripheral cells from entering the CNS. Tight junctions produce two functionally distinct cyto-anatomical aspects: the luminal side facing the circulation and the abluminal side facing the brain parenchyma. Both aspects are highly sensitive to cytokines, especially proinflammatory TNF-α, IL1-β, and IL-6, as well as TH17 and TH9 cytokines, which contribute in opening selected "gates" through the BBB [6, 55–57].

Whereas the central nervous system is considered an immune-privileged tissue protected specifically by the BBB, upon infection or traumatic brain injury, the BBB can be breached. A variety of immune cells – myeloid and lymphoid – are recruited to the affected area and mount and direct a vigorous CMI surveillance. Therefore, there exist specific signals that direct autoreactive T cells past the BBB and into particular sites of the brain parenchyma. Case in point, a mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis, has established one possible mechanism of pathogenesis in which regional neural stimulations modulate BBB locally to allow invasion of autoreactive T cells. This gate can be experimentally manipulated systemically via activation of regional neurons [58]. It is possible and even probable that similar manipulations via cranial nerves may alter BBB permeability locally as well. Moreover, viral infection, such as HIV and EBOV, could similarly open specific gates within the BBB.

We have proposed [6] that viral infection, including EBOV and HIV, may induce an initial activation of monocytes/macrophages and dendritic cells, which in turn will release proinflammatory cytokines, which will alter, as noted, the integrity of the plasma membrane of endothelial cells [59]. These transient changes in the integrity of the membrane at the molecular level (e.g., NF-κB, JAK/STAT signaling pathway) will be sufficient to open significant gates in the tight junctions, and allow the transpiration of virions and virus-infected myeloid cells [6].

In brief, and as we discussed elsewhere [1, 6, 20] and is supported by others [60, 61] inflammation, mediated in part by chemokine activity and the release of proinflammatory cytokines, contributes to the breakdown of the brain microvascular endothelial cells that constitute the BBB, thus increasing the potential for continued viral invasion into the CNS by HIV, as well as by EBOV, Zika, and related families of viruses.

31.3 CER in HIV/AIDS and EVD: Lessons Learned from Neuro-AIDS

31.3.1 Translational Effectiveness: Identifying the Best Evidence Base

Modern contemporary healthcare is driven by two intertwined forces, which are recognized as translational research –namely, going from the patient to the laboratory and back to the patient in order to design and administer a patient-centered

treatment intervention that is consonant with the fundamental biopathology, and translational effectiveness – namely, the identification of the best available evidence, the best evidence base (BEB) for the most cost-effective treatment intervention that also maximizes benefits to the patients and minimizes risks, and that is tailored to the patient's needs and wants, medical, and dental history, and psychobiological (cf., psychoneuroendocrine-immune) unique characteristics. Taken together, the transaction between translational research and translational effectiveness yields the timely and clinically critical perspective of translational science in healthcare, which ranges across all pathological traits (e.g., psychopathological personality disorders) and states (e.g., HPA-CMI deregulation consequential to major depression or other psychological disorder, IRIS consequential to HAART treatment of HIV/AIDS patients, and the like) [49, 62].

Although the driver of translational effectiveness is the investigational approach designated as comparative effectiveness research (CER), and its research design consists in the research synthesis process [49, 63], one fundamental question of the philosophy of science of translational healthcare remains and can be stated briefly as follows: does CER drive translational effectiveness – that is, does the identification of the best evidence base drive its utilization in specific clinical settings in logic-based evidence-based clinical decision making, or rather does translational effectiveness drive CER – that is, does the information obtained by means of translational research and analysis of translational effectiveness outcomes research determine the search and consensus of the best evidence base.

This is not a moot point, particularly in the context of patient-centered, effectiveness-focused, and evidence-based treatment interventions for patients with HIV/AIDS, neuro-AIDS, EBOV infection, EVD, putative neuro-EVD, and conceivably the next level of global health concern: Zika infection and its pathological *sequelae*, including, we defend, neuro-Zika.

The process of crafting a PICOTS research question/hypothesis based on the patient characteristics uncovered during the initial patient-clinician contact (P), potential novel and control interventions (I & C), toward the sought clinical outcome (O) within a given timeline (T) and clinical setting (S) is at the very core of the CER process. Thence, it is obtained through a systematic process of inclusion and exclusion criteria, the bibliome (namely, the collection of systematic reviews, meta-analyses, clinical trials and observational studies) that specifically responds to PICOTS. Concerted protocols follow to establish the level of the evidence to quantify the quality of the evidence with duly validated instruments, and to obtain an acceptable sampling statistical analysis of the findings. The CER process concludes with a quantitative – namely, meta-analysis – and a qualitative consensus of BEB – namely, the RAND protocol for critical assessment and evaluation of the clinical observations [49, 63].

Undoubtedly, translational effectiveness does not end with CER. The consensus of BEB thus obtained must then be (1) disseminated to the patient, caregivers and other stakeholders, including the clinical and allied healthcare staff, and (2) cogently utilized toward optimizing evidence-based clinical decision making in practice. The question at hand here reflects fundamentally how to establish BEB in the profession

and the patients and stakeholders it serves, how to get clinicians across varied clinical settings (i.e., private practice, community healthcare, university teaching clinics) to become active in and involved in understanding and utilizing CER products for maximizing the benefit of the patient, and how we can monitor and evaluate the success rate of evidence-based clinical treatment interventions for, say, patients with HIV/AIDS, IRIS, neuro-AIDS, EBOV, EVD, putative neuro-EVD, and the related pathologies we have discussed throughout this chapter. The query is as complex as it is timely and critical for the afflicted patients we serve.

The goal of translational science on healthcare, and specifically of translational effectiveness, is not utopian. It is not a hypothetical. It is a real achievable goal – one that necessitates concerted and assiduous effort aimed not only at obtaining BEB through systematic CER protocols, but utilizing BEB in logic-based, evidence-based, clinical decision-making paradigms [64] that put the patient's needs, wants, benefit, and well-being first.

Utilization of BEB in a translational effectiveness modality will require changing long-held beliefs by clinicians, habits, and drives. That is a tall order, but it is not an impossible endeavor. Behavioral psychologists and cognitive psychologists have long defined and characterized how we develop engrained thought patterns, habits, and drives, and how these can be modified, changed, and improved. One technique that is emerging as most promising involves mindfulness intervention, through which people, individually or under the supervised guidance of a professionally trained psychologist, can identify and analyze the destructive or negative habit to be corrected, and engage a progressive process of self-empowerment toward changing that aspect of the psychological inner world. The process of mindfulness self-examination (namely, meditation) can be applied to reactivity to stress and anxiety, to learning, memory, and cognition, social behaviors, motivation, and a variety of other patterns of behaviors.

In brief, mindfulness meditation consists of a well-established intervention protocol that can be taught and learned to become an effective long-term technique for controlling and correcting stress, anxiety, undesirable drives, and habits, etc. Mindfulness meditation involves deliberately being aware of moment-to-moment changes in one's sensations, feelings, and thoughts, and guides the individual to focus on the moment present, and on the present state of mind, perception, tension, apprehension, and anxiety. It directs the person to notice the breathing pattern, the rapid heartbeat, and other state and trait sensations that would otherwise be perceived as unpleasant or alarming. By focusing on these experiences, the person learns to accept them, to own them, and eventually to control them psycho-emotionally non-judgmentally. The person is empowered to focus calmly on new occurrences, feelings, and perceptions by simply associating with the sense of calm and focus developed in the previous mindfulness meditation moment. The person becomes aware of the positive aspects of every mindfulness moment-present experience by a progressive feeling of empowerment, of settling into a steady and reassuring rhythm, and therefore a gratifying sense of control attributable to the wellness perception. In brief, mindfulness calls for the patient's mental position to be focused on a given experience (e.g., one's breathing or heartbeat) separate from the source of the anxi-

ety and associated emotion in the moment-present. It elicits and facilitates a skillful (mindful) – response of being fully aware in a given situation. Mindfulness is more effective and longer lasting than controlled breathing/relaxation and other similar interventions [17, 19, 62, 65, 66].

In brief, mindfulness protocols involve the following:

1. Place: Ideal characteristics of a good meditation place include somewhere neither too dark nor too light, and neither too hot nor too cold, with few noises or distractions (i.e., pagers and cell phones off).
2. Posture:
   (a) Sitting upright, lower back and stomach relaxed, neck balanced, head tilted gently forward to open the airways. Hands resting on thighs or knees. Focus on breathing: sitting and noting breathing in, breathing out, and other things such as the muscles being used to "sit," the muscles used to expand the lungs, as well as any sensations, mental feelings, thoughts, hearing a noise, smelling a smell, etc., all while adding extra actions as they are noticed.
   (b) Walking – Gentle stationary walking is most beneficial because it makes mindfulness the easiest to incorporate in daily experiences. Hands relaxed by the side. Focus on moving: walking and noting movements such as "lifting foot, putting down, shifting weight, slowing down, stopping, turning around...", and so forth, adding extra more subtle actions and sensations as they are noticed.
3. Meditation:
   (a) Relax in chosen posture. Develop a sense of calm focus. Settle into the meditation by becoming aware and examining the four foundations of mindfulness (i.e., the four frames of reference to study, one at a time, methodically but gently.
      (i) The first foundation: mindfulness of the body (i.e., body-mindfulness). Focus on "in and out" breathing, and progressively become aware of the body's composite nature by mentally noting, focusing, and exploring component parts of the body such as head, hair, skin, teeth, muscles, bones, heart, stomach, etc. Focus on what they are, where they are, what they are dependent on, what they do, etc. Focus on each part of the body, become fully aware of it, and study it as an image or in any other form it may come to the mind, including its physical characteristics (e.g., solid, liquid, temperature). Become aware of the movements the body parts can do, and the body reacts and interacts with events, tiredness, and muscle tension, and other phenomena. Become aware that the body struggles against all physical experiences, but is guided and rescued by the mind.
      (ii) The second foundation: mindfulness of physical feelings and sensations (i.e., body states meditation). Focus on how and when sensations occur and note whether they are pleasant, unpleasant, or neutral. Study

how the mind and body are acting and interacting with these feelings. Scan the body up and down to examine sensations and then let them go gently and nonjudgmentally to pass onto another part of the body. Be aware and watch the energy flow. Gain tolerance of the body and understanding of its nature, mentally let go of the stress that arises and become aware of how to relax the body to reduce tension.

(iii) The third foundation: mindfulness of mental states. Examine thoughts, fantasies, ideas, dreams, images, etc., and how they arise, evolve, change, and dissolve. Note how they arise dependent on feelings, outside distractions, thoughts, and impulses, and how they can be controlled by concentration. Let the mind wander, and gently and nonjudgmentally bring it back to the focus of the meditation.

(iv) The fourth foundation: mindfulness of consciousness (i.e., meta-mindfulness). Be conscious of the state of consciousness. Meditate on the state of meditation. Temper or gently change the state of consciousness as needed based on the mindful awareness of the body, the body states, and the mental states – for example, introduce compassion when feeling depressed, goodwill when feeling angry, appreciation when feeling dissatisfaction; recognize what is needed when the body or the mind are in a state of tiredness, focused or unfocused energy, states, peacefulness, anxiety, greed, anger, want or need, happiness, joy, grief.

(b) Awareness: Take note of feelings and mindful observations either verbally or mentally (recommended because it builds concentration and forces recall, which should be in writing in a journal). Note events, perceptions, sensations, images, and reactions. Gain insight and see how and what the mind sees, and translate into brief sentences, phrases, and single words as necessary in writing to label the moment experience and to further the knowledge of the mind (without getting absorbed in the story: it is about recalling the experience not about the story-telling). The aim is toward silent awareness and eventually wordless awareness.

(c) Abandonment: Let go. An integral part of all four foundations is to let go of any stressful states in the mind and body that arise, without judging or condemning them (note that being kind to oneself and pragmatic in releasing stress without judging oneself is as important as it is hard to practice). As the mind seeks refuge from reality in fear, distress, bewilderment, confusion and condemnation, mindfulness restores the mind in a state of open, unbiased, balanced, and peaceful awareness. Let go to attain peace of mind.

4. Practice:

(a) Detachment – everything as an independent process, stress free
(b) Continuity – carry mindfulness across to all day-to-day activities outside
(c) Release – release fear, greed, loathing, ignorance nonjudgmentally
(d) Peacefulness – mindfulness restores peacefulness to the mind

Drives and habits are often part of a silent agenda, which reflects in part perceptions of the demands of the environment contrasted to perceptions and reality contact about one's abilities, strengths, limitations and weaknesses – namely, the person-environment fit model [3, 50, 67]. In brief, drives and habits reflect, from a psychological standpoint, an implicit set of attitudes that can be modified by simple techniques, such as behavioral reciprocity. Reciprocal actions are important in social psychology because they often explain the maintenance, or the change of social norms. As a community of professionals wishing to improve healthcare practice by changing its norms to evidence-based, effectiveness-focused, and patient-centered care, it will behoove us to become more aware of the powerful force of reciprocity: people feel obligated to return a favor regardless of whether they like the person who originally gave the favor and even if they did not want the favor. Reciprocal actions are distinct from altruistic actions and from social gift giving. Rather, reciprocity is centered more on trading favors than making a negotiation or a contract with another person. As a social construct, reciprocity relates to the fact that in response to friendly actions, people are frequently generally open and cooperative, and willing to reciprocate. Case in point, token gifts (e.g., free lunches) that pharmaceutical companies make to physicians and dentists in the hope of having them prescribe their pain medication to the patients, rather than their competitors.

Therefore, and not in contrast to mindfulness intervention but complementary to it, might the integration of BEB in clinical interventions for HIV/AIDS and EVD patients be as simple as that to engage clinicians in translational effectiveness simply by engaging in some reciprocity modality – namely, visit clinicians at their practice, bring free lunches for their staff, initiate them to the CER protocol and give them a gift card for shopping at the Mall as a token thank you for their beginning to walk the walk of evidence-based healthcare practice. Might this be all it takes to actualize the changes obtained through mindfulness "restructuring" of the clinicians' and stakeholders' views on treatment intervention?

The concerted development of mindfulness-targeted interventions for the purpose of changing the attitudes of clinicians and stakeholders to CER toward optimizing evidence-based clinical practice [68] and the role of reciprocity as concrete modes of actualizing such changes are the next frontier in translational healthcare for patients with HIV/AIDS, neuro-AIDS, EBOV, putative neuro-EVD, and related conditions we foresee in the near future (case in point, Zika).

31.3.2 Preparing for the Next EVD Outbreak

As of July 2015, there have been more than 11,268 deaths from the 2014 Ebola outbreak. Although we are in better shape than we were in 2014–2015 with regards to containing and controlling a new Ebola outbreak, we are by no means out of the woods yet. One of the most challenging aspects of EBOV infection and EVD remains the risk of transmission to healthcare providers, consequential to inappropriate use of personal protective equipment. Approximately 10–15% of the deaths

from Ebola have been healthcare providers that caught the virus from treating patients. This remains a grave concern, which the healthcare community was able to contain relatively early on in the case of HIV/AIDS. This is one of the areas of EVD containment where research in telehealth (*vide infra*) can help.

Another domain where research must be sustained in order for the healthcare community to be alert and ready for the next Ebola outbreak is vaccine development and testing. A recent study has established the efficacy and the effectiveness of a novel Merck rVSV-ZEBOV recombinant replication-competent vesicular stomatitis virus-based vaccine raised against the surface glycoprotein of Zaire EBOV. In a cluster randomized trial, the vaccine effectiveness was found to be promising (75.1% [CI^{95}: 7.1–94.2; $p = 0.1791$], and 76.3% [CI^{95}: 15.5–95.1; $p = 0.3351$]), as was its efficacy (100% [CI^{95}: 74.7–100.0; $p = 0.0036$]) [69]. We are, it appears, better prepared now for an outbreak of EVD, compared to 2013–2014. However, and in light of the parallels between HIV and EBOV, we have outlined earlier, the question remains as to whether we are indeed adequately prepared to face the physiopathological consequences that will manifest in EBOV seropositive asymptomatic patients, and which we predict to be as serious and complex as what is observed in asymptomatic HIV seropositive patients, in patients with IRIS, and in patients with neuro-AIDS.

31.3.3 Telehealth and Comparative Individual Patient Effectiveness Research (CIPER)

Telehealth is a component of the healthcare system that connects patients and healthcare providers through the use of communication technologies [49, 63]. Since 2005, specialty infectious disease teleconsultations have been provided through Army Knowledge Online. According to the Association of Military Surgeons, from 2005 through 2008, infectious disease teleconsults ranked second in the total number of online consults.

Technologies for that specific purpose vary in their nature and application, including but not limited, to video consultations, online health records, sensors that monitor movement, medical devices, and computer-based information systems to enhance the access.

In brief, telehealth can be one of five types:

(a) Telecare
(b) Electronic health records
(c) Decision support systems
(d) Web-based packages
(e) Assistive information technologies [70]

The healthcare provider can perform teleconsultation and telediagnosis through the use of these electronic applications [71]. Telehealth enables patients who live in rural areas or far away from healthcare services to receive the best available treatment and care in a cost-effective modality. Telehealth serves as a mode for health-

care providers to connect and treat patients in need with the proper communication technology in place.

With improving technology, telehealth has much potential as a healthcare service, particularly in situations such as Ebola outbreaks in Western Africa or other tropical region. By substantially reducing the cost of healthcare delivery and increasing instant access to providers without the need to travel, telehealth technologies improve the quality of healthcare given to the patients in inaccessible communities, and raise patient and healthcare provider's satisfaction [70, 71].

Implementation of telehealth communication technologies for HIV/AIDS and neuro-AIDS patient populations can be optimized through the use of an electronic application across the five domains listed earlier. The same benefits of telehealth can also be obtained with EBOV-infected patients, and perhaps even more so because Ebola outbreaks are prevalent in African countries where poverty and dilapidated healthcare structure limit patient access to clinical services. Telehealth can vastly improve the well-being of EBOV seropositive and EVD patients in remote areas by bringing in a direct source of communication to providers in another area.

Patients during Ebola outbreaks are often times quarantined due to the infectious nature of the disease. Telehealth allows physicians and nurses to stay a safe distance away from those infected to prevent transmission of the virus from other vectors while providing diagnoses and treatment assistance via electronic devices. That is, in part, the reason why teleconsultation, a low-cost and low-bandwidth exchange of information between health specialists and patients when specialists are not available, is among the most common type of telehealth service in developing countries [70, 71].

Telehealth has shown great promise across a variety of health problems, including HIV/AIDS and neuro-AIDS. It, therefore, has great potential in helping contain future Ebola outbreaks as well. But, this will be obtained only if concerted research is sustained in this field, which must include the development of faster and more user-friendly technologies [70, 71]. Improved telehealth technologies will enable seamless interconnectedness among medical professionals and direct access to patients in critical needs.

As we prepare new global challenges of Ebola, the need for telemedicine is clear. When implemented effectively, telehealth will greatly increase the treatment and care for Ebola patients, as seen previously in HIV/AIDS patients experiencing neurological complications.

One aspect of telecare that is emerging with increasing relevance to situations of infectious diseases, such as Ebola, is that it ensures individual patient care, and individual patient clinical data. Consequently, one important development in this area involves new tools and protocols to analyze and interpret individual patient data [49, 63, 72].

The term individual patient data refers to the availability of raw data for each study participant in each included trial, as opposed to aggregate data (summary data for the comparison groups in each study). Reviews using individual patient data require collaboration of the investigators who conducted the original trials, who must provide the necessary data. From a methodological standpoint, the domain of individual patient data gathering, analysis, and inference needs to specify the individual patient data

outcomes under study – namely, individual patient data outcomes research. This requires a cogent characterization of the variables to measure, the analyses to plan and the type of data (i.e., qualitative vs. quantitative; categorical vs. continuous) to gather. Thence will derive the type of analyses – usually longitudinal repeated-measures type analyses [49, 63, 73] – that will be most appropriate and informative.

That is altogether relatively simple for a research methodologist and biostatistician involved in the type of study outlined here. What becomes several orders of magnitude more complex is the performance of a research synthesis design, described earlier, for the purpose of a PICOTS-driven systematic review and meta-analysis with individual patient data. In that case, the comparative effectiveness research paradigm is integrated within the construct of individual patient data analysis and inference, thus generating a novel, and fast emerging subdomain of the field of translational effectiveness, which has been termed Comparative Individual Patient Effectiveness Research (CIPER).

CIPER is designed to compare effectiveness outcomes research obtained from independent patient data analyses and inferences. The protocol follows that outlined above for CER. But, the problem arises at the level of analysis of the quantitative consensus. Indeed, and as discussed in greater depth elsewhere [49, 63], whereas many standard statistical packages exist to perform the necessary analyses of individual patient data from individual studies, meta-analyses of such data sets are unwieldy and time-consuming because commercially available softwares are not currently available that support the direct analysis, pooling, and plotting of independent patient data in meta-analytical. These are large data sets, often as complex as what is today referred to as "big data," the analysis of which in translational science is still at its infancy [74].

Practically speaking, individual patient data can rarely be analyzed directly in RevMan, the Review Manager (RevMan) softwares used for preparing and maintaining meta-analyses in Cochrane Reviews (current version: 5.2.5; http://ims.cochrane.org/revman/download). The data need to be first analyzed outside of this software, and summary statistics for each study may be entered into RevMan. The SAS package, "SCHARP," can perform analysis of each study – not yet determined as fixed or random model meta-analyses – by pooling results and tabulating time-to-event individual patient data. As CIPER continues to evolve, driven by the need of clinical situations such as HIV/AIDS, neuro-AIDS, EBOV, EVD, and putative neuro-EVD, biostatistics adequate and pertinent to the urgent needs of this domain of translational effectiveness will also surely evolve in parallel.

31.3.4 Convergent Translational Science for HIV/AIDS and EVD

Convergence medicine is a novel derivative of convergence science. The discipline involves the trans-disciplinary integration of diverse fields with traditional medical practice to improve translational science in healthcare [49]. The convergent view of the

translational research – translational effectiveness transactions seeks the integration of Western science and traditional medical traditions across the world, namely, Ayurvedic medicine [75], traditional Chinese medicine, and other traditions.

Promoting the role of convergence science in medicine is important for two principal reasons.

(a) A convergent viewpoint may further assist the medical profession in developing research and clinical innovations, educational frameworks, and a workforce that can more optimally tackle future, complex healthcare challenges. These include rising rates of infectious diseases, such as HIV/AIDS and EVD and their *sequelae* (i.e., neuro-AIDS, putative neuro-EVD), chronic diseases, and age-related disorders through population aging and lifestyle factors; rising healthcare expenditures through population aging; chronic clinical situations, such as the increasingly worrisome antimicrobial resistance; health effects of climate change, rapid urbanization and social inequality, and the like. These problems share diverse and polyfactorial etiology, manifestations, and consequences extending across seemingly unconnected sectors that are best considered through the convergence lens of healthcare.
(b) The convergent perspective is important to enhance the concerted engagement of the healthcare professions – medical doctors, doctors of dental medicine or surgery, pharmacists, nurses, and clinical psychologists – with nonclinical innovations, from molecular biology to BEB and CER consensus findings, from genomic profiling to novel biostatistical paradigms, from health information technology to telehealthcare, from new diagnostic tools to sociocultural determinants of health and disease, which apply to all clinical conditions, not the least of which are HIV/AIDS and EVD, and neuro-AIDS, putative neuro-EVD *sequelae*.

Case in point, traditional Chinese medicine includes herbal medicines, in addition to acupuncture/acupressure, manual therapies, spiritual therapies, and Qi exercises. Research has shown that certain herbal extracts from Indian medicine that may subdue the activity of four major drug targets of Ebola virus (VP24, VP30, VP35, and VP40) [76]. This observation is important because VP24 is known to counteract the host interferon system [77] and VP30 functions in the production of viral mRNAs [78]. VP35 is a virulent factor with multiple functions, including antagonizing antiviral signaling pathways and hindering secretion of host interferon, making this protein the dominant virulent protein [79].

Molecular docking analysis of natural ligands against Ebola viral receptors demonstrated that the best docked energy for VP24 was the natural ligand Limonin, Samarcandin, and Gummosin. Limonin, which is the active part of *Citrus bergamia* with known antiviral activity against retroviruses (i.e., HIV, and putatively EBOV and Zika as well) [80], is a vital component of *Syzgium aromaticum*, a medicinal plant commonly used in traditional Chinese medicine for a variety several diseases. Limonin Samarcandin and Gummosin are found in *Ferula assa-foetida*, a spice with high therapeutic values across diverse traditional medicines.

For VP30, minimum theoretical binding energy was found for Curcumin, which is endowed with a wide range of reported therapeutic properties antiviral properties against HIV and putatively other retroviruses [81]. Mahanimbine (from the skin of red grapes, *Vitis vinifera*), which also binds to Ebola VP30, has promising antiviral activity against HIV and related virus families [82].

In brief, there is great potential for traditional and herbal medicine, known to have significant effects upon HIV, to be used with a certain degree of confidence in the treatment of Ebola. As noted earlier, the utilization of traditional medicines, including herbal medicine, will be most effectively used in a convergence mode in conjunction with Western medicine to provide optimum treatment interventions for patients infected with HIV, EBOV, Zika, or related retroviruses, and to control and contain more effectively *sequelae* such as neuro-AIDS and putative neuro-EVD.

31.4 Conclusion: Toward Neuro-EVD and Beyond

Taken together, the lessons learned from HIV/AIDS will be most useful as we face similar issues with related retroviruses. Case in point, the fast emerging Zika virus disease (ZVD), or perhaps more accurately Zika virus syndrome, is a constellation of symptoms caused by the Zika virus.[1] Whereas HIV is well controlled by HAART, despite the unforeseen emergence of IRIS noted above, and whereas the latest Ebola outbreak of 2014–2015 was contained, despite the putative unforeseen consequences in asymptomatic EBOV seropositive patients we discussed in this chapter, including the specter of putative neuro-EVD, the cases of ZVD infections and associated Zika virus syndrome are fast increasing worldwide at pandemic levels. Similar arguments to those present above with respect to the extent to which our concerted experience with HIV/AIDS will aid in better containing future Ebola outbreaks and their consequences to public health can be entertained presently in the case of the ongoing spread of ZVD.

The Zika virus syndrome *per se* is generally a mild disease and most people infected with the virus may actually show no or relatively few symptoms. Only 20% of infection with ZVD are symptomatic, in contrast with HIV infection. Aggressive ZVD infection is, for the most cases, lethal, again in contrast with HIV. Nonetheless, it is now clear that over three quarters of ZVD infection leads to cases that are either ZVD+ asymptomatic or that show minor symptoms, such as feverish perception without actual fever elevation, muscle, eye, and joint pains and aches, nausea, and occasionally vomiting, lethargy, and fatigue, difficulty in cognitive functioning,

[1] The Zika virus was first discovered in 1947 in the Zika Forest in Uganda. By 1952, the first human cases of Zika infection were detected. In the last half century, outbreaks of Zika have been reported in tropical Africa, Southeast Asia, and the Pacific Islands. In May 2015, the Pan American Health Organization (PAHO) issued an alert regarding the first confirmed Zika virus infection in Brazil. On February 1, 2016, the World Health Organization (WHO) declared Zika virus a Public Health Emergency of International Concern (PHEIC).

such as memory, recall, focus, and concentration, and conjunctivitis – all symptoms of the "sickness behavior" in consequence to the effect of proinflammatory cytokines on the hypothalamus [9]. The question must be posed now as to whether important psycho-physiological lessons can be learned from asymptomatic HIV seropositive patients that may benefit asymptomatic ZVD seropositive patients, as we discussed earlier in the case of EBOV seropositivity.

As the medical establishment increasingly uncovers the best evidence base for successful treatment intervention in the aggressive acute cases of EBOV or ZVD infection, beneficial outcomes will mount, death from EVD or Zika virus syndrome will be prevented, pathological signs of active infectious disease will be curbed, and cohorts of survivors will fast increase, and join the cohorts of asymptomatic HIV/AIDS patients with subclinical emergence of HIV infection of the brain and related neuro-AIDS. The lessons learned from the latter will benefit translational healthcare of EBOV and of Zika victims.

Case in point and as mentioned earlier – but the important point deserves to be reiterated – because it is clear that HIV is never cleared from the infected organism, and that it can hide in immune privileged areas, such as the lymph nodes, the semen, the brain, the eyes, and other sites, it is also to be expected that EBOV, Zika, and other related retroviruses may find a similar escape route and hide in similar organs and tissues. HIV infection of the central nervous system ensues and propagates the well-recognized neuropathology we now associate with neuro-AIDS. It is, therefore, possible and even likely that EBOV and ZVD will soon be found to infect the central nervous system and to induce putative neuro-EVD and putative neuro-Zika.

Indeed, it is clear already that ZDV causes important pathology of established and of developing neurons – hence the catastrophic delay in central nervous development in newborns born of ZDV-infected mothers (i.e., congenital Zika syndrome, whose most evident symptom is microcephaly). In adults, ZDV may mimic or lead to serious neurological conditions, such as Guillain-Barré syndrome,[2] a serious progressive generalized weakness and paralysis consequential to immune activation of various etiology, including viral infections (e.g., Zika).[3]

[2] First described in 1916 by French neuropathologists Georges Giullain (1876–1961) and Jean Alexandre Barré (1880–1967), with their physiologist colleague André Strohl (1887–1977).

[3] The onset of Guillian-Barré is relatively rare even among at risk populations, such as ZDV+ patients. Its etiopathology remains to be elucidated, although we, on the basis of the cellular immune and neuroendocrine evidence discussed in this chapter, are poised to entertain the hypothesis that poor regulation of Tregs, as modulated by TH17 and TH9 cytokines and the regulation thereof by the HPA axis, may well play a critical and timely role in the prognosis of Guillian-Barré symptomatology. Giullain-Barré is generally not reversible, as the neural damage is often irreparable. When it is, our corollary hypothesis suggests, it will result from normalized TH17/TH9 modulation of Tregs consequential to normalized HPA-CMI regulation. The health-related quality of life (HRQL) is significantly lowered in patients with Guillain-Barré syndrome. Only about 20% of patients can resume walking walk unaided six months after an attack, and most patients continue to experience some extent of chronic pain, fatigue and difficulty with work, education, hobbies and social activities for months to years, regardless of whether HRQL scores improve significantly in the first year. Our second corollary hypothesis proposes that the anxiety associated with these

In conclusion, it is evident that there is much to be learned and applied from HIV/AIDS to Ebola, and to the current acute emergency of Zika infection. Diagnostic characterization of the constellation of signs and symptoms known as the post-Ebola syndrome and Zika virus syndrome are increasingly common as the medical establishment improves in promptly and successfully treating EBOV and Zika infection. Specifically, in the case of Ebola, it is now clear that half of the patients who lived through an acute Ebola attack later reported muscle and joint aches and problems. Two-thirds had neurological difficulties and memory loss, and 60% reported eye problems within 1 year of an acute Ebola bout. Other important symptoms include psychological problems ranging from depression to anxiety, fatigue, loss of appetite, and related "sickness behaviors." Taken together, the indices outlined in this chapter converge in pointing to a serious neural involvement in Ebola viral disease.

Conflict of interest The authors report no conflicts of interest.

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impairments further exacerbates HPA-CMI alterations. Lastly, and based on our discussion above, we propose that mindfulness intervention will be found to be beneficial to patients with Giullain-Barré syndrome in conjunction with antiviral treatments aimed at curbing ZDV and EBOV.

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Chapter 32
Sequence Accuracy in Primary Databases: A Case Study on HIV-1B

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Keywords Sequencing error • Sequence ambiguity • Sequence uncertainty • Misinterpretation • Sequence variation • Database • Next-generation sequencing • Sequence accuracy of HIV • Sequencing errors in HIV • Sequence accuracy in primary database • Pitfalls of sequencing • Sequence uncertainties in databases • Next-generation sequencing data • HIV-1 sequence variation

Core Message

This chapter introduces the history of sequencing methods, their advancements and also addresses the accuracy of the deposited sequences in primary public databases. The source of errors, frequency, errors due to sequencing and assembly, and their quality are discussed. Misinterpretations of sequence analysis and errors due to differences in the sequencing technologies are also addressed. All these ambiguities makes data analysis a more challenging task and it is very difficult to distinguish true variants from sequencing errors. This alarms data collection and analysis for future studies for understanding pathogenesis, drug resistance, and geographical variations.

32.1 Introduction: History of Sequencing and Advances

Although the protein-sequencing technique (Edman degradation method) was developed in 1950 [1], the technique was slow to become popular and the first protein sequenced was insulin by Fred Sanger in 1955. In contrast, DNA sequencing

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methods were invented only in the mid-1970s, but showed rapid widespread growth. In 1972, Fiers and his coworkers developed RNA sequencing; they sequenced the MS2 bacteriophage genome [2, 3]. The complimentary "plus and minus strand" method was developed by Sanger and Coulson in 1975 [4]. Two methods were also published almost simultaneously, namely, the chain-termination method [5] and the chemical degradation method [6] and both techniques became popular. However, the chain-termination technique subsequently earned greater significance due to the use of easy chain-termination in automated sequencing [7].

The discovery of thermostable DNA polymerases, revolutionized DNA sequencing methods. In addition thermal cycle sequencing [8] gained importance when compared to traditional chain-termination sequencing, primarily because of the choice of starting material – it uses native double-stranded DNA instead of single-stranded DNA. Moreover, much smaller quantities of sample are required and hence this method avoids amplification via in vivo cloning. The use of fluorescent labels and radioactive labels gave rise to in situ hybridization nucleic acid target detection techniques in cells and in tissues. The use of fluorolabels has been critical for the evolution of sequencing methodology [9]. This led to the development of automated base reading. An ever-increasing need for affordable sequencing has further accelerated the development of high-throughput and massively parallel DNA sequencing methodologies that has produced tsunamis of sequences [10]. This greatly reduced the expense of DNA sequencing, beyond the potential of standard dye-terminator methods and resulted in a plethora of sequences to be recorded [11].

In vitro cloning to amplify separate DNA molecules is an integral step in most sequencing approaches because molecular detection techniques are not very sensitive for single molecule sequencing. To avoid this issue, emulsion or bridge PCR can be used. Emulsion PCR, methods developed by Margulies et al. [12] isolate separate DNA molecules with the aid of primer-coated beads in aqueous droplets dispersed in an immiscible phase. Following this, a polymerase chain reaction (PCR) is carried out that coats each bead with copies of amplified DNA that is then immobilized for later sequencing. This method was commercialized as Polony ("polymerase colony") sequencing [13] by 454 Life Sciences (Branford, CT) and SOLiD sequencing, (developed by Agencourt, later Applied Biosystems, now Life Technologies (Waltham, MA). An alternative to in vitro cloning is bridge PCR, in which DNA fragments are amplified using primers attached to a solid surface [14] that form "DNA colonies or clusters." This technique is used in the Illumina sequence Analyzers (San Diego, CA). Single molecule method like Helicos (Helicos BioSciences Corporation, Cambridge, MA) is an exception, as it applies bright fluorophores and laser excitation, to detect bases and it also eliminates the need for molecular amplification.

Massively parallel signature sequencing (MPSS) was developed in the 1990s. This was the first of the so-called "next-generation sequencing" technologies. MPSS uses an adapter ligation method, reading the sequence every four bases, referred to as a "bead-based method." Because the technique employed was complex, it was decided to perform MPSS only "in-house" by Lynx Therapeutics (Heidelberg, Germany) and was not commercialized. However, later, Lynx Therapeutics amalgamated with Solexa that had been adopted by Illumina in 2004 and developed sequencing-by-synthesis (SBS). This technology allows the incorporation of desired

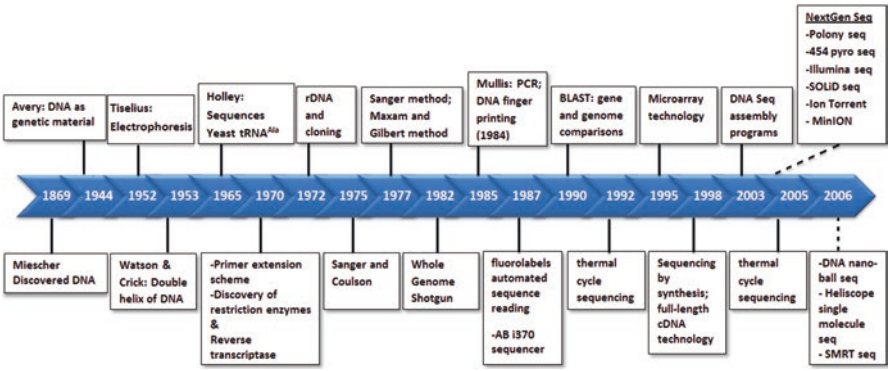

Fig. 32.1 Evolutionary timeline of sequencing methods. Methods after 2006 (in *dashed lines*) indicate the list of sequencing methods that are available at present and undergo rapid change

dNTP, and then it is cleaved so as to allow the next base. Because this minimizes incorporation bias, the end result is synthesis of true base-by-base referred as SBS. The essential features of the MPSS output are used for the "next-generation" data approaches. In 2004, a parallelized version of pyrosequencing was marketed by 454 Life Sciences [15]. It decreased the expense of sequencing by sixfold in comparison with automated Sanger sequencing and it was "second" of the new generation sequencing technologies. This technology allows for intermediate read length and price per base compared to Sanger sequencing on the one hand and Solexa and SOLiD on the other hand. In 2005**,** Polony sequencing method was developed at Harvard Medical School (Boston, MA) and successfully completed a full *E. coli* genome in the same year [13].

During the past several years, many additional techniques have been developed that exhibit greater speed and efficiency in revealing sequences of genes and genomes. The cost of sequencing was reduced through the use of next-generation sequencing technologies, which included a suite of sequencing platforms, such as Illumina sequencing [16], SOLiD sequencing [17], and PacBio sequencing [18], among others. Next-generation sequencing was utilized to sequence whole genomes, resequencing, DNA–protein interactions (ChIP-sequencing), transcriptome profiling (RNA-Seq) and epigenome characterization. Resequencing is important because the genome level variations of a representative species will not represent the true variations of other individuals of the same species.

The timeline of sequencing methods with important milestones is depicted in Fig. 32.1.

Applied Biosystems (now Life Technologies) introduced the SOLiD sequencing technique that employs sequencing-by-ligation. This technique has some limitations for sequences containing palindromic repeats. However, the resulting sequence quantity and length are comparable to Illumina sequencing [11]. Ion Torrent Systems Inc. (now owned by Life Technologies) uses a novel semiconductor-based detection system and a standard sequencing chemistry. This technique detects the release of hydrogen ions during the polymerization of DNA in contrast to optical-based detection systems in other sequencing systems.

Another high-throughput sequencing technology uses DNA nanoball sequencing [19] to sequence the entire genome of an organism. The methodology employed is similar to rolling circle replication, which amplifies small fragments of a whole genome via DNA nanoballs. Following that, unbound sequencing by ligation is used to read the sequence.

Heliscope sequencing [20] is a method of single-molecule sequencing developed by Helicos Biosciences. It employs poly-A tail adapters and follow extension-based sequencing with the aid of fluorescent-labeled nucleotides to read nucleotides one at a time. The use of SBS approach is referred as single molecule real time (SMRT) sequencing. There are many sequencing methods that are currently under development, which includes sequencing through hybridization [21], nanopore [22], tunneling currents [23], mass spectrometry [24], microfluidics [25], microscopy-based techniques [26], RNAP [27], and in vitro virus high-throughput sequencing [28].

32.2 Databases, Errors, and Complications

The recent advances in high-throughput and massively parallel DNA-sequencing technologies have dramatically produced large volumes of gene and genomic data in public databases. These data were generated through many different sequencing technologies and often have not been recorded with an organized, consensus, and uniform methodology. Hence, they have wide ranges of platform-specific error rates and biases that are inherent in each sequencing system used. The integrity of this stored data is critical for research and development including basic research, clinical research, epidemiology, and diagnostics. There are many statistical measures that endeavored to address such error rates. However, none of these measures addresses uncertainties during experimentation and there are no international standards that have been developed toward this purpose.

It is difficult to model the various sources of errors and to represent the diverse types of uncertainties: the scope is very wide. Broadly, there are two major types of quantitative uncertainty, “aleatory” and “epistemic” [29]. Aleatory uncertainty is inherent to most molecular systems, such as quantity variations every time measurements are taken, unable to predict precisely, and are not reproducible [30]. By contrast, epistemic uncertainty arises due to limited sampling, inaccuracy in measurements, or lack of understanding about the underlying processes that influence a quantity [30]. In the context of DNA sequencing, aleatory uncertainty stems up from inherent errors of enzymes such as reverse transcriptases [31] and polymerases [32], whereas epistemic uncertainty emerges from errors including automation; for example, sparse sequence data and poor base detection. In principle, aleatory uncertainty (variability) can sometimes be reduced through repeated experimentation; it cannot be reduced through experimental efforts, whereas epistemic uncertainty can be minimized by additional experimental efforts. Importantly, the underlying differences between these uncertainties have practical implications [29]. It is also pointed out that added to this, validation of software tools is also required, and such analyses should be performed and described alongside each sequencing project.

Depositing sequence information in public databases has become mandatory for publication, basic research, and biomedical research. Most scientific journals will neither accept nor publish work without nucleotide sequence information that is made available to the public by depositing sequences in any one of the three major international sequence databases. The International Nucleotide Sequence Database Collaboration (INSDC) (http://www.insdc.org) at Heidelberg, Germany, shares sequence information among three primary databases: GenBank (GenBank) at National Center for Biotechnology Information (NCBI) at National Institutes of Health (NIH), Bethesda, MD, the European Molecular Biology Laboratory (EMBL) at Heidelberg, Germany, and the DNA DataBank of Japan (DDBJ) at Shizuoka prefecture of Japan. The INSDC administers a wide variety of sequences including raw data, alignments, assemblies, contextual information, and experimental design. In addition, there are databases that derive sequence information from primary databases, including Los Alamos National Laboratories (LANL) (Los Alamos, NM) (http://www.lanl.gov) that houses an international HIV database that works with GenBank, as well as immunology, vaccine, hepatitis C virus (HCV), and hemorrhagic fever viruses (HFV) databases.

In general, databases are curated and considered reliable. In this chapter, we address the important question of how reliable are the sequence entries in the primary public databases? Although experts curate the sequence information, it should be noted that all these databases are compiled manually. Hence, there are more chances of errors and inconsistencies in the sequencing approaches used by the submitting authors around the world that are for the most part unavoidable. Submitting authors have control over deposited entries and would have to modify erroneous entries that otherwise remain in the databases for many years. Moreover, entries including erroneous entries are constantly exchanged along with the annotated sequences among the various related databases as well as among researchers. It is difficult to constantly and immediately detect and eliminate incorrect sequences from the databases and to correct them.

As pointed out by Jones et al, in 2007, the fundamental limitation of sequence information is that only a small set of data in the GO database, for example, have been characterized by direct confirmatory experiments, whereas the majority are annotated based on sequence similarity with few characterized sequences [33]. Caution should be exercised before analyzing sequence annotations alone or misinterpretation may result. If an entry is not supported by published peer-reviewed research, it lacks information regarding the methodology used for sequencing, and quality of the sample, etc. For example, a protein from *Deinococcus radiodurans* DR2227 was mistakenly annotated as "phosphonopyruvate decarboxylase" [34]. Based on sequence similarity, a homologous protein (OrfZZ) from *Streptomyces hygroscopicus* was provisionally annotated as "probable phosphonopyruvate decarboxylases" [35] in 1994. However, there was no experimental evidence to support the annotation. Five years later, it was confirmed as a "thiamine pyrophosphate-dependent enzyme" [36] and replaced the original sequence entry with the corrected version GenBank (accession no. D37809.2), the sequence was renamed OrfZZ (BAA93685). Nevertheless, as a result in the 5 years' of time the original entry was used in several databases and many genomes such as *A. fulgidus*, *A. aeolicus*, *M.*

jannaschii, *T. maritima*, and other prokaryotes were annotated as having "phosphonopyruvate decarboxylase." Several new entries in Genbank were also mistakenly annotated as "phosphonopyruvate decarboxylase" based on sequence similarity. A detailed analysis of OrfZZ showed that they belong to alkaline phosphatase superfamily and could function as phosphomutases, for example, phosphoglycerate mutases [37]. This prediction was confirmed experimentally [38, 39].

Nonetheless, quality control of the overwhelming sequences deposited in the primary databases remains cumbersome to deal with. For instance, Fabret et al. [40] stated that there are 3% errors in the deposited sequences of GenBank as well as in EMBL. Kristensen et al. [41] also established a quantitative measure of errors. Felsenfeld et al. described the accepted standards for the quality of a given sequence in three parameters: accuracy, contiguity, and fidelity. A completed sequence should be 99.99% accurate and should not have more than one error per 10,000 bases [42]. Contiguity is the length vs. ambiguities in the assembled sequence. Fidelity is the precision of the assembly within the sequence although a decisive standard has not been set as yet. The final sequence should not have any gaps or ambiguities. In regions where gaps are inevitable, the size of the gaps, orientation of flanking region, and the efforts made to close the gaps are to be mentioned in the sequence entry record or in corresponding publication. Wesche et al. [43] suggested that sequences with errors deposited in the public databases affect the research outcomes for at least three reasons: (1) failure to detect sequence features due to automated annotation as well as sequencing errors, (2) spurious annotation of single nucleotide polymorphisms, and (3) inappropriate evolutionary inferences especially while estimating the rate of nucleotide substitution or recombination [44]. These lead to complications in intraspecific or interspecific comparisons between closely related taxa.

32.2.1 Sources of Errors

Uncertainties arise from sequencing due to (1) sample variability, (2) experimental procedures, and (3) classification of specimens that derive from clinics. Sample variability arises mainly from inherent errors including sample extraction from tissues of different genotypes, sampling large populations to study allele frequencies, and from different expression levels of RNA. Experimental procedures may produce errors due to contamination, sequence amplification, library preparation, sequencing methodologies, chimeric reads, alignment and assembly algorithms, and apparatus failures. There is often lack of uniformity and consensus in the classification of specimens that derive from clinics. Not all clinicians always agree on the various clinical and pathological classifications and even when they do agree, clinical and pathological findings may evolve over time due to changes in diseases or due to changes in the tests and procedures used for diagnostic criteria. The source of sequencing errors ranges from errors in sample handling to automation in sequencing and assembly. The overview of such errors is presented in Fig. 32.2.

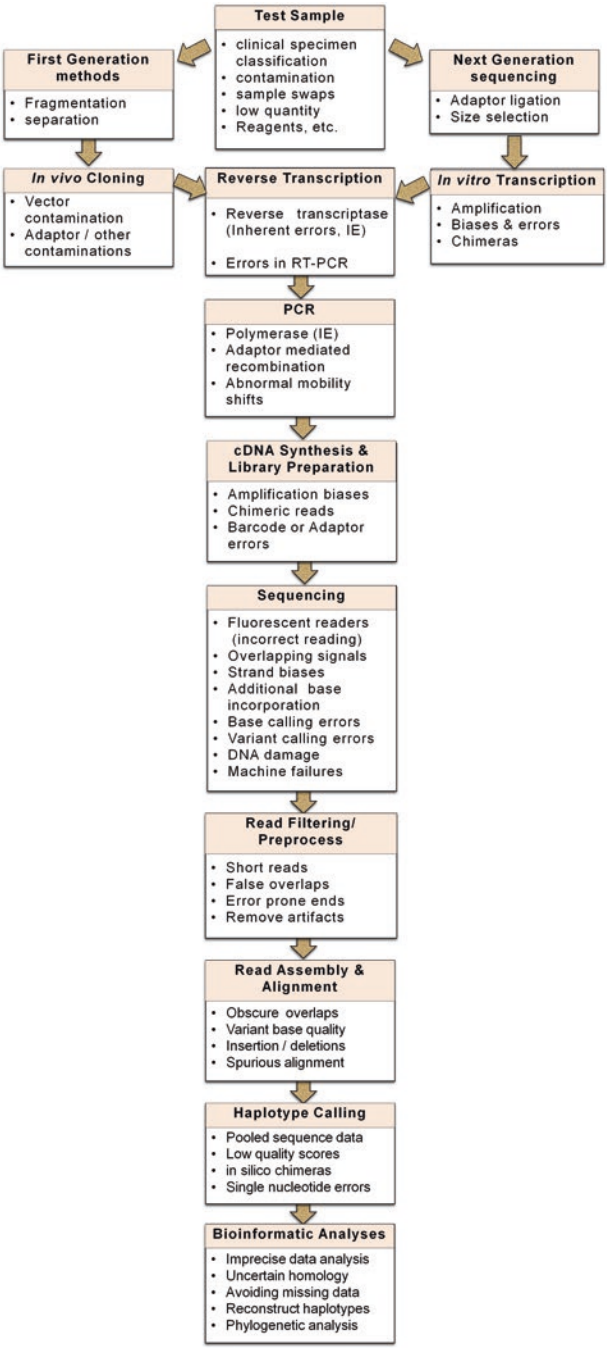

Fig. 32.2 Sources of sequencing errors

32.2.1.1 Sample Preparation

Errors originating during sample preparation are due to manual or automated errors including labeling and sample mix-up, sample contamination by exogenous DNA from other specimen or extraction kits [45, 46], reagents [47, 48], vials, tubes, and other containers [49], sample handling [50] including sample and specimen swaps, DNA/RNA degradation due to storage conditions or enzyme contamination, and sample cross-contamination [51]. In the case of viruses, insufficient sample quantity (having low titers) and the sample yield may not be sufficient as required by the particular PCR or other amplification methods required prior to sequencing [52]. However, during PCR and other amplification methods, errors may also be introduced; for instance, incorrect bases may be incorporated in PCR or other amplification cycles.

32.2.1.2 In Vivo Cloning

It was found that 0.23% of 20,000 sequences of the GenBank Release 63 had accidental incorporation of vectors from cloning. This may be due to editing errors or vector contamination during cloning. Lamperti et al. [53] attributed the presence of vector sequences due to (1) extension of the read into the vector, (2) rearrangement of restriction site and its ambiguous boundary between vector and cloned sequence, and (3) incorrect incorporation of an intermediate vector into the sample sequence during subcloning. In these cases, a step for careful and specific vector trimming is necessary to eliminate any vector sequences.

32.2.1.3 In Vitro Transcription and Reverse Transcription

The study of HIV since its discovery is an important case in point for preserving database integrity, accuracy, and utility. For example, transcripts of the RNA virus, HIV-1, in infected cells undergoes RNA editing [54]. There are multiple purine-to-purine and pyrimidine-to-pyrimidine substitutions that occur throughout the genome including in spliced HIV-1 mRNAs [55]. The HIV proviruses contain distinct mutations owing to error-prone reverse transcriptase that can lead to mutational inactivation of motifs that regulate subsequent life-cycle events including either splicing or RNA packaging. HIV-1 reverse transcriptase does not correct these errors by proofreading; fidelity assays indicated that it is the least accurate enzyme described to date and has an average error rate of 1 in 1700 nucleotides. This is one-tenth as accurate (1/17,000) as the isolated polymerase of avian myeloblastosis virus (AMV) and 1/30,000 in case of polymerases from murine leukemia viruses (MLV). Moreover, the exceptional diversity of HIV-1 is attributed to its error-prone reverse transcriptase [56].

32.2.1.4 PCR

In classical sequencing methodology that usually includes a PCR step, exogenous DNA may also get amplified along with sample DNA and contaminate the sequencing results. A blank sample prepared without the genome to be sequenced may provide characterization of the contaminants; however, the lack of input DNA will result in fewer reads. Hence, the blank control may be prepared with carrier DNA with a known sequence. PCR is also involved in an NGS library preparation step, for which DNA is first subject to random shearing both in Roche-454 and in Illumina sequencing. Then, appropriate fragments are sorted based on their size; chimera formation may occur at this step. Ligation of DNA is also done using oligonucleotide adapters that provide immobilization and primer binding sites of the fragments for clonal amplification [57, 58].

During PCR, amplification errors may be introduced either prior to sequencing or during library preparation; both can induce biases and errors. For example, differences in primer binding affinities can result in amplification bias [52]. Measurement of amplification bias of individual sequence reads indicated that the observed frequencies differed by 2–15 fold compared to confirmed frequencies. In some cases, the bias was up to 100-fold [59]. Chimeras can lead to nonexistent false templates. Some reads may also contain adaptor sequences that in turn induce adaptor-mediated recombination, which appears in the aligned sequence data as indels [60].

In a deep sequencing analysis, Zagordi et al. studied a mixture of HIV clones and found that the chimera rate in PCR was 1.9% [61], whereas in studies not employing deep sequencing, the chimera rates were found to vary from 1% to 5%, depending on elongation times [62]. Moreover, polymerases in PCR have their own inherent error rates, inducing errors that may mimic confirmed variants [52]. It was inferred that the error rate of polymerase per base was 2.5×10^{-3} [61]. However, other studies showed that the error rates ranges from 10^{-3} to 10^{-6} [32]. Taq-like polymerases introduce sequence errors due to strong bias toward A to G and T to C substitutions [63]. While sequencing the whole genomes of RNA viruses, the fragment overlaps are also amplified before ligating with adapter sequence [64, 65]. Usually for RNA viruses RT-PCR is employed, although it can be performed separately before regular PCR amplification [66]. Additionally, during sequence library preparation there are chances of obtaining additional errors due to amplification biases, adapter errors, chimeric reads, and machine/technology failures [29].

32.2.1.5 Sequencing

McElroy et al. [52] reviewed error rates in NGS platforms including Roche-454 and Illumina. The error rates in Roche-454 were originally estimated for experimental samples at 4% and for test fragments at 0.6%. This may be due to some "clonally" amplified fragments in the sequencing libraries that are not actually clonal. They may originate from two or more fragments and thereby result from amplification errors [12]. Subsequently, using the Roche-454 Huse et al. (2007) estimated the

error rates for the GS20 sequencer: for experimental data it is 0.49%, and for test fragments it is at a lower rate, 0.1% [67]. It should be noted that the Roche-454 test fragments were neither subjected to library preparation nor PCR amplification steps before sequencing. This could be the reason for lower error rates. Error analysis was performed using the Roche-454 test approach on the GS-FLX Titanium platform and showed an average error rate of 1.07%. In some cases, the local maximum error rates are greater than 50% [68]. This may be due to the presence of homopolymers, GGC sequences, read length, inverted repeats, and spatial location within the picotiter plate [60, 68, 69]. It was observed in Roche-454 that the error rate has been shown to increase with read lengths [60]. This depends on several biological factors like organism and genomic region to be analyzed as well as technical factors like intensity of light emission, the position of picotiter plate with respect to the chemical flow and camera position [68]. It is also recognized that insertion/deletion errors (indels) are associated with homopolymers and cause errors in Roche-454 sequencing. It is likewise evident that Ion Torrent sequencing also sustains with such errors [70]. Illumina sequencing also have downstream error rates that are heterogeneous due to GGC sequences, inverted repeats, and homopolymers. The run average error rates vary between 0.31% and 1.66% [71], even when performed on the same machine [60]. An increased read length results in increased error rates. It was observed that in some cases, the error rate is 10-fold higher at the 3′ end than at the 5′ end [71].

32.2.1.6 Base-Call Errors

Errors may also arise from the incorrect reading of bases by automatic film or fluorescence readers during the assembling of the sequence data obtained through shotgun sequencing [40]. This may be alleviated by manual film readings by experts at a higher cost of time and effort, instead of which more stringent parameters could be applied to automated algorithms used in construction of the sequences. Fabret et al. [40] also suggested that since errors are based on the sequence being analyzed, error-prone regions may be identified using local sequence properties. During base imaging and sequencing there are likelihoods of superfluous base insertions due to each successive single sequencing cycle, overlapping signals, strand biases, DNA damage, and sequence complexity [29, 71].

32.2.1.7 Read Filtering/Preprocessing

In order to reduce sequencing errors, filtering is usually done before or during alignment to retain only high-quality reads. Just by eliminating the reads of nonspecific nucleotides of GS20 data, the average error rate was decreased from 4.7% to 0.24%. Filtering steps vary with sequence data and with sequencing platform, for example, there were no errors in 82% of Roche GS20 reads, whereas in the Roche-454 GS-FLX platform, 89.1% of the reads contained errors [68]. Throughout filtering of

reads, a decrease in error was observed instead of increasing coverage and depth that increases the problem. Alternatively, the error-prone ends can be trimmed but of course may decrease read lengths. However, the available software and filtering parameters vary and are not always clearly described in the literature. This makes the comparison of results very difficult [72].

32.2.1.8 Read Assembly and Alignment

Sequence alignment itself introduces bias and error. When the study aims to compare samples, then alignment to a known reference is appropriate in examining HIV-1 evolution within host. However the use of inappropriate (distantly related) sequences disrupts the alignment. This can be overcome by generating a consensus sequence that is representative of all the clonal populations followed by performing an iterative alignment with the reference sequence [73] with some replacements of single nucleotide variations at every iteration. An alternative is to perform Sanger sequencing of the same sample, as in [64]. However, this is only possible for small genomes. If the clonal populations diverge significantly from the consensus sequence, then the clonal reads lead to misalignment resulting in bias and error. The presence of coverage gaps for rare variants introduces additional challenges in the assembly process, making rare genomes unreachable by assembly tools [74].

32.2.1.9 Haplotype Reconstruction Errors

Haplotype reconstruction involves assembling longer genomic segments from short, overlapping reads. However, chimeras may form while reconstructing haplotypes of long read lengths. This problem is also aggravated by single nucleotide errors, creating misleading paths and generates more chimeras as well as nonchimeric haplotypes. These technical artifacts can easily be mistaken for real variants, and hence increase the diversity estimates, and thus complicate the problem if the study is focused on recombination and evolution [75]. For an overview of the capacities and limitations of available haplotype reconstruction programs, refer [76].

32.2.1.10 Bioinformatics Analysis

The large amount of sequencing data obtained by NGS place substantial pressures on information technology and computational data analysis in terms of storage, quality control, mapping, error correction, single nucleotide variant (SNV) calling, haplotype reconstruction, diversity estimation, and data integration [61, 77–81]. Errors in choice of analytical methods used by bioinformaticians can result in poor sequence alignments resulting in difficulties to map and annotate [29, 77]. Bioinformaticians utilize various algorithms and statistical approaches to quantify uncertainties as well as to correct errors. There are multiple tools and techniques

available to compare laboratory sequences with reference sequences so as to infer similarities and variations among them [82, 83]. There are data preprocessing steps that use data-quality thresholds and avoid low-quality sequences. Data analysis usually starts by filtering reads based on quality scores. The rationale for this initial filtering step is that low-quality reads contribute disproportionally to the overall error rate, that is, most errors occur on a few reads [67]. It was shown that the optimized filtering has reduced the error rate in detecting genomic variation up to 300-fold [84]. Most sequencing technologies quantify detection reliability using probability scores that recognize bases more likely to be correct [85, 86]. However the currently available methods have drawbacks due to uncertainty and errors in DNA-sequencing data. There are no standards and software available to quantify epistemic uncertainties. Even when quantified, analytic methods are not generally incorporated into subsequent analyses and calculations. Correcting for local variants in error rates within lanes, possibly produced by bubbles in flow cells, also warrants further investigation [89].

32.2.2 Sequencing Technologies: Features and Errors

It was reported that all NGS platforms introduce sequencing errors and it is difficult to achieve uniform coverage and, in practice, the read coverage often varies by orders of magnitude. All NGS platforms report quality scores, defined as $Q = -10 \log_{10} p$, where p is the error probability [77], together with the called bases, but the calibration of these scores is challenging [88, 89] and there is no consensus on how to compare scores across platforms [81]. For instance, correlation of coverage and errors among the three different NGS platforms, 454/Roche, Illumina, and ABISOLiD is fairly weak [90]. These technologies typically produce billions of base calls per experiment, translating to millions of errors [91]. A comparative analysis on Illumina MiSeq platform and Ion Torrent PGM, reported that the Illumina MiSeq platform is more suited for detecting variant sequences than the Ion Torrent PGM, whereas the Ion Torrent platform has a shorter turnaround time. The output data of both sequencing platforms were processed in silico and used to count the number of reads with C/T and A/T. Illumina MiSeq slightly overestimated and Ion Torrent PGM slightly underestimated the expected percentage of tracer mutations [72].

An overview of NGS platforms and type of errors that are inherent to sequencing technologies are listed in Table 32.1.

The major sources of errors for Illumina are substitution-type miscalls [93]. Sleep et al. [87], modeled error probabilities of G➔T substitution in Illumina Genome Analyzer (GA), and found it is very high. The transition C➔A is also high. These errors are due to the combined effects of cross-talk and T fluorophore accumulation. Whereas the Illumina HiSeq data showed a different profile in that error rates along the read are substantially lower overall. The substitution G➔T is higher at the beginning of the reads but becomes lower moving along the read, which is in contrast to the reverse error, T➔G, which increases towards the end. They have also

Table 32.1 Sequencing technologies and the inherent error types

Platform	Manufacturer	Library construction/ sequencing	Throughput (per machine run)	Error type reported	Error rate (%)	Depth (virus)	Reference
Sanger	Sanger 3730xl	PCR/dideoxy chain-termination	~96 K reads @ 650 bp	Substitution	0.1	–	[5]
454 GS Junior	Roche	Emulsion PCR/ pyrosequencing	~135 K reads @ ~ 520 nt	Indels	0.38	7 K	[23]
GS-FLX Titanium	Roche	Emulsion PCR/ pyrosequencing	~1 M reads @ ~ 500 nt	Indels/substitution	1.07	50 K	[60]
MiSeq	Illumina	Bridge/synthesis	~11 M reads @ ~ 500 nt	Indels/substitution	0.1	165 K	[23]
GAIIx	Illumina	Bridge/synthesis	~640 M @ 100 nt	Indels/substitution	5.85	6 M	[60]
HiSeq 2000	Illumina	Bridge/synthesis	~6G reads @100 nt	Indels/substitution	8.2	60 M	[69]
Ion Torrent PGM	Life technologies	Emulsion PCR/ligation	~2 M reads @ ~ 121 nt	Indels	1.5	24 K	[23]
SOLiD	Life technologies	Emulsion PCR/synthesis	~120 M reads @ ~ 50 nt	AT bias	0.01	1 K	[24, 25]
RS	Pacific biosystems	SMRT	~200 K reads @ ~ 2000 nt	Indels/substitution	14	40 K	[26, 92]
tSMS	Helicos		~1G reads @35 nt	Indels/substitution	3	3 M	[20]

proposed a model of sequencing errors that is intrinsic to the Illumina sequencing technologies and that does not rely on the availability of a reference genome for error detection. However, like most other methods the reported method also identifies at least 96.64% of errors in the example PhiX data set. Moreover, the reported model may not account for some errors occur during reverse transcription or library amplification. These errors are difficult to identify without a reference genome.

Schirmer et al. [94] showed that PhiX is not suitable for reference genome. Because the reference sequence should match with similar experimental design, the same library preparation method, forward and reverse primers and sequenced on the same run. The adapters used represent a specific library preparation method. This is the best strategy to identify errors, motifs, read alignment, and error removal potential for data sets with similar experimental design. However, for commercial reasons PhiX is used as a reference genome to increase the data quality of low diversity samples and to optimize the cluster map generation.

Sequencing from single cells is gaining importance for biomedical applications. Recently, a comprehensive comparison of single cell variation detection performance based on different whole genome amplification methods: multiple displacement amplification (MDA), degenerate-oligonucleotide-primed PCR (DOPPCR), and multiple annealing and looping-based amplification cycles (MALBAC) were compared and concluded that MDA and MALBAC can be used for high genome coverage and for the detection of SNVs in disease research [95].

32.2.3 Overcoming Errors

Among sources of errors and uncertainties described (in Sect. 2), it is impossible to avoid uncertainties and errors as they are "aleatory" in nature and integral to sequencing. For instance it is reported that there is a substitution error rate of around 6% in the GC-rich motif stretches [60]. Such motifs promote single stranded DNA folding and affect DNA polymerase action by slippage or stalling [96]. As discussed PCR not only introduces base substitution errors, but also produces cross-overs among templates [97]. It was found that the base substitution error rate for the non-PCR sample was 0.05%, whereas for the PCR-amplified sample it was 0.25%. The PCR-associated increase of the error rate was highly significant ($P < 10^{-6}$, Wilcoxon rank-sum test) [61]. PCR biases also have a significant impact for instance HIV-1 deep sequencing analysis of the protease gene bias was encountered due to the use of the primer tag system [59]. Sequencing and resequencing may precisely characterize errors but may not decrease allelic variability [29]. To minimize the error rate, each step requires careful handling, starting from biological sample retrieval to storage and analysis. Some simple measures to control cross-sample contamination during laboratory work include exposing laboratories to UV light in-between the experiments to reduce contamination errors. There are some improved ways to overcome intrinsic and important problems. Ten possibilities follow.

1. It is important to infer individual error profiles for different sequencers, library preparation methods, and sequencing types to handle miscalls [94]. Especially for viral diversity estimation, where uniform coverage and error correction are critical, complementary sequencing strategies involving more than one platform may be more efficient than increasing the coverage on a single platform [81].
2. Contaminating vector sequences can be identified using the VecScreen tool (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) that makes comparisons with known restriction maps. LUCY [82] is used to identify and trim vector sequences in sequenced genomes. It uses base quality scores obtained from programs like PHRED [98] and TraceTuner [99] and identifies stretches of sequences with continuous high-quality base scores.
3. Dependence on PCR techniques is no longer required when using emerging PCR-free single molecular sequencing methods such as Pacific Bioscience's RS [92] platform and Oxford Nanopore Technologies' GridION [100]. However, currently even these new technologies are error prone and with innovative methods such as circular sequencing, future error rates should decrease. In addition, a PCR-free Illumina based hepatitis C virus deep sequencing method succeeded, covering 99% of the HCV genome with an average read depth over 50× [101]. The same methodology was recently adapted for direct sequencing of RNA from HIV and other RNA viruses [102].
4. Increasing sequencing depth has a positive effect on the discovery of rare variants and improves the overall assembly accuracy. The use of primers situated near the genomic termini during the cDNA synthesis stages have been shown to improve the number of termini sequences, but have not completely resolved the problem [103]
5. For sequence alignment a modified Smith–Waterman algorithm [104] is employed so as to match with the reference sequences. There are several statistical approaches including Bayesian inference [83, 105], frequentist inference [106], and others [107] including the latest approaches to handle aberrant alignments using local sequence assembly to detect variations in reference sequences [108–110]. Moreover, high fidelity techniques use hybrid-sequencing methods that correct error-prone long and short reads [111, 112]. These techniques correct alignment artifacts and enable sequencing of larger genomes whose reference sequence is not known. It was also suggested to use consensus rather than a reference sequence that was shown to improve the alignment quality, especially if their divergence is high [93].
6. The selection of appropriate algorithms is essential to overcome errors. For example, the Bowtie algorithm [113] does not absolve any gaps or reads with homopolymer errors and leads to mismatches and hence the alignment fails. Another program, SOAP2 does not permit more than two mismatches in the aligned reads [114]. This may not solve the issue, especially for longer reads or in case of HIV-1, where errors are predominant. Even though software programs generate a "best alignment," they may not be "true alignments." Hence, both assembly and alignment are error prone and the best way to deal with is to find the appropriate approach that is suitable for the samples, the purpose of the study, and the experimental strategies [115, 116].

7. Various programs are available for assessing read quality and filtering reads, software such as BIGpre [117] and AmpliconNoise [118] remove PCR duplicates. Other programs such as ConDeTri [119] and SolexaQA [120] trim the error-prone ends of reads. This can also remove low-quality and adapter sequences. There are programs available that offer solutions for single nucleotide variation with calling and automated filtering. They employ cut-off based filtering to differentiate improved true variant identification from errors. An alternative, quality score independent approach is implemented in the program Short Read Assembly into Haplotypes (ShoRAH) [61]. A similar approach is used in V-phaser [121].
8. Avoiding chimeras due to PCR in haplotype reconstruction is a key challenge. Algorithms for removing chimeras are in development, for instance, the Perseus algorithm within the program AmpliconNoise [122]. Finally, it is been suggested that independent of the method used, manual inspection of results is a recommended standard practice [52]. A convenient tablet is available for this purpose with features like zooming of the entire contig in real time and can import various data formats and handle both 454 and Solexa data [123].
9. The best strategy for removing errors from the sequencing data is to include a mock community in a sequencing run. For instance, Mangul et al. [74] introduced a high-fidelity sequencing protocol and an accurate viral population assembly method (VGA) to simulate the data in presence of recombinations and point mutations and suggest that their method can accurately assemble viral population. Experiments on both real and synthetic HIV data sets generated with various sequencing parameters and distribution assumptions suggest that VGA is suitable for clinical applications. This can supplement ultradeep sequencing technologies to discover ultrarare viral variants in the population.
10. A more generalized software solution is recommended that provides robust results by incorporating accurate representations of uncertainties in sequencing and analyses. It is also suggested to actually include imprecise or poor quality data for the analysis because heuristic filtering approaches usually discard them [29]. Well-known statistical approaches may not be applicable always for errors that are nonrandom and are systematic in nature. Systematic errors are also due to instrumental bias in calibrations and measurements [124]. Additional methods are in demand to infer such complicated problems. Robust Bayesian [125, 126] inference is one such method that allows imprecise sample data or epistemic uncertainties to be modeled [29]. The selection of appropriate experimental design and experimental controls, biases, and errors can be minimized [52].

There is a demand for elucidating uncertainty in sequence data. Special attention is required for the quality of sequence data, as it is obvious that even a single nucleotide change may lead to serious human disease [127, 128]. This further alarms data collection efforts and inferences derived from error-prone DNA-sequencing technologies. With the issues of sequencing technologies, cost-effectiveness becomes a question that impacts on the science of error rates [29]. For example, even if corporations have in house validation of large scale sequencing data, the commercial

enterprise may prevent publically disclosing or publishing their error rates and reporting specific data processing steps and procedures used in their analyses. This may be based on protection of intellectual property, trademarks, and patents. Moreover, there is the as yet unresolved issue of patenting human DNA sequences for commercial purposes that then obviates the possibility of public analyses of such sequences that may relate to other relevant and related families of sequences. Consequently, these further impacts on public clinical and health as well as the scientific peer review process. Furthermore, many software programs used for aligning sequences and detecting variants are closed-source and their algorithms for instance Novoalign [129] or GATK HaplotypeCaller [130] describe only the general steps without explicit description or published information. Most software tools that are partially validated against in house data may not perform equally on real data [109]. Therefore, time and effort consuming empirical work are required for characterizing sequence error rates that originate from such sources, if possible. Moreover, the commercial enterprise though at times providing consulting services, generally require fees for analytical services that are not affordable to most researchers.

32.3 HIV-1: Polymorphism

Errors in deposited sequences of databases may lead to incorrect inferences about the conserved and variable sequence patterns, coding regions, polymorphisms, primer design, and may also result in inaccurate determination of evolutionary relationships with increasing branch lengths [52, 131]. These are more crucial in case of viral sequences due to high mutation and replication rates, especially in HIV [132]. As is well known, there are two major groups of HIV namely, HIV-1 and HIV-2. HIV-1 is predominant and spread worldwide. HIV-2, although originally confined to West Africa, has spread to other countries including the USA, India, and Europe [133].

As an example, HIV-1 is subdivided into four groups M, N, O, and P. Group M is responsible for most infections in the pandemics that are further subdivided into subtypes, designated by upper-case alphabets from A to K (excluding E and I). Sub-subtypes are usually designated by alpha numerals, for example, A1, A2, F1, and F2, whereas the subtypes B and D are closely related but not designated as sub-subtypes. This large diversity is further amplified by recombination among these different types and subtypes. This complexity in viral subtype spectrum of classification further exacerbates the issues discussed in the current chapter, problems in sequence error production and detection [134].

When a host cell is infected by two or more virions, termed “superinfection,” there is a potential possibility to transcribe and pack recombinants of two viral genomes into the same virion. There are various degrees of genetic variation within an infected individual as well as between infected individuals [135]. The rate of superinfection of host cells is higher with two to three cross-overs per genome per replication cycle and the estimated recombination rate is approximately the same

order of magnitude as the mutation rate of reverse transcriptase [136]. This generates recombinant forms, which carry mosaic genomes. During the last three decades it was evident that many RNA viruses recombine with different types and subtypes as well as acquiring genes from their hosts [137]. Many studies describe the mechanisms of recombination through a copy-choice model [138]. Recombinant strains often acquire increased pathogenicity. These recombinant forms are referred as "circulating recombinant forms" (CRF). However, there are recombinant forms with unknown ancestry that are indicated by a prefix "U" [31]. Some CRFs predominate in pandemics.

The understanding of CRFs at the molecular level is central to control the pandemic spread as well as to weaken viral replication. It is estimated that as many as 10^{10} viruses can be produced in an infected individual daily. Possibly, 10^{7}–10^{8} of these are capable of infecting new host cells. The viral reverse transcriptase has a very high nucleotide misincorporation rate on the order of 10^{-5} to 10^{-4} per site per replication [139]. The high mutation rate that result include frameshifts, insertions or deletions, stop codons, and other mutations that compromise the production of free and mature virions. The rate of substitution varies across the genome, with the substitution rate being lowest in pol, intermediate in vif, vpr, tat, vpu, and gag, and highest in rev, nef, and env [135]. Mutation is the key for HIV's adaptation to the host immune response as well as to escape antiretroviral drugs. The deleterious changes accumulate in HIV genomes and spread beneficial combinations of these mutations [139].

