Franco Borruto · Marc De Ridder *Editors*

HPV and Cervical Cancer

Achievements in Prevention and Future Prospects

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Foreword

It is with immense pleasure that I present the book of Professors Franco Borruto and Marc De Ridder. The book is a sum of the current available information on HPV, given by world experts in this scientific field. I shall nonetheless mention the Editors Marc de Ridder, internationally reknown immunologist whose scientific contribution on HPV is of absolute value, and Franco Borruto, whose academic curriculum was already appreciated by my teacher Professor G.B. Candiani. Professor Franco Borruto, a dear friend and collaborator, was assigned to me as co-worker at the University of Verona. He is now part of Montecarlo's Medical Association and has joined the Professor A. Treisser's team at the Princess Grace of Monaco Hospital. He was one of the advocates for the HPV vaccination in the Principality of Monaco and is actively involved in a governmental campaign of the Princier Palace for the prevention of cervical cancer.

I am pleased to present this book, which will have its well-deserved success, and will represent an important instrument for the enthusiasts of this discipline and of this specific subject.

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Preface

The Greek philosopher and mathematician Pythagoras (580–495 BC) developed the idea that the resonance of the harmony of the world resides in numbers: "everything is numbers, numbers are harmony". Indeed, if one analyses *a posteriori* the diverse elements of our life, each of them are linked to precise or presumed dates and therefore to numbers.

Results of scientific research are often expressed in quantitative data and therefore in numbers. Again, events or encounters that changed the course of each of our lives is frequently associated to single points in time and therefore to a number.

In 1979, before a congress in Tokyo, Japan, I (FB) met professor H. zur Hausen(°). This encounter dramatically changed my perception and concepts of oncogenesis and of the viral origin of cancer in women. It completely reoriented my professional and scientific practice. During this very same trip to Tokyo, I travelled with professor De Broux. Thanks to this memorable trip we became good friends. After my Tokyo experience, I decided to go abroad and practiced in different countries.

Many years later, Dr. J. Monsonego created the Eurogyn Conference and the Human Papilloma Virus (HPV) community and is now considered an important congress. The authors are linked by the friendship of this HPV community and by the heritage of professor H. zur Hausen.

My co-editor, M. De Ridder and I met at a HPV Pan European Advisory Board in September 2007. A brotherly friendship was born during this meeting and the idea rose between us to help the transmission of knowledge on HPV to the scientific community worldwide.

His scientific input and his extraordinary personality was a gift for me: without him, this book would never have been realized. He also helped me in taking important decisions in my life. From the beginning I felt the need to honor special people, including women in my profession, they asked me for help. In a sense, they have contributed to my personal life and my career. They are in me, a part of me, they changed me, and invited me to do the same with those who were my students and that I could pass on what I knew.

I thank the president of the "Ordre des Médecins" of the Principality of Monaco, professor A. Treisser and my colleagues of Princess Grace Hospital who welcomed me and where I'm now living.

HPV infections and their clinical consequences are a major disease burden.

This book aims to provide an updated comprehensive summary of the available data in this multidisciplinary field. The editors received a flattering esteem on this book from professor H. zur Hausen.

The different contributing experts have managed to pull together the most recent information, updated in depth reviews on different aspects of HPV infections:

Infection and cervical cancer: an old fact with a recent solution
Human papillomavirus: natural history of a viral infection in the genesis of cancer
The immunobiology of human papillomavirus – associated oncogenesis
The epidemiology of cervical cancer
The Basic Elements of a Correct Diagnosis: from cytohistopathology to screening
Human papillomavirus DNA testing: What, How, When
Colposcopic appearance of HPV infection
Multiple aspects of human papillomavirus infections
Prevention of HPV- associated diseases in the United States
Immunogenicity, efficacy, effectiveness and overall Impact of HPV vaccines
Treatment, follow-Up, and prevention of human papillomavirus infection and cervical cancer
Therapeutic vaccines for HPV infection
Perspectives on therapeutic HPV vaccines: Where are we now?

The reader will discover how fast interesting new data has been generated recently not only on the immune-biology of this infection, but also on the more clinical aspects like the diagnostic approaches and techniques, the success of primary and secondary prevention, the follow-up in vaccinated cohorts or individuals and the various treatment options. Experienced clinicians will appreciate our wish to give them the most up-to-date trial data results for the clinical management of their patients. Last but not least, two reviews on summarizing the data and future prospects for therapeutic HPV vaccination.

All over the world scientists and clinicians will continue to inform the public and health officials highlighting the importance of preventive medicine as a "best choice" to preserve health, and reduce suffering, hence defending the largest possible access to preventive medicine.

We want to express our sincere gratitude to the authors for their contribution to the understanding of diagnosis, treatment and prevention of HPV infections and also for raising the awareness on the importance of preventive medicine.

This book is dedicated to all the women and girls around the world and in particular the ones who entered our lives for professional or sentimental reasons.

Franco Borruto and Marc De Ridder

(°) In the mid 1970s, he published his article on HPV presence in cervical cancer which at time was not considered credible… (many believed it was Herpes simplex). In 2008 he received the Nobel prize in Physiology or Medicine for his discovery on HPV viruses being capable of inducing cervical cancer.

The discoveries of the three Nobel Laureates in Physiology or Medicine 2011 – Bruce A. Beutler, Jules A. Hoffmann and Ralph M. Steinman – have revealed how the innate and adaptive phases of the immune response are activated and thereby provided novel insights into disease mechanisms. Their work has opened up new avenues for the development of prevention and therapy against infections, cancer, and inflammatory diseases.

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Chapter 1
Infection and Cervical Cancer: An Old Fact with a Recent Solution

Lutz Gissman

Ever since the first report about the incidence of cancer in different populations published in nineteenth century Italy, the infectious etiology of cervical cancer has been suspected. Rigoni-Stern reported that this tumor is more frequent in married women or widows than in nuns (Rigoni 1987). Clearly, this and many subsequent epidemiologic studies could not reveal the nature of the infectious agent. Due to the often reported coexistence with syphilis and gonorrhea, Treponema pallidum and Neisseria were discussed as cause (Rotkin 1973). A long-time suspect was the Herpes Simplex Virus type 2 (HSV 2), yet prospective studies finally excluded its role in cancer and demonstrated that the previously reported link was due to a confounding effect since the HSV 2-specific antibodies that had been detected more frequently in cancer patients than in control were simply a measure for the number of sex partners (Vonka et al. 1984a, b).

Harald zur Hausen, who was convinced of the infectious etiology of cervical cancer, constantly failed to detect HSV 2 DNA in tumor cells and never accepted the hit-and-run hypothesis (the virus being required only initially to start the process of malignant progression). Therefore, he started to look for other candidates. He identified the group of papillomaviruses due to the well-documented oncogenic capacity of the cottontail rabbit papillomavirus that causes squamous cell cancer when experimentally inoculated into domestic rabbits. But there were also reports of malignant conversion of papillomavirus-associated benign tumor in humans, such as genital warts, laryngeal papillomas and lesions in patients with epidermodysplasia verruciformis (zur Hausen 1976). Almost at the same time, potential precursors to cervical cancer virus particles and viral antigens had been found (Meisels et al. 1977).

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The first experiments performed by the zur Hausen group failed to confirm the suggested link (zur Hausen et al. 1974). In these experiments the investigators used DNA from the virus causing skin warts (later designated as HPV types 1 or 2) as a molecular probe to search for papillomavirus DNA in cancer cells. Later we learned that there is too much difference between the cutaneous HPV types in nucleotide sequences and the high-risk types such as HPV 16 to permit detection by nucleotide hybridization performed in vitro after extraction of total cellular DNA. The researchers were also unable to find the viral DNA in genital warts, suggesting heterogeneity of human papillomaviruses that was already suspected in an earlier study when virus isolated from skin warts and condylomata acuminata revealed clear antigenic differences (Almeida et al. 1969; zur Hausen 1976; zur Hausen et al. 1975).

To further study a putative plurality of human papillomaviruses, particles were purified from skin warts, their DNA extracted and characterized by restriction enzyme digestion and by hybridization experiments. This straightforward strategy permitted the identification of the first five HPV types (Gissmann and zur Hausen 1976; Gissmann et al. 1977; Orth et al. 1977, 1978) but soon reached its limits: for unknown reasons, the production of virus particles strongly varies across HPV-type and was found to be minute in non-keratinizing warts such as condylomata acuminata (Grussendorf-Conen et al. 1983). Since papillomaviruses cannot be propagated experimentally in sufficient quantities, characterization of the particle and its DNA depended entirely upon clinical materials. Some progress was made when the particular physical structure of papillomavirus structure was exploited, which permitted a separation from the bulk of cellular DNA using specific conditions of centrifugation. By the aid of this method, sufficient viral DNA for an initial characterization was recovered from a genital wart. The data demonstrated the existence of a new type (later named HPV 6) (Gissmann and zur Hausen 1980). More exhaustive characterization of the viral DNA and its biologic property, such as sequencing and screening for homologous genomes in other biopsies, required the amplification via molecular cloning that had become available at the time (de Villiers et al. 1981). The next obvious HPV-related disease virus was laryngeal papillomatosis. However, due to the very small size of such lesions, the method of direct isolation of viral DNA was not applicable. On the other hand, it was shown that the DNA of closely related HPV-types cross-hybridize under certain experimental conditions ("low stringency") (Law et al. 1979) and it was possible to detect HPV sequences related to but different from HPV 6 in laryngeal papillomas. To obtain this DNA cloning of the viral genomes as part of the total cellular DNA via a genomic library generated in recombinant bacteriophage was chosen. To identify the HPV-positive clones the same low-stringency conditions were applied. By this experimental protocol HPV 11 and later on 16, 18 and other high-risk types were identified and molecularly cloned (Boshart et al. 1984; Durst et al. 1983; Gissmann et al. 1982; Lorincz et al. 1986; Naghashfar et al. 1987). A plethora of subsequent studies demonstrated the regular presence of HPV DNA in the tumor cells leading to the concept of HPV being the necessary cause of cervical cancer (Walboomers et al. 1999). From a purist point of view, this statement might be hyperbolic, since it means that not a single case of cervical cancer exists without the involvement of HPV. On the other hand, there

is not the slightest doubt that the overwhelming majority of cervical cancer worldwide is actually caused by HPV. A strong argument is the existence and expression of HPV DNA in cell lines derived from this tumor that has been in culture for many years (the most famous example being HeLa (Boshart et al. 1984)). Moreover, it was shown that the constant expression of early genes in tumor cells is required for the maintenance of their proliferative state and their tumorigenicity (von Knebel et al. 1990, 1992).

There were also early epidemiologic studies that suggested causality of HPV infection in development of cervical cancer. For example, the presence of HPV DNA within a cervical lesion was shown to favor its progression (Schneider et al. 1987; Syrjanen et al. 1985). However, it took a few more years before a causal link between high-risk HPV and cervical cancer was finally accepted by all groups of experts working in the HPV field (Munoz and Bosch 1992; zur Hausen 1989).

1 New Papillomaviruses were Identified on the Basis of DNA

After the first identification of HPV DNA in cancer cells (Durst et al. 1983) it was questioned whether these sequences were of viral origin. Despite the size of the cloned genomes (about 8 kbp), which was characteristic for a papillomavirus genome, it was unclear whether these sequences would really stem from an infectious agent that was the cause of cervical cancer. In fact, unlike cases of well-characterized particles taken from papillomaviruses in cattle, rabbits, and also from human skin warts (Klug and Finch 1965), virus replication in the mucosa is very low, hence the virus cannot be characterized by standard transmission electron microscopy or other methods of structural biology. Therefore, the identification of novel papillomaviruses was different from classical procedures in virology with particles being isolated from different sources, then typically propagated in cells in culture, and subsequently characterized by analysis of the particles. Epidemiologic studies clearly supported the infectious nature of the high-risk HPV DNA e.g. by demonstrating transmission of the same HPV type between couples (Hernandez et al. 2008).

Molecular proof that the high-risk genomes actually carry the information of a papillomavirus and are most likely part of an infectious agent was obtained after the complete nucleotide sequence had been determined (Cole and Danos 1987; Seedorf et al. 1985). The genomic organization (all open reading frames located on one strand, two coding regions separated by an about 1 kbp untranslated region) was identical to the sequence of HPV 1 and the cottontail rabbit papillomavirus that had been isolated from virus particles (Danos et al. 1982; Giri et al. 1985). This observation was substantiated by functional analyses of individual genes, e.g. demonstrating the transforming capacity of E6 and E7 and most importantly, the ability of L1 and L2 to form virus-like particles (Ganguly and Parihar 2009; Schiller and Lowy 2001). This information paved the way for the development of the highly efficient HPV vaccines that have been on the market for the past several years (Harper 2009).

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Chapter 2
Human Papillomavirus: Natural History of a Viral Infection in the Genesis of a Cancer

Franco Borruto and Ciro Comparetto

1 Introduction

Human Papillomaviruses (HPV) are simple, non-enveloped, double-stranded deoxy-ribonucleic acid (DNA) viruses belonging to the Papillomaviridae (Fig. 2.1). More than 200 genotypes have been identified, causing benign (low-risk, LR-HPV) or malignant (high-risk, HR-HPV) cutaneous or mucosal lesions. The LR-HPV-6 and HPV-11 provoke genital warts, while the HR-HPV-16 and HPV-18 can cause cervical cancer. HPV genome includes several open reading frames that encode proteins involved in viral DNA replication (early, E1 and E2), viral gene expression regulation (E2), virus assembly (E4), and the immortalization and transformation of infected epithelial cells (E5, E6, and E7, HR-HPV only). The open reading frames (late, L1 and L2) encode the two capsid proteins (Fig. 2.2). HPV targets stem cells of the squamous epithelium. The complete life cycle involves three phases, with sequential expression of viral genes leading to viral DNA replication and to the production of highly infectious virions. Viral DNA integration occurs with HR types and leads to the overexpression of two viral oncoproteins, E6 and E7. These proteins, in combination with E5, promote the immortalization and transformation of infected cells (Prétet et al. 2007). Extensive laboratory and epidemiological evidence demonstrate that HPV is the major cause of cervical squamous cell carcinoma (SCC), its precursor lesions (cervical intraepithelial neoplasia, CIN), and several other benign and malignant clinical manifestations including genital warts, condylomata

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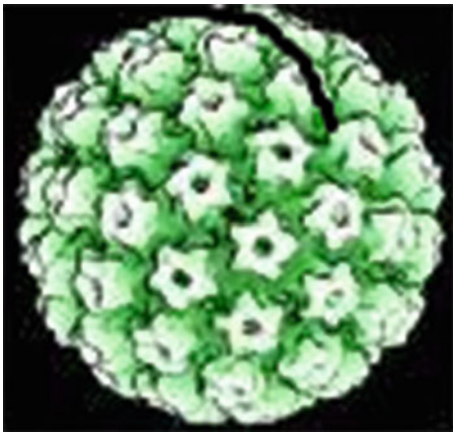

Fig. 2.1 Human Papillomavirus

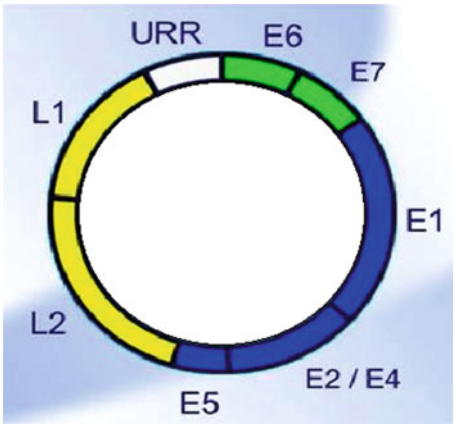

Fig. 2.2 HPV genome

acuminata, Bowenoid papulosis, vaginal, vulvar, and anal intraepithelial neoplasia (VAIN, VIN, and AIN) and carcinoma, penile intraepithelial neoplasia (PIN) and carcinoma, and other squamous neoplasias of the head and neck districts. In addition, mother-to-child transmission is responsible for recurrent laryngeal and pulmonary papillomatosis in infants. The relevance and high level of scientific interest surrounding HPV are related to the oncogenic potential of some viral types belonging to this family and the possibility to influence the incidence of various tumor forms like cervical carcinoma, improving the efficacy of specific screening programs or defining preventive strategies like vaccination (Lillo 2005).

The most familiar HPV manifestation in the genital tract, the venereal wart (condyloma acuminatum) has been recognized since ancient times, and known to be a sexually transmitted disease (STD). In 1976, two other morphologically distinct HPV lesions were described in the uterine cervix, known currently as flat and inverted condyloma. Subsequently, these HPV lesions were shown to be a STD and, in addition, frequently associated with concomitant CIN and carcinoma in situ (CIS)

lesions, and occasionally with invasive cervical carcinomas as well. These morphological findings, substantiated by the increasing number of reports of malignant transformation of HPV lesions, as well as data from animal experiments and epidemiological surveys, have lent support to the concept that HPV is involved in the development of cervical (and other) human SCC. Further evidence has been provided by the discoveries of HPV structural proteins (viral antigens) and HPV type 11 DNA in lesions of CIN, as well as HPV-16 and HPV-18 DNA predominantly in invasive cervical carcinomas. HPV-16 and HPV-18 are HPV types with DNA shown to exist integrated in the host cell DNA. Cervical (and other) HPV lesions have been the subject of intense study utilizing epidemiological, morphological, immunohistochemical, biochemical, and molecular biological methods (recombinant gene technology) to provide further evidence of the causal relationship between HPV and cancer. Prospective follow-up studies have also explored the natural history of cervical HPV lesions as well as the factors (e.g. immunological, epidemiological, synergistic actions) that modify it (Syrjänen and Syrjänen 1985). Links between HPV and cervical cancer were first suspected almost 30 years ago. DNA of specific HPV types has since been found in almost all cervical cancer biopsies. HPV oncogenes that are expressed in these cells are involved in their transformation and immortalization, and are required for the progression towards malignancy (zur Hausen 2002). HR-HPV types 16 and 18 DNA were initially identified in 1983–1984. Subsequently, the DNA of several other HR-HPV types has been identified. HPV-16 is present in more than 50% of cervical cancer biopsies, and HPV-18 is close to 20%. Some geographic variations exist in the prevalence of HR-HPV types, e.g. HPV-45 is more frequently observed in equatorial Africa, whereas types 52 and 58 have been more often found in East Asia. Molecular, as well as epidemiological studies, demonstrate that HR-HPV are indeed the causative agents for cervical cancer, they are also involved in other anogenital cancers, and in 25–30% of oropharyngeal carcinomas (zur Hausen 2008).

Since the last century, epidemiological studies of cervical carcinoma have shown a close link with sexual activity and in particular with promiscuity starting at an early age. Etiological research has therefore concentrated on identifying sexually transmitted pathogens. In more recent years studies have focused on the apparently significant role of Herpes Simplex Virus (HSV) and particularly HPV in the etiopathogenesis of this tumor. After the first cytohistological findings, the HPV-cervical cancer link has been confirmed by electron microscopy, immuno-histochemical studies, and hybridization of viral DNA. The identification of different HPV types presenting varying degrees of oncogenic risk offers the prospect of reaching a reliable prognosis on the basis of the particular virus identified in the lesion (Penna et al. 1989). So, although the existence of disease associated with HPV has been documented for centuries, it has only been within the past four decades that we have recognized the clinical diversity and significant morbidity and mortality associated with HPV infections. The original lack of interest and non-availability of in vitro culture systems has hampered research. However, with the advent of molecular diagnostic techniques, strong evidence suggested that HPV plays a major role in the development of specific anogenital cancers, including cervical, vaginal, vulvar, penile, and anal (Moscicki 1992).