Next-generation DNA sequencing (NGS) opened up possibilities to study evolution of pathogens especially the use of "ultradeep sequencing" to detect evolving viral populations, drug resistance, evolutionary dynamics, immune escape mutations, and genomic variations over time [52]. However, currently the NGS technologies have significant error rates and it is very difficult to distinguish true variants among the experimental errors. It is reported that the diversity among viral genomes in an HIV population can vary from 3% to 20% depending on regions [140, 141]. The sequence variations of HIV-1 are also due to RNA editing, hypermutation, error-prone reverse transcriptase, and error-prone polymerase, all of these contribute to aleatory uncertainty (as described in Sect. 2 of this chapter).

Gall et al. [142] determined the accuracy and frequency of in vitro recombination. They prepared a mix of RNA from two plasma samples (1:1) containing HIV-1 subtype B (viral load, 360,000 copies/ml) and subtype C (viral load, 120,000 copies/ml), respectively. One amplicon from the mixed population was amplified and then reamplified in 96-well plates, less than 20% of the wells yielded a product. The reamplified products (64) were sequenced separately and included the control subtypes of B and C. It was found that 46/64 (71.88%) products were of subtype B, and 17/64 (26.56%) products were of subtype C, and a single product with an intersubtype B-C recombinant sequence corresponding to an in vitro recombination rate of 1.56%. This can be mistaken for viral recombination or lead to an overestimation of the viral diversity. Such in vitro recombination might depend on various factors such as the amount of template DNA, specific PCR conditions, and during PCR amplification [62].

It is an elusive problem for computers to identify variants within the context of technological and sequencing errors and it has become a key challenge to bioinformaticians. In addition to these challenges, there are problems in converting "quantity into quality." Even though a qualified researcher annotates a carefully sequenced genome, the reliability may be retained for one or ten protein sequences, and thereupon fail. Be it as it may, this remains the case even if sequences are scaled up to 10,000-fold with automated annotation.

32.3.1 Sequence Uncertainties in Public Databases: A Case Study on HIV-1B Sequences

In this study, the data set analyzed is filtered from a compilation of HIV-1B protein sequences of blood isolates from different geographical locations, (documented along with their accession ID in volume 1 of the Global Virology series published in 2015, and refer to Table 24.5 of Chap. 24 [143]). There are 845 sequences of Env, 482 sequences of Gag, 545 sequences of Nef, 341 sequences of Pol, 305 sequences of Rev, 310 sequences of Tat, 497 sequences of Vif, 404 sequences of Vpu, and 471 sequences of Vpr makes a total of 4200 sequences. These sequences were scanned for ambiguities in their amino acids. A PERL script was written to detect the character "X" that refers to any of the 20 amino acids. In addition, other ambiguous characters such as B, O, J, U, Z, were also identified, however, not represented in the graphs (Figs. 32.3b, 32.4, 32.5, 32.6, 32.7, 32.8, 32.9, 32.10, and 32.11b) because the uncertainties were relatively lesser and were not complicated. As these proteins are simple translations from the entire HIV-1 genome, the uncertainties in their protein coding sequences (*vis a vis* corresponding nucleotide triplets) were assessed using IUPAC letter codes (Table 32.2) along with low quality bases or errors (designated as any nucleotide, "N"). The ambiguous sequences of proteins as well as of nucleotides with their accession numbers are listed in Table 32.3. Although few sequences in the table seems less ambiguous as in case of purine-to-purine substitution (designated as "R") that may be due to the presence of quasispecies, their corresponding proteins are ambiguous as it may code for any amino acid (designated as "X"). This may hinder protein sequence annotation.

In general, the sequence positions containing such residues are treated as "missing data" by automated sequence alignment programs. However, the current study is focused only on the uncertainties and less informative sites present in nucleotide sequences of HIV-1B (blood isolates). The sequences contain many N's (any nucleotide) across the sequences that may reflect true variations or rates of substitution (synonymous or nonsynonymous), frameshift mutations, low quality bases or frameshifts due to sequencing errors and uncertainties. It is awkward to discriminate one from other due to differences in the sequencing strategies used. The sequencing errors are mainly due to incorrect reading of bases (mis-call). To portray these errors and uncertainties, column-wise comparisons of the collected sequences were performed to illustrate such sites. The consensus sequence was portrayed in the graph

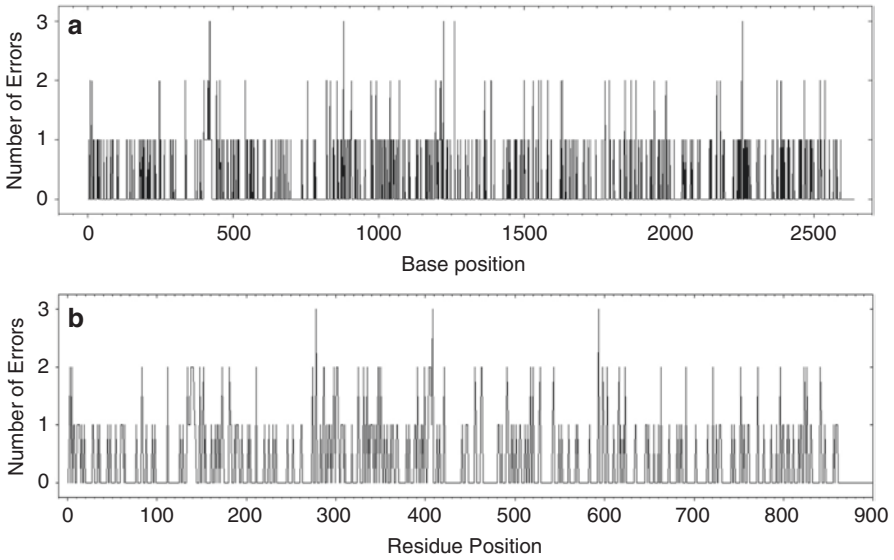

Fig. 32.3 The frequency of error distribution is plotted for Env (**a**) nucleotide and (**b**) protein sequence

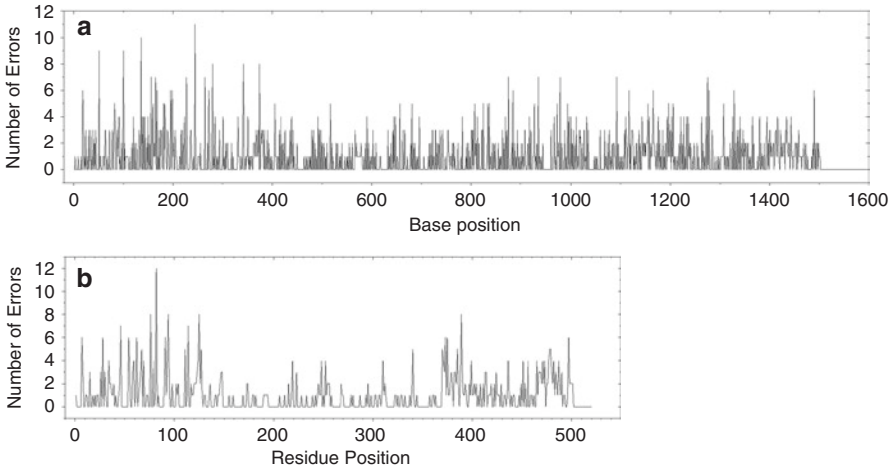

Fig. 32.4 The frequency of error distribution is plotted for Gag (**a**) nucleotide and (**b**) protein sequence

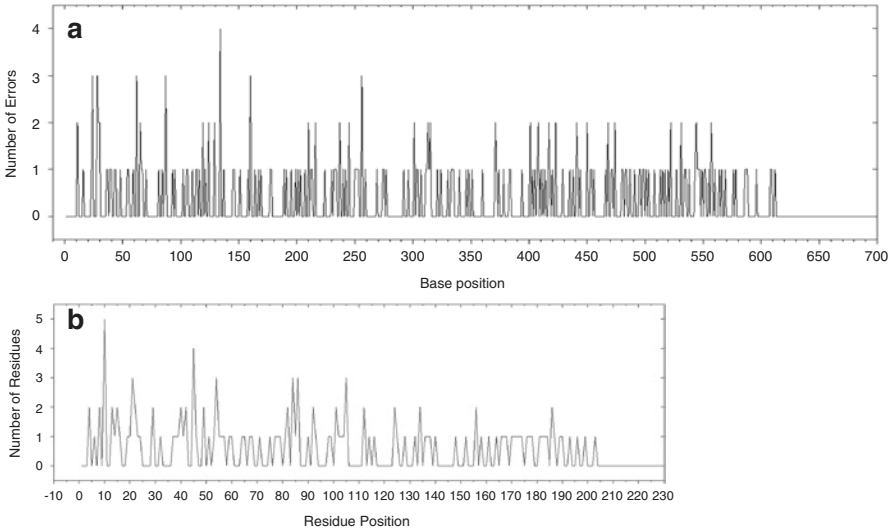

Fig. 32.5 The frequency of error distribution is plotted for Nef (**a**) nucleotide and (**b**) protein sequence

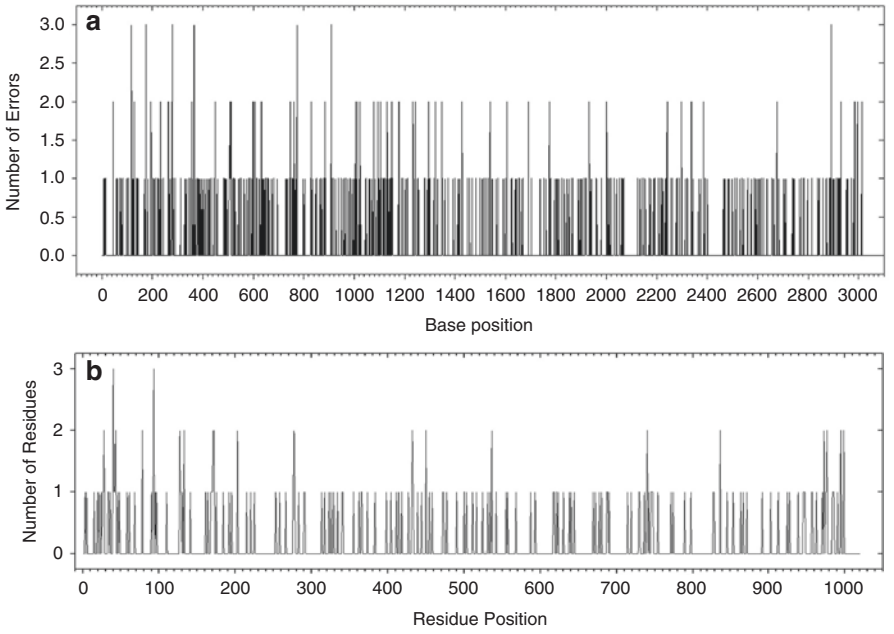

Fig. 32.6 The frequency of error distribution is plotted for Pol (**a**) nucleotide and (**b**) protein sequence

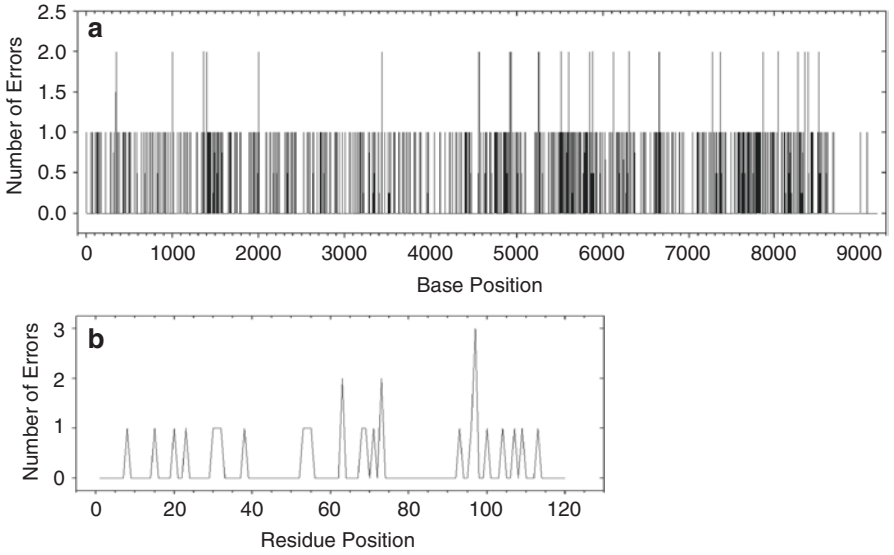

Fig. 32.7 The frequency of error distribution is plotted for Rev (**a**) nucleotide and (**b**) protein sequence

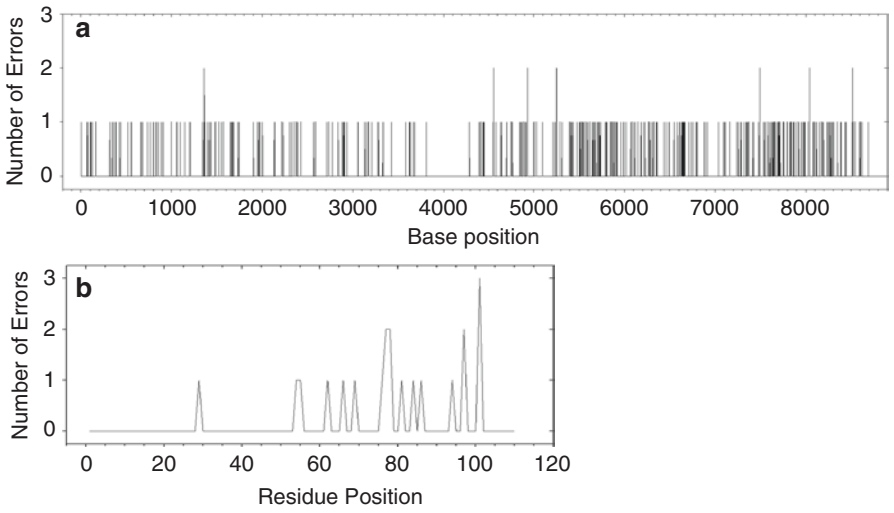

Fig. 32.8 The frequency of error distribution is plotted for Tat (**a**) nucleotide and (**b**) protein sequence

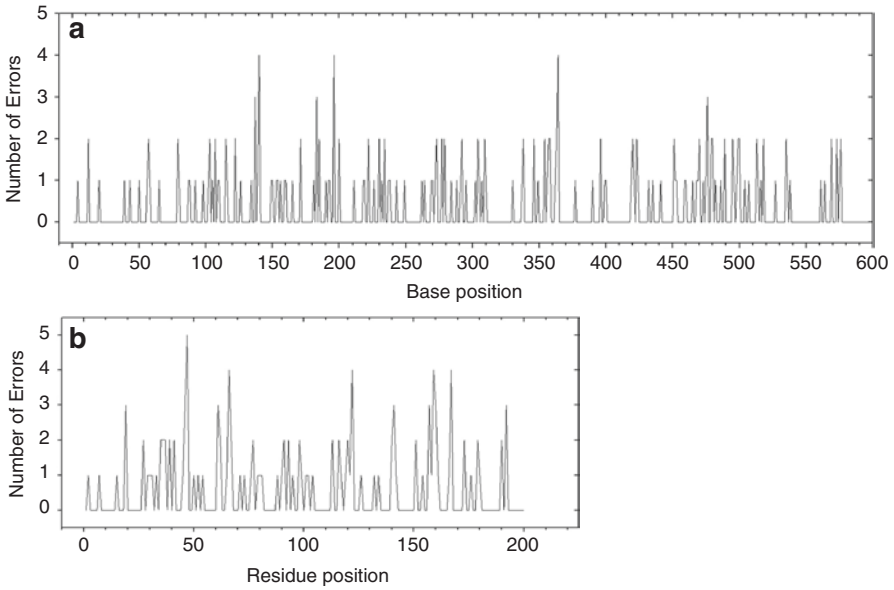

Fig. 32.9 The frequency of error distribution is plotted for Vif (**a**) nucleotide and (**b**) protein sequence

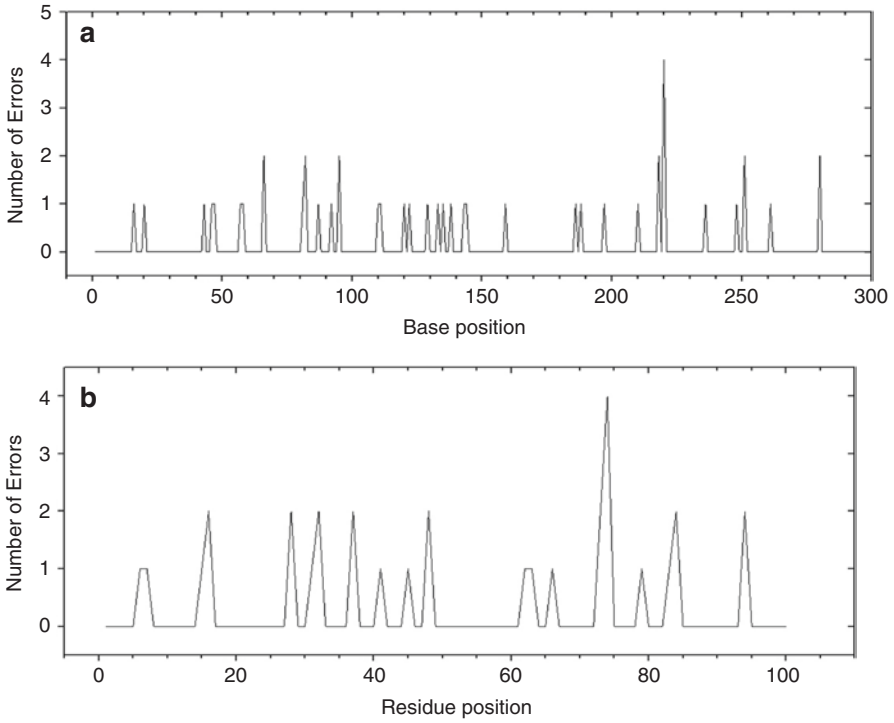

Fig. 32.10 The frequency of error distribution is plotted for Vpr (**a**) nucleotide and (**b**) protein sequence

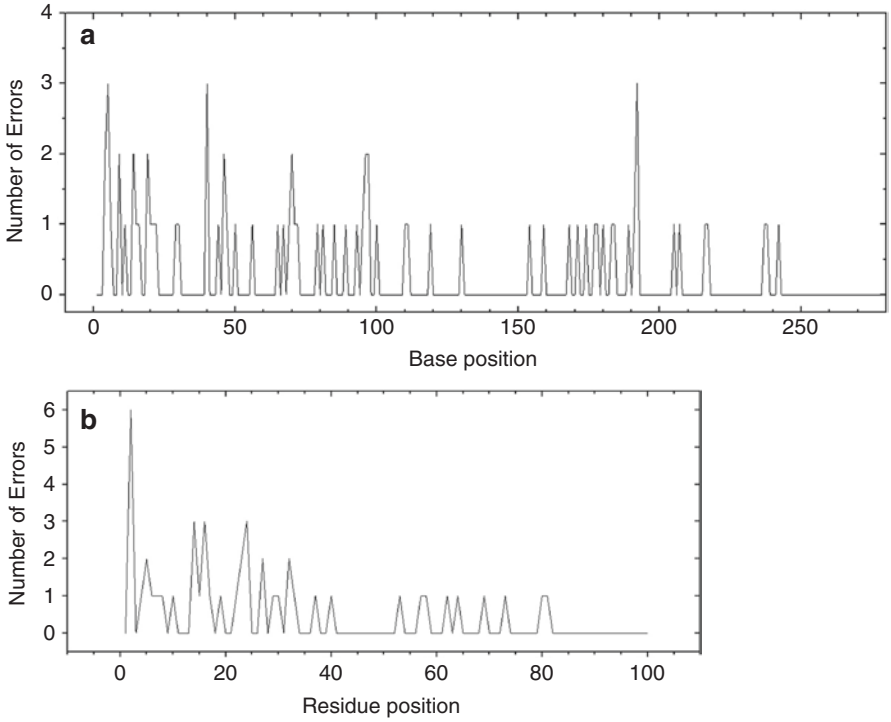

Fig. 32.11 The frequency of error distribution is plotted for Vpu (**a**) nucleotide and (**b**) protein sequence

Table 32.2 IUPAC ambiguity codes for nucleotides

Code	Description
M	AC
R	AG
W	AT
S	CG
Y	CT
K	GT
V	ACG
H	ACT
D	AGT
B	CGT
N	ATGC

Table 32.3 HIV-1 B proteins and corresponding coding sequences containing ambiguous residues

Gene (source)	Geographical location	Proteins (Accession ID)	Ambiguous residues	Coding gene (Accession ID)	Ambiguous bases
Env (peripheral blood mononuclear cells, PBMC)	Australia	ABI20201.1	BJX	DQ676870.1	WRYMK
		ABI20237.1	B	DQ676874.1	R
		ABI20354.1	X	DQ676887.1	RWK
		AAD03223.1	X	AF042103.1	S
	Argentina	ABD39426.1	X	DQ383750.1	RY
		ABD39444.1	X	DQ383752.1	YRMK
	Denmark	ABV00798.1	B	EF514708.1	NSWYR
	Jamaica	ADG57040.1	X	HM030561.1	Y
		ADG57057.1	X	HM030563.1	RMY
	Netherlands	ADD12388.1	X	GU455483.1	R
	Paraguay	AEK79381.1	X	JN251906.1	YRMWK
	South Africa	ACO50040.1	X	FJ647145.1	SY
	South Korea	ABB96430.1	X	DQ295193.1	Y
	Spain	ACJ37064.1	X	EU786672.1	WRYM
		ACJ37077.1	X	EU786674.1	RKY
		ACJ37100.1	X	EU786677.1	MR
		ACJ37109.1	X	EU786678.1	K
		ADV58152.1	X	HM469972.1	RKWHSYM
		ADV58175.1	X	HM469975.1	Y
		ADV58245.1	J	HM469983.1	YWRKSM
Env (blood)	Russia	AAT76857.1	X	AY682547.1	R Y M
		AAV30097.1	B	AY751406.1	RYKM
		AAV30106.1	X	AY751407.1	R
		AAV90745.1	X	AY819715.1	RYM
Env (plasma)	Thailand	ABD04233.1	X	DQ354114.1	R
	Australia	ADC55386.1	X	GU367397.1	S
		ADC55387.1	X	GU367398.1	Y
	Brazil	AEW28047.1	X	JN687739.1	R
		AEW28063.1	X	JN687741.1	R
	Germany	AGK62361.1	B	KC247954.1	YDRWMS
		AGK62388.1	BJZ	KC247981.1	RYSWKMN
		AGK62431.1	BJ	KC248024.1	RYSWKMV
		AGK62443.1	BJZ	KC248036.1	RYSWKMVD
	Japan	BAG31067.1	X	AB428556.1	RYKM
		BAG31112.1	X	AB428561.1	Y

(continued)

Table 32.3 (continued)

Gene (source)	Geographical location	Proteins (Accession ID)	Ambiguous residues	Coding gene (Accession ID)	Ambiguous bases
	Spain	ACY70072.1	X	FJ670524.1	R
		ACY70081.1	X	FJ670525.1	SRY
		ADO32878.1	X	GU362885.1	Y
	Thailand	AFU26520.1	X	JX446798.1	R
		AGK29559.1	X	KC749062.1	R
	USA	AAQ97441.1		AY331282.1	R
		AAQ97467.1	B	AY331285.1	RK
		AAQ97485.1	X	AY331287.1	KYR
		AAQ97495.1	X	AY331288.1	KRY
		AAQ97504.1	[a]	AY331288.1	KRY
		ABF47478.1	X	DQ487188.1	W
		ABF47505.1	X	DQ487191.1	KM
		ACS91559.1	X	GQ256646.1	N
		ADC55400.1	X	GU367411.1	YRHBK
		ADZ35142.1	X	JF320460.1	R
		AFZ63360.1	X	JX864047.1	R
Gag (blood)	Russia	AAT76855.1	B	AY682547.1	RY
		AAV30090.1	X	AY751406.1	RY
		AAV90743.1	X	AY819715.1	RYWM
Gag (PBMC)	Argentina	AAL07549.1	X	AF332867.1	MRYW
	Australia	ABI20194.1	X	DQ676870.1	MRY
		ABI20230.1	X	DQ676874.1	R
		ABI20320.1	J	DQ676884.1	RKYM
		ABI20347.1	X	DQ676887.1	MYR
	Germany	ADF45347.1	JZ	GU390464.1	RSMYWK
		ADF45349.1	J	GU390466.1	RYSWM
		ADF45354.1	X	GU390471.1	R
		ADF45361.1	X	GU390478.1	RSYKWMS
		ADF45363.1	X	GU390480.1	R
	Spain	ACJ37072.1	X	EU786674.1	RY
		ACJ37079.1	X	EU786675.1	WSYM
		ADV58246.1	X	HM469983.1	SMWYK
		AFN61964.1	X	JX140653.1	Y
	USA	AAA86730.1	X	U26546.1	WKMYNR
		AAO63135.1	X	AY206648.1	R
		AAO63143.1	X	AY206656.1	R
		ADF45403.1	X	GU390520.1	RYKM
Gag (plasma)	Argentina	ACI05325.1	X	FJ155079.1	RYWM
		ACI05375.1	X	FJ155132.1	RYSM
		ACI05413.1	JZ	FJ155175.1	RYSWKM
		ACI05447.1	B	FJ155211.1	RM

(continued)

Table 32.3 (continued)

Gene (source)	Geographical location	Proteins (Accession ID)	Ambiguous residues	Coding gene (Accession ID)	Ambiguous bases
	Canada	ABY78040.1	X	EU241938.1	RMYW
		ABY78041.1	B	EU241939.1	RYWKM
		ABY78256.1	X	EU242154.1	RYSKM
		ABY78268.1	X	EU242166.1	RYWM
		ABY78287.1	J	EU242185.1	RYKM
		ABY78536.1	X	EU242434.1	RYWM
		ABY78553.1	X	EU242451.1	RYKM
		ABY78559.1	X	EU242457.1	RYSKM
		ABY78565.1	J	EU242463.1	RYSWKM
		ABY78575.1	X	EU242473.1	RYWKMDH
		ABY78576.1	X	EU242474.1	RYSWKMV
		ABY78579.1	Z	EU242477.1	RYSWK
		ABY78588.1	X	EU242486.1	RYKMH
		ABY78592.1	B	EU242490.1	RYWKMBD
		ABY78594.1	X	EU242492.1	RYM
		ABY78596.1	X	EU242494.1	RYWM
		ABY78600.1	X	EU242498.1	RYWKMD
		ABY78602.1	J	EU242500.1	RYWKM
		ABY78604.1	X	EU242502.1	RYW
		ABY78606.1	X	EU242504.1	YW
		ACB36692.1	X	EU517762.1	RYM
		ACN94821.1	X	FJ667249.1	N
	China	ACT76434.1	X	FJ531384.1	RYKM
		ACT76444.1	X	FJ531390.1	R
		AEY77155.1	X	JQ234979.1	RKM
		AFZ77625.1	X	JQ900845.1	RM
		AFZ77690.1	X	JQ900910.1	RYM
		AFZ77722.1	X	JQ900942.1	RYSWK
	Germany	ACN94809.1	JZ	FJ667236.1	RYSWKM
		ACN94816.1	X	FJ667243.1	RYSM
		ACN94818.1	X	FJ667245.1	K
	Jamaica	ACN80948.1	X	FJ211789.1	RYS
	Japan	AFR66788.1	X	JX264269.1	RY
		AFR66854.1	X	JX264375.1	R
		AFR66862.1	X	JX264393.1	RYW
		BAG31060.1	X	AB428556.1	RY
	Spain	ADO32883.1	X	GU362886.1	R
	Thailand	AEO17558.1	X	JN248344.1	R
		AFU26582.1	X	JX446808.1	R
	USA	AAQ97443.1	X	AY331283.1	R
		AAQ97461.1	X	AY331285.1	W

(continued)

Table 32.3 (continued)

Gene (source)	Geographical location	Proteins (Accession ID)	Ambiguous residues	Coding gene (Accession ID)	Ambiguous bases
		AAQ97470.1	X	AY331286.1	RW
		AAQ97569.1	X	AY331297.1	Y
		ABM05980.1	X	DQ996250.1	V
		ACB36692.1	X	EU517762.1	RYM
		ACB36710.1	J	EU517780.1	RYWK
		ACB36723.1	X	EU517793.1	RYM
		ACB36732.1	X	EU517802.1	RY
		ACB36739.1	X	EU517809.1	RY
		ACB36742.1	X	EU517812.1	R
		ACB36744.1	X	EU517814.1	RYSWKH
		ACF93239.1	X	EU873003.1	RYWM
		ACG55531.1	J	EU864070.1	RYSWKM
		ACG55541.1	J	EU864080.1	
		ACG55543.1	X	EU864082.1	RYWMV
		ACU50315.1	X	GQ371221.1	RYKN
		ACU50508.1	X	GQ371414.1	RYK
		ACU50659.1	X	GQ371566.1	RYWM
		ACU50854.1	X	GQ371763.1	RYSWKMN
		ADC55431.1	X	GU367442.1	RYKM
		ADW26877.1	X	GU561116.1	RYMH
		ADW26991.1	X	GU561230.1	RYWK
		ADW27005.1	X	GU561244.1	RY
		ADW27023.1	J	GU561262.1	RYSWKM
		ADW27040.1	J	GU561279.1	RYSWKMH
		ADW27045.1	X	GU561284.1	Y
		ADW27063.1	X	GU561302.1	RY
		ADW27064.1	J	GU561303.1	RYM
		AFH37780.1	X	JN685321.1	RYKM
		AFH37796.1	J	JN685337.1	RYWM
		AFH37803.1	X	JN685344.1	RYWKMH
		AFH37810.1	X	JN685351.1	RYWKM
		AFH37814.1	X	JN685355.1	RYK
		AFH37816.1	X	JN685357.1	RW
		AFH37829.1	X	JN685370.1	RYSW
		AFH37833.1	X	JN685374.1	RYK
		AFH37836.1	J	JN685377.1	RYWM
		AFH37838.1	X	JN685379.1	RYW
		AFZ62941.1	X	JX863921.1	R
	South Korea	ABB96432.1	X	DQ295194.1	R
Nef (blood)	USA	AAA79604.1	X	U24461.1	R
	Russia	AAV30098.1	X	AY751406.1	RYWKM
		AAV30107.1	X	AY751407.1	W

(continued)

Table 32.3 (continued)

Gene (source)	Geographical location	Proteins (Accession ID)	Ambiguous residues	Coding gene (Accession ID)	Ambiguous bases
Nef (plasma)	Aus	ADC67110.1	X	GU367447.1	D
	Brazil	AEW28056.1	X	JN687740.1	R
	Japan	BAG31068.1	X	AB428556.1	RYM
	Spain	ADE34342.1	X	GQ372990.1	Y
	Thailand	AFU26500.1	X	JX446795.1	R
		AFU26521.1	X	JX446798.1	R
	USA	ABM05995.1	X	DQ996265.1	RN
		ABY53234.1	X	EU327403.1	N
		ACS91545.1	X	GQ256632.1	N
		ACU55757.1	X	GQ372325.1	RYKM
		ACU55915.1	X	GQ372483.1	RYSWMN
		ACU55971.1	X	GQ372539.1	RYWKM
		ACU56252.1	X	GQ372820.1	RYWM
		ADB03665.1	J	GU046578.1	RYM
		ADB03683.1	X	GU046596.1	RY
		ADC67111.1	X	GU367449.1	N
		ADZ32964.1	X	JF320069.1	R
		AEW28492.1	X	JN687847.1	R
	Canada	AAU89984.1	X	AY701254.1	RYN
		AAU89986.1	X	AY701256.1	DV
		AAU89987.1	X	AY701257.1	M
		AAU89993.1	X	AY701263.1	RYMD
		AAU89994.1	X	AY701264.1	RYSK
		AAU89996.1	X	AY701267.1	RKMD
		AAU90000.1	X	AY701271.1	RYKM
		AAU90001.1	X	AY701272.1	S
	Argentina	ABD39445.1	X	DQ383752.1	Y
		ABY49071.1	X	EU312168.1	R
		ABY49087.1	X	EU312184.1	RY
	Australia	ABI20283.1	X	DQ676879.1	RYKV
	Denmark	ABV00799.1	X	EF514708.1	SK
	France	AAA44943.1	X	M58245.1	N
		AAA44969.1	X	M58271.1	N
	Italy	AAD39184.1	X	AF147728.1	RYM
		AAD39186.1	X	AF147730.1	RYSWK
		AAD39189.1	X	AF147733.1	RYW
		AAD39191.1	X	AF147735.1	RW
		AAD39193.1	X	AF147737.1	RY
	Jamaica	ADG57022.1	X	HM030559.1	R
		ADG57058.1	X	HM030563.1	RYSW
	Netherlands	AAX86721.1	X	AY970946.1	N
	Paraguay	AEK79317.1	X	JN251896.1	RK

(continued)

Table 32.3 (continued)

Gene (source)	Geographical location	Proteins (Accession ID)	Ambiguous residues	Coding gene (Accession ID)	Ambiguous bases
	Russia	AAS01347.1	X	AY500393.1	RWKM
	Spain	AAR20513.1	X	AY444316.1	N
		AAR20515.1	X	AY444318.1	R
		ACJ37103.1	X	EU786677.1	W
	USA	AAA86737.1	X	U26546.1	D
		ABY81214.1	X	EU180818.1	N
Pol (blood)	Cyprus	AEB52476.1	X	JF683741.1	RYWKM
		AEB52574.1	J	JF683753.1	RYW
		AEB52699.1	X	JF683769.1	M
		AEB52767.1	B	JF683778.1	RY
		AEB52827.1	X	JF683785.1	RYM
		AEB52916.1	X	JF683796.1	RYW
	Russia	AAT76856.1	X	AY682547.1	RYWM
		AAV30091.1	X	AY751406.1	RYKM
		AAV30100.1	X	AY751407.1	RY
		AAV90744.1	B	AY819715.1	RYWM
Pol (PBMC)	Germany	CAA50426.1	X	X71109.1	RYWM
	Argentina	ABD39420.1	X	DQ383750.1	YK
		ABD39438.1	X	DQ383752.1	RM
	Aus	ABI20195.1	BJ	DQ676870.1	RYSWM
		ABI20231.1	X	DQ676874.1	RM
		ABI20321.1	X	DQ676884.1	RYM
		ABI20348.1	X	DQ676887.1	RYM
	Denmark	ABV00739.1	X	EF514702.1	RY
		ABV00748.1	X	EF514703.1	R
		ABV00793.1	X	EF514708.1	RYM
	Georgia	ABB29349.1	X	DQ207940.1	W
	Jamaica	ADG57019.1	X	HM030559.1	RY
		ADG57052.1	X	HM030563.1	RYWM
	Paraguay	AEK79312.1	X	JN251896.1	YM
	Spain	ACJ37060.1	X	EU786672.1	RYK
		ACJ37080.1	X	EU786675.1	RYKM
		ACJ37096.1	B	EU786677.1	RYK
		ADV58154.1	X	HM469972.1	RSWKM
		ADV58247.1	X	HM469983.1	KM
	Uruguay	AEK79466.1	X	JN235965.1	R
Pol (plasma)	Aus	ADC67095.1	X	GU367473.1	R
	Brazil	AEW27528.1	X	JN687657.1	Y
		AEW27533.1	X	JN687658.1	R
	China	AEY77193.1	X	JQ235017.1	RKM
		AFV39528.1	X	JQ302625.1	RM

(continued)

Table 32.3 (continued)

Gene (source)	Geographical location	Proteins (Accession ID)	Ambiguous residues	Coding gene (Accession ID)	Ambiguous bases
		AFZ77826.1	X	JQ901046.1	RY
		AFZ77842.1	X	JQ901062.1	RYW
		AFZ77877.1	X	JQ901097.1	R
	Japan	BAG31061.1	[a]	AB428556.1	RYWK
		BAG31088.1	X	AB428559.1	R
		BAG31115.1	X	AB428562.1	Y
	Peru	ADZ32731.1	X	JF320035.1	Y
	Spain	ACY70077.1	X	FJ670525.1	R
		ACZ82302.1	X	FJ853620.1	R
		ACZ82320.1	X	FJ853622.1	R
		ADE34335.1	X	GQ372990.1	R
	Thailand	AEO17593.1	X	JN248348.1	R
		AFU31744.1	X	JX447787.1	R
	USA	AAQ97570.1	X	AY331297.1	R
		ACS91546.1	X	GQ256633.1	N
		ACU55196.1	Z	GQ371764.1	RYSWKD
		ACU55221.1	X	GQ371789.1	RYKM
		ACU55279.1	X	GQ371847.1	RYS
		ACU55516.1	X	GQ372085.1	RYKM
		ACU55684.1	X	GQ372253.1	RYSWKM
		ACU55747.1	B	GQ372316.1	RYSWKMN
		ADC67105.1	X	GU367483.1	YW
		AEW27585.1	X	JN687669.1	R
		AFZ62942.1	X	JX863921.1	RY
Rev (blood)	Russia	AAT76858.1	X	AY682547.1	RYWKM
		AAV30095.1	X	AY751406.1	RYWKMD
		AAV90748.1	B	AY819715.1	RYWM
Rev (PBMC)	Argentina	ABD39424.1	X	DQ383750.1	RYK
		ABD39442.1	X	DQ383752.1	RYKM
	Australia	ABI20199.1	X	DQ676870.1	RYSWKM
		ABI20253.1	X	DQ676876.1	RYSWKMH
		ABI20280.1	J	DQ676879.1	RYSWKMV
		ABI20316.1	X	DQ676883.1	RYSWKM
		ABI20352.1	X	DQ676887.1	RYWKM
	Jamaica	ADG57055.1	X	HM030563.1	RYSWKM
	Paraguay	AEK79379.1	X	JN251906.1	RYWKM
Rev (plasma)	Japan	BAG31092.1	J	AB428559.1	RYM
	Thailand	ABD04231.1	X	DQ354114.1	RY
		AEO17597.1	X	JN248348.1	RYWKM
	USA	AFZ62929.1	X	JX863919.1	RYKM
Tat (blood)	USA	AAA79680.1	X	U24487.1	Y
Tat (PBMC)	Argentina	ABD39441.1	X	DQ383752.1	RYKM

(continued)

Table 32.3 (continued)

Gene (source)	Geographical location	Proteins (Accession ID)	Ambiguous residues	Coding gene (Accession ID)	Ambiguous bases
	Australia	ABI20198.1	X	DQ676870.1	RYSWKM
		ABI20279.1	X	DQ676879.1	RYSWKMV
		ABI20315.1	X	DQ676883.1	RYSWKM
		ABI20351.1	X	DQ676887.1	RYWKM
Tat (plasma)	Japan	BAG31091.1	X	AB428559.1	RYM
	Thailand	AEO17596.1	X	JN248348.1	RYWKM
	USA	AFZ62928.1	X	JX863919.1	RYKM
Vif (blood)	Russia	AAV30092.1	X	AY751406.1	RY
		AAV90746.1	B	AY819715.1	RY
		AAA79672.1	X	U24486.1	RM
Vif (PBMC)	Australia	ABI20196.1	J	DQ676870.1	RYM
		ABI20313.1	J	DQ676883.1	YM
	France	AAD37885.1	X	AF143118.1	N
	Jamaica	ADG57053.1	X	HM030563.1	RY
	Paraguay	AEK79313.1	X	JN251896.1	Y
	Spain	ACJ37074.1	X	EU786674.1	RSKM
		ADV58181.1	X	HM469975.1	W
	Uruguay	AEK79467.1	X	JN235965.1	K
Vif (plasma)	China	AEI88153.1	X	HQ215554.1	R
	Japan	BAG31062.1	X	AB428556.1	R
	Netherlands	AGO97607.1	X	KF301744.1	M
		AGO97621.1	X	KF301748.1	RWKM
	Spain	ACJ37121.1	X	EU786680.1	R
		ADE34336.1	X	GQ372990.1	R
		ADO32840.1	X	GU362881.1	R
	Thailand	AEO17484.1	X	JN248335.1	R
	USA	AAP74163.1	X	AY308760.1	S
		AAP74172.1	X	AY308761.1	S
		AAP74182.1	X	AY308762.1	YM
		AAQ97490.1	X	AY331288.1	RYW
		ACB20979.1	X	EU518054.1	YM
		ACB20983.1	X	EU518058.1	RM
		ACB21008.1	X	EU518083.1	RKM
		ADC55476.1	X	GU367548.1	RW
		ADF86692.1	B	GU729587.1	RYWM
		ADF87137.1	[a]	GU730033.1	RKM
		AFI49418.1	X	JQ409032.1	RYWM
		AFI49419.1	X	JQ409033.1	RYWM
		AFI49420.1	X	JQ409034.1	RYM
		AFI49421.1	X	JQ409035.1	RYM
		AFI49422.1	X	JQ409036.1	RYWM

(continued)

Table 32.3 (continued)

Gene (source)	Geographical location	Proteins (Accession ID)	Ambiguous residues	Coding gene (Accession ID)	Ambiguous bases
		AFI49423.1	X	JQ409037.1	RYWM
		AFI49424.1	X	JQ409038.1	RYM
		AFI49425.1	X	JQ409039.1	RYW
		AFI49426.1	X	JQ409040.1	RY
		AFI49427.1	X	JQ409041.1	RYS
Vpr (PBMC)	Argentina	ABD39440.1	X	DQ383752.1	RY
	Australia	ABI20314.1	X	DQ676883.1	RK
	Canada	AAW88732.1	X	AY910541.1	N
	Jamaica	ADG57054.1	X	HM030563.1	R
	Spain	ACJ37098.1	X	EU786677.1	Y
	USA	AAR24638.1	X	AY444331.1	RM
		AFJ32702.1	X	JQ928102.1	N
Vpr (plasma)	Brazil	AEW27530.1	X	JN687657.1	R
	Japan	BAG31063.1	X	AB428556.1	Y
	Netherlands	AGO97608.1	J	KF301744.1	RYM
	Spain	ADE34319.1	X	GQ372988.1	R
	USA	ACB36885.1	X	EU518105.1	RY
		ADC55492.1	X	GU367564.1	R
		ADF85088.1	X	GU730621.1	RY
		ADF85288.1	X	GU730822.1	RYW
		ADF85416.1	X	GU730950.1	RYS
		ADF85528.1	X	GU731062.1	RM
Vpu (blood)	Russia	AAT76862.1	X	AY682547.1	RYKM
		AAV90749.1	B	AY819715.1	R
Vpu (PBMC)	Jamaica	ADG57021.1	X	HM030559.1	RWK
	Netherlands	AAX86755.1	X	AY970950.1	N
	Spain	ACJ37076.1	B	EU786674.1	RYK
Vpu (plasma)	Argentina	ABM67811.1	J	EF119863.1	RYWM
		ABM67852.1	X	EF119910.1	RYS
		ABM67878.1	X	EF119941.1	RYW
	Japan	BAG31066.1	X	AB428556.1	RS
	Thailand	AFU26621.1	X	JX446817.1	R
	USA	ACB29483.1	X	EU517753.1	RY
		ADF84534.1	X	GU730069.1	R
		ADF84634.1	X	GU730169.1	RWK
		ADF84776.1	X	GU730311.1	RYWM
		ADF84886.1	X	GU730422.1	RYM
		ADF85016.1	B	GU730554.1	RYK

[a]No ambiguities

to show the frequency of distribution (Figs. 32.3, 32.4, 32.5, 32.6, 32.7, 32.8, 32.9, 32.10, and 32.11). The consensus sequence is obtained by the linear ordering of the nucleotides at each site that contains uncertainties, "N." It should be noted that it is a consensus sequence and hence it may not correspond to a real HIV-1B sequence. The analysis shows that the positions of "N" are highly variable and spread throughout the sequence. The frequency of substitutions is sufficiently high and is an indicative of sequencing errors. The sequence length heterogeneity was observed especially in the variable regions encoding gp120. The ambiguities of HIV-1 nucleotide as well as protein sequences are illustrated (Figs. 32.3, 32.4, 32.5, 32.6, 32.7, 32.8, 32.9, 32.10, and 32.11).

In the analyzed data set (Fig. 32.3), uncertainties in Env protein sequence (Fig. 32.3b) range from 1 to 47. Out of the analyzed sequences, the maximum number of ambiguities was recorded for Env plasma isolate from Germany (AGK62388.1), which contain uncertainties "X" 47 times (Fig. 32.3a) throughout the sequence (out of 865 residues). In the corresponding coding sequence (KC247981.1), there are 97 ambiguities out of 2598 bases, 28 "Rs" (represent either "A" or "G"), 17 "Ys" (either "C" or "T"), 6 "Ws" (either "A" or "T"), 5 "Ss" (either "C" or "G"), 6 "Ms" (either "A" or "C") and 2 "Ks" (either "G" or "T"), and 33 "Ns" (any nucleotides). The authors of the deposited sequence indicated that their study has certain limitations due to indeterminate regions in their HIV env population sequences and lack of correlation of virological response with some baseline variable [24]. Such numerous sequence ambiguities are not useful in variability studies. Therefore, such sequences should be discarded from sequence analysis, which make spurious alignments and uncertain phylogenetic relationships. However, it should be decided according to the purpose of the study. It is to be noted that we have eliminated such sequences while deriving a consensus sequences in the previous study [143].

The ambiguity in the protein sequence of Env (AGK62388.1) was compared with the reference genome (HXB2) and residues annotated. The ambiguous residues at positions 5, 13, 16, 19, 28, 45, 48, 84 and 126 correspond to the residues E, L, W, M, S, K, T, V, and V, respectively. Serine at the 28th position (S-28) is in the Env signal peptide end (and in another reading frame it is at the Vpu end) and V-126 is near to the V1 loop start. A continuous stretch of ambiguous residues from 134 to 141 may probably represent a pattern "KNDTNTNS" in the V1 loop. Positions 165–172 and 189–191 are also ambiguous and present in the V2 loop. Positions 274, 295, 298, and 302 correspond to residues E, T, E, and T, respectively. The residue T at position 302 indicates the V3 loop start. Ambiguous positions at 310, 318, 341, 347, 351, 361, 389, 391, and 392 correspond to residues K, R, K, K, S, I, T, T, and Q, respectively. The positions 389, 391, and 392 are an ambiguous pattern CXTXX and mark the V4 loop start. The conserved pattern in the reference corresponding to this and is "CNSTQ" at the V4 loop start. Ambiguity in positions 403–405 is in the highly variable V4. The ambiguous position 686 corresponds to residue "N," toward the gp41 transmembrane domain as well as the following residues (687-WLWYIKLF-694) is well conserved marking the gp41 transmembrane domain. At positions 702, 729, and 730 corresponds to residues V, H, and L, respectively. This marks the end of Tat/Rev intron and the start of Tat/Rev exon 2. The last ambiguous residue is at 842 that correspond to "V" and it is towards the end of gp41 and gp160.

Similarly other proteins were scanned for sequence ambiguities and annotated with the reference genome (HXB2). Uncertainties in the Gag protein sequence (Fig. 32.4b) ranged from 1 to 21. The maximum number of ambiguities was recorded for peripheral blood mononuclear cells (PBMC) isolate from Germany (ADF45361.1) yielding 21 ambiguities out of 505 residues. The corresponding coding sequence (GU390478.1) contains 46 ambiguities (Fig. 32.4a) out of 1518 bases. The ambiguous residue positions at 7, 15, 76, 114, 378, and 382 corresponds to the residues V, R, R, K, I, and R, respectively. R-382 marks the start of Gag P7 nucleocapsid as well as the Gag-pol fusion TF protein. The ambiguities from 385-XX-NQ-XXX-391 correspond to 385-FR-NQ-RKI-391. The ambiguous residue positions at 400, 413, 465, 469, 471, 475, 477, 482, 484, and 488 corresponds to the residues E, K, E, R, G, T, P, E, I, and L, respectively.

Uncertainties in Nef protein sequences (Fig. 32.5b) range from 1 to 10. The highest is observed for plasma isolate from the USA (ACU55971.1) as well as Canada (AAU89996.1) each had 10 ambiguities out of 206 and 210 residues, respectively. The corresponding coding sequence of the USA sequence (GQ372539.1) contains 25 ambiguities (Fig. 32.5a) out of 621 bases. The ambiguous residue positions at 14, 21, 45, 134, 136, 166, 168, 173, 184, and 203 correspond to the residues P, R, S, V, Y, H, V, M, R, and F, respectively. The residue F at position 203 is involved in TCF-1 alpha binding.

Uncertainties in Pol protein sequences (Fig. 32.6b) range from 1 to 19. The maximum number of ambiguities is recorded for plasma isolate from the USA (ACU55747.1), with 19 ambiguities out of 1003 residues. The corresponding coding sequence (GQ372316.1) contains 29 ambiguities (Fig. 32.6a) out of 3012 bases. However, it was observed that the highest ambiguities for Pol nucleotide sequence is 34 (plasma isolate from the USA, GQ371847.1) and in the corresponding protein is only six residues. This trend was not observed for the proteins discussed above. The ambiguous residue positions at 15, 42, 78, 169, 170, 171, 432, 687, 688, 742, 828, 854, 903, 913, 939, 946, 948, 976, and 977 correspond to the residues E, S, A, P, G, M, R, Y, L, N, I, F, K, E, R, R, L, P, and R, respectively.

Uncertainties in Rev protein sequences (Fig. 32.7b) range from 1 to 4. The maximum number was observed for PBMC isolate from Australia (ABI20352.1), there were four ambiguities out of 116 residues. The corresponding coding sequence (DQ676887.1, join (5189.. 5264, 7625..7899)) contains 38 ambiguities (Fig. 32.7a) out of 8687 bases. However, it was observed that the highest ambiguities for Rev nucleotide sequence is 91 (Blood isolate from Russia, AY751406.1) and in the corresponding protein it is only 1 residue. The ambiguous residue positions at 8, 20, 55, and 69 correspond to the residues S, K, I, and E, respectively. The residue K at position 20 is in Rev exon 1 and the residue I at position 55 marks the end of Tat (in another reading frame).

Uncertainties in Tat protein sequences (Fig. 32.8b) range from 1 to 5. The maximum number was observed for PBMC isolate from Australia (ABI20351.1), there were five ambiguities out of 101 residues. The corresponding coding sequence (DQ676887.1, join (5050.. 5264, 7625..7715)) contains 38 ambiguities (Fig. 32.8a) out of 8687 bases. However, it was observed that the highest ambiguities for Tat nucleotide sequence is 87 (PBMC isolate from Australia, AY751406.1) and in the

corresponding protein it is three residues. The ambiguous residue positions at 54, 62, 66, 97, and 101 correspond to the residues Q, S, Q, T, and D, respectively.

Uncertainties in Vif protein sequence (Fig. 32.9b) range from 1 to 18. The maximum number is for plasma isolate from the USA (AFI49422.1), there were 18 ambiguities out of 192 residues. The corresponding coding sequence (JQ409036.1) contains 26 ambiguities (Fig. 32.9a) out of 579 bases. The ambiguous residue positions at 31, 39, 41, 46, 47, 67, 91, 93, 113, 116, 120, 122, 140, 151, 157, 173, 176, and 179 correspond to the residues V, W, Y, S, P, T, K, R, D, S, I, K, N, A, K, R, K, and K, respectively.

Uncertainties in Vpr protein sequences (Fig. 32.10b) range from 2 to 5. The plasma isolate from the USA (ADF85088.1) has five ambiguities out of 96 residues. The corresponding coding sequence (GU730621.1) contains eight ambiguities (Fig. 32.10a) out of 291 bases. The ambiguous residue positions at 32, 45, 74, and 79 correspond to the reference R, H, I, and S, respectively.

Uncertainties in Vpu protein sequence (Fig. 32.11b) ranges from 1 to 6. The plasma isolate from the USA (ADF84634.1) has six ambiguities out of 82 residues. The corresponding coding sequence (GU730169.1) contains seven ambiguities (Fig. 32.11a) out of 249 bases. The ambiguous residue positions at 2, 6, 7, 14, 24, and 33 correspond to the reference Q, I, V, V, S, and I, respectively. The residue S at position 24 is in the transmembrane domain, whereas the residue I at position 33 is located after the TM domain.

In summary, the uncertainties of all genes in HIV-1B are significantly elevated. In the Env coding gene, the sequence ambiguities are consistent throughout. Uncertainties are more frequent in Gag and are consistent throughout Nef. In the first half of Nef, there are two to three ambiguities with a large peak (four ambiguities) at 135th base and in the second half there are one to two errors. In Pol, ambiguity is consistent and very dense throughout. This may affect the reverse transcriptase encoding domain and may be associated with variable resistance to RT inhibitors. For Tat and Rev ambiguity is less but consistent throughout with one or two ambiguities. In Vif there are large peaks around positions 150, 200, and 370. In Vpr it is sparse and a large peak at the 220th position with four ambiguities. In Vpu, there are three peaks one at the beginning one around the 50th base and around the 200th base. Ambiguity is consistent in Env and at some positions, there are two to three ambiguities along its length. This may correspond to cell-tropism during infection [144]. The error rate of HIV-1 genome does not correlate with the substitution rates. The substitution rate varies across the genome and it is reported that the substitution rate is lowest in pol, intermediate in gag, and relatively high in env [138]. The error rates analyzed are given in decreasing order, Gag> pol >env >nef >vif >rev >tat >vpu >vpr (Figs. 32.3, 32.4, 32.5, 32.6, 32.7, 32.8, 32.9, 32.10, and 32.11). In the case of HIV-1 protein sequences, the ambiguous amino acids in Env are frequently spread throughout the sequence. In case of Gag, it is more frequent in first 100 residues, in Vif it is prominent in every 50 residues, in Pol it is less but consistent throughout, in Rev Tat and Vpr it is less, in Vpu it is predominant in the N-terminus, and in the first half of Nef it is greater than in the second half.

32.4 Conclusions

Data bases face several problems as described in the current chapter. On the one hand, the DNA, RNA, and protein sequences that they contain have specific sequence variations that result from many decades of research. On the other hand as described, there are uncertainties and ambiguities in the sequences. Of great difficulty, furthermore, is the ability to maintain accuracy and reproducibility, which are required for the clinical diagnostics, research, and development. Thus, there is a need to focus additionally on the reliability of the data for these purposes. This is possible only if there are regulations and requirements for data submission and of course that the methods are developed to detect and possibly correct the problems ascertained. More stringent descriptions and requirements are thus needed for quality and accuracy and the definition of what should be required at any time based on the current technologies and bioinformatics. This approach progress toward reducing the risks of errors and uncertainties as well as uninformative sequence sites and promote the submission of quality sequences. From the data set analyzed, it is clear that the sequences listed in the Table 32.2, contain uncertainties and none of them is updated or annotated as deduced from their "accession.version" details. It is well known that even a single nucleotide change may lead to human disease. Thus, it is a serious issue, when degree of quality or erroneous sequence data are not labeled or subjected to further analysis and then later used and assumed to be error free. This forewarns data collection efforts and inferences. Currently, the NGS technologies have significant error rates and it is very difficult to distinguish true variants from the errors.

It is obvious that the ambiguous base "N" represents "any nucleotide" that appears predominantly in the terminals due to base-call errors but in the analyzed data set "Ns" were spread throughout the sequence. These sequences are not informative for any bioinformatics analysis and persist in the public databases and create confusions during analysis. There are many limitations associated with these sequences. For instance, it was observed that plasma isolates of HIV-1 Env protein sequences from Germany (AGK62361.1), Australia (ADC55386.1) and Brazil (AEW28063.1) have uncertainties or ambiguities as mentioned above. Future studies should be cautious in handling such sequences especially on analyzing mutations. Of course, it is a component in the physical and biological sciences that errors occur; however, methods to reduce technological-related errors and methods for their detection and reduction all need improvement.

Germany (AGK62361.1), Australia (ADC55386.1), and Brazil (AEW28063.1) have uncertainties "X" at positions 204, 506, and 655, respectively. Mutations at these positions are reported for drug resistance to HIV-1 Attachment Inhibitor [145–147]. Similarly, in Pol uncertainties are consistent throughout. This might have practical effects on the reverse transcriptase encoding domain and hence variable resistance to RT inhibitors. Future studies should be cautious in handling such sequences especially on analyzing mutations. In the future, some of these

challenges can be tackled by upcoming third- and fourth-generation sequencing technologies, like single molecule or direct RNA sequencing. It should also be noted that validation of software tools is also required and such analyses should be performed and described alongside each sequencing project.

Conflict of interest The authors report no conflicts of interest.

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Chapter 33
Amyloidogenic Pattern Prediction of HIV-1 Proteins

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Keywords HIV-1 • CNS • Brain • HIV associated dementia • Cognitive decline • Hydrophobic • Amyloidogenic patterns • Hydrophobicity • Average packing density • Aggregation propensity scale • Beta strand contiguity • Hexapeptide conformational energy • Misfolding • Neurocognitive complications of HIV-1 • HIV-1 associated dementia (HAD) • HIV-1 associated neurodegeneration • Amyloid forming potential of HIV-1 • Self-aggregating amyloids and neurodegeneration • Amyloidogenic pattern prediction of HIV-1 brain isolates • Inter-molecular pairing and ambiguities in secondary structure propensities • Misfolding leading to aggregation • Amyloidogenicity assessment of HIV-1 proteins

Core Message

This chapter discusses proteins encoded by HIV-1 brain isolates and their predicted amyloidogenic regions. Amyloidogenic patterns were predicted by consensus prediction methods based on hydrophobicity, average packing density, aggregation propensity scale, beta-strand contiguity, and hexapeptide conformational energy and compared

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with the published literature. The study of HIV-1 protein amyloidogenic patterns, secondary structure, and property conservation benefits tracking HIV-1-associated dementia and cognitive decline. This methodology can be used to track infection and can also be applied to neurodegenerative disorders including amyotrophic lateral sclerosis (ALS), Alzheimer's, Parkinson's, Huntington's, and prion diseases.

33.1 Introduction

Neurodegeneration in general is characterized by progressive loss of structure or function of neurons inducing neuronal cell death [1]. Diseases including Parkinson's, Alzheimer's, and Huntington's diseases (PD, AD, HD, respectively), as well as amyotrophic lateral sclerosis (ALS), result in progressive degeneration and remain incurable [2]. Protein aggregation in the brain and inclusion body formation are linked to these diseases, while smaller oligomeric forms of misfolded proteins are involved in their onset [3]. Human immunodeficiency virus 1 (HIV-1) infection is known to be associated with neurologic complications leading to dementia in a significant number of patients [4]. During early infection, the ability of HIV-1 to colonize and compartmentalize in different organs within the body, also adapting to their particular environments and different selective pressures, enables the virus to form separate viral populations in these organs including the central nervous system (CNS) and thus associated with HIV-encephalopathy as well as neuropathology [5, 6].

Several studies have discussed parallels among different neurodegenerative diseases with respect to unusual protein assemblies [7–9]. Proteins generally fold into a particular three-dimensional (3D) conformations of their native state in which they are functional. Nevertheless, newly synthesized proteins may not fold properly, or correctly folded proteins may suddenly misfold. In these cases, the absence of protein refolding, or inability to degrade the unfolded or misfolded protein, leads to accumulation of protein clumps [10, 11]. Case in point, amyloid fibrils are the most typical types of aggregation observed in connection with neurodegeneration. Amino acid hydrophobicity, charge, as well as their structural propensities guide formation of secondary structures and serve as intrinsic parameters that are important for identification of the tendency of a peptide to form amyloid fibril with compact β-sheets [12–15]. Several studies suggest neurodegenerative diseases, including AD and PD, share commonalities with the development of HAND/HAD [16, 17]. Brain tissues studied from HIV-1-infected patients indicate the presence of amyloid-beta (Aβ) plaques similar to those in AD and Lewy bodies in case of PD [18, 19].

33.2 Role of HIV-1 Proteins in Neurodegeneration and Amyloidogenicity

Post neuroinvasion by HIV-1, viral proteins released by infected macrophage and microglial cells serve as significant components of HAD pathogenesis. They are associated with neurotoxic effects, neuronal dysfunction, and cell death [20, 21].

Proteins including Trans-Activator of Transcription (Tat), negative regulatory factor (Nef), viral protein R (Vpr), and envelope glycoprotein (gp120–gp41) play roles in neuronal apoptosis [22, 23]. HIV-1 gp120 interacts with chemokine receptors including CC-chemokine receptor 5 (CCR5), CC-chemokine receptor 3 (CCR3), and CXC-chemokine receptor 4 (CXCR4) expressed on the surfaces of neurons and glial cells [24–27] and thus induces neuronal apoptosis by activating certain intracellular signal transduction pathways [22, 28]. Disruption of calcium homeostasis in neurons by gp120 in turn is associated with apoptosis pathways such as the destruction of mitochondrial membrane integrity leading to release of cytochrome c and activation of caspases and endonucleases [29]. Perturbation of calcium-regulated systems of endoplasmic reticulum (ER) induced by gp120 and glutamate-related hyperactivation of the N-methyl-D-aspartic acid (NMDA) receptor also due to activated macrophages and microglial cells both trigger ER stress [30–32]. Moreover, ER stress is linked with multiple neurodegenerative diseases as it may be involved early in the process of cellular apoptosis [33, 34]. Additionally, gp120 has been demonstrated to alter gene expression patterns in astrocytes and neurons, suggesting that alterations in cellular processes may contribute to neuronal damage [35, 36]. The mechanisms of neurotoxicity induced by gp41, a transmembrane protein that anchors gp120 in the viral envelope, involve activation of inducible nitrous oxide synthetase (iNOS), NO formation, glutathione depletion, and disruption of mitochondrial function [37, 38]. HIV-1 accessory protein, viral protein R (Vpr), is a 14-kDa, 96-amino-acid nuclear protein that has been isolated from the CSF of patients with HAD [39]. A study by Jones et al. [40] reports that Vpr protein and its transcripts were detected in the brains of HIV-infected persons and proposed Vpr is neurotoxic. Soluble Vpr was associated with neuronal apoptosis, involving cytochrome c extravasation by increasing mitochondrial permeability, p53 induction, and activation of caspase-9 [40]. Nef, a major determinant of lentiviral infection, modulates a number of signaling pathways and is critical in disease progression. It is known to play an important role in HAD pathogenesis. Nef is found predominately in astrocytes within the brain of HIV-1-infected patients [41]. Nef-induced chemokine ligand 2 (CCL2)/monocyte chemotactic protein-1 expression by infected astrocytes facilitates the trans-endothelial migration of HIV-1-infected monocytes into the CNS [42, 43]. Tat protein, also known as transactivating nuclear regulatory protein, is secreted by HIV-1-infected cells which binds to the cellular factors and facilitates their phosphorylation thus effectively enhancing viral transcription [44]. It has been identified in the brains of HIV-1-infected individuals with known CNS infection and is known to trigger oxidative stress-dependent apoptosis of neurons both in vitro and in vivo [45–47].

HIV-1 proteins possess amyloidogenicity in several studies. HIV-1 Tat inhibits neprilysin which is an Aβ degrading enzyme, thus leading to elevated levels of Aβ in the brains of HIV-1-infected individuals [48]. HIV-1 Tat is also associated with destruction of endolysosomal structure and function, increasing neuronal Aβ generation [49]. Along with HIV-1 Tat, the gp120 protein promotes release of Aβ and accumulation of amyloid aggregates [50]. HIV-1 gp120 also induces β-amyloid precursor protein APP accumulation causing axonal injury to corpus callosum [51]. Possibly, amyloid fibrils derived from HIV-1 proteins themselves might pro-

mote viral entry and enhance HIV-1 infection [52]. For instance, peptides derived from semen, the fragments of prosthetic acid phosphatase (PAP), termed semen-derived enhancer of virus infection (SEVI) and semenogelins 1 and 2 (SEM1 and SEM2), are known to assemble into amyloid fibrils and enhance viral infectivity by promoting target cell attachment [53, 54]. The ectodomains of simian immunodeficiency virus (SIV) and HIV gp41 (e-gp41) are noted to accumulate as highly stable and high molecular weight extracellular aggregates in the brains of the infected patients linked with dementia and thus associated with neurological damage [55]. Enhancing peptides (EP) derived from HIV-1 gp120 co-receptor-binding region forms amyloid fibrils and possesses the ability of enhancing viral infectivity by enriching virus binding properties, facilitating membrane interactions and promotes viral entry [56]. These studies shed light on the fact that amyloid fibrils originate in the host environment. The host-derived viral proteins trigger formation of amyloid fibrils that are likely to aggregate. However, aggregation propensities vary among proteins due to sequence variations and environmental conditions [57, 58].

In this chapter, we predict possible amyloidogenic regions of HIV-1 proteins (from brain isolates) that have the potential to form amyloid fibrils and study the intrinsic properties of these proteins that promote protein aggregation.

33.3 Amyloidogenicity Prediction

There are various Internet-online tools available to predict amyloidogenic regions from protein sequence data. These tools reflect the complex biological mechanisms associated with amyloidosis. There is a high correlation between the relative aggregation rates for a wide range of polypeptides and their physicochemical features such as charge, propensity of secondary structure formation, and hydrophobicity [57, 59]. In order to determine the aggregation propensity of proteins, there are specific segments of peptides that can tend to aggregate, providing an indication of sequence dependence of aggregation properties [60]. The available tools take into account various parameters of the proteins responsible for amyloidogenicity including hydrophobicity, aggregation propensity scale, β-strand contiguity, average packing density, and hexapeptide conformational energy [61–80]. Table 33.1 summarizes the utility of each tool used in our study with its URL.

In our study, we have used consensus prediction results of servers such as *Fold Amyloid*, *AGGRESCAN*, *Waltz*, *TANGO*, *MetAmyl*, *AMYLPRED2*, and *PASTA*. Each of these programs has its own assumptions and implementations for predictors. They vary in their algorithms used for identification of aggregation-prone sites. *Fold Amyloid* makes the prediction of amyloid regions on the basis of two important properties, expected probability of backbone-backbone hydrogen bonds formation and expected packing density of residues. These two properties are considered to be primarily responsible for formation of amyloid fibrils. Regions with strong expected packing density are considered to be involved in amyloid formation [61, 62].

Table 33.1 Amyloidogenicity prediction web servers with their URL

Web server	URL	Method predicts
Fold Amyloid	http://bioinfo.protres.ru/fold-amyloid/	Amyloidogenic region based on HH bond formation and expected packing density
AGGRESCAN	http://bioinf.uab.es/aggrescan/	Amyloid regions as "hot spots" based on aggregation-propensity scale
Waltz	http://waltz.switchlab.org/	Ordered amyloid-forming regions in functional amyloids
TANGO	http://tango.crg.es/	Aggregation nucleating regions based on physicochemical properties of secondary structure formation
MetAmyl	http://metamyl.genouest.org/	Amyloidogenicity of sequence
AMYLPRED 2.0	http://aias.biol.uoa.gr/AMYLPRED2/	Consensus amyloid regions based on prediction of 5 out of 11 successful methods
	AmyloidMutants	Structural and mutational landscapes of amyloid fibrils using an ensemble algorithm
	Amyloidogenic pattern	Scans query sequence for presence of amyloidogenic pattern
	Average packing density	Regions with strong expected packing density
	Beta-strand contiguity	Propensities for β-strand formation
	Hexapeptide conformational energy	Strands forming stacked β-sheet structure
	NetCSSP	Predicts of contact-dependent secondary structure
	Pafig	Amyloid-forming hexapeptides based on support vector machines
	SecStr	Possible conformational switches between helix and sheet

(continued)

Table 33.1 (continued)

Web server	URL	Method predicts
	AGGRESCAN	
	TANGO	
	Waltz	
PASTA 2.0	http://protein.bio.unipd.it/pasta2/	β-strand intermolecular pairing between the possible peptides

Galzitskaya et al. [63] presented a positive relation among packing density, hydrophobicity, and β-sheet propensity. The statistics of the observed number of contacts per residue, i.e., packing density and hydrogen bonds observed in protein structures, were analyzed by constructing a database of spatial structures of proteins having <25% sequence identity. The packing density was calculated for each amino acid residue. Two residues were considered close to one another if any pair of their non-hydrogen atoms were at a distance <8 Å. Subsequently, the mean observed packing density and probability of hydrogen bond formation for each of the 20 amino acids was calculated. Then, profiles of packing density and probability of hydrogen bond formation for each protein sequence were compiled using the scale of deduced expected values. The prediction accuracy was about 80% for amyloidogenic and 72% for non-amyloidogenic peptides, when tested for 144 amyloidogenic and 263 non-amyloidogenic peptides [62]. *AGGRESCAN* predicts the aggregation-prone segments in protein sequences and allows analysis of the effect of mutations on protein aggregation propensities and the comparison of the aggregation properties of different proteins or protein sets [60]. It calculates aggregation "hot spots" in sequences based on aggregation-propensity scale for natural amino acids based on aggregation-propensity values per amino acid termed as aaAV, or a3v, that are derived from experimental data [64]. The program then calculates a4v, which is average of aggregation-propensity values per amino acid (a3v's) over a sliding window and assigns to central residue of the window. A plot of a4v over the entire sequence defines the aggregation profile of the sequence. A segment of a protein sequence is considered as a putative aggregation hot spot (HS) if there are five or more consecutive residues with an a4v larger than a threshold value without any aggregation-breaker residue such as proline. The hot spot threshold (HST) was defined as the average of the a3v of the 20 natural amino acids weighted by their frequencies in the Swiss-Prot database [65]. Several other parameters were calculated and described, including average a4v for each hot spot, the area of the aggregation profile above the HST, the total area (the HST being the zero axis), and the area above the HST of each profile peak identified as a hot spot [60]. *Waltz* uses a position-specific scoring matrix to determine the amyloid-forming sequences. It distinguishes between ordered amyloid-forming structures from that of amorphous β-sheet aggregates and thus provides identification of amyloid-forming regions in functional amyloid regions based on knowledge that their functionality depends on a high intrinsic degree of order [66]. The amorphous aggregates do not have an

ordered intermolecular interaction in contrast to ordered cross β-structures and are common to most proteins. These two categories of aggregates not only differ in kinetics of fibril formation but also differ in certain biological features such as chaperone affinities and degradation pathways [67–69]. *TANGO* predicts aggregation nucleating regions in proteins as well as the effect of mutations and environmental conditions on the aggregation propensity of these regions. It utilizes a statistical mechanics algorithm that is built upon physicochemical properties of secondary structure formation (β-sheet formation) with an assumption that aggregate core regions are often buried. This calculation was tried using dataset of 179 peptides of more than 20 proteins including new set of 71 peptides derived from human disease-related proteins, including prion protein, lysozyme, and beta2-microglobulin. TANGO was able to correctly predict the experimentally determined regions, identifying 65 of the 71 peptides (91%) giving a correlation of 0.70 [70]. *MetAmyl* is considered to be highly statistically informative of amyloid hot spots. It is based on a logistic regression model that aims at weighting predictions from a set of accepted algorithms. It is also a resource in highlighting the effect of point mutations in human amyloidosis [71]. *AMYLPRED2* is a consensus-based prediction algorithm that provides "aggregation-prone" regions by combining the results of different tools and thus imparting greater sensitivity and specificity for the results obtained [72]. AMYLPRED2 combines the results of various tools including *AGGRESCAN*, *TANGO*, *Waltz*, *AmyloidMutants*, *amyloidogenic pattern*, *average packing density*, *beta-strand contiguity*, *hexapeptide conformational energy*, *NetCSSP*, prediction of amyloid fibril-forming segments (*Pafig*), and *secondary structure prediction (SecStr)* (possible conformational switches). *AmyloidMutants* predicts the structural and mutational landscapes of amyloid fibrils using an ensemble algorithm [73]. *Amyloidogenic pattern* scans the query sequence for the presence of amyloidogenic pattern identified as highly related to the formation of amyloid fibrils {P}-{PKRHW}-[VLSCWFNQE]-[ILTYWFNE]-[FIY]-{PKRH} [74]. *Average packing density* detects both amyloidogenic and disordered regions in protein sequence. Regions with strong expected packing density are associated with amyloid formation, while the ones with weak expected packing density correspond to disordered region [63]. *Beta-strand contiguity* predicts the β-strands in the amyloid fibril core depending on the amino acid sequence. It is based on calculation on average β-strand propensity score for peptide windows of varying sizes (from 4 to 20 residues) within the sequence [75]. *Hexapeptide conformational energy* results are based on Zhang et al. [76], identification of peptide segments that fit as β-strands in a stacked β-sheet structure based on the solved microcrystal structures obtained from the peptides GNNQQNY and NNQQNY. *NetCSSP* is an artificial neural network (ANN)-based algorithm that predicts contact-dependent secondary structure propensity in protein sequences grounded on the premise that local secondary structure of a protein can change depending on the tertiary environment such as intermolecular interactions and misfolding [77]. *Pafig* identifies hexapeptides associated with amyloid fibrils based on support vector machines [78]. *SecStr* predicts potential conformational switches in a protein sequence both as α-helices and β-sheet strands. It is based on the result of at least three out of six secondary structure prediction programs to

predict a particular region as both [79]. Prediction of Amyloid Structure Aggregation (*PASTA2.0*) predicts the portions of a given input sequence that are more likely to form the cross-β core of fibrillar aggregates [80]. The server provides information about intrinsic disorder and secondary structure predictions that match the aggregation data. The algorithm is based on the observation that the ability to form the cross-β structure is an inherent characteristic of polypeptides [81] and the same mechanism is responsible for β-sheet formation both in globular proteins and in cross-β aggregates. PASTA predicts the interacting portions of a protein that are stabilizing the cross-β structure employing an energy function based on the propensities of two residues to be found within a β-sheet facing one another on neighboring strands. It has been re-derived on a larger dataset of globular protein domains involving more than 424 peptides with experimental information about aggregation. The predictions take into account structural characteristics of the sequences and predict protein dimer formation. The server with a false-positive detection rate of <5% and with a sensitivity of 40% has high specificity. The examination of point mutations is possible with the use of a mutate option, and the effects on the aggregation ability of a protein sequence can be studied. It provides a graphical representation of the disordered versus aggregation probability as well as aggregation versus helix or strand forming probabilities [80] (Fig. 33.1).