The identification of a specific marker cell, the koilocyte, has led to studies of frequency and biologic significance of neoplastic lesions of the uterine cervix associated with HPV. By molecular virology techniques, many types of HPV have been identified and their tissue affinity determined. Types 6, 11, 16, 18, and 31 are most commonly associated with anogenital lesions, among them a broad spectrum of CIN. Current evidence suggests that lesions associated with HPV types 6 and 11 are potentially less harmful to the patient than lesions associated with HPV types 16 and 18 (which have been identified also in invasive cervical carcinomas and cell lines derived therefrom). A factor that complicates the issue still further is the observation that HPV DNA of all four types has been identified in 11% of women and 5.5% of men free of disease. Infection with multiple viral types (including types 16 and 18) is common in this apparently healthy population. Although HPV must be considered as a transforming virus, current evidence suggests that infection with the virus is per se an insufficient condition for the development of precancerous lesions or cancer of the uterine cervix and that other factors may be necessary for these events to take place. Some of these other possible factors are age, repeated infections, and the immune status of the patient (Koss 1987).

Clinical and subclinical HPV infections are the most common STD in the world, and most sexually active individuals are likely to be exposed to HPV infection during their lifetimes. More than 40 genotypes of HPV infect the epithelial lining of the anogenital tract and other mucosal areas of the body. Of these, 15–16 types are considered to be HR-HPV types. Persistent infection with HR-HPV is now unequivocally established as a necessary cause of cervical cancer and is likely to be responsible for a substantial proportion of other anogenital neoplasms and upper aero-digestive tract cancers. LR-HPV types are also responsible for considerable morbidity as the cause of genital warts. Youth and certain sexual characteristics are key risk factors for HPV acquisition and persistence of HPV infection, but other mediating factors include smoking, oral contraceptive (OC) use, other STD (e.g. Chlamydia, HSV), chronic inflammation, immuno-suppressive conditions including Human Immunodeficiency Virus (HIV) infection, parity, dietary factors, and polymorphisms in the human leukocyte antigen (HLA) system. Not surprisingly, these factors are also established or candidate co-factors identified in epidemiologic studies of cervical cancer. HPV transmissibility and molecular events in HPV-induced carcinogenesis have been the focus of recent multidisciplinary epidemiologic studies (Trottier and Franco 2006).

2 Pathogenesis and Natural History

Natural history models of HPV infection and disease have been used in a number of policy evaluations of technologies to prevent and screen for HPV disease (e.g. cervical cancer, anogenital warts), sometimes with wide variation in values for epidemiologic and clinical inputs. Published data meeting review eligibility criteria were most plentiful for natural history parameters relating to the progression and

Fig. 2.3 Disease progression of cervical cancer (modified from: From Oncogenic HPV to Cervical Cancer. http://www.epgonline.org/page.cfm/pageId/941)

regression of CIN without HPV typing, and data concerning the natural history of HPV disease due to specific HPV types were often lacking. Epidemiologic evidence to support age-dependency in the risk of progression and regression of HPV disease was found to be weak. No data were found on the duration of immunity following HPV infection. In the area of clinical management, data were observed to be lacking on the proportion of clinically manifest anogenital warts that are treated and the proportion of cervical cancer cases that become symptomatic by stage. Knowledge of the natural history of HPV disease has been considerably enhanced over the past two decades, through the publication of an increasing number of relevant studies. However, considerable opportunity remains for advancing our understanding of HPV natural history and the quality of associated models, particularly with respect to examining HPV age- and type-specific outcomes, and acquired immunity following infection (Insinga et al. 2009).

Precursor lesions of invasive cancer of uterine cervix begin at the squamocolumnar junction (SCJ). On this zone in permanent transformation (transformation zone, TZ), HPV gives condylomatous lesions, pure or associated with neoplasic transformation of the epithelium. For 50 years, various histological classifications have been proposed. First, four groups have been designed: light, moderate, severe dysplasia, and CIS. Secondly, Richart proposed the CIN classification with three grades (1–3) according to their severity. Progression from CIN 1 to CIN 3 and invasive carcinoma is admitted and is consistent with the concept of lesional continuum (Fig. 2.3). However, because of the elevated rate of spontaneous regression of CIN 1, this is probably a lesion of very low potential aggressivity and its role as a precursor is uncertain. Now, two groups of different evolutivity are currently considered: low-grade (LG) and high-grade (HG) lesions. The last ones, at the opposite of the first, are monoclonal, have major epithelial abnormalities with sometimes abnormal mitoses, and are frequently aneuploid. Aggressivity depends on the persistence of HR-HPV more than on progressive morphologic transformation. By integrating in host genoma, it induces modifications on cellular cycle proteins. Revelation by immunohistochemistry (IHC) brings help to diagnosis of HG lesions when traditional morphology is ambiguous (Tranbaloc 2008). The TZ of the cervix is the most frequent target of the HR-HPV types. Depending on the nomenclature used, cancer precursors are subdivided on the basis of their morphologic presentation into dysplasias (mild, moderate, and severe), CIN (1, 2, and 3), or LG and HG squamous

intraepithelial lesions (L-SIL and H-SIL). H-SIL (i.e. moderate and severe dysplasias, CIN 2 and 3 lesions) are recognized universally as cancer precursors. L-SIL (i.e. very mild dysplasia and mild dysplasias, condylomata, and CIN 1 lesions), have shown that one of the most important denominators of their cancer potential is the presence of intermediate and particularly HR-HPV types. HPV typing provides the most rational basis for selecting women with L-SIL to be colposcoped and treated or given follow-up treatment with Papanicolaou (Pap) smears (Ferenczy and Jenson 1996).

The HPV origin of cervical cancer has been suggested by Dr. Harald zur Hausen (winner of the Nobel Prize in Physiology or Medicine 2008) in 1976 and then confirmed by fundamental and epidemiological studies. Indeed, the proportion of invasive cervical cancers found to contain HR-HPV DNA reached more than 95%, the HPV-negative women being not risky. The progression of dysplastic lesions is closely linked to the persistence of viral infection. The determinants affecting persistence of HPV infection can be divided into viral factors (types and variants, viral load, viral DNA integration, and viral E6/E7 messenger-RNA, mRNA, expression), host-related factors (immune response, genetic susceptibility), and environmental factors (OC, smoking, diet) (Mougin et al. 2006). Genetic events underlying the mechanism of anogenital carcinogenesis have become increasingly understood. A host cell-mediated intracellular control down-regulating specific HPV genes (E6, E7) in replicating normal cells appears to be interrupted in cancer cells, probably due to structural modifications of the respective host cell genes acquired in the course of HPV DNA persistence. Since genital HPV infections are ubiquitous, co-factors that modify controlling host cell genes are likely to determine the different geographic rates of cervical cancer incidence (zur Hausen 1989). In fact, although infection appears to be necessary for the development of malignant tumors, it is clearly not sufficient. Additional factors probably affecting host cell genes engaged in the control of HPV functions seem to be required for conversion of premalignant changes into invasive growth (zur Hausen 1990). Moreover, additional modifications of host cell genes appear to be required for malignant progression of infected cells. The expression of viral oncoproteins in cells infected by HR-HPV types (e.g. HPV-16, HPV-18), in contrast to LR-HPV types (e.g. HPV-6, HPV-11), results in chromosomal instability and an accumulation of mutational events. These "endogenous" modifications seem to be most important in the pathogenesis of premalignant lesions and tumor progression. Exogenous mutagens should act as additional co-factors (zur Hausen 1991).

The great majority of these tumors carry integrated HPV DNA sequences and constitutively express two early viral genes, E6 and E7. DNA transfection studies show that these same genes can co-operate to immortalize human epithelial cells in vitro and that immortalized cells subsequently acquire a malignant phenotype through additional cellular genetic changes. Cell lines established from HPV-positive tumors also express E6 and E7, upon which continued tumor cell proliferation appears to depend (Gissmann 1992). Their expression emerges as necessary but not sufficient factors for malignant conversion. Besides stimulating cell proliferation, theses cell lines are responsible for the genetic instability of the infected cells and

regulate their transcriptional and functional activity. Mutational modifications of the latter appear to be required for malignant progression (zur Hausen and de Villiers 1994). In vitro transformation studies indicate that these viruses can transform primary mammalian cells in cooperation with Ha-ras. This may mean that these viruses have at least a c-myc-like activity that is consistent with their ability on their own to immortalize primary keratinocytes. The fact that HPV-6 can also transform mammalian cells at a low frequency in cooperation with Ha-ras indicates the need to elucidate differences in virus-cell interactions between benign HPV types 6 and 11 and the types associated with severe disease 16 and 18 (McCance 1988).

Risk factors for the presence of HPV are high number of sexual partners, early cohabitarche, young age at first delivery, suppression and alteration of immune status, young age, and hormonal influences. While the fact of a high number of sexual partners exclusively increases the risk of HPV infection, it is not known whether the other factors lead to either an increased risk for HPV infection and/or to HPV-associated neoplasia. Sexual transmission is the main pathway for genital HPV, however vertical, peripartal, and oral transmission are also possible. Seroreactivity against genital HPV may be due to an active infection or the result of contact with HPV earlier in life. Antibodies against the HPV-16 E7 protein indicate an increased risk for cervical cancer. Compared with humoral response, cellular immune response is probably more important for regression of genital HPV infection: impaired cellular response is characterized by depletion of T helper (Th)/inducer cells and/or Langerhans cells (LC) and impaired function of natural killer (NK) cells and/or the infected keratinocyte. In condylomata, replication and transcription of viral nucleic acids and antigen production coincide with cellular differentiation. Regression or persistence of subclinical and latent genital HPV infections as observed in longitudinal investigations show a constant come-and-go of HPV presence. Subclinical or latent cervical infections with HR-HPV types have an increased risk for the development of HPV-associated neoplasia (Schneider 1993). The data on the etiological factors enable us to suggest a synergism between the various factors associated with the pathogenesis of cervical cancer. Infection of the cervix by HPV 16/18 results in persistence of viral DNA. The persistent HPV DNA undergoes disruption at the E2 region, when integrates into the host genome. The transcriptional products E6 and E7 oncoproteins bind to and cause the degradation of p53 and retinoblastoma (Rb) tumor-suppressor gene products. It is possible, at that point, that other co-factors may be involved in the progression toward a precancerous or cancerous condition. Those co-factors may include:

1. Cigarette smoking, by introducing co-carcinogens to the tissue or by suppressing the local or systemic immune resistance similar to the effect of depressed immune resistance seen in acquired immunodeficiency syndrome (AIDS) or immunosuppression of transplant patients
2. Hormones, by enhancing growth of HPV and transformation of HPV infected cells

3. Low serum vitamin levels, leading to decreased tissue resistance
4. Other infections causing local inflammation and the production of free radicals

These elements can eventually bring to cervical cancer (Bornstein et al. 1995). The role of nutritional factors in biochemical interactions that are part of an oncogenic process or inhibit free radical proliferation have attracted considerable interest in relation to molecular mechanisms and the natural history of human cancer. Epidemiologic and experimental studies have drawn attention to the association between dietary micronutrient deficiencies and the incidence of neoplastic and malignant lesions. In the last two decades, the roles of retinoids, carotenoids, tocopherols, water soluble antioxidant vitamins, and allegations of anti-tumor properties in the daily dietary consumption of fresh fruits and green leafy vegetables have captured the attention of an increasingly sensitive diet and health care conscious public, the biochemical community, and industrial food producers (Romney et al. 1995).