The brain-derived sequences of HIV-1 proteins, Env (P12488), viral infectivity factor (Vif) (P20877), regulator of expression of virion proteins (Rev) (P12485), group-specific antigen (Gag) (P20873), Nef (P12479), and viral protein U (Vpu) (P12516) were obtained from the Swiss-Prot (http://www.uniprot.org/) database. Three additional sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and used for the analysis, Pol (AAB05599.1), Tat (ABL10649.1), and Vpr (AAD03202.1). These representative sequences are a subset of over 400 brain-derived protein sequences from different geographical locations and were analyzed for sequence variations. This was compiled and published in the volume 1 of *Global Virology* [81]. The representatives of the compiled sequences are subjected to amyloidogenic prediction servers mentioned in Table 33.1. Amyloid regions predicted in at least five web servers are considered as consensus results that are provided in Table 33.2 and were used for further analysis.

33.3.1 Structural Proteins

There are eight amyloidogenic regions predicted in the Env protein sequence spanning from 20–43, 169–182, 278–289, 407–425, 531–537, 661–705, and 747–783 to 831–840. Region 1 (20–43) is a signal peptide region and marks the start of gp120, while region 2 (169–182) falls within the variable region V2, and the region 4 (407–425) lies partly at the end of variable region V4. These findings are in agreement with Tan et al. (2014); they have shown that the experimentally identified amyloidogenic peptides of HIV-1 gp120 co-receptor promoted HIV-1 infection [56]. Regions

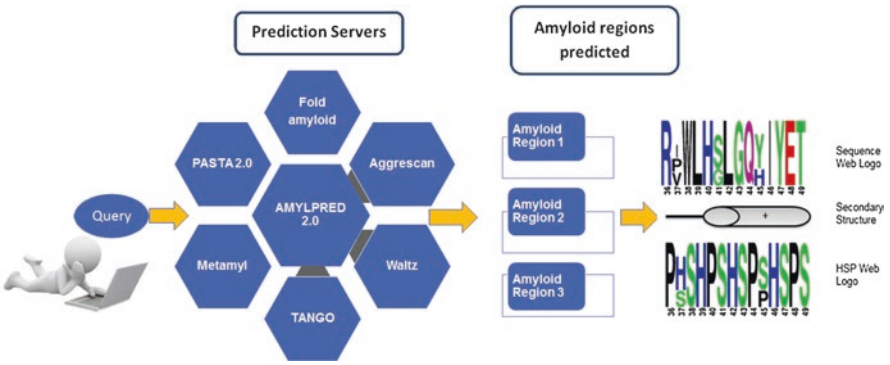


Fig. 33.1 The query sequence is submitted to the seven prediction programs. The programs provide the positions of predicted amyloid regions in the query sequence in the form of hits. Results of these programs are compiled and consensus amyloid regions are displayed. Secondary structure is predicted for these consensus amyloid regions in PsiPred and is then mapped to sequence and HSP web logos generated to study variations

661–705, 747–783, and 831–840 are present in gp41. The stretch 660–708 shows parallel aggregation with energy from −25.54 to −27.45 in the PASTA2.0 prediction findings (measured in PASTA Energy Units (PEU); 1PEU = 1.192 Kcal/mol).

33.3.2 Enzymatic Proteins

There are nine consensus amyloid regions predicted in the Pol protein spanning 101–123, 331–351, 524–541, 681–692, 702–719, 774–806, 810–823, 886–901, and 913–921. Parallel pairing was observed in PASTA2.0 in the region 787–804 with energy from −8.27 to −10.13PEU. In the case of the Vif protein, four consensus amyloid regions predicted spans 5–31, 59–72, 104–116, and 143–156. Parallel aggregation is observed in the predicted region of 5–13 and 7–31 with energy between 6.26 and −8.68PEU. Parallel aggregation is observed in the region having stretch of ordered residues.

33.3.3 Regulatory Proteins

The two consensus amyloid regions predicted in the Rev protein span 1–14 and 45–55. Some aggregation is observed in the predicted areas with a decrease in disorder levels in PASTA2.0 results. Parallel pairing was observed in the region 1–14 with energy −5.73 PEU in PASTA2.0 predictions. The region has an ordered stretch of residues. The consensus amyloid region predicted in the Tat protein spans 19–47. PASTA2.0 results show antiparallel aggregation in the region of 19–40 with energy

Table 33.2 The predicted amyloid regions from HIV-1 protein sequences. Regions predicted by at least five out of seven servers are included in the table

Protein name accession	Position	Predicted amyloid regions by at least five out of seven programs	Fold Amyloid	Aggrescan[b]±	Waltz[b]	TANGO[b]±	MetAmyl	AMYLPRED2 Consensus	PASTA
NEF brain P12479	Region 1 121–140 Region 2 145–159 Region 3 190–198	ILDLWVYHTQGYFPDWQNYT VRYPLTFGWCFKLVP REVLQWRFD	67–71 122–128 151–159 192–197 201–206	8–12 91–101 122–130 149–158 192–198	8–13 65–72 98–103 109–116, 121–140 147–158, 171–177, 192–198 *Waltz*± No predicted regions	150–155	40–47 52–60 92–102 111–116 125–134 155–160 190–198	98–101 112–113 122–130 149–158 192–198	121–129
VPU brain P12516	Region 1 3–35	PLQISAIVALVVAAIIAIVVWSIALLEYR	6–35	4–31 65–70	5–31 *Waltz*± 5–31	6–26	4–27	5–31	6–28
VIF brain P20877	Region 1 5–31 Region 2 59–72 Region 3 104–116 Region 4 143–156	WQVMIVWQVDRMRIRTWNSLVKHHMYIS LGDARLVITTYWGLH DQLIHLYYFDCFS GSLQYLALTALIKP	5–13 15–20 26–31 37–42 63–72 87–92 104–114 146–153	5–14 25–29 31–37 61–71 105–116 121–129 144–155	5–11 19–25 35–41 53–58 62–71 103–129 143–152 *Waltz*± 5–11 103–113	5–13 64–72 147–154	5–14 23–28 62–70 81–88 94–100 105–110 124–133 138–147 149–154	5–14 62–72 105–114 122–129 145–154	5–13 7–31

VPR brain AAD03202.1	Region 1 36–49 Region 2 56–76	RPWLHGLGQHIYET GVEAIIRILQQLLFIHFRIGC	16–22 36–40 44–48 59–74	56–75	15–23 42–73 *Waltz*± 43–51 65–72	56–70	42–47 56–76 79–84	46–48 56–76	57–74
REV brain P12485	Region 1 1–14 Region 2 45–55	ELLKTVRVIKFLYQ SGWILSTFLGR	7–14 23–37 46–55	1–14 45–55 91–96	9–14 42–54 *Waltz*± No predicted regions	47–54	4–14 42–55 82–89 91–96	7–14 46–55	2–13
TAT brain ABL10649.1	Region 1 19–47	KTACTNCYCKKCCFHCQVCFTTKGLGISY	24–28 30–39	28–46	34–44 65–71 *Waltz*± No predicted regions	–	22–48 62–70	29–41	19–40

(continued)

Table 33.2 (continued)

ENV brain P12488	Region 1 20–43 Region 2 169–182 Region 3 278–289 Region 4 407–425 Region 5 531–537 Region 6 661–705 Region 7 747–783 Region 8 831–840	MLLGILMICSATDKLWVTVYYGVP KEYALFYKLDIVPI TNNVKTIIVQLNE PITLPCRIKQIINMWQEVG[a] LTLTVQA KWASLWNWFNITNWLWYIKIFIMIVGGLIG LIVFAVLSIVNRVR GFLALFWVDLRSLFLFSYHRLRDLLLIVTRIVELLGR RAFRAILHIP	11–29 33–44 66–71 109–114 124–130 172–181 191–195 214–218 223–227 283–287 346–351 369–374 380–384 387–395 418–422 445–449 480–486 513–518 531–535 537–543 551–555 560–569 574–583 589–593 617–627 635–641 663–687 689–703 749–755 757–766	15–42 69–73 111–117 119–123 125–131 170–179 194–201 215–219 221–226 254–262 280–291 316–321 343–349 352–357 371–390 412–426 441–448 479–488 510–524 526–544 562–575 577–581 586–604 634–643 660–705 745–800 806–816 829–839	20–42 49–57 67–72 87–96 108–113 125–144 153–160 167–179 197–203 221–229 256–264 273–297 312–323 355–362 370–403 414–424 439–450 510–545 547–570 585–593 598–625 631–643 662–714 745–765 769–780 787–805 807–818	19–28 34–41 171–175 284–288 680–689,693–701 747–764 770–781	22–29 33–46 48–56 122–132 154–164 175–182 191–205 213–218 221–226 238–260 268–275 278–289 291–297 312–322 340–348 354–363 369–374 385–390 397–402 408–420 424–429 439–448 480–488 500–505 531–537 540–547 562–570 593–605 634–643 677–704	20–27 34–41 172–175 283–288 381–383 417–420 444–448 531–535 542–543 635–641 680–689 693–701 747–754 758–764 769–780 833–837	661–705

			768–780 786–801 830–839		832–836 *Waltz*± 36–41 89–96 170–178 221–227 312–323 378–396 634–643 662–704 758–765 769–774 795–801		758–763 769–782 807–819 824–832 836–841		
GAG brain P20873	Region 1 75–92 Region 2 151–160 Region 3 258–285 Region 4 366–380	LTSLYNTVATLYCVHQRI TLNAWVKVIE VGEIYKRWIILGLNKIVRMYSPVSILDI AMSQVTNPATIMMQR	17–21 29–37 77–81 83–90 153–158 169–174 247–251 262– 269, 274–278 281–285 298–303 315–323 375–380	77–90 151–161 171–176 258–285 297–301 317–321 333–338 366–380 434–438 490–495	5–10 33–39 75–86 128–140, 143–149, 151–160 171–177 182–191, 201–206, 243–252, 258–284, 306–311, 372–377, 429–438, 493–498 *Waltz*± 75–86	78–88 155–160	6–12 32–37 75–92 124–130 134–139 142–148 153–160 188–196 236–241 269–274 278–287 309–315 318–325 351–356 366–372 487–493	31–38 75–90 134–145 152–160 170–175 247–251 258–285 320–324 366–372 434–435 493–495	78–89 261– 285 (265– 274)

(continued)

Table 33.2 (continued)

POL brain AAB05599.1	Region 1 101–123 Region 2 331–351 Region 3 524–541 Region 4 681–692 Region 5 702–719 Region 6 774–806 Region 7 810–823 Region 8 886–901 Region 9 913–921	KMIGGIGGFIKVRQYDQILIEIC PDIIIYQYMDDLYVGSDLEIG TEAVQKIANESIVIWGKI IKKEKVYLAWVP QVDKLVSAGIRKVLFLDG GIWQLDCTHLEGKIILVAVHVASGYIEAEVIPA QETAYFLLKLAGRW HLKTAVQMAVFIHNFK ERIVDIIATDI	54–64 110–114 116–122 139–148 230–234 241–245 269–273 283–287 297–303 313–317 333–338 340–344 362–369 382–389 423–427 492–497 535–539 554–567 578–583 635–641 656–662 686–692 713–718 787–794 813–818 844–849 891–899 916–921 936–943 981–985	1–6 58–72 101–134 140–155 184–193 244–248 264–272 282–287 299–304 313–323 333–341 364–369 416–436 442–448 531–541 560–574 577–587 591–596 651–661 681–691 706–719 746–752 783–804 812–823 839–855 890–901 913–921	30–43 50–63 65–72 106–112 115–122 136–158 188–194 266–272 281–294 297–306 311–323 331–351 395–400 404–431 444–452 469–475 489–503 526–538 553–575 577–586 591–597 621–626 633–643 646–663 672–682 686–691 713–719 737–744 747–755 774–806	128–133 333–337 534–539 686–690, 787–799 812–817 891–897	30–35 47–55 64–73 108–113 115–123 126–136 138–154 162–167 186–194 211–220 243–248 257–277 281–287 299–304 313–319 332–339 395–401 405–411 422–431 443–448 468–475 490–498 524–539 590–600 608–615 619–627 645–663 671–678 686–691 702–716	118–120 535–537 686–690 714–716 787–794 815–817 892–897 916–920	787–794 787–804

					810–820 832–841 843–849 864–869 892–899 905–921 931–941 962–968		746–756 767–772 786–804 825–830 834–844 860–866 875–881 886–899 906–911 914–923 931–937 960–984		
					WALTZ± 188–193 267–272 332–340 422–427 470–475 490–498 526–538 646–651 657–662 843–849 893–899 962–968				

[a]experimentally predicted amyloid region
[b]Results according to Amylpred2.0 parameters
± – Results of individual server

of −8.62 PEU for this brain isolate. This antiparallel aggregation site lies within the disulfide bonding region of Tat protein and thus is indicative of cross-beta pairing as positively detected by the program. However, the possible role of disulfide region in aggregation is not well understood as it varies depending on neighboring amino acids and solution conditions.

33.3.4 Accessory Proteins

There are three consensus amyloid regions predicted in the Nef protein spanning 121–140, 145–159, and 190–198. Parallel aggregation was observed in PASTA2.0 within regions 121–129 with energy of −5.34 PEU. The region 113–129 mediates dimerization and is involved in Nef-PTE1 (potential T cell epitopes) interaction; Nef induces downregulation of CD4 and Major Histocompatibility-I (MHC-I) and thus in turn is involved in enhancement of infectivity and prevents viral super infection [83]. The two consensus amyloid regions predicted in Vpr protein span 36–49 and 56–76. Parallel aggregation was observed in PASTA2.0 within the regions from 53 to 74 with pairing energy between −6.38 and −8.46 PEU. The region 36–51 falls in the oligomerization region of Vpr, while 53–74 precedes the H(S/N)RIG (histidine-serine/asparagine-arginine-isoleucine-glycine) motif. This motif is involved in Vpr-induced activation of the ataxia-telangiectasia and Rad3-related(ATR) pathway and in turn arrests cells in the G2 phase of the cell cycle [84]. The consensus amyloid regions predicted in Vpu protein span from 3 to 35. Parallel aggregation was observed in PASTA2.0 within the predicted region with energy between −18.37 and −20.69 PEU. This region is a transmembrane helical region.

SecStr predicted an α-helix to β-sheet transition in the predicted region. The four consensus amyloid regions predicted in the Gag protein span 75–92 that lie within the Matrix protein p17, as well as for 151–160, 258–285, and 366–378 that lie within the capsid protein p24. Parallel aggregation was observed in PASTA2.0 within the regions 78–89 with energy −6.27 PEU and 261–285 with energy from −6.62 to −7.64 PEU. The region 366–378 lies in the spacer peptide 1 that is generated when the protein is cleaved by the viral proteases. However, no parallel pairing was observed in this region.

33.4 Hydrophobicity and Sequence Variation

Amyloid formation is a process of transition of soluble polypeptides into insoluble amyloid via a partially unfolded intermediate. This thermodynamically unfavorable state rapidly converts into stable amyloid fibrils [80, 85–88]. As mentioned earlier, the factors including sequence, hydrophobicity, charge, and secondary structure govern the amyloid-forming potential of each protein. Some amyloid-forming polypeptides are characterized by glutamine repeats as in the case of yeast and prions.

Lopez and Serrano (2004) have experimentally derived a consensus pattern associated with the amyloid-forming fibrils by performing mutagenesis analysis on de novo-designed amyloid peptide STVIIE [58, 74]. The derived sequence pattern {P}-{PKRHW}-[VLSCWFNQE]-[ILTYWFNE]-[FIY]-{PKRH} is validated through in silico scanning of already known amyloid proteins. In addition, they studied the role of the position dependence of certain residues affecting the nature of amyloid fibrils [74]. Not only the amyloid-forming proteins differ largely in sequence and structure, but they are also observed to be mutation sensitive. Therefore, one cannot solely rely on the presence of consensus patterns and must consider other factors that drive aggregation. It has been established that hydrophobicity correlates with aggregating potential of polypeptides as in the case of amylin and β-amyloid that are found in Alzheimer's disease, wherein hydrophobic interactions play a significant role in influencing fibrillogenesis [89, 90]. Aromatic amino acids are observed to have an increased propensity to form amyloids, a characteristic promoted by hydrophobicity, β-sheet propensity, and planar geometry [91–93].

Mutations and their role in amyloidogenesis have been extensively studied to obtain insights with respect to amyloid fibril formation and to identify mutations that may circumvent misfolding of the protein. For instance, 19 mutations have been identified in *Apolipoprotein A-I* associated with amyloidosis in a study performed by Rowczenio et al. [94], while Gessel et al. (2012) studied alterations in amyloid β-protein oligomerization in cases of familial Alzheimer's disease [95]. Another study by Smaoui et al. (2015) predicted mutations affecting amylin stability and amyloidogenicity [96]. Even a single mutation is observed to promote altered dimer formation in the case of amyloidogenic light chain protein AL-09 contributing fibril structure [97]. Moreover, mutations in the certain proteins inhibit the self-assembly process and hence prevented fibril formation [98]. Therefore these polypeptide sequences appear to contain sensitive sites in amyloid regions that even with a single mutation can alter aggregation rates, whereas mutations in other areas of these proteins do are less effective [13, 99].

In our study, we have estimated the hydrophobicity of the representative HIV-1 proteins using ProtScale on the basis of Kyte-Doolittle values (http://web.expasy.org/protscale/). The predicted regions of the corresponding HIV-1 proteins were represented as highly hydrophobic "*H*," moderately hydrophobic "*S*", and polar "*P*" amino acids , referred to as *HSP* strings to display the hydrophobicity index of the peptide. Amino acids such as isoleucine (I), valine (V), leucine (L), phenylalanine (F), cysteine (C), methionine (M), and alanine(A) have hydrophobicity values between 1 and 4.5 and are highly hydrophobic, designated by "H." Amino acids such as glycine (G), threonine (T), serine (S), tryptophan (W), tyrosine (Y), and proline (P) have hydrophobicity values between −0.4 and −1.6 and are moderately hydrophobic, designated by "S," while amino acids such as histidine (H), glutamic acid (E), glutamine (Q), aspartic acid (D), asparagine (N), lysine (K), and arginine (R) have hydrophobicity values between −3.2 and −4.5 and are polar, designated as "P."

The predicted amyloid regions in the representative HIV-1 proteins described in Table 33.2 are mapped to the sequence logo generated for over 400 brain-derived

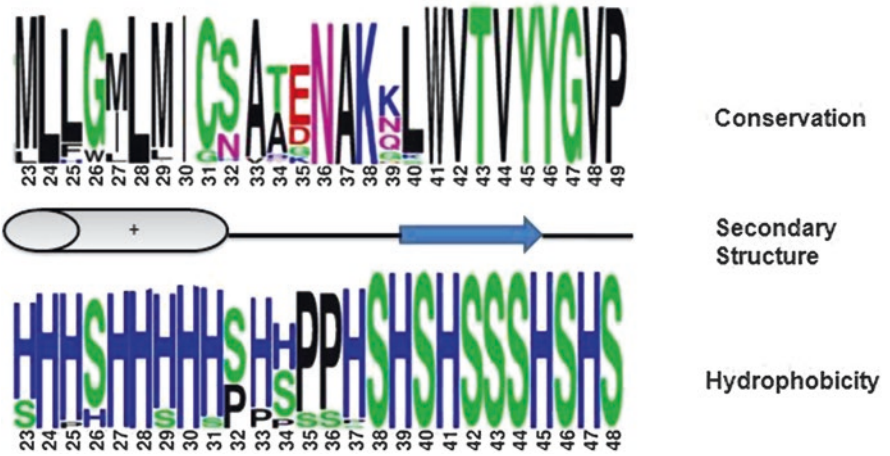


Fig. 33.2 Env protein amyloid region 1, 20–42. The figure indicates change in hydrophobicity at some positions due to residue substitutions (unless otherwise stated, show sequence conservations (*tall characters*), semiconserved substitutions (*stacked with similar colored characters*), and variations (*stacked with different colors*) in sequence web logo, and their corresponding HSP pattern is also presented to indicate the change in hydrophobicity along with PsiPred predicted secondary structures (α-helix and β-sheet)

protein sequences published in the volume 1 of *Global Virology* [82]. These logos give a graphical representation of the multiple sequence alignment representing stack of symbols for each position in the alignment denoting sequence conservation (http://weblogo.berkeley.edu/). The mapped regions of the sequence logos represent the predicted amyloid regions of entire group of brain-derived HIV-1 proteins from different geographical locations, thus making it reproducible. The mapped regions are represented as HSP strings and the corresponding web logos are presented in Figs. 33.2–33.28. The sequence variations identified are essential to understand whether the change in sequence affects the surrounding hydrophobicity and helps to further study contribution of hydrophobicity in amyloidogenicity [89, 90]. The significant variations and their corresponding HSP pattern are presented as sequence logos along with PsiPred predicted secondary structures in Figs. 33.2–33.28.

For the structural protein Env, the predicted sites 20–42 and 661–705 show the presence of highly hydrophobic and moderately hydrophobic residue stretches separated by a few polar amino acids. The hydrophobicity indexes of these regions are from 2.0 to 3.0 and 2.0 to 3.2, respectively, using Kyte-Doolittle plots. The hydrophobic stretches are almost conserved for this site as displayed in the web logo. The sites 531–537, 747–783, and 831–839 are hydrophobic in nature with hydrophobicity indexes from 1.8 to 2.5, 1.8 to 2.6, and 1.0 to 2.0, respectively. However, the experimentally verified site 407–425 (amyloidogenic) is composed of an HSP string with hydrophobicity index of 0.0–1.0. Similarly, the remaining sites 169–182 and

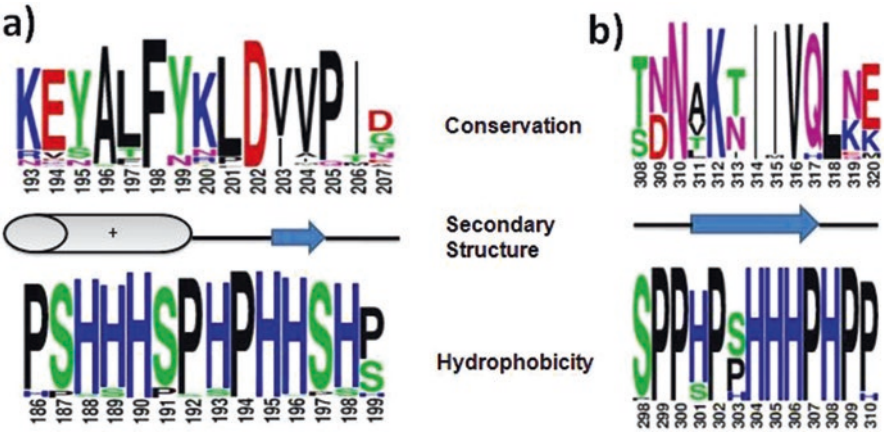


Fig. 33.3 (**a**) Env protein amyloid region 2, 169–182. (**b**) Env protein amyloid region 3, 278–289. The figure indicates change in hydrophobicity at some positions due to residue substitutions

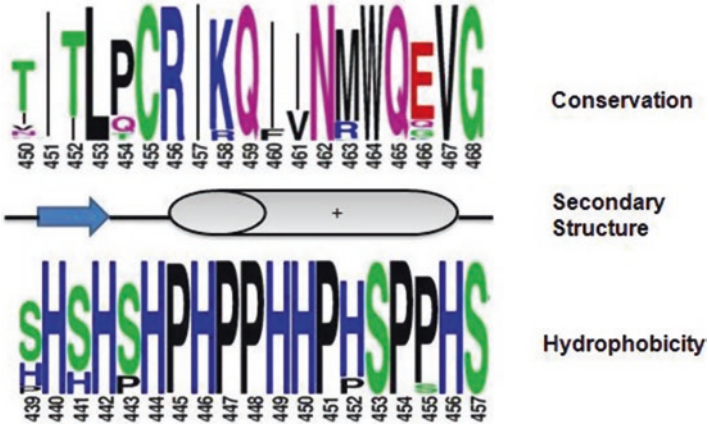


Fig. 33.4 Env protein amyloid region 4, 410–425. The figure indicates change in hydrophobicity at some positions due to residue substitutions

278–289 showed hydrophobicity indexes of 0.0–1.2. Significant variations are observed at positions 23, 25, 26, 29, and 31–37 in region 1 (Fig. 33.2), characterized by H ⇔ P, S ⇔ H, and S ⇔ P substitutions. Similar variations are observed in region 2 (Fig. 33.3a) at positions 186–189, 191–193, and 197–199. Region 3 (Fig. 33.3b) and 5 (Fig. 33.5) appears to be conserved with variations only at positions 301, 303, and 568, respectively. Variations in region 4 (Fig. 33.4) are at positions 439, 441, 443, 452, and 455. In region 6 (Fig. 33.6), the residues at position 710–741 are quite conserved with respect to hydrophobicity, while the stretch 697–709 is variable with S ⇔ P substitutions at positions 697, 700,706, and 709. S ⇔ P transitions are seen at position 794 of region 7 (Fig. 33.7) and 815 and 867 of region 8 (Fig. 33.8),

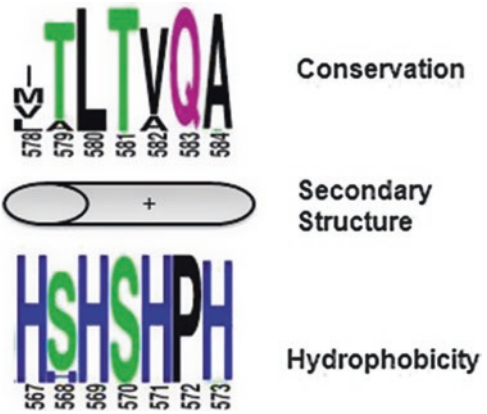


Fig. 33.5 Env protein amyloid region 5, 531–537. The figure indicates slight change in hydrophobicity with respect to residue substitutions

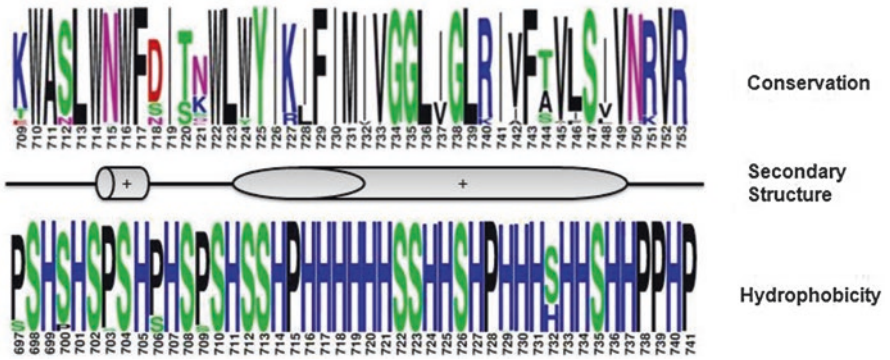


Fig. 33.6 Env protein amyloid region 6, 661–705. The figure indicates change in hydrophobicity at some positions due to residue substitutions

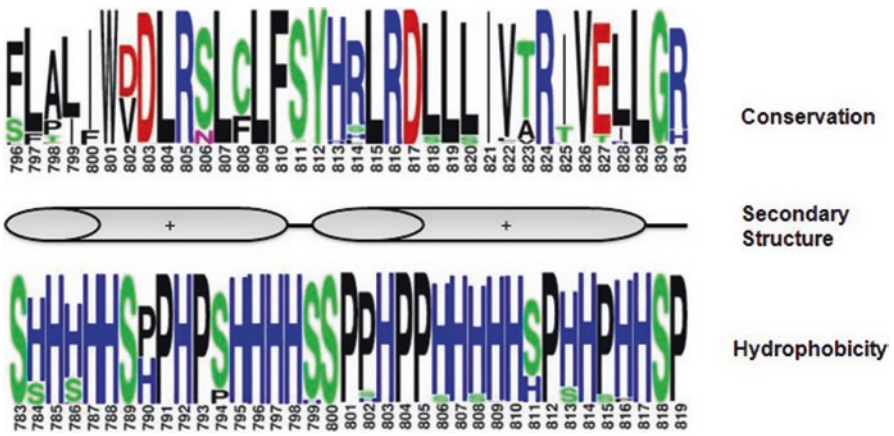


Fig. 33.7 Env protein amyloid region 7, 747–783. The figure indicates change in hydrophobicity at some positions due to residue substitutions

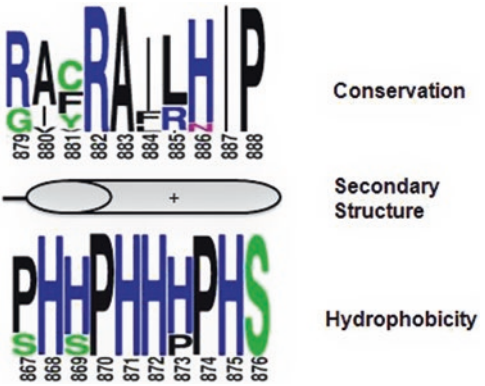


Fig. 33.8 Env protein amyloid region 9, 831–840. The figure indicates change in hydrophobicity at some positions due to residue substitutions

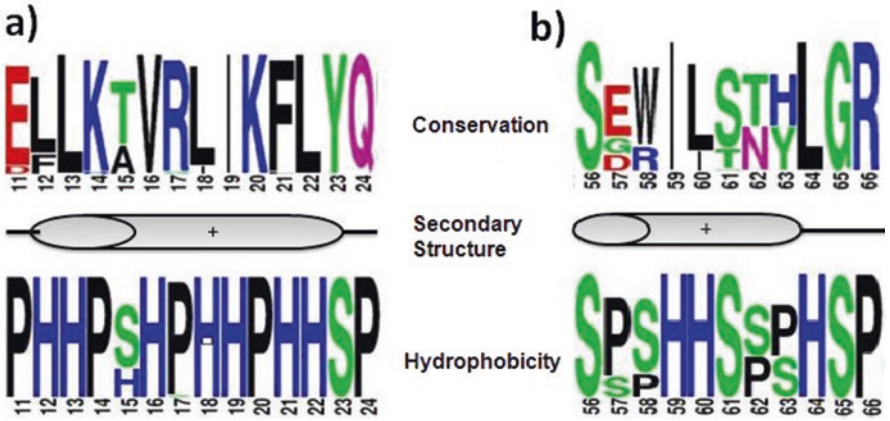


Fig. 33.9 (**a**) Rev protein amyloid region 1, 1–14. (**b**) Rev protein amyloid region 2, 45–55. The figure indicates change in hydrophobicity that are more in (**b**) compared to (**a**), with respect to residue substitutions

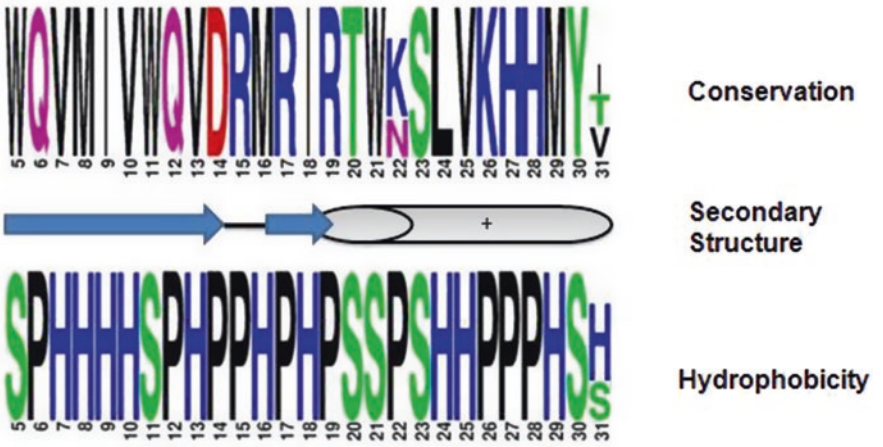


Fig. 33.10 Vif protein amyloid region 1, 5–31. The figure indicates no change in hydrophobicity with respect to residue substitutions

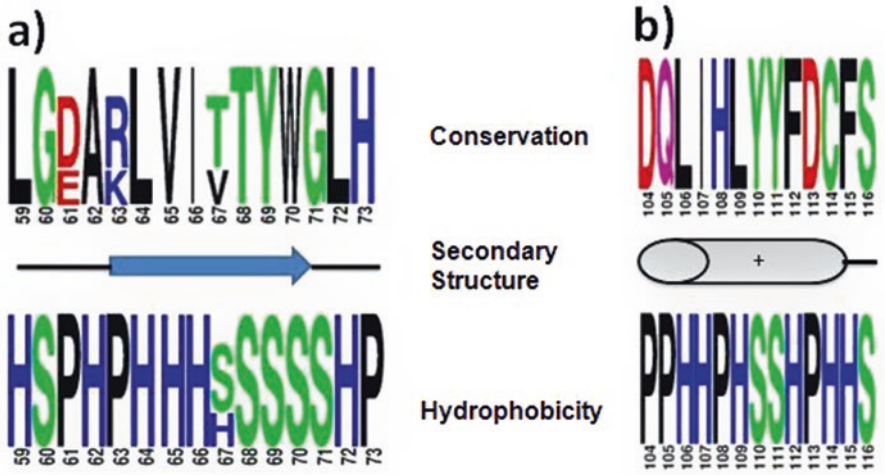


Fig. 33.11 (**a**) Vif protein amyloid region 2, 59–72. (**b**) Vif protein amyloid region 3:104–116. The figure indicates no change in hydrophobicity with respect to residue substitutions

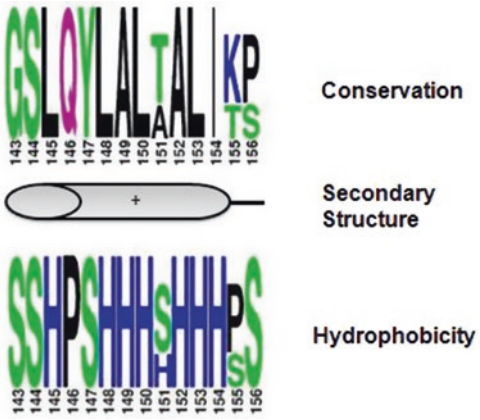


Fig. 33.12 Vif protein amyloid region 4, 143–156. The figure indicates change in hydrophobicity at some positions due to residue substitutions

while H ⇔ P transitions are observed at 790 of region 7 and 873 of region 8. Positions 784, 786, 806, 808, 811, and 869 are characterized by H ⇔ S substitutions. For regulatory protein Rev, the predicted amyloid region 1 (Fig. 33.9a), 1–14, consists of highly hydrophobic residues separated by polar residues, while the region 2 (Fig. 33.9b), 45–65, is moderately hydrophobic in nature. In accordance to the Kyte-Doolittle plot, the region 1–14 has a hydrophobicity score from 0.5 to 1.0 and region 45–65 has score from 0.5 to 1.2. The sequence and HSP logo shows significant variations at position 15, 57, 58, 62, and 63. However, the T ⇔ A transition at position 15 does not reflect major transitions in the HSP string, while variations at 57, 58, 62, and 63 indicate S ⇔ P transitions. In the case of Vif protein, the region 1 (Fig. 33.10) 5–31 and region 3 (Fig. 33.11b) 104–116 consist of HSP residues, while region 2, 59–72 (Fig. 33.11a), and region 4, 143–156 (Fig. 33.12),

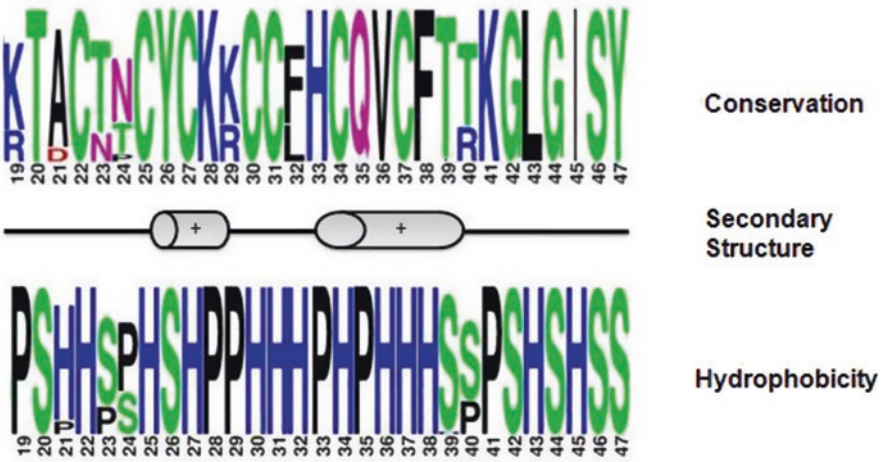


Fig. 33.13 Tat protein amyloid region 1, 19–47. The figure indicates change in hydrophobicity at some positions due to residue substitutions

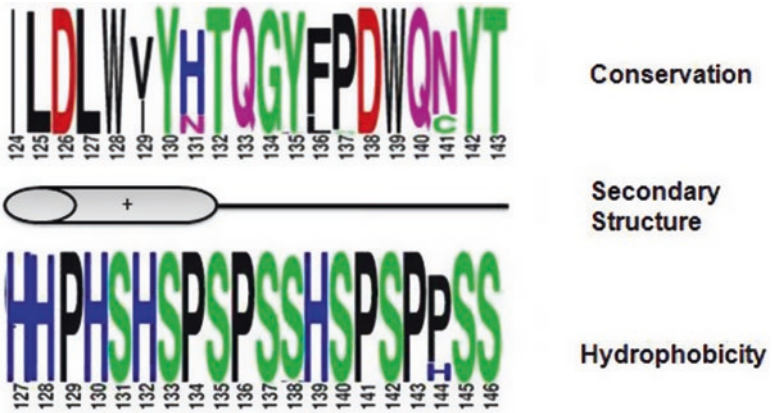


Fig. 33.14 Nef protein amyloid region 1, 121–140. The figure indicates change in hydrophobicity at some positions due to residue substitutions

are composed of stretches of continuous hydrophobic residues. Kyte-Doolittle plots give a hydrophobicity score of 0.5–1.2 and 0.5–1.0 for region 1 and 3, respectively. While regions 2 and 4 have a score of 1.0 to 1.5. Significant variations are observed at positions 31, 67, 151, and 155 including S ⇔ P and S ⇔ H transitions. However, the lesser variation can be attributed to small sample size of Vif protein. For Tat protein, the predicted region 1 (Fig. 33.13), 19–47, shows a stretch of hydrophobic residues with interrupting polar amino acids. The hydrophobicity score of this region is from 0 to 1.5. Significant variation is observed at positions 21, 23, 24, and 40 characterized by H ⇔ P and S ⇔ P transitions. In the case of the accessory proteins Nef, the predicted region 1, 121–140 (Fig. 33.14), consists of moderately

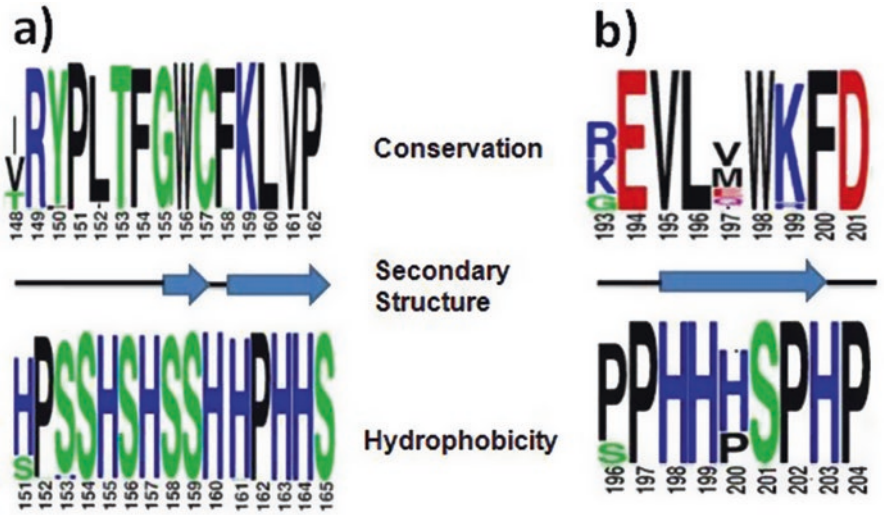


Fig. 33.15 (**a**) Nef protein amyloid region 2: 145–159. (**b**) Nef protein amyloid region 3: 190–198. The figure indicates change in hydrophobicity that are more in (**b**) compared to (**a**), with respect to residue substitutions

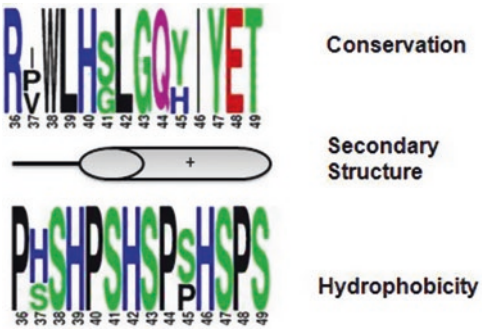


Fig. 33.16 Vpr protein amyloid region 1: 36–48. The figure indicates change in hydrophobicity at some positions due to residue substitutions

hydrophobic and polar residues with a hydrophobicity index of 0.1–0.7. The region 2, 145–159 (Fig. 33.15a), is quite hydrophobic with hydrophobicity index of 0.5–1.2. Region 3, 190–198 (Fig. 33.15b), consists of hydrophobic as well as polar residues with a hydrophobicity index of −1 to −0.5. Significant variation is observed at position 141 that has a transition N ⇔ C reflecting H ⇔ P transition and position 148 characterized by H ⇔ S transition and 196 and 200 indicating S ⇔ P to H ⇔ P transitions, respectively. For the Vpr protein, the predicted region 1, 36–48 (Fig. 33.16), and region 2, 56–76 (Fig. 33.17),consist of hydrophobic residues as well as polar residues with a hydrophobicity index ranging from −0.5 to 0 and 1 to 1.7, respectively. Significant variations were observed at positions 37 and 45 having H ⇔ S transition and S ⇔ P transitions, respectively.

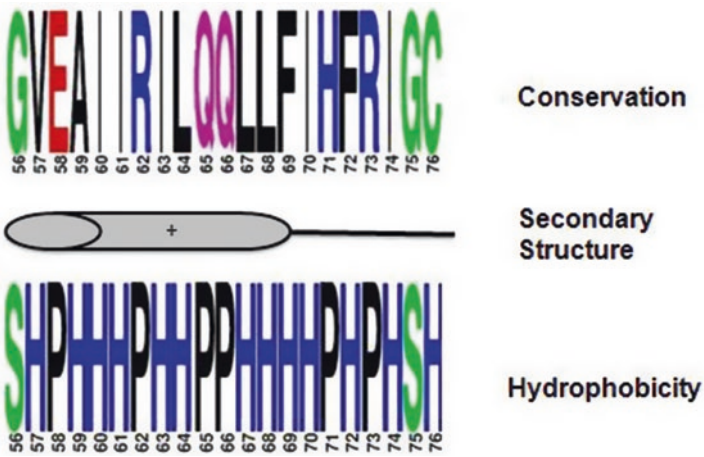


Fig. 33.17 Vpr protein amyloid region 2, 56–76. The figure indicates no change in hydrophobicity, and there are no substitutions

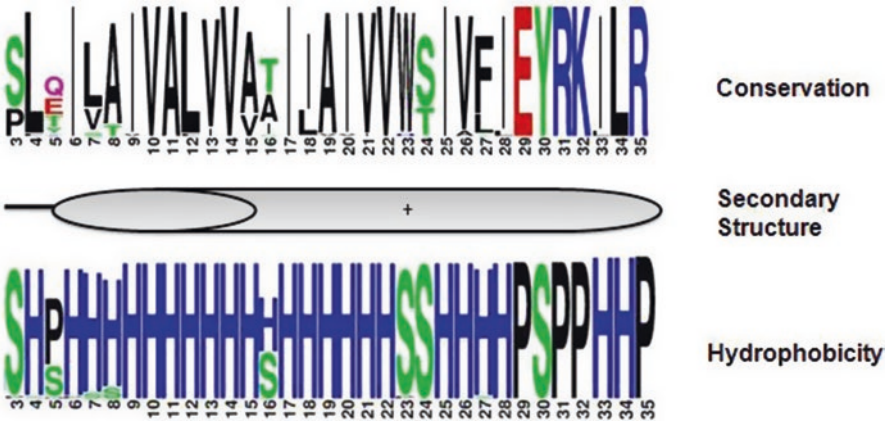


Fig. 33.18 Vpu protein amyloid region 1, 3–35. The figure indicates less change in hydrophobicity due to residue substitutions

In the Vpu protein, the predicted region 1, 3–35 (Fig. 33.18), is a long stretch of highly hydrophobic residues as well as the residues having high potential to form amyloid structures with a hydrophobicity score of 2.5–3.5 observed in Kyte-Doolittle plot. Significant variations are observed at the position 5; there is H ⇔ P substitution, while at positions 7, 8, and 16 are H ⇔ S substitutions. In Gag protein region 1, 75–92 (Fig. 33.19a); region 2, 151–160 (Fig. 33.19b); region 3, 258–285(Fig. 33.20); and region 4, 366–380 (Fig. 33.21), show the presence of highly hydrophobic and moderately hydrophobic residues separated by polar residues with a hydrophobicity index of 0.5 to 1.5, 1.5 to 2.2, 1.0 to 2.0, and 0.5 to 1.5, respectively.

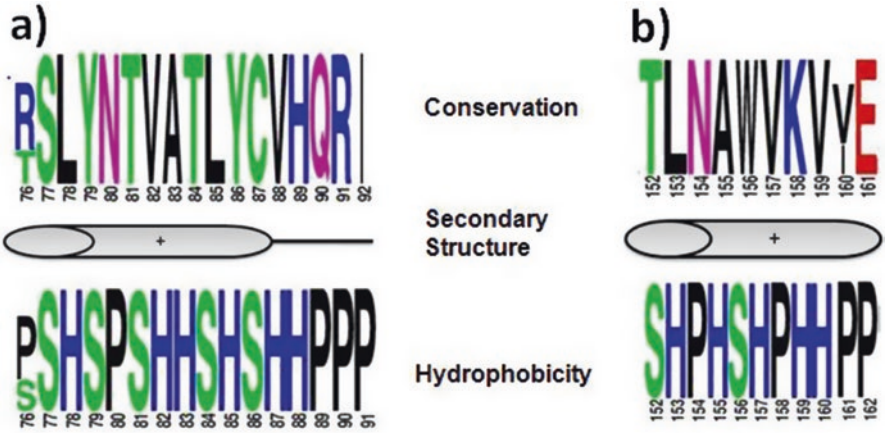


Fig. 33.19 (**a**) Gag protein amyloid region 1, 75–92. (**b**) Gag protein amyloid region 2, 151–160. The figure indicates no change in hydrophobicity in (**b**) compared to (**a**), with respect to residue substitutions

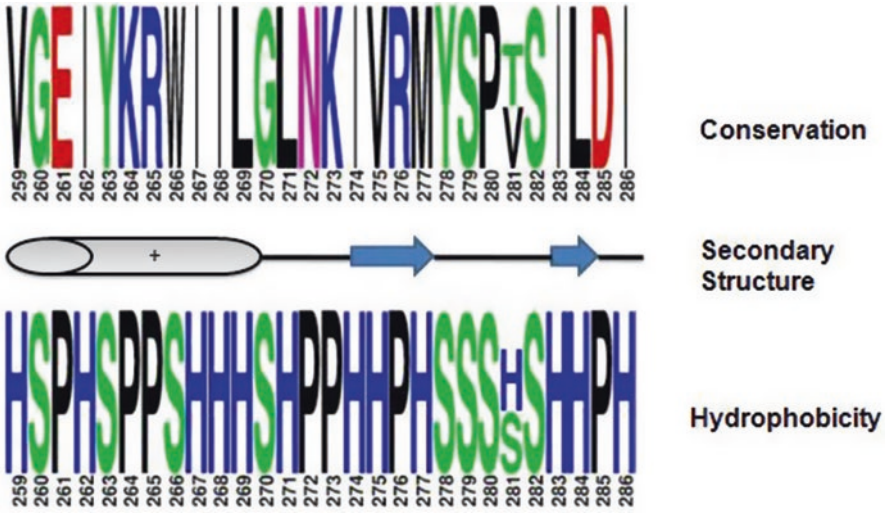


Fig. 33.20 Gag protein amyloid region 3, 258–285. The figure indicates no change in hydrophobicity due to residue substitutions

No H ⇔ P substitutions were observed in these regions, while S ⇔ P substitution is observed at position 76 of region 1, and S ⇔ H substitution is observed at positions 281 of region 3. In case of Pol protein, region 1, 101–123 (Fig. 33.22), and region 6, 774–786 (Fig. 33.26), have a stretch of hydrophobic residues with hydrophobicity score from 1.0 to 2.0 and 0.5 to 3.0, respectively. The other regions 2, 331–351

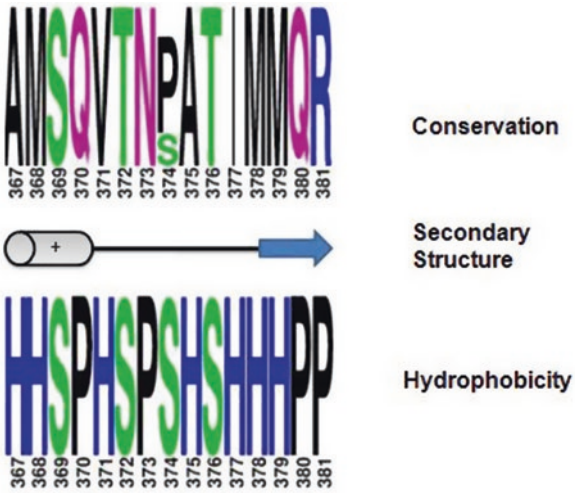


Fig. 33.21 Gag protein amyloid region 4, 366–380. The figure indicates no change in hydrophobicity due to residue substitutions

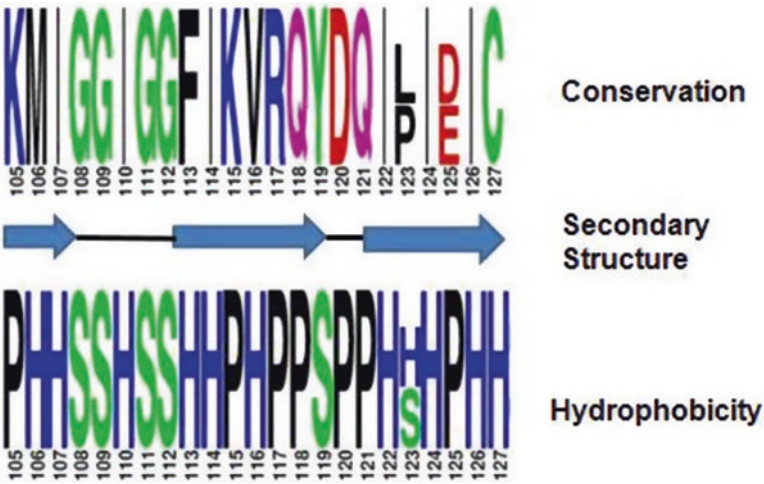


Fig. 33.22 Pol protein amyloid region 1, 101–123. The figure indicates slight change in hydrophobicity due to residue substitutions

(Fig. 33.23); region 3, 524–541 (Fig. 33.24a); region 4, 681–692 (Fig. 33.24b); region 5, 702–719 (Fig. 33.25); region 7, 810–823 (Fig. 33.27); region 8, 886–901(Fig. 33.28a); and region 9, 913–921(Fig. 33.28b), have hydrophobicity score from 0.0 to 2.0 consisting of highly hydrophobic, moderately hydrophobic, and polar residues. No significant variations are observed affecting the hydrophobicity.

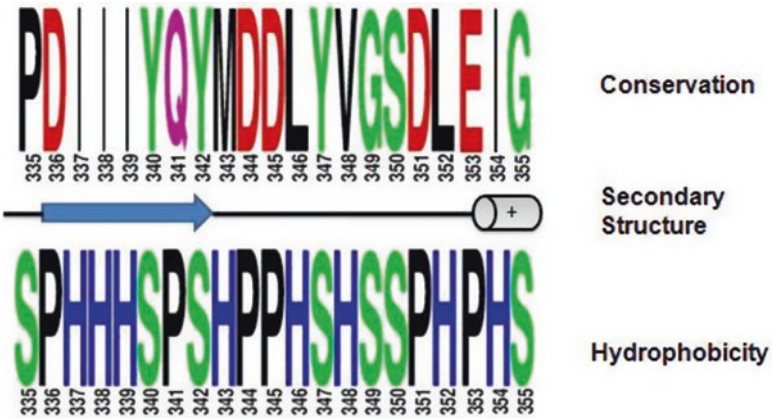


Fig. 33.23 Pol protein amyloid region 2, 331–351. The figure indicates no change in hydrophobicity due to residue substitutions

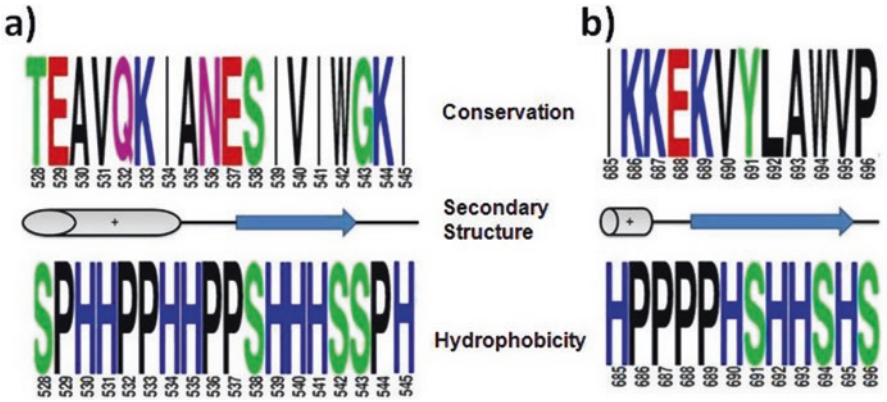
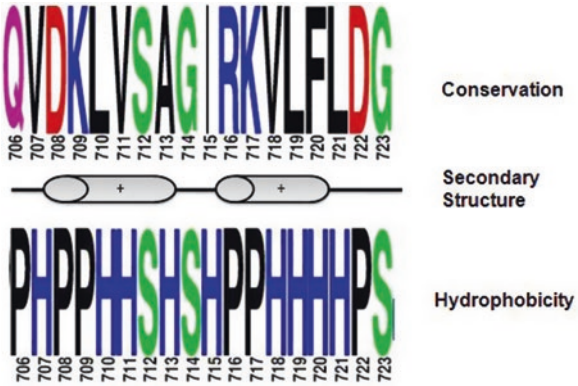


Fig. 33.24 (**a**) Pol protein amyloid region 3, 524–541. (**b**) Pol protein amyloid region 4, 681–692. The figure indicates no residue substitutions

Fig. 33.25 Pol protein amyloid region 5, 702–716. The figure indicates no residue substitutions



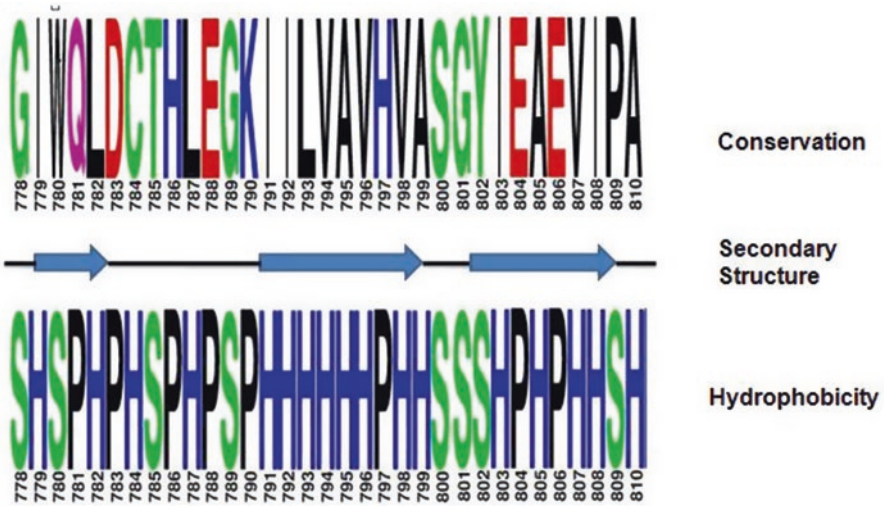


Fig. 33.26 Pol protein amyloid region 6, 774–806. The figure indicates no residue substitutions

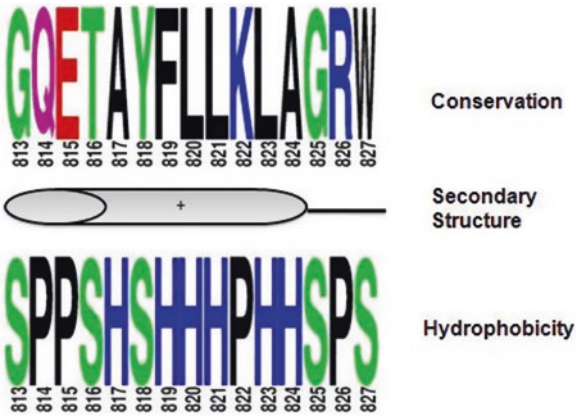


Fig. 33.27 Pol protein amyloid region 7, 810–823. The figure indicates no residue substitutions

This may be due to lesser number of variations inferred from the web logos, which in turn is due to a lesser sample size.

33.5 Predicted Patterns and Their Secondary Structure

The secondary structures were predicted for the representative HIV-1 protein sequences using PsiPred (http://bioinf.cs.ucl.ac.uk/psipred/) and Chou-Fasman methods (http://cho-fas.sourceforge.net/). PsiPred incorporates two feed-forward neural networks, previously trained from known structures and known alignments

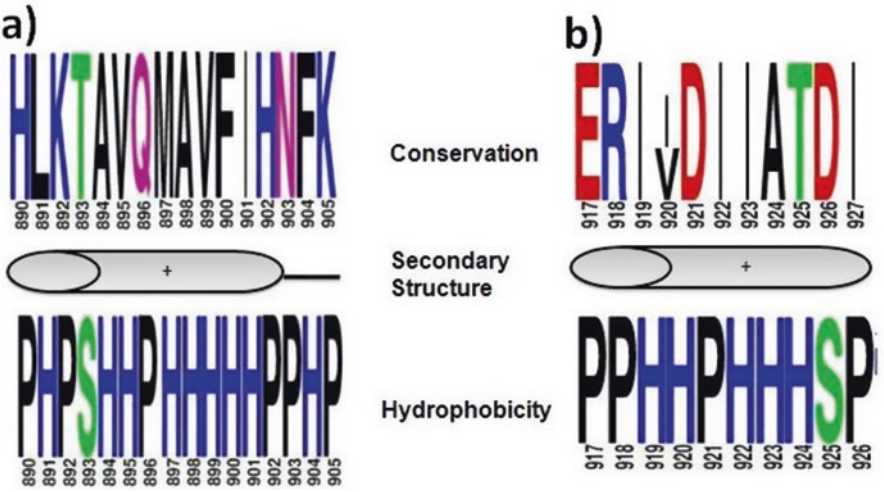


Fig. 33.28 (**a**) Pol protein amyloid region 8, 886–901. (**b**) Pol protein amyloid region 9: 913–921. The figure indicates no residue substitutions

to have a characteristic sequence-structure pattern that performs analyses on results obtained from position-specific iterated BLAST (PSI-BLAST) [100]. Those patterns are used to predict the secondary structure of the sequence submitted. PsiPred has a per residue accuracy (Q3) of ∼78% [101]. Chou-Fasman is an empirical method based on analyses of the relative frequencies of each amino acid in α-helices, β-sheets, and turns based on known protein crystal structures solved with X-ray crystallography. For each amino acid in a particular secondary structure type, a set of probability parameters were derived, and these are used to estimate the probability that a given sequence of amino acids would form a helix, a β-strand, or a turn in a protein [102]. The accuracy of the method is about 50–60% in predicting the correct secondary structures though lesser than the machine learning methods, it serves essential for obtaining individual amino acid propensities to form a particular secondary structure in the query protein sequence [103]. Due to differences in the prediction methodology employed in these two methods, their results may possibly differ for a set of protein sequences. The PsiPred predicted secondary structures of representative HIV-1 proteins are incorporated in Figs. 33.2, 33.3, 33.4, 33.5, 33.6, 33.7, 33.8, 33.9, 33.10, 33.11, 33.12, 33.13, 33.14, 33.15, 33.16, 33.17, 33.18, 33.19, 33.20, 33.21, 33.22, 33.23, 33.24, 33.25, 33.26, 33.27, and 33.28. The Chou-Fasman prediction was performed on ten randomly selected Nef, Vpu, Rev, Tat, Env protein sequences from brain isolates, four brain-derived sequences of Vif and Gag, three brain-derived sequences of Vpr, and two of Pol protein that were retrieved from GenBank (www.ncbi.nlm.nih.gov/genbank/) including the representative sequences presented earlier (Table 33.2). The average percentages of α-helix, β-sheet, and turn-forming residues in the predicted amyloid regions are showed in Table 33.3 along with the individual percentages for representative protein sequences.

Table 33.3 Individual and average percentages of residues forming of α-helix, β-sheet, and turns as predicted by Chou-Fasman program

Protein	H	Avg helix	E	Avg beta sheet	T	Avg turn
VPR region 1 36–46	0.0	14.26	64.3	69.03	14.3	9.5
VPR region 2 56–76	81.0	81	66.7	66.7	0.0	0
VPU region 1 3–35	86.2	86.2	89.7	89.7	0.0	0
VIF region 1 5–31	92.9	89.12	75.0	66.45	0.0	0
VIF region 2 59–72	13.3	28.3	53.3	53.3	6.7	6.7
VIF region 3 104–116	15.4	15.4	76.9	76.9	7.7	7.7
VIF region 4 143–156	71.4	69.62	64.3	64.3	7.1	7.1
NEF region 1 121–140	35.0	32.5	90.0	90	5.0	5.5
NEF region 2 145–159	46.7	46.7	86.7	86.7	0.0	0
NEF region 3 190–198	77.8	84.46	55.6	54.48	11.1	11.1
TAT region 1 19–47	H: 0.0	4.48	86.2	81.04	3.4	3.75
REV region 1 1–14	92.9	92.9	78.6	75.74	7.1	7.1
REV region 1 45–55	54.5	14.55	54.5	34.55	9.1	13.65
ENV region 1 20–43	75.0	75.61	91.7	82.3	4.2	3.77
ENV region 2 169–182	92.9	87.2	64.3	55.72	7.1	7.82
ENV region 3 278–289	69.2	67.66	53.8	45.38	7.7	7.7
ENV region 4 407–425	52.6	56.82	84.2	87.48	0.0	0
ENV region 5 531–537	85.7	69.4	71.4	66.48	0.0	0
ENV region 6 661–705	88.6	89.98	84.1	81.71	0.0	0
ENV region 7 747–783	75..7	73	89.2	88.39	2.7	2.7
ENV region 8 831–840	70.0	49	0.0	18	10.0	10

(continued)

Table 33.3 (continued)

Protein	H	Avg helix	E	Avg beta sheet	T	Avg turn
GAG region 1 75–92	0.0	0	83.3	83.3	5.6	8.35
GAG region 2 151–160	80.0	80	80.0	80	0.0	0
GAG region 3 258–285	71.4	67.85	89.3	89.3	3.6	5.35
GAG region 4 366–378	86.7	86.7	80.0	80	6.7	6.7
POL region 1 101–123	91.3	91.3	56.5	56.5	4.3	4.3
POL region 2 331–351	57.1	57.15	52.4	52.4	9.5	9.5
POL region 3 524–541	88.9	88.9	55.6	55.6	16.7	16.7
POL region 4 681–692	83.3	83.3	41.7	41.7	16.7	16.7
POL region 5 702–719	88.9	88.9	83.3	83.3	5.6	5.6
POL region 6 774–806	93.9	93.9	87.9	87.9	12.1	12.1
POL region 7 810–823	92.9	92.9	64.3	64.3	14.3	14.3
POL region 8 890–901	93.8	93.8	68.8	68.8	0.0	0
POL region 9 913–921	90.9	90.45	54.5	52.25	9.1	9.6

In the Env protein sequence, regions 20–43 consist partially of helix with confidence value of 06 to 0.9, coil with confidence value of 0.5 to 0.9, and β-sheet with confidence value of 0.5 to 0.9 in PsiPred prediction. Similarly, region 169–182 consists of initial helix with a confidence value of 0.4 to 0.7, then coil with confidence value of 0.6 to 0.9, and β-sheet with confidence value of 0.5 to 0.7, and for region 407–425 consists of coil with confidence value 0.5 to 0.9, β-sheet with confidence value of 0.6 to 0.8, and helix with confidence value of 0.5 to 0.9. Regions 531–537, 747–783, and 831–839 have helical conformations with confidence values of 0.7 to 0.9, 0.4 to 0.9, and 0.7 to 0.9, respectively. Region 661–705 has α-helix with confidence value of 0.4 to 0.8 and coils with confidence value ranges from 0.4 to 0.8 according to PsiPred, and region 278–289 consists of a β-sheet with a confidence value of 0.7 to 0.9. Chou-Fasman analysis shows a greater percentage of helix-forming residues in regions 169–182, 531–537, and 831–839. It shows a greater percentage of residue forming β-sheets in regions 20–43, 407–425, and 747–783, while approximately similar percentage of residues forming helix or sheet in regions 280–289 and 661–705. Regions 1–14 and 45–55 of the Rev proteins have helical

conformations in PsiPred prediction with a confidence value of 0.6 to 0.9 and encompass higher percentage of helix-forming residues in these regions according to the Chou-Fasman results. Vif protein region 5–31 forms partial β-sheet with confidence value ranges from 0.3 to 0.9 for region 5–17, and helix structure with confidence value between 0.5 and 0.7 for regions 104–116 and 143–156 favors helix conformations in PsiPred with confidence value ranges from 0.3 to 0.6 and 0.4 to 0.8, respectively. Region 59–72 has β-sheet conformation with confidence value of 0.5–0.9. Chou-Fasman predicts a greater percentage of helix-forming residues in region 5–31 and greater percentage of sheet-forming residues in regions 59–72 and 104–116. However, the average percentage of residues forming helix and sheet is similar for region 143–156. For Tat protein, region 19–47 is predicted to be partial helix with confidence 0.3 to 0.7 and coil with confidence value 0.5 to 0.9, while Chou-Fasman predicts higher percentage of β-sheet-forming residues.

PsiPred predicts partial helix with a confidence value of 0.5 to 0.6 and coil with a confidence value of 0.7 to 0.9 for Nef protein in regions 121–140. For region 145–159, partial β-sheet with a confidence value of 0.4 to 0.5 and with a confidence value of coil 0.5 to 0.7 and for region 190–198, partial β-sheet with a confidence value of 0.5 to 0.7 and coil with a confidence value between 0.4 and 0.6 was predicted. Region 121–140 has higher percentage of helix-forming residues according to Chou-Fasman prediction. Region 145–159 has partial sheet-forming and helix-forming residues, while region 190–198 is helix but encompassing a small section having residues forming either helix or sheet. The initial part of Vpr region 36–48 is predicted by PsiPred to be a coil structure with a confidence value of 0.4 to 0.8, and remainder of the structure is helix with a confidence value of 0.3 to 0.5; region 56–76 is an initial helix with a confidence value of 0.4 to 0.9 and then remains a coil structure having confidence value of 0.4 to 0.8 in PsiPred prediction. Chou-Fasman analysis shows the presence of β-sheet residues in region 36–48 while region 56–76 has residues forming both helix and β-sheet. PsiPred predicts helix in the Vpu protein region 3–35 with a confidence value of 0.6 to 0.9, while Chou-Fasman results show presence of residues having propensities to form both helix and β-sheet structure.

For Gag protein, regions 75–92 have helical conformation with a confidence value of 0.4 to 0.8, while region 151–160 is partial coil with confidence value of 0.2 to 0.6 and partial sheet with confidence value of 0.5 to 0.7. Regions 258–285 and 366–380 consist of helix with confidence value of 0.5 to 0.9, β-sheet with confidence value of 0.5 to 0.6, and coil with confidence value of 0.4 to 0.9 according to PsiPred results. The Chou-Fasman results show region 75–92 consists of helix-forming residues, and regions 151–160, 258–285, and 366–380 encompasses residues forming both helix and sheet. PsiPred prediction for Pol protein shows helical conformation for regions 810–823 with a confidence value of 0.6 to 0.9, region 890–901 with a confidence value of 0.4 to 0.9, and region 913–921 with a confidence value of 0.5 to 0.8. Region 101–123 consists of β-sheets with confidence value of 0.5 to 0.9 and coils with a confidence value of 0.5 to 0.9. Similarly, region 774–806 consists of β-sheets with confidence value of 0.5 to 0.9 and coils with a confidence value of 0.6 to 0.9. Region 702–719 consists of helix with a confidence value of 0.3 to 0.7 and

coil with confidence value of 0.4 to 0.9. Region 331–351 consists of initial β-sheet with a confidence value of 0.6 to 0.9 followed by coil conformation with a confidence value of 0.4 to 0.9 and helix with a confidence value of 0.7 to 0.9. Similarly, region 524–541 consists of a helix with confidence value of 0.7 to 0.9 followed by coil with a confidence value of 0.4 to 0.5 and then a sheet with confidence value of 0.5 to 0.9 followed by coil with a confidence value of 0.7 to 0.9. Region 681–692 is also predicted to encompass helix with confidence value of 0.8 to 0.9, β-sheet with confidence value of 0.5 to 0.9 and coil conformation with a confidence value of 0.6 to 0.8. Chou-Fasman results show the presence of higher percentage of residues forming helix in regions 524–541, 681–692, 810–823, 890–901, and 913–921, while the remaining regions consisted of residues forming both helix and sheet. The differences in the individual and average values in Chou-Fasman results presented in Table 33.3 are attributed to the sequence variations observed at predicted sites.

33.6 Amino Acid Propensity Values and Their Ambiguity

Estimating the secondary structure and amino acid propensities to form diverse secondary structures, i.e., α-helix or β-sheet, is an important aspect while understanding amyloidogenicity of a protein. It is observed that several amyloid-forming proteins enclose α-helix/β-sheet discordance. A segment predicted to form a β-sheet may actually exist as an α-helix structure, while segment predicted as α-helix may consist residues having higher propensity to form β-sheet and therefore displays ambiguities in the residual propensity values, and such discordant stretches are found to be associated with amyloid formation [104]. Amino acid substitutions may play a significant role in discordant regions, the ones predicted to be β-sheets but exist as helix. Substitution of certain residues in this stretch to statistically helix-favoring residues has been demonstrated to inhibit the fibril formation in a study performed by Karllberg et al. [104]. It may be postulated that proteins with discordant regions form unfolded helix intermediates. The high β-sheet propensity of residues in these regions implies that they are less likely to refold into a helical conformation and form β-sheets leading to aggregation [104]. Such amyloid-forming proteins consisting mainly of a helical structure in native state have been detected to make a transition from α-helix to β-strand before or during fibril formation as seen in the case of prion protein where the conversion of PrP^{C}(cellular) to its fibrillary counterpart PrP^{Sc}(scrapie) and is accompanied by reduction in α-helix content and increase in β-sheet structure [105]. Moreover, the folding of proteins in native state is primarily guided by the side-chain and intramolecular interactions, whereas the amyloid structure is formed mainly due to hydrogen bonds and main chain intermolecular interactions with possible unfavorable side-chain contributions [106, 107]. The protein is thermodynamically stable in native state if its free energy (G) is lower than the amyloid state; thus, its transition from a state of lower free energy to a state of higher free energy is less likely. The stability of amyloid state (ΔG) is dependent on the protein concentration, and at a critical concentration,

the stability of amyloid state becomes equivalent to that of native state [108]. At concentrations above this critical value, the protein is likely to be stable in amyloid form than in native state. In such situations, the native state of protein is kinetically metastable and the protein converts into thermodynamically more stable aggregates by overcoming the kinetic barriers responsible for their existence in soluble state [108, 109].

Amyloid β-peptide (Aβ) fibril formation associated with Alzheimer's disease also involves α-helix to β-strand conversion [110]. Theoretical and experimental studies of the primary structure of the hydrophobic peptide beta A4 have demonstrated that the C-terminal region adopts β-strand structure, while the N-terminal region has residues permitting different secondary structures. This region can exist as α-helical or β-strand conformation depending on the environmental condition, pH, and hydrophobicity surrounding the molecule [90]. A similar phenomenon is observed in the case of pulmonary surfactant-associated lipopolypeptide (SP-C). Where the SP-C is composed of α-helix that is thermodynamically unstable in monomeric form and the peptide irreversibly forms aggregates with β-sheet structure [111]. It has been demonstrated that substitution of all valines to leucines (amino acid with helix-forming propensity higher than valine) in discordant region of this protein abolishes the fibril formation, asserting the role played by the amino acid propensity values [104]. The monomeric α-synuclein of Parkinson's disease is also postulated to bind the membranes in helical form, while it is shown in vitro that the unfolded monomer can aggregate into oligomeric forms leading to stable β-sheet structures [112]. The other proteins associated with amyloid formation are known to have either a β-sheet structure or deficient in an experimentally determined three-dimensional structure [113].

In an attempt to identify discordant regions, the amino acid propensity values were predicted from the TANGO web server that provides propensities for each amino acid in the sequence to form either β-sheet or α-helix. The randomly selected HIV-1 protein sequences as well as the representative sequences were subjected to the TANGO server and propensities of residues to form α-helix and β-sheet at corresponding positions were calculated. Table 33.4 provides the propensities of the residues to form α-helix and β-sheet in the predicted amyloid regions of the representative HIV-1 protein sequences along with the average propensities of residues at respective predicted regions. For Env protein region 1, 20–43, the residues have more propensity to form sheet rather than helix, while PsiPred shows the presence of helix and sheet in this region. For region 2, 169–182, the initial residues have a greater propensity to form α-helix, and the later part has residues having greater propensities to form β-sheet, in accordance to PsiPred results. Region 3, 278–289, and region 4, 407–425, consist of residues having greater propensities to form β-sheet. Region 5, 531–539, consists of residues having greater propensities to form sheet however predicted as helix in PsiPred. Region 6, 661–705, and region 7, 747–783, consist of residues having mixed propensities to form α-helix and β-sheet, while residues in region 8, 831–840, have greater propensities to form helix in accordance with PsiPred results. Residues in the Rev region 1, 1–14, are predicted to be helix in PsiPred and have higher propensities to form helix, while residues in

region 2, 45–55, have higher propensities to form β-sheet. For Vif protein, region 1, 5–31; region 2, 59–72; and region 3, 104–116, residues have higher propensity values for forming β-sheet rather than helix, while region 4, 143–156, has higher propensity values to form helix. However, region 3, 104–116, is predicted to be helix PsiPred. Tat protein region 1, 19–47, has residues with higher propensity to form β-sheet and has the same conformation in PsiPred result. Nef protein region 3, 190–198, is predicted to be β-sheet in PsiPred, while this region shows the presence of residues having a greater propensity to form an α-helix. The residues in the region 1, 121–140, of the Nef protein has higher propensity to form β-sheet than helix, while region 2, 145–159, has β-sheet-forming residues as predicted by PsiPred. Region 2, 56–74, of the Vpr protein exhibits a strong helix-forming potential, while the residues in region 1, 36–48, has β-sheet-forming propensities in contrast to the helix conformation predicted by PsiPred. The residues in the Vpu protein region 3–35 has a higher propensity to form a β-sheet than an alpha-helix as seen in Table 33.4, while a helix conformation is predicted in PsiPred. Also SecStr program of AMYLPRED2.0 has predicted the possible conformational switch in this region indicating a possibility of helix to sheet transition leading to amyloid fibril formation. The residues in region 1, 75–92, and region 2, 151–160, of Gag protein are predicted to have helix conformation in PsiPred, while the results in Table 33.4 show higher propensity values of the residues to form β-sheet than helix. The remaining segments of Gag protein consist of residues having mixed propensity values to form α-helix or β-strand. Pol protein shows that region 5, 702–719; region 7, 890–901; and region 9, 913–921, that are predicted to be helix in PsiPred include residues having higher propensities to form β-strand than helix, while residues in other regions are having propensity values in accordance to the PsiPred results and no significant discordance is observed.

33.7 Comparison with Standards

Amyloid fibrils have a compact polymerized structure demonstrably cross β-sheets perpendicular to fibril axis, which makes it difficult for proteases to access and breakdown the amyloid, thus accumulating and leading to amyloidosis [66, 114–117]. Accumulation of intermediate states is critical for the transition from native state to amyloid state. Such partially unfolded conformations resulting from destabilized proteins are highly prone to aggregations with favorable thermodynamic requirements [108, 118–120]. More than 20 known amyloid-forming proteins are retrieved from Swiss-Prot database, and their amyloid regions are presented in Table 33.5. These proteins that form amyloids have variable sequences, and native three-dimensional structures suggesting that the overall amino acid composition must play a crucial role in influencing a shared phenomenon of amyloidogenicity [67, 118], where in factors like hydrophobicity, low net charge, and residual propensity to form helix or sheet have significant involvement [121, 122]. Earlier studies suggest that low mean hydrophobicity and net

charge are the characteristics of the unfolded protein [118], while the insoluble fibril form is associated with increased hydrophobicity, low net charge, and significant propensity to form secondary structure [59] and is considered to be a prominent factor to form accumulating aggregates.

Hydrophobicity in amyloid fibrils aids interactions with cellular membrane of target cells [123]. Amyloid fibrils are thus postulated to play a significant role in viral infection by enhancing virulence due to boosted viral attachment and facilitating entry into the host [53, 54, 56]. The hydrophobic stretches in the central and C-terminal parts of Aβ42 as observed from Table 33.5 are found to be responsible for aggregation in contrast to the N-terminal region (residues 1–16) of Aβ42, which is mostly polar and does not appear to promote aggregation [124]. Such continuous hydrophobic stretches are observed in certain amyloid regions predicted in brain-derived HIV-1 proteins in our study. In order to develop an insight of the hydrophobicity content of the experimentally determined amyloid fibrils, the fibril sequences are represented into a HSP strings. A sequence logo is created to have an understanding of hydrophobic pattern present in these fibrils (Fig. 33.29). The HSP logo of these fibrils suggests major transitions between highly hydrophobic and moderately hydrophobic residues along with some polar residue substitutions highlighting the fact that amyloid formation is a mutation sensitive phenomenon [94–99].

The representatives as well as randomly selected sequences were analyzed and amyloid regions predicted in them were converted to HSP strings. The percentages of highly hydrophobic, moderately hydrophobic, and polar residues were calculated for datasets, i.e., for experimentally determined amyloid fibrils and predicted amyloid regions of brain-derived HIV-1 proteins in our study, respectively. The amyloid fibrils of protein transthyretin, a pulmonary lung surfactant protein C (SP-C) and insulin, consisted of >70% highly hydrophobic residues (H); the percentage in other proteins is in the range of 25–70% and 15% in protein semenogelin as depicted in Fig. 33.30. The average percentage of highly hydrophobic residues in the predicted amyloid regions of brain-derived HIV-1 proteins ranged from 25% to 72%, where average percentages of highly hydrophobic residues in Vpu region 1 are >70%, in Vpr region 2 it is >60%, and Vif region 4; Rev region 1; Env region 1; Env regions 5, 6, 7, and 8; and Pol region 6, 8, and 9 have >50% of "H" residues as observed in Fig. 33.31. However, the percentages vary in individual sequences according to the residue variations at the predicted sites.

To verify whether the hydrophobicity and polarity differ between the predicted (A) and experimental groups (B), an independent samples T-test was carried out online (http://www.physics.csbsju.edu/stats/), and the predicted amyloid patterns of brain-derived HIV-1 isolates (group A) and experimentally determined amyloid fibrils (group B) were compared by considering a null hypothesis (H_0: there is no difference between the groups, A and B). The statistical analysis of "*H*" residues for group A1 versus group B1 ($N = 34$) indicated that there is no statistically significant difference between the two groups, group A1 ($M = 46.4$, SD = 9.87) and group B1 ($M = 43.9$, SD = 14.5), t (66) = 0.804, $p = 0.42$ (≥ 0.05). Therefore, we fail to reject the null hypothesis that there is no difference between the groups, A1 and B1 (Fig. 33.29). The statistical analysis of "*S*" residues for group A2 versus group B2

Table 33.4 Propensities of residues to form helix and sheet in the amyloid-forming regions of HIV-1 proteins as predicted by TANGO program

Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix	Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix
NEF region 1 121–140	I	0.7	0.7	0.11	0.14	VIF region 1 5–31	W	2.3	2.3	0.00	0
	L	1.5	1.51	0.11	0.14		Q	2.7	2.7	0.00	0
	D	1.6	1.64	0.11	0.19		V	4.9	4.9	0.00	0
	L	1.6	1.64	0.00	0.05		M	5.2	5.2	0.00	0
	W	1.6	1.36	0.00	0.05		I	8.6	8.6	0.00	0
	V	4.4	3.2	0.00	0.05		V	8.3	8.3	0.00	0
	Y	4.7	3.51	0.00	0.05		W	8.0	8.0	0.44	0.44
	H	4.6	3.6	0.00	0.05		Q	4.5	4.5	0.44	0.44
	T	1.6	1.6	0.00	0		V	1.9	1.9	0.44	0.44
	Q	0.9	0.89	0.00	0		D	0.5	0.5	1.41	1.49
	G	0.1	0.1	0.00	0		R	0.8	0.8	1.41	1.49
	Y	0.2	0.2	0.00	0		M	5.2	5.12	1.41	1.49
	F	0.2	0.2	0.00	0		R	8.5	8.38	1.25	1.33
	P	0.2	0.2	0.00	0		I	8.8	8.68	1.10	1.18
	D	0.4	0.4	0.00	0		R	5.3	5.22	1.10	1.18
	W	1.3	1.3	0.00	0		T	7.7	8.98	0.24	0.33
	Q	2.0	2	0.00	0		W	7.4	8.6	0.24	0.33
	N	4.0	4	0.00	0		N	6.5	7.74	0.24	0.27
	Y	3.7	3.7	0.00	0		S	1.1	1.1	0.10	0.06
	T	3.0	3	0.00	0		L	0.9	0.94	0.10	0.06
							S	2.7	2.7	0.10	0.06

NEF region 2 145–159	V	3.5	3.98	0.00	0		K	2.6	2.6	0.10	0.06
	R	3.6	4.08	0.00	0		H	2.7	2.7	0.10	0.06
	Y	3.6	4.04	0.00	0		H	1.4	1.4	0.00	0
	P	0.1	0.1	0.00	0		M	2.0	1.92	0.00	0
	L	3.8	3.49	0.00	0		Y	2.3	2.76	0.00	0
	T	4.7	4.37	0.11	0.10		I	2.9	0.13	0.00	0
	F	4.9	4.57	0.11	0.10						
	G	1.1	1.09	0.11	0.10	VIF region 2 59–72	L	0.0	0	0.00	0.00
	W	2.0	2.01	0.11	0.10		G	0.0	0	0.00	0.00
	C	5.5	5.5	0.11	0.10		D	0.1	0.16	0.00	0.00
	F	6.7	6.71	0.11	0.10		A	0.3	0.36	0.00	0.00
	K	5.8	5.85	0.11	0.10		T	0.8	0.84	0.00	0.00
	L	2.3	2.3	0.00	0		L	2.8	2.74	0.00	0.00
	V	1.1	1.06	0.00	0		V	6.3	8	0.00	0.00
	P	0.2	0.19	0.00	0		V	9.0	10.74	0.00	0.00
							T	10.2	12.78	0.00	0.00
NEF region 3 190–198	R	0.2	0.42	7.84	7.32		T	11.5	12.22	0.00	0.00
	E	0.6	0.82	7.84	7.33		Y	8.9	9.52	0.00	0.00
	V	2.7	3.52	7.84	7.20		W	5.7	5.56	0.00	0.00

(continued)

Table 33.4 (continued)

Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix	Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix
	L	3.7	4.05	7.71	7.10		G	0.7	0.68	0.00	0.00
	Q	4.4	4.51	7.06	6.45		L	0.3	0.3	0.00	0.00
	W	3.8	2.7	5.04	4.89		H	0.0	0.32	0.00	0.00
	R	5.8	3.16	2.53	2.39						
	F	5.4	3.11	1.78	1.31	VIF region 3 104–116	D	0.1	0.1	2.67	2.37
	D	4.2	2.29	2.12	1.54		Q	0.3	0.3	2.39	2.15
							L	0.7	0.78	2.28	2.062
VPR region 1 36–49	R	0.0	1.5	0.00	0		I	1.9	2.96	2.14	1.95
	P	0.0	2.6	0.00	0		H	4.4	3.62	2.14	1.95
	W	0.4	2.96	0.00	0		T	9.9	4.78	1.81	1.65
	L	0.5	1.63	0.00	0		Y	9.9	3.74	1.70	1.56
	H	0.6	0.66	0.00	0		Y	8.5	4.2	0.79	0.73
	G	0.2	0.33	0.00	0		F	7.2	7.38	0.00	0
	L	0.1	0.23	0.00	0		D	7.1	7.32	0.00	0
	G	0.0	0.13	0.00	0		C	9.0	9.06	0.00	0
	Q	0.5	0.5	0.00	0		F	5.2	5.04	0.00	0
	H	3.7	2.03	0.00	0		S	4.0	3.78	1.94	3.11
	I	4.5	2.76	0.00	0						
	Y	4.4	2.66	0.00	0	VIF region 4 143–155	G	0.5	0.5	0.47	0.90

	E	1.4	1.4	0.00	0		S	0.7	0.7	3.32	3.32
	T	1.4	1.4	0.00	0		L	2.1	2.06	3.52	3.46
							Q	3.0	3.02	3.95	3.77
VPR region 2 56–74	G	0.1	0.1	37.20	37.59		Y	3.0	3	4.26	4.00
	V	0.7	0.7	41.30	41.73		L	1.8	1.8	4.66	4.30
	E	0.7	0.7	44.70	45.16		A	1.0	0.98	5.94	5.38
	A	1.0	0.96	46.32	46.81		L	1.1	0.94	6.05	5.27
	I	2.2	2.16	47.02	47.52		T	1.2	1	5.81	5.32
	I	6.3	6.23	47.09	47.61		A	1.4	1.18	5.81	5.29
	R	7.3	7.2	47.21	47.74		L	1.4	1.22	5.70	5.16
	I	6.1	6.03	47.40	47.93		I	1.3	1.44	5.33	4.83
	L	2.0	2	47.40	47.93		K	1.1	1.3	5.33	5.27
	Q	0.8	0.8	46.74	47.28						
	Q	0.4	0.4	44.36	44.93	REV region 1 1–14	E	0.0	0.02	0.00	18.50
	L	0.6	0.6	38.47	39.10		L	0.2	0.29	0.00	18.53
	L	0.7	0.7	37.65	38.30		L	0.3	0.4	0.00	18.54
	F	0.8	0.8	35.76	36.43		K	1.0	0.9	0.00	18.45
	I	2.2	2.16	24.81	25.6		T	7.9	5.93	0.09	17.56
	H	3.5	3.43	24.68	25.47		V	15.9	9.35	0.09	17.31
	F	6.8	6.7	19.12	19.96		R	17.7	9.76	0.09	17.24
	R	5.6	5.5	15.29	16.18		V	13.3	5.22	0.09	13.12

(continued)

Table 33.4 (continued)

Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix	Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix
	I	4.2	4.13	8.63	9.60		I	7.4	3.86	0.09	12.00
	G	0.5	0.5	8.56	9.52		K	4.7	3.01	0.09	11.87
	C	0.8	0.86	3.04	3.30		F	3.3	3.02	0.09	8.66
							L	1.1	0.94	0.09	6.42
TAT region 1 19–47	K	0.4	0.34	0.00	0		Y	2.3	2.03	0.09	5.39
	T	0.9	0.83	0.00	0		Q	1.7	1.53	0.00	1.62
	A	1.0	1.01	0.00	0						
	C	1.5	1.31	0.00	0	REV region 2 45–55	S	5.7	4.2	0.00	0.75
	T	1.5	1.44	0.00	0.01		G	5.5	0.91	0.00	0.76
	N	3.7	3.96	0.11	0.09		W	0.2	1.04	0.00	0.76
	C	5.8	6.33	0.11	0.09		I	0.6	2.57	0.00	0.76
	Y	6.3	6.7	0.11	0.09		L	1.9	3.04	0.00	0.76
	C	4.3	4.36	0.11	0.09		S	2.0	2.36	0.00	0.75
	K	1.4	1.51	0.11	0.09		T	2.2	1.64	0.00	0.58
	K	3.1	2.53	0.11	0.08		F	4.1	1.62	0.00	0.09
	C	8.2	8.32	0.00	0		L	4.3	1.38	0.00	0
	C	10.4	10.18	0.00	0		G	3.6	0.6	0.00	0
	F	12.0	11.95	0.00	0		R	0.4	0.07	0.00	0
	H	7.5	6.8	0.00	0						
	C	6.1	5.57	0.00	0	ENV Region 2 169–182	K	0.8	0.7	3.17	1.23

	Q	2.0	1.99	0.00	0		E	0.9	0.75	3.34	1.48
	V	2.1	2.11	0.00	0		Y	1.1	1.15	3.01	1.33
	C	2.2	2.24	0.00	0		A	1.1	1.15	2.68	1.19
	F	3.6	3.7	0.00	0		L	1.4	1.35	2.54	1.13
	T	5.2	5.12	0.00	0		F	1.9	1.67	2.29	1.03
	T	4.3	4.18	0.00	0		Y	2.9	3.22	1.83	0.94
	K	2.7	2.52	0.00	0		K	3	3.77	1.12	0.49
	G	0.1	0.1	0.00	0		L	4.2	5.25	0	0.07
	L	0.1	0.1	0.00	0		D	4.7	5.02	0	0.07
	G	0.2	0.2	0.00	0		I	4.7	4.55	0	0.07
	I	4.3	4.3	0.00	0		V	2.4	2.12	0	0.07
	S	4.9	4.9	1.03	1.0312		P	0.6	0.57	0	0
	Y	5.0	5	1.03	1.0312		I	0.1	0.25	3.17	0.79
ENV region 1 20–43	M	2.3	2.55	0.23	0.68	ENV region 3 278–289	T	3.4	2.55	0	0.15
	L	2.4	2.72	0.23	0.68		N	2.1	1.37	0	0.15
	L	1.3	1.42	0.23	0.68		N	2.3	1.22	0.69	0.51
	G	0.2	0.52	0.23	0.73		V	3.5	1.8	0.69	0.49
	I	3.2	2.4	0	0.59		K	4.1	2.35	0.69	0.49
	L	3.9	3.02	0	0.52		T	4.1	3.47	0.69	0.54
	M	5	3.77	0	0.52		I	9.7	9.8	0.69	0.44
	I	2.6	2.22	0	0.49		I	12.1	12.27	0.69	0.44
	C	5.5	3.32	0	0.54		V	13.9	14.1	0.69	0.44

(continued)

Table 33.4 (continued)

Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix	Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix
	S	5.2	2.9	0.30	0.42		Q	7.8	7.95	0.69	0.44
	A	4.7	2.52	0.30	0.26		L	4.7	4.97	0.28	0.22
	T	1	0.62	0.30	0.28		N	0.8	1	0.18	0.19
	D	0.2	0.17	0.39	0.36						
	K	0.3	0.52	0.39	0.36	ENV region 5 531–537	L	3.5	6.57	0.43	0.27
	L	0.5	0.8	0.39	0.32		T	5.4	8.55	1.03	0.60
	W	0.9	1.15	0.28	0.25		L	7.1	10.05	1.03	0.60
	V	18.8	18.72	0.08	0.14		T	14.1	14.17	6.45	3.97
	T	23.1	23.05	0.08	0.14		V	12.8	12.55	6.45	3.97
	V	24.3	24.45	0.08	0.14		Q	9.9	9.7	6.55	4.02
	Y	7.8	7.75	0.08	0.14		A	1.3	1.42	6.22	3.79
	Y	2.6	2.6	0	0						
	G	1	0.82	0	0	ENV region 6 661–705	K	0.5	1.25	6.97	4.94
	V	0.3	0.3	0	0		W	0.8	1.5	5.44	3.97
	P	0.1	0.1	0	0		A	1.1	1.42	4.24	3.08
							S	1	0.9	3.97	2.73
ENV region 4 407–425	P	0	2.97	0	0.28		L	0.9	0.8	3.38	2.26
	I	19	19.4	0	0.28		W	1.9	1.95	2.94	1.89
	T	19.2	19.02	0	0.56		N	2.7	2.72	3.71	4.36
	L	19.2	17.87	0	0.56		W	3.4	3.27	2.32	3.08

	P	0.2	1.55	0.08	0.56		F	6.3	5.07	1.94	2.88
	C	2.4	2.72	0.08	0.45		N	7.4	5.2	4.18	6.15
	R	4.9	3.92	0.08	0.46		I	7	4.82	3.52	5.99
	I	6	4.8	0.08	0.44		T	3.2	2	5.20	8.28
	K	3.9	3.22	0.08	0.44		N	0.9	0.7	6.41	9.45
	Q	1.9	1.87	0.08	0.21		W	0.7	0.6	6.82	9.72
	I	1.7	1.72	0	0.12		L	0.7	0.65	6.82	9.62
	I	5.9	5.85	0	0.10		W	1.2	1.15	7.06	9.81
	N	5.9	5.85	0.12	0.41		Y	2	1.92	6.95	9.61
	M	5.1	5.07	0.12	0.41		I	6.2	6.02	6.60	9.05
	W	1.4	1.42	0.12	0.34		K	6	5.82	6.60	9.05
	Q	1.1	1.07	0.12	0.34		I	5.7	5.52	5.53	7.60
	E	1	0.95	0.25	0.40		F	2	1.95	5.53	7.60
	V	0.5	0.37	0.25	0.43		I	3.8	3.75	3.83	5.35
	G	0.2	0.17	0.25	0.43		M	4.6	4.5	3.83	5.35
							I	6.5	6.37	2.66	3.73
ENV region 7 747–783	F	0.6	0.47	0.32	0.27		V	4.5	4.37	2.66	3.73
	L	0.9	0.8	0.32	0.27		G	2.8	2.75	2.66	3.73
	A	1.1	1.15	0.32	0.27		G	0.1	0.1	1.30	1.84
	L	0.9	1.4	0.32	0.27		L	0.1	0.12	0	0.04
	F	1.5	1.65	0.32	0.27		I	0.3	0.32	0	0
	W	2.1	1.7	0.12	0.22		G	0.3	0.32	0.49	0.27

(continued)

Table 33.4 (continued)

Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix	Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix
	V	3.8	2.05	0	0.52		L	4	3.87	0.49	0.27
	D	3.2	1.85	5.03	3.64		R	8.5	8.02	0.49	0.27
	L	3	1.95	5.03	3.61		I	12.9	12.17	0.49	0.27
	R	1.3	1.3	5.03	3.61		V	10.9	10.55	0.49	0.27
	S	1.2	1.37	5.03	3.61		F	7.2	11	0.49	0.27
	L	1.3	1.67	5.03	3.61		A	3.4	9.02	0.24	0.22
	F	1.5	1.75	5.03	3.53		V	4.4	10.22	0.24	0.22
	L	1.7	1.82	3.53	2.60		L	4.9	7.85	0.24	0.22
	F	6.9	6.85	2.74	2.02		S	4.7	6.1	0.83	0.71
	S	8.3	8.35	2.65	2.18		I	3.1	4.82	0.70	0.68
	Y	9	9.05	2.24	1.83		V	1.9	2.65	0.70	0.68
	H	3.8	3.8	2.17	1.98		N	1.4	2.12	0.96	0.86
	R	2.2	2.2	2.41	2.46		R	1.9	1.92	0.96	0.79
	L	1.1	1.1	2.36	2.53		V	4	3.95	0.96	0.70
	R	0.7	0.7	2.48	2.73		R	3.9	3.9	0.96	0.70
	D	0.2	0.2	4.34	4.74						
	L	0.9	0.77	4.34	4.74	ENV region 8 831–840	R	1.2	1.8	1.76	11.65
	L	1.5	1.57	4.34	4.79		A	0.7	2.02	1.55	10.08
	L	2.7	2.57	4.67	5.48		F	2.3	2.37	1.24	9.05
	I	5.5	5.5	4.98	5.82		R	2.3	1.77	0	4.77
	V	6.1	5.95	5.44	6.24		A	2.4	2.1	0	3.21
	T	5.6	5.47	7.07	7.83		I	1.4	1.25	0	2.41
	R	6.2	4.25	7.79	8.54		L	6.7	6.32	0	1.80

	I	7.2	5.65	7.49	8.52		H	6.8	6.52	0	1.32
	V	10.2	8.82	7.60	8.57		I	6.2	5.95	0	0.19
	E	6.1	6.57	7.60	8.57		P	0.6	0.6	0	0
	L	3.9	3.92	7.60	8.47						
	L	0.1	0.1	7.60	8.47	GAG region 2 151–160	T	1.9	1.95	2.79	2.75
	G	0.1	0.1	7.22	8.05		L	2.2	2.2	2.79	2.75
	R	0.1	0.1	5.05	5.70		N	2	2	3.36	3.16
							A	0.5	0.5	2.18	1.98
GAG region 1 75–92	L	1.1	1.35	0.10	0.23		W	1.3	1.3	1.81	1.60
	T	1.6	1.72	0.10	0.23		V	15.8	15.75	1.14	0.94
	S	1.3	1.37	0.10	0.34		K	17.6	17.35	1.14	0.94
	L	1.2	0.92	0.10	0.40		V	19.3	19.4	0.65	0.45
	Y	1.5	1.17	0.10	1.40		I	5.4	5.65	0.65	0.45
	N	2.1	1.57	0.12	1.49		E	3	3.45	0.65	0.45
	T	5.5	4.17	0.30	1.59						
	V	5.7	4.45	0.30	1.59						
	A	5.2	4.2	0.30	1.59	GAG region 4 366–380	A	0.3	0.3	0	0
	T	2.4	2.1	0.30	1.57		M	0.5	0.5	0	0
	L	2.9	2.45	0.30	1.25		S	0.6	0.6	0	0.05

(continued)

Table 33.4 (continued)

Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix	Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix
	Y	4.9	4.02	0.30	1.10		Q	1	1	0	0.05
	C	5.5	4.62	0	0.48		V	3.7	3.42	0	0.05
	V	6	5.8	0	0.09		T	3.6	3.5	0	0.05
	H	4	4.32	0	0.09		N	2.9	2.82	1.95	1.81
	Q	2.6	3.17	0	0.09		P	0	0.37	1.95	1.85
	R	3.5	3.12	0	0.07		A	0.7	0.9	1.95	1.80
	I	7.6	7.22	0	0.07		T	3.5	3.67	2.06	1.91
							I	7	7	2.06	1.91
GAG region 3 258–285	V	0.1	0.1	0.49	0.49		M	6.5	6.47	2.06	1.91
	G	0.1	0.1	2.35	2.35		M	3.8	3.8	1.59	1.48
	E	0.6	0.6	2.35	2.35		Q	0.4	0.4	1.36	1.27
	I	0.9	0.9	2.35	2.35		R	0.2	0.2	0.92	0.83
	Y	1.8	1.8	2.35	2.35						
	K	1.4	1.4	2.35	2.35	POL region 1 101–123	K	0.6	0.6	0	0
	R	1.6	1.6	2.21	2.21		M	0.7	0.7	0	0
	W	1.4	1.4	1.51	1.51		I	0.8	0.8	0	0
	I	6.9	6.9	0.95	0.95		G	0.2	0.2	0	0
	I	7.5	7.5	0.95	0.95		G	0.1	0.1	0	0
	L	6.8	6.8	0.95	0.95		I	0.1	0.1	0	0
	G	1.3	1.3	1.09	1.09		G	0.1	0.1	0	0
	L	0.3	0.3	0.53	0.53		G	0.1	0.1	0	0

	N	0.3	0.3	0.72	0.72		F	1.2	1.2	0	0
	K	1.7	1.7	0.72	0.72		I	8.5	8.5	0	0
	I	6.1	6.1	0.72	0.72		K	10	10	0	0
	V	9.7	9.7	0.72	0.72		V	11.1	11.1	0	0
	R	8.9	8.9	0.72	0.72		R	5.5	5.5	0	0
	M	5.6	5.6	0.60	0.59		Q	5.3	5.35	0	0
	Y	2.2	2.2	0.42	0.42		Y	4.1	4.15	0	0
	S	1.5	1.55	0	0		D	2.6	2.75	1.54	0.77
	P	0.3	0.35	0	0		Q	2	1.75	1.54	0.77
	V	3.3	1.85	0	0		I	3	1.8	1.54	0.77
	S	3.6	2.1	2.46	1.49		L	2.9	1.5	1.54	0.77
	I	4	2.5	2.46	1.49		I	6.3	5.85	1.54	0.77
	L	3	3.05	2.46	1.49		E	4.8	5.25	1.54	0.77
	D	4.5	4.6	2.46	1.49		I	5.5	6.05	1.31	0.65
	I	6.1	6.25	2.46	1.49		C	1.4	1.4	1.31	0.65
POL region 2 331–351	P	0	0	0	0	POL region 3 524–541	T	0.9	0.9	2.43	2.43
	D	1.3	1.3	0.28	0.28		E	0.1	0.1	2.93	2.93
	I	6.1	6.1	0.28	0.28		A	0.5	0.5	3.04	3.04
	I	8	8	0.28	0.28		V	1.1	1.1	3.04	3.04
	I	7.8	7.8	0.28	0.28		Q	1.3	1.3	3.04	3.04
	Y	8.2	8.2	0.28	0.28		K	2.1	2.1	2.75	2.75
	Q	7.3	7.3	0.28	0.28		I	1.6	1.6	2.38	2.38

(continued)

Table 33.4 (continued)

Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix	Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix
	Y	7.1	7.1	0.09	0.09		A	1.4	1.4	2.38	2.38
	M	2.1	2.1	0	0		N	0.1	0.1	2.63	2.63
	D	1	1	0	0		E	0	0	1.60	1.60
	D	0.4	0.4	0	0		S	0.6	0.6	0.81	0.81
	L	0.9	0.9	0	0		I	8.2	8.2	0.64	0.64
	Y	1.6	1.6	0	0		V	9.9	9.9	0.64	0.64
	V	1.5	1.5	0	0		I	9.9	9.9	0.64	0.64
	G	0.8	0.8	0	0		W	2.4	2.4	0.64	0.64
	S	0.1	0.1	0.17	0.17		G	0.8	0.8	0.64	0.64
	D	0.2	0.2	0.39	0.39		K	0.3	0.3	0.37	0.37
	L	1.5	1.55	0.39	0.39		I	0.2	0.2	0	0
	E	1.6	1.6	0.39	0.39						
	I	1.4	1.45	0.39	0.39	POL region 5 702–719	Q	0.4	0.4	0.47	0.47
	G	0.1	0.1	1.07	0.73		V	0.6	0.6	0.47	0.47
							D	0.6	0.6	0.68	0.68
POL region 4 681–692	I	0.9	0.9	8.52	8.52		K	0.8	0.8	0.68	0.68
	K	0.9	0.9	8.52	8.52		L	1.3	1.3	0.68	0.68
	K	0.8	0.8	5.41	5.41		V	3.3	3.3	0.57	0.57
	E	0.5	0.5	1.28	1.28		S	2.8	2.8	1.11	1.11
	K	1.2	1.2	0.28	0.28		A	2.2	2.2	0.96	0.96
	V	4.4	4.4	0	0		G	0.3	0.3	1.08	1.08

	Y	4.9	4.9	0	0		I	0.9	0.9	0.64	0.64
	L	4.2	4.2	0	0		R	1.4	1.4	0.64	0.64
	A	1.1	1.1	0	0		K	2.2	2.2	0.64	0.64
	W	0.6	0.6	0	0		V	3	3	0.64	0.64
	V	0.4	0.4	0	0		L	3.1	3.1	0.64	0.64
	P	0.1	0.1	0	0		F	3.1	3.1	0.50	0.50
							L	1.6	1.6	0.16	0.16
POL region 6 774–806	G	0	0	0	0		D	1	1	0.25	0.25
	I	0.9	0.9	0	0		G	0.3	0.3	0.64	0.64
	W	2.7	2.7	0	0						
	Q	3.3	3.3	0	0	POL region 8 886–901	H	0.7	0.7	0.39	0.39
	L	4.7	4.7	0	0		L	0.8	0.8	0.39	0.39
	D	3.3	3.3	0	0		K	1.1	1.1	0.26	0.26
	C	4.9	4.9	0	0		T	1.3	1.3	0.13	0.13
	T	3.7	3.7	0.15	0.15		A	1.6	1.6	0.13	0.13
	H	3.6	3.6	0.15	0.15		V	5.8	5.8	0.13	0.13
	L	1.4	1.4	0.15	0.15		Q	5.2	5.2	0.13	0.13
	E	0.5	0.5	0.15	0.15		M	5.1	5.1	0.13	0.13
	G	0.1	0.1	0.38	0.38		A	1.1	1.1	0.13	0.13
	K	1.3	1.3	0.38	0.38		V	3.4	3.4	0.13	0.13

(continued)

Table 33.4 (continued)

Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix	Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix
	I	6.7	6.7	0.23	0.23		F	3.7	3.7	0.13	0.13
	I	9.3	9.3	0.23	0.23		I	3.3	3.3	0	0
	L	8.7	8.7	0.23	0.23		H	1.3	1.3	0	0
	V	9.3	9.3	0.23	0.23		N	1.4	1.4	0	0
	A	7.2	7.2	0.23	0.23		F	2.1	2.1	0	0
	V	14.6	14.6	0.14	0.14		K	0.7	1.35	0.39	0.19
	H	10.3	10.3	0.14	0.14						
	V	11.3	11.3	0	0	POL region 9 913–921	E	0.1	0.1	0.09	0.09
	A	3.5	3.5	0	0		R	4.7	4.85	0.09	0.09
	S	2	2	0	0		I	5.5	5.6	0.09	0.09
	G	0.1	0.1	0	0		V	9.8	10.25	0.09	0.09
	Y	1.1	1.1	0	0		D	6.5	6.8	0.09	0.09
	I	2	2	0	0		I	8.4	8.7	0	0
	E	2	2	0	0		I	5.2	5.2	0	0
	A	1.2	1.2	0	0		A	3.8	3.75	0	0
	E	1	1	0	0		T	1.3	1.3	0	0
	V	1.6	1.6	0	0		D	0.6	0.6	4.23	4.23
	I	1.4	1.4	0	0		I	0.7	0.7	4.23	4.23
	P	0.6	0.6	0	0						
	A	0	0	0	0	VPU region 1 3–31	P	0.1	0.66	1.42	2.37
							L	1.5	2.53	1.42	2.43

POL region 7 810–823	G	0.1	0.1	4.53	4.53		Q	2.3	3.63	1.42	2.91
	Q	0.1	0.1	4.53	4.53		I	3.7	4.36	1.42	2.99
	E	0.1	0.1	4.64	4.64		S	2.4	2.87	1.42	3.29
	T	0.8	0.8	7.06	7.06		A	2.2	2.24	1.42	3.5
	A	1.1	1.1	7.06	7.06		I	5	5.03	1.42	3.53
	Y	1.9	1.9	7.19	7.19		V	7.7	7.29	1.42	3.60
	F	1.9	1.9	6.65	6.65		A	7.4	6.96	1.42	3.67
	L	1.8	1.8	6.39	6.39		L	4.9	4.61	1.19	3.52
	L	2.1	2.1	6.29	6.29		V	5.7	7.3	0.83	3.25
	K	1.7	1.7	6.16	6.16		V	6.5	8.35	0.83	3.30
	L	1.6	1.6	5.59	5.59		A	5	9.45	0.83	3.27
	A	0.3	0.3	5.20	5.20		A	1.8	4.88	0.65	3.38
	G	0.1	0.1	4.12	4.12		I	3.3	5.41	0.41	3.09
	R	0.1	0.1	1.80	1.80		I	5.2	4.36	0.41	3.09
	W	0.1	0.1	0.48	0.47		A	5.1	3.85	0.41	2.94
							I	9.8	9.47	0.25	2.59
							V	10	10.38	0.44	2.75
							V	11.8	12.09	0.53	2.78
							W	6.2	6.76	0.53	2.91
							S	4.1	5.37	1.36	3.68
							I	3.8	7.49	1.36	2.89
							A	2.5	7.35	1.87	3.49

(continued)

Table 33.4 (continued)

Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix	Amyloid region	Sequence	Beta	Avg beta	Helix	Avg helix
							L	2.3	6.86	2.62	4.24
							L	0.9	4.68	3.99	5.67
							E	1.2	3.27	7.47	9.01
							Y	2.2	3.79	7.58	8.96
							R	1.9	2.21	7.63	8.72
							K	1.6	2.11	7.55	8.46
							L	0.6	2.85	7.46	8.26
							L	0.5	2.54	7.46	8.23
							R	0.7	2.47	7.06	7.79

Table 33.5 List of experimentally identified amyloid fibrils retrieved with their Swiss-Prot IDs with their HSP strings

Serial number	Protein name	Protein ID	Amyloid fibril
1	Beta amyloid [138–142]	P05067	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
			PHPHPPPSSSPHPPPPHHHHHPPHSSPPSHHHSHHHSSHHHH
2	Lactadherin Medin [143, 144]	Q08431	RLDKQGNFNAWVAGSYGNDQWLQVDLGSSKEVTGIITQGARNFGSVQFVA
			PHPPPSPHPHSHHSSSSPPPSHPHPHSSSPPHSSHHSPSHPPHSSHPHHH
3	Apolipoprotein A-I [145]	P02647	DEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLDNWDSVTSTFSKLREQLGP VTQEFWDNLEKETEGLRQEMSKDLEEV
			PPSSPSSSPPHPPHHSHSHPHHPPSSPPSHSPHPSSHHSPPHPHPHHPPSPSHSSSHSPHPPPHSSHSPPHS PPHPPPSPSHPPPHSPPHPPH
4	Apolipoprotein C-II [146]	P02655	MSTYTGIFTDQ
			HSSSSSHHSPP
5	Atrial natriuretic factor [147]	P01160	SLRRSSCFGGRMDRIGAQSGLGCNSFRY
			SHPPSSHHSSPHPPHSHPSSHSHPSHPS
6	Cystatin [148, 149]	P01034	SFQIYA
			SHPHSH
7	Amylin [150–154]	P10997	KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY
			PHPSHSHHSPPHHPHHHPSSPPHSHHHSSSPHSSPSS
8	Beta 2 microglobulin [155–158]	P61769	SNFLNCYVSGFHPSDIEVDLLKN
			SPHHPHSHSSHPSSPHPHPHHPP
			WSFYLLYYTEFTPTEKDEYACRVNHVTLSQ
			SSHSHHSSSPHSSSPPPPSHHPHPPHSHSP
9	Transthyretin [159–161]	P02766	CPLMVKVLDAV
			HSHHHPHHPHH
			YTIAALLSPYS
			SSHHHHHSSSS

(continued)

Table 33.5 (continued)

Serial number	Protein name	Protein ID	Amyloid fibril
10	Gelsolin [162–164]	P06396	ATEVPVSWESFNNGDCFILDLGNNIHQWCGSNSNRYERLKATQVSKGIRDNERSGRAR
			HSPHSHSSPSHPPSPHHHHPHSPPHPPSHSSPSPPSPPHPHSPHSPSHPPPPPSSPHP
11	Lysozyme [70, 165]	P61626	RCELARTLKRL
			PHPHHPSHPPH
			LANWMCLAKW
			HHPSHHHHPS
			IFQINS
			HHPHPS
12	Keratoepithelin [166]	Q15582	FSMLVAAIQSAGLTETLN
			HSHHHHHHPSHSHSPSHP
13	Calcitonin [167]	P01258	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP
			HSPHSSHHHSSSSPPHPPHPSHSPSHHSHSHS
14	Alpha-synuclein [168–174]	P37840	EGVLYVGSKTKEGVVHGVATVAEKTKEQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFV
			PSHHSHSSPSPPSHHPSHHSHHPPSPPPHSPHSSHHHSSHSHHHPPSHPSHSSHHHHSSHH
15	Prolactin [175]	P01236	GAARCQVTLRDLFDRAVVLSHYIHNLSS
			SHHPHPHSHPPHHPPHHHHSPSHPPHSS
			RYTHGRGFITKAINS
			PSSPSPSHHSPHHPS
16	Serum amyloid A [176, 177]	P0DJI8	RSFFSFLGEAFD
			PSHHSHHSPHHP
17	Lactoferrin [178]	P02788	NAGDVAF
			PHSPHHH

18	Semenogelin I [179]	P04279	QKGGSKGRLPSEFSQFPHGQKGQHYSGQKGKQQTESKGSFSIQYTYHVDANDHDQSRKSQQYDLN ALHKTTKSQRHLGGSQQLLHNKQEGRDHDKSKGHFHRVVIHHKGGKAHRGTQNPSQDQGNSPSGKGI SSQYSNTEER
			PPSSSPSPHSSPHSPHSPSPPSPPSSSPPSPPPSPSPSSHSHPSSSPHPHPPPPSPPSPPSPHPHHPPSSPSPPP HSSSPPHHPPPPPSPPPPPSPSPHPPHHHPPPSSPHPPSSPPSSPPPSPSSSSPSHSSPSSPSPPP
19	Tau [180, 181]	P10636	KVQIINKKLD
			PHPHHPPPHP
			VQIVYK
			HPHHSP
20	Pulmonary surfactant-associated lipopolypeptide (SP-C) [111, 182]	P11686	FGIPCCPVHLKRLLIVVVVVVLIVVVIVGALLMGL
			HSHSHHSHPHPPHHHHHHHHHHHHHHHHSHHHHSH
21	proBNP brain natriuretic peptide [183]	P16860	KMVLYTL
			PHHHSSH
22	ODAM [184]	A1E959	MPYVFSFKMPQEQGQMFQYYPVYMVLPWEQPQQTVPRSPQQTRQQQ
			HSSHHSHPHSPPPSPHHPSSSHSHHHSSPPSPPSHSPSSPPSPPPP
23	Insulin [185, 186]	P01308	LVEALYL
			HHPHHSH
			LYQLEN
			HSPHPP

(continued)

Table 33.5 (continued)

Serial number	Protein name	Protein ID	Amyloid fibril
24	Beta-lactoglobulin [187]	P02754	DIQKVAGTWY
			PHPPHHSSSS
			KYLLFCMENS
			PSHHHHHPPS
			SLACQCLVRTP
			SHHHPHHHPSS
			MHIRLSFN
			HPHPHSHP
25	Acylphosphatase-2 [188, 189]	P14621	RVQGVCFRMYTEDEARK
			PHPSHHHPHSSPPPHPP
			SKLEYSNFSIRY
			SPHPSSPHSHPS

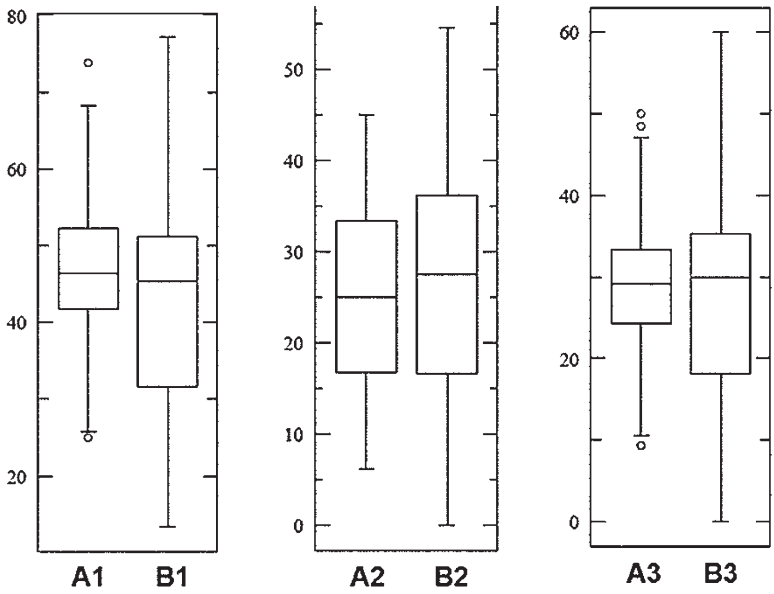


Fig. 33.29 Box and whisker plot of the data shows the comparison of estimated means of predicted HIV-1 proteins (**a**) as well as the experimental groups (**b**) of amyloid fibrils. Where A1 and B1 correspond to highly hydrophobic "H" residue composition, A2 and B2 correspond to moderately hydrophobic "S" residue composition, and A3 and B3 correspond to polar "P" amino acid composition. The plots indicate that the median (*middle quartile*) of both the groups A and B lies in the same range with some amount of variation in the range of distribution

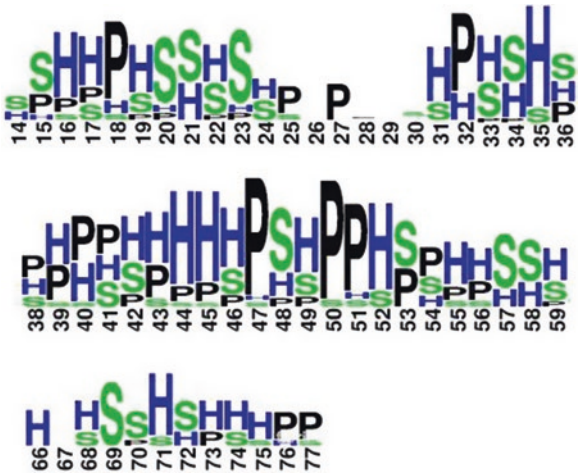


Fig. 33.30 HSP string web logo of experimentally identified amyloid-forming fibrils showing presence of highly hydrophobic and moderately hydrophobic residues and polar residues with S ⇔ P, H ⇔ P, and H ⇔ S transitions

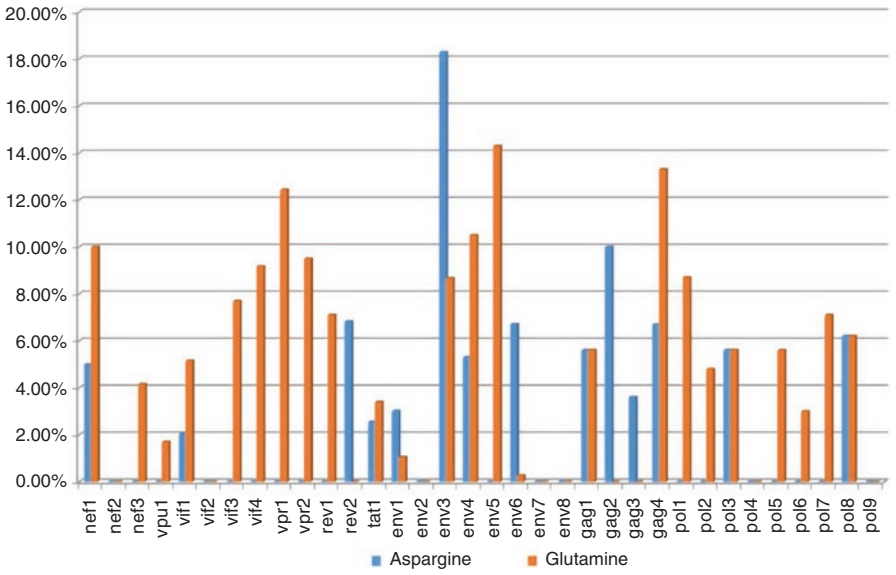


Fig. 33.31 Average percentage of asparagine and glutamine residues in predicted amyloid regions of brain-derived HIV-1 protein sequences

(N = 34) indicated there is no statistically significant difference between the two groups, group A2 (M = 25.5, SD = 10.4) and group B2 (M = 26.9, SD = 12.7), t (66) = 0.504, p = 0.62 ($\geq$ 0.05). Therefore, we fail to reject the null hypothesis (Fig. 33.29). The statistical analysis of "*P*" residues for group A3 versus group B3 (N = 34) indicated there is no statistically significant difference between the two groups, group A3 (M = 24.26, SD = 9.48) and group B3 (M = 29.1, SD = 12.7), t (66) = 0.371, p = 0.71 ($\geq$ 0.05). Therefore, we fail to reject the null hypothesis (Fig. 33.29). The T-test results show that the hydrophobicity and polarity content between the predicted amyloid regions of brain-derived HIV-1 proteins (group A) and the experimentally determined amyloid fibrils (group B) do not differ significantly, thus suggesting a possible role of hydrophobic and polar residues in determining amyloidogenicity (Fig. 33.32).

The most manifest hydrophobic stretch is seen in the amyloid site predicted in Vpu protein region (3–31), having a long continuous stretch of hydrophobic residues as seen in Fig. 33.18. The sequence as well as structural properties of this stretch has a striking similarity to the amyloid fibril of pulmonary lung surfactant protein C (SP-C). This region has a continuous stretch of hydrophobic residues as well as a helix conformation as observed in the PDB structure 1SPF; however, it has been observed that this region undergoes structural transition of α-helix into β-sheet aggregates. This may be attributed to the change in surroundings affecting the thermodynamic stability or effect of point mutations on thermodynamic stability [111]. A pH-dependent study by Dluhy et al. has shown that deacylated SP-C adopts an

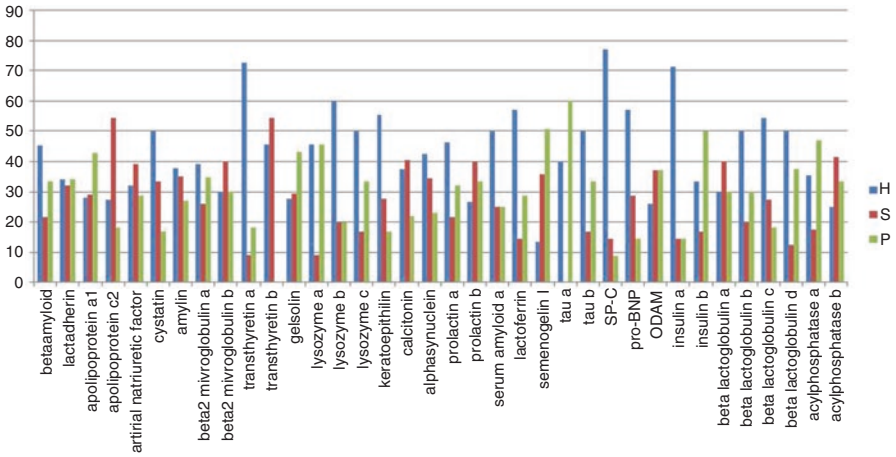


Fig. 33.32 Percentage of highly hydrophobic (*H*), moderately hydrophobic (*S*), and polar (*P*) residues in experimentally identified amyloid fibrils

α-helical conformation at low pH; however, with increase in pH, it undergoes an α-helix to β-strand conformational change [125]. Region 1, 3–31, of VPU has a helical conformation (PDB structure, 1VPU), while the residues in this region indicate propensities to form both α-helix and β-sheet as predicted by Chou-Fasman server and SecStr program in AmylPred2.0. It should be noted that the amyloid region predicted in Vpu and the hydrophobic stretch of SP-C, which are a part of transmembrane protein, relate to β-amyloid and is also derived from a transmembrane region of its precursor protein [126] and comprises α-helix to β-sheet discordance, depending upon the surrounding milieu conditions [90]. A similar case is seen in α-synuclein where its amyloid-like region comprises stretches of hydrophobic residues and it is proposed to exist in α-helix and β-sheet forms in equilibrium [127]. There are various other experimentally identified amyloid fibrils that display the presence of continuous amyloid stretches as observed in Table 33.5. The other proteins recognized to contain continuous hydrophobic residue stretches were Env (regions 20–42, 661–705), Vif (regions 59–72, 143–156), Nef (region 145–159), and Gag (regions 75–92, 258–285). However, other predicted regions are characterized by hydrophobic residues along with interrupting polar residues, an observation that is encountered in the case of well-known amyloid fibrils (Table 33.5).

Pawar et al. [92] defined the intrinsic propensities for the aggregation of individual amino acids in their study which was performed on experimentally predicted amyloid proteins. According to these findings, it is observed that the highly hydrophobic residues designated as "H" (isoleucine, valine, leucine, phenylalanine, cysteine, methionine, and alanine) and moderately hydrophobic residues designated as "S" (glycine, threonine, serine,tryptophan, and tyrosine) have aggregation propensity values in the range of −5.08 to 2.92; however, proline has the lowest amy-

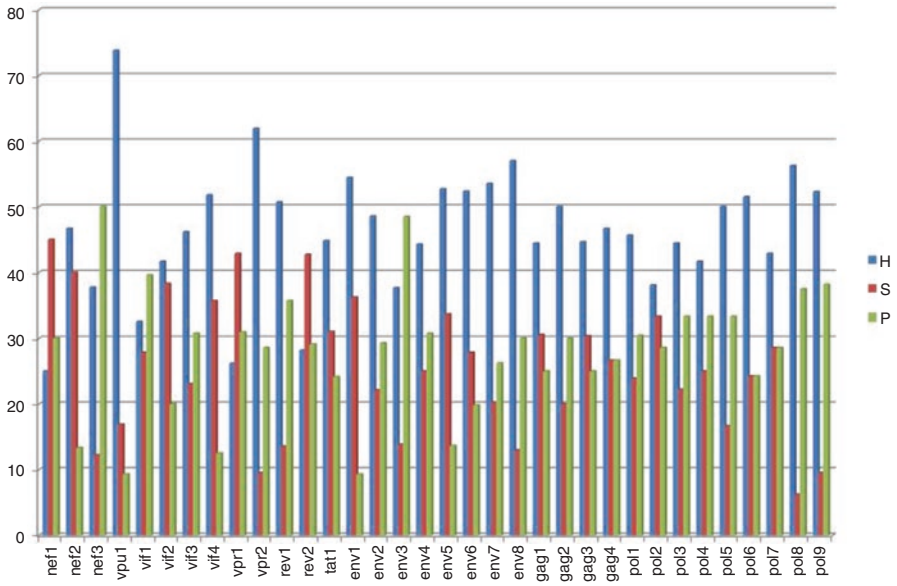


Fig. 33.33 Average percentage of highly hydrophobic (*H*), moderately hydrophobic(*S*), and polar residues (*P*) in predicted amyloid regions in brain-derived HIV-1 proteins

loid propensity of −11.96. The amino acids tryptophan, phenylalanine, cysteine, tyrosine, isoleucine, and valine possess the highest amyloid propensities suggesting an important role of aromatic residues in promoting aggregation in coordination to their hydrophobicity values [93]. It should be noted that proline residues introduce slight twists in the polypeptide backbone and thus would predict proline's antithetical effect on beta sheet planarity in amyloid. The polar residues designated as "P" (glutamine, glutamate, aspartate, asparagine, lysine, and arginine) have lower aggregation propensity values ranging from −11.93 to −6.00; histidine has variable propensity values at different pH levels.

In contrast to the appearance of hydrophobic residues in the amyloid-forming regions, the glutamine-rich polar polypeptides are associated with some inherited, neurodegenerative amyloidoses [128]. Expanded CAG repeats give rise to uninterrupted polyglutamine (polyQ) stretches and are subject to increased aggregation in the case of Huntington's disease [129, 130]. In the current study of predicted amyloid regions of HIV-1 proteins, no such polyQ stretch was identified. Prion formation is seen to be primarily driven by amino acid composition such as glutamine-/asparagine-rich domains that are overrepresented in eukaryotic genomes [131, 132]. It has been reported that glutamine supports the β-hairpin fold by forming interstrand hydrogen bonds rendering it irreversible, thus indicating that toxicity correlates with glutamine content [133]. The glutamine/asparagine content in the identified amyloid regions of HIV-1 proteins was investigated (Fig. 33.33). The glutamine content in some of the identified amyloid regions was in the range of 2–15%, while asparagine content was in the range of 2–19%. However, Nef region 3, Vpu

region 1, Vif regions 3 and 4, Vpr regions 1 and 2, Rev region 1, Env region 5, and Pol regions 1, 2, 5, 6, and 7 lack asparagine residues, while Rev region 2 and Gag regions 2 and 3 lack glutamine residues. Moreover, Nef region 2; Vif region 2; Env regions 2, 7, and 8; and Pol region 4 contained neither glutamine nor asparagine.

33.8 Conclusions

Amyloid-forming proteins are likely to share common features with respect to similar kinetics and thermodynamics of progression, prevalence of disorder of protein structure, and molecular characteristics of final aggregated states of the proteins, i.e., amyloid-like fibrils. It is known that the amyloid-forming proteins possess characteristics of being disordered and that these proteins, in certain cases, fail to assume their native forms, transition into intermediate states and due to intrinsic and extrinsic factors misfold into insoluble fibrils accumulating as aggregates [134]. Thus, prediction algorithms usually identify the amyloid-forming regions indicating an ordered structure. As observed in Table 33.2, PASTA2.0 identified such regions in brain-derived HIV-1 proteins (Nef 121–129, Vpu 6–28, Vif 5–13 and 7–31, Vpr 57–74, Rev 2–13, Tat 19–40, Env 661–705, 78–89 and 261–285, and Pol 787–804) as ordered stretches that form possible pairing and aggregation. It is important to note that in addition to proteins associated with amyloid diseases, studies have shown that diverse proteins not associated with any disease can aggregate into fibrils under destabilizing circumstances [135–137]. Studies suggesting roles of a few HIV-1 proteins in progression of disease served a possible course toward supporting the amyloidogenicity of these proteins [52–56]. On this basis, to better comprehend the role of these proteins in relation to neurotoxicity in HAD patients, it is beneficial to characterize their possible amyloidogenic behavior in brain-derived HIV-1 proteins and compare them to isolates from blood and the other compartments. The identification based on various factors including average packing density, β-sheet propensity, aggregation propensity, hydrophobicity, and sequential motifs supports the presence of amyloid-forming regions in the brain and other derived HIV-1 proteins with possible roles in accumulation and enhancement of toxicity. Hydrophobicity is an important parameter, and the majority of the predicted amyloid regions were observed to have hydrophobicity indexes above 0.5 from Kyte-Doolittle hydrophobicity plots. On analyzing the residues in the predicted regions for amyloid-forming propensities given by Pawar et al. [92], it is observed that the majority of the predicted regions have residues with higher to moderate aggregation propensities. Primarily, regions with hydrophobic stretches showed the presence of residues with higher amyloid propensity values suggesting that hydrophobicity may contribute to amyloidogenicity; though, it may not be the sole factor. Also, no significant difference was observed in the hydrophobicity and polarity content of predicted HIV-1 amyloid regions and experimentally determined amyloid fibrils emphasizing on additional examination of HSP patterns shared by these regions. The high mutation rate in HIV-1 forms the background for H ⇔ S, H ⇔ P, and S ⇔

P transitions observed in the sequence logos of proteins. The substitutions responsible for changes in the hydrophobicity content of the regions need to be analyzed further for its effect on the structural properties of these proteins and thus aid in tracking virus-associated toxicity and pathogenesis.

Conflict of interest The authors report no conflicts of interest.

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Chapter 34
On the Potential for Multiscale Oscillatory Behavior in HIV

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Core Message
Nonlinear dynamics and inherently multiscale properties of the HIV-host system impede our ability to effectively intervene in the disease process. Bursty or oscillatory behavior of the virus along with its other strategies to avoid elimination by the host immune system has significant implications for diagnosis and control of infection. Mathematical modeling of HIV-host multiscale interactions and dynamics offers an opportunity to coherently integrate available experimental data, reveal nontrivial emergent properties of the system, and systematically explore optimal intervention strategies.

34.1 Introduction

The life cycle of human immunodeficiency virus type 1 (HIV-1) is complex and inherently multiscale [1, 2]. The virus has developed various strategies to avoid elimination by the host immune system [3–6]. These strategies result in intricate dynamical behaviors of the host-virus system. HIV-1 exploits the host organism at multiple levels during the course of infection. In this chapter, we discuss two levels of host-virus dynamical interactions. Namely, we consider the within-host organism and the within-host target cell (e.g., T cells or macrophages) HIV dynamics (Fig. 34.1). Within-host organism HIV dynamics represents the host-virus interaction at the cell population level (Fig. 34.1A), whereas within-host target cell HIV dynamics represents the host-virus interaction at the intracellular level (Fig. 34.1B). These two scales of host-virus interactions are tightly interlinked and shape the overall dynamical properties of the host-virus system.

The host-HIV interaction at the cell population level can be viewed and modeled as a collection of different target cell types with different properties (e.g., half-lives,

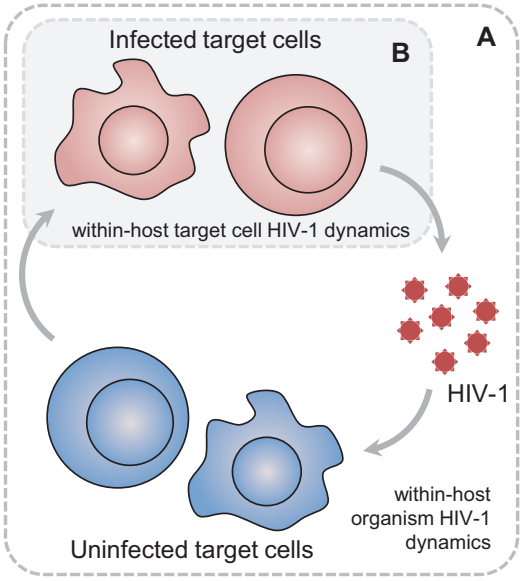

Fig. 34.1 Schematic representation of the link between the within-host organism and within-host target cell HIV dynamics. (*A*) The within-host organism HIV dynamics is considered at the cell population level. (*B*) The within-host target cell HIV dynamics is considered at the intracellular level. Uninfected target cells (e.g., T cells or macrophages) can be infected by viruses and converted to latently or productively infected target cells. HIV dynamics at both scales are intertwined and interdependent

probabilities of being infected). The target cells can be infected by HIV and converted to infected cells also with different properties (e.g., latently or productively infected) [7]. The host-HIV interaction models at the cell population level can be used to systematically analyze the rates of HIV infection and replication, the rate of HIV particle clearance, and different properties of infected cells [7–9] and to explore intervention strategies and appearance of drug-resistant virus populations [10, 11].

The host-HIV interaction at the intracellular level can be viewed and modeled as a system of interacting host and HIV molecular components within the target cell [12–17]. The intracellular host-HIV interaction models can be used for systematic analysis of the transient properties of the viral replication processes, such as, reverse transcription, integration of proviral DNA into the host genome, transcription, RNA maturation and transport from the nucleus to the cytoplasm, synthesis and transport of viral proteins to the cell membrane, and viral particle assembly. There are multiple nonlinear molecular mechanisms of intracellular HIV-1 replication. One such mechanism is positive regulation of virus replication by Tat protein via the antitermination of genomic RNA transcription on the trans-activation response (TAR) element of the proviral DNA (Fig. 34.2). Another mechanism is based on interference with the splicing of full-length (9 kb) RNA and incompletely spliced (4 kb) RNA molecules through their active transport from the nucleus to the cytoplasm [18].

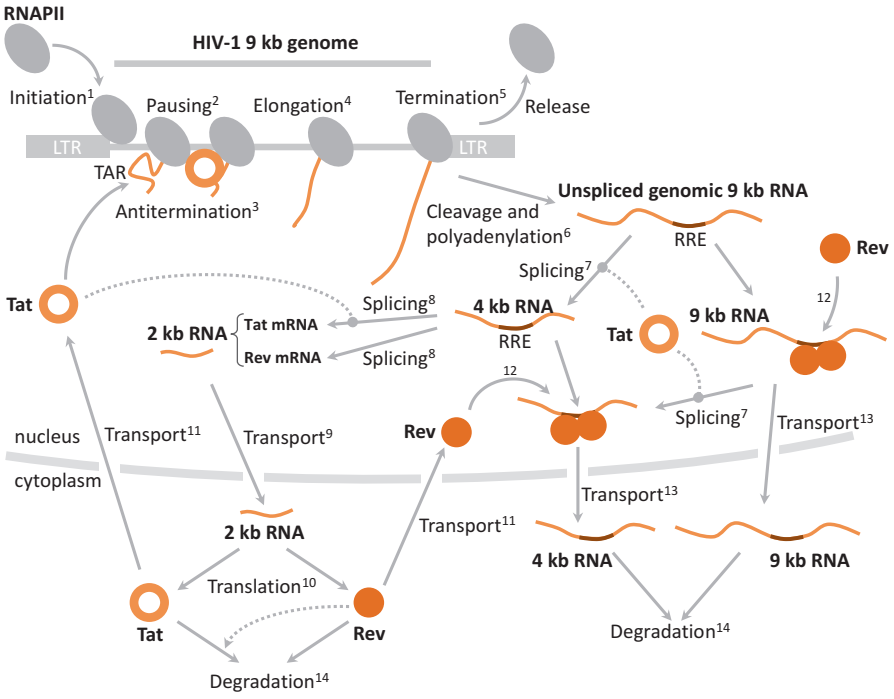

Fig. 34.2 Schematic representation of the regulation of HIV-1 replication by Tat and Rev, *dotted arrows* represent a positive regulation. *Dotted oval arrow* represents a negative regulation. The description of the numbered processes is presented in the text (Modified from Likhoshvai et al. [17])

The Tat and Rev proteins are synthesized by infected cells at early stages of HIV-1 ontogenesis. Their mRNAs are fully spliced (2 kb) RNAs [19] and can be transported into the cytoplasm without delay by RNA transport machinery [18]. The production of the Tat protein leads to an augmentation of full-length genomic RNA transcription by at least 25- to 100-fold [20–25]. This full-length HIV-1 genomic RNA encodes Gag and Gag-Pol proteins, which are essential for the formation of virus particles in the cytoplasm [26]. Since there are no molecular mechanisms of nuclear-cytoplasmic transport of intron-containing RNA in the cells of higher organisms, the export of intron-containing RNA to the cytoplasm is mediated by HIV-1 Rev proteins (Fig. 34.2). The Rev protein contains a nuclear localization sequence (NLS) and a nuclear export sequence (NES), which control the shuttling of Rev between the nucleus and cytoplasm [27, 28]. Its appearance in the nucleus followed by an interaction with the Rev response element (RRE) leads to the assembly of a high-affinity complex on unspliced (9 kb) or incompletely spliced (4 kb) viral mRNA [29] and the export of the above classes of the viral mRNAs out of the nucleus. This results in a downregulation of the generation of the completely spliced mRNAs and, therefore, the overall synthesis of the Rev and Tat proteins [30].

34.2 Network Motifs for Oscillatory Behavior of Within-Host Organism and Within-Host Target Cell HIV Dynamical Systems

There are positive and negative feedback loops in the within-host organism and within-host target cell HIV dynamical systems (Fig. 34.3). At the cell population level, HIV-1 enhances its viral titer by infecting the target cells and converting them into productively infected cells. At the same time, HIV is actively reducing the number of uninfected target cells that can be viewed as an indirect negative feedback regulation (Fig. 34.3a). At the intracellular level, the positive feedback loops are dictated by the self-replicating molecular mechanisms of the virus [31]. One of the positive feedback loops is the transcriptional activation of HIV-1 components including Tat-encoding mRNA by Tat through TAR element. There is also a negative feedback regulation of Tat and Rev mRNA synthesis by Rev (Figs. 34.2 and 34.3b). Moreover, the negative feedback loop in this system is reinforced by the negative regulation of Tat mRNA synthesis in the nucleus by the Tat protein through inhibition of splicing 9 kb and 4 kb RNAs to 2 kb mRNAs encoding Tat as well as by the positive regulation of the Tat protein degradation in the cytoplasm by Rev and the HIV nucleocapsid protein (NC) [32–34]. NC is translated from the full-length viral genomic RNA as a part of the Gag polyprotein and subsequently processed by a viral protease.

It is known that molecular regulatory systems with negative feedback loops or network architectures comprising linked positive and negative feedback loops can potentially have an oscillatory behavior or a limit cycle [35–39]. There are multiple theoretical studies of both natural and synthetic gene regulatory networks that demonstrate an important role for negative feedback loops in the oscillatory behavior of the

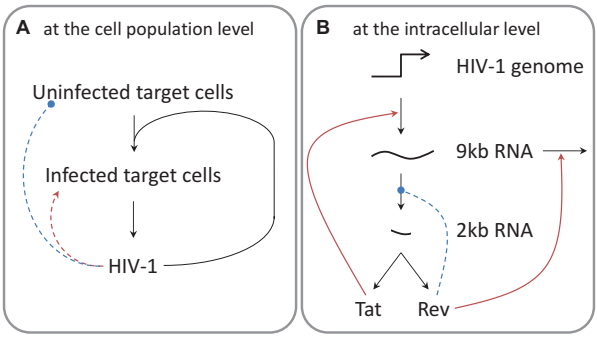

Fig. 34.3 Positive and negative feedback loops in the within-host organism (**a**) and within-host target cell (**b**) HIV dynamical systems. *Red solid and dashed arrows* represent direct and indirect, respectively, positive regulation. *Blue oval dashed arrows* represent indirect negative regulation

system [40–44]. Examples of such systems include oscillation of NF-κB protein in the immune response [45–47], the oscillatory behavior of Hes1 and Hes7 proteins and their regulation of the formation of somites in developing vertebrate embryos [48, 49], and the oscillation of p53 and its regulation of apoptosis [50]. Thus, the regulatory program of HIV-1 ontogenesis has all of the prerequisites for oscillatory regimes in the production of viral particles at both the cell population and intracellular scales. In this chapter we will briefly review mathematical models of the within-host organism and within-host target cell HIV dynamical systems and describe theoretical results that support the existence of oscillatory dynamics of HIV-1 at both scales.

34.3 Sustained Oscillations in the Basic Within-Host Organism HIV Model

One of the early within-host organism models, known as the standard model, was used by Perelson and Nelson [51] and by Nowak and May [52] to model HIV. It was successful in numerically reproducing early stages of the HIV lifecycle in its target, the CD4+ T cells, following an infection event. The global behavior of the standard model was first investigated analytically in [53] and will be reviewed here. The global stability of a disease steady state was proved under certain conditions, using powerful second compound matrix methods developed by Muldowney [54] and by Li and Muldowney [55]. It also established the possibility of sustained oscillations in particular regions of the parameter space.

After HIV enters its target, a T cell, it makes a DNA copy of its viral RNA. The viral DNA is then inserted into the DNA of the T cell, which will henceforth produce viral particles that can bud off the cell to infect other uninfected T cells. A concise summary in chemical reaction notation of these processes is

$$T + V \rightarrow T^* \rightarrow NT$$

where N is the expected number of viral particles that bud off an infected T cell over its lifetime. These mathematical models assume mass action kinetics for both of the reactions above.

The standard model has three state variables: T, the concentration of uninfected T cells; T^*, the concentration of productively infected T cells; and V, the concentration of free virus particles in the blood. The interaction between these cells and virus particles is then given by the following equations [51–53]:

$$\dot{T} = f(T) - kVT \tag{34.1}$$

$$\dot{T}^* = kVT - \beta T^* \tag{34.2}$$

$$\dot{V} = N\beta T^* - \gamma V - kVT \tag{34.3}$$

The functional form of f is defined differently by different authors:

1. Perelson and Nelson [51] take

$$f(T) = f_1(T) = \delta - \alpha T + pT\left(1 - \frac{T}{T_{\max}}\right).$$

2. Nowak and May [52] use $f(T) = f_2(T) = \delta - \alpha T$.

The parameters α, β, γ, δ, k, N, p, and $T_{\max}$ are positive and have the following interpretation:

1. α, β, and γ are death rates for uninfected T cells, infected T cells, and virus particles, respectively.
2. k is the contact rate between uninfected T cells and virus particles.
3. δ represents a constant production of T cells in the thymus.
4. N is the average number of virus particles produced by an infected T cell during its lifetime.
5. In the case $f = f_2$, healthy T-cell proliferation is neglected, and only the thymus serves as a source of newly produced healthy T cells. In the case $f = f_1$, healthy T cells are assumed to proliferate logistically. The parameters p and $T_{\max}$ are the growth rate and carrying capacity, respectively, associated with a logistic growth of uninfected T cells in the absence of virus particles, infected T cells, and other sources such as the thymus. This logistic proliferation is a simplification of the more biologically realistic term $pT\left(1 - \frac{T + T^*}{T_{\max}}\right)$, and a model that includes this term instead has since been considered in [56]. Another simplification is that logistic proliferation of the infected T cells has been neglected, but this has also been considered since then, namely, in [57].

The simplifications regarding T-cell proliferation have important mathematical consequences, because the resulting system turned out to be a three-dimensional

competitive system [58], which opened up a whole arsenal of tools to analyze the model [53].