The most common manifestation of sexually transmitted HPV infection is a non-papilliferous hyperplasia in the TZ. Such subclinical papilloma viral infections (SPI) closely resemble CIN and were previously misdiagnosed as such. In essence, the triage of the abnormal Pap smear amounts to the differentiation between benign warty proliferation, minor-grade dysplasia, and full-thickness CIN (Reid 1987). HPV infection of metaplastic epithelium in the cervical TZ is relatively common, producing latent infection in susceptible persons. The epidemiological characteristics of SPI and cervical cancer are essentially identical and there is a strong clinico-pathological association between condylomata and anogenital carcinomas. Tissue culture cells have been transformed from a normal to a neoplastic phenotype by animal Papillomaviruses, with the successful identification of viral genomic sequences in tumor cells. SPI commonly co-exists with foci of CIN. Two lesions linked with a discernible spectrum of morphologic change see areas of apparent transition. It is postulated that cervical neoplasia arises by progression from benign viral hyperplasia, through varying stages of koilocytotic atypia with associated dysplasia, to unremarkable CIS. Invasion is presumed to reflect the emergence of an aggressive heteroploid clone, an age-related decline in host immune surveillance, or an interaction of both factors (Reid 1983). Problems associated with HPV infection of the cervix and lower female genital tract include the definition of HPV infection and its distinction from HPV-associated neoplasia, the distinction of HPV infection from reactive epithelial changes induced by other infections, and the transmission of HPV infection via the male partner. The available evidence indicates that there are two distinct intraepithelial processes in the cervix associated with HPV. One is the classical condyloma and its counterpart in immature epithelium, atypical immature metaplasia. The other is CIN, which, like classical infection, may be mature (CIN with koilocytosis) or immature (HG-CIN or CIS). Molecular hybridization studies indicate that HPV-6 and HPV-11 are most commonly detected in the former, whereas HPV-16 and HPV-18 DNA are most common in the latter and in invasive cancer (Crum and Levine 1984). It is therefore suggested that cervical lesions should be diagnosed as flat condyloma if they contain HPV types 6 or 11 and as CIN if they are confined to the epithelium and contain HPV types 16 or 18 or other

types associated with neoplasia. Patients with a Pap smear or clinical evidence of HPV infection in the genital tract should be examined colposcopically, invasive cancer should be ruled out, and the HPV-induced lesions should be identified and eradicated (Richart 1987).

From the clinical standpoint, the most important distinction is between HPV-related disease (condyloma or CIN) and reactive changes associated with other pathogens, such as Chlamydia. The former should be removed from the cervix, whereas the latter should be medically treated or followed. It is stressed that therapy should not hinge upon the histological distinction of HPV infection from neoplasia and that all lesions should be removed, by conservative means if possible. This is underscored by the fact that a high proportion of CIN lesions contain areas identical to condyloma and those lesions with deep endo-cervical canal involvement, including some with features suggesting condyloma could be treated by cone biopsy to exclude the presence of invasive cancer. The management of the male partner is still unsettled. A large percent of the male partners of these patients have penile lesions and should be included in diagnostic and therapeutic protocols of the women with genital HPV infections or neoplasms (Crum and Levine 1984). It is now clear that the relation between HPV infection and cervical neoplasia is more complex than initially realized. Molecular virologic data suggests preferential distributions of LR and HR-HPV types in CIN that tend to correlate with the morphologic appearance. Thus, mild and moderate dysplasia (CIN 1 and 2) contains a diverse distribution of HPV types, including a minority that have an HR of malignant potential. The presence of associated viral changes can be considered and added to the diagnosis, e.g. "moderate dysplasia (CIN 2) with evidence of Papillomavirus infection". Treatment should be the same for all intraepithelial lesions, regardless of the presence of morphologic evidence of HPV (Brescia et al. 1986).

Pathologic and epidemiologic studies performed over the past decades have provided evidence that the development of SCC of the cervix is a multistep process involving a precursor pre-invasive stage. Infection by a variety of HPV types may result in active viral intranuclear replication without integration into the cellular genome. This episomal form of infection is manifested morphologically by the development of mild dysplasia, CIN 1 with koilocytosis, and acanthosis. Approximately 20 different HPV types have been associated with CIN 1 lesions, whereas HG dysplasia and CIS (CIN 2 and 3) are associated with only a few viral types. LG lesions are differentiated and have a LR of progression to cancer, whereas HG lesions are characterized by nearly complete or complete loss of squamous maturation and HR of progression to invasive cancer. Based on the biologic dichotomy of an infectious and a neoplastic process and the segregation of HPV types into two groups, a modification of the CIN classification into L-SIL and H-SIL in accordance with the Bethesda System has been proposed (Ambros and Kurman 1990). CIN has been traditionally defined as a continuum of intraepithelial squamous abnormalities that exhibit nuclear atypia in all epithelial layers and possess some potential for progression to invasive carcinoma if not removed. Efforts to subdivide this spectrum into categories of low and high cancer risk have been based previously on the strong association between CIN 3 (CIS) and subsequent invasive carcinoma.

However, in practice, this distinction has been discouraged because CIN 1 and 2 may be associated with CIN 3 and a small proportion may progress to invasive carcinoma. As HPV have emerged as potential markers for subdividing precursor lesions, HR-HPV types have been associated with all grades of CIN, whereas LR-HPV types have segregated primarily in lesions closely resembling condylomata. The place of condyloma in the spectrum of CIN, as well as the precise definition of CIN 1, has been controversial. Some Authors distinguish condyloma from CIN 1 and other use similar criteria for the diagnosis of both. Currently, the trend among pathologists and cytopathologists is to classify CIN 1 as a process either identical to or closely resembling condyloma (LG), and CIN 2 and 3 as lesions falling within the spectrum of CIN as classically described (HG). When using cervical lesion morphology as an endpoint in chemoprevention studies, investigators must understand that "morphologic progression" of CIN may not be synonymous with biologic progression. Discrepancies between HPV type and morphology exist, and cytology and histology provide variable, and at times conflicting, information (Crum and McLachlin 1995). Because of the fact that any meaningful classification should bear a close relationship to the biological behavior of the lesions, the usefulness of all new classifications of cervical precancerous lesions can only be established by well-controlled prospective follow-up studies. However, several methodological and conceptual problems are encountered in the natural history studies conducted during the past several decades. The recognition of the association between HPV and CIN has further complicated the assessment of the natural history of cervical precancerous lesions. Results from the early prospective follow-up studies are remarkably consistent, however. It is obvious that the probability of a cervical precancerous lesion to progress into an invasive disease increases with the severity of the atypia. Another distinct prognostic factor is HPV type, HPV-16 lesions possessing a significantly higher risk for progression than infections by other HPV types. The follow-up data also indicate, however, that even the HG lesions may spontaneously regress, which should have important implications in therapy. The continuous problem still remains. These natural history observations only apply to a large series of women but are of little help in predicting the disease outcome in individual women (Syrjänen 1996).

Each HPV type is preferentially associated with specific clinical lesions and has an anatomic site preference for either cutaneous or mucosal squamous epithelium. Infection appears to begin in the basal cells. Early gene expression is associated with acanthosis, and late gene expression is associated with appearance of structural antigens and virions in nuclei of cells of the granular layer, usually koilocytotic cells. Malignant transformation of warts and papillomas appears to be related to a variety of factors:

1. Infection by certain HPV types (HPV 5, 8, 16, 18, 31, 33, and 35)
2. Decreased cellular immunity to HPV-associated antigens
3. Interaction with co-factors such as other microorganisms or sunlight

Spontaneous regression or successful treatment of the benign lesions appears to depend on either naturally acquired or iatrogenically related stimulation of HPV

type-specific immunity. The humoral antibody response to HPV particles is important in preventing infection. In contrast, the local events surrounding regression of warts and condylomata are primarily associated with specific cell-mediated immunity. Local cell-mediated immune responses, particularly cell-associated soluble mediators and stationary macrophage-like cells, are especially important in the host's immune response to mucosal infections (Jenson et al. 1991). However, it has recently become apparent that HPV is highly prevalent in the general population, including a substantial number of cytologically normal women. Although HPV detection is often transient in these individuals, it is not known whether the virus is truly eliminated or whether it remains below the threshold of detection in a latent state (Morrison 1994).