The model always has a disease-free steady state $E_0 = (T_0, 0, 0)$, where T_0 is the positive root of the function f, i.e., $f(T_0) = 0$. There may be a second, chronic disease steady state $E_e = \left(T_e, T_e^*, V_e\right)$, if and only if the value of the basic reproduction number

$$R_0 = \frac{kNT_0}{\gamma + kT_0}$$

is larger than 1.

If $R_0 > 1$, then E_0 is unstable, and Smith and De Leenheer [53] provide conditions under which E_e is globally stable.

However, when $f = f_1$, there exist regions in parameter space (e.g., when kT_{max} and p are sufficiently large, see [53]), where E_e is unstable with a two-dimensional unstable manifold (caused by the linearization having a pair of imaginary eigenvalues with positive real part), and a one-dimensional stable manifold (by the linearization having a real, negative eigenvalue). In this case, there exists at least one orbitally asymptotically stable periodic orbit. Every solution, except those with initial data on the one-dimensional stable manifold of E_e or on the invariant T-axis, converges to a nontrivial periodic orbit. In other words, in this case, the model exhibits sustained oscillations (Fig. 34.4).

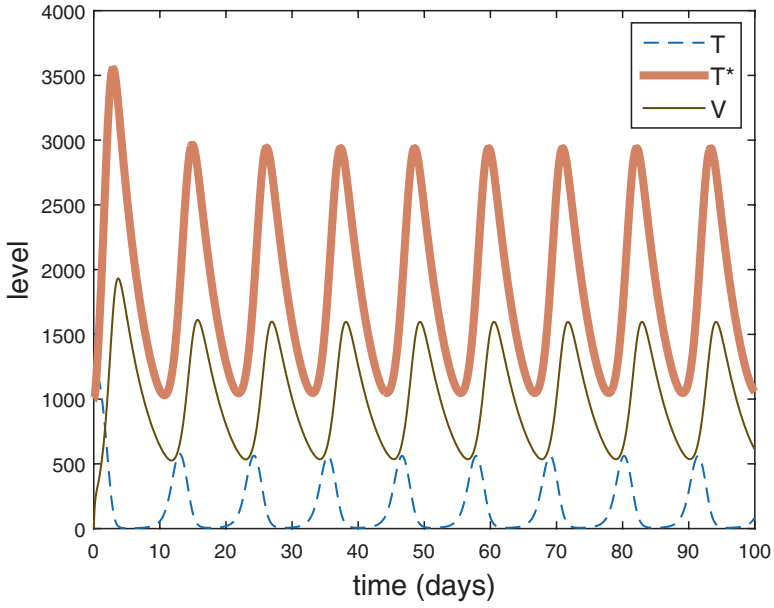

Fig. 34.4 Periodic solution for the basic within-host organism HIV model ($f = f_1$). Parameters: δ = 5 day^{-1} mm^{-3}, α = 0.03 day^{-1}, p = 3 day^{-1}, T_{max} = 1700 mm^{-3}, β = 0.2 day^{-1}, γ = 3.0 day^{-1}, k = 0.003 mm^3 day^{-1}, and N = 9. T is the level of uninfected T cells; T^* is the level of productively infected T cells; and V is the level of free virus particles in the blood

34.3.1 *Comments on* $\mathbf{R}_0$

The above formula for R_0 is obtained following the procedure outlined in [59]. In fact, technically speaking that procedure would yield the square root of the expression given above. However, since R_0 is used to determine the local stability or instability of the disease-free steady state (stable if $R_0 < 1$ and unstable if $R_0 > 1$), it does not matter whether the square root is applied or not. Similar comments explain the difference between R_0 given above and the formula for R_0 given in [53]; the latter was derived before the now accepted procedure in [59] was known to the authors of [53].

34.4 Sustained Oscillations of the Within-Host Target Cell HIV Model: The Tat-Rev-Mediated Regulation of HIV-1 Replication

Here we review a mathematical model for Tat-Rev-mediated regulation of HIV-1 replication. This model was originally developed and used to examine the dynamics of the accumulation of Tat and Rev proteins and the viral RNA in an infected macrophage, that is, persistently producing the virus particles [17]. In this model, two specific hypotheses on the recycling (nuclear import/export cycle) of the HIV-1 Rev protein were considered. The first hypothesis is that Rev is released from the export complex and binds to importin-β in the cytoplasm [18]. The second hypothesis is that Rev returns into the nucleus directly at the nuclear pore complex without the export of Rev to the cytoplasm [60, 61]. The mathematical model was calibrated using published experimental data. It predicts the existence of oscillatory dynamics which depends on the efficacy of the interaction between the Tat protein and TAR and on the transport kinetics regulated by the Rev protein [17]. Below, we describe the formal representation of this model.

34.4.1 *A Mathematical Model of the Tat-Rev-Mediated Regulation of HIV-1 Replication*

The mathematical model of the Tat-Rev-mediated regulation of the intracellular HIV-1 replication is specified using the biochemical systems formalism [62]. Two elementary types of reactions, bimolecular and monomolecular, are considered. Below we discuss some standard notation to represent the chemical reactions. A bimolecular reaction can be formally presented as follows:

$$A + B \leftrightarrow C : k_1, k_2 \tag{34.4}$$

where A, B, and C are concentrations of reactants in the reaction; k_1 and k_2 are rate constants of the forward and reverse reactions, respectively. According to the law of mass action, the system of ordinary differential equations (ODEs) corresponding to bimolecular reaction (34.4) can be written as follows:

$$\frac{dA(t)}{dt} = \frac{dB(t)}{dt} = -\frac{dC(t)}{dt} = -k_1 A(t)B(t) + k_2 C(t) \tag{34.5}$$

These equations describe the local rates of changes in concentrations of reactants A, B, and C in a fixed volume.

A monomolecular reaction can be formally presented as follows:

$$[a]A \to [b_1]B_1 + \ldots + [b_n]B_n : k \tag{34.6}$$

where A and B_i are concentrations of the reaction reactant and products, respectively; k is the reaction rate constant; a and b_i are the stoichiometric coefficients. The system of ordinary differential equations (ODEs) corresponding to monomolecular reaction (34.6) can be written as follows:

$$\frac{dA(t)}{dt} = -akA(t), \frac{dB_i(t)}{dt} = b_i kA(t), \quad i = 1,\ldots,n \tag{34.7}$$

The system (34.4) determines the local rates of changes in concentrations for reaction reactants in a fixed volume. To simplify the notation of monomolecular reactions, we will omit the stoichiometric coefficient of 1. The value of $a = 0$ corresponds to a reaction, where reactant A plays a role of an infinite resource for the reaction products.

34.4.2 Elementary Subsystems of the Tat-Rev Model

The mathematical model of a Tat-Rev-mediated regulatory network of HIV-1 replication consists of the following 14 elementary subsystems (Fig. 34.2):

1. The initiation of transcription from the HIV-1 proviral long terminal repeat (LTR) promoter, $\text{LTRP}_{\text{HIV1}}$, leading to formation of the elongation complex $\text{RNAPII}_{\text{TAR}}$ that is prone to termination at a TAR element is formally presented as follows:

$$[0]\text{LTRP}_{\text{HIV1}} \to \text{RNAPII}_{\text{TAR}} : k_{\text{transcr,ini}}\text{proV}$$

where $k_{\text{transcr,ini}}$ is the rate constant of the transcription initiation, and proV is the number of proviral DNA genomes in the cell.

2. The passage of TAR element by RNA polymerase II (RNAPII). It is assumed that RNAPII is terminated at the TAR element and forms short RNAs *microRNA* with probability λ. Thus, the termination of RNAPII transcription at the TAR element is avoided, and the elongation complex $\mathrm{RNAPII}_{\mathrm{DNAunit}_1}$ is formed with probability $(1-\lambda)$. This process can be formally presented as follows:

$$\mathrm{RNAPII}_{\mathrm{TAR}} \rightarrow [1-\lambda]\mathrm{RNAPII}_{\mathrm{DNAunit}_1} + [\lambda]\mathrm{microRNA} : k_{\mathrm{delay}}$$

where k_{delay} is the rate constant for the RNAPII exit from the pausing site at the TAR element.

3. The Tat-dependent antitermination of transcription at the TAR element is described using the following two reactions:

$$\mathrm{RNAPII}_{\mathrm{TAR}} + \mathrm{Tat}_{\mathrm{nuc}} \leftrightarrow \mathrm{Tat}_{\mathrm{TAR}} : k_{\mathrm{assoc,Tat_{TAR}}}, k_{\mathrm{dissoc,Tat_{TAR}}}$$

$$\mathrm{Tat}_{\mathrm{TAR}} \rightarrow \mathrm{RNAPII}_{\mathrm{DNAunit}_1} + \mathrm{Tat}_{\mathrm{nuc}} : k_{\mathrm{antiterm}}$$

The first reaction describes the interaction of the complex $\mathrm{RNAPII}_{\mathrm{TAR}}$ at the TAR element with the nuclear fraction of the Tat protein $\mathrm{Tat}_{\mathrm{nuc}}$ resulting in the formation of a $\mathrm{Tat}_{\mathrm{TAR}}$ complex. The second reaction describes Tat-dependent antitermination leading to the formation of the elongation complex, $\mathrm{RNAPII}_{\mathrm{DNAunit}_1}$, and the release of the Tat protein. $k_{\mathrm{assoc},Tat_{TAR}}$ and $k_{\mathrm{dissoc},Tat_{TAR}}$ are the rate constants for the association and dissociation of Tat protein with the TAR element, respectively; k_{antiterm} is the rate constant of transcriptional antitermination by the Tat protein at the TAR element.

4. To model a delay in the synthesis of 9 kb RNAs, a chain of n_{DNAunit} reactions was introduced to formally present transcription elongation from the TAR element to the transcription terminator site. This process can be written as follows:

$$\mathrm{RNAPII}_{\mathrm{DNAunit}_i} \rightarrow \mathrm{RNAPII}_{\mathrm{DNAunit}_{i+1}} : k_{\mathrm{transcr,elong},i}, \quad i = 1,\ldots,n_{\mathrm{DNAunit}}$$

where $\mathrm{RNAPII}_{\mathrm{DNAunit}_i}$ is the level of elongating complexes at the i-th segment of the proviral DNA; $k_{\mathrm{transcr,elong},i}$ is the transcription elongation rate constant at the i-th segment. It is assumed that the lengths of the segments and the transcription rates are the same.

5. The transcription termination finalizing the elongation of the last $(n_{\mathrm{DNAunit}}+1)$-th segment of the proviral DNA and the release of the precursor molecule of the nuclear 9 kb RNA, $\mathrm{pre9kbRNA}_{\mathrm{nuc}}$, is formally presented as follows:

$$\mathrm{RNAPII}_{\mathrm{DNAunit}_{n_{\mathrm{DNAunit}}+1}} \rightarrow \mathrm{pre9kbRNA}_{\mathrm{nuc}} : k_{\mathrm{transcr,term}}$$

where $k_{\mathrm{transcr,term}}$ is the transcription termination rate constant of the nuclear 9 kb RNA.

6. The maturation of the 9 kb mRNA primary transcript into the mature 9 kb mRNA form, $9\text{kbRNA}_{\text{nuc}}$, is modeled as follows:

$$\text{pre9kbRNA}_{\text{nuc}} \rightarrow 9\text{kbRNA}_{\text{nuc}} : k_{\text{modif}}$$

where k_{modif} is the rate constant of the primary 9 kb RNA maturation process.

7. The splicing of the 9 kb RNA leading to the formation of 4 kb RNA, $4\text{kbRNA}_{\text{nuc}}$, in the nucleus is represented by:

$$9\text{kbRNA}_{\text{nuc}} \rightarrow 4\text{kbRNA}_{\text{nuc}} : k_{\text{splicing,94}}$$

where $k_{\text{splicing,94}}$ is the rate constant for the splicing of 9 kb to 4 kb mRNAs.

8. The alternative splicing of 9 kb and 4 kb RNAs to 2 kb mRNAs, which encode Tat, $2\text{kbRNA}_{\text{nuc}}$, and Rev, $2\text{kbRNA}_{\text{nuc}}$, proteins, is described as follows:

$$x\text{kbRNA}_{\text{nuc}} \rightarrow \left[\delta_{x,\text{Tat}}\right] 2\text{kbRNA}_{\text{Tat,nuc}} + \left[\delta_{x,\text{Rev}}\right] 2\text{kbRNA}_{\text{Rev,nuc}} : k_{\text{splicing,x2}}, \quad x \in \{9,4\}$$

where $k_{\text{splicing},x2}$ is the rate constant for the splicing of 9 kb and 4 kb to 2 kb mRNAs; $\delta_{x,\text{Tat}}$ and $\delta_{x,\text{Rev}}$ are the fractions of 2 kb RNAs, $2\text{kbRNA}_{\text{Tat,nuc}}$ and $2\text{kbRNA}_{\text{Rev,nuc}}$, respectively, produced by the alternative splicing of 9 kb and 4 kb mRNAs.

9. The transport of 2 kb mRNAs into the cytoplasm is described as follows:

$$2\text{kbRNA}_{\text{X,nuc}} \rightarrow 2\text{kbRNA}_{\text{X,cyt}} : k_{\text{transport,2kbRNA}_{\text{X}}}, \quad X \in \{\text{Tat}, \text{Rev}\}$$

where $2\text{kbRNA}_{X,\text{cyt}}$ is the concentration of 2 kb mRNAs encoding Tat and Rev in the cytoplasm; $k_{\text{transport,2kbRNA}_X}$ is the rate constant of the nuclear export of 2 kb RNAs of the Tat and Rev proteins.

10. The synthesis of Tat and Rev proteins in the cytoplasm is described as a chain of reactions considering the initiation, elongation, and termination of the translation:

$$[0] 2\text{kbRNA}_{X,\text{cyt}} \rightarrow 2\text{kbRNA}_{X,\text{transl}_{,\text{elong}_i}} : k_{\text{transl,ini},X}$$

$$2\text{kbRNA}_{X,\text{transl}_{,\text{elong}_i}} \rightarrow 2\text{kbRNA}_{X,\text{transl}_{,\text{elong}_{i+1}}} : k_{\text{transl,elong}_i}, \quad i = 1,\ldots,n_X,$$

$$2\text{kbRNA}_{X,\text{transl}_{,\text{elong}_{n_X+1}}} \rightarrow \text{prot}_{X,\text{cyt}} : k_{\text{transl,term},n_X+1}, \quad X \in \{\text{Tat}, \text{Rev}\}$$

where $k_{\text{transl,ini},X}$ is the rate constant of translation initiation; $k_{\text{transl,elong}_i}$ is the rate constant of translation elongation for the i-th segment, and $k_{\text{transl,term},n_X+1}$ is the rate

constant of translation termination for the (n_X+1)-th segment. The first reaction describes the initiation of the translation of Tat and Rev proteins in the cytoplasm resulting in the formation of the elongation complex $2\text{kbRNA}_{X,\text{transl}_{\text{elong1}}}$. The next n_X reactions describe the translation elongation for Tat and Rev proteins. The reaction chains were used to reproduce a delay in the synthesis of Tat and Rev. The set $2\text{kbRNA}_{X,\text{transl}_{\text{elong}i}}$ characterizes the number of elongation complexes on the i-th segment of the viral mRNAs. The last reaction describes the translation termination associated with the formation of Tat and Rev. $\text{prot}_{X,\text{cyt}}$ denotes the amount of Tat and Rev proteins in the cytoplasm.

11. The transport of Tat and Rev proteins from the cytoplasm to the nucleus is modeled by the equation:

$$\text{prot}_{X,\text{cyt}} \rightarrow \text{prot}_{X,\text{nuc}} : k_{\text{transport,prot}_X}, X \in \{\text{Tat}, \text{Rev}\}$$

where $\text{prot}_{X,\text{nuc}}$ is the abundance of Tat and Rev in the nucleus, and $k_{\text{transport,prot}_X}$ is the protein-specific rate constant for the transport of Tat and Rev from the cytoplasm to the nucleus.

12. The formation of the complexes of the Rev proteins with 9 kb and 4 kb RNAs is described as a sequence of n_{Rev} reactions resulting in the production of n_{Rev}-th complex. It is assumed that the complexes of Rev with 9 kb and 4 kb RNAs can take place both in the nucleus and the cytoplasm. The corresponding set of equations reads:

$$x\text{kbRNA}_y + \text{prot}_{\text{Rev}_y} \leftrightarrow \text{Rev}_1 x\text{kbRNA}_y : k_{\text{assoc,Rev}_1 X_y}, k_{\text{dissoc,Rev}_1 X_y}$$

$$\text{Rev}_i x\text{kbRNA}_y + \text{prot}_{\text{Rev}_y} \leftrightarrow \text{Rev}_{i+1} x\text{kbRNA}_y : k_{\text{assoc,Rev}_{i+1} X_y}, k_{\text{dissoc,Rev}_{i+1} X_y}$$

$$i = 1, \ldots, n_{\text{Rev}} - 1, x \in \{9, 4\}, y \in \{\text{nuc}, \text{cyt}\}$$

where $\text{Rev}_i x\text{kbRNA}_y$ is the level of complexes containing i molecules of the Rev proteins with either 4 kb or 9 kb RNAs in the nucleus or in the cytoplasm, respectively; $k_{\text{assoc,Rev}_{i+1} X_y}$ and $k_{\text{dissoc,Rev}_{i+1} X_y}$ are the association and dissociation rate constants, respectively, for the binding of Rev to 4 kb or 9 kb RNAs at the stage of the (i + 1)-meric complex formation in the nucleus or the cytoplasm.

13. The Rev-dependent transport of 9 kb and 4 kb RNAs from the nucleus ($\text{Rev}_i x\text{kbRNA}_{\text{nuc}}$) to the cytoplasm ($x\text{kbRNA}_{\text{cyt}}$) followed by the release of the Rev protein in the nucleus (at the nuclear pore) and in the cytoplasm is described as follows:

$$\text{Rev}_i x\text{kbRNA}_{\text{nuc}} \rightarrow x\text{kbRNA}_{\text{cyt}} + [\gamma_i]\text{prot}_{\text{Rev}_{\text{nuc}}} + [i - \gamma_i]\text{prot}_{\text{Rev}_{\text{cyt}}} : k_{\text{transport},X,i}$$

$$i = 1, \ldots, n_{\text{Rev},x} - 1, \quad x \in \{9, 4\}$$

where $k_{\text{transport},X,i}$ is the rate constant of the nuclear export of the i-meric complex of Rev with 9 kb or 4 kb RNAs; γ_i is the fraction of Rev proteins released from the i-meric complex at the nuclear pore; $(i - \gamma_i)$ is the fraction of Rev proteins released from the i-meric complex in the cytoplasm.

14. The degradation of the 9 kb, 4 kb, and 2 kb RNAs in the nucleus and the cytoplasm is described by the following first-order reactions:

$$x\text{kbRNA}_y \rightarrow \varnothing : k_{\text{degr},x\text{kbRNA}_y}$$

$$2\text{kbRNA}_y \rightarrow \varnothing : k_{\text{degr},2\text{kbRNA}_y}$$

$$\text{Rev}_i x\text{kbRNA}_y \rightarrow [\gamma_i]\text{prot}_{\text{Rev}_{\text{nuc}}} : k_{\text{degr},\text{Rev}_i x\text{kbRNA}_y}$$

$$\text{prot}_{X_y} \rightarrow \varnothing : k_{\text{degr},\text{prot}_{X_y}}$$

$$i = 1,\ldots,n_{\text{Rev},x} - 1, \quad x \in \{9,4\}, X \in \{\text{Tat},\text{Rev}\}, y \in \{\text{nuc},\text{cyt}\}$$

where $k_{\text{degr},x\text{kbRNA}_y}$ is the 9 kb and 4 kb RNA degradation rate constants in the nucleus and the cytoplasm; $k_{\text{degr},prot_{X_y}}$ are rate constants of the specific degradation of the Tat and Rev proteins; and $k_{\text{degr},\text{Rev}_i x\text{kbRNA}_y}$ are degradation rate constants for 9 kb and 4 kb RNAs in the i-meric complex with the Rev protein in the nucleus and cytoplasm.

Model parameters were directly estimated from the published experimental data or indirectly from physical and chemical properties of the model processes and components (Table 34.1). Below we present some of those estimates.

Transcription The transcription elongation rate in eukaryotic cells ranges from 25 to 60 nucleotides/s [66, 76, 78]. A similar estimate for HIV-1 (~33 nucleotides/s) was obtained using a reporter construct integrated at specific transcription site of the viral genome [79, 80]. The basal transcription rate starting from the HIV-1 promoter in nonactivated cells is assumed to be ~40 nucleotides/s, whereas transcription initiation is assumed to be ~0.25 events/min.

The exit rate of RNAPII from the transcription elongation pausing state is formally quantified by the parameter k_{delay}. The value of this parameter is estimated to be 1/min assuming that the duration of pausing is ~1 min. Pausing time is assumed to be longer than the time of the transcription through the TAR element by RNAPII without pausing, which ranges from 1 to 2 s. The later estimate results from the base length of the TAR element being 59 nucleotides and an elongation rate of about 25–60 bases/s [66, 76]. In the absence of Tat protein, RNAPII located at the TAR element can spontaneously leave the pausing site and either continue the transcription or terminate it. We assumed that in the absence of Tat, the pausing leads to transcription termination for about 99 out of 100 RNAPII molecules. RNAPII can also exit the pausing site and continue the transcription in the presence of Tat protein due to its interaction with the secondary structure of the TAR element on

Table 34.1 Model parameter used for numerical simulations of the Tat-Rev-mediated regulation of HIV-1 replication

Subsystem number	Parameter notation	Units	Reference value	Reference
1	$k_{\text{transcr,ini}}$	Transcription initiation/(min genome)	0.25 (for nonactivated cell)	[63, 64]
			25 (for activated cell)	
	$\text{LTRP}_{\text{HIV1}}$	Elements/nucleus	1	Assigned
2	k_{delay}	1/min	1	Assigned
	λ		0.99	Estimated
3	$k_{\text{assoc},Tat_{TAR}}$	Elements/(nucleus min)	0.0017	[65]
	$k_{\text{dissoc},Tat_{TAR}}$	1/min	1	
	k_{antiterm}	1/min	60	Assigned
4	$k_{\text{transcr,elong},i}$, $i = 1, \ldots, n_{\text{DNAunit}}$	1/min	5.33	Derived
	n_{DNAunit}	Dimensionless	20	Assigned
5	$k_{\text{transcr,term}}$	1/min	60	Assigned
6	k_{modif}	1/min	60	Assigned
7	$k_{\text{splicing,94}}$	1/min	0.0415	[66–68]
8	$k_{\text{splicing,42}}$	1/min	0.0415	
	$k_{\text{splicing,92}}$	1/min	0.0207	Assigned
	$\delta_{9,\text{Tat}}=\delta_{4,\text{Tat}}$	Dimensionless	0.115	Estimated from [69]
	$\delta_{9,\text{Rev}}=\delta_{4,\text{Rev}}$	Dimensionless	0.115	
9	$k_{\text{transport,2kbRNA}_X}$, $X \in \{\text{Tat, Rev}\}$	1/min	0.0767	[28]
10	$k_{\text{transl,ini},X}$, $X \in \{\text{Tat, Rev}\}$	1/min	10	Assigned
	$k_{\text{transl,elong}_i}, X \in \{\text{Tat,Rev}\}$	1/min	18	Derived
	$k_{\text{transl,term},n_X+1}$, $X \in \{\text{Tat, Rev}\}$	1/min	60	Assigned
	$n_X, X \in \{\text{Tat, Rev}\}$	Dimensionless	20	Assigned
11	$k_{\text{transport,prot}_X}, X \in \{\text{Tat,Rev}\}$	1/min	0.347	[70]

(continued)

Table 34.1 (continued)

Subsystem number	Parameter notation	Units	Reference value	Reference
12	$k_{\text{assoc,Rev}_i X_{nuc}}$, $i = 1, \ldots, n_{\text{Rev}} - 1$, $x \in \{9, 4\}$	Copy/(nucleus min)	0.59	[29]
	$k_{\text{assoc,Rev}_i X_{cyt}}$, $i = 1, \ldots, n_{\text{Rev}} - 1$, $x \in \{9, 4\}$			
	$k_{\text{dissoc,Rev}_i X_{nuc}}$, $i = 1, \ldots, n_{Rev} - 1$, $x \in \{9, 4\}$	1/min	8.4	
	$k_{\text{dissoc,Rev}_i X_{cyt}}$, $i = 1, \ldots, n_{\text{Rev}} - 1$, $x \in \{9, 4\}$			
13	$k_{\text{transport},x,1}$, $x \in \{9, 4\}$	1/min	0	[71]
	$k_{\text{transport},X,1}$, $i = 2, \ldots, n_{\text{Rev},x} - 1$, $x \in \{9, 4\}$	1/min	0.0767	[28]
	γ_i, $i = 1, \ldots, n_{\text{Rev},X}$	Dimensionless	12	Quantified following the recycling in the nuclear pore hypothesis
12,13	$n_{\text{Rev},x}$ $x \in \{9, 4\}$	Dimensionless	12	[29, 72, 73]
14	$k_{\text{degr},X\text{kbRNA}_y}$, $X \in \{\text{Tat, Rev}\}$, $y \in \{\text{nuc, cyt}\}$	1/min	0.0029	[74]
	$k_{\text{degr,2kbRNA}_y}$, $y \in \{\text{nuc,cyt}\}$			
	$k_{\text{degr,Rev}_i X\text{kbRNA}_y}$ $i = 1, \ldots, n_{\text{Rev},x} - 1$, $x \in \{9, 4\}$, $y \in \{\text{nuc, cyt}\}$			
	$k_{\text{degr,prot}_{X_{nuc}}}$, $X \in \{\text{Tat,Rev}\}$	1/min	0.000722	[75]
	$k_{\text{degr,prot}_{X_{cyt}}}$, $X \in \{\text{Tat,Rev}\}$	1/min	0.00289	
Auxiliary parameters	$r_{\text{transcr,elong}}$	Nucleotides/min	2400	[66, 76]
	$r_{\text{transl,elong}}$	Nucleotides/min	1800	[77]
	$9\text{kbRNA}_{\text{nuc}}$	Nucl	9000	[19]
	$2kbRNA_{\text{nuc}}$	Nucl	2000	

Modified from Likhoshvai et al. [17]

synthesized RNA. The availability of Tat in the nucleus activates the synthesis of 9 kb RNA by up to 100-fold [23–25].

The antitermination efficacy in the model is described by the parameter k_{antiterm}. This parameter is estimated to be 60 min^{-1}. Parameters $k_{\text{assoc},Tat_{TAR}}$ and $k_{\text{dissoc},Tat_{TAR}}$ represent the association and dissociation rate constants, respectively, for Tat protein with the secondary structure of the partially synthesized RNA at the TAR element region (see Table 34.1). The published data provide an estimate for the ratio $K_{d,\text{Tat}} = k_{\text{dissoc,Tat}_{\text{TAR}}} / k_{\text{assoc,Tat}_{\text{TAR}}}$ with a range of 100–400/μM [65, 81]. The dissociation rate constant is assumed to be $k_{\text{dissoc},Tat_{TAR}}$ =1/min. It was derived using a value of $K_{d,\text{Tat}}$=100/μM with the volume of the nucleus set to 100 μm^3.

Splicing The characteristic time of splicing the pre-mRNAs during transcription is about 5–10 min and does not depend on intron size [66–68].

Transport The transport of 2 kb mRNA from the nucleus to the cytoplasm is carried out by endogenous cellular mechanisms. Active transport through the nuclear pore is a relatively fast process (10–100 molecules per the pore per second) [82, 83]. It is formally described in the model as a monomolecular reaction. The transport of 9 kb and 4 kb RNAs out of the nucleus is mediated by a Rev-dependent mechanism. The binding of Rev protein to the RRE site of the intron-containing HIV-1 mRNA takes place sequentially, and the assembled oligomeric complex, Rev/RRE, can contain up to 12 molecules of Rev protein [29, 72, 73]. The transport of Tat and Rev back to the nucleus is mediated by endogenous cellular mechanisms, i.e., a nuclear localization sequence (NLS) signal. The transport kinetics of Tat was experimentally measured, and the specific rate constant of nuclear import is ~0.3 min^{-1} [70]. Here, Tat and Rev nuclear import rate constants are set to 0.347 min^{-1}.

Translation The average ribosome density per codon in eukaryotic cells is 0.017 [77, 84]. Taking into account a length of Tat/Rev mRNA of about 2000 bases, the number of available ribosomes at this segment can be estimated to be ~11. The translation elongation rate of mRNA depends on the initiation rate, and the initiation rate in the model is assumed to be ~10 events/min. Thus, the translation elongation rate constant is ~30 nucleotides/s.

mRNA Stability The stability of HIV-1 mRNA in the nucleus and the cytoplasm is about the same [85] with a half-life for Tat mRNA ranging from 4 to 5 h and for Rev mRNA from 4 to 13 h [74, 86].

Stability of Proteins The half-life of Rev is assumed in the model to be 4 h in the cytoplasm and 16 h in the nucleus [75].

The parameters of the mathematical model of the Tat-Rev-mediated regulation of HIV-1 replication used for numerical simulations are presented in Table 34.1.

The overall rate of change for every model variable is calculated by adding or subtracting the rates of the elementary subsystems where the current variable is produced or consumed, respectively. The initial value problem for the model equations was solved numerically using Gear's method based on backward differentiation formulas [87] implemented in Fortran [17]. The model with parameter values

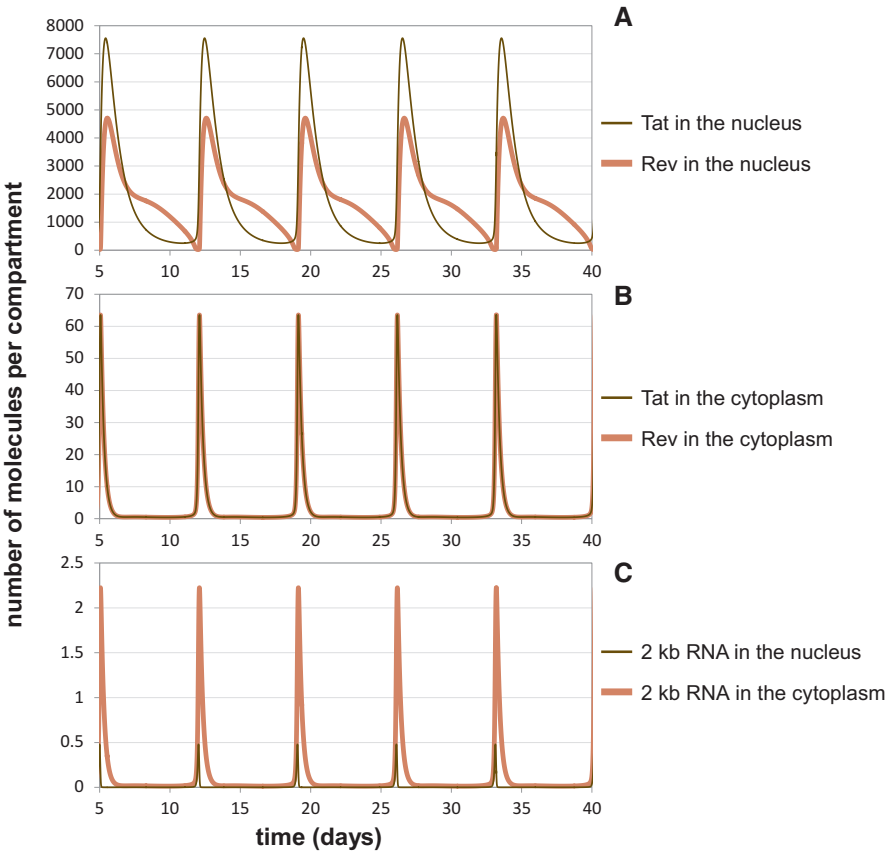

Fig. 34.5 Kinetics of the viral RNA and proteins synthesized in an activated cell with one provirus copy. (**a**) The abundance of free Tat molecules (not bound to RNA at the TAR element) and Rev molecules (not bound to 9 kb RNA and 4 kb RNA) in the nucleus. (**b**) The abundance of free Tat and Rev proteins in the cytoplasm (their kinetics is identical as the corresponding model parameters for these two proteins are identical, and the nuclear export of Rev to the cytoplasm is not considered). (**c**) The abundance of 2 kb RNA molecules in the nucleus and cytoplasm encoding the Tat and Rev proteins

presented in Table 34.1 predicts sustained oscillations of HIV components in the within-host target cell system controlled by the Tat-Rev regulatory circuit (Fig. 34.5).

It should be noted that the oscillatory pattern in Fig. 34.5 appears as a limit cycle. The period of the cycle is rather long, being more than 150 h. The amplitude of oscillations in the concentrations of Tat and Rev proteins and the 2 kb RNAs encoding Tat and Rev over one period is quite significant. The simulations further predict that the 2 kb RNA molecules are present in trace amounts and are amenable to detection during a rather short-time window of about 10 h as compared to the cycle period. The above solutions were computed under the assumption that Rev-regulated

export of 9 kb RNA and 4 kb RNA from the nucleus to the cytoplasm does not lead to the exit of Rev protein from the nucleus to the cytoplasm, which is in agreement with the hypothesis of Rev recycling at the nuclear pore [60]. The formation of complexes of the de novo synthesized Rev proteins with the 9 kb RNA and 4 kb RNA molecules in the cytoplasm compartment was not considered.

34.5 Parameter Sensitivity Analysis for Periodic Solutions for the Basic Within-Host Organism and Within-Host Target Cell HIV Models

Theoretical analysis of the basic within-host organism model [53] and the parameter sensitivity analysis of the basic within-host target cell HIV model reveal that both models have oscillatory dynamics within a wide range of parameters. For example, the basic within-host organism model has periodic solutions within a wide range of death rates for the uninfected target cells (Fig. 34.6). For certain parameter sets, this model exhibits oscillatory dynamics with half-lives of the uninfected target cells varying from less than a day to more than 100 days. This range of half-lives covers different groups of potential target cells, including short-lived (e.g., T cells) and long-lived (e.g., macrophages) ones. Similarly, the basic within-host target cell model has periodic solutions within a wide range of half-life times for Tat and Rev proteins (Fig. 34.7). This model can exhibit oscillations with half-lives of Tat and Rev proteins in the nucleus and the cytoplasm varying from a few hours to a few hundred hours. The period and amplitude of oscillations can be different for different half-lives of the viral regulatory proteins. The kinetic properties and potential oscillatory dynamics of the basic within-host target cell model were previously systematically investigated [17]. Particularly, it was shown that this model has robust oscillatory dynamics for wide ranges of kinetic parameters of such processes and quantities as: (1) The nuclear export of the HIV-1 Rev protein to the cytoplasm; (2) The variation of provirus copy numbers; (3) The abundance of Rev protein in the complexes with 9 kb RNA and 4 kb RNA; (4) The transcription antitermination at the TAR element; (5) The oligomerization of Rev protein and the complex transport; (6) The 2 kb RNA translation initiation efficacy; (7) The Rev-mRNA stability

For example, the antitermination of transcription in the proposed model [17] is characterized by four parameters: the rate constant for the exit of RNAPII from the pausing site at TAR element (k_{delay}), the rate constant of the antitermination efficacy (k_{antiterm}), the forward, and the reverse rate constants of Tat protein binding to the secondary structure at the TAR element ($k_{\text{assoc},Tat_{TAR}}$ and $k_{\text{dissoc},Tat_{TAR}}$) (see Table 34.1). The ratio $K_{d\,,\,\text{Tat}} = k_{\text{dissoc},Tat_{TAR}} / k_{\text{assoc},Tat_{TAR}}$ was experimentally estimated as 10 nM [19]. In order to characterize the impact of these parameters on transcription antitermination at the TAR element and the overall model dynamics, the ratio $K_{d\,,\,\text{Tat}}$ and other two parameters (k_{delay} and k_{antiterm}) were varied as follows: k_{delay} = 0.25–10 min^{-1}, $k_{\text{antiterm}}/k_{\text{delay}}$ = 1–60, $k_{\text{dissoc},Tat_{TAR}}$ = 0.05–32 min^{-1}. Table 34.2 summarizes the results of parameter sensitivity analysis on the impact of Tat-dependent antitermination

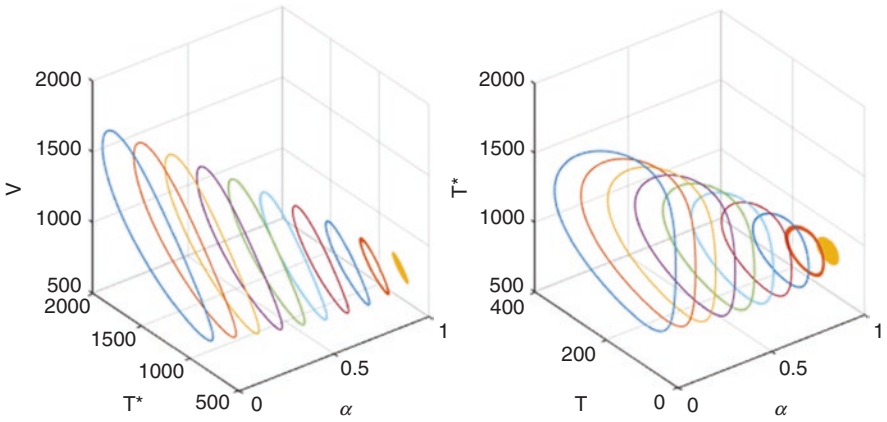

Fig. 34.6 Parameter sensitivity analysis for periodic solutions for the basic within-host organism HIV model ($f = f_I$). Parameter α was varied between 0.01 and 1 day^{-1}. Other parameters were fixed as follows: δ = 10 day^{-1} mm^{-3}, p = 3 day^{-1}, T_{max} = 1500 mm^{-3}, β = 0.24 day^{-1}, γ = 2.4 day^{-1}, k = 0.0027 mm^{3} day^{-1}, and N = 10. T is the level of uninfected T cells; T^* is the level of productively infected T cells; and V is the level of free virus particles in the blood

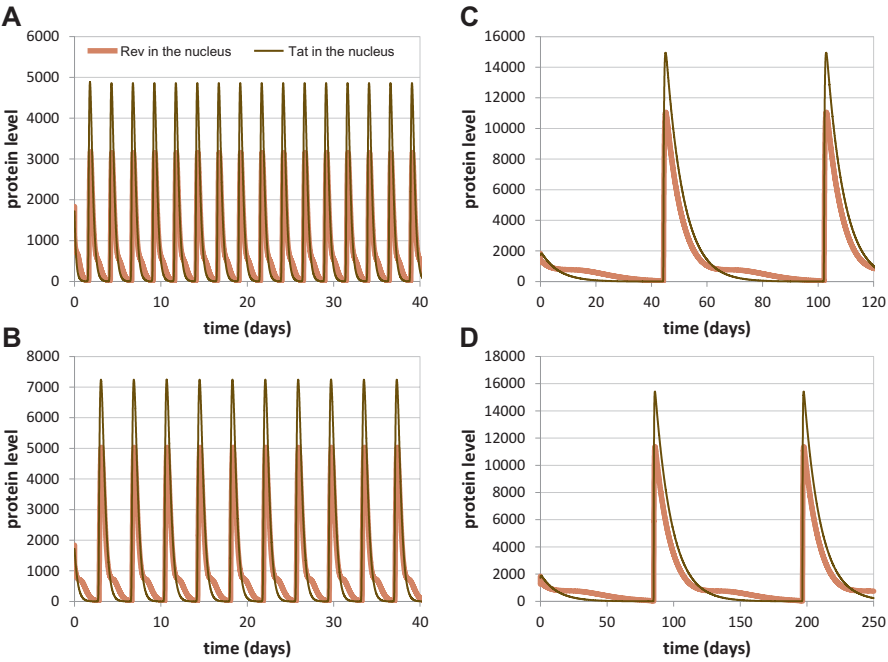

Fig. 34.7 Parameter sensitivity analysis for periodic solutions for the basic within-host target cell HIV model. Half-life time ($\tau_{1/2}$) of Tat and Rev in the nucleus and the cytoplasm were varied. (**a**) $\tau_{1/2}$ = 2.6 h for Tat and Rev both in the nucleus and the cytoplasm. (**b**) $\tau_{1/2}$ = 3.2 h for Tat and Rev both in the nucleus and the cytoplasm. (**c**) $\tau_{1/2}$ = 104 h for Tat and Rev both in the nucleus and the cytoplasm. (**d**) $\tau_{1/2}$ = 208 h for Tat and Rev both in the nucleus and the cytoplasm. γ_i = 0. Other parameters of the model used for these numerical simulations are presented in Table 34.1

Table 34.2 The impact of the Tat-dependent antitermination efficiency on the kinetics of the synthesis of viral components in the activated cell

Constant of the delay of RNA polymerase II on TAR element k_{delay} (min^{-1})	Regimes of the system functioning for different values of the dissociation constant of Tat-TAR complex $k_{dissoc,Tat_{TAR}}$ (min^{-1})									
	32	16	8	4	2	1	0.5	0.25	0.1	0.05
10	o[a]/o[b]/s[c]	o/o/o	o/o/o	o/o/o	o/o/o	s/s/s	s/s/s	s/s/s	s/s/s	s/s/s
4	o/s/s	o/s/s	o/s/s	o/o/o	o/o/o	o/o/o	s/s/s	s/s/s	s/s/s	s/s/s
1	o/s/s	o/s/s	o/s/s	o/s/s	o/s/s	o/o/o	o/o/o	o/o/o	s/s/s	s/s/s
0.25	o/s/s	o/s/s	o/s/s	o/s/s	o/s/s	o/s/s	o/s/s	o/o/o	o/o/o	s/o/o

Note: *o* stands for oscillation; *s* stands for steady state
[a]The value of the antitermination efficiency constant $k_{antiterm} = k_{delay}$
[b]$k_{antiterm} = 10*k_{delay}$
[c]$k_{antiterm} = 60*k_{delay}$

efficiency on the kinetics of the synthesis of viral components in the activated cell. The simulations were performed for a single virus genome copy since the calculations for higher copy numbers are not fundamentally different.

These computational results suggest the following:

1. The parameter space region corresponding to the oscillatory dynamics is rather large.
2. The parameters are not independent with respect to their impact on the HIV-1 replication system behavior. For example, for a decreased value of the pausing delay of RNAPII at the TAR element, the oscillatory dynamics is observed at higher values of the dissociation of the Tat-TAR complex. The oscillatory regime is shifted from the higher to lower values of $k_{dissoc,Tat_{TAR}}$ with the increase of k_{delay} (see Table 34.2).
3. An increase in the proviral copy number extends the parameter region, for which the model has oscillatory dynamics of the viral proteins (the data are not shown).
4. The considered parameter values are in the physiological range and may well belong to the oscillatory domains of the model parameter space.

Thus, the analysis of both the within-host organism model and the within-host target cell HIV models revealed their high potential to generate oscillatory dynamics. In the case of the basic within-host organism HIV model, the virus persists in the host and the model solutions approach either a chronic disease steady state or a periodic orbit if the basic reproduction number $R_0 > 1$. If $R_0 < 1$, then the virus is cleared and the disease dies out [53]. In the case of the basic within-host target cell HIV system, the oscillatory dynamics of the model essentially depends on a Rev protein shuttling mechanism, the stability of the Rev mRNA, and the interaction parameters of Rev protein with the RRE site on the intron-containing RNA [17].

Taking into account that the parametric domain corresponding to the oscillatory mode of HIV-1 replication is quite large, we hypothesize that the predicted phenomenon is not just a modeling artefact but may take place under certain conditions in an infected target cell or in a host cell population. One of the indirect indications in favor of this hypothesis is the ability of HIV-1 to establish a long-term persistent production of the infectious particles in humans [88]. We speculate that the identified oscillatory dynamics of HIV-1 replication at both the intracellular level and at the level of cell populations can be one of the possible mechanisms for the maintenance of the prolonged within-host organism or within-host target cell virus persistence.

34.6 Discussion

Mathematical modeling of within-host pathogen dynamics has flourished over the past few decades [89]. These models have been used to describe the dynamics of various infectious diseases, such as HIV [7, 17, 90, 91], HCV [92, 93], HTLV [94, 95], IAV [96–98], HDV [99], HSV [100], CMV [101] as well as tuberculosis [102, 103] and malaria [104, 105] infections. Testing specific hypotheses based on clinical data is often difficult since samples cannot always be frequently taken from patients and because techniques for detecting the pathogen may not be accurate. This only amplifies the importance of mathematical models in this area of research.

In this chapter we summarized several theoretical studies on the potential for oscillatory behavior of HIV infection at molecular and cellular levels. These studies emphasize some aspects of nonlinear and inherently multiscale properties of the host-virus system. These properties of the virus, along with others, such as rapid mutation, hiding viral surface components from neutralizing antibodies, proviral latency, removal of cell-surface receptors, and destruction of immune effectors [3], may potentially be the result of coevolution with the host immune system. These properties may also constantly drive and shape the ability of the virus to avoid immune eradication.

Consider the oscillatory behavior of the viral components from the viewpoint of a biological advantage. In our discussion, we will assume the broadly accepted paradigm stating that if a specific feature of a biological system stably persists through many generations, then this feature provides a certain evolutionary advantage in the struggle for survival. Now, the question is what type of benefit would the cycling dynamics of viral components production give to the virus as compared to a steady state one? In our view, the real advantage is that the likelihood of the survival of the HIV-1-infected cell population should increase.

Consider the population of infected macrophages bearing the provirus in a latent form. When these cells activate, viral RNAs and proteins will be synthesized, and virus particles will be assembled and bud from infected cells. These cells become a target for the immune system, with the likelihood of the cell to be recognized by cytotoxic T lymphocytes (CTLs) being proportional to the concentration of the

virus-specific antigens that are expressed on the cell surface by major histocompatibility complex (MHC) class I molecules. It is clear that in the absence of specific hiding mechanisms, the probability of the infected cell being recognized and destroyed will increase as the viral replication rate increases. Therefore, after some time most of the infected cells would become highly vulnerable to immune system, probably leading to a complete elimination of the infected cell population.

If we assume that the HIV-1 life cycle follows an oscillatory behavior, virus production by every infected cell will cycle and, unless there is a synchronization mechanism, the infected cell population will cycle asynchronously. This implies that at any given time, only a fraction of infected cells will be actively producing virus, with the rest of them staying in a silent mode of viral replication. Obviously, the cells characterized by a low-level viral replication will be less recognizable for the immune system as compared to the active producers. Thus, the immune system will recognize and destroy only a fraction of infected cells at any given time. Therefore, the oscillatory dynamics of viral ontogenesis should increase the survival of the virus under the selection pressure of the immune system.

An additional mechanism contributing to long-term viral persistence in an infected cell may be linked to oscillations in the level of the viral regulatory protein Nef. It has been shown that Nef induces a reduction of MHC class I molecules at the cell surface via endocytosis [106, 107]. This in turn reduces the efficacy of the recognition and killing of infected cells by CTLs leading to a latent infection characterized by long-term low levels of viral replication.

There are additional advantages to a virus exhibiting an oscillatory phenotype, including another phenomenon that we call the “recovery effect.” Active viral production by the infected cell over a long period can lead to the exhaustion of its resources and, finally, to death. Therefore, the alternating phases of high- and low-level virus replication should allow the cell to replenish consumed resources and avoid dying. Thus, the recovery effect should increase virus survival and grant an evolutionary advantage.

Therefore, oscillatory dynamics of the viral components at both molecular and cellular levels can provide evolutionary advantages enabling the survival of a fraction of the infected target cells even under constant pressure by the host immune system. This type of ontogenesis dynamics, independent of other specific protection mechanisms evolved by the virus, can contribute to long-term persistent production of the virus in humans, a remarkable emergent property of HIV.

34.7 Conclusions

Theoretically predicted oscillatory behaviors of HIV at both within-host organism and within-host target cell levels necessitates the development of multiscale models that integrate intracellular and cell population host-viral dynamical systems [91, 108]. These models will allow a systematic exploration of potential host-virus

system oscillations and/or pulsatile and bursting behaviors of the virus at different scales, their potential interference or amplification, heterogeneity of the virus behavior within different target cell types (e.g., T cells or macrophages) or target cell subpopulations, and stochastic properties of the multiscale interaction between virus and the host immune system. Multiscale mathematical modeling of the within-host HIV dynamical system combined with relevant experimental measurements at cell population and single-cell [109] levels, especially with the ever improving super-resolution fluorescence microscopy [110–112], promises to facilitate the comprehensive understanding of this complex host-virus system and develop rational interventions into disease processes.

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Conflict of interest The authors report no conflicts of interest.

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Chapter 35
Short Oligopeptide T-Cell Epitopes in HIV-1/AIDS Vaccine Development: Current Status, Design, Promises and Challenges

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Core Message

The development of a HIV-1/AIDS vaccine is a technological challenge over the last two decades for several reasons. Gp120 trimer spike is highly promising for recombinant vaccine development. However, the production of the native spike trimer complex is difficult. The development of an HLA-restricted short peptide T-cell epitope as an alternative vaccine type for HIV-1/AIDS is promising. There are about283 CD8+ and 197 CD4+ gp160 ENV-derived short peptides known to bind to a range of HLA alleles at the LANL HIV molecular database. There is also about 10,000 HLA alleles defined at a sequence level and are made available at the IMGT/HLA database (maintained at the EBI, UK). This forms a basis to select a cocktail of relevant short peptides covering a wider range of HLA alleles using database search and model prediction followed by in vitro and in vivo analysis for consideration as vaccine candidates.

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35.1 HIV-1/AIDS Vaccine: Progress and Current Status

HIV-1/AIDS is one of the life-threatening diseases in the world. The development of an HIV-1/AIDS vaccine is in progress over the last two decades, and it remains indefinable. Verma et al. updated the importance of an effective vaccine for the prevention of the disease in addition to currently available antiretroviral (ARV) drugs [1]. The vaccine candidates developed so far do not show significant efficacy in clinical trials as reviewed recently by Shin [2]. This is true in the case of VaxGen (a Genentech offshoot company) AIDSVAXgp120 vaccine [3] and MRKAd5 HIV-1 Gag/Pol/Nef (STEP trial, a Merck Inc. initiative) [4]. RV144 (Env-gp120, Gag and Pro) vaccine (Thai trial vaccine) showed moderate efficacy in clinical trials [5, 6]. The development of an effective vaccine is possible through the complete understanding of the HIV biology-related immunity. It is known that HIV vaccine development is obstructed by viral mutation, glycosylation, complex structural conformation, viral-human homologous proteins and potential molecular mimicry. Haynes et al. summarized that improved knowledge on the biology of virus, structure of envelope trimer and CD8+ T-cell-controlled neutralizing antibody helps in the development of a vaccine [7]. Several strategies have been formulated towards the development of an effective vaccine for HIV-1/AIDS over the last decade. This is based on information available thus far regarding sequence, structure, function and immunobiology of HIV-1 in relation to the corresponding human host. The RV144 formulation uses single gp120 recombinant protein. However, the viral envelope is a glycosylated trimer spike protein. Therefore, it is important to mimic the native structural conformation of the spike taking surface mutation into context to induce relevant immune response. This is based on the principle of sequence (mutation) to structure (shape) to conformation (changes) to immune response. The cloning, expression, refolding and assembly of a gycosylated gp160 trimer spike ENV are biotechnological challenges [83, 84]. The assembly of individual gp120 into a trimer spike complex architecture is an intricate protein-protein interaction problem. It should be noted that a number of commercial corporations are working towards the production of a stable spike ENV complex with less success. Pancera et al. reported the structural resolution of HIV-1 trimer spike pre-fusion complex captured in a mature closed state by antibodies PGT122 and 35O22 [9]. This is highly promising for recombinant vaccine development despite huge technological challenges in producing a native trimer conformation of the spike protein towards vaccine application. Huang et al. debate on a target candidate selection approach using data from several HIV-1 vaccine trials [21]. There is much interest in ENV as a vaccine candidate post-RV144 trail. Edlefsen et al. [18] reported a comprehensive sieve analysis of sequences related to the RV144 vaccine (Env-gp120, Gag and Pro) clinical trial. This describes methods and tools applicable to analyse infection genomes in vaccine efficacy trials for considering diverse pathogen mutants. Sowmya et al. described HIV-1 ENV surface mutations using polarity properties among available clade, blood, and brain sequences [43] in the perspective of NeuroAIDS as reviewed elsewhere by Shapshak et al. [44].

Musich and Robert-Guroff review recent developments in prime/boost ENV vaccine strategies and highlight complex factors in the induction of adaptive and protective immunity [26]. Gorman et al. engineered ontogeny-specific antigens (ENV

trimers with chimeric V1V2s) that interacted with inferred ancestor and intermediate antibodies providing a general means for eliciting antibodies of a desired class [11]. Bradley et al. showed that oligomeric TF (transmitted/founder) ENVs elicit autologous antibodies to tier-2 HIVs [12]. The HIV ENV is heavily glycosylated. This facilitates the spread and survival of the virus. Therefore, the importance of a specific carbohydrate-based ENV vaccine as updated by Liu et al. is intriguing [16]. Baden et al. showed increased EnvA antibody with moderate immune response in healthy individuals administered with Ad26.EnvA (adenovirus serotype 26 with an HIV-1 envelope A insert) and Ad35.EnvA (adenovirus serotype 35 with an HIV-1 envelope A insert) vaccines in homologous and heterologous combination [22]. Dai et al. showed in vitro binding properties associated with in vivo activation for functional archiving of antigen-specific B cells elicited by a complex YU2 gp140-foldon trimers glycoprotein antigen immunization in non-human primates [25].

Cicalca et al. highlight the use of HIV-1 envelope (ENV) glycoprotein gp120 and discuss its biology for interactions with cell surface receptors as a potential vaccine candidate target [8]. Sadanand et al. review the structural characteristics of broadly neutralizing antibodies against HIV-1 towards a potential vaccine development strategy [10]. This is contextual to vaccine development. Ruane et al. showed that a nasally delivered dendritic-targeted α-CD205-p24 (GAG) vaccine generates HIV-specific immune responses in the GI tract towards a preventative approach [13]. Jacobson et al. administered the Profectus Biosciences multiantigen (MAG) HIV-1 DNA vaccine (encodes Gag/Pol, Nef/Tat/Vif and ENV with IL-12) by electroporation combined with intramuscular injection (IM-EP) and showed increased CD4(+) but not CD8(+) T-cell responses [15]. Dinges et al. show F4/AS01B (fusion—F4–p24, RT, Nef, p17) vaccine induced polyfunctional F4-specific CD4 T cells but did not reduce HIV-1 viral load among antiretroviral therapy (ART)-naive HIV-1-infected adults [24].

A number of other issues such as cost, multiple trials and use of effective adjuvants are to be considered in vaccine development. Harmon et al. highlight that vaccine efficacy and cost per regimen are critical in achieving cost-effectiveness, with cost per regimen being particularly critical in low-income countries and at lower efficacy levels [27]. Tarimo et al. suggest that multiple trials are needed from different geographical sites particularly in sub-Saharan Africa where most HIV infections occur with different HIV-1 subtypes and frequent mutations [28]. Hou et al. showed the use of cyclophilin A as a genetic adjuvant in multiplex DNA immunization strategies for promoting HIV-1 Gag-specific cellular immunity through Gag-CyPA interaction in mouse models [29]. Liu et al. [30], Glass et al. [31] and Caucheteux et al. [32] showed the use of nanomaterials as potential adjuvant materials in HIV vaccine development.

The decreased immune response in HIV-infected individuals or HIV-exposed infants to other vaccines such as yellow fever [23, 33], influenza [34–36], hepatitis B [37], human papilloma virus (HPV) [38] and pneumococcal polysaccharide [39] is described in recent studies, and it shows the interferences caused by HIV exposure. However, this is not true in adults with well-controlled HIV infection [40, 41]. It should also be noted that four-dose or double-dose schedule of HBV vaccine [42] and pneumococcal polysaccharide vaccine [41] achieves very high seroconversion rates in HIV-infected adults/children who have no or mild immunosuppression.

This is a huge concern as it also obstructs the prevention of other viral infections where specific vaccines are available and, hence, warrants the development of an HIV-1/AIDS recombinant vaccine. The production of an ENV trimer SPIKE is a biotechnology challenge for years. Hence, design and development of alternative vaccine type for HIV-1/AIDS such as the short peptide vaccines is highly imperative. The promises and challenges in short peptide viral vaccine design are described elsewhere by Kangueane et al. [45]. A framework for an HIV gp120 peptide vaccine for NeuroAIDS is illustrated elsewhere by Kangueane et al. [46]. This is a promising solution for HIV-1 vaccine development in the context of HIV-1/AIDS, NeuroAIDS and personalized vaccination.

35.2 Principles of Short Peptide Vaccine Design

The key step in T-cell-mediated short peptide vaccine design is HLA allele-specific restriction of antigen peptides (Fig. 35.1). Hence, prediction of HLA-peptide binding using molecular modelling-driven homology procedures and side-chain packing simulations was tested for large dataset of known HLA-restricted peptides by Kangueane et al. [47] and Kangueane and Sakharkar [48]. This methodology was promising for allele-specific prediction of HLA-peptide binding. Therefore, it was of

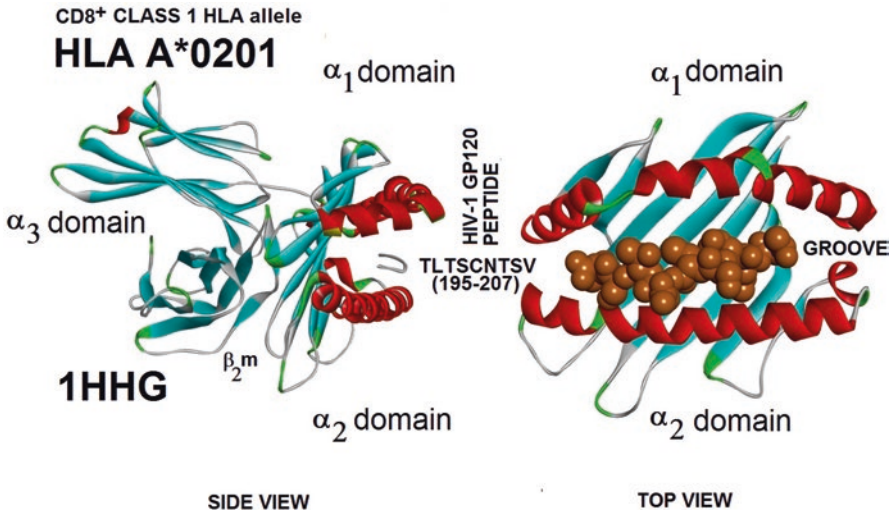

Fig. 35.1 Crystal structure of HIV-1 GP120 (195–207) short peptide TLTSCNTSV (9-mer) bound to HLA A*201 allele structure (PDB ID: 1HHG) is shown. The HIV-1 GP120 nonapeptide is bound to the groove formed by α_1 and α_2 domains in HLA A*201 allele structure. This is the critical step in T-cell-mediated immune response, and this forms the basis for short peptide-based vaccine design and development. Structures for several such HIV peptides bound to known HLA alleles are given in Table 35.1. HIV RT/GP120/GAG/NEF peptides bound to HLA A*0201, B*5101, B*5301 and DR1 are tabulated

further interest to study HLA-peptide binding and its interface properties using known X-ray structure complexes as described elsewhere by Kangueane et al. [49] and Adrian et al. [50]. Public databases of HLA-peptide structures were also developed as a community-driven effort by Govindarajan et al. [51] to further motivate such study. The overlapping functional effect among HLA alleles was also demonstrated using structure-driven sequence features by Zhao et al. [52]. The overlapping HLA function defined using supertypes was shown using structure-driven sequence features by Kangueane et al. [53] for class I alleles and by Mohanapriya et al. [54] for class II alleles. These analyses provided valuable insights to the development of structure-driven pocket-based HLA-peptide binding prediction for class I alleles by Zhao et al. [55] and for class II alleles by Mohanapriya et al. [56]. This resulted in the development of a web server (developed using PERL script) called T-Epitope Designer by Kangueane and Sakharkar [57]. This is made available in the public domain at http://www.bioinformation.net/ted/. The description of structural basis for HLA supertypes by Kangueane and Sakharkar [58] and the visual difference in electrostatic distribution at the binding groove among alleles by Kangueane and Sakharkar [59] are encouraging to the context. It should be noted that there are several other parallel methods for commercial and non-commercial research and development with varying degrees of merits and demerits. However, we restricted our emphasis to T-Epitope Designer in this chapter (Fig. 35.2).

35.3 Short Peptide Vaccine for HIV-1/AIDS: Promises

35.3.1 HIV-1 GP160 ENV-Specific T-Cell Epitopes

There are a large number of known CD8+ and CD4+ T-cell epitopes made available at the LANL HIV Molecular Immunology Database [http://www.hiv.lanl.gov/content/immunology/]. This data was gathered over a period of few decades of extensive research followed by all-inclusive literature mining. This is an invaluable resource for the research and development of a HIV-1/AIDS short peptide vaccine (Fig. 35.2).

35.3.2 CD8+ Class I-Restricted T-Cell Epitopes

There are 1588 *class I-restricted* short peptides, and about 283 of them are GP160 ENV specific at the LANL HIV database. This set was known to be restricted with one or more class I HLA alleles (A1, A2, A3, A11, A23, A24, A25,A26, A29, A30, A31, A32, A33, A68, B7, B8, B14, B15, B18, B27, B35, B38, B40, B42, B44, B51, B53, B55, B56, B57, B58, B60, B61, B63, Cw1, Cw3, Cw4, Cw6, Cw7, Cw8, Cw12, Cw15). This data was assessed on April 01, 2015 from LANL.

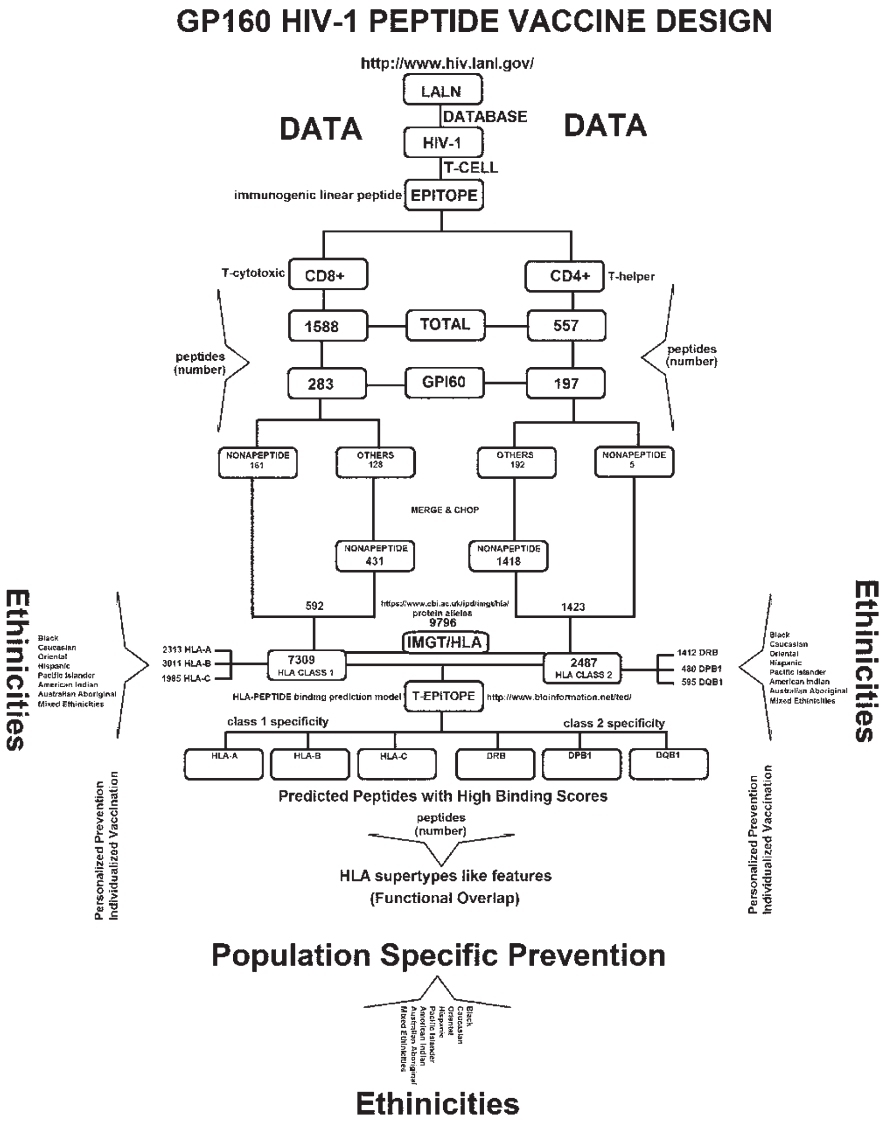

Fig. 35.2 A workflow for the analysis and prediction of HLA allele-specific binding of gp160 peptide binding is shown. Thus, a strategy for the design and evaluation of a global peptide vaccine is illustrated

Table 35.1 Structures of HIV-specific peptides bound to known HLA alleles (downloaded from PDB)

S	Code	Allele	Peptide sequence	L	Source	R (Å)	Year	Group	Country	State
1	1I1F	A*0201	FLKEPVHGV	9	HIV-1 RT	2.8	2000	Collins EJ	USA	North Carolina
2	1AKJ	A*0201	ILKEPVHGV	9	HIV-1 RT	2.6	1997	Jakobsen BK	UK	Oxford
3	1HHG	A*0201	TLTSCNTSV	9	HIV-1 GP120	2.6	1993	Wiley DC	USA	Massachusetts
4	1I1Y	A*0201	YLKEPVHGV	9	HIV-1 RT	2.2	2000	Collins EJ	USA	North Carolina
5	1E27	B*5101	LPPVVAKEI	9	HIV-1	2.2	2000	Jones EY	UK	Oxford
6	1A1M	B*5301	TPYDINQML	9	HIV-2 GAG	2.3	1998	Jones EY	UK	Oxford
7	1A1O	B*5301	KPIVQYDNF	9	HIV-1 NEF	2.3	1998	Jones EY	UK	Oxford
8	1SJH	DR1	PEVIPMFSALSEG	13	HIV-1	2.2	2004	Stern LJ	USA	Cambridge

Protein databank (PDB) entries were downloaded from http://www.rcsb.org/
S Serial Number, *Code* PDB code, *L* length of peptide, *R* Resolution

35.3.3 CD4+ Class II-Restricted T-Cell Epitopes

There are 557 *class II-restricted* short peptides, and about 197 of them are GP160 ENV specific at the LANL HIV database. This set was known to be restricted with one or more class II HLA alleles (DR, DR2, DR6, DRB1 and DP4). This data was assessed on April 01, 2015 from LANL.

35.3.4 Known HLA Alleles

There are a total of 9796 known HLA alleles consisting of 7309 class I and 2487 class II as on October 6, 2015 at IMGT/HLA [https://www.ebi.ac.uk/ipd/imgt/hla/]. There are also about 167 non-HLA alleles and 43 confidential alleles at IMGT/HLA in this update.

35.3.5 Epitope Design Using HLA-Peptide Binding Prediction

Kangueane and Sakharkar [57] have described T-epitope designer [http://www.bioinformation.net/ted]. This provides a platform to predict the binding of selected gp160-specific nonapeptides to 9796 HLA class I alleles and 2487 HLA class II alleles as illustrated in Fig. 35.2.

35.4 Short Peptide Vaccines: Current Status

One of the novel and promising vaccination methods under extensive research and development is peptide vaccine. There are several factors that contribute to the use of peptide vaccine than the conventional vaccine. Tremendous progress has been made in the development of short linear peptide vaccine during the last decade. This is based on allele-specific binding of HLA with antigen peptides [45]. Sun et al. showed the high thermal stability of self-assembled peptides than the traditional recombinant proteins requiring cold storage as vaccines [60]. It is a promising alternative solution where traditional vaccine strategies are not successful. The potential of short peptides as vaccine candidates for cancer [61–69], arthritis [70], candidiasis [71], enterotoxigenic *Escherichia coli* (ETEC) [72], contraceptive [73], Crimean Congo haemorrhagic fever virus [74], foot and mouth disease [75], group A streptococci (GAS) [76] and peanut allergy [77] is demonstrated. Small peptides by themselves are weak to induce antibody responses. Hence, the use of adjuvants such as toll-like receptor (TLR) ligands [78], curcumin-polyethylene glycol conjugate (CUR-PEG) [79], C12 polylysine platform [80] and immune-tolerant elastin-like polypeptide (iTEP)-based nanoparticle (NP) [81, 82] to improve immune response by short peptides is shown in several studies.

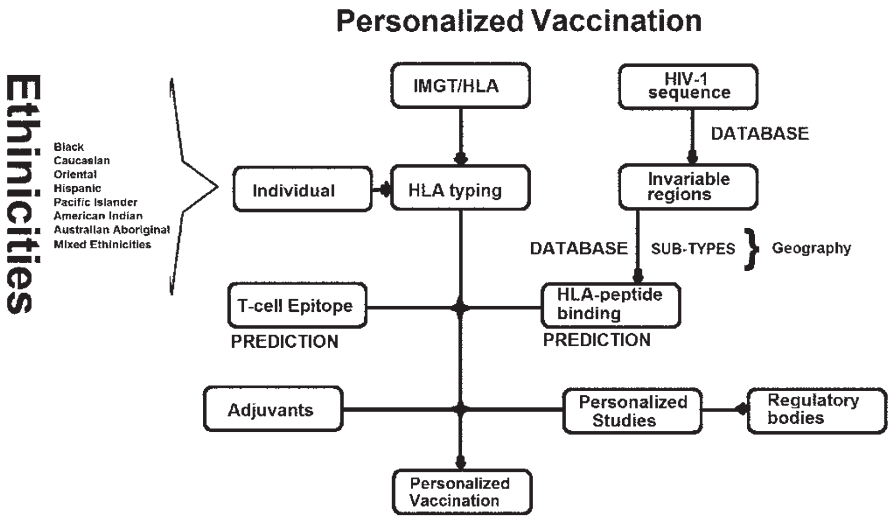

Fig. 35.3 A workflow for the design of gp160-specific personalized vaccine candidates is illustrated. The feasibility of a personalized AIDS vaccine strategy is shown. This is based on HLA typing and evaluation of identical regions among prevalent HIV-1 subtypes in the region followed by HLA-peptide binding studies

35.5 HIV-1/AIDS Short Peptide Vaccine

Short peptide vaccines are based on HLA-restricted T-cell epitopes (Fig. 35.1). There are 1588 HIV-1-specific class I-restricted and 557 class II-restricted short peptides gleaned from literature and made available at the LANL HIV molecular database (Fig. 35.2). This is an impressive collection and has provided valuable yet diversified information on HIV-1-related T-cell immunology. Impressive observation on HIV-specific T-cell-restricted short peptide-mediated immune response using immunogens tHIVconsvX [14], Pol [17], adenovirus type 5 (Ad5) [19], Vacc-4x (p24(Gag)) [20] and pooled peptide among STEP (HVTN 502) trial participants is noted [23]. A combination of mosaic and conserved viral regions provides a better coverage of global HIV-1 variants than other T-cell vaccines [14]. It should be noted that short peptide (high stability and shelf life)-based vaccination is also a reality for the personalized prevention of the disease where the prevalent susceptibility of the infectious agent is clearly ascertained at a molecular level, while HLA typing is feasible for the individual (Fig. 35.3). Short peptide immunogenic potential is demonstrated in the case of other genetic diseases such as cancer [61–69].

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Chapter 36
HIV-1 Envelope (ENV) GP160 Trimer Protein Complex SPIKE as a Recombinant Macromolecular Assembly Vaccine Component Candidate: Current Opinion

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Keywords HIV-1 • Envelope protein • Glycoprotein • Trimer • Spike • Conformation • Vaccine

Core Message
The HIV-1 ENV GP160 glycoprotein trimer complex described as the viral spike is a recognized promising vaccine candidate for HIV-1/AIDS in recent years. The production of viral spike mimicking the in vivo conformation taking mutational variation across different viral clades into consideration in the context of glycoproteomics (study of glycol-proteins) remains a bottleneck. Knowledge gained thus far on ENV trimer using information gleaned from data derived using techniques from several disciplines of biotechnology including molecular biology (sequencing), sequence analysis (mutational study), immunobiology (epitope discovery), genetic engineering (cloning and expression), biochemistry (purification and refolding) and biophysics (X-ray, NMR, electron microscopy (EM), molecular models) is highly hopeful towards the development of a viable vaccine component. An interdisciplinary approach connecting knowledge points on several aspects of the viral spike is critical in the design and development of a prevention candidate for HIV-1/AIDS.