As we have said before, the E6-E7 oncoproteins of HR HPV-16-18 bind specifically, and with high affinity, to cellular tumor suppressor gene products p53 and pRb, in contrast to LR HPV- 6–11 types. This bond disturbs the cell cycle and results in chromosomal instability, aneuploidy, and is the probable starting point of the integration of viral DNA to the host genome. These endogenous modifications are reported to the morphological and colposcopical events of CIN and seem to be most important in the pathogenesis of cervical cancer precursors lesions and tumor progression (Monsonego 1995). The viral oncogenes E6 and E7 are required for the initiation and maintenance of the malignant phenotype in HPV-positive cancers. Proteins coded by these genes are multifunctional and interfere with important cell cycle regulatory proteins. Expression of viral oncogenes is tightly controlled in non-differentiated keratinocytes by at least two signaling cascades, one operative at the functional level and the other at the transcriptional level. The latter has been partially characterized (zur Hausen 1999). However, only a small proportion of CIN infected with HR-HPV will progress to invasive cervical carcinoma, which indicates the involvement of additional factors. An important emerging viral factor is naturally occurring intratypic sequence variation. Such variation has been used to study the geographical spread of HPV, but there is increasing evidence that it may be important in determining the risk of development of neoplastic disease. The collected data indicate that different HPV variants have altered biochemical and biological properties and represent an additional risk factor in the development of SIL and invasive carcinoma of the cervix. This may be relevant not only to the biology of HPV infection and its association with squamous neoplasia, but also to the use of HPV typing in clinical practice (Giannoudis and Herrington 2001). Binding of HPV E6 and E7 oncoproteins to tumor-suppressor genes p53 and Rb follows integration of highly oncogenic HPV into host-cell chromosomes. This process results in impaired tumor-suppressor gene function, involving DNA repair, decreased apoptosis, and eventual cell immortalization. Mutations causing chromosomal alterations, loss of heterozygosity, and proto-oncogene and telomerase activation in immuno-permissive individuals have important roles in virus-induced cervical carcinogenesis. The so-called non-European variants of HPV-16 and HPV-18 may increase the degradation potential of p53. HPV-16 is polymorphic and, although the evidence is controversial, the Arg/Arg genotype of p53 could have greater susceptibility to HPV E6 degradation than the other genotypes. The coincident

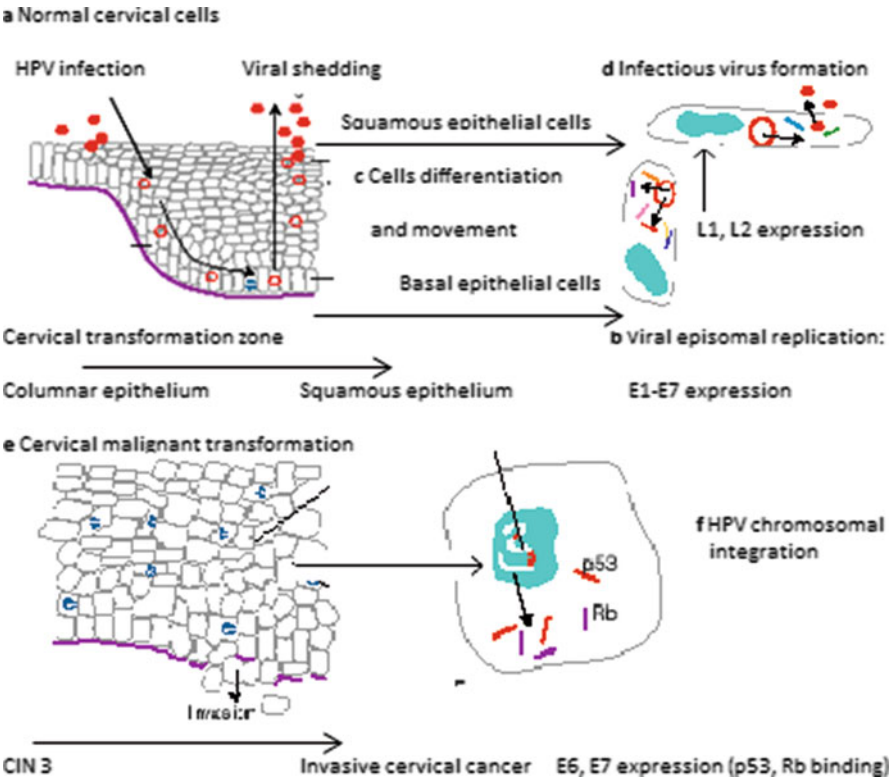

Fig. 2.4 HPV infection and replication in cervical epithelial cells (modified from: HPV-16 and HPV-18. Expert Reviews in Molecular Medicine. http://www-ermm.cbcu.cam.ac.uk/smc/fig001smc.htm. http://www.stanford.edu/group/virus/papilloma/2005/papilloma10.html)

interplay between the non-European genomic variants of HPV-16/18 and p53 Arg/Arg may explain, at least in part, the persistence of HPV infection and tumor progression in women with cervical neoplasia (Ferenczy and Franco 2002).

The productive life cycle of HPV is linked to epithelial differentiation (Fig. 2.4). Papillomaviruses are thought to infect cells in the basal layer of stratified epithelia and establish their genomes as multicopy nuclear episomes. In these cells, viral DNA is replicated along with cellular chromosomes. Following cell division, one of the daughter cells migrates away from the basal layer and undergoes differentiation. In highly differentiated suprabasal cells, vegetative viral replication and late gene expression are activated, resulting in the generation of progeny virions. Since virion production is restricted to differentiated cells, infected basal cells can persist for up to several decades or until the immune system clears the infection. As we have said, the E6 and E7 genes encode viral oncoproteins that target p53 and Rb, respectively. During the viral life cycle, these proteins facilitate stable maintenance of episomes and stimulate differentiated cells to re-enter the S phase. The E1 and E2 proteins act as origin recognition factors as well as regulators of early viral transcription. The functions of the E5 and E1-E4 proteins have been implicated in modulating late

viral functions. The L1 and L2 proteins form icosahedral capsids for progeny virion generation. The characterization of the cellular targets of these viral proteins and the mechanisms regulating the differentiation-dependent viral life cycle remain active areas for the study of these important human pathogens (Longworth and Laimins 2004). Although HR-HPV is a necessary cause for cervical cancer, additional epigenetic events are required for malignant transformation. HPV-mediated transformation of human epithelial cells has been recognized as a multi-step process resulting from deregulated transcription of the viral oncogenes E6 and E7 in the proliferating cells. Interference of E6 and E7 with cell cycle regulators induces genetic instability, which drives the continuous selection of oncogenic alterations providing cells with a malignant phenotype. Early genetic events during cervical carcinogenesis associated with immortalization include deletions at chromosomes 3p, 6, and 10p, whereas amongst others a gain of chromosome 3q, loss of chromosome 11, and epigenetic alterations such as inactivation of the tumor suppressor in lung cancer 1 (TSLC1) gene represent later events associated with tumor invasion (Steenbergen et al. 2005).

The modal time between HPV infection occurring in the late teens or early 20s and pre-cancer peaking around 30 years of age is 7–10 years. Women detected with invasive cancers tend to be an average 10 years older than women with HG disease. The natural history of cervical cancer reveals that infection with HR types may lead to L-SIL or H-SIL. HG lesions may progress to cervical carcinoma if not treated. The purpose of screening, in addition to detecting cervical cancers at an early stage, is to detect and remove HG lesions and thus prevent the potential progression to cervical carcinoma. Early detection of cervical neoplasia is possible with regular Pap smears performed from 21 to 70 years of age. In case of abnormal Pap smear, a biopsy performed under colposcopy will allow the diagnosis of cervical lesion (Heard 2005). There are four major steps in cervical cancer development: infection of metaplastic epithelium at the cervical TZ, viral persistence, progression of persistently infected epithelium to cervical pre-cancer, and invasion through the basement membrane of the epithelium. Infection is extremely common in young women in their first decade of sexual activity. Persistent infections and pre-cancer are established, typically within 5–10 years, from less than 10% of new infections. Invasive cancer arises over many years, even decades, in a minority of women with pre-cancer, with a peak or plateau in risk at about 35–55 years of age. Each genotype of HPV acts as an independent infection, with differing carcinogenic risks linked to evolutionary species. The new HPV-oriented model of cervical carcinogenesis should gradually replace older morphological models based only on cytology and histology (Schiffman et al. 2007). HPV-16 infection, however, is very common in young sexually active women, but the majority mount an effective immune response and clear infection. Approximately 10% of individuals develop a persistent infection, and it is these cohorts who are at risk of cancer progression, with the development of HG precursor lesions and eventually invasive carcinoma. Effective evasion of innate immune recognition seems to be the hallmark of HPV infections, since the infectious cycle is one in which viral replication and release is not associated with inflammation. Furthermore, HPV infections disrupt cytokine expression and signaling with the E6 and E7 oncoproteins particularly targeting the type I interferon

(IFN) pathway. High doses of IFN can overcome the HPV-mediated abrogation of signaling, and this may be the basis for the therapeutic effects on HPV infections of immune-response modulators such as the imidazoquinolones that induce high levels of type I IFN by activation of toll-like receptor (TLR) 7. Using the unique W12 model of cervical carcinogenesis, some of these IFN-related interactions and their relevance in the selection of cells with integrated viral DNA in cancer progression have been investigated. These data show that episome loss associated with induction of antiviral response genes is a key event in the spontaneous selection of cervical keratinocytes containing integrated HPV-16. Exogenous IFN-beta treatment of W12 keratinocytes in which the majority of the population contain episomes results only in the rapid emergence of IFN-resistant cells, loss of episome-containing cells, and a selection of cells containing integrated HPV-16 in which the expression of the transcriptional repressor E2 is down-regulated, but in which E6 and E7 are up-regulated (Stanley et al. 2007).

Although it is now widely admitted that a persistent infection with a HR-HPV type is necessary for the development of HG-CIN and invasive disease, whether persistent HPV infections are characterized by the continuing detection of HPV, or by a state of viral latency during which the virus remains undetectable only to reappear later remains unknown. The distinction between a persistent and transient infection is arbitrary, depending from both the time of sampling in relation to the natural history of the infection and the interval between samples. The longitudinal studies show that "recurrent" HPV infections offer no evidence that the recurrent episode is correlated with re-emergence of the same strain or another strain of the same genotype (wild or variant), but the sequential detection of other HPV type is common. The studies offer no evidence of competition between HPV types but frequently show an increased risk of acquisition of new HPV types in patients already infected compared with those who are HPV-negative (Denis et al. 2008).