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36.1 HIV-1/AIDS Vaccine Trials

The development of an HIV-1/AIDS vaccine has been a great challenge over the last decade with no significant efficacy in clinical trials [1]. The unsuccessful clinical trial outcomes from VaxGen (a Genentech offshoot company) AIDSVAXgp 120 vaccine [2] and MRKAd5 HIV-1 Gag/Pol/Nef (STEP trial, a Merck Inc., initiative) [3] have been discussed in detail. This is due to viral mutation, viral protein glycosylation (e.g. ENV GP160), protein structural architecture and viral-human molecular mimicry. The moderate efficacy shown in RV144 (Env-gp 120, Gag and Pro) vaccine (Thai trial vaccine) clinical trials [4, 5] is promising. Data from several HIV-1 vaccine trials [6] set the pathway for vaccine target selection. There is much interest in ENV as a vaccine candidate post RV144 trial. Moreover, sequence comparison of HIV to human proteome selects ENV GP160 with least homology [7]. Hence, HIV-1 ENV GP160 trimer protein complex SPIKE (Fig. 36.1) has been a vaccine candidate for consideration in recent years.

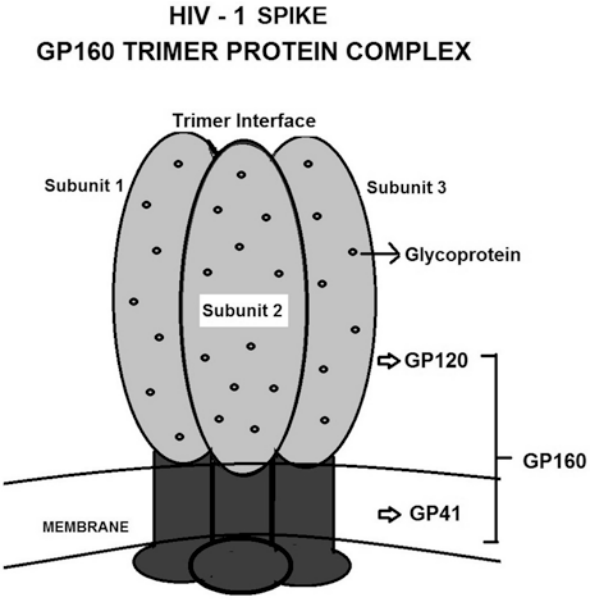

Fig. 36.1 HIV-1 spike protein complex is illustrated. The structure of HIV-1 spike is made of the viral envelope (ENV). It is a trimer protein complex of three GP160 protein monomers forming a trimer interface to shape the spike structure. The ENV protein is glycosylated with glucose moieties as illustrated. The current challenge is to develop a macromolecule assembly of the functional spike complex through biotechnological advances with potential immunological epitopes having vaccine-related features

36.2 Expression, Purification and Characterization of ENV Trimer

There is an increased momentum to synthesize ENV trimer despite heavy biotechnological challenges (Fig. 36.2). Native like HIV-1 ENV GP160 trimer protein complex with glycan shield SPIKE is a challenging platform for biochemical, structural and immunological improvements towards vaccine design, development and validation of an effective vaccine [8–10]. A number of factors influencing the stability of HIV-1 ENV make it nontrivial to design and purify a soluble recombinant native-like trimer [11]. The role of disulphide bonds in protein folding, glycosylation, modification and their association to form the native conformation of HIV-1 ENV trimer is also described [12]. Purification of HIV-1 GP160 (GP120/GP41) trimer complex by Strep-tag method resulted in cleaved, uncleaved, fully and partial glycosylated trimers is known [13]. The importance of a cleaved GP160 (GP120/GP41) over an uncleaved GP160 for correct glycosylation through an appropriate pathway towards generating the three-blade propeller-shaped trimers is shown. The isolation of diverse soluble Env trimers with native-like (NL) structure presents technical challenges, and it is overcome using an epitope-independent size exclusion lectin affinity chromatography in comparison with SOSIP (engineering trimer-stabilizing mutations) [14].

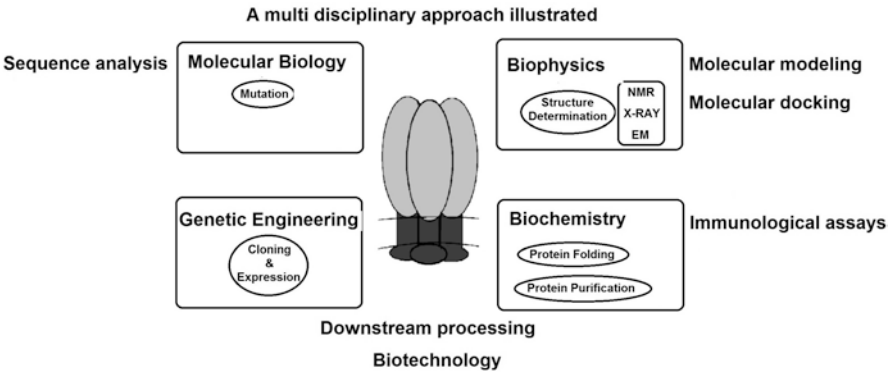

Fig. 36.2 Schematic representation of a multidisciplinary approach towards the production of an immunologically effective SPIKE protein complex is illustrated. The development of a SPIKE protein complex, a vaccine product enclosing a vast degree of known mutations across several known clades considering the type of protein (membrane type) structure and its production through cloning, expression and refolding, requires advancements from several disciplines of biotechnology and bioinformatics

36.3 GP160 Mutations

The LANL database contains over 39,000 GP160 variant sequences across different clades and subtypes (Fig. 36.3). HIV-1 ENV surface mutations using polarity properties among available clade, blood and brain sequences [15] in the perspective of NeuroAIDS [16] have been described. The large number of known variants of GP160 sequences is a great challenge towards the development of a viable vaccine component.

36.4 GP120/41 and Structures

The interactions of ENV with cell surface receptors as a potential vaccine candidate target are also discussed [17]. Oligomeric TF (transmitted/founder) ENVs elicit autologous antibodies to tier-2 HIVs [18]. The upstream and downstream synthesis of an ENV GP160 trimer SPIKE is a challenge [19, 20]. It is critical to imitate the native conformation of the spike taking surface mutation and glycosylation into context for relevant immunity. The gathering of individual gp160 into a trimer spike complex architecture is a difficult protein-protein interaction problem to formulate. A large number of GP120 (Table 36.1) and GP41 (Table 36.2) structures in complex with several forms of neutralizing antibodies and cluster of differentiation (CD) are known using several techniques (X-ray, NMR, EM) of biophysics (Fig. 36.4) towards the understanding of the molecular mechanism of viral infection despite huge variation across clades and subtypes (Fig. 36.3).

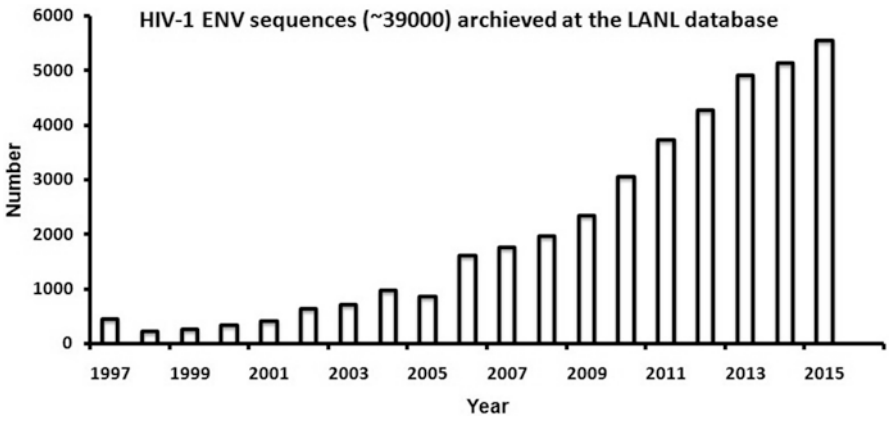

Fig. 36.3 Year-wise archive of HIV-1 ENV sequence representing clades (A-K) and others at the LANL HIV database [25] is shown

Table 36.1 HIV-1 ENV GP120-related structures made available at the protein databank (PDB). A number of GP120 structures are solved in complex with neutralizing antibodies towards understanding its molecular immunology mechanism. http://www.rcsb.org/pdb

Group	PDB ID	Country	Method
Bjorkman, P.J.	3LQA, 3U7Y, 4JDT, 4JPV, 4JPW, 4P9H, 5CAY, 5FEC, 5I9Q, 5IGX	USA	XRD
Haynes, B.F.	4JAN, 4OLU, 4OLV, 4OLW, 4OLX, 4OLY, 4OLZ, 4OM0, 4OM1, 5F96, 5F6J, 5F9O, 5F9W, 5KG9	USA	XRD
Kong, X.P.	3MLS, 3Q6F, 3Q6G, 4D9L, 4JO1, 4JO2, 4JO3, 4OAW, 4XMK, 4XML, 4ZTO, 4ZTP	USA	XRD
Kwong, P.D.	1G9M, 1G9N, 1GC1, 1RZ7, 1RZ8, 1RZF, 1RZG, 1RZI, 1RZJ, 1RZK, 1YYL, 1YYM, 2B4C, 2NXY, 2NXZ, 2NY0, 2NY1, 2NY2, 2NY3, 2NY4, 2NY5, 2NY6, 2NY7, 2QAD, 3HI1, 3IDX, 3IDY, 3JWD, 3JWO, 3NGB, 3SE8, 3SE9, 3TGQ, 3TGR, 3TGS, 3TGT, 3TIH, 4DVR, 4DVS, 4DVT, 4DVV, 4DVW, 4DVX, 4H8W, 4I3R, 4I3S, 4J6R, 4JB9, 4JZW, 4JZZ, 4K0A, 4LAJ, 4LSP, 4LSQ, 4LSR, 4LSS, 4LST, 4LSU, 4LSV, 4R4F, 4R4H, 4R4N, 4RFN, 4RFO, 4RWY, 4RX4, 4RZ8, 4S1Q, 4S1R, 4S1S, 4TVP, 4XMP, 4XNY, 4XNZ, 4XVS, 4XVT, 4YDI, 4YDJ, 4YDK, 4YDL, 4YFL, 5TE4, 5TE6, 5TE7	USA	XRD
Smith, A.B.	4DKO, 4DKP, 4DKQ, 4DKR, 4I53, 4I54, 5F4L, 5F4P, 5F4R, 5F4U	USA	XRD
Wilson, I.A.	1NAK, 2F58, 3F58, 3TYG, 4JM2, 4JPK, 4R2G, 4RQS, 5IDL, 5IES, 5IF0, 5KZC, 4JPJ	USA	XRD
Pazgier, M.	4YC2, 4YBL, 5FCU, 5KJR	Canada	XRD
Martin, L.	2I5Y, 2I60, 4KA2	France	XRD
Weissenhorn, W.	2XA3	France	XRD
Takenaka, A.	4DEN	Japan	XRD
Morris, L.	5 T33	South Africa	XRD
Bajaj C	3 J70	USA	EM
Subramaniam, S.	3DNL, 3DNN, 3DNO	USA	EM
Caffrey, M.	1MEQ	USA	NMR
Borremans, F.A.	1 CE4	Belgium	NMR
Anglister, J.	1QNZ, 2ESX, 2ESZ, 2 L87	Israel	NMR

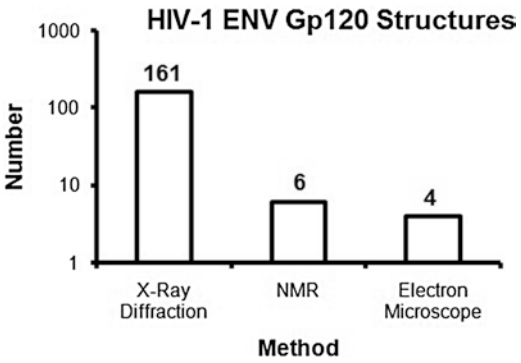

Fig. 36.4 Distribution of HIV-1 ENV GP120 protein structures determined in complex several neutralizing antibodies and CD4 molecules from the protein databank (PDB) [26] determined using X-Ray, NMR and EM (electron microscope)

Table 36.2 HIV-1 GP41-related X-ray, EM and NMR structures made available at the protein databank (PDB). http://www.rcsb.org/pdb

Group	PDB ID	Country	Method
Delmedico MK.	2ZFC	USA	XRD
Kwong, P.D.	1TJG, 1TJH, 1TJI, 3JWD, 3JWO, 4G6F, 4NGH, 4NHC, 4NRX, 4TVP, 4U6G, 5F89, 5IQ7, 5IQ9, 5MP6	USA	XRD
Margulies, D.H.	3ECB	USA	XRD
Kim, P.S.	1AIK, 1GZL, 1I5X, 1I5Y, 2Q3I	USA	XRD
Bjorkman, P.J.	5D9Q	USA	XRD
Carter, D.C.	1GNE	USA	XRD
Chance, M.R.	3K9A, 3LEX, 3LEY	USA	XRD
Chen, B.	3P30	USA	XRD
Wlodawer, A.	3MA9, 3MAC, 4KHT, 4KHX,	USA	XRD
Dwyer, J.J.	2OT5, 3CP1, 3CYO	USA	XRD
Gellman, S.H.	3O3Y, 3F4Y, 3F4Z, 3F50, 3G7A, 3O3X, 3O3Z, 3O40, 3O43	USA	XRD
Haynes, B.F.	3MNW, 5U3J, 5U3K, 5U3L, 5U3M, 5U3N, 5U3O	USA	XRD
Kay, M.S.	2R3C, 2R5B, 2R5D, 2SIV	USA	XRD
Lu, M.	3 U91, 4HLR, 3UIA, 1 CE0, 1DF4, 1DF5, 1DLB, 1CZQ, 1SZT, 1JPX, 1JQ0, 1 K33, 1 K34, 3G9R, 3GWO, 3H00, 3H01, 1QR8, 1QR9	USA	XRD
Root, M.J.	5KA5, 5KA6	USA	XRD
Wilson, I.A.	4XAW, 4XBE, 4XC1, 4XC3, 4XCF	USA	XRD
Ward, A.B.	5FUU	USA	XRD
Wiley, D.C.	1FAV, 1ENV	USA	XRD
Wilson, I.A.	1TZG, 2FX7, 2FX8, 2FX9, 3FN0, 3G7A, 3O3X, 3O3Z, 3O40, 3O43, 3OZ9, 5C7K, 5CCK, 5JS9, 5JSA	USA	XRD
Nieva, J.L.	3EGS	Spain	XRD
Conejero-Lara, F.	4R61	Spain	XRD
Fujii, N	2ZZO, 3AHA	Japan	XRD
Carfi, A.	2CMR	Italy	XRD
Weissenhorn, W.	2X7R, 4B50	France	XRD
Muirhead, H.	1NLD	England	XRD
Jiang, S.	5CMU, 5CMZ, 5CN0	China	XRD
Chong, H	3VGX, 3VGY, 3VH7, 3VIE	China	XRD
Liu, X.	3WFV	China	XRD
Zhang, R.	5HFL, 5HFM, 5H0N	China	XRD
Pai, E.F.	1U8H, 1U8I, 1U8J, 1U8K, 1U8L, 1U8M, 1U8N, 1U8O, 1U8P, 1U8Q, 1 U91, 1 U92, 1 U93, 1 U95, 2F5B, 2P8L, 2P8M, 2P8P, 3D0L, 3D0V, 3DRO, 3DRQ, 3DRT, 3IDG, 3IDI, 3IDJ, 3IDM, 3IDN,	Canada	XRD
Tamm, L.K.	2PW1, 2PW2	Canada	XRD

(continued)

Table 36.2 (continued)

Group	PDB ID	Country	Method
Bajaj, C.	3 J70	USA	EM
Bjorkman, P.J.	5THR	USA	EM
Kwong, P.D.	5U1F	USA	EM
Subramaniam, S.	4CC8	USA	EM
Ward, A.B.	3J5M, 5ACO	USA	EM
Waring, A.J.	1ERF, 1P5A	USA	IR
Caffrey, M.	1MEQ	USA	NMR
Chou, J.J.	5JYN	USA	NMR
Reinherz, E.L.	2ME1, 2ME2, 2ME3, 2ME4, 2PV6	USA	NMR
Tamm, L.K.	2PJV	USA	NMR
Haynes, B.F.,	2LP7, 2M7W	USA	NMR
Wingfield, P.T.	2ARI	USA	NMR
Bax, A.	2MK3	USA	NMR
Nieva, J.L.	2M8M, 2M8O, 2NCS, 2NCT, 2MG1, 2MG2,2MG3	Spain	NMR
Anglister, J.	1LB0, 1LCX	Rehovot	NMR
Pessi, A.	1MZI	Italy	NMR
Kirchhoff, F.	2L6S, 2L6T, 2JNR	Germany	NMR
Muller, S.	1IM7, 1J8N, 1J8Z, 1J9V, 1JAA, 1JAR, 1JC8, 1JCP, 1JD8, 1JDK	France	NMR
Vogel, H.J.	1JAU, 1JAV	Canada	NMR

36.5 EM ENV Trimer Structures

The commercial interest by a number of corporations towards the production of a stable HIV-1 ENV GP160 trimer spike has already been observed. The structural resolution of HIV-1 trimer spike pre-fusion complex captured in a mature closed state by antibodies PGT122 and 35O22 is interesting [21]. Data on several HV-1 ENV GP120/GP41 structures towards the design of an effective vaccine target is a clear reality in the near future [22]. A 4.19 Å resolution EM structure of HIV-1 GP160 (GP120/GP41) trimer spike provides valuable insight into its design and development (Fig. 36.5). There are nine interfaces of three types. These include each three of GP120-GP120, GP41-GP41 and GP120-GP41 interfaces. The GP120-GP120 interface residues are predominantly polar in nature (Fig. 36.6). However, the GP41-GP41 interface is equally polar and non-polar in nature (Fig. 36.6). This implies that the interface consists of 50% polar and 50% non-polar residues. Similarly, the GP120-GP41 interface is also equally polar and non-polar in nature (Fig. 36.7). The stability of the nine interface trimer complex is critical for an immunologically viable conformational structure with epitope specificity. A combined illustration of GP120-GP120 and GP41-GP41 interfaces is shown for more clarity (Fig. 36.8). It is of interest to study the conformational changes in these interfaces among 39,000 known ENV variants. This will help in the development of

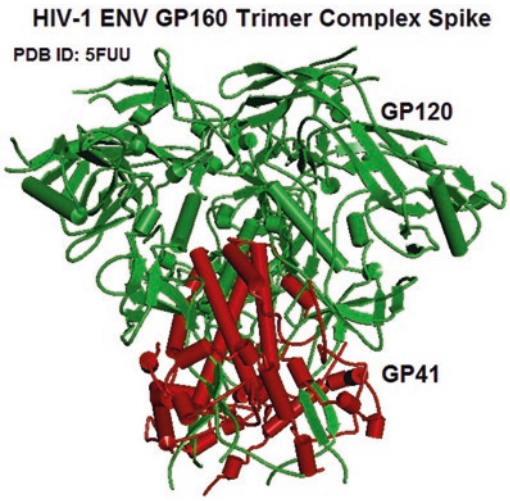

Fig. 36.5 HIV-1 ENV GP160 (GP120/GP41) trimer complex spike protein (PDB ID 5FUU) determined using EM at 4.19 Å resolution schematically represented using Discovery Studio™ software [28]. Each ENV GP160 is cleaved into GP120 and GP41. GP120 interacts with GP41 non-covalently in their cleaved state. Each of GP120 and GP41 interacts among themselves forming a trimer complex

an invariant epitope among clades. The conformational states of a soluble, uncleaved HIV-1 envelope trimer at 20 Å resolution using cryo-electron microscopy (EM) are now made available showing compact conformation [23]. Nonetheless, the direction of correct glycosylation in cleaved GP120/GP41 is also noted [13]. The application of these data in combination for the development of an immunologically viable vaccine component is evident.

36.6 Glycosylated HIV-1 ENV Spike

The HIV-1 ENV is heavily glycosylated, and the distribution of carbohydrate molecules in the trimer complex is shown (Fig. 36.9). This facilitates the spread and survival of the virus. Therefore, the importance of a specific carbohydrate-based ENV vaccine is intriguing [24]. It should be noted that there are about 39,000 known ENV variants at the LANL database. The conformational variation on the interface of these variants is evident. Subsequently variation in glycosylation among known variants within different clades is also envisioned. Nevertheless, this is highly promising for recombinant vaccine development despite huge technological challenges in producing a fully glycosylated native trimer conformation of the spike towards vaccine application.

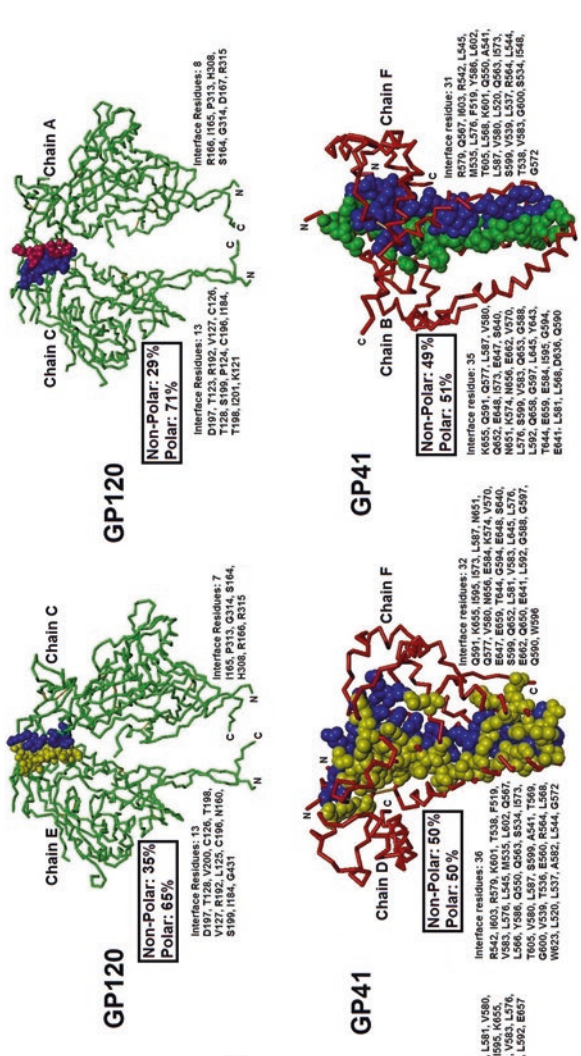

Fig. 36.6 The interaction between AC, CE and AE chains of GP120 and the interactions between BD, DF and BF chains of GP41 are shown using the EM structure with PDB ID: 5FUU. The interface residues existing between these interactions are displayed in CPK (Corey, Pauling, Kultin) representation using Discovery Studio™ Visualizer [28]. The percentage abundance of polar and non-polar residues at the interfaces of GP120-GP120 (predominantly polar) and GP41-GP41 (equally polar and non-polar) in the trimer complex is shown. The interface residues are identified using the change in accessible surface area (ASA) upon complex formation as described elsewhere by Jones and Thornton [29]. ASA was calculated using the software tool surface racer [27] with a probe radius of 1.4 Å

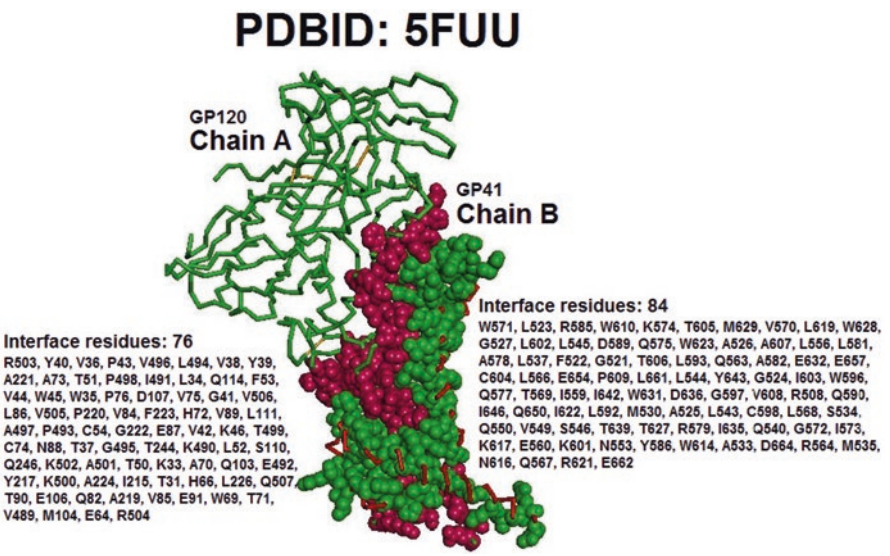

Fig. 36.7 The interface residues (equally polar and non-polar) existing between GP120 and GP41 are displayed in CPK (Corey, Pauling, Kultin) representation using Discovery Studio™ Visualizer [28]. The interface residues are identified using the change in accessible surface area (ASA) upon complex formation as described elsewhere by Jones and Thornton [29]. ASA was calculated using the software tool surface racer [27] with a probe radius of 1.4 Å

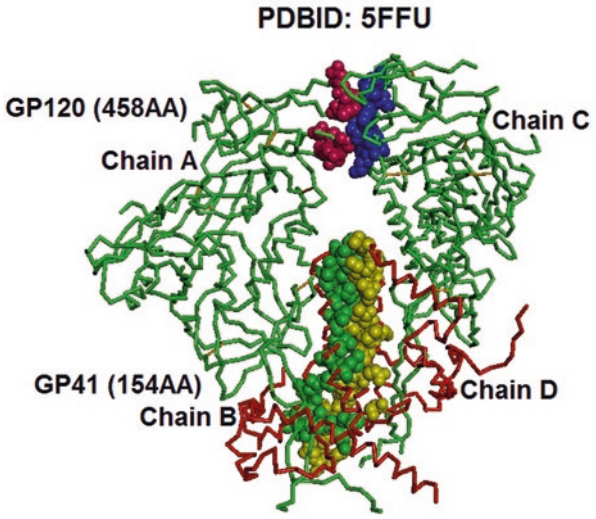

Fig. 36.8 The interface between GP120-GP120 and GP41-GP41 is shown together in CPK (Corey, Pauling, Kultin) representation using Discovery Studio™ Visualizer [28]. See Fig. 36.6 for interface residues. The interface residues are identified using the change in accessible surface area (ASA) upon complex formation as described elsewhere by Jones and Thornton [29]. ASA was calculated using the software tool surface racer [27] with a probe radius of 1.4 Å

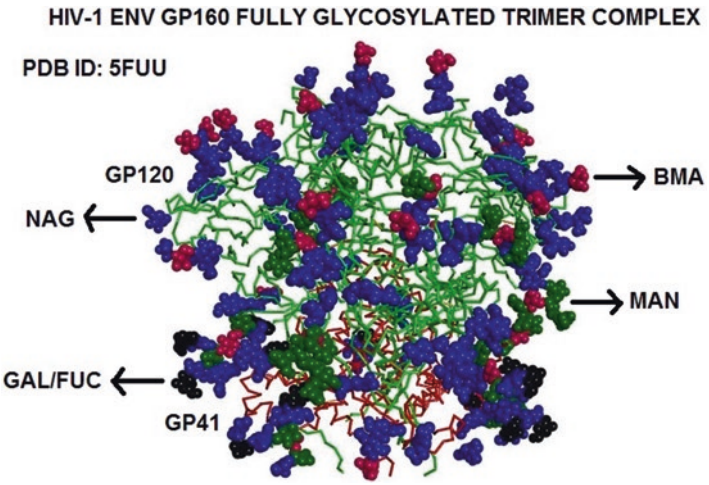

Fig. 36.9 CPK (Corey, Pauling, Kultin) representation of HIV-1 ENV GP160 fully glycosylated trimer complex spike with labelled sugar moieties is shown using Discovery Studio™ Visualizer [28]. Sugar moieties expanded as NAG (N-acetyl-D-glucosamine), BMA (beta-D-mannose), MAN (alpha-D-mannose), GAL (beta-D-galactose) and FUC (alpha-L-fucose)

36.7 Conclusion

The design, synthesis, development and production of a biochemically stable, structurally understood and immunologically viable HIV-1 ENV GP160 (GP120/Gp41) trimer spike complex as a vaccine component are clearly nontrivial. The synthesis of a HIV-1 ENV GP160 (GP120/Gp41) trimer spike complex mimicking the exact native conformation after optimal glycosylation in consideration with known 39,000 GP160 known mutants across clades is a great challenge. The HIV-1 ENV GP160 (GP120/Gp41) trimer spike EM structure resolved at 4.19 Å resolution shows nine interfaces with three different types. Their characteristic features are interesting towards the development of a stable yet effective trimer complex. The GP41-GP41 and GP41-GP120 interfaces are characteristics of equal polar to non-polar residues, and the GP120-GP120 interfaces are predominantly polar in nature. This is a critical information towards the development of a stable and viable vaccine component.

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Conflict of interest The authors report no conflicts of interest.

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Chapter 37
Gene Therapy Blueprints for NeuroAIDS

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Keywords Gene Therapy • NeuroAIDS • HIV-1B • Provirus • Adenovirus • Adeno-Associated Virus (AAV) • Retrovirus/Lentivirus • CRISPR/CAS9 • RNA editing • Trojan horse • BioBrick plasmids • Cre-LoxP recombinant • Transcription activator-like effector nuclease (TALEN) • Zinc-finger • Nucleases (ZFN) • Single-cell labeling of endogenous proteins by CRISPR/Cas9-mediated homology-directed repair (SLENDR) • Engineered homing endonucleases (HE) • siRNA • Nanosystem deliveries • Inteins • Synthetic genomics • Host gene • CXCR5 • CXCR4 • Neuron • Recombinase • Blood-brain barrier (BBB).

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Core Message
Early work produced evidence for CNS involvement after HIV-1B infection.

Many host proteins, RNAs, and genes have been implicated in brain involvement.

This chapter summarizes gene therapy methods to neutralize effects of HIV-1 infection-related deleterious host genes and disease susceptibility and enhance health-promoting genes. This chapter proposes early work should be done carefully, to reduce the risk of causing malfunction of genes and network pathways. We describe several of the genes known to be involved in neuroAIDS, for potential application for various techniques of gene therapy. With more than 179,962 publications using gene therapy, as of February 27, 2017, where are all the clinically appropriate products applying all this knowledge? Ethical considerations of gene modification are discussed as well.

37.1 Introduction

With the extensive progress that has occurred in neuroAIDS research and treatment [1], then why write a chapter such as this? The reasons include that worldwide, with the plethora of various strains of HIV, the work described here serves as a paradigm for the continued global quest against HIV disease spread and neuropathogenesis. Moreover, the array of technologies and target genes assembled in this chapter provides an overview of where twenty-first-century molecular biotechnology may lead, utilizing gene transformation and optimization against neuroAIDS and related disorders. The research is very promising, offering glimpses at potential cures for HIV-infected patients. Several applications of gene therapy describe host gene modifications, which make host cells resistant to HIV infection and replication. Moreover, methods and targets are specified, where gene therapy in neuroAIDS is appropriate, germane, and feasible.

37.1.1 Gene Therapy and Synthetic Biology

37.1.1.1 Gene Therapy as a Subcategory of Synthetic Biology

The interdisciplinary branch of biology combined with engineering is called synthetic biology, which involves engineering models used to build artificial biological systems. Synthetic biology and engineering scientists design and construct new biological components, devices, and systems for therapeutic advances. Their work can rewrite the existing natural biological systems in humans that result from nearly a billion years of natural selection and evolution [2]. In other words, the purpose of synthetic biology is to redesign and fabricate biological components and artificial systems, thus creating life in a sense, ab initio [3]. Synthetic biology intersects

genomics when scientists combine chemical synthesis of DNA with the widely expanding expertise and knowledge of genomics to enable researchers to quickly manufacture catalogued DNA sequences and assemble them into new partial and functional genomes. Applying these systems in controlled environments can further lead to understanding biology through the lens of engineering (bioengineering) [4]. In this chapter, the applications to date of synthetic biology and genomics are enumerated to craft new systems to further understand the complex virology and host genetic machinery associated with neuroAIDS and HIV-associated neurocognitive disorders (HAND).

37.1.1.2 Rewriting: Refactoring Processes

Refactoring is a term that comes from engineering, where it is used to describe the process of improving the internal design of software. In general terms, the goal of refactoring is to improve the internal structure of an existing system while simultaneously maintaining external system function. For example, in synthetic biology, refactoring could be used to improve the DNA of bacteriophages so that they encode heat-resistant genes while still maintaining their ability to replicate and invade bacterial cells. Many reviews and discussions of refactoring concepts have been published [5, 6]. Such principles are applicable to human studies and neuroAIDS, specifically, with far-reaching consequences.

37.1.1.3 Historical Breakthroughs

Since the 1970s, genetic engineering has had tremendous biotechnology breakthroughs. For example, bacteria were bioengineered, capable of digesting petroleum components. These were the first patented microorganism used to clean up oil spills [7]. In 2000, a synthetic biological circuit[1] was created [8]. In 2004, a microchip-based technology was constructed for multiplex gene DNA synthesis, which enabled synthesis of 21 genes coding for the *Escherichia coli* 30S ribosomal subunit proteins [9]. In 2007, genome transplantation was described, transplanting the complete genome as pure DNA, which produced the donor phenotype [10]. In the following year, 2008, the J. Craig Venter Institute synthetized and assembled the first bacterial genome (*Mycoplasma genitalium*) [11]. They synthesized and assembled the first single-cell eukaryotic organism in 2010 [12].

[1] Interacting genes and proteins can compose genetic circuits. These can empower cells to individually counter environmental and signal inputs. Moreover, communication and decision-making can ensue.

37.2 Methods of Gene Therapy in NeuroAIDS

37.2.1 Antiretroviral Therapy

The age of antiretroviral therapy (ART) produced remarkable controls of the progression of AIDS, allowing patients to live longer, on the one hand, and, on the other hand, increasing the frequency of chronic complications of AIDS. Suffice it to say, with the further development of highly active ART (HAART), HIV-positive patients live longer, as is evident in children and adolescents as well as adults. However, complications include infections and neuroAIDS pathology (as reviewed in several chapters in this book), as well as the incipient problem of HIV reservoirs [1, 13, 14].

Going beyond the ART approach to the problem of HIV infection, within the past several years, numerous novel technologies emerged that have been applied, modified, and engineered toward abrogating HIV infection. For example, recognition of specific DNA target sequences coupled with "molecular machinery" enables site-specific editing of host and virus genes and their expression. These include technologies such as recombinase, siRNA, ZFN, TALEN, and CRISPR/Cas9, which have been described, in part, in several prior reviews [15, 16].

37.2.2 Genome Editing/Genome Editing with Engineered Nuclease Technology

37.2.2.1 Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/Cas9 System

Structure and Mechanism

A recent and novel method of gene therapy was discussed by geneticists Jennifer Doudna and Emmanuelle Charpentier who coinvented a new technology that they proposed to facilitate the cure of genetic diseases. The structure and mechanism will be discussed here briefly, to recognize its applications in the use of gene therapy for neuroAIDS. CRISPR was discovered through the study of a bacterial natural adaptive immune system, allowing bacteria to detect and destroy foreign intrusive viral DNA [17]. Since its discovery, CRISPR has been used due to its possible simplicity, applicability, and adaptability. However, to the current views, it is not really a simple system to apply and involves many exceedingly complex assumptions and component. Nevertheless, from the simpler point of view, CRISPR is composed of a guide RNA (gRNA) and a non-specific CRISPR-associated endonuclease, i.e., Cas9 [18, 19]. The gRNA is a short synthetic RNA composed of a scaffold sequence necessary for Cas9 binding including a user-defined approximately 20-nucleotide spacer/targeting sequence, which defines the genomic target to be modified. Thus, one can change the genomic target of Cas9 by changing the

targeting sequence present in the gRNA. This targeting sequence of approximately 20 nucleotides must hold true to its conditions of uniqueness compared to the rest of the genome and location immediately upstream of a protospacer adjacent motif (PAM) [18].

Modifications to the Cas9 enzyme have extended the application of CRISPR to selectively activate or repress target genes, purify specific regions of DNA, and even image DNA in live cells using fluorescence microscopy. After the expression of the Cas9 protein and the gRNA forms a riboprotein complex, Cas9 undergoes a conformational change, activating for DNA-binding conformation, in turn, leading to the spacer sequence remaining free to bind to the target DNA. The Cas9-gRNA complex binds to a genomic sequence with a PAM, producing a sequence at the 3′ end of the gRNA; consequently, the targeting sequence begins to anneal to the target DNA [18, 20]. Upon binding, Cas9 undergoes a second conformational change, resulting in a double-stranded break within the DNA target. The DSB is then repaired by the nonhomologous end-joining (NHEJ) pathway or homology-directed repair (HDR) pathway. The former is an active repair mechanism but leads to small nucleotide insertions or deletions at the DSB site. Moreover, NHEJ-mediated DSB repair is imperfect and often results in disruption of the open reading frame of the gene; HDR can be used to generate specific nucleotide changes or edits ranging from a single nucleotide change to large insertions [18].

CRISPR/Cas9 Applications Related to HIV Infection

The CRISPR technology has been used to remove the entire HIV-1 genome from infected human CD4+ T cells. Dr. Khalili and colleagues used CD4+ T cells obtained from HIV-1-infected patients and reduced viral load ex vivo by using CRISPR/Cas9 delivered via lentiviruses. The verification of such removal was obtained using genome-wide assessment, which confirmed complete excision of the integrated copies of viral DNA. Another discovery presented was the introduction of lentivirus-delivered Cas9/gRNAs A/B into primary cultured human CD4+ HIV-1-infected T cells, which leads to a significant reduction in the numbers of viral copies [21, 22].

In the case of a CD4(+) T-cell leukemia cell line, gene targets required for HIV infection – but not required for cell survival for CRISPR technology – are put forward including sulfation of CCR5 as well as T-cell aggregation [23]. In cell culture, using RNA interference (RNAi) screening methods, nearly 1000 host genes were implicated in HIV infection. However, using screens based on CRISPR, five genes were identified as of primary importance biologically: CD4, CCR5, tyrosylprotein sulfotransferase 2 (TPST2), solute carrier family 35 member B2 (SLC35B2), and activated leukocyte cell adhesion molecule (ALCAM) [24].

Remarkably, HIV-1 shows the ability to escape the new CRISPR technology, reviewed by Harper [25]. Although initial studies on the effects of various applications of the CRISPR/CAS9 system indicated possible inhibition of HIV replication, unfortunately, this system also reverses HIV latency. Several studies indicate HIV

mutates and circumvents and abrogates inhibition [26–29]. As a preferable method to vitiate HIV escape, it was suggested that instead of targeting a single site in the HIV genome, it targets two sites simultaneously [30, 31]. Attacking HIV DNA and HIV RNA using CRISPR technology and siRNA, respectively, can both result in virus escape. However, attacking two targets simultaneously reduces the prospect of the escape problem [32].

As a proof of concept, various gene modification techniques were published, intended to characterize the interactions of HIV proteins with the host proteome. Methods included electroporation of CRISPR/Cas9 ribonucleoproteins (RNPs) to edit the genomes of primary CD4+ T-cell gene resulting in CCR5 and CXCR4 knockout cells. The cells exhibited tropism-dependent resistance to HIV infection. Tropism-independent resistance was accomplished by LEDGF and TNPO3 knockouts. Furthermore, they demonstrated that CRISPR/Cas9 RNPs, simultaneously, could edit multiple genes. By screening, they also identified several candidate dependency/restriction factors of 45 HIV integrase-associated genes [33].

However, due to the ability of HIV to mutate extensively, it remains to be determined what sort of extensive dynamic modifications may be required further to counteract HIV mutations that circumvent HIV therapies. Possibly, multiple CRISPR applications need to be applied consecutively or simultaneously to eliminate HIV from its reservoirs in patients, inhibit any remaining virus from replicating, and induce CD4- T cell and macrophage knockouts (as well as other reservoirs of HIV infection) that are resistant to HIV eradication. Moreover, there is preliminary evidence that multiple genes could be simultaneously edited by the CRISPR/Cas9 technology [33]. Furthermore, their adaptation of the technology to a high-throughput platform opens the door to a widespread use of the new technology among pharmaceutical and clinical domains.

Additional Applications: *Class 2 Type VI CRISPR-Cas Effector (C2c2)*

Researchers at the Broad Institute of Massachusetts Institute of Technology and Harvard University (Cambridge, MA) recently commenced working on RNA-guided targets [34]. The group categorized the class 2 type VI CRISPR-Cas effector (C2c2) [35]. C2c2 is an RNA-guided enzyme capable of targeting and degrading RNA, from the bacterium *Leptotrichia shahii*, providing an interference against RNA phage. C2c2 complements the CRISPR/Cas9 system and has the capacity to target only RNA to carry out genomic instructions, thus offering the ability to manipulate RNA precisely. Therefore, the C2c2 effector can be used to develop new RNA-targeting tools potentially creating an alternative to the use of small interfering RNA in CRISPR [35, 36]. This technology suggests greater specificity, with a wider range of applications in the study of genetic editing. In another study, a phage virus that infects major freshwater bacteria was used as

follows. The cyanophage N-1 encodes a CRISPR array, like the DR5 family of CRISPR, found in cyanobacteria. This presence of CRISPR implies that CRISPR transfer is possible with a phage among related cyanobacteria, providing resistance to infection with phages. This may further expand the CRISPR technology toward unanticipated subtleties of molecular specificity and target repertoire [37–39].

37.2.2.2 Zinc-Finger Nucleases

Zinc-finger nucleases (ZFN) are very powerful chimeric nucleases with gene-editing modification tools. Umov et al. reported the use of zinc-finger proteins, engineered to recognize a specific unique chromosomal site and create double-stranded breaks. This in turn creates specific sequence alterations. Zinc-finger nucleases enhance the very low efficiency of required homologous recombination events, thus modifying DNA-binding specificity of the zinc-finger nucleases [40–42].

The CCR5 receptor protein gene, a coreceptor for entry of HIV-1 into T cell, has significance due to its implication in viral disease. In 20% of Caucasian populations, a CCR5-Δ32 deletion prevents the CCR5 chemokine receptor protein from permitting HIV access to the host cell [43]. Naturally homozygous individuals for the CCR5-Δ32-deleted allele consequently are resistant to HIV infection. In a clinical trial, this gene-editing technique was shown to be safe and effective in humans. ZFNs were used to target a gene in immune cells of 12 people infected with HIV, which increased their resistance to virus infection. CCR5-modified autologous CD4 T-cell infusions were shown to be safe within the scope of the study [41]. Mutation of even a subset of such cells allows reconstitution of the T-cell arm of the immune system with resistant cells upon retransplantation. In this study, the CCR5 gene was modified as follows. A 24-base pair composite site was the target for binding by two ZFNs. This site is actually upstream from the well-known naturally occurring CCR5-Δ32 mutation and is in the domain of the CCR5 gene's first transmembrane portion. The ZFN pair produced DNA double-stranded breaks. These breaks are successively repaired within the cell, and this results in insertions of random sequences that damage the CCR5 gene as an HIV coreceptor without abrogating its immune function. This work was based on a prior procedure in which the CCR5 gene was mutated in CD4+ T cells and produced an HIV-resistant population, by designing a series of ZFNs targeting the human CCR5 gene [44]. The study also resulted in HIV-1-infected mice with the ZFN-modified CD4+ T cells, having lower viral loads and higher CD4+ T-cell counts than the mice engrafted with wild-type CD4+ T cells. To be completely effective, both CCR5 alleles would have to be inactivated in the same cell. A clinical trial showed that only one patient was heterozygous and took longer to become HIV resistant than the CCR5-disrupted homozygous patients [45]. Repeated application of the ZFN procedure may likely increase the frequency of modified cells [40].

37.2.2.3 Engineered Homing Endonucleases

Novel homing endonucleases (HEs) were used to specifically target (~22 base pair) DNA sequences and induce double-stranded breaks. The double-stranded DNases that have large, asymmetric recognition sites (12–40 base pairs) and coding sequences are often embedded in either introns or protein inteins.[2] These long recognition sites guarantee raised specificity as well as preventing off-target effects. The breaks are subsequently repaired by error-prone nonhomologous end joining, thus allowing targeted disruption of essential viral genes [44, 46–54].

A therapeutic approach was devised that aims to cure cells of latent HIV infection by rendering latent virus incapable of replication and pathogenesis. They attempted this via targeted cellular mutagenesis of essential viral genes, using a homing endonuclease to introduce DNA double-stranded breaks (DSB) within the integrated proviral DNA, which is followed by triggering of the cellular DNA damage response and error-prone repair. Their work demonstrates that homing endonucleases can efficiently and selectively target an integrated reporter lentivirus within the human cellular genome, leading to mutation in the proviral DNA and loss of reporter gene expression [55].

37.2.2.4 Transcription Activator-Like Effector Nucleases (TALENs)

Transcription activator-like effector nucleases (TALENs) are naturally occurring proteins, which contain DNA-binding domains composed of a series of 33–35 amino acid repeat domains [56]. The large size of TALENs may limit their delivery by size-restricted vectors such as recombinant adeno-associated virus (AAV). However, these vectors have been shown to accommodate ZFN genes. Their specificity is determined by two hypervariable amino acids that are known as the repeat variable diresidues (RVD) [57]. TALEN repeats can be combined to recognize virtually any user-defined sequence indicated by various assembly methods. TALENs are also capable of correcting the underlying cause of the disease, therefore permanently eliminating the symptoms with precise genome modifications [15]. The HIV-targeted transcription activator-like effector nucleases (HT-TALENS) were developed by altering a commonly used plant pathogen protein. A transcription activator-like effector nuclease (TALEN) approach was used, which more specifically engineered a custom TALEN pair of HIV-targeted TALENs (HT-TALENs) to specifically reach a highly conserved region in the transactivation response (TAR) element of HIV-1 proviral DNA. A plasmid DNA construct was developed for HT-TALENs using the Joung Lab REAL Assembly TALEN kit [18], which demonstrated the DNA template cleaved by TALENs – the HIV-1 proviral target site, in vitro. The TALEN plasmids effectively introduced mutations and inactivated the GFP reporter, under control of HIV-1 TAR, a highly conserved sequence in the LTR. HIV-1 proviral DNA was then

[2] Inteins are protein introns, parts of proteins that self-excise analogously to DNA genome and messenger RNA introns. Inteins were discovered decades ago and have resurfaced as a twenty-first-century bioengineering tool.

incapable of producing detectable Gag expression. That TALEN variants could cleave methylated as well as unmethylated DNA was demonstrated as well [58].

37.2.2.5 Engineering Molecular Trojan Horses to Circumvent the Blood-Brain Barrier

HIV-1 penetrates the blood-brain barrier (BBB) early after infection and can be found in macrophages resident in the brain [59–63]. However, genetic therapy has shown to be a challenge for neuroAIDS, because of the brain's physiologic protection against foreign and toxic molecules. The blood supply to the brain is separated through tightly packed cell layers that form the physiologic barrier, the BBB. Because of the BBB, many therapies for AIDS can reach other organs of the body but are blockaded from reaching the brain. Due in large part to this physiologic barrier, researchers have developed technologies to penetrate the brain tissue of patients suffering from degenerative disorders including neuroAIDS for genetic therapy. The molecular Trojan horse technology was developed to create genetic engineering proteins for ferrying therapeutic molecules across the BBB, via endogenous receptor-mediated transport [64–66].

In a phase I open-label nonrandomized clinical trial for therapy against HIV, a strain of HIV was produced in vitro that carried DNA sequences that were inhibitory to subsequent rounds of HIV replication. With the use of a vector, called VRX496, a laboratory-modified HIV was disabled and used to function as a Trojan horse, carrying a gene that prevents new infectious HIV from being produced. In this study, HIV is added to immune cells that have been removed from the patient's blood by apheresis, then purified, genetically modified, and expanded by culturing. Via the HIV vector, VRX496, and its antiviral cargo, the vector delivers an antisense RNA molecule for the HIV envelope gene to the T cells. The antisense gene is permanently integrated into the cellular DNA. This approach enables patients' own T cells, which are targets for HIV, to inhibit HIV replication [67].

37.2.2.6 Viral Vectors and Gene Trapping

Lentiviruses, CD4 T Cells, and Macrophages

Another method of genetic therapy is the use of lentiviral vectors, which are capable of transducing dividing and nondividing cells that constitute the targets of HIV-1 infection such as resting T cells, dendritic cells, and macrophages. Their ability to block HIV-1 replication occurs by several mechanisms that include sequestration of the regulatory proteins Tat and Rev; their inhibitory ability can be enhanced by expression of anti-HIV-1 genes [68]. Levine et al. evaluated the safety of a lentiviral vector at the clinical level for HIV-related responses and immune effects in a phase I open-label nonrandomized clinical trial. The conditionally replicating lentivirus expressed an HIV envelope-specific antisense gene. The five chronically infected patients in the trial had previous failure to at least two ART regimens. These patients

were subject to one intravenous infusion of autologous genetically modified CD4 T cells. The patients exhibited no adverse effects. One patient showed an unceasing lowered virus load, and the other four patients showed unchanging virus loads. Constant or increased CD4 counts were found in four individuals. Gene transfer remained continuous. Four subjects showed improved immune function – insertional mutagenesis had not occurred after 21–36 months [68].

Additionally, lentiviral vectors can be used to genetically modify human macrophages to carry the anti-HIV regulatory genes without causing adverse effects to the cells themselves or the brain. It was reported that with transient disruption of the BBB, using a bradykinin or a hypertonic mannitol solution, the efficiency of CNS uptake of modified macrophages was improved. These cells contained defective lentiviral vectors expressing enhanced green fluorescent protein (GFP) as a reporter [69]. Thus, lentiviruses may be used as viral vectors when gene delivery requires crossing the BBB.

Adeno-associated Viruses and the BBB

Adeno-associated viruses (AAVs) are small viruses that, when infecting humans, can cause a mild immune response [70, 71]. When used as a vector, AAV can produce protracted and stable expression, in addition to incorporating its genome in the host cell under some conditions [72]. AAV has a limitation – since it packages single-stranded genomes, it requires host-cell DNA synthesis. The use of a self-complementary adeno-associated virus (scAAV) can eliminate this problem by packaging both strands and forming double-stranded DNA [73]. As a related issue of the use of viruses as vectors for which there is prevalent herd immunity, virtually all humans have immunity to both adenovirus and HSV. However, if the DNA is encapsulated in PEGylated immunoliposomes (PILs), it can produce a net anionic charge, in contrast to cationic liposomes. The PEGylated liposomes, however, encounter difficulty crossing the BBB in vivo, in contrast to viral vectors [65, 66, 74].

Simian Vacuolating Virus 40 Vectors

Recombinant simian vacuolating virus 40 vectors (rSV40) transduce both dividing and quiescent cells efficiently, and so Strayer et al. tested them for their ability to deliver anti-HIV-1 transgenes to terminally differentiated human NT2-derived neurons (NT2-N) [75, 76]. Their study transduced >95% of immature as well as mature human neurons efficiently, without detectable toxicity and without requiring selection. The vectors used in these studies, SV(RevM10) and SV(AT), respectively, carried the cDNAs for RevM10, a trans-dominant mutant of HIV-1 Rev, and human alpha-1 antitrypsin. The NT2-N treated with these vectors strongly resisted challenge with different strains of HIV-1, and viral protection continued throughout the 11-week study. Consequently, this may be a practicable approach for gene delivery for the treatment of CNS diseases, including neuroAIDS [77, 78].

37.2.2.7 BioBrick Plasmids

BioBrick plasmids have been used in microbial engineering, a goal of synthetic biology to find processes of engineering biological systems. BioBrick parts are DNA sequences, with their own structure and function, capable of incorporation into living cells, to construct new biological systems [79]. Some of these BioBrick parts include promoters, terminators, and operators, which can then form a device, with capabilities of performing high-level tasks once constructed into systems. Reshma designed a new set of parts capable of encoding useful vector functions with the use of BioBrick assembly vectors from BioBrick parts [80]. BioBrick vectors can also be used to allow quick assembly of multigene pathways into several vectors and cloned gene into vectors with different features with fast mobilization. This was achieved with a primary BioBrick developed by separated discrete regions, cloning sites, and affinity tags using additional restriction enzymes, serving as a template for other BioBrick vectors that also demonstrated functional capability, in vivo [81]. Thus, potentially, in neuroAIDS, it can be incorporated into viral-unique metabolic pathways.

37.2.2.8 Oligonucleotide Delivery

Oligonucleotide delivery into cells is frequently problematic due to its precipitation. Among a variety of applications, oligonucleotides have been used that are complimentary to the HIV-1 transactivation responsive (TAR) element that block Tat-dependent transactivation. They describe a procedure, with oligonucleotides delivered by peptide disulfide conjugation [82].

37.2.2.9 Translocatable Drug-Resistance Elements

Translocatable drug-resistance elements can make recognizable mutations by inserting themselves in different sites of prokaryotic genomes; these elements must insert within a structural gene or an operon. They have been shown to be useful in the isolation of mutants, construction of strains, and other genetic manipulations. A list of the properties found in translocatable drug-resistance elements summarized genetic manipulations of bacteria [83].

37.2.2.10 Transposable Retroviral Elements

Human endogenous retrovirus makes up a wide group of retro-element part of the human genome, possibly representatives of previous retroviral infection with the potential to provide undesired immune responses [84]. Transposable elements, upon integration and inheritance, may have evolved toward functions in regulation of host genes [85].

37.2.2.11 Xeno DNA

It has been proposed to change the chemical composition of living cells by additions and modifications of the genetic code. Thus, efforts to create artificial biodiversity with the synthetic design beyond the 20-amino acid building blocks, allowing alternative reading of the genetic code [86]. One of the consequences would be the development of safe strains with functionalities such as a genetic firewall, which can potentially be of biosafety. The goal is to design chromosomes with modified DNA and RNA, the xeno nucleic acids, which have a variety of sequence and structural changes [86]. It is argued that differences in their chemistry and structure may block exchange of genetic information with the external "natural" environment, i.e., these xeno nucleic acids are its genetic firewall properties [87].

37.2.2.12 HIV Recombinase

One of the first applications of HIV recombinase utilized a recombinant HIV-1 clone in conjunction with the Cre-loxP recombination system [88]. The HIV-1 clone with the loxP motif, which was maintained during replication, and with the use of two types of HIV-1 permissive cells expressed CRE and subsequently resulted in a substantial reduction in virus replication. Ten years following this strategy, a tailored HIV recombinase was reported that recognized a sequence within the HIV-1 long terminal repeats (LTR), which led to the possibility of recombinase excision of integrated HIV proviral DNA from the genomes of infected cells [89]. Subsequently, an HIV recombinase was used to remove the HIV-1 provirus from patient-derived cells, in conjunction with reduced infection in humanized mice [90]. Due to HIV persisting in the cell as a proviral reservoir, a substrate-specific recombinase was used to recognize a sequence present in the long terminal repeats. This recombinase can remove integrated provirus from infected cells in vitro and in vivo, with the potential of curative clinical application [91].

37.3 Genetic Targets and Sequences

A plethora of human genes has been implicated in the occurrence and progression of neuroAIDS – not all of which by any means interact directly with HIV-1 infection itself or directly with HIV-1 proteins and nucleic acids. Thus, we view such host genes, both as harbingers of neuroAIDS, for which the development of gene therapy is appropriate, and as reviewing host genes for which gene therapy is imminent or has commenced.

37.3.1 NeuroAIDS-Related Genes

We mention how several genes analyzed in Dr. Levine's chapter in this book have been subject to gene modification techniques [92]. An important aspect of neuroAIDS is to understand the genes involved in the pathophysiology and to establish if they differ based on the degree of neurocognitive impairment. However, in the case of HAND, there are no heritable neurocognitive deficits to serve as a starting point to explore genetic contributors to this process. Hence, the focus has been on variants of genes involved in numerous biological processes that significantly impact risk of neurocognitive impairment, the course of the disease, response to ARV medications, and those associated with putative biomarkers of HAND [93]. The National NeuroAIDS Tissue Consortium (NNTC) performed a brain gene expression array study to elucidate genes involved in HAND. In this study, by Gelman et al., four groups of patients were examined: (A) uninfected controls, (B) HIV-1-infected subjects with no substantial neurocognitive impairment (NCI), (C) infected with substantial NCI without HIV encephalitis (HIVE), and (D) infected with substantial NCI and HIVE. The results of this study noted that at least two patterns of brain gene expression mediate HAND. In type 1 neurocognitive impairment (patient in group D) seen in patients with HIVE, there was notable upregulation of interferon-gamma genes (IFRGs), antigen presentation, the complement system, and *CD163* antigen. Hundreds of downregulated neuronal transcripts in neocortical neurons also were observed in type I impairment – importantly, the GABAergic system. In type 2 neurocognitive impairment (group C), there was low brain HIV burden, virtually no evidence of increased IFRG responses, no downregulation of transcripts in neocortical neurons, and a relative paucity of regulated transcripts overall. However, one pattern of brain injury was recognized in this group with transcripts characteristically expressed by vascular- and perivascular-type cells being regulated in the *neostriatum*. Brain microvascular endothelial cells (ECs) are exposed directly to blood plasma and are potentially vulnerable to systemic and metabolic anomalies including inflammation. (ECs conduct trans-vascular signaling from the plasma compartment to brain cells such as neurons and astrocytes via the neurovascular unit, often in response to infection or inflammatory and metabolic events.) Plasma components that drive trans-vascular changes and are linked with HAND include plasma lipopolysaccharide (LPS) released by gut bacteria and soluble CD14 antigen [92, 94]. In the following sections, we will discuss some of the representative genes and how they have been used in gene therapy, using some of the methods previously stated in this chapter.

37.3.1.1 C-C Chemokine Receptor Type 5

A gene of great interest related to gene therapy is the C-C chemokine receptor type 5 (CCR5). A zinc-finger nuclease was used to target a gene in immune cells of 12 patients infected with HIV. The technique increased immune cell resistance to HIV

without destroying the gene's function. Moreover, this technique maintained clinical safety and effectiveness for these patients. Targeted gene editing can be accomplished with ZFNs, designed to cleave DNA at specific sites. A hairpin ribozyme was produced that reduced cellular CCR5 mRNA and cell surface CCR5, when introduced into PM1 cells[3] by transduction with recombinant AAV. The ribozymes effectively protect the cells from infection by CCR5 HIV-1 strains (non-syncytium-inducing clinical isolates) commensurate with a reduction in CCR5 mRNA [95, 96]. In another study, a ribozyme that targets CCR5 mRNA was designed with specificity toward the CCR5 mRNA. By design, the CCR5 mRNA-targeting ribozyme lacks complementarity to other members of the chemokine receptor gene family [97]. In addition, the use of stable VA1-ribozyme chimeric transcripts resulted in CCR5 beta-chemokine receptor downregulation, subsequently resulting in reduced in vitro HIV infection [98]. These methods should be useful in both studies of CCR5 receptor function and with therapeutic intervention against HIV-1 infection.

37.3.1.2 CXCR4

As communicated in Dr. Levine's chapter in this book, the CXCR4 receptor and its chemokine ligand, CXCL2, perform many functions essential for neuronal survival and neuronal-glial communication during neuroinflammation [92]. An example of gene alteration through HIV coreceptor modifications follows. HIV-1-resistant CD4(+) cells were engineered through modification of the CXCR4 coreceptor via ZFNs. This was accomplished through ZFN adenovirus delivery, which disrupted the CXCR4 mRNAs by cleavage and nonhomologous DNA end joining [99, 100]. This method was an alternative to the use of cell-integrated lentivirus-expressing CXCR4 targeted by short hairpin (shRNAs). When CD4(+) T cells were challenged with HIV-1 that utilized CXCR4 for entry, they maintained stable disruption, HIV resistance, and cell enrichment, in vitro as well as in vivo (in mice). Thus, mice with engrafted modified CD4(+) T cells compared to mice without the modification showed lower virus loads [100].

37.3.1.3 Monocyte Chemoattractant Protein-1

The monocyte chemoattractant protein-1 (MCP-1, also known as C-C motif chemokine ligand 2 (CCL2)) is one of the members of the chemokine family [97]. MCP-1 is widely expressed in many tissues and cell types by various inflammatory cells, including astrocytes and microglia. MCP-1 specifically recruits monocytes and infiltration of HIV-infected leukocytes into the CNS [97, 101, 102]. MCP-1 is involved in several disease processes of the CNS, where there is a marked

[3] PM1 cells are a human T-cell line, which has exceptional susceptibility to infection by primary HIV-1 isolates.

proinflammatory response.[4] CNS monocytes, monocyte-derived cells, microglia, and CNS macrophages are prominently involved in the CNS inflammatory response [103, 104]. In addition, many of these cells produce HIV-1 gene products such as Tat and gp120, among others, which are neurotoxic [105–107].

In vitro models of the BBB demonstrate inflammatory processes involved in the passage of HIV-1 across the BBB [108–110]. As a model of the BBB, coculture method of endothelial cells and astrocytes examined the mechanism of Tat-facilitated monocyte transmigration. Tat may expedite recruitment of monocytes into the CNS by inducing MCP-1 expression in astrocytes. Moreover, treatment with HIV-1 Tat on the astrocyte side induced significant MCP-1 levels [111].

Gene transfer techniques using a dominant inhibitory MCP-1 gene were used to attenuate infarct size in a murine model of ischemic stroke. To negate the effects of MCP-1-associated inflammation and damage, the MCP-1 gene was modified by deleting seven amino acids (numbers 2–8) at the N-terminus of the protein; the deletion mutant is termed 7ND, which abrogates the signaling pathway, in vivo, for MCP-1/CCR2. A heterodimer is produced in vivo between the wild-type MCP-1 protein and the defective 7ND protein. This heterodimer binds to the MCP-1/CCR2 receptor and entirely obstructs chemotaxis of monocytes mediated, in vitro. 7ND was delivered by an adenovirus vector and was effective in vivo, to reduce the effects of ischemic stroke [112]. This work highlights the potential for such a strategy to combat the inflammatory effects of HIV-1 brain infection.

In an animal model study for neuroAIDS, intracerebral gene delivery significantly protected neurons from gp120-mediated neuronal toxicity. Caspase 3, 6, 8, and 9 production in neurons, coupled with neuronal apoptosis, occurred at elevated levels in the *caudate-putamen* of rats following injection into the *caudate-putamen* of HIV-1 gp120. However, prior gene delivery of glutathione peroxidase (GPx1) or Cu/Zn superoxide dismutase (SOD1), which are antioxidant enzymes, reduced caspase production. Antioxidant enzymes were delivered by SV40-derived vectors [113].

Thus, gene therapy approaches to counteract or prevent the MCP-1 and other inflammatory cytokine-mediated as well as reactive oxygen species-mediated neurotropic and neurovirulent effects of HIV are feasible.

37.3.1.4 Nef and Host Genes

The Nef gene was targeted using an inducible lentivirus vector expressing F12Nef in a modified HIV-1 genome. This was done with a previously cloned and sequenced defective nef gene in an HIV-1 genome (F12HIV-1). Expression of this HIV-1 nef blocks release of HIV-1 by interfering with viral assembly and release. Three amino acids were mutated and reduced HIV replication. This nef was fused with a nerve

[4] A review of the presence of numerous cytokine and chemokine mediators of neuroinflammation and neurodegeneration includes HIV encephalitis among other inflammatory diseases [103].

growth factor receptor segment; expression of the fusion protein via a lentivirus vector resulted in inhibition of HIV replication [114].

37.3.1.5 HIV-1 Tat and Host Genes

Despite the use of ART, Tat, the transactivator of the virus is still produced and interacts with host genes to alter their expression. The tat-induced cytokine secretion in macrophages, including monocyte chemoattractant protein-1/CCL2, mediates neuroinflammation and is highly expressed in the CNS of HIV-infected people with HAND and also increased the expression of CCR5 and CXCR4 on macrophages. The first randomized, double-blind, placebo-controlled, phase II cell-delivered gene transfer clinical trial was conducted in 74 HIV-1-infected adults who received a tat-vpr-specific anti-HIV ribozyme (OZ1). The study demonstrated no statistically significant difference in viral load between the OZ1 and placebo group at the primary endpoint (average at weeks 47 and 48), but time-weighted areas under the curve from weeks 40–48 to 40–100 were significantly lower in the OZ1 group. This study indicated that cell-delivered gene transfer is safe and biologically active in individuals with HIV and can be developed as a conventional therapeutic product [115].

37.3.1.6 Beta-Amyloid

Beta-amyloid deposition has been implicated in many forms of neurodegenerative disorders including HIV. In one of the earlier studies among 97 cases of AIDS patients, a statistically significant increase in argyrophilic deposits was noted in the brain [116]. Despite the use of HAART, another study showed an overall increase in beta-amyloid deposits, possibly due to disruption of axonal transport of the amyloid precursor protein (APP). This has been corroborated by a study that examined the frequency of HIV-associated brain pathology and the presence of alpha-synuclein and beta-amyloid deposits [117]. The beta-amyloid deposits were found to be in nearly all of HIV+ cases (35/36). Despite these increases of beta-amyloid deposits, in an apparent paradox, HIV-associated brain pathology was present in only 10% of cases [118].

37.3.1.7 Recombinant Adeno-associated Viruses, HAND, and Other Neurological Diseases

As mentioned above, recombinant adeno-associated virus (rAAV) is used in gene-based therapy, and one of its desirable features is that it is nonpathogenic and has reduced immunogenicity. rAAVs have been used to deliver genes to a broad range of cells including the muscles, retina, liver, lung, and brain. Recent developments in gene therapy-based approaches, particularly in rAAVs, have provided new tools to

study Alzheimer's disease (AD) and other neurodegenerative disorders, including Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, spinal muscular atrophy, Rett syndrome, and HAND. These studies include humans and animal models of human diseases. For example, rAAV has been used to deliver antibodies against HIV proteins in mice and monkey models of HIV infection [70, 72, 73, 119–122]. Limitations of rAAV include trying to limit rAAV delivery to target specific tissue areas alone and to improve concentrations of gene therapy vehicle, which reach their targets.

37.3.1.8 Leptin

Leptin is an adipocyte-derived hormone that is thought to have a facilitator role on the hippocampus. CSF levels of leptin may be a potential marker for HIV-associated dementia. Huang et al. demonstrated that leptin levels in CSF and serum samples from HIV-positive men correlated with impaired learning and memory performance. Leptin's role in cognition is also being studied in another form of dementia – Alzheimer's disease (AD). The pathologic hallmark of AD includes senile plaques for which beta-amyloid peptide is a major component. Beta-amyloid peptide is thought to be toxic to neurons and synapses and induces neurite degeneration which leads to decrease neurite length. These processes likely contribute to cognitive decline in AD. A lentivirus vector was constructed, expressing leptin protein in a self-inactivating HIV-1 vector (HIV leptin), and delivered by intracerebroventricular administration to an APP/PS1 transgenic model of AD. After 3 months of treatment, there were decreased cerebral cortex and hippocampal beta-amyloid peptide (Abeta) deposits as well as prevention of the reduction of cerebral cortex and hippocampal neurite length [123, 124].

37.3.1.9 Alpha-Synuclein

The human synuclein family consists of three members – alpha-, beta-, and gamma-synuclein. Alpha-synuclein is a 140-amino acid protein that aggregates into fibrillary forms likely precipitated by increased cellular oxidative stress. These fibrillary forms are known as Lewy bodies, the morphologic hallmark of Parkinson's disease. Most Lewy bodies occur sporadically though mutations in the alpha-synuclein gene have been demonstrated in familial cases of Parkinson's disease and Lewy body dementia. Khanlou et al. used the National NeuroAIDS Tissue Consortium and examined the substantia nigra of 73 clinically well-characterized HIV-infected individuals aged 50–76 years old and found neuritic alpha-synuclein expression in 16% (12/73) of the *substantia nigra* of the HIV+ cases. Among the HIV-negative controls, no alpha-synuclein was detected [118]. The increased prevalence of alpha-synuclein may be a marker of increased risk of neurodegenerative disease. This may be a future target for therapeutic development to prevent possible cause of cognitive impairment for HIV-infected individuals.

37.3.1.10 Tau

Tau is a microtubule-associated protein that is essential in the formation and stabilization of microtubules and in the movement of organelles down the axons and dendrites. Inflammation can cause phosphorylation of tau, and this phosphorylated form has been associated with neurodegeneration. Several epidemiologic studies correlated CSF tau levels among HIV-infected individuals with cognitive dysfunction. Green et al. showed no correlation with the quantity of CSF Tau for HIV-infected individuals presenting with cognitive dysfunction; however, they could correlate elevated CSF tau with poor outcome in six of eight patients. Another study by Brew et al. showed elevated CSF total tau and phosphorylated TAU among HIV patients with HAND. Significant correlation between HAND and CSF tau levels was demonstrated. These findings suggest that mechanisms that decrease CSF tau levels or prevent the phosphorylation of tau could be potential targets for HAND [125–128].

37.3.1.11 Presenilin-1 and Presenilin-2

Presenilins are a group of transmembrane proteins that constitute the catalytic subunits of the gamma-secretase intramembrane protease complex. Presenilin-1 (PSEN-1) gene and presenilin-2 (PSEN 2) gene encode presenilin-1 protein and presenilin-2 protein, respectively. Mutations in presenilin have been associated with familial Alzheimer's disease. Presenilins form part of the gamma-secretase complex, a membrane protease that processes amyloid peptide protein to the beta-amyloid peptide found in AD plaques. They may also play a role in central nervous system inflammation. Increased PS2 protein was found in HIV-associated dementia brain, and the role of presenilin in HAND, inflammation, and cytokine production was evaluated [129–132].

37.3.1.12 Apolipoprotein E4

Apolipoproteins are a class of proteins that bind lipids to form lipoproteins and facilitate their transport. Apolipoprotein E (ApoE) is a class of apolipoproteins that is believed to serve as the principal cholesterol transporter in the brain. It also serves a role in injury repair in the brain. In the CNS, it is mainly produced by astrocytes and transports cholesterol into neurons via ApoE receptors, which are members of the LDL receptor gene family. ApoE exists in three isoforms – ApoE2, ApoE3, and ApoE4. Genome-wide association studies have confirmed that the epsilon4 allele of ApoE is the strongest genetic risk factor for AD [133]. Among people living with HIV, ApoE4 enhances HIV-1 cell entry in vitro, and the ApoE 4/4 genotype accelerates HIV disease progression. The effect of lipid profiles and ApoE4 alleles was studied on cognitive decline in a cohort of ART-adherent HIV-positive and HIV-negative men aged 50–65 years. Elevated cholesterol and ApoE4 genotype are

independent risk factors for cognitive decline in ART-adherent HIV (+) men aged >50 years [134]. Mechanisms that target ApoE4 may be potential therapies for cognitive decline in persons living with HIV.

37.3.1.13 Amyloid Precursor Protein

Amyloid precursor protein (APP) is an integral membrane protein found concentrated in the synapses of neurons. It has been implicated in regulation of synapse formation, neural plasticity, and iron export and is the precursor molecule for beta-amyloid, which is the main component of amyloid plaques, the pathologic hallmark of Alzheimer's disease. HIV1 tat protein is produced by the HIV tat gene and is an important regulator of viral transcription via transactivation of the HIV-1 long terminal repeat promoter [135]. Several studies suggest that HIV-1 tat protein plays an important role in the accumulation of beta-amyloid in the brains of AIDS patients. It was shown that HIV-1 tat directly interacts with APP and modulates the trafficking and processing of the protein, resulting in increased Aβ production. This work provides new insights into the role of HIV-1 viral proteins in the pathogenesis of HAND in the post-HAART era and could be used as potential therapy in HIV-associated neurocognitive disorders [135].

37.3.1.14 Ad5-gag, Ad5-Pol, and Ad5-Nef

Adenovirus serotype 5 (Ad5) live viral vectors have been used in several animal and human HIV vaccine trials as delivery vehicles of viral genes. These HIV genes include genes coding for structural proteins or group-specific antigens (Gag), the virus polymerase enzyme (pol), and the accessory regulatory protein negative factor (nef) [136]. Ad5 vectors expressing these HIV-1 genes (gag, pol, and nef) as target antigens have been shown to induce cell-mediated immunity against the transgene products (peptides) utilized by the HIV for replication and ongoing propagation. Studies have also shown that the quality of cell-mediated immune response corresponds to the degree of control of viral replication [137, 138]. Thus, such targeting of HIV genes and the respective peptides can be used to prevent viral replication [139]. Further understanding and characterization of the cell-mediated immunity against HIV, which can be enhanced through therapy targeted at these genes (and perhaps many more), could have a significant impact on viral load control and eventual development of an effective HIV vaccine.

37.3.1.15 HLA-DR

HIV incorporates human lymphocyte antigen class II (HLA-DR) proteins into its viral envelope as it buds from the host-cell plasma membrane [140–142]. Following such incorporation into the HIV-1 envelope, HLA-DR retains its several functions

including antigen presentation and as an adhesion molecule whose natural receptor is CD4 molecule on cell surfaces [143]. Thus, it appears that the presence of host-cell-derived HLA-DR in the HIV-1 envelope increases the propensity of HIV interactions with host cells. This serves HIV by increasing its infectivity toward CD4 expressing cell lines and hence overall pathogenesis of the HIV/AIDS. This mechanism is particularly important in the pathogenesis of neuroAIDS since the neurotropic effect of HIV targets CD4+ cells of the CNS. Gene therapy strategies utilizing hematopoietic stem cells transduced with self-inactivating lentiviral vectors have successfully delivered targeted genes to microglial and dendritic cells of the CNS in the mouse model [144]. Subsequent studies therapeutically administered a single gene clone of HLA-DR and have shown successful neuroprotective and anti-inflammatory effects also in the murine model [145, 146]. Thus, therapeutic strategies targeting the HLA-DR gene to counteract the infectivity and pathogenicity of the HIV in the CNS could be one of several gene therapy approaches that could be realized soon.

37.3.1.16 Dopamine Transporter

The level of dopamine and its metabolite homovanillic acid is reduced in the CSF of HIV-infected individuals even in the absence of neurocognitive impairment [147, 148]. The HIV gp120 and transactivator of transcription protein (Tat) have driven the activation of the caspase system, and the ensuing apoptosis in dopaminergic synaptic terminals is believed to be at least one of the mechanisms for the reduced dopaminergic activity observed in AIDS-related neurocognitive disorder [149]. The dopamine transporter, also known as dopamine active transporter (DAT), belongs to the solute carrier-6 (SLC-6) family of sodium- and chloride-dependent facilitators of dopamine transport across the synaptic cell membrane. DAT localizes on the cell membrane and the different domains of the transporter (extracellular, transmembrane, as well as the intracellular carboxy and amino terminals) have specific functions in modulating the level of pre-and postsynaptic dopamine levels [150]. More specifically, DAT removes dopamine from the synaptic cleft and deposits it into cells for storage, thus reducing the dopaminergic signal. The function of the transporters in general is regulated by conformational changes determined by the polypeptide chain (among others). Recent advances in stem cell and gene therapy techniques have raised a distinct possibility with respect to DAT – a recent study in a mouse model for PD has shown the potential of a knock-in strategy to selectively enrich for DAT-containing neurons in vivo in mice and in vitro in cell culture [151].

37.3.1.17 Catechol-O-Methyltransferase

Dopamine biosynthesis and degradation and its overall regulation play an important role in cognitive cortical brain function [152, 153]. Optimal levels of dopamine are essential for proper cognitive function. Malfunction of a gene for one of the major enzymes required for the degradation of dopamine, catechol-O-methyl transferase

(COMT), is at least partly accountable for the pathogenesis of neurobehavioral deficits seen in HIV [154, 155]. HIV has been shown to significantly increase the expression of COMT and reduce the expression of dopamine receptor D4 [156]. Studies of the COMT gene have revealed functional polymorphism involving substitution of valine with methionine that reduced COMT activity associated with an enhanced cognitive performance [157, 158]. Experiments conducted using neuroblastoma cells have substantiated this observation in that inhibition of COMT by the commercially available anti-Parkinson's drug – tolcapone – conferred protective effect against HIV-associated neuronal damage [156, 159]. It will thus be of much interest to investigate the dopaminergic and COMT genes and metabolic pathway as a potential target in the prevention and therapy of neuroAIDS.

37.3.1.18 Brain-Derived Neurotropic Factor

BDNF is a member of the neurotropic family of growth factor peptides expressed in various parts of the central and peripheral nervous system. Its function supports the survival of existing neurons and promotes differentiation and formation of new neurons and synapses. BDNF is highly active in the hippocampus, cerebral cortex, and basal forebrain areas of the brain that are central to memory and learning [160–162]. BDNF has also been shown to be neuroprotective by blocking neurotoxins and counteracting the effect of traumatic brain injury through inhibiting caspase-3 activation [163, 164]. Thus, abnormal neurotropic regulation involving BDNF and its receptor tyrosine kinase receptor B (trkB) is one of the mechanisms of infection-related neurodegeneration and neuronal cell death [165]. The HIV viral envelope gp120 in turn has been shown to activate a caspase-dependent apoptotic pathway in brain cells, thus having a causal role in neuronal cell death and loss of cognitive function that accompanies HIV infection [119, 120]. In vitro and in vivo experimental evidence has shown that this gp120-induced neurotoxicity can be reduced by brain-derived BDNF. In one such study, a lentiviral-mediated gene transfer of human BDNF plasmid was constructed and characterized, and then a high-titer lentiviral vector was harvested and used to transduce human neuronal cell lines, primary cultures of human peripheral monocyte-derived macrophages (hMDM), and murine myeloid monocyte-derived macrophages (mMDM), showing that conditioned media containing BDNF had a high-level, stable expression of the neuroprotective factor BDNF and protection of neuronal and monocytic cell lines from TNF-alpha and HIV-1 Tat-mediated cytotoxicity [166]. Thus, further understanding and in vivo application of such a strategy could be one of the gene therapy options for preventing and treating neuroAIDS.

37.3.1.19 Secreted Anti-HIV Tat Single-Chain Antibodies (scFv)

Transactivator of transcription (Tat) is an HIV protein that enhances the viral gene transcription and hence viral replication and infectivity [167]. In addition to its direct role in transcription, Tat is also taken up by various types of cells including cells of

the CNS and causes direct neurotoxicity through activation of apoptotic pathways [168]. Moreover, Tat reprograms immature dendritic cells to express chemoattractants for activated T cells and macrophages that are the targets of HIV-1 infection, fueling activation of a cascade of inflammation and apoptosis that culminates in neuronal death and eventual loss of neurocognitive function [169, 170]. Thus, counteracting the effect of the HIV Tat protein has been one of the molecular strategies to neutralize or mitigate the pathogenesis of HIV in the brain. Induction of anti-Tat immune response through Tat toxoid- or Tat sequence-derived vaccines resulted in an immune response. However, there was conflicting data on the level of protection conferred by this immune response [171–173]. More recent advances in antigen delivery methods that utilized the full-length sequence of the HIV Tat protein have shown efficacy in successfully neutralizing Tat transactivation function. This holds promise as a future potential preventive and therapeutic option [174, 175].

37.3.1.20 Soluble Tumor Necrosis Factor Receptor

One of the main mechanisms of neurotoxicity that accompanies the invasion and replication of the HIV in the CNS is increased release of inflammatory cytokines by CNS macrophages, microglial cells, and astrocytes. Such overstimulation (excitotoxicity) of neurons leads to overproduction of free radicals that may be responsible for neurodegeneration [176, 177]. One of the major cytokine mediators of this neurotoxicity is tumor necrosis factor-α (TNF-α). Counteracting the effect of TNF-α thus will be an attractive approach to promote neuroprotection and prevent the development of HIV-associated neurocognitive disorder which according to some estimates occurs in up to >50% of HIV-infected patients [178]. Studies done in human embryonic microglial cell lines and cerebellar brain tissue harvested from Sprague Dawley rats have shown some promise in blocking TNF-α. More specifically, these cells transduced with a human codon-optimized gene encoding the soluble TNF receptor (sTNFR)-Fc fusion protein transfer via plasmid containing an expression cassette for sTNFR-Fc have shown that sTNFR-Fc can block TNF-α, HIV-1 Tat, and gp120-mediated toxicity with neuronal cells [179]. Thus, lentivirus vector-mediated sTNFR-Fc expression could be an effective neuroprotective strategy in neuroAIDS. However, such strategies need to overcome current difficulties with ex vivo transduction and delivery of the target gene to the CNS via trans-BBB migration.

37.3.1.21 Tumor Necrosis Factor-1 alpha (TNF-1 alpha)

Secretion of proinflammatory cytokines including TNF-1 alpha is one of the hallmarks of HIV infection and replication within the CNS. In addition to direct neurotoxicity, TNF-1 alpha interacts with cell surface receptors and increases the blood-brain barrier permeability, further enhancing the chance for HIV-infected monocytes to enter the CNS [180, 181]. Successful neutralization of the effect of

TNF-1 alpha has been achieved experimentally by using lentiviral-delivered gene vector to produce a soluble receptor fusion protein [179]. An animal model of inhibition of TNF-1 alpha signaling was also shown to prevent the HIV protein Tat and attenuates its neurotoxicity in hippocampal neurons [182]. Thus, counteracting the TNF-1 alpha and/or its receptor is a potential gene therapy target for neuroAIDS.

37.3.1.22 Chemokine (C-C Motif) Receptor-2

Chemokines are small molecule peptide members of the cytokine family that play vital roles in activation and migration of leukocytes by binding to specific G protein-coupled receptors [183, 184]. Chemokine receptors are widely distributed over several tissues and cell types and are also expressed in non-hematopoietic cells such as neurons, astrocytes, glial, and endothelial cells [183]. Thus, there has recently been increasing interest about their roles in mediating inflammatory responses in the central nervous system (CNS). The chemokine CCL2 and its receptor CCR2 are known to have a role in controlling the migration and infiltration of monocytes and macrophages in the CNS and hence in the pathogenesis of various neurodegenerative disorders [185, 186]. Upregulated gene expression for CCL2 is believed to enhance HIV-1 replication based on evidence showing that CCL2 serum concentration is significantly elevated in viremic compared to aviremic HIV patients and seronegative individuals, indicating a positive correlation between HIV viremia and CCL2 [187]. Thus, inhibition of CCL2 production and depleting the receptor from cell surfaces through gene therapy could provide a new therapeutic intervention in rendering cells refractory to HIV infection, thereby mitigating the neurodegenerative consequences of neuroAIDS. To this end, several strategies have been suggested; one approach is to target the coreceptor protein using a genetically engineered chemokine with a carboxy terminal endoplasmic reticulum retention sequence which will trap CCR2 (cc motif-2) and prevents its expression on the cell surface [188, 189].

37.3.1.23 Chemokine (C-C Motif) Ligand-3

Chemokine (C-C motif) ligand-3 (CCL3L1) is a potent HIV-suppressive chemokine and a ligand for the HIV receptor CCR5. The number of duplicated genes coding for CCL3L1 varies among individuals and populations, and the number correlates with susceptibility to infection by HIV. There is evidence showing that possession of a low CCL3L1 copy number was a major determinant of enhanced HIV susceptibility among individuals (Gonzalez et al. *Science*, [190]). siRNA techniques utilizing recombinant SV vectors targeted at CCRS have been experimentally shown to prevent HIV infection of macrophages and microglial cells [78, 191]. Although challenges remain due to high viral mutation rates and concern about the potential immunosuppressive effect on the host, directed approaches targeting more specific sequences of viral genotypes and residues of the receptor peptides hold great promise as future gene therapy [192].

37.3.1.24 Stromal Cell-Derived Factor-1 (SDF-1)

The stromal cell-derived factor-1 (SDF-1) is a chemokine extensively expressed in many tissue and cell types. Its functions include activation of leukocytes and initiation of the cascade of inflammatory response mediators that includes TNF and interleukins such as IL1 [193, 194]. SDF-1 is also the ligand for chemokine receptor 4 (CXC4), which is one of the coreceptors for HIV [95, 96]. Gene therapy strategies targeting the inhibition of cell surface expression of SDF-1 have been shown to make lymphocytes resistant to HIV infection [188]. When current limitations in scaling this approach to genome-wide application are overcome, this therapeutic approach could have major contribution toward prevention and treatment of HIV.

37.4 Future Studies in NeuroAIDS Gene Therapy

37.4.1 Neuronal DNA Editing Advances

On the one hand, CRISPR system use in brain cells has been limited by the normal physiologic progression found in human brain development, which leads to early cessation of cellular replication. On the other hand, mature brain cell self-repair using nonhomologous end joining (NHEJ) decreases with age [195]. Using single-cell labeling of endogenous proteins by CRISPR/Cas9-mediated homology-directed repair (SLENDR), the CRISPR/Cas9 system can be used to rapidly edit neuronal DNA [196]. SLENDR and CRISPR/Cas9 were delivered to neuronal cells through electroporation [197].[5] Using these techniques, many endogenous proteins in the brain were localized by inserting a sequence encoding a fluorescent protein for a target gene. SLENDR maps protein subcellular localization with micrometers to nanometer resolution in the brain [196]. Electroporation and insertion of the CRISPR/Cas9 system were used in prenatal models, where brain cells are still developing and dividing. Thus, the broken DNA was repaired via homology-directed repair (HDR) and cellular mechanism to repair double-stranded DNA lesions, providing researchers with the opportunity to precisely modify genes consistent with the tempo of various brain cell divisions. SLENDR can possibly be applied to neuroAIDS for the use of CRISPR.

In addition, there remains potential for next-generation Cas9 methods and its use with lentivirus-mediated delivery and with nanoparticles. These advances may potentially create more versatile methods and approaches for the permanent removal of the proviral DNA and eradicating HIV-1 [16].

[5] Electroporation induces pores in cell membranes using controlled electrical pulses [197].

37.4.2 Mutants: Tripartite Motif-Containing Protein 5

Tripartite motif-containing protein 5 (TRIM5a) is a protein containing RING finger domains. A mutant (R332G-R335G TRIM5αhu) efficiently restricted HIV-1 infection by interfering with the uncoating process, thus preventing reverse transcription and infection. In addition, lymphocytes expressing this mutant had a survival advantage in comparison with the unmodified parental cells. The study supported mutation of TRIM5a, to exhibit potential for HIV-1 gene therapy [198]. RING finger domains bind two zinc ions via their cysteine residues and function as part of the ubiquitin ligation pathway of the 26S proteasome involved in protein degradation. The human genome contains more than 380 RING motifs. Of relevance to the brain is that RING finger domain mutations are involved in the pathogenesis of Parkinson's disease, and RING finger motifs have been associated with HAND progression as well [93, 199].

A Rev-responsive element (RRE) decoy gene was produced and inserted into bone marrow-derived CD34(+) cells from HIV-1-infected children. The study utilized retroviral-mediated transfer of the RRE decoy in a clinical trial that evaluated the safety and feasibility of placing the treated cells back into the children. The patients showed no evidence of adverse effects. Low levels of gene-containing leukocytes in peripheral blood samples were still detected after 1 year, demonstrating a potential for performing gene therapy for HIV-1 using hematopoietic cells [200].

37.4.3 Engineered Homing Endonucleases (HE)

Current HAART is unable to fully cure patients with HIV infection, partially due to HIV reservoirs residual in various locations in the body, as a provirus in several cell types, and moreover in the CSF and brain [1]. Additional recent publications confirm the importance of HIV CSF escape and the issue of privileged reservoir foci of HIV replication, even in the presence of HAART [201–204].

Some work to eradicate HIV-1 uses disabling techniques for the virus and still maintains cell survival. The use of homing endonucleases (HE) targets base pair sequences in HIV. This technique can introduce DNA double-stranded breaks in the HIV proviral DNA, thereby losing gene expression [55]. The long-term goal using HEs is to achieve virologic cures of HIV, by targeting and mutating the integrated HIV such that it is rendered incapable of further replication, gene expression, and pathogenesis [205].

37.4.4 Nanotechnology, Inhibitory Molecule Carriers, and Therapeutic Gene Delivery

The delivery of therapeutic medicines is limited by intense protection of the brain by the BBB. Consequently, the use of nanotechnology is being evaluated to deliver effectively therapeutic molecules to the brain and target HIV. Such delivery to the

brain includes ARV with a relatively noninvasive approach in neuroAIDS [206, 207]. Advances have been made for single-walled nanotubes, dendrimers, fusion proteins, and peptide-antibody conjugates for therapeutic delivery [208].

A fusion protein was used to deliver siRNA to T cells in vivo [209]. Using CD4- and CD8-specific siRNA delivery caused RNAi responses demonstrating no adverse effects – cytotoxicity – or immune response. Similarly, siRNA knockdown of the Gag gene can inhibit HIV replication in primary T cells with the use of a protamine-antibody fusion protein-based siRNA [210]. In another study, carbon-nanotube transporters were used to deliver siRNAs specific for CXCF4 and CD4 into human T cells and peripheral blood mononuclear cells, resulting in the knockdown of the CXCR4 receptors on these cells [208, 211]. In another study, HIV-infected humanized mice viremia, an excellent model for HIV-1 infection and disease, was suppressed with an arginine peptide conjugated to an antibody that delivered siRNA to T cells. The siRNAs were specific for CCR5 and reduced viral replication, which prevented CD4 T-cell loss [212, 213].

Additional animal models of therapeutic delivery have expanded the prospects of anti-HIV methods in neuroAIDS. The use of magnetically guided CNS delivery made several potential therapies possible. For example, magnetoelectric nanocarriers have been exploited as a noninvasive and nontoxic tool for therapeutic drug delivery to the brain (as well as to several other C57Bl/J mice organs). Anti-HIV drugs, such as tenofovir, have been used with this technology, resulting in increased drug loading, good BBB transmigration, and in vitro antiviral efficacy, and ultimately may be therapeutic for humans, as well as improving patient compliance to therapy [214, 215]. It should be noted as well that utilizing such nanotechnology beads with ubiquitous tissue and cell dissemination could be a method for penetrating and delivering gene therapy as well as HAART to pockets and reservoirs of HIV replication that remain after classic therapies have been used and may fail.

Apitoxin, bee venom, contains about 40–50% melittin[6] and had inhibitory effects via nanoparticle delivery on CCR5 and CXCR4 HIV-1 strains. Under conditions that were HIV inhibitory, there were no deleterious effects of melittin nanoparticles on vaginal epithelial cell viability. The melittin nanoparticles were delivered by low-speed centrifugation. Additionally, and interestingly, free melittin was not inhibitory for viral strains, whereas free melittin was detrimental to vaginal epithelial cell viability [216, 217]. Thus, the use of melittin reflects the possibility of a wider use of bee as well as wasp venoms for biotechnological and therapeutic antiviral and antimicrobial goals, in general [218].

Several additional uses of nanoparticle and vector delivery have been fabricated as well. This is illustrative of the use of shared technology – illustrating gene and drug therapy. TAK-779 is an active HIV-1 fusion inhibitor. SDC-1721 is a fragment of TAK-779 and is inactive. However, when SDC-1721 is bonded to 2 nm diameter gold particles, it is comparably active as TAK-779. This illustrates the ability of nanoparticles of gold to produce active from inactive drugs. Silver has also been used to

[6] The melittin 26-amino sequence oligopeptide is GIGAVLKVLTTGLPALISWIKRKRQQ. Melitt in disrupts the HIV envelope by inducing holes [216].

inhibit HIV-1. Silver nanoparticle HIV-1 interactions are size dependent; 1–10 nm particles demonstrate optimum binding. Binding is probably due to gp120 knob binding, and indeed the silver nanoparticles inhibit virus-cell binding [208, 219–222].

Overall, target-specific next-generation genome modification technologies including TALENs, ZFNs, and CRSPR/Cas9-RNA-guided endonuclease expertise will greatly boost the attack on HIV-1 and AIDS [16].

As one may anticipate, the use of combined nanoparticle-vector gene therapy to improve delivery under hitherto difficult circumstances is already under investigation [223]. This should greatly improve gene therapy and medication delivery system specificity, efficiency, effectiveness, feasibility, and affordability as well as minimize side-effects.

37.5 Ethical Issues

During the 2015 TED conference in London, CRISPR/Cas9 cocreator Dr. Jennifer Doudna reviewed how the CRISPR/Cas9 technology works and, in discussion, requested a global pause of the scientific community. Dr. Duodena's goal was to spark a discussion on the risks and potential complications of CRISPR/Cas9 technology. This is applicable to many genetic engineering technologies, including CRISPR. CRISPR's ease of use and inexpensiveness most likely are the causes of its widespread use. Concerned with the possible misuse of this technology, Doudna proposed to examine, assess, and discuss possible intended and unintended consequences of the growing technology analogously to the pause in cloning of the 1970s [17].

Several ethical issues were also reviewed related to using gene editing and CRISPR technology, in relation to social and ethical concerns, and their use with humans [15, 224]. The first concern is the general question of biological ethics and the balance of risks and benefits. In relation to CRISPR technology, an important case in point is the problem of off-target mutation – e.g., higher frequency of off-target effects in larger genomes [225, 226]. Mean genome lengths based on genome NCBI searches indicate 2.989, 2.671, and 1.391 billion bp, respectively, for the human, mouse, and zebrafish genomes [227]. One possible explanation is the inability of CRISPR to avoid cleaving identical or highly homologous DNA sequences in larger genomes, in turn leading to cell death. This may be in part due to sequence duplications in larger genomes. Off-target alterations were located by examination of partially mismatched sites induced by RNA-guided nucleases (RGNs). The off-target effects were produced at higher frequencies than the originally intended on-target effects [228]. Thus, there are indications for caveats in the use of gene editing technologies. Nonetheless, the literature (PubMed) indicates expanding use of these technologies (See Tables 37.1 and 37.2).

Consequently, Rodriguez focuses on the issue of ecological disequilibrium, to which CRISPR/Cas9 technology may lead – for example, the possibility of mutations due to off-target effects can continue when in germ line cells and may even

Table 37.1 Select genes and pathways for gene therapy in neuroAIDS

Method	Application example	References
CRISPR[a]/Cas9 system	HIV-1 genome removal in mice	[15, 16, 21, 22, 232]
CRISPR[a]/Cas9 system	Gene-editing CD4+ T cells, CCR5/CXCR4 knockout	[33]
C2c2[b] effector	RNA targeting/degradation	[35, 36]
Zinc-finger nucleases (ZFN)	Specific sequence alterations of CCR5-Δ32 deletion	[15, 16, 21, 22, 45]
Engineered homing endonucleases	Proviral DNA mutation, reporter gene expression	[55]
Transcription activator-like effector nucleases (TALEN)	HT-TALEN[c] mutation HIV-1 proviral DNA	[15, 16]
Molecular Trojan horses	HIV-disabled Trojan horse deliver antisense RNA molecule	[67]
Lentiviruses	Tat and Rev sequestration	[68]
Viral vectors with macrophages/monocytes	MDM[d] modification and bradykinin-enhanced CNS uptake	[69]
Adeno-associated viruses	Crossing BBB	[65, 66, 74]
Recombinant SV40	Transduced human neurons, viral protection	[77, 78]
BioBrick plasmids	Multigene pathway quick assembly, cloned gene mobilization	[81]
Oligonucleotide delivery	Cell delivery by cationic peptide thiol linkage	[82]
Translocatable drug-resistance elements	Mutant isolations, construction of strains	[83]
Transposable retroviral elements	Potential host gene regulation	[84]
Xeno DNA	Genetic firewall	[87]
HIV recombinase	Reduced HIV viral replication, HIV-1 provirus removal	[89, 91]

Refer to the text for discussions of each method
[a]*CRISPR* Clustered regularly interspersed short palindromic repeats
[b]*C2c2* Class 2 type VI CRISPR-Cas9 effector
[c]*HT-TALEN* HIV Targeted transcription activator-like effector nucleases
[d]*MDM* Monocyte-derived macrophages

increase in each generation [224]. Ecosystem disequilibrium consequences in part due to the modifications of the sequences may lead to the disappearance of whole populations treated with such targeted by gene modifications. This possibility requires scientific oversight.

In addition, do-it-yourself biology, biohacking, and bioterrorism, which can potentially escalate to disregard for ethical standards or even be used for criminal purposes, need to be prevented [224, 229, 230]. Another question, the obverse side of the coin, is whether the research, for example, on CRISPR/CAS9 leading to pharmaceutical patents will then result in restrictions and limitations of original research, with questionable progress in clinical effectiveness, or will research remain com-

Table 37.2 Publications in PubMed for gene therapies (as of 2-27-2017)

Method	Number of publications	url
All methods	179,962	https://www.ncbi.nlm.nih.gov/pubmed/?term=gene+therapies
siRNA	81,345	https://www.ncbi.nlm.nih.gov/pubmed/?term=siRNA
Lentiviruses	7462	https://www.ncbi.nlm.nih.gov/pubmed/?term=lentivirus+gene+transfer
CRISPR	5157	https://www.ncbi.nlm.nih.gov/pubmed/?term=CRISPR
AAV	3260	https://www.ncbi.nlm.nih.gov/pubmed/?term=gene+transfer+AAV
SV40	724	https://www.ncbi.nlm.nih.gov/pubmed/?term=SV40++gene+transfer
TALEN	688	https://www.ncbi.nlm.nih.gov/pubmed/?term=TALEN
Zinc finger Nuclease	582	https://www.ncbi.nlm.nih.gov/pubmed/?term=zinc+finger+nuclease
C2c2	92	https://www.ncbi.nlm.nih.gov/pubmed/?term=c2c2
BioBrick	51	https://www.ncbi.nlm.nih.gov/pubmed/?term=biobrick

Abbreviations are defined in the text

petitive, open, and peer-reviewed? The point and counterpoint of such deliberations are applicable widely in genetic engineering. However, there appear to be little public discussions of the pros and cons. It should be noted that an extensive detailed review by Dr. J. Yu is an important step in the analysis of an understanding of the complex human and ethical issues involved in the widespread growing use of genetic engineering technologies [231].

37.6 Conclusions

In this chapter, we summarize various types of gene therapy methods available in recent years for application in neuroAIDS. We also present a brief overview of synthetic biology, a branch of biology capable of designing and constructing new biological components that can potentially lead to creating new systems for further understanding the complex host genetic organization and molecular virology associated with neuroAIDS. We also mention studies of these genes using gene therapeutic methods. Variants of such genes have been shown to be involved in various molecular and biological processes, course of disease, response to antiretroviral medications (ARVs), as well as several associated and putative biomarkers of HAND. Furthermore, we discuss future studies in gene therapy, for example, the expanding use of the CRISPR technology in brain cells. Additionally, nanoparticles are being developed to achieve penetration of the BBB for therapeutic delivery of medications.

The DNA-editing capability of the CRISPR/Cas9 technology remains an astonishing twentieth-century discovery, in addition to the invention of PCR by Kary

Mullis as well as work on the human genome project. However, surprisingly, HIV-1, although only 9000 bases in length (compared to the human three billion nucleotide genome), in addition to demonstrating an ability to escape any therapies that have been pitched at it, including chemotherapy, immune therapy, and siRNA, also shows ability to escape the new CRSPR genetic modification excision-replacement technology. We also address the ethics and responsibilities of human genome editing.

An abundance of human genes has been implicated in the incidence and progression of neuroAIDS – not all of which by any means interact directly with HIV-1 infection, HIV-1 proteins, or HIV-1 nucleic acids – but whose expression is nonetheless associated with clinical signs and symptoms of neuroAIDS as well as brain damage. This chapter reviews such host genes both as harbingers of neuroAIDS for which the development of gene therapy is appropriate and as reviewing host genes for which gene therapy is imminent or has already commenced. It should be further noted that there is no prime or "holy grail" gene identified yet, implicated in neuroAIDS incidence or pathogenesis. Therefore, the development of gene therapy methods that simultaneously attack multiple faulty genes is appropriate and is under development. Table 37.1 summarizes gene therapy methods. Table 37.2 shows numbers of publications for several of these methods.

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Conflict of interest The authors report no conflicts of interest.

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Chapter 38
Biostatistical Concerns in HIV Research

Robert C. Duncan

Keywords Measurement scales • Populations • Population distributions • Parameters of distributions • Sample description • Random samples • Sample selection • Derived distributions • Hypothesis tests on means • Multiple comparisons • Relationships among variables • Comparing proportions • Risk analyses • Survival analysis • Design of experiments • Things not to do • Populations • Sampling • Testing means and proportions • Experimental design

Core Message
Proper statistical design and analysis are essential for successful biomedical research. Effective use of statistical methods calls for close collaboration among researchers and biostatisticians. There must be clear and effective communication so that research goals are understood by the biostatistician and that the statistical needs to achieve those goals are understood by the researcher. The material presented in this chapter is intended to provide a foundation for the researcher to interact with the biostatistician in the development of a research plan.

38.1 Introduction

Human immunodeficiency virus (HIV) research involves the complete spectrum of scientific investigation, ranging from basic science (laboratory) studies, clinical trials, and the perusal and reduction of existing data from the literature and large data sets. Almost all such studies require a close collaboration between the researcher and a consulting biostatistician for data analyses and interpretation. The statistical

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methods employed for a particular investigation, as well as the inferences therefrom, depend upon the conditions under which the data were gathered and prepared for analysis. To this end, it is vitally necessary that the researcher and the biostatistician are able to communicate clearly. To select the proper statistical methods the biostatistician must have a clear understanding of "how the data got on the data sheet." To understand the scope and limitations of the statistical analyses, the researcher must understand why the particular methods were chosen, the feature(s) of the data to which the analyses were directed, and the assumptions about the properties of the data and the experimental procedures which guided the biostatistician to the choice of methods.

During graduate training, a biostatistician is fortunate to have completed coursework in the undergraduate medical curriculum (microanatomy, neuroanatomy, biochemistry, physiology) as well as the required mathematical and statistics curriculum. This background prepares the biostatistician well to interact with medical researchers. Unfortunately, not all biostatisticians are trained in the "bio" aspects of their profession. Consequently, HIV researchers are advised to be prepared to explain their studies to such an extent that it is clear that the biostatistician understands their scientific basis as well as the specific aims involved. On the other hand, it cannot be expected that the researcher is a statistical expert. At a minimum, the HIV researcher should be familiar with statistical terminology as well as the purpose and limitations of common statistical procedures. In particular, the researcher should be aware of misappropriate applications of procedures which have become all too common in the research literature.

In the following, statistical concepts and their applications and misapplications will be developed as heuristically as possible while maintaining accuracy and continuity. It will be assumed that the reader is, at least superficially, cognizant of the ordinary statistical techniques used in medical research. These include (1) hypothesis tests used when comparing mean values (Student *t* tests, analyses of variance [ANOVA]); (2) procedures for testing relationships among two or more variables (simple linear and multiple regression and correlation, analysis of covariance [ANCOVA]); (3) chi-squared tests when comparing binomial and multinomial proportions; and (4) the comparison of rates and hazard ratios (logistic regression, survival analysis). The main focus will be on providing a framework for the interaction of the researcher with the consulting biostatistician and making the researcher aware of the common errors and misapplications of statistical techniques.

38.2 Measurement Scales

The basic concept in the development of statistical methodology is that of an observational unit on which on which one or more variables can be measured. Examples of observational units include clinical patients, laboratory animals, and chemical preparations. Examples of variables include gender, weight, and concentration. For our purposes we will assume that observational units are uniquely identified and that each unit can be observed one or more times. Observed values of variables,

which are nonnumerical (e.g., gender) are said to be qualitative and measured on a nominal scale. Since statistical procedures usually involve some numerical manipulation of observed values a numerical code for the qualitative attributes can be developed (e.g., female = 1, male- = 0). The only comparison that can be made between observational units is whether they have equal observed values. There is no concept of direction or distance between scale values. If observed values are numeric and not qualitative then they are termed to be quantitative. Quantitative values are measured on three scales of increasing mathematical complexity. The next scale above the nominal is the ordinal scale. In this scale, an observed value can be classified as smaller, equal, or larger than another observed value, but distance between scale values has no meaning. As an example, an immune assay might be coded as 0, 1, 1+, 2, 2+. (More will be said about such coding later.) Nominal and ordinal scales are said to be discrete. When observed values can have any numerical value within a given range, which may have infinite as well as finite lower and upper limits, the scale is said to be continuous. If the difference between (the "distance between") two scale values is preserved under an additive transformation then the values are said to be measured in an interval scale. Time as measured on a 12 h clock represents an interval scale. From one o'clock until three o'clock is 2 h. From four o'clock to eight o'clock is 4 h. Further, the ratio of differences is meaningful. We see that $(3-1)/(8-4) = 2$, which captures the fact that the second interval is twice as long as the first. Finally, the highest measurement scale is the ratio scale. Ratio scales are characterized by having a true zero, which none of the other scales possess. Examples of ratio scales include mass, length, and electric charge. In this scale, ratios of values have interpretable meanings. Time as measured on a 24 h clock represents a ratio scale since there is a true zero (midnight = 0). Thus, 14 o'clock is twice as far from midnight as is 7 o'clock. All of the usual mathematical techniques can be applied to both interval scales and ratio scales. As we will see in the sequel, this is not true of the two lower scales. The use and misuse of the ubiquitous mathematical transformations whose validity depend on the scale of measurement will be covered thoroughly in following sections.

38.3 Populations

Observational units are ordinarily thought of as belonging to a population which is defined by known and observable characteristics. As an example, one might speak of the population of males "who currently reside in the US and are known to be HIV positive." This defines a finite population whose members could, at least theoretically, be individually identified and enumerated. Such a population is not static but changes as new members are added and old members deleted. To avoid these issues, membership could defined "as of a date certain" or "within a specified time period." A smaller population could be defined as "all of the HIV positive patients currently enrolled in a specific hospital outpatient program." A possibly smaller population could be defined "all of the HIV positive patients currently enrolled in a specific hospital outpatient program and have agreed to participate in a clinical trial." The

purpose of the clinical trial would be to make scientific inferences about the treatments in the clinical trial, and to extend these inferences to the outpatient program members and to the national population. It is important to note that none of these three populations are defined to include all of the positive HIV since the HIV status of some may not be known and some HIV positives may decline the clinical trial. Also, it is not clear from the definitions how closely the populations might resemble each other. In biological populations such as those above, values of certain variables are fixed and unchanging (e.g., gender, ethnicity, etc.) while others are variable over time (e.g., blood pressure, viral load, etc.). It is usually assumed that a nonfixed observed variable represents the usual value for that observational unit.

Other populations can be defined that exist only in potential but that can be called into being by certain manipulations. Examples include the tossing of a fair coin, the roll of a die, the roll of a pair of dice, and dealing bridge hands. The single toss of a coin has two possible outcomes, usually named H for "heads" and T for "tails." If order by toss is observed, two tosses ($n = 2$) of a coin has four possible outcomes, (H_1, H_2), (H_1, T_2), (T_1, H_2), and (T_1, T_2). The pattern of subscripts can be used to identify unique observational units. For a "fair" coin each outcome is equally likely. This collection of four ordered outcomes represents the "population units" and the "observed values" associated with two tosses of a fair coin. If one were to repeat a single coin toss numerous times and tabulate the frequency with which heads (or tails) appeared then one could make an informed judgment about whether the coin being used is fair since one would expect heads to appear in approximately 50% of the tosses.

38.4 Population Distributions

For scientific purposes (as opposed to health management purposes) the individual values of variables associated with population units are usually of little interest. Instead, it is the distribution of variable values and the parameters, which describe the distribution which are of interest. For discrete variables (those described by nominal or ordinal measurement scales) the distribution is given by a table or a mathematical formula which assigns the relative frequency of occurrence within the population to each of the individual discrete values. As an example, consider the distribution of ethnicity in a clinical population where ethnicity is described as White non-Hispanic (WNH), White Hispanic (WH), and Black (K). The parameters to describe this distribution are the proportions of ethnicities: P_1 WNH, P_2 WH, and P_3 B, where $\sum_i P_i = P_1 + P_2 + P_3 = 1$. These parameters, which add to 1.00, describe completely the distribution of ethnicity in the population. Consider a population of N observational units, each of whom has associated two discrete variables (e.g., gender and ethnicity). Then a table of gender by ethnicity could be constructed and the relative frequency of each cell of the table could be calculated. Thus, P_{ij} represents the gender i and ethnicity j, $i = 1, 2$, and $j = 1, 2, 3$, and $\sum_{i,j} P_{ij} = 1$. The population distribution of combined gender and ethnicity is completely described by the

parameters P_{ij}. If the table proportions are added across rows then the row marginal totals are the relative frequencies of the row variable. These frequencies describe the distribution of the row variable in the population. Similarly, the column marginal frequencies describe the distribution of the column variable in the population. Among the mathematically described distributions of discrete variables, the most important are the binomial and its multivariate analog, the multinomial. The use of these will be demonstrated in later sections.

For continuous variables (those described by interval and ratio measurement scales), the distributions are described by mathematical functions. For any continuous distribution, between any two unequal values there is an infinite number of possible values so that relative frequencies of individual specific values are zero. Thus, only ranges of values with unequal upper and lower limits have nonzero relative frequencies. This feature of continuous distributions will be illustrated later. Among the continuous distributions whose properties are used in the statistical analyses of research data are the normal, the chi-square, the student *t*, the Fisher–Snedecor *F*, all of which will be shown to be mathematically related. Other distributions frequently used are the logistic and the log-normal. Specific mathematical formulae for distributions of interest will be presented after the discussion parameters of distributions below.

38.5 Parameters of Distributions

Distribution parameters are defined as mathematical entities with numerical values which express properties of the distribution. The parameters of major interest in statistics involve measures of location and dispersion. Location refers to the central tendency of the variables associated with the distribution. Dispersion refers to the spread of variable values, such dispersion ordinarily being measured as a distance from a measure of central tendency. Definitions will be given first and the numerical examples will be given to illustrate the definitions.

Measures of central tendency for the distribution of a given variable include:

Arithmetic mean (or simply, mean) – The expected value of the variable. For a finite population the expected value of the variable X_i is calculated by multiplying each value by its relative frequency and then summing all of these products. If all variable values are listed separately for each of the *N* observational units then the expected value is given by the sum of all values measurements divided by *N*. Thus, $\mu = \frac{1}{N}\sum_i X_i$ = Arithmetic mean. (Greek symbols are used for population parameters.) For a continuous population the expected value is calculated using the mathematical formula $f(X)$ representing the distribution by an integral (the continuous analog of a finite sum) as $\mu = \int_{+\infty}^{-\infty} Xf(X)\mathrm{d}X$. It will be noted that the mean is analogous to the center of gravity as defined in physics (i.e., the mean is

the center of the distribution by weight). For these computations to be valid, the data must be measured on the interval or ratio scales.

Median – The middle value that separates the higher half from the lower half of the data set. For a finite population with N an even number we have median = (XN/2 + XN/2 + 1)/2. If N is odd, then the median is given by the middle value. Thus, in both cases, there is an even number of observations above and below the median. For continuous data the median is given by median = m where m is found from $\int_{m}^{-\infty} Xf(X)\mathrm{d}X = \int_{+\infty}^{m} Xf(X)\mathrm{d}X = 0.5$. In the continuous case, the median is the point above and below which 50% of the population is found. The median is the center of the distribution by count. The median and the mode are the only measures of central tendency that can be used for ordinal data.

Mode – The most frequent value in the data set. It sometimes happens that researchers desire to locate all points which show a higher frequency than do the points to either side of them (i.e., the data show multiple "peaks"). In this case, the distribution is said to be multimodal. If all of the observations are distinct then there is no mode. For continuous data the mode (or modes) are best found graphically. This will be illustrated below. The mode is the only central tendency measure that can be used with nominal data.

Geometric mean (GM) – For finite populations the Nth root of the product of the data values, where there are N values. Thus, GM = $(X_1 \times X_2 \times X_3 \ldots \times X_N)1/N$ Although the geometric mean is involved in some advanced statistics topics, our interest is best served by restriction our consideration to finite populations and samples. The geometric mean finds its application in the study of pharmacokinetics and chemical residue analyses such as pesticide exposure. The geometric mean is appropriate for expressing the central tendency of variables measured as ratios, such as chemical concentrations. It will be noted that the logarithm of the harmonic mean is equal to the arithmetic mean of the logarithm of the X values. That is, $\mathrm{Log}\left(\mathrm{HM}\right) = \frac{1}{N}\sum_i \log\left(X_i\right)$. This measure is valid only for data that are strictly positive and measured on the interval or ratio scales.

Harmonic mean (HM) – For finite populations the reciprocal of the arithmetic mean of the reciprocals of the data values. Thus, $\mathrm{HM} = N / \sum_i 1/X_i$. The harmonic mean finds its application in the study of rates and rate-like measures. Here too we will restrict our attention to finite populations and samples. This measure too is valid only for data that are strictly positive and measured on the interval or ratio scales.

There are other measures of central tendency used in advanced statistical techniques which are beyond the scope and intent of this presentation.

Common measures of statistical dispersion include:

Variance – The expected value of the sum of the squared deviations of each variable value from the population mean. For finite distributions the variance is $\sigma^2 = \frac{1}{N}\sum_i \left(X_i - \mu\right)^2$. For continuous distributions $\sigma^2 = \int_{+\infty}^{-\infty} \left(X - \mu\right)^2 f\left(X\right)\mathrm{d}X$.

Standard Deviation – The square root of the variance. Standard Deviation = $(\sigma)^{1/2} = \sigma$. For two distributions of the same single variable the larger standard deviation indicates the distribution with the larger spread.

Range – The range is defined as the largest variable value minus the smallest variable value.

38.6 Sample Description

Sample summary statistics are computed to and displayed in tabular form to describe the samples. These computations usually include sample sizes, sample means, sample variances or standard deviations, and sometimes minima, maxima, and ranges. It is sometimes desired to pool, or aggregate, the data over various groups. In particular, a sample mean is calculated as $\bar{X} = \frac{1}{N}\sum_i X_i$.

The standard way that presentations of the shape of statistical distributions of samples are presented in textbooks is by frequency tables or histograms, which are based on aggregating the data into specified intervals. It has been my experience that a better way is to present sample data distributions is by plotting the each data point by its cumulative frequency of occurrence. This is accomplished by ordering the n observed data points from low to high and labeling each data point with its position in the sorted array. Then the approximation of cumulative frequency p for any value is then given by $p = (\text{position} - 0.5)/n$. For the expression as a percentage one would simply multiply p by 100. The graph or table from this exercise is called the empirical distribution. This computation of p, rather than $p = \text{position}/n$, avoids assigning either 0 (0%) to the lowest or 1 (100%) to the highest position and does not require the specification of the beginning and ending points in a frequency table. It is well known that different choices of intervals can have a dramatic impact on the visual inferences from histograms. This does not happen when the empirical distribution is used. For data to illustrate sampling procedures and computations involving sample estimates, a table of 100 observations of a standard normal distribution with a mean $\mu = 0$ and a standard deviation $\sigma = 1$ was computed using the NORM.INV (probability, mean, standard deviation) in Microsoft Excel. Any normal distribution $N(Y, \mu, \sigma)$ can be converted to the standard normal $N(X, 0, 1)$ by the transformation $X = (Y - \mu)/\sigma$. Thus, all properties of sampling from a normal distribution can be investigated using the standard normal. The probabilities given, and used in the NORM.INV function, were computed as probability = (position − 0.5)/100 where position refers observations 1–100 in the data table. The X values in Table 38.1 will serve as a finite population to be sampled in the illustration of statistical procedures.

Shown below in Fig. 38.1 is the plot of the data in Table 38.1. This chart will be used to illustrate many aspects of sampling from a normal distribution. A cumulative probability plot of the any normal distribution, like Fig. 38.1, is sigmoid in shape, symmetric about the mean, and is characterized by the association of certain multi-

Table 38.1 Values of the standard normal (0, 1) distribution by probability

Probability (position)	*X*	Probability (position)	*X*	Probability (position)	*X*	Probability (position)	*X*
0.005 (1)	−2.58	0.255 (26)	−0.66	0.505 (51)	0.01	0.755 (76)	0.69
0.015 (2)	−2.17	0.265 (27)	−0.63	0.515 (52)	0.04	0.765 (77)	0.72
0.025 (3)	−1.96	0.275 (28)	−0.60	0.525 (53)	0.06	0.775 (79)	0.76
0.035 (4)	−1.81	0.285 (29)	−0.57	0.535 (54)	0.09	0.785 (79)	0.79
0.045 (5)	−1.70	0.295 (30)	−0.54	0.545 (55)	0.11	0.795 (80)	0.82
0.055 (6)	−1.60	0.305 (31)	−0.51	0.555 (56)	0.14	0.805 (81)	0.86
0.065 (7)	−1.51	0.315 (32)	−0.48	0.565 (57)	0.16	0.815 (82)	0.90
0.075 (8)	−1.44	0.325 (33)	−0.45	0.575 (58)	0.19	0.825 (83)	0.93
0.085 (9)	−1.37	0.335 (34)	−0.43	0.585 (59)	0.21	0.835 (84)	0.97
0.095 (10)	−1.31	0.345 (35)	−0.40	0.595 (60)	0.24	0.845 (85)	1.02
0.105 (11)	−1.25	0.355 (36)	−0.37	0.605 (61)	0.27	0.855 (86)	1.06
0.115 (12)	−1.20	0.365 (37)	−0.35	0.615 (62)	0.29	0.865 (87)	1.10
0.125 (13)	−1.15	0.375 (38)	−0.32	0.625 (63)	0.32	0.875 (88)	1.15
0.135 (14)	−1.10	0.385 (39)	−0.29	0.635 (64)	0.35	0.885 (89)	1.20
0.145 (15)	−1.06	0.395 (40)	−0.27	0.645 (65)	0.37	0.895 (90)	1.25
0.155 (16)	−1.02	0.405 (41)	−0.24	0.655 (66)	0.40	0.905 (91)	1.31
0.165 (17)	−0.97	0.415 (42)	−0.21	0.665 (67)	0.43	0.915 (92)	1.37
0.175 (18)	−0.93	0.425 (43)	−0.19	0.675 (68)	0.45	0.925 (93)	1.44
0.185 (19)	−0.90	0.435 (44)	−0.16	0.685 (69)	0.48	0.935 (94)	1.51
0.195 (20)	−0.86	0.445 (45)	−0.14	0.695 (70)	0.51	0.945 (95)	1.60
0.205 (21)	−0.82	0.455 (46)	−0.11	0.705 (71)	0.54	0.955 (96)	1.70
0.215 (22)	−0.79	0.465 (47)	−0.09	0.715 (72)	0.57	0.965 (97)	1.81
0.225 (23)	−0.76	0.475 (48)	−0.06	0.725 (73)	0.60	0.975 (98)	1.96
0.235 (24)	−0.72	0.485 (49)	−0.04	0.735 (74)	0.63	0.985 (99)	2.17
0.245 (25)	−0.69	0.495 (50)	−0.01	0.745 (75)	0.66	0.995 (100)	2.58

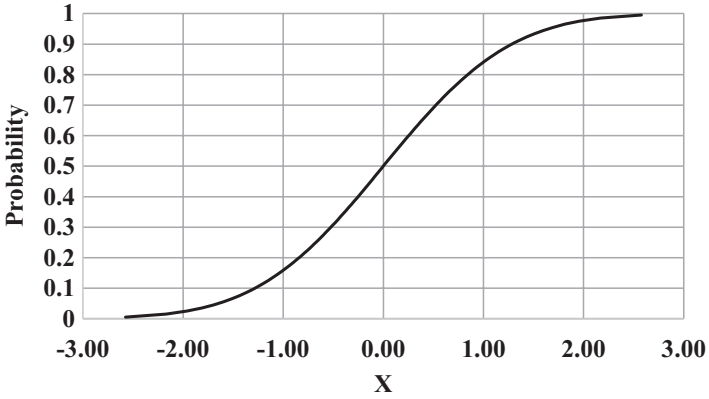

Fig. 38.1 Probability that a single sample is less than *X*

ples of the standard deviation above and below the mean with common probabilities used very frequently in statistical analyses. The median is located at zero standard deviations above (or below) the mean since 50% of the observations are above (or below) the mean. Thus, the mean and median are equal in a normal distribution. Below the X value equal to $\mu - 1.96\sigma$ (which is -1.96 in Table 38.1 since $\mu = 0$ and $\sigma = 1$) are to be found 2.5% of sample values (probability = 0.025). Similarly (as can be checked in Table 38.1) $\mu + 1.96\sigma$ is the point above which 2.5% of values are to be found (probability = 1–0.975). This means that μ and σ are sufficient to calculate the relative frequency (probability) for any range of values of the observed variable. By supposing that any variable under study is sufficiently approximately described by an underlying normal distribution, we can use sample estimates of μ and σ to estimate the distributional properties from which the sample was drawn.

Included in the statistical functions in Excel are programs to manipulate several commonly used statistical distributions. The approach described above can be used in Excel to generate the value of a variable X given a probability by "*.INV (p, parameter list)", or the probability given X by "*.DIST(X, parameter list)", from continuous distributions. The particular distribution name is used in the position shown by the asterisk. These distributions include the standard normal, the normal, the Student t, the chi-squared, the F, the log normal, the beta, and the gamma. These tables and their use can also be found in most textbooks on statistics.

38.6.1 Random Samples

The definition of populations indicates there is a "tag" or "name" for each observational unit as well as one or more observed values for the variables associated with that unit. For a finite population and a small number of variables, it is possible to model the population in such a way that random samples can be selected and the observed variables recorded for analysis. During my training for my PhD (1962–1966) we used a round Quaker Oats box containing 500 wooden Bingo markers. On each marker, a number was written to represent the observed value for that observational unit. To generate a random sample of size n the box was shaken, n markers were withdrawn by hand without looking in the box, and the value from each marker recorded. The markers were then replaced in the box to be ready for subsequent sampling. It is important to note that the variable values were not known to the sampler as the sample was constructed and that each observational unit had an equal probability of being selected. Any sampling procedure must be an exact analog of this procedure in order to be truly "random." One way to insure that the process is random is to construct a list of observational units and then select from this so that each unit has an equal likelihood of being in the sample. The list of units in Table 38.1 is given by the ordered position numbers. For any finite population, say subjects enrolled in a clinical trial, each subject could be numbered before any observations are made. This would provide an unordered list for any subsequent actions or variable measurements.

A random sample from a finite (discrete) population can be generated by any means that produces a selection of unit names from a list that each unit has an equal probability of being in the sample. Fortunately, surrogate lists for both finite and infinite populations can be constructed in a way that allows the use of computer generated random numbers for sample selection. As an example of a finite population, consider setting up a pilot study based on a large group of clinic patients. A list of medical record numbers of patients eligible for the study could be generated from computerized patient records. This list may either be computerized or on paper. Assuming that the total number of subjects, *N*, in this list is larger than the number needed for the pilot study, a random sample from the list is desired to provide patients to be interviewed for possible enrollment. If the pilot study needs n subjects, one might plan to interview more than *n* patients because of refusals or other reasons of exclusion.

There are computer programs (e.g., in SAS, R, and SPSS) which biostatisticians use to provide the researcher randomization lists for their studies. For researchers who are not used to using sophisticated statistical programs there is a simpler way. Random sampling can be performed using the randbetween (min, max) function in Microsoft Excel. This program will be used in the following examples so that the reader can repeat these examples and try his/her own if desired. The procedure for generating the surrogate list is as follows: (1) number the position of each of the subjects in the total list from 1 to *N*. The position number is a unique "label" or "ID" for each subject (note that this list does not have to be computerized); (2) in a cell, say A2, of an empty column in an Excel spreadsheet type "= randbetween(1,*N*); (3) select this cell and drag it down to select 2*n* cells; (4) copy these cells by entering "ctrl v" (when the numbers appear, all cells will show as "selected"); (5) without doing anything else, paste these cells into their current position using the "paste values" selection on the paste tool; (6) with the cursor select the top number in this list and on the Data tool bar select and enter "Remove Duplicates." The remaining numbers comprise a random sample of IDs from the total list.

Steps (4) and (5) above are necessary because the function randbetween (bottom,top) is a volatile function in Excel. A volatile function is executed whenever any action is taken on the spreadsheet in which it appears. If the "paste values" were not carried out, then any action on the sheet would generate new random numbers. Step (6) is necessary because the initial selection can include an ID more than once. As an example, if the patient Mr. Smith is the 147th patient on a list of 200 patients and ID = 147 appears on the random list, then Mr. Smith is included in the sample. Importantly, this method of selection has nothing to do with any characteristics of the subjects comprised by the total list.

The information given above serves to illustrate how random sampling is developed. It is not likely that an HIV researcher would want to construct samples in this way, although it might be useful for learning purposes to repeat some of the examples given below. This information has been provided to familiarize the researcher with the process and to emphasize the necessity for

the sample selection not to depend in any way on the variable values under study. The only way to guarantee unbiased estimates is to develop random samples where every population member has an equal likelihood of being in the sample.

38.6.2 Sample Selection and Description

For purposes of verisimilitude, a set of 100 simulated systolic blood pressure assessments are shown in Table 38.2. These were constructed to be distributed normally with a mean value of 124 and a standard deviation of 9 by transforming the values in Table 38.1 by the computation SBP = $9X + 124$. The data were then randomly ordered so that they might resemble data gathered from a clinical population. These data will serve to illustrate sample selection and description, sample estimates, study design, and hypothesis tests in subsequent sections.

Table 38.2 Systolic blood pressure (SBP) of 100 subjects in a clinical study

Subject	SBP	Subject	SBP	Subject	SBP	Subject	SBP
1	117	26	125	51	132	76	125
2	124	27	119	52	114	77	125
3	121	28	130	53	121	78	110
4	126	29	133	54	122	79	127
5	117	30	122	55	117	80	126
6	136	31	101	56	128	81	124
7	112	32	122	57	134	82	127
8	117	33	121	58	111	83	132
9	128	34	122	59	116	84	129
10	119	35	108	60	133	85	135
11	112	36	114	61	134	86	110
12	118	37	120	62	120	87	121
13	127	38	119	63	142	88	123
14	131	39	140	64	125	89	131
15	123	40	106	65	118	90	123
16	138	41	119	66	123	91	116
17	139	42	135	67	131	92	116
18	115	43	120	68	124	93	123
19	131	44	147	69	120	94	126
20	125	45	132	70	136	95	127
21	113	46	134	71	130	96	104
22	113	47	144	72	130	97	138
23	114	48	137	73	124	98	129
24	109	49	128	74	129	99	128
25	126	50	115	75	118	100	129

Table 38.3 Random sample of 10 subjects from 100 subjects enrolling in a clinical trial

ID	37	11	54	57	17	98	95	30	56	8
SBP	120	112	122	134	139	129	127	122	128	117

Table 38.4 Random sample of size $n = 10$

Source	Mean	Std. dev.	Range
Population	124.0	9.00	46
Sample	125.0	8.04	27

The data in Table 38.2 were entered in an Excel spreadsheet to be used to illustrate the properties of random samples. To begin, the Excel program randbetween (1100) was used to randomly select 10 ID numbers from Table 38.2. The sample data are shown in Table 38.3.

In order to describe the sample, we must compute the statistics used to estimate the corresponding population values and to assess the most likely type of distribution from which the sample was drawn. Since this is a learning situation, we know population parameters and the distribution. Ordinarily we would not have this information but only the information contained in the sample. Sample estimates of central tendency are computed using the same formulas defining population central tendency above. The only difference is that the sample size n is substituted for the population size N. The sample mean is $\bar{X} = \frac{1}{n}\sum_i X_i$. The calculation of the range is still the same (maximum − minimum), but the calculation of variance (and hence the standard deviation) is different. In the development of statistical theory, it was found that in order for a sample estimate to be an unbiased estimate, the sample variance must be calculated as $S^2 = \frac{1}{n-1}\sum_i \left(X_i - \bar{X}\right)^2$. By unbiased it is meant that the average value over all possible estimates is equal to the true value. For sample estimates the Greek symbols used for population values are replaced by Roman letters. These calculations compared to population values are shown Table 38.4.

It will be noticed that the sample gave a close estimate of the true mean value, but not as good an estimate of the standard deviation, and a worse estimate of the range.

An important property of sample estimates is that the closeness of their approximation to population values increases as sample size increases. This is shown in Table 38.5 for repeated samples of increasing sample size from Table 38.2. The data in Table 38.5 show the variability of repeated estimates of the population mean (124) and the effect of sample size on these estimates.

It will be noted that the individual sample means and their overall mean get closer to the population mean with increasing sample size. Moreover, the variations of the sample means, as indicated by the standard deviations of the individual values, decreases as sample size increases. This important property is used extensively in the planning of experiments in order to make sure that estimates will have the precision necessary to meet experimental objectives.

Table 38.5 Normal means by sample size

	Sample size			
Sample	5	10	15	20
1	131.6	126.1	123.0	126.1
2	127.4	126.5	125.2	126.4
3	125.6	119.1	124.0	124.8
4	128.6	123.3	120.3	125.8
5	124.4	123.2	126.1	125.3
6	124.4	121.9	124.3	124.9
7	126.4	121.2	119.3	124.9
8	117.6	125.7	121.2	125.7
9	120.0	121.1	126.4	120.2
10	134.4	118.2	123.4	122.4
Mean	126.0	122.6	123.3	124.6
Std. Dev.	4.97	2.87	2.41	1.90

Table 38.6 Normal standard deviations by sample size

	Sample size			
Sample	5	10	15	20
1	4.51	7.05	7.18	9.57
2	11.19	9.13	5.60	9.55
3	9.74	14.66	8.55	9.20
4	7.02	7.53	9.67	8.17
5	8.17	6.21	10.15	9.99
6	10.09	9.71	7.33	7.96
7	8.41	8.11	5.81	8.06
8	9.86	5.93	8.78	6.44
9	15.72	6.17	7.73	11.34
10	11.41	8.46	11.65	6.44
Mean	9.61	8.30	8.24	8.67
Std. Dev.	2.97	2.58	1.91	1.56

The same behavior is true for sample estimates of the population standard deviation (9) as demonstrated in Table 38.6. As we will see later, the convergence of sample estimates means to the population value happens sooner (lower sample sizes) than does the convergence of estimates of variation.

A very important feature of sampling from a normal distribution is that the estimated sample mean and the estimated sample standard deviation are independent. That is, larger (smaller) sample means are not associated with larger (smaller) sample standard deviations. This is shown in Fig. 38.2 for the samples shown in Tables 38.5 and 38.6. The red dotted line is the true population standard deviation. The independence of sample means and standard deviations is true only for the normal distribution.

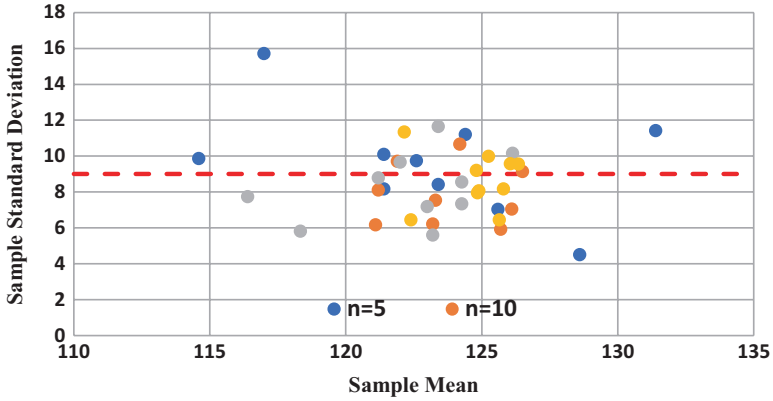

Fig. 38.2 Relationship of normal sample estimates

38.7 Derived Distributions

It is clear from the discussion in the last section that new populations can be generated from existing populations by repeated sampling from the parent population and recording new variables as functions of the sample values in each sample. These derived populations, often referred to as sampling distributions, are the foundation of statistical hypothesis testing. We will first describe the important derived distributions and then show how they are applied in experimental investigations.

By far, the normal distribution is the most important for statistical analyses. Fortunately there is a property of sampling that can make normal theory hypothesis testing applicable to samples from almost any parent population. The central limit theorem states that, given any random variable X, discrete or continuous, with finite mean μ and finite variance σ^2, then, regardless of the shape of the population distribution of X, as the sample size n gets larger, the sampling distribution of $\bar{X}$ becomes increasingly closer to normal, with mean μ and variance σ^2/n. This means that for population distributions of any shape or size, the estimates of mean and variance (standard deviation) can be used in normal theory tests. This will be illustrated by sampling from the exponential distribution, shown in Fig. 38.3, which is highly asymmetrical and very different in shape form a normal distribution.

The exponential distribution, symbolized as EXP($X|\ \lambda$), has probability density function is given by $f(X) = \lambda e(-\lambda X)$, and cumulative function given by $F(X) = 1 - e\ (-\lambda X)$, where $0 \leq X \leq \infty$ and $\lambda > 0$. For the exponential distribution we have $\mu = 1/\lambda$, and $\sigma^2 = 1/\lambda^2$, which means that the population mean and standard deviation are equal. The cumulative probability plot for an exponential distribution with $\lambda = 1$, similar to the plot for the standard normal, is shown in Fig. 38.3. It will be appreciated that this form is nothing like that shown in Fig. 38.1 for the standard normal, and thus might require a large samples to approach the sample mean to approach a normal distribution. Sample means and sample standard deviations for repeated samples of increasing sample size are shown in Table 38.7.

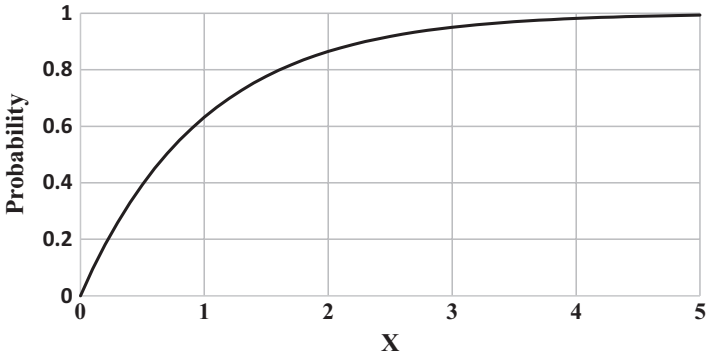

Fig. 38.3 Probability that a single sample is less than *X*

Table 38.7 Exponential sample means and standard deviations by sample size

	$n = 5$		$n = 10$		$n = 15$		$n = 20$	
Sample	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
1	1.07	0.929	1.2	1.179	1.3	0.988	0.9	0.919
2	1.44	1.236	1.0	0.868	1.4	1.393	0.9	1.106
3	1.10	1.044	0.8	0.582	0.6	0.621	1.2	0.462
4	1.07	1.077	1.1	1.007	1.0	0.793	1.1	1.115
5	0.63	0.382	1.3	1.332	0.8	0.584	1.5	1.743
6	1.28	1.015	0.8	0.800	0.8	0.675	0.7	0.587
7	1.11	0.968	1.6	1.522	1.0	0.836	1.0	0.707
8	1.19	0.499	1.6	1.645	0.7	0.708	1.0	1.018
9	2.22	2.742	1.4	1.812	0.7	0.536	1.1	0.747
10	1.22	1.547	0.5	0.618	1.2	1.336	1.2	0.794
11	0.42	0.468	0.6	0.364	1.0	0.710	0.8	0.738
12	1.30	1.122	0.9	0.582	1.2	1.051	1.1	0.594
13	1.41	0.802	0.9	0.479	0.7	0.443	1.4	1.060
14	1.18	0.990	1.1	0.823	1.6	1.546	0.8	0.695
15	1.11	1.057	1.0	0.579	1.0	1.028	1.0	0.642
16	1.30	0.625	0.7	0.760	1.4	1.286	1.0	1.009
17	1.01	0.814	0.5	0.617	1.3	1.289	1.2	0.818
18	0.34	0.232	1.0	1.094	1.3	1.172	0.7	0.566
19	1.61	2.265	1.1	1.341	0.7	0.677	0.9	1.087
20	0.94	0.444	0.7	0.642	0.8	0.900	1.0	0.768

The overall means and the standard deviations for the data in Table 38.7 are shown in Table 38.8.

It will be noted that as sample size increases the means approach the true population mean ($\mu = 1$) and the true population of $\sigma / \sqrt{n}$ (which is called the standard error to signify that it refers to a distribution of mean values rather than to a distribution of individual values). The standard errors would be $1/\sqrt{5} = 0.447$,

Table 38.8 Summary of the data in Table 38.7

	$n = 5$	$n = 10$	$n = 15$	$n = 20$
Mean	1.147	0.988	1.017	1.016
Std. err.	0.4054	0.3174	0.3062	0.2101

$1/\sqrt{10} = 0.316$, $1/\sqrt{15} = 0.258$, and $1/\sqrt{20} = 0.224$ respectively. However, the means converge to the true sample mean much more rapidly than do the standard deviations. As a rule of thumb, the minimum sample size for stable estimates of sample variance (or standard deviation) is 30 observations.

For the sample size $n = 20$ the distribution of sample means is nearly normal as shown in Fig. 38.4.

As noted above, the normal is the only distribution for which estimates of mean and standard deviation are independent. As we will see, this has implications for almost every statistical test since the criterion for judging differences among sample means is related to the inverse of the standard error of the difference. If larger mean values were implied larger standard deviations and smaller mean values implied smaller standard deviations, then the normal-theory statistical tests would be invalidated. Figure 38.5 shows the relationship of the estimates in Table 38.7. The red dotted line is the true population standard deviation.

Clearly, the sample estimates are not independent, and reflect the fact that the mean and standard deviation are equal in the exponential distribution.

The major distributions derived from the normal which are used in statistical analyses include the Student *t*, chi-squared and the *F*. The Student *t* is defined by the sample mean and the sample standard error (SE) computed from a sample of size *n* from a normal distribution. The standard error is given by the sample standard deviation, *S* divided by the square root of the sample size. That is, $\mathrm{SE} = S/\sqrt{n}$. Recall that the sample variance was defined as $S^2 = \frac{1}{n-1}\sum_i \left(X_i - \bar{X}\right)^2$. The student is $t = \left(\bar{X} - \mu\right)/S/\sqrt{n}$, or as $t = \overline{(X - \mu)/\mathrm{SE}}$. Clearly, the numerator of the *t* distribution represents a single sample from the derived distribution of sample means from a normal population with mean μ and standard error $\sigma/\sqrt{n}$. Since the expected value of $\bar{X}$ is μ, in repeated samples, the numerator would be distributed normally around a mean of zero if we were sampling from a distribution whose mean was truly μ. But the *t* is a ratio, and the variation of the denominator also enters into the distribution. Thus, the *t* does not show only the variation of the numerator, but also reflects the variation in the denominator. As we saw above in the samples from the exponential distribution, the variance of estimates of standard deviations depends on sample size, with such variations decreasing with increasing sample size. Consequently, the Student *t* has another parameter to take into account, the sample size *n*. For reasons not clear, Student *t* values are tabulated not by *n* but rather by $n - 1$, the divisor of the sums of squares $\sum\left(X_i - \bar{X}\right)^2$ used in the calculation of the sample variance S^2. This means that the smallest sample for which a *t* can be

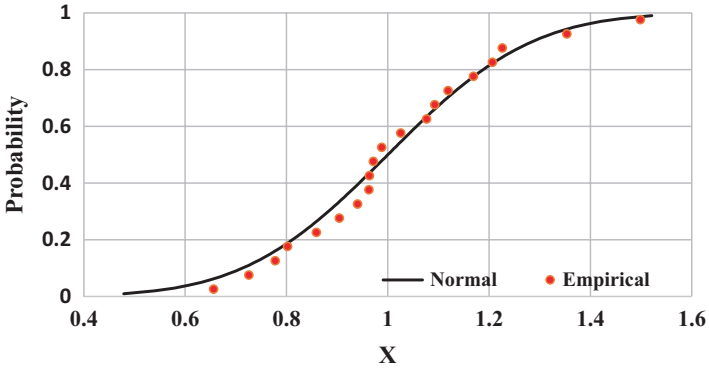

Fig. 38.4 Distribution of sample means, $n = 20$

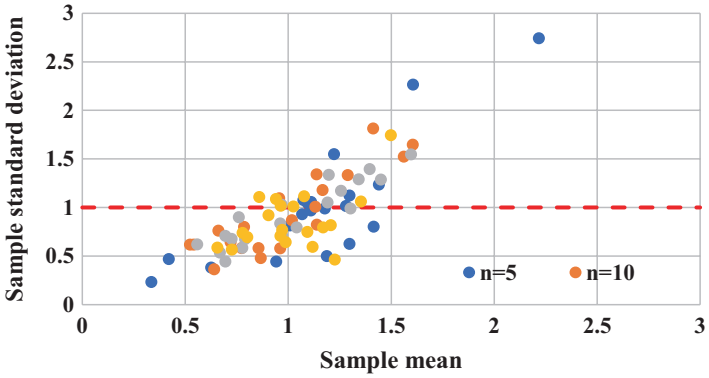

Fig. 38.5 Relationship of exponential sample estimates

computed is $n = 2$. This factor, $n - 1$, has a special name the **degrees of freedom** of the sample. For small sample sizes the t is more spread out than the standard normal. But, as we saw from the data in Table 38.8, the standard error of samples goes toward zero as sample size increases and the student starts to resemble a normal distribution. Some statisticians consider that after $n = 30$, the t is very nearly the same as the standard normal z distribution. However, it is never wrong to use the t regardless of sample size. The properties of the t distribution will be explored further when discussing hypothesis testing.

The chi-squared distribution, which we will use extensively for testing hypotheses about proportions of subjects falling into the categories of discrete distributions, is based on the derived distributions of standardized squared deviations of standard normal values around the true mean. If a random sample of size n is selected from a normal with mean μ and standard deviation σ, the quantity $\chi^2 = \Sigma\,[(X_i - \mu)/\sigma]^2 = 1, 2, \ldots, n$ is considered a single value from the derived distribution of the sums of

squares of a standard normal. It will be noted that the χ^2, as was true for the t, reduces the sample to a single value as a defined function of sample values. This is the format for all statistical hypothesis tests. One hypothesizes parameters for populations, draws random samples from those populations, and computes a single statistic from the samples, and tests how likely that statistic would be given the hypothesized parameters. As was true for the t distribution, the properties of the χ^2 depend on the size of the random sample, which means that the smallest sample for which a χ^2 can be computed is $n = 2$. If repeated sample of size n were drawn and the χ^2 for each sample calculated, the shape of each derived distribution would be tabulate by the degrees of freedom $n - 1$.

The F distribution is defined as the ratio of two χ^2 variables. If X were a χ^2 variable with $n - 1$ degrees of freedom and Y were a χ^2 variable with $m - 1$ degrees of freedom, then the ratio $F = X/Y$ is distributed as an F with $n - 1$ and $m - 1$ degrees of freedom. We will see later that the F statistic is used to test hypotheses about whether two or more means come from populations with the same mean value (i.e., whether $\mu_1 = \mu_2 = \ldots = \mu_k$, $k = 2, 3, \ldots, k$).

For the forgoing distributions, although population means may be expected to unequal, it is necessary to assume that the populations are distributed at least approximately normally, and that population variances (standard deviations) are, at least approximately equal. If not, the hypothesis test based on these distributions will not be valid.

38.8 Hypothesis Tests on Mean Values

Single Means Testing whether a mean of a single sample from a population is sufficient evidence to reject or not reject the hypothesis that the sampled population is distributed with a true mean value μ is the fundamental application of normal theory hypothesis testing. The process involves: (1) having a defined population from which random samples can be drawn; (2) specifying the hypothesized population mean value μ_o; (3) deciding on the statistical test to be applied to the sample results, which is ordinarily the Student t test, $t = (\bar{X} - \mu_o)/\text{SE}$; (4) deciding on how far the computed t value must be from zero (either positively, negatively, or either) in order to reject the hypothesis that the true population mean is μ; and (5), collecting the sample and computing the t value and comparing it to the distance decided on in step (4). Note that steps (1) thru (4) must be done before sample values are examined. The specified mean value μ_o is referred to as the null hypothesis, or H0: $\mu = \mu_o$. The Alternate hypothesis can take on three forms. Either HA: $\mu \geq \mu_o$, HA: $\mu \leq \mu_o$, or HA: $\mu \neq \mu_o$, If the null hypothesis is true then the repeated t values described in step (3) would be distributed with a mean of zero and the associated Student t probability distribution. If however the true mean value were described by either of the three alternate hypotheses, then the computed t values from repeated samples would not be centered on μ_o but on some larger (or smaller) value. Consequently, in the derived distribution of repeated samples there would be an increased number of larger

(smaller) t values than expected under the H0. This fact is the basis of the definition of sufficient evidence for rejecting or not rejecting H0. This is done by setting the probability level to describe one's confidence that observed data supports H0.

Sufficient evidence is quantified by defining confidence levels in terms of the probability distribution of t values under H0. This entails defining ranges of t values and noting within which range an observed t value falls. One set of regions would lead to not rejecting H0. The other decision regions would lead to rejecting H0. The only requirement for the regions would be that the rejection regions have total probability α.

The confidence regions can arise from one of three scenarios, either HA: $\mu \geq \mu_o$, HA: $\mu \leq \mu_o$, or HA: $\mu \neq \mu_o$. If HA: $\mu \geq \mu_o$ is true then the rejection region for H0 is all t values such that $t \leq t_\alpha$, where t_α is the point below which $100\alpha\%$ of the t values from repeated samples would fall. If the observed t value falls in this region the inference is that the observed $\bar{X}$ is too small to have come from a population with $\mu \geq \mu_o$. Similarly, if HA: $\mu \geq \mu_o$ is true, the rejection region for H0 is all t values such that $t \leq t_{1-\alpha}$ ($\bar{X}$ is too large). If HA: $\mu \neq \mu_o$ is true, the rejection region for H0 is all t values such that $t_{\alpha/2} \leq t \leq t_{1-\alpha/2}$ ($\bar{X}$ is too small or $\bar{X}$ is too large). Note that $t_{\alpha/2} = -t_{1-\alpha/2}$ since the t distribution is symmetric about a mean of zero. The first two cases are called directional hypotheses, or one-tailed tests. The third case represents a two-tailed test. In all three cases, the probability is α of rejecting H0 if it is true. In practice, the t values are not compared. Rather, the probability associated with the sample t value is looked up (in a table or in the computer program being used) and compared to the α level chosen for testing the null hypothesis. If the observed probability is less than or equal to such that probability that the observed X would less than or equal to α then the null hypothesis H0 is rejected. There is another way to express this process using sample estimates of mean and standard error. A lower $\bar{X}$ value, say $\overline{X_L}$ could be computed such that the probability that the observed $\bar{X}$ is less than equal to $\overline{X_L}$ is $\alpha/2$. This value is given by $X_L = \bar{X} - t_{1-\alpha/2}$ (SE). Similarly, $X_U = \bar{X} + t_{\alpha/2}$ (SE). The range (X_L, X_U) is called the $100(1 - \alpha)$ % confidence interval for μ. If the hypothesized mean is in this interval then H0: $\mu = \mu_o$ is not rejected.