In conclusion, current data implicating the role of HPV infections in SCC may be summarized as follows:

1. Animal models have shown that Papillomaviruses can induce malignant transformation
2. HPV involvement in both benign and malignant human SCC has been demonstrated by morphological, immuno-histochemical, and DNA hybridization techniques
3. HPV infections in the genital tract are venereally transmitted and are associated with the same risk factors as cervical carcinoma
4. The natural history of cervical HPV lesions is similar to that of CIN, namely, they have the potential to develop into CIS
5. Malignant transformation of HPV-induced lesions seems to depend on virus type and the physical state of its DNA, e.g. whether or not it is integrated in the host cell DNA
6. Malignant transformation most probably requires synergistic actions between HPV and chemical or physical carcinogens, or other infectious agents

7. Genetic disposition (at least in animals) significantly contributes to malignant transformation
8. Immunological defense mechanisms of the host are probably capable of modifying the course of HPV infections (Syrjänen 1986; Syrjänen 1987)

The tumor biology of cervical pre-cancer is unusual. A large variety of individually distinct forms crudely divided into slight, moderate, severe dysplasia, and CIS exist. Virtually all contain genital HPV either as infectious virions or as episomal or integrated DNA. HPV, which occurs as hundreds of types, subtypes, and variants, has a high prevalence in all human populations. Most males are symptomless reservoirs, whereas a proportion of infected women develop condyloma, pre-cancer, and subsequently, in a minority, invasive cancer. HPV infection has unequivocal features of a STD. Risk of pre-cancer is statistically related to infection with genital HPV, but differences in risk between populations with high and low prevalence of HPV are larger than expected from a direct correlation. Findings fit with HPV as a major risk factor, but other factors must also be operative. These may include shifts in number of target cells, depending on regeneration and infection by various microorganisms, hormones, smoking, and immunity. Genital condyloma, which is not precancerous, is caused by LR-HPV types, typically 6 or 11, in analogy with papilloma formation in skin and mucosa in a large variety of species. This benign lesion is the hallmark of mammalian HPV pathology and a source of inter-individual spread of virus. Slight dysplasia is heterogeneous. Many lesions seem to be polyclonal, self-limited cell proliferative responses to infection with LR-HPV. Small proportions are associated with either simultaneous presence or subsequent development of HG dysplasia, CIS, or invasive cancer. Evidence exists for two mechanisms: clonal selection of cells with increasingly undifferentiated phenotypes, and independent development of different morphological types of pre-cancer. HR-HPV, typically 16 or 18, is preferentially associated with HG dysplasia and CIS, either because it increases risk of clonal progression to these forms or induces them de novo. Severe dysplasia, CIS, and invasive cancer always present as monoclonal lesions. Genetic links indicate that these pathologies arise by clonal selection from less advanced precursors. The number of potential target cells for pre-cancer confined to a narrow TZ is small. Risk of pre-cancer and malignant transformation per target cell is therefore probably far higher than in any other human tissue subject to cancer. Spontaneous mutation rate and physicochemical carcinogens seem insufficient for the creation of a malignant phenotype in cells of the TZ. Currently, HPV is the only strong candidate for such a feat. The following mechanisms play a role: overexpression of viral E6 and E7 genes, often triggered by disruption of control elements upon integration of viral DNA into the cellular genome, activity of specific (E6) configurations in certain HPV variants, inactivation of p53 with decreased capacity for DNA repair, and enhanced likelihood of accumulation of "transforming" mutations and viral integration at sites controlling function of cellular oncogenes and/or suppressor genes. Target cells within the TZ have the capacity for bidirectional (squamous and/or glandular) differentiation. HPV types seem to drive cells preferentially in different directions after infection/transformation. LR-HPV types are almost always associated

with squamous differentiation, HPV-16 usually also with squamous differentiation and HPV-18 with adenosquamous or adenomatous differentiation (Pontén and Guo 1998). HPV have also been detected in a wide range of asymptomatic controls, indicating that other events are required for development of neoplasia such as viral persistence and/or altered expression of viral genes, often following integration of the viral genome. This leaves the two major viral oncogenes, E6 and E7, directly coupled to viral enhancers and promoters, allowing their continued expression after integration. HR-HPV E6 proteins bind p53 and direct its rapid degradation, whereas E7 proteins bind and inactivate the Rb protein. A range of putative co-factors has been implicated in progression: HLA type, immunosuppression, sex steroid hormones, and smoking. Most of these co-factors appear to influence progression to CIN 3. The natural history includes progression to CIN 3 in 10% of CIN 1 and 20% of CIN 2 cases, whereas at least 12% of CIN 3 cases progress to invasive carcinoma. Cervical glandular intraepithelial neoplasia (CGIN) often coexists with squamous CIN, and the premalignant potential of HG-CGIN is not in doubt, but the natural history of LG-CGIN remains uncertain (Arends et al. 1998).

Cervical cancer and its precursor lesions are unique in that we can study the natural history of one disease at two different levels:

1. By assessing the clinical lesions
2. By analyzing the viral events of HPV infections, their prime etiological agent

The natural history of CIN has been well established by a large number of prospective cohort studies covering over 25,000 patients, and the figures for regression, persistence, and progression are well established. The outcome of HPV infections is far more complex with at least six distinct patterns being demonstrated in long-term cohort studies. There is little doubt that the mechanistic explanation for HPV clearance is by specific immunological reactions, where competent humoral and cell-mediated immune mediators are needed. To understand this process in detail still necessitates a substantial amount of clinical and laboratory research. In general, HPV outcomes follow the pattern where a dynamic balance exists between incident infections and virus clearance. Following a rapid accumulation of incident infections after onset of sexual activity (women <20 years of age), there is a transition of this balance in favor of virus clearance soon after age 25. This explains the constantly declining age-specific prevalence of HPV infections until menopause. Failure to eradicate the virus at post-menopause is not uncommon, however, explaining the deep second peak in HPV prevalence now reported in many different populations. The importance of HPV clearance/non clearance (persistence) has been recognized recently, and the number of studies addressing these issues has increased substantially during the past few years. The data are now rather unanimous concerning the times and rates (usually expressed per 1,000 women/months at risk, WMR) of HPV clearance. On the other hand, data are still incomplete and in part inconsistent as to the co-factors that regulate these events. A wide variety of variables have been explored as potential co-determinants and/or predictors of HPV clearance. Until now, all efforts attempting to identify suitable biomarkers as such predictors, have been disappointing, but fortunately, this is a largely unexplored

area as yet. Similarly, data on the two extremes of life, i.e. early infancy and post-menopause, is still far too fragmentary to enable creating a comprehensive view on how these viral infections behave in early life, and what makes many women incapable of clearing their virus at post-menopause. Both issues are of utmost importance and have widespread clinical implications: we need to know how and why some infants and children contract HR-HPV infections well before the onset of their sexual activity, to be able to select the proper targets for prophylactic HPV vaccination. Similarly, we need to know why some women over 55 years of age are likely to remain HR-HPV carriers, while the vast majority successfully clears their infection well before the menopausal age. Early detection of cervical cancer precursors among these elderly HR-HPV positive women past the usual age of organized screening remains a major challenge also in the future (Syrjänen 2007; Deligeoroglou et al. 2009; Huh 2009; Cardiff and Borowsky 2010; Simon et al. 2010; Slama et al. 2010; Veldhuijzen et al. 2010; Bodily and Laimins 2011).

3 Epidemiology of HPV Infection and Cervical Cancer

The epidemiology of cervical cancer presents a number of unique challenges, mainly with respect to disentangling correlated factors and to elucidating biological mechanisms. Several epidemiologic leads can be pursued through biochemical and molecular techniques. The evidence links certain HPV types to cervical abnormalities, including cancer, and newly developed probes can be incorporated into epidemiologic studies to evaluate an array of risk factors. Endocrine and metabolic assays may be helpful in clarifying the role of exogenous and possibly endogenous hormones. The effects of cigarette smoking may be further evaluated by studying constituents of tobacco smoke and their metabolites in cervical mucus. Finally, the relationship of diet to cervical cancer should be assessed by examining the levels of micronutrients, trace minerals, and other nutritional indices in body tissues and fluids, as well as through interview data. Through studies that focus on disease stage and time-related events, it could be possible to clarify the multi-stage processes involved in cervical carcinogenesis, and those factors that may inhibit as well as promote transition rates. Research into dietary factors may lead in time to nutritional intervention. Investigation by cell type should also be pursued to define the epidemiology of the rarely occurring adenocarcinomas and adenosquamous carcinomas of the cervix. Finally, preventive strategies should be targeted to HR populations, especially those of the lower socioeconomic classes and with limited access to medical care. The need for a renewed focus on epidemiology and prevention is emphasized by recent increases in exposure to several postulated risk factors, including sexual promiscuity, OC, and smoking (Brinton and Fraumeni 1986). The question of whether HPV infection causes, or at least contributes to, the development of cervical carcinoma has been a topic of much scrutiny and controversy over the past decades. The sexual mode of transmission of genital HPV and detection of viral DNA in cervical tumors suggest a causal role at some stage during malignant progression.

To some extent, it seems that the epidemiological data accumulated so far has served to confuse as much as to clarify the issue, and the distribution of genital HPV in the general population appears to be more complex than originally anticipated. The development of cervical cancer is a multistage process, and HPV infection alone is clearly not sufficient for full malignant transformation. Nevertheless, identification and control of HPV infection may be of critical importance in the diagnosis and treatment of this disease (Vousden 1989).