Clearly, this approach to hypothesis testing reduces to a process that resembles a coin toss. The coin is labeled "Reject H0" on one side and "Don't Reject H0" on the other. The corresponding probabilities are α and $1-\alpha$ if H0 is true. This paradigm is true for all statistical hypothesis tests, no matter how complicated the manipulations of the data are in the computation of the test statistic.

Two Means The Student t test can also be applied to decide whether there is sufficient evidence to reject or not reject the null hypothesis of whether the true means of two populations could be equal. The starting point in this case is to consider the difference $d = (\bar{X}_1 - \mu_1) - (\bar{X}_2 - \mu_2)$. Under the null hypothesis H0: $\mu_1 = \mu_2$ we have $d = \bar{X}_1 - \bar{X}_2$. The Student t is given by $t = (\bar{X}_1 - \bar{X}_2)/\mathrm{SE_d}$, where $\mathrm{SE_d}$ is computed from the pooled variance $S_p^2 = [((n1 - 1)\, S_1^2 + (n2 - 1)\, S_2^2)/(n1 + n2 - 2)]$. Then, the standard of the difference is given by $\mathrm{SE_d} = S_p(1/n1 + 1/n2)$. The rejection

regions are defined as above for whatever alternate hypothesis is chosen among HA: $\mu_1 \geq \mu_2$, HA: $\mu_1 \geq \mu_2$, or HA: $\mu_1 \neq \mu_2$. As given above, a $100(1-\alpha)\%$ confidence interval could be computed the difference in sample mean values and the pooled standard error. If this interval contains the value zero then the null is not rejected.

Notice that the so-called deflation factor, $(1/n_1 + 1/n_2)$, which is related to the harmonic mean of the sample sizes, governs how much the pooled standard deviation is reduced by the joint sample size. It can be shown that the deflation factor is a minimum when $n_1 = n_2$. Thus, the comparison of two means would be more precise (smaller standard error) when sample sizes were equal. As an example, suppose one has funds to pay for 40 laboratory analyses of blood samples from an experiment to compare a specific chemical in a treated group against the same chemical from an untreated (control) group in whom the chemicals distribution is very well known. Since the value expected from the control group is well known, it might be tempting to have a smaller number of subjects (say 10) as controls and a larger number (say 30) in the experimental group. The deflation factor is then (1/10 + 1/30) = 0.1333. If the allocation had been into equal groups, then the deflation factor would be (1/20 + 1/20) = 0.1000. The effective sample size is given by the harmonic mean, which is $n = 2 / (1/10 + 1/20) = 15$. As a check, (1/15 + 1/15) = 0.1333. Thus, by allocating unequal numbers to the samples, the precision of the experiment is as if 30 subjects had been studied instead of 40.

Two or More Means The F statistic, computed in what is called an analysis of variance (ANOVA) can be used to test whether a group of k normal populations with identical variances σ^2 can be assumed to have equal true mean values. The null hypothesis is H0: $\mu_1 = \mu_2 = \ldots = \mu_k$. The alternate hypothesis is that in at least one pair $\mu_i \neq \mu_j$, $i \neq j$. The rationale of the F test is to compare the variation among the group means to the pooled variation of the individual values within the different experimental groups. The variation among the means is sometimes referred to as the among sum of squares (SSA), while that variation based on individual values is called the within sum of squares (SSW). If H0 is true, then the population distributions would exactly overlay each other, in which case the different mean values would act like individual observations and the SSA and SSW could both be used to estimate the pooled population variance. If at least one pair of the means came from populations which were separated from each other, these two means would show excess variation. The overall mean in the calculations is defined as $\overline{\overline{X}} = \frac{1}{N}\sum_{ij} X_{ij}$, where $N = n1 + n2 + \ldots + n_k$, $i = 1,2, \ldots,k$, and $j = 1,2, \ldots, n_i$. The SSW = $\sum_i \left(n_i - 1\right) S_i^2$. To convert the SSA and the SSW into a chi-squared statistics each must be divided by its degrees of freedom, which is $k - 1$ (the number of means -1) for SSA and $\sum_i \left(n_i - 1\right) = N - k$ for SSW. SSA/$(k - 1)$ is usually called the treatment mean square (TMS) by statisticians since separate groups are usually defined by receiving a different "treatment" in the study. SSW/$(N - k)$ is called the error mean square (EMS) and represents the pooled estimate of the population variance σ^2. TMS/σ^2 has a chi-squared distribution with $k - 1$ degrees of freedom. EMS/σ^2 has a chi-squared distribution with $N - k$ degrees of freedom. The ratio (TMS/ σ^2)/ (EMS/ σ^2) = TMS/

EMS has an F distribution with $k - 1$ numerator degrees of freedom and $N - k$ denominator degrees of freedom. This is usually denoted as $F_k - 1, N - k$. Note that if only two means are being compared then the numerator degrees of freedom is one and the denominator degrees of freedom is $n - 1$. Thus we have $F_1, n - 1 = t^2_{n-1}$, or an F with a one degree of freedom numerator is the same as a Student t squared. The σ^2 in the numerator has been divided out by the σ^2 in the denominator. This cancellation of what would have been intractable nuisance parameters is why one must assume equal variances among the sampled populations. It will be noted that the statistical comparison among several means has been reduced to a single value to be used in the hypothesis test. As presented, the F test is an omnibus test in the sense that a significant F test of three or more mean values does not specify for which pair(s) of means significant difference applies.

Usually, when the F statistic is used the results of the calculations and the inferences therefrom are presented in what is called an analysis of variance (ANOVA). The ANOVA can be characterized as follows: "The analysis of variance is a numerical procedure which partitions total variation into recognized sources of variation." We will give numerical examples of various ANOVAs, including experiments with two or more classifications for experimental groups, in the section Design of Experiments.

38.8.1 Multiple Comparisons Between Pairs of Means

When comparing three or more mean values one might be tempted to perform Student t tests on each pair of means instead of performing an ANOVA. The problem with this approach arises from what is known as alpha inflation. When multiple comparisons are made among sample mean values from populations with identical means, the probability of declaring at least one pair of means to be significantly different increases exponentially with the number of means involved. If k means are compared pairwise, then there are $k(k - 1)/2$ comparisons and the probability of falsely declaring at least one pair significantly different is not α but $\alpha^* = 1 - (1 - \alpha)k$. Three means, with $\alpha = 0.05$ would lead to three comparisons and $\alpha^* = 0.143$. Five means would have 10 comparisons and $\alpha^* = 0.401$. Since it is neither useful nor satisfying to say "We found a statistically significant difference among the mean values tested." and nothing more, pairwise tests are necessary.

The solution is found if modifying the tests used to decrease or remove the alpha inflation. Multiple comparison rests in common use include the Scheffe, the Neuman–Keuls, and the Tukey. The Tukey test, sometimes called the honestly significant difference (HSD) does not inflate the chosen α level. These tests, however, are felt to be sometimes too conservative.

There are two alternatives. One, due to Fisher, is called the least significant difference (LSD) test. It has been shown that if an F test of three or more means yields a significant result (i.e., the probability of the computed F statistic is less than or equal to the chosen significance level α) then at least one pair of means would also

be significant at the α-level by the Student t test. The LSD can be used following a significant ANOVA to find the one or more pairs of means responsible for the significant F test. If the overall ANOVA F test is not significant, then the LSD is not applied. Thus the chosen experiment wise alpha level is preserved.

Another way to preserve the chosen alpha level is to use the Bonferroni correction. In this approach, the alpha levels for each of the pairwise comparisons are chosen such that their sum is equal to the overall alpha. Thus, if three pairs of means are to be compared we would choose each α_i such that $\alpha_1 + \alpha_2 + \alpha_3 = \alpha$. The usual choice is to divide the overall α by the number of tests. For three tests we would have $\alpha^* = 1 - (1–0.05/3)3 = 0.0492 < 0.05$. If it is desired to test one pair with more power (see the section Design of Experiments for a discussion of the meaning of power and power calculations), then one α_i can be larger than the others. Suppose we set $\alpha_1 = 0.04$ and $\alpha_2 = \alpha_3 = 0.005$. Then we would find also that $\alpha^* = 1 - (1–0.04)(1–0.005)(1–0.005) = 0.0496 < 0.05$. The Bonferroni correction is very common in medical research literature.

38.9 Relationships Among Variables

Experimental units rarely have only a single variable of interest. It is usual to limit multivariable measures of association to continuous variables, although these measures are often compared for similarity or dissimilarity across categories defined by discrete variables. The distinction will be shown in the examples in the followings sections. Relationships can be investigated whether they involve only a pair of variables or multiple variables. Analyses involving two variables are usually referred to as simple regression. When the analysis involves the elucidation of the relationship of one variable to two or more other variables, the analysis is usually referred to as a multiple regression.

Pairs of Variables The most common approach for investigating two-variable associations is to ask whether the data support the inference that there is a dependency relationship between the variables and, if so, describing its functional form. Usually one variable is symbolized as Y and the other as X and the data are collected as n pairs Y_i, X_i. The question is then asked "What is the mathematical function that best describes changes in Y as X increases from its lowest to its highest value?" Statisticians have answered the question of the best function by proposing various functions and then determining how the data should be used to estimate the parameters of these functions using the techniques of differential calculus. Among the common functional forms are polynomials represented by one or more terms in a power series, exponential functions, trigonometric functions, and combinations of these. A power series would have the form $Y_i = \beta_0 + \beta_1 \boldsymbol{X}_i + \beta_2 X_i^2 + \ldots$. The exponential could have the form $Y_i = \beta_0 \operatorname{Exp}(\beta_1 \boldsymbol{X}_i)$. The trigonometric function could have the form of $Y_i = \beta_0 + \beta_1 \mathbf{Sin} \boldsymbol{X}_i$. The computational task is to use the data to find estimates of the parameters β_0, β_1, β_2, … which provide the best fit to the data. It will be appreciated that the choice of the function(s) to be fitted to the data must

be carefully considered, both as to how well the data might be represented by the chosen function(s) and as to whether the estimated parameters adequately describe the features of the data being explored in the experiments. These issues should be thoroughly discussed by the researcher and the statistician before the data are gathered.

In all of these forms, the normal theory statistical models require that we consider that the X values are fixed and measured without error and that the distribution of Y values under repeated observation at a particular X value is distributed normally with mean μ_x and variance σ^2. That is, the mean is particular to the X at which Y was measured and the variance σ^2 is constant across X values. The reason for these seemingly strong restrictions (i.e., fixed X values and common variance) is that the mathematical difficulties of treating the X values as random variables are almost insurmountable, and that statistical tests would be invalidated if the variances were different. In practice, this means that in order to repeat an experiment we would need to find subjects with the same values as in the first experiment. Fortunately, in most cases the effect of ignoring the requirement for X values to be fixed and measured without error has little effect on inferences from the data.

Simple Linear Regression The simplest function relating Y to X is the simple linear model [1] (a truncated power series) of $Y_i = \beta_0 + \beta_1 X_i + \in_i$. The quantity to be minimized is then the sum of the squared errors $\Sigma\hat{}\,(\in_i)^2 = \Sigma\hat{}(Y_i - \beta_0 + \beta_1 X_i)^2$. The final estimates for the parameters yield the model $\hat{Y} = \hat{\beta}_0 + \hat{\beta}_1 X$. If the data pairs are plotted with X on the abscissa (horizontal axis) and Y on the ordinate (vertical axis), then the continuous line drawn from the model would have an intercept given by $\hat{\beta}_0$ and slope given by $\hat{\beta}_1$. To test whether the estimated intercept and slope are consistent with hypothesized values Student t-tests are ordinarily used. These and other issues will be discussed later. Methods of whether two or more data sets exhibit similar slopes will be discussed later in the section Design of Experiments.

38.10 Covariance and Correlation

An important assessment of the linear relationship between two variables involves the estimation of their covariation and their correlation. The covariance between n random sample of pairs of two variables X and Y is defined as $S_{XY} = \Sigma\,(X - \bar{X})(Y - \bar{Y})/(n-1)$. If Y tends to be above its mean when X is above its mean, then the covariance is positive. If Y tends to be below its mean when X is above its mean, then the covariance is negative. If Y tends to be equally above and below its mean when X is above its mean then the covariance tends to zero. If the covariance is zero then Y is said to be independent of X. The covariance measures how X and Y vary together. The correlation between X and Y measures the strength of the covariation of X and Y. To compute the correlation one must firs compute the standard deviations of X and Y. These are $S_X = \Sigma\,(X - \bar{X})^2/(n-1)$ and $S_Y = \Sigma(Y - \bar{Y})^2/(n-1)$ respectively. The correlation is defined as the ratio of the covariance to the product of the standard deviations, $r = S_{XY}/S_X S_Y$. It can be shown that the range of

the correlation is from minus one to plus one, $-1 \leq r \leq 1$. If r is negative, the Y decreases as X increases. If r is positive then Y increases as X increases. If r is near zero, the Y does not vary linearly with X. The correlation and the slope of a linear model measure different things. The slope defines how fast the fitted line increases (or decreases) and the correlation measures how close the observed points lie to the fitted line. Although the correlation coefficient r can be tested against the null hypothesis that it is zero with the Student t test, better practice is to test the regression coefficient β_1 against zero and, if it is significant, infer that the correlation is significant.

Clearly, both estimates of covariance and correlation can be computed regardless of the true functional relationship of Y and X. One should always plot the data to interpret the model being used. As an example, if the data represented the ascending left arm of a quadratic curve, with only a little of the data coming from the descending right arm, then a linear model might test to be significant in slope and correlation, but it would not be the "best" model and could lead to false conclusions. There is one instance when correlation alone can be computed without a plot to yield a valuable interpretation. Regardless of whether simple linear regression has been involved or multiple regression (see later) has been involved, the result is to express the results as Y = "Model." Using the model, for every observed Y_i the associated Y_i can be computed. The correlation between these two variables can be calculated. It has a special name, R, and its square R^2 is used to measure the effectiveness of the model in fitting the data. Called "cap R square" by statisticians, R^2 lies between zero and one and its value measure the proportion of the variation between the observed Y values accounted for by the model. If a linear regression model were being used and all of the observed Y values fell exactly on the computed line, the R^2 would equal one and the model would account for 100% of the observed variation.

Multiple Variables It is not unusual to measure a number of variables, some of which are known (or suspected) to be directly related to the condition under study and some of which may have an influence on those directly related. Out of the total set of observations a single variable Y of major interest is selected for closer scrutiny together with a set of k variables, X_1, X_2, …, X_k which may be explanatory for the target variable. The analysis often involves choosing a subset from the k variables, which are significantly predictive of the target variable. Such analyses are based on the techniques of multiple regression [1]. The model to be fitted is $Y_i = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2i} + \ldots + \beta_k X_{ki}$. The statistical analyses depend on the correlations of Y with each of the X variables as well as the pairwise correlations among the X variables. In this formulation, the linearity of the data should be checked. The relation between the target variable Y should be approximately linear with each of the X variables and with each of the pairs of X variables (i.e., planar). If any nonlinear effects are detected, then data transformations on the X values to achieve approximate linearity should be investigated.

There are several approaches to the statistical analyses of multiple regression models. One is to fit the model with all of the X variables and test the overall model against the null hypothesis that the model does not account for a significant proportion of the variation found among the Y values. This would involve an omnibus

F test, which does not refer to any particular β among the model estimates. This approach might be used if there exists prior evidence that the X values chosen together are explanatory of the Y values. The other common approach is to perform what is known as a stepwise regression analysis. The goal is to choose a small subset of predictive variables from the total set so that the resulting regression model is simple, yet have good predictive ability.

There are two types of stepwise regression. In forward stepwise regression, the X variables are added to the model one at a time, starting with that X variable, say X_i, which is most highly correlated with Y. This variable is then checked against the criterion for entering the model, called F-to-enter. The process is to compute the F statistic of the estimated regression coefficient and compare it to the F-to-enter. The F-to-enter is specified by the statistical significance level which the researcher wants each estimated regression coefficient to have in the final model. Often, this significance level (probability) is set at $\alpha E = 0.05$, although sometimes a less restrictive level is chosen. If the F value for the regression coefficient for the first variable, β_i, does not exceed the F-to-enter the process stops with no significant associations indicated. If X_i is entered into the model, then all of the other X variables are searched to find the one which has the highest F-to-enter conditional on the X_i being in the model. At this second step, if no variable is found, which exceeds the F-to-enter, the process stops with the model indicating a significant effect for the first X variable. If the second variable is entered, say X_j, then, because their mutual influence might weaken each other's predictive power when both are in the model, the F values for null hypotheses that regression coefficients $\beta_i = 0$ and $\beta_j = 0$ are compared to a value called F-to-remove αR. If F-to-remove is not exceeded by the computed F for any β being tested, that variable is removed from the model. Usually, the significance level of F-to-remove is larger than that for F-to-enter to avoid repeated looping in the computations. This process selects a model in which each of the included variables has some predictive value in the presence of the other variables in the model.

In backward stepwise regression, the process is to fit all of the of explanatory variables to the model and then remove variables one by one if they fail to pass the F-to-remove criterion. This process selects a set of variables for which the model has the specified predictive value. Each separate variable is not necessarily predictive, either alone or in the presence of the included variables.

A technique, which is not often used but is sometimes useful, is an all subsets regression. In this procedure, regressions are calculated for all single predictor variables, all pairs of predictor variables, all triples of predictor variables, and so on until all predictor variables are included. The criterion for the choice of the model is the maximum R^2, the correlation between the observed and predicted Y values as defined above. This produces the best model without requiring statistical significance for the various predictors. Usually, only models with a significant R^2, say $p \leq 0.05$ would be considered to be adequate.

One of the common uses of multiple regression is to "adjust" the differences in means (or rates and proportions) between two groups in an analysis called the analysis of covariance (ANCOVA) [3]. The ANCOVA is best illustrated with a simple linear regression model which compares the sample means, $\overline{Y}_1$ and $\overline{Y}_2$, of two

Table 38.9 Data for covariance analysis

Group 1		Group2	
X	*Y*	*X*	*Y*
1.0	4.5	4.2	5.1
2.1	7.2	5.5	8.2
3.4	7.4	6.1	7.3
4.2	7.5	7.3	7.7
5.0	10	8.0	10.2

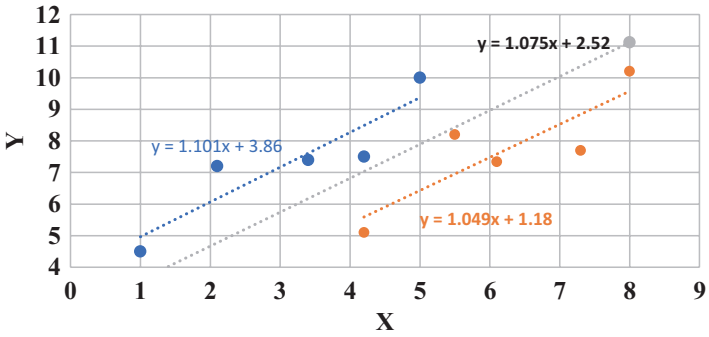

Fig. 38.6 Covariance adjustment of means

groups after adjusting for differences between the groups on a common predictor variable *X*. The data in Table 38.9 represent a sample of two groups of subjects for which a response variable *Y* has been measured along with a variable *X* which is known to influence *Y*.

The *Y* means and standard errors are $\overline{Y}_1 = 7.32$, $SE_1 = 0.871$, and $\overline{Y}_2 = 7.71$, $SE_1 = 0.818$. The difference in the mean values is $\overline{Y}_2 - \overline{Y}_1 = 0.39$. The pooled standard error of the difference in means is SEd = 0.1.19. Thus, $t = 0.39/1.19 < 1$, not significant. The picture of the data are shown in Fig. 38.6 below.

We see from Fig. 38.6, the data from Group two has been displaced from Group 1 because it has different *X* values. Thus, the true difference between groups has been "confounded" by the relationship of *Y* with *X*. The regression slopes are very similar between the two groups, so a common regression fitted to the pooled data is appropriate. This is shown by the black dotted line. If we projected the data in Group 1 forward along the slope given by the black line to the mean of all of the *X* values and projected the Group 2 data to the overall *X* mean back along the same slope, we would see that the adjusted differences in their means would be given by difference in the two intercepts. The overall mean *X* value is 4.68. The difference in the *Y* means adjusted to a common *X* value should be $d = 3.86–1.18 = 2.68$. For Group 1 the projected *Y* mean would be $Y_1 = (1.075 \times 4.68 + 3.86) = 8.89$. Similarly, for Group 2 $Y_2 = (1.075 \times 4.68 + 1.18) = 6.21$, and $Y_1 - Y_2 = 2.68$. The estimated *Y* values for each group are given by $Y_{1j} = (1.075 \times X_{1j} + 3.86)$ and $Y_{2j} = (1.075 \times X_{2j} + 1.18)$,

respectively. The error mean square is computed as EMS = $[\Sigma_{ij}(Y_{ij} - \hat{Y}_i)2]/(n1 + n2–2)$. Finally, the standard error for the difference in adjusted mean values is given by SEd = $[EMS(1/n_1 + 1/n_2)]1/2$. Making these computations, we find that $SE_d = 0.653$ and the *t*-statistic for the difference in adjusted means is $t = 2.68/0.653 = 4.10$. The *t* has 6 degrees o freedom, so the probability for rejecting the null of no difference is $p = 0.0032$. Thus, the adjusted means are significantly different, while the unadjusted means were not. In practice, it is found that when unadjusted means are not significantly different the adjusted means sometimes are, and when unadjusted means are different the adjusted means sometimes are not.

The analysis of covariance can be applied when there are multiple groups being compared and when there are several variables for which adjustment is necessary. There are two conditions to be met before the adjustments can be made. The relationship between the response variable, *Y*, and all of the explanatory variables must be jointly linear and the regression coefficients for each *X* variable must be equal across groups. In other words, the only effect of the *X* variables is to displace the group *Y* variables linearly along similar lines.

38.11 Comparing Proportions

Very often there is experimental interest in comparing the percent distribution of a population or populations across categories of interest [2, 3]. When only two categories are involved in the comparison the underlying distribution is the binomial. The familiar model for the binomial is repeated tosses of a coin and testing a null hypothesis that the true proportion of heads observed is compatible with a specified value, H0: $P = P_0$. Usually, the alternate hypothesis is HA: $P \neq P_0$. There is an exact distribution for exactly *X* occurrences in n tosses of the coin, namely $p(X) = \binom{n}{X} P^X (1 - P)^{n-X}$, $X = 0, 1, 2\ldots, n$. The cumulative probability of observing $X = k$ or fewer heads in n tosses is given by adding the individual probabilities, $P(X \leq X_k) = \sum_{k}^{i=0} p(X_i)$. The sample estimate for the true population proportion is $p = X/n$ and the sample estimate for the standard error of *p* is $[pq/n]^{1/2}$. Often the quantity *q* is defined as $q = (1 - p)$.

The binomial distribution can also be expressed in terms of the cumulative number "positive" outcomes (e.g., improved response in rehab, testing positive for a disease, or being female). If a sample on n responses from a population with a true proportion of positive responses of *P* were tabulated, then the cumulative probability of observing X_L or fewer responses is given by the cumulative probability $p(X \leq X_L)$. For a two-tailed test with the probability α of rejecting H0 when it is true one would find the lower number of success X_L such that probability that the observed *X* would less than or equal to $\alpha / 2$, and the upper number of success X_U such that probability that the observed *X* would greater or equal to $\alpha / 2$. If it

Table 38.10 Disease presence (1) or absence (0) in 20 subjects

Subject	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Disease	0	1	1	0	0	1	0	1	1	1	0	0	1	1	0	1	1	1	0	1

is found that $X_L < X < X_U$ then the null hypothesis would not be rejected. This is a somewhat awkward process to employ and not directly extendable to the comparisons of proportions among two or more populations or to the comparisons of multinomial populations. Fortunately, the process that is routinely used to make such comparisons can be developed through the subterfuge of a derived distribution for repeated the estimates of the true population proportion. As an example, suppose we want an estimate the total rate at which a certain disease is found in members of a given population (i.e., the rate without taking other variables into account.). If we code the presence of the disease as $Y = 1$ and the absence of the disease as $Y = 0$, then we can use these codes to compute sample estimates of the population mean and standard error of the mean. An example of such coding is shown in Table 38.10.

The sample mean is $p = \Sigma\ Y_i\ /n = 0.60$ (the sample mean $\overline{Y}$), and the sample standard error is SE = $(pq/n)^{1/2} = 0.1095$. If the standard error were computed by the usual formula for a observations from a random sample, we would have $S_2 = \Sigma\ (Y_i - \overline{Y})^{\ 2}/(n - 1)$ and the standard error as SE = $S/n^{1/2}$. If the standard error were calculated in this way we would find SE = 0.1124. It can be shown that $(pq/n)^{1/2} = [(n - 1)/n]^{1/2}\ S/n^{1/2}$. (Check: (19/20)1/2(0.1124) = 0.1095.) Thus, as n increases estimated p values from repeated samples of size n behave more and more like the estimated $\overline{X}$ estimates discussed above in the section Derived Distributions. The estimate p becomes normally distributed with sample means approaching P and sample standard errors approaching $(PQ/n)^{\ 1/2}$. It has been found that it is mathematically simpler to deal with the observed number of "successes" X rather than with the sample proportion p. If p is approximately normally distributed with mean P and standard error $(PQ/n)^{1/2}$, the X is distributed normally with mean nP and standard error $(nPQ)^{1/2}$. This is based on the principle of the propagation of error governing the transformation of variables. In the case that the transformation is multiplying by a constant, say $Y = aX$ where X is distributed with mean μ and variance σ^2, then Y will have a mean $a\mu$ and variance $a^2\sigma^2$.

We will use the fact that a sum of standard normal deviates (i.e., distributed with mean zero and standard deviation one) are distributed as a chi-squared distribution as discussed in the section Derived Distributions above. The rapidity of the approach to normality depends both on the sample size n and the true population proportion P. The rule that is general use in general use is to have $nP \geq 5$ and $n(1 - P) \geq 5$ in order for the chi-squared to be used (when P is unknown, the sample p is substituted so that we must have $np \geq 5$ and $n(1 - p) \geq 5$). This raises the possibility that distributions derived from the normal could be used for testing hypotheses about proportions. Such is the case and the distribution to be used is the chi-squared distribution.

For the data in Table 38.10, if the null hypothesis is that the disease rate in the sampled population is $P = 0.5$, then $nP = n(1 - P) = 5$ and the chi-squared approxi-

Table 38.11 Observed disease rate

Disease	Yes	No	Total
N	12	8	20
%	60	40	100

mation is valid. The z statistic for the observed sum versus the expected sum is given by $z = (12–10)/(20 \times 0.5 \times 0.5)^{1/2} = 0.89443$.

Statistical theory shows that if the data from Table 38.10 is aggregated as shown in Table 38.11, a simpler method can be used to test hypotheses about sample proportions. This formulation is based on the equivalence of the chi-squared statistic with a sum of squared z statistics (i.e., $\chi^2_{n-1} \sim \sum_{n}^{1}(z_i)^2$). This process of finding the expected values E_i for each cell for a table such as Table 38.8 and computing a chi-squared statistic using the observed values for each cell. The chi-squared would then have degrees of freedom equal to the number of cells minus one. In Table 38.8, there are two cells (Yes in cell 1, No in cell 2) and the observed values $O_1 = 12$ and $O_2 = 8$. The expected values for the cells are computed from the null hypothesis H0: $P = 0./5$. For cell 1 $E_1 = 0.5(20) = 10$ and for cell 2 $E_2 = 0.5(20) = 10$ also. Note that the sum of expected values equals the total. The computation of the chi-squared is $\chi^2 = \Sigma$ all cells $(O_i – E_i)^2/E_i$. Using the data shown in Table 38.8 we have $\chi^2 = (12–10)2/10 + (8–10)2/10 = 0.80$, with 1 degree of freedom. Note that for the computations for z above we also have $z_2 = (0.89443)^2 = 0.80$, verifying that the two methods of computation have equivalent results. Both tests would lead to not rejecting the null.

The single sample use of the chi-squared test for more than two categories is an obvious extension. For k categories there would be k cells and the null hypothesis would need to specify the proportions of the total sample expected for at least $k - 1$ of these categories. The last proportion would then be fixed since the proportions have to add to one. The computed χ^2 would have $k - a$ degrees of freedom. Often the null hypothesis is that all of the proportions are equal. The alternate hypothesis would be that at least one pair of proportions are not equal. This would lead to an omnibus test similar to that described above for testing three or more mean values with the F test. If a significant χ^2 were found, it would not refer to any particular pair of proportions.

The tests of proportions can easily be extended to comparing rates between two groups in a fashion similar to the extension of the Student t test for comparing two means. The data in Table 38.12 represent symbolically the responses to a particular question on a survey to determine whether positive responses differed by gender.

Quite often a series of such tables are used to display response of two groups, say treated and control, under various other categorical classifications. The entries in the body of the table, n_{ij} are called "cell totals", also called the observed values O_{ij}. The entries on the right side, $ni.$, are called "row marginal totals", the entries at the bottom, $n.i$, are the "column totals", and $n..$ is the "grand total." The rationale for computing the expected values in the cells is as follows: if males and female truly responded Yes at the same rate then the best estimate of that rate would be $n.1/n..$, the total Yes responses divided by the total of all responses. Using this rate estimate

Table 38.12 Symbolic responses by gender

	Yes	No	Total
Male	$n11$	$n12$	$n1.$
Female	$n21$	$n22$	$n2.$
Total	$n.1$	$n.2$	$n..$

the expected number of males answering Yes would be $n1.n.1/n.. = E_{11}$. Thus we can compute a χ^2 statistic based on the observed and expected as before: $\chi^2 = \Sigma_{ij}(O_{ij} - E_{ij})^2/E_{ij}$. This chi-squared statistic has degrees of freedom (df) given by the product $(r - 1)(c - 1)$, where r is the number of rows and c is the number of columns in the body of the table. The χ^2 computed for Table 38.9 would have one degree of freedom. These formulas can be extended to any r by c table and usually are the formulas used in computer programs. There is a simpler form of computation that is useful for hand calculation that provides insight into calculations we will do later. In this form, the computation uses table entries directly. The formula is $\chi^2 = (n11n22 - n21n12)2n.. / (n1.n.2n.1n.2)$. The χ^2 would be exactly zero if the "cross product" terms $n11n22 - n21n12$. The ratio of these terms, called the odds ratio, is used very frequently in epidemiological studies. We see that if the odds ratio $n11n22/n21n12 = 1$, indicating that females would be equally as likely as males to respond Yes, then the $\chi^2 = 0$, and males and females would have exactly the same rate of positive responses. The odds ratio, OR, will appear below in a discussion of Logistic Regression.

The validation that the simplified computational form given above leads to a χ^2 distribution could be accomplished if one were to specify reasonably large fixed numerical total $n..$ in Table 38.12, construct all possible tables consistent with that total, and the tabulate the cumulative distribution for the computed χ^2 values for each table. (Note that, since the grand total is fixed, only three table entries can be freely chosen.) It would be found that the empirical distribution would be a close fit to a one-degree of freedom χ^2. One of the major drawbacks of this formulation of the analysis of 2 × 2 tables is that data cannot easily be pooled across stratifying categories. As an example, one might separate Table 38.9 by White versus Non-White respondents. If one wanted a pooled estimate of the rate of Yes responses, it would be mathematically intractable in the current framework. However, there is an approach which is more fruitful. This approach, referred to as the Mantel–Haenzel (MH) , or sometimes as the Cochran–Mantel–Haenzel (CMH), is to assume that the marginal totals, both row and column, in each 2 × 2 table was a fixed value rather than a random value. This would mean that only one cell in the table was free to vary since the row, column, and grand totals are considered to be fixed. This changes the distribution of the table entries from the multinomial distribution with three table probabilities random, to the hypergeometric distribution with one cell entry distributed randomly. The calculation of the Mantel–Heanzel chi-squared statistic is slightly different from that calculated above. The shortcut formula to be used to calculate it is χ^2 (MH) = $(n11n22 - n21n12)2(n.. - 1)/ (n1.n.2n.1n.2)$. Thus, it will always be slightly smaller than the regular $\chi 2$, but the difference is becomes negligible for increasing sample size. Simulated of responses are shown in Table 38.13.

Table 38.13 Responses by gender

	Yes	No	Total
Male	16	23	39
Female	26	17	43
Total	42	40	82

Table 38.14 Response to rehabilitation regimens

	Return of function (%)			
Treatment	None	Partial	Complete	Total
A	6 (20.0)	16 (53.3)	8 (26.7)	30
B	8 (26.7)	15 (50.0)	7 (23.3)	30
C	17 (56.7)	8 (26.7)	5 (26.7)	30
Total	31 (34.4)	39 (43.3)	20 (22.2)	90

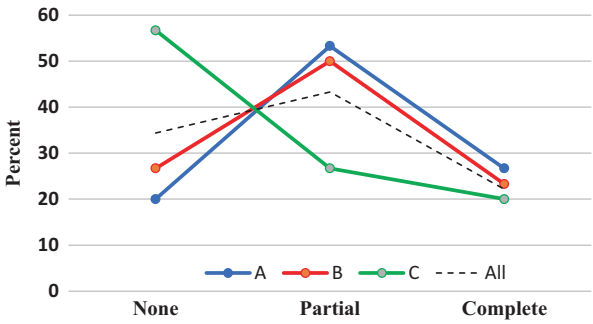

Fig. 38.7 Response to rehabilitation regimen

The chi-squared value is $\chi^2 = (16 \times 17–26 \times 23)2 \times 82/ (42 \times 40 \times 39 \times 43) = 3.09$, $p = 0.079$. The corresponding Mantel–Haenzel chi-squared is $\chi^2 = (16 \times 17–26 \times 23)2 \times 81/ (42 \times 40 \times 39 \times 43) = 3.06$, $p = 0.081$. The Mantel–Haenzel chi-squared approach is frequently used to pool estimates of rates across strata comprising a series of 2×2 tables.

The underlying features of the chi-squared analyses can best be illustrated by using higher dimensional tables. The data shown in Table 38.14 illustrate the expression of the responses as percentages. This allows plotting of the data and a more clear interpretation of the analyses.

Looking at the percent responses in Table 38.14 leaves the impression that treatments *A* and *B* show similar responses, while the responses for treatment *C* shows an elevated lack of response with a decrease in partial response. The percentages are plotted in Fig. 38.7.

The dotted line in Fig. 38.7 is that when responses are combined over treatment. If the null hypothesis that the three treatments would have equal effects were true, then all three individual lines would be expected to fall close to the dotted line. From Fig. 38.7 we can infer that regimens *A* and *B* have similar results, but regimen

Table 38.15 Return of some function

Treatment	Yes	No	Total
A	6	24	30
B	8	22	30
A + *B*	14	46	60
C	17	13	30
Total	31	59	90

C differs from both *A* and *B*, especially for no response and partial response. The computed chi-squared for these data is $\chi^2 = 4.17$, 4 df, $p = 0.036$.

A common procedure when overall statistical significance is found in a cross-classification table such as Table 38.14, subcomparisons are made within subtables of interest. In this case, one might be interested in comparisons among regimens within response groups. One approach, suggested by the data, would be to recast the data into three separate tables of response (Yes, No) by regimen, partial response (Yes, No) by regimen, and complete response (Yes, No) by regimen. This would seem to be statistically acceptable since the overall analysis showed a significant difference whose basis should be investigated. The most fruitful procedure would seem to be to test whether responses to regimens *A* and *B* are similar, and, if so, test regimen *C* responses against *A* and *B* combined. The table describing the return of some function versus no return of function is shown in Table 38.15.

Comparing regimen *A* versus regimen *B* yields $\chi^2 = 0.37$, 1 df, $p = 0.002$. Comparing *A* + *B* versus *C* yields $\chi^2 = 9.84$, 1 df, $p > 0.002$. The comparison within the partial response group shows no difference between regimens *A* and *B* but *C* is significantly different from both combined. Regimen *A* does not differ from *B* among complete responders and *C* does not differ from *A* and *B* combined.

38.12 Risk Analysis

Risk Ratio Disease-oriented studies often involve the estimation of the probability of observing the disease rate at which a disease develops in subjects who are otherwise alike but differ in the presence or absence of a specified condition [2, 3]. The condition could a new treatment versus the standard treatment (control) or a sham treatment (placebo). Study results are usually expressed as the relative risk (sometimes called the risk ratio). Such a study is started with a group of subjects, all without the disease, the subjects are randomized (usually in equally sized groups) to treatment or control, and then followed for the planned duration of the study while being monitored for development of the disease. Risk is defined for each group as the proportion of subjects in that group who developed the disease during the study. The number of new cases in a specified period of time is often called the disease incidence rate. If the risk in the treatment group is RT and that among the controls is RC, then the relative risk is RR = RT/RC. If RR = 1, then the risk is equal between

Table 38.16 Disease outcome

Risk	Present	Absent
Treated	*a*	*b*
Control	*c*	*d*

the groups. If RR < 1, then the risk is greater in the controls than in the treated. If RR > 1, then the risk is greater among the treated than the controls. The usual way of displaying data from a relative risk study is shown in Table 38.16.

The computation of the relative risk is RR = [*a*/(*a* + *b*)] / [*c*/(*c* + *d*)]. As is often true for ratios in statistics, it is easier to express results as the natural log (log to the base *e*) of the ratio. It has been shown that the natural log of the relative risk, Ln(RR), is distributed approximately normally when each of the expected values in the table are five or more. The mean of Ln(RR) is estimated from the table as $\hat{RR}$ = Ln(RT/RC) and its standard error as SE($\hat{RR}$) = {*b*/[*a*(*a* + *b*)] + *d*/[*c*(*c* + *d*)]}$^{1/2}$. The 95% confidence interval for the RR is given by Exp[$\hat{RR}$ ± 1.96 SE($\hat{RR}$)], where Exp(*x*) = e^x.

It will be appreciated that a study to estimate relative risk requires a good deal of effort. One must establish a study group whose members are free of the disease and who will be available for follow-up for the duration of the study. Follow-up requires staffing and facilities to examining the subjects at various periods during the study. If any subjects are lost to follow-up, then the estimation of risk is severely compromised because data have been "lost" from both the numerator and the denominator of the formula defining risk. There are two ways to analyze data when there is loss to follow-up. One way is to express the numerator as the status of the subject at the time of the last observation and to calculate the "person-time" for that subject as the time elapsed from the start of the study until the last observation. Such data are often expressed in terms of person-years. Then the total number of new cases of the disease in a group is divided by the total person-time for all subjects in that group. Instead of incidence rate this ratio is called the person-time incidence rate, or incidence density rate. There are approximate statistical methods to deal with person-time data, including Poisson regression. The other approach is to cast the data in terms of a survival analysis. Both of these methodologies are quite complex and should be used only in close collaboration with a biostatistician who is knowledgeable in the subject.

Odds Ratio Another very popular method of assessing risk is based on using the Odds ratio (OR) as an estimate of relative risk. Odds ratios based on the cross-sectional assessment of disease and exposure among cases and controls has been shown to yield appropriate estimates of odds ratios. The relative risk (RR) and the odds ratio (OR) are the two most widely used measures of association in epidemiology. The direct computation of relative risks is feasible if meaningful estimates of prevalence (the number of cases in a population, new or existing, observed over a specified period) or incidence are available. Prospective or cohort study designs allow for the direct calculation of relative risk from incidence. The situation is more complicated for case–control studies. If meaningful estimates of prevalence or inci-

dence are not available, the OR provides a valid effect measure. In Table 38.16, the "odds" that a subject in the treated group would develop the disease is defined as a/b. The odds that a subject in the control group would develop the disease is c/d. Consequently the odds ratio is OR = $(a/b)/(c/d)$. As defined above the relative risk was shown to be RR = $[a/(a+b)] / [c/(c+d)]$. If the presence of the disease in both groups is such that $a \ll a+b$ (a is much less than b) and $c \ll c+d$, the RR $\cong (a/b)/(c/d)$ = RR. The relation between RR and OR can be expressed exactly as OR = RR × (1 − RC)/(1 − RT). For a small RC, say 0.001, the OR and the RR are very nearly equal up to a RR ≤ 10. However, if the RC is as large as 0.1 then the RR must be less than or equal 2.5 for the OR to closely approximate the RR. More will be said about this later.

The most productive use of the OR is in using the logistic regression model to compare risk between two groups while simultaneously adjusting the model for differences between the groups on other variables associated with risk. For Table 38.16, the odds of getting the disease for the treated group was computed as "odds" = a/b. If we divide numerator and denominator by $a = b$, then we would have "odds" = (probability of developing disease) / (probability of not developing disease). Or, symbolically, odds = $p / (1 - p)$. In a case–control study, we would say that it was the odds of observing the disease. In fact, for any dichotomous random variable Y, which can be coded as 0, 1 a meaningful odds and odds ratios can be formed. Moreover, if Y depends on one or more random variables $X = X_1, X_2, \ldots$ so that $p(Y = 1 | X = x_1, x_2, \ldots)$, that is the probability that $Y = 1$ changes for different values X, we can write $p(Y = 1 | X) = 1/[1 + \text{Exp}[-(\beta_0 + \Sigma \beta_i X_i)]]$, where $p(Y = 1 | X)$ means "the probability that $Y = 1$ given a specific set of values for X. The expression in the denominator, $1 + \text{Exp}[-(\beta_0 + \Sigma \beta_i X_i)$, is the cumulative Logistic distribution probability for that X. The odds that $Y = 1$ is given by $p(Y = 1 | X) / [1 - p(Y = 1 | X)] = \text{Exp}(\beta_0 + \Sigma \beta_i X_i)$. From this we see that the Log (Odds) is given by Log (Odds) = $\beta_0 + \Sigma \beta_i X_i$. Computer programs performing logistic regression fit the model defined by the Log (Odds). In this way, odds can be explained by the concomitant variables and also odds ratios can be formed and explained. These analyses are somewhat complicated and should be discussed with a biostatistician if their use is anticipated.

38.12.1 Survival Analysis

When the rate at which an outcome occurs is of interest the techniques of survival analysis are usually invoked [4]. The outcome of interest is very commonly death following a disease diagnosis so that the term life table analysis is also used. As an example, consider a study of the length of survival after a diagnosis of a specific cancer. The object might be to see whether a new treatment is associated with a prolonged survival (i.e., slower rate of death) as compared to the standard treatment. After treatment patients are followed until either death, loss to follow-up, or study termination. The relevant measurements are time from start of the study until death (all causes mortality), time until death for a specific cause, or time until the patient

is censored from the study. Patients are considered to have been "censored" if they are lost to follow-up or are still alive at study termination. The usual way of analyzing such data is using Kaplan–Meier estimate of the survival function. The Kaplan–Meier is a nonparametric method used to estimate the survival function from time-to-event data. In medical research, the Kaplan–Meir estimates are often used to measure the fraction of patients living for a certain amount of time after treatment. It is also possible to estimate survival functions using complex mathematical models. The event does not have to be death, but can be any definite event, which is distributed over time from a specific starting time.

Censoring due to lack of follow-up can cause severe problems with accurate estimation, especially if the number censored is large or the censoring is not randomly distributed across time. Also, patients can be lost to follow-up by death to a cause other than the cancer under study. If this is the case the "competing risks" analysis should be performed. Survival studies require that resources be allocated to study management to insure as low loss-to-follow-up as possible and the collection of complete data at every return visit on all patients. Whenever a survival analysis is to be undertaken there should be close collaboration on study design and analysis with study biostatisticians.

38.13 Design of Experiments

The principles and procedures of good experimental design [5] are among the most important considerations in clinical and basic science research. The results from a badly designed experiment can lead to faulty conclusions, delay the advancement of the science involved in the study, and waste precious resources. Every facet of a study, from conceptualization to analysis and reporting, must be carefully thought out and executed in order to maximize the return on the investment of time and resources. The tasks addressed in good design include the development of inclusion and exclusion criteria for study participants, sample size calculations to insure adequate precision of statistical estimates, random allocation of subjects to treatment arms, and blinded assessments of study outcomes. Where possible, a placebo treatment should be employed for one arm of the study even if the control arm is a standard, well known treatment.

Inclusion and Exclusion Criteria Very specific criteria should be developed for enrolling or not enrolling subjects into the study. This is necessary so that the "population of inference" for the study results is clearly defined. Even when these criteria are reported, there is an unfortunate tendency for study results to be applied outside the scope of inference as defined by study participants. Reported conclusions from study results should always be restricted to the population studied and cautions about wider applications should be given. Among the inclusion/exclusion criteria is usually an informed consent to be in the study. If the subject consents and meets the other inclusion criteria, then he/she is enrolled. If the subject declines to consent, then he/she is excluded. The preliminary data gathered during the potential enrollment process on those who refuse to participate should be captured and made

Table 38.17 Statistical truth table

Test	H0 True	HA True
Not	No error	Type II error
Reject H0	$(1 - \alpha)$	β, C_β
Reject	Type I error	No error
H0	α, C_α	$1 - \beta$

available to the data analysts. These data can be used to examine whether those who declined and those who accepted are dissimilar in meaningful ways.

Sample Size Calculations The number of subjects in a study should, in theory, be determined based on two sets of criteria. On is the chosen probability of rejecting the null hypothesis H0 when it is true and on the chosen probability of not rejecting the alternate hypothesis HA when it is false. The other set is based on the costs of making the wrong decision. These probabilities and costs are shown in Table 38.17.

In statistical decision theory, whenever the costs for making the wrong decision can be determined, the values for α and β would be chosen to minimize the sum of the costs $C_\alpha + C_\beta$. This might arise, say, in the manufacturing of ball bearings. Each ball bearing radius must be within specification limits or it cannot be used by the buyer. If a quality control inspection were put in place at the factory production line, then the line would be shut down for repair if the quality test failed. The test failure could be a false decision (Type I error) and the owner would lose the cost C_α for shutting down the line when it was working properly plus the loss of production. If the line was producing faulty ball bearings and the quality test failed to detect this (Type II error), then the owner would suffer a cost C_β for having to replace the faulty product. Thus, both α and β would be functions of the prevailing costs. In medical research, and in science in general, it is usually very difficult to determine these costs. As an example, what is the cost of saying a new treatment is not better than the standard treatment when it truly is? What is the cost of replacing a treatment with one which is no better? As a default, the Type I error level used in practice has come to be no larger than $\alpha \leq 0.05$ and the Type II error level used in practice has come to be no larger than $\beta \leq 0.20$. Sometimes, when enough data have been accumulated to do really definitive experiments, both α and β levels will be made smaller.

The quantity $1 - \beta$ has a special name: $100 \times (1 - \beta)\%$ is called the power of the experiment to detect a prespecified difference between two measures. The closer these two measures the larger the study needs to be to detect a difference. As an example, in comparing mean values the expected overlap of individual data points would depend on the separation of the true mean values. To achieve a Student t large enough for sample means to be statistically significant, the standard errors of the sample means would need to be less than a certain value. Recall that the standard errors decrease by the deflation factor of $(1/n1 + 1/n2)^{1/2}$ applied to the pooled standard deviation from the two samples. Also, the deflation factor is minimized when the sample sizes are equal. Thus, given the difference to be detected and an estimate of the pooled standard deviation, the sample size required to give the desired power can be calculated. There are statistical programs, which biostatisticians use to compute power for the various types of estimates and analyses discussed above. A good

study design always has power calculations done before enrollment and data collection.

Random Allocation Random allocation of subjects from a collection of subjects into k "treatment" groups does not necessarily represent k random samples from a population. The only situation where this would be true would be when the collection of subjects was itself a random sample from a specified population. Ordinarily in clinical studies, a set of inclusion criteria, neglecting informed consent, are defined and a source, or sources, of subjects meeting these criteria is identified. As an example, a clinic, or clinics, may be treating the kind of subjects one wishes to study. Clinic populations may not necessarily represent any clear extra-clinic population. There are dynamics, which decide clinic attendance, and often these are not well understood or even known. The result of this ambiguity is that the extension of the results of clinical studies to larger populations depend mostly on nonstatistical arguments that to do so is proper. The result of such considerations is that one should think the collection of known study eligible (including consent) subjects as a complete population, which has been randomly partitioned into treatment groups. Since it is usual to examine possibly eligible subjects only until a precalculated number are enrolled, not even the total collection of the possibly eligible subjects is in the final population of inference. However, all of the desired properties of randomization do accrue to inferences to the known study-eligible study group.

The major purpose of random allocation of subjects to treatment groups is to employ an unbiased estimation process. Bias is defined in terms of the expected value of an estimate over all possible randomizations of subjects to the treatment groups. That is, it the process of estimation that examined for bias, not the outcome of any particular randomization. One should not say that a sample is biased. Samples are not biased or unbiased, estimation processes are. This approach is based on the fundamental underpinning of statistics, which is the distribution of estimates from a very large (preferably infinite) number of experiments. The unbiased nature of estimating population means by sample means can be easily illustrated. Suppose one had a group of four rats to be randomized into two groups, one group to be treated and one group as control. Suppose further that the null hypothesis was absolutely true, the treatment has no effect. The possible data that could be gathered would be represented by all possible differences in sample means that could arise from a random partition of the rats into two equal groups. Suppose the individual rat measurements were 1.4, 2.2, 3.6, and 4.0 respectively. The overall mean value is 2.8. The data in Table 38.18 show the mean values that result from all possible partitions of these four rats into two groups of size $n = 2$.

Although no particular allocation has a difference in means of zero, the overall difference in means is zero. Note that the extreme differences are quite large and different from zero. This is the pattern that would be true in any comparison of allocations with more members which led to the rejection of the null hypothesis when there was no treatment effect. Only rarely, if ever, should a study not be randomized. If this happens, special care should be taken in the interpretation and presentation of the results.

Table 38.18 Random partitions of four rats into two groups

Sample	Control	$\overline{X1}$	Treated	$\overline{X2}$	Diff.
1	1.4, 2.2	1.8	3.6, 4.0	3.8	2.0
2	1.4, 3.6	2.5	2.2, 4.0	3.1	0.6
3	1.4, 4.0	2.7	2.2, 3.6	2.9	0.2
4	2.2, 3.6	2.9	1.4, 4.0	2.7	−0.2
5	2.2, 4.0	3.1	1.4, 3.6	2.5	−0.6
6	3.6, 4.0	3.8	1.4, 2.2	1.8	−2.0
Overall		2.8		2.8	0.0

Blinded Assessments Since all subjects are informed of the possible effects of the treatments to which they may be allocated, and the study staff are all familiar with these possible effects, the knowledge of the particular treatment which a subject gets might influence the behavior of the subject and the assessment of the subject's response by the staff. Sometimes it is possible to "blind" the subject to the treatment assigned, and also require that the assessment of the subject's response is to be made only by staff "blinded" to the treatment assigned. If these are both done, then the study is said to be "double blinded." Of course, it is sometimes possible that the subject would know that they had received an active treatment, or even know what the actual treatment is. If only the assessment is blinded the study is said to be "single blinded." At a minimum, every study should have blinded assessment.

Placebo Control A placebo treatment is supposed to be completely indistinguishable in outward appearance and application from the active treatment to which it is being compared. Chemical formulations (pills, liquids, etc.) and some mechanically mediated treatments lend themselves to the use of a placebo. Other treatments do not. Care must be taken that experimental subjects do not recognize that they have ben randomized to a placebo arm. The purpose of the placebo is to account for the fact that subjects sometimes respond to the act of being treated even if the treatment has no biological effect.

The study design that has the highest regard among clinical researchers is "a randomized, double blind, placebo controlled study" whose methods include clear inclusion and exclusion criteria and sample size calculations. The fewer of these elements reported in a publication of study results, the less confidence readers have in the conclusions.

38.14 Examples of Randomizations

The randomization scheme defines the analysis, which will be used to summarize study results. When planning a study it is often useful to lay dummy data sheets and summary tables to display the form the data collection and reduction will take. This helps to discover whether anything has been overlooked and clarify that the planned data will answer all study questions.

In the following examples, it is assumed that randomization lists by condition and either subject ID (or order of enrollment) will be prepared by the consulting biostatistician and given to a study staff member not in contact with study subjects. This person will be called when eligible subjects are identified for the condition to be assigned to the subject. This step helps blind the "hands on" study staff from knowing beforehand, which condition will be assigned.

One-Way Classification When the proposed study intends to compare outcomes of two or more experimental conditions using only the conditions themselves for identifying groups, the study is defined as a one-way classification. If a total group of N subjects are to be randomized to k conditions, then the only restriction on the randomization is that $n1 + n2 + \ldots + nk = N$. There are usually two choices, either all n_i are fixed and equal or the n_i are random. In the first case, each condition would be recorded on a list N/k times beside a uniformly distributed random number between 0 and 1. Adjacent to each random number is a patient ID. The list is then sorted by the random number column to distribute the conditions randomly accross patients. When subjects are assigned to condition according to this list, the assignment will be random. In the second case (unequal n), one sample at a time will be selected from the list of conditions and recorded in sequence on the list of IDs or enrollment numbers until all patients are asigned. This will produce a scheme where the group sample sizes are random. The first case is referred to as a restricted randomization, whereas the second case is unrestricted randomization. It may be recalled that the equal n scenario has higher statistical power than the unequal n case. The analysis would be performed us a one-way ANOVA followed by any desired pairwise comparisons.

N-Way Cross-Classification It might be desired to use other classifications of subjects rather than just by the assigned condition. As an example, one might want to take gender into account. If so, then the data would be tabulated in condition by gender two-way tables. Randomization lists would be generated for each gender separately. If gender were not balanced (i.e., an equal number of males and females) and the condition randomization lists within gender were completely at random, one might find widely varying sample sizes by condition and gender. This would lead to unequal precision of between gender and among condition comparisons, which might not be desirable. It is usually a better approach to compute the sample sizes needed for the desired power for all major comparisons anticipated. To minimize the total sample size, balanced gender and condition sample sizes should be used in a restricted randomization. The analysis of this study would involve a two-way ANOVA to test overall differences among conditions, between genders, and whether the responses among conditions have the same pattern between genders. If the patterns are not the same between genders, then it is said there is a gender by condition interaction. The concept of interaction will be discussed further below. Desired pairwise comparisons could follow the overall analysis.

Repeated Measures Designs When a subject is measured more than once, either over time under the same conditions, or under different conditions without regard to

time, the data reflect a repeated measures design. The analysis of such data requires special techniques because repeated measures within a subject would be expected to be correlated. As an example, if a subject showed a systolic blood pressure higher than the group average on the first measurement, then his/her systolic pressure might be expected to be higher than the group mean on the second measure if there were no intervening events. Correlated observations have a smaller variation than uncorrelated (independent) observations. The estimates of experimental error must take this into account. As an example, consider a study where two experimental conditions are to be observed at baseline and once a month for 2 months. Thus, each subject will yield three observations, baseline, month 1, and month 2. The randomization would be only to the conditions, not to the times of observation. Besides random variation the analysis would recognize three sources of variation. These would be between conditions, within subjects over time, and whether responses over time were similar for the two conditions. The analysis is called a repeated measures ANOVA. The interpretation of such studies should discussed with the consulting biostatistician.

A special feature of repeated measures designs is that all subjects must be measured at all times. If a subject has missing data, the analysis is compromised since that subject cannot be included. For this reason repeated measures studies require more effort by study staff to get all measurements.

38.15 Things Not To Do

There are fashions in the statistical analysis of studies reported in the literature, which perpetuate bad methodology, which may obfuscate or even invalidate findings. The problem is exacerbated by the recommendations for the use of these methodologies in statistical texts. To avoid some of these problems it is necessary to know just what feature of the data is being tested by any statistical test used and to be aware of the effect of manipulations (numerical calculations or coding of data). Some of these situations are discussed below.

Adjustment of Data It is in fashion that responses can be "adjusted" for initial (baseline) values by dividing the treated response of a subject by the baseline value for that subject before treatment. Sometimes, the adjustment is to divide the change between final and initial values by the initial values. These are referred to as "percent of baseline" and "percent change from baseline", respectively. This adjustment produces ratios, not percentages. Ratios and percentages have different behaviors mathematically and statistically. A similar situation arises in when one measurement is "adjusted" another by division, say dividing brain chemical determinations within a subject by the creatinine level for that subject. Unfortunately, there is only one relationship between the numerator and the denominator for which such adjustments are correct. The relationship must be such that a plot of the numerator against the denominator is linear and through the origin. That is, the linear model $Y = \beta X$ fits

Table 38.19 Adjustment of response *Y* by initial value *X*

Group 1			Group2		
X	*Y*	*Z = Y/X*	*X*	*Y*	*Z = Y/X*
1.0	4.5	4.50	4.2	5.1	1.21
2.1	7.2	3.43	5.5	8.2	1.49
3.4	7.4	2.18	6.1	7.3	1.20
4.2	7.5	1.79	7.3	7.7	1.05
5.0	10	2.00	8.0	10.2	1.28

the data for each group. The slopes β would be similar if there is no treatment effect and different if there were a treatment effect. This can be seen by rewriting the adjusted data. Suppose there are two groups being compared with data pairs $Y_{1i}/X_{1i} = \beta_{1i}$ in Group 1 and $Y_{2j}/X_{2j} = \beta_{2j}$ in Group 2. The statistical test would be a Student t test of the difference in means $d = \bar{\beta}_2 - \bar{\beta}_1$. Under the null hypothesis we would the difference d to be near zero. That is, a plot of the raw Y_1 and Y_2 values against their corresponding X_1 and X_2 values would lie along the same strait line through the origin. If d tests not to be zero, then there would be two lines, each through the origin but with different slopes. Any other relationship between Y and X would introduce a distortion in the "adjusted" values and bias the comparisons. It is to be carefully noted that an adjustment of responses by dividing final value by initial values, or (final – initial)/initial, is not an adjustment of sample mean values as in the ANCOVA but is a transformation to a new variable for analysis. The overarching concept for any numerical adjustment of data is that the adjustment has consequences. These consequences must be carefully studied before they are applied. To illustrate the effects of adjustment of final values by dividing by the initial values, the data in Table 38.9, used to introduce the analysis of covariance, have been recast in Table 38.19 as if it were composed from data for a two group intervention intended to be controlled by the division of the response by initial (baseline) values.

The first thing to notice in Table 38.19 is that the Group 1 mean for the adjusted values would be larger than the similarly adjusted Group 2 mean. This is exactly reversed from what was found by the covariance adjustment shown in Fig. 38.6. Second, it is clear that the variation of the values for Group 1 are larger than those for Group 2. A moment's reflection shows that this will always be the case if the two response data sets do not essentially overlap each other. The data groups nearer the origin will have more dispersed ratios than data groups farther than the origin. The two methods of adjustment, covariance, and ratio, will give similar results if and only if the response data lies along a single line through the origin. If it is appropriate to do a covariance analysis, it will always provide the proper adjustments.

As was seen in the section on the Analysis of Covariance above (Fig. 38.6), the relationship between the response variable and the explanatory variables must be linear (or can be made linear with suitable transformations) and the individual regression coefficients must be homogeneous across groups. If this is not the case, then no covariance adjustment can be made. Further, if there is a significant relationship between

the response variable and the explanatory variables but changes from group to group, this should be considered a finding of the study and establishing why this is so might prove fruitful. As an example, suppose that a certain chemical increases with age in a nonlinear fashion early on, and become constant after a given age. Also suppose that, among a subset of subjects, this pattern has shifted, showing the nonlinear rise in earlier years and becoming constant at an earlier age. Such a response pattern cannot be "adjusted for age" by any methodology. Subjects at the same age not acting alike in regard to the chemical levels, and mean values cannot be adjusted to a common age. The message from such data is that subjects in the subgroup are acting older than their calendar age in regard to the chemical. Unfortunately, the "kneejerk" reaction is automatically to "adjust" for variables such as age, gender, ethnicity, and so on, without looking carefully at the relationships revealed by the data.

Confidence Intervals Once the sample mean and the sample standard error are computed one can construct a $100(1 - \alpha)\%$ confidence interval using the Student t for either a single mean or the difference in means for two means. For proportions, the same can be done using the standard normal z statistic. The proper interpretation for a confidence interval is that $100(1 - \alpha)\%$ of such intervals so constructed would contain the true mean. It is not proper to say that "$100(1 - \alpha)\%$ of the time, the true mean would be in the constructed interval." It appears that some authors in the scientific literature think that reporting confidence intervals is to be preferred to simply reporting sample statistics and their associated probability levels. In fact, the two approaches use the same sample information in their development. Consider the test at a given α-level of the null hypothesis H0: $\mu = \mu_o$ versus the alternate hypothesis HA: $\mu \neq \mu_o$. The Student t would be computed as $t = (\overline{X} - \mu_o)/\mathrm{SE}$, the critical t-value $t(1 - \alpha/2)$ [read as "t-value tabulated at the probability of (1-alpha/2)"] would be looked up and compared to the computed t. If the absolute value of the computed t (sign ignored) were greater or equal to the critical t then H0 would be rejected. Now consider how a $100(1 - \alpha)$ % confidence would be computed. The critical t would be looked up as in the hypothesis test. Since there is a one-to-one and onto relationship between $t(1 - \alpha/2)$ and α (one t for every α, and one α for every t) $t(1 - \alpha/2)$ and α are interchangeable with respect to information. The confidence interval is computed as $(X_L, X_U) = (\overline{X} - \mathrm{SE}\, t(1 - \alpha/2),\ \overline{X} + \mathrm{SE}\, t(1 - \alpha/2))$. If the value μ_o, as given by the null hypothesis, falls on between X_L and X_U the null hypothesis is not rejected. If μ_o falls outside the interval (X_L, X_U), then the null hypothesis is rejected. Thus, we see that the same six numbers used in the hypothesis test and in constructing the confidence interval, namely, the sample size, the mean, the standard error, the hypothesized mean, the alpha level, and the critical t value. The only additional information the confidence interval provides is the range of values for which the null hypothesis would not be rejected. After the study has been analyzed, one might behave as if the true mean value could be in the range given by the confidence interval.

There is a common error in the use of confidence intervals. This error can be found in the scientific literature as well as in statistical textbooks. It is often, erroneously, said that if two confidence intervals overlap, then the sample mean values use

to construct them are not significantly different. Also, it is often, also erroneously, said that if two confidence intervals do not overlap, then the sample mean values use to construct them are significantly different. Both of these statements can, with a little effort, be shown not to be true. In fact, they are more often not true than true. The reason for this is twofold. One reason is that the degrees of freedom and the critical values of the statistics for computing the single and combined confidence intervals are not the same. In the individual computations, two critical values would be looked up, one with $n1–1$ degrees of freedom and the other with $n2–1$ degrees of freedom. Thus, different distributions of test statistics are involved and there is some "slack" between them to allow for one or the other result. Also, when the combined comparison is made, the standard error is different from the individual standard errors. This also allows for some "wiggle room" for the results to go both ways. This invalid use of confidence interval should be avoided.

Coding Values Although coding continuous numerical variables into ordinal scale values (e.g., 0, 1, 1+, 2, 2+, …) is useful for presentation and easy interpretation, such coding can represent a significant loss of information. As an example, consider the coding into age groups of a response variable that increases linearly with age. If the consultant statistician had the observed ages, then an ANCOVA could be investigated. If a covariance analysis were appropriate, then the error term used to compare means would be the residual error of the responses around the fitted covariance model, which is nearly always a large reduction over unadjusted error. If the only data the consultant received were the coded values, then the only analysis would be an ANOVA using age groups as a classification variable in the analysis. The error term used for the comparison of means would be based on the pooled variance within age groups comprising subjects with different ages in each group. This error term would almost certainly be larger than that from an ANCOVA, thus reducing the power of the comparisons. Coded tables can always be prepared for presentation after analyses are done, but proper analyses cannot always be done with coded values.

Correlation The interpretation of a correlation between two variables can be tricky. A study that measures two variables, say X and Y, at the same time only once between a defined group of subjects yields only a cross-sectional interpretation of the relationship between the variables. If Y and X are positively correlated, then, if later measures were taken on similar subjects, one would expect higher (lower) Y values to appear with higher (lower) X values. Such data say nothing about what would happen to a subject's Y value if his/her X value were changed. This would need to be determined in a controlled experiment. Also, "correlations" between current measurements and future health outcomes (e.g., those with higher X values at baseline had a higher mortality rate over the subsequent years) do not necessarily mean that there would be a benefit in reducing current X values.

Another problem can arise when two initially uncorrelated variables are transformed by a third variable. The transformed variables can be spuriously correlated through the mathematical manipulation. A simple example is shown in Table 38.20.

The initial correlation of X and Y in Table 38.20 is $r_{XY} = 0.0159$, $p = 0.9518$. None of the pairs (X, Y), (X, Z), or (Y, Z) are significantly correlated with each other.

Table 38.20 Induced correlation

X	Y	Z	$U = X/Z$	$V = Y/Z$
24.6	82.5	50.6	0.49	1.63
17.9	55.0	89.1	0.20	0.62
17.4	2.5	79.1	0.22	0.03
6.6	75.4	51.4	0.13	1.47
9.1	89.1	64.4	0.14	1.38
76.5	41.4	23.1	3.31	1.79
85.8	73.7	23.0	3.73	3.20
19.2	48.3	23.6	0.81	2.05
83.2	68.5	71.0	1.17	0.96
71.2	56.9	32.4	2.20	1.76

However, the transformed variables $U = X/Z$ and $V = Y/Z$ are significantly correlated $r_{UV} = 0.702$, $p = 0.0118$. The interpretation of such a relationship is not at all clear.

Nonparametric Tests To say that a statistical test is non-parametric (or alternatively distribution free) means that the test relies neither on the shape of the distributions being sampled nor the estimation of the parameters defining those distributions [6]. Instead, such tests use properties of the samples to infer features of the sampled distributions. Nonparametric tests are invoked when the population distributions are not known (or safely assumed) or when the specific feature being assessed has no normal-theory test. Frequently used nonparametric tests in clinical research include:

- Empirical distributions estimate the probability distribution function of the population
- Frequency tables (or histograms) estimate the probability density function of the population
- Cochran's Q tests whether k raters assign a 1 from 0/1 in equal proportions to n rated objects
- Friedman two-way ANOVA by Ranks tests whether k treatments in repeated measures design have identical effects
- Kaplan–Meier method estimates the event function from time-to-event data
- Kolmogorov–Smirnov test tests whether a single sample comes from a specified distribution, or whether two samples come from the same distribution
- Kruskal–Wallis one-way ANOVA by ranks tests whether three or more independent samples come from the same distribution
- Mann–Whitney U or Wilcoxon rank sum test tests whether one population is stochastically larger than another population
- McNemar's test tests whether row and column marginal frequencies equal in a table of responses to two conditions (Condition 1 × Condition 2) where each subject is measured for both conditions
- Sign test tests whether the signs of the differences in responses of matched pairs have an equal number of positives and negatives
- Spearman rank correlation measures the correlation of the ranks of two variables ranked separately

- Wilcoxon signed rank test tests whether matched pair samples come populations which are stochastically of the same size

There are other nonparametric tests. If it is desired to test features not listed here, consult the study biostatistician. Since the Mann–Whitney U/Wilcoxon rank sum test is so commonly applied, a fuller discussion of this test will be given.

A very general formulation is to assume that:

- Observations independent (not correlated)
- The data are measured on at least an ordinal scale
- If Y_1 is a single sample from population 1 and Y_2 a single sample from population 2, then the null hypothesis is H0: $P(Y_1 > Y_2) = P(Y_2 > Y_1)$
- Alternate hypotheses are HA: $P(Y_1 > Y_2) \neq P(Y_2 > Y_1)$, $P(Y_1 > Y_2) < P(Y_2 > Y_1)$, and $P(Y_1 > Y_2) > P(Y_2 > Y_1)$. The first leads to a two-tailed test, the others to a one-tailed test

The test is based on jointly ranking all observations (from 1 to $n1 + n2$) and then summing the ranks assigned to each sample. If the null is true the difference in rank sums should be small. If the null is not true, then the sum of one of the set of ranks will be larger than the other sum.

There are two problems with how the rank sum test is used in the literature. The first problem is that sample values are examined and deemed to be from nonnormal populations, making normal-theory tests inapplicable. A moment's reflection will show that, for moderate sample sizes, if two-sample means came from the same normal population but tested significantly different. Then a larger number of observations from one sample came from a different side of the common mean value than did those in the other sample. Such individual samples might not appear to be normally distributed. Moreover, what if one knew that the samples were from normal populations but they appeared to be nonnormal. Which test is to be used? However, the major problem is that is relatively easy to construct two quite large samples, each of which appear to be distributed almost exactly normal, with identical medians and almost equal means, which a judged not to be significantly different on by the Student t test, and yet a judged to be significantly different by the Mann–Whitney U test. The Mann–Whitney U test (Wilcoxon rank sum test) does not test for differences in means or medians. It is not the nonparametric analog of the Student t test. For an assessment of the parlous state of the understanding of this test, an Internet search and perusal is eye-opening.

Multiple Regression Analyses – The most pernicious problem with analyzing the influence of candidate predictor variables on a response variable is that, especially in large studies, some records will be missing values for some of the predictor variables. A multiple regression analysis requires that there is complete data on all subjects included in the analysis. A common approach is to select the largest subset of data, which has complete data. If an analysis of these data show a subset of variables (i.e., not all variables in the starting analysis) should be used to model the response variable a question arises. Is there a larger subset of the data that has complete data on the reduced set of variables? As an example, suppose 47 of 62 total subjects had complete data on all of the predictor variables X_1, X_2, and X_3. The analysis of this subset showed

Table 38.21 Interaction present

	Condition	
Gender	A	B
Male	10	25
Female	5	10

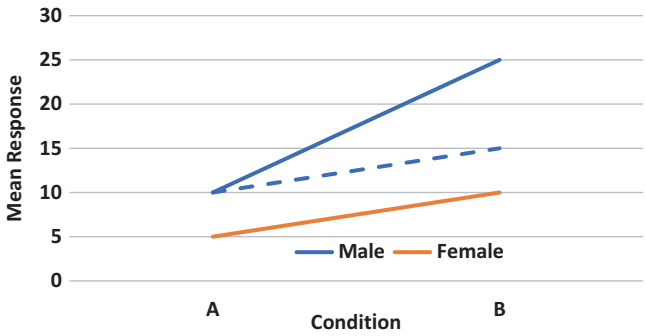

Fig. 38.8 Interaction versus no interaction

that only X_1 and X_2 should be included in the model. It is found that 54 of the total 62 subjects had complete data on X_1 and X_2. Suppose an analysis of these 54 observations did not yield the same regression coefficients and probabilities as those from the analysis of the 47 subjects? It is not clear how this state of affairs should be resolved or reported. Missing values in a study often introduce such conundrums.

Statistical Interaction Statistical interaction is easy to define but difficult to deal with. By statistical interaction is meant the phenomenon that the mean response of groups by levels of one classification are not parallel across the levels of a cross-classification. This is easiest to display in a plot of mean responses in a 2 × 2 table. Data depicting interaction are shown in Table 38.21.

The increase across conditions is 15 for males but only 5 for females. If the differences across classifications is significant, there is said to be interaction present. In this case, there is Gender × Condition interaction. The difference in parallel and nonparallel responses are shown if Fig. 38.8.

Had the male responses been as shown by the dotted line, then there would be no interaction. Interaction is important because it often means a comparison of overall effects (called "main effects") is not appropriate. Here, an overall comparison of conditions would be confounded by the differences in responses by gender and an overall comparison of gender would be confounded gender differences within condition. The best analysis would be to test for what are called "simple effects." These Condition within Gender and Gender within Condition. Any analysis which shows interaction must be approached with care.

Logistic Regression There are two situations in which care must be taken in using logistic regression [2]. One is the recognition that the logistic estimate of the odds ratio is a good estimate of relative risk if, and only if, relative risk is small, say less

than 0.05. Odds ratios can increase dramatically if the true relative risk is large. The other situation is that the log-logistic transformation of the ratio $p/(1-p)$ (called the "log-odds") must be linear in all of the variables being analyzed. Suppose there were one exposure variable (yes, no) and another explanatory variable X (yes, no). The computer output to a logistic regression analysis would have three lines for the source of variation. One for Exposure, one for X, and one for Exposure by X interaction. If the Exposure by X interaction has a significant p value, the p values given in the computer output for Exposure and X cannot be interpreted as they stand. In particular, the p value for Exposure refers only to the Yes category of the X variable and is not a pooled estimate over both X categories as it would be if there were no significant interaction. When there is interaction in a logistic regression analysis, work closely with the statistical consultant to parse the results carefully.

38.16 Conclusions

It is anticipated that some parts of the material presented here will be more useful than others. The intention is not to make statisticians out of clinical or basic science researchers, but instead to give researchers some insights not ordinarily presented in courses on statistics. As a final note, the author wishes to emphasize that a well thought out design, followed by careful data collection, and productive interactions with the consulting statistician, will almost surely lead to clear findings and successful publications.

This chapter discusses the impact of the use of biostatistics on HIV research. HIV research is like cancer research in that advances in treatment efficacy are often incremental in nature with cumulative improvements over time. This requires that experimentation must be carefully controlled and as precise as possible. A proper understanding experimental design and statistical analyses is necessary to achieve these goals.

Conflict of interest The authors report no conflicts of interest.

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Index

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