The reliable assessment of epidemiology of genital HPV infections is hampered by a number of technical problems. Until recently, because of the lack of tissue-culture systems, methods based on morphological approaches (i.e. macromorphology, colposcopy, cytology, and histopathology) played a central role in HPV diagnosis, supplemented by immuno-histochemical and DNA-hybridization techniques and most lately by DNA amplification with polymerase chain reaction (PCR). Because of the fact that these morphological techniques are extremely difficult to standardize, they are subject to major inter-laboratory variations, both inter-observer and intra-observer. Further confusion in the field is created by the complex biological behavior of HPV infections. As established by long-term prospective follow-up studies, clinical progression and regression are significantly related to the grade of the lesion at the time of diagnosis ($p < 0.00001$ and $p = 0.0005$, respectively), as well as to the type of HPV ($p = 0.0012$). Most importantly, however, genital HPV infections seem to run an extremely fluctuating course, a passage from a manifest to a subclinical or latent infection being frequently encountered in individual patients when examined at 6-month intervals over prolonged periods. This explains the significantly divergent prevalence figures reported in different series (ranging from 2% to 80%), which are completely dependent on the technique used to analyze the presence of HPV, i.e. whether through:

1. PAP smear
2. Biopsy
3. DNA hybridization
4. PCR amplification

The first two are capable of disclosing only manifest (clinical) infections, the latter two also (at least theoretically) the subclinical and latent ones. In an unselected population of 22-year-old females, the prevalence of clinical (i.e. detectable by PAP smear) HPV infections was about 3%, and the adjusted annual incidence was 8.0%. According to estimates of the lifetime risk, up to 79% of females will contract at least one HPV infection between the ages 20 and 79 years. When related to the long-term trends in invasive cervical cancer, it is evident that this 79% life-time risk of becoming HPV-infected or even the observed 15% of clinical progression rate for HPV infections in the prospective follow-up study by no means signify an identical risk of developing cervical cancer (i.e. $0.79 \times 0.15 = 11\%$). It seems likely that in countries where mass-screening programmes exist (and precancerous lesions are traced and eradicated), the high prevalence of HPV infections is not necessarily reflected as an increased prevalence of invasive cervical carcinomas. The distinction of lesions at risk for malignant transformation from those regressing spontaneously

will have major implications in therapeutic considerations of genital HPV infections (Syrjänen 1989; Syrjänen and Syrjänen 1990). Measurement errors have been an important concern in studies of HPV and anogenital cancers. Misclassification of HPV infection status is a possible explanation for incoherent findings in previous epidemiological studies purporting to show an etiological role for HPV in cervical cancer. Even low levels of misclassification of HPV infection can cause severe underestimation of HPV prevalence in field surveys, bias the association between HPV and sexual activity, and impair the ability to control statistically the relation between sexual activity and neoplasia by viral status. Some simple numeric formulae allow the correction of prevalence rates and epidemiological measures of effect, such as the odds ratio (OR) and the relative risk (RR), under conditions of misclassification constraint. These formulations have been used to correct estimates from recent epidemiological studies using hypothetical misclassification scenarios in order to obtain clues on the magnitude of the underlying relationship between HPV and cervical cancer (Franco 1992).

Cervical cancer is the most common cancer in developing countries and the sixth most common in developed countries. In all areas, it is more frequent among women of low socioeconomic status, it is associated with multiple sexual partners and early age at first sexual intercourse, and screening reduces both incidence and mortality. According to population-based surveys in industrialized countries, men of low socioeconomic status report fewer sexual partners than men of high socioeconomic status but there is no clear indication that the same is true of women of low socioeconomic status. In case–control studies, HPV and all other STD were more prevalent among women in low socioeconomic strata. Number of sexual partners and particularly contacts with prostitutes were higher among husbands of women of low socioeconomic status. Other potential risk factors for the disease, such as smoking and OC use, and also cervical cancer screening (Pap smears), were more common in women of high social strata. Women with no schooling had a three to fivefold higher risk of having cervical cancer compared with women who had achieved a higher educational level. After adjustment for sexual behavior, HPV DNA status, history of Pap smears and husband's contact with prostitutes, this association was considerably reduced. These results are indicative that socioeconomic differences in the incidence of cervical cancer can be partly explained by differences in the prevalence of HPV DNA. Men's sexual behavior and particularly contacts with prostitutes might be a major contributor to the higher prevalence of HPV DNA among the poor (de Sanjosé et al. 1997). Worldwide, cancer of the cervix is the second leading cause of cancer death in women: each year, an estimated 500,000 cases are newly diagnosed. Among populations, there are large differences in incidence rates of invasive cervical cancer: these reflect the influence of environmental factors, screening Pap tests, and treatment of pre-invasive lesions. The HR-HPV subtypes 16, 18, 31, 33, and 51 have been recovered from more than 95% of cervical cancers. There have been made great strides in understanding the molecular mechanism of oncogenesis of this virus, focusing on the action of the E6 and E7 viral oncoproteins. These oncoproteins function by inactivating cell cycle regulators p53 and Rb, thus providing the initial event in progression to malignancy.

Cervical cancers develop from precursor lesions, which are termed SIL and are graded as high or low, depending on the degree of disruption of epithelial differentiation. Viral production occurs in LG lesions and is restricted to basal cells. In carcinomas, viral DNA is found integrated into the host genome, but no viral production is seen. The well-defined pre-invasive stages, as well as the viral factors involved at the molecular level, make cervical carcinoma a good model for investigating immune therapeutic alternatives or adjuvants to standard treatments (Schoell et al. 1999).

Studies on risk factors for pre-cancerous lesions of the uterine cervix have shown strong association with sexual practice, as we have seen. Women with multiple sexual partners and intercourse at early age are at HR. A role of male partners in further enhancing the risk has been identified. All these support the hypothesis relating to a sexually transmissible etiological agent. The risk factors for pre-cancerous lesions of cervix are grouped into genital, sexual, chemical, dietary, and life factors. HPV is the major infectious etiological agent associated with the development of pre-cancerous lesions of cervix. Other co-factors such as multiple sexual partners of the male as well as the female and early age of first intercourse are also involved at the critical etiological step of progression from LG to HG lesions. The role of other infectious agents in terms of supportive or interactive effects is not clear. No independent effect for HSV type 2 on risk is observed. Other risk factors include cigarette smoking, OC usage, certain nutritional deficiencies, and poor personal hygiene. However, it is not clear whether these factors operate independently from HPV. There is no consistency in the independent effect of these factors on the development of LG to HG lesions of cervix. There is a similarity in the patterns of risk between pre-cancerous lesions of the cervix and cervical cancer. Monogamy, late commencement of sexual activity, personal hygiene, and use of barrier contraceptive methods help towards primary prevention (Murthy and Mathew 2000).

The hypothesis of a geographical correlation between HPV detection rates and incidence of cervical cancer has been investigated in studies of various types. However, results from these studies are equivocal, in contrast to findings concerning other suspected risk factors that seem to correlate well with the cervical cancer incidence. Possible explanations include:

1. Greater sensitivity of ecological studies to cumulative exposures such as lifetime number of sexual partners, lifetime smoking, and seroprevalence of HSV-2, than to HPV DNA prevalence which does not reflect cumulative exposure to HPV
2. Misclassification in the HPV diagnosis leading to wrong prevalence estimates

In future research, it will be important to establish the sensitivity and specificity of the different methods and conduct intra- and inter-laboratory validation studies in order to standardize methods. In spite of the limitations of cross-sectional studies, the measurement of HPV prevalence and its correlation with, for example, sexual behavior is still valuable for our understanding and interpretation of the role of HPV infections (Kjaer and Jensen 1992). Numerous studies of the epidemiology of cervical cancer have shown strong associations with religious, marital, and sexual patterns. It is well established that women with multiple partners and early ages at first

intercourse are at HR. Number of steady partners and frequent intercourse at early ages further enhance risk, supporting hypotheses regarding a vulnerable period of the cervix and a need for repeated exposure to an infectious agent. HPV is the major infectious etiological agent. Other speculative risk factors for cervical cancer include cigarette smoking, OC usage, and certain nutritional deficiencies. Cervical cancer incidence trends correlate with the population prevalence of various venereally transmitted agents. In addition, a number of studies highlight the need for considering not only female influences on risk of cervical cancer, but also male factors, since the sexual behavior of the male consort appears to play an important role (Brinton 1992). HPV is the most common diagnosed STD in the United States of America (USA). Although the majority of sexually active adults will be infected with HPV at least once in their lives, sexually active women less than 25 years of age consistently have the highest rates of infection. Besides youth and gender, common risk factors for HPV infection and clinical sequelae of infection include high number of sexual partners and co-infection with Chlamydia trachomatis or HSV. Most HPV infections are cleared by the immune system and do not result in clinical complications. Clinical sequelae in cases of LR-HPV infection consist of genital warts, and clinical manifestations of HR-HPV infection include abnormal Pap test results, L-SIL, H-SIL, and cervical cancer, which carry significant morbidity and/or mortality. Genital warts and abnormal Pap test results are often significant sources of psychosocial distress. Currently, there are neither effective means of preventing HPV transmission nor cures for clinical manifestations: infection can only be prevented via complete sexual abstinence, while treatment for clinical sequelae such as genital warts and cytologic abnormalities consists of removing the problematic cells and watching for recurrence. This method consumes significant health care resources and is costly. New prophylactic HPV vaccines promise to dramatically reduce the incidence of HPV infection, genital warts, and cytologic abnormalities (Ault 2006).

Epidemiological studies on the association between HPV and cervical cancer using HPV DNA hybridization methods to assess the presence of viral markers have yielded compelling evidence that HPV has a causal role in the disease: the association is strong, consistent, and specific to a limited number of viral types. A dose–response relationship has been reported between increasing estimated viral load and risk of cervical cancer. Indirect evidence suggests that HPV DNA detected in cancer cells is a good marker of HPV infection occurring before cancer development. An increased risk for progression to more advanced CIN lesions has been reported among HPV-16/18-positive women as compared to women with other HPV types or to women without any viral DNA (Muñoz and Bosch 1992). So, the evidence implicating specific HPV types in the etiology of cervical cancer is strong enough to establish a causative role. HPV infection of the cervix affects the developing immature metaplastic cells of the TZ. Cervical neoplasia can be viewed as the interaction of HR-HPV and immature metaplastic epithelium. Once maturity is reached, there is minimal risk of subsequent development of cervical squamous neoplasia. Exposure to HPV is an extremely common event, especially in young sexually active women. Yet, despite frequent HPV exposure at that phase of life in which the cervical TZ is at its most vulnerable, established expressed disease

is relatively uncommon. Most studies in which the natural history of CIN is not altered by cervical biopsy reveal a progression rate from LG to HG-CIN of less than one third. Where viral type is taken into account, however, the progression rate from normal but HR-HPV-infected cervical epithelium to CIN 2 or 3 is higher. Despite this, most cervical abnormalities will not transform into invasive cancer, even if left untreated. The variance between the high rate of HPV infection, the intermediate rate of CIN and the relatively low rate of cervical cancer establishes a stepwise gradient of disease of increasing severity with decreasing prevalence. In an immunocompetent host, HPV infection alone does not appear to be sufficient to induce the step from HG-CIN to invasion. Epidemiological studies indicating that HPV infection with oncogenic viral types is far more common than cervical neoplasia suggest the necessity of co-factors in cervical carcinogenesis. The long time lag between initial infection and eventual malignant conversion suggests that random events may be necessary for such conversion, and the spontaneous regression of many primary lesions suggests that most patients are not exposed to these random events. Potential co-factors, as we have seen, include cigarette smoking, hormonal effects of OC and pregnancy, dietary deficiencies, immuno-suppression, and chronic inflammation. In those women who develop cervical cancer, malignant progression is rarely rapid, more commonly taking many years or decades. Malignant progression has been documented in patients who presented initially with only LG HPV-induced atypia. On the other hand, progression may be a misnomer, as "apparent" progression may really represent adjacent "de novo" development of HG-CIN. Although most cervical cancers contain HR-HPV types, up to 15% of such cancers test negative for HPV, raising the possibility that a few, usually more aggressive, cervical cancers may arise from a non-viral source (Cox 1995).

As we have seen, epidemiologic and laboratory data suggest that cervical cancer typically arises from a series of causal steps. Each step can be studied separately in the hope of better etiologic understanding and improved cancer prevention. The earliest identified etiologic step is infection of young women with specific types of venereally transmissible HPV. Cervical HPV infections often lead to L-SIL (mildly abnormal Pap smears). HPV infections and their associated lesions are extremely common among young, sexually active women. The infections typically resolve spontaneously even at the molecular level within months to a few years. Uncommonly, HPV infections and/or LG lesions persist and progress to HG lesions. The risk factors for progression are mainly unknown but include HPV type and intensity, cell-mediated immunity, and reproductive factors. Nutritional factors or co-infection with other pathogens may also be involved at this apparently critical etiologic step between common L-SIL and uncommon H-SIL. Except for advancing age, no epidemiologic risk factors have been found for the next step between H-SIL and invasive cancer. At the molecular level, invasion is associated with integration of viral DNA. Based on worldwide research, the steps in cervical carcinogenesis appear to be fundamentally the same everywhere, with a central role for HPV infection. The importance of etiologic co-factors like smoking, however, may vary by region (Schiffman and Brinton 1995).

Numerous studies have attempted to determine associations between micronutrients and risk of CIN and cervical cancer. Studies that were conducted before a reliable test for assessing HPV infections was available may have resulted in misclassification because of differences in assay sensitivity, which could have led to residual confounding. Another limitation in previous studies may be related to methodologic limitations such as the proper choice of controls for case–control studies. Since cervical cancer does not develop in the absence of HR-HPV infections, only controls exposed to HR-HPV should be included in studies that investigate co-factors for CIN or cervical cancer. Also, the recruitment of subjects for these studies had been based on screening programs that used different approaches such as cytology, colposcopic impression, or biopsy to identify pre-neoplastic cervical lesions. Recent studies have demonstrated that some of these approaches could lead to substantial under detection and misclassification of preneoplastic lesions of the cervix. Recent studies that addressed these issues have demonstrated that folate is an important micronutrient in cervical cancer prevention via its influence on HR-HPV and the development of CIN. Carefully designed ongoing studies are expected to generate data on whether folate-related biomarkers could be used to identify subjects who are at risk of developing cervical cancer and whether folate supplementation will be beneficial in preventing cervical cancer in women exposed to HR-HPV (Piyathilake 2007). Studies of nutritional predictors of cervical neoplasia to date have been limited by inadequate HPV measures, which compromise the interpretations of findings. Current research using accurate measures of HPV will be most revealing. Nonetheless, agreements in findings from previous studies suggest a role for nutritional factors in some or all stages of cervical neoplasia. Low vitamin C and carotenoid status are associated fairly consistently with both cervical cancer and precursors, whereas results for vitamin E status are less consistent. The effect of folate status may be restricted to early preneoplastic cervical lesions and not to more advanced disease. Current research is addressing nutritional influences on HPV infection and persistence and on progression of cervical disease (Potischman and Brinton 1996). Folate deficiency appears to play a crucial role early in cervical carcinogenesis by facilitating the incorporation of HPV genomes at a fragile chromosomal site. Thereafter, multiple nutritional, hormonal, and immunologic factors probably interact in a synergistic manner to determine whether the cell line becomes immortalized and invasive (Butterworth 1992).

The establishment of HPV infection as a major cause of cervical neoplasia has resulted in major efforts to develop prophylactic HPV vaccines for prevention of cervical neoplasia. Cervical cancer and the other HPV-associated cancers constitute a major public health burden and eradication of the major causative infection is certainly the most appealing long-term preventive measure. Nevertheless, the effects of preventive HPV vaccination will need to be estimated and compared for cost-efficacy with other primary prevention and with secondary prevention programs. However, estimating the effects of preventing a causative exposure is complicated when the exposure is a transmissible infection. The spread of the epidemic is dynamic and may change over time depending e.g. on the changes in human behavior. Depending on the circumstances, prevention of an infection may have

either greater or lesser effects than the prevention of a non-infectious exposure. Estimating the time trends in HPV infections and the underlying trends in the risk of cervical neoplasia is important for estimating effects of interventions. Trends in detection rates of cervical cancer precursor lesions are consistent with an increase in the background cervical cancer risk. From the 1960s to 1980s, there has been a major increase in HPV seroprevalences over time. Increasing trends are also seen for other STD and smoking. Several studies indicate the existence of interaction between benign and oncogenic HPV types, thus making the relationship between the incidences in HPV infections and in cervical neoplasia complex. The increase in cervical cancer is paralleled by increases in HPV infection, other STD, smoking, and changes in screening practices, all of which may have contributed. Prediction of the effect on cervical cancer incidence of changes in HPV incidences is complicated by the existence of several risk factors, the protective effect of screening, and by the population dynamics of HPV infections (Dillner 2000). Effective primary and secondary cancer prevention programmes are key to improve public health. Cervical cancer is preventable if high-quality screening programmes, diagnosis, and treatment are offered to female populations at high coverage. Nevertheless, it continues to be a public health problem, and screening programmes need improvements. HPV has been firmly established as the necessary cause of virtually all cervical cancer cases. To date, we count two clinically validated and approved HPV technologies available to prevent cervical cancer and other diseases caused by these carcinogenic viruses: prophylactic vaccines for primary prevention, and HPV DNA tests for secondary prevention, to detect life threatening infections by carcinogenic HPV types, allowing timely diagnosis and clinical management of precancerous lesions. The new technologies will help improve the health of the public if made widely accessible. Similar to vaccination programmes, systematic and well organized cervical screening programmes, with high-quality validated HPV tests, can save more lives than ever and improve women's health in an effective manner (Pagliusi 2007).

An in vitro multistage genital epithelial cell model for cervical cancer that parallels the in vivo neoplastic process has been developed using recombinant HPV DNA and genital cells. HPV-16-immortalized genital cells are responsive to the genotoxic action of known chemical carcinogens (polycyclic hydrocarbons, alkylating agents, or cigarette smoke condensate), but are not converted to malignancy. Ras oncogene and human HSV-2 did convert HPV immortalized cells to malignancy, whereas human HSV-6 infection only increased HPV expression. HIV did not infect genital cells (DiPaolo et al. 1996). The effect of smoking on HPV activity and subsequent dysplasia and neoplasia remains controversial. Several recent large studies demonstrated that smoking was associated with a greater incidence of cervical, vulvar, penile, anal, oral, and head and neck cancers in a dose-dependent fashion, while other studies did not show any correlation between smoking and cervical dysplasia after multivariate adjustment. Recent studies have also indicated that smoking may be more closely related to HG lesions of the cervix and vulva. This data provides evidence of an association between HPV, smoking, and cancer. Progression of dysplasia likewise seems to be associated with smoking. Several groups have attempted to discern whether the connection between smoking

and cervical cancer is from local immunosuppression and/or from direct carcinogenic effects (Moore et al. 2001). Smoking has long been suspected to be a risk factor for cervical cancer. However, not all previous studies have properly controlled for the effect of HPV infection, which has now been established as a virtually necessary cause of cervical cancer. There is an excess risk for smoking among HPV positive women (OR 2.17, 95% confidence interval, CI, 1.46–3.22). When results are analyzed by histological type, an excess risk is observed among cases of SCC for current smokers (OR 2.30, 95% CI 1.31–4.04) and ex-smokers (OR 1.80, 95% CI 0.95–3.44). No clear pattern of association with risk is detected for adenocarcinomas, although the number of cases with this histologic type is limited. Smoking increases the risk of cervical cancer among HPV positive women. The few previously conducted studies of smoking and cervical cancer have adequately controlled for HPV infection. Recent increasing trends of smoking among young women could have a serious impact on cervical cancer incidence in the coming years (Plummer et al. 2003).

Appropriately sophisticated statistical approaches are crucial for addressing the increasingly complex set of critical questions that follow from the recognition that HPV is a necessary causal factor for cervical cancer. Cervical cancer researchers have defined the major stages of cervical carcinogenesis, with HPV infection as the necessary cause. Focus of etiologic studies is shifting from establishing causality to determining risk factors for HPV persistence and neoplastic progression using serially collected biomarkers. Prevention-oriented epidemiology and trials of new screening strategies and vaccines will rely on surrogate endpoint biomarkers (SEB) because we cannot let women develop cancer when it can be prevented. Future epidemiologic and prevention studies of HPV infection and cervical carcinogenesis will exploit subtle pathologic distinctions and will employ improved measurements of complex molecular biologic phenomena (Wacholder 2003).

Despite advances in early detection and prevention of cervical cancer, women living in rural areas have had consistently higher rates of cervical cancer mortality than their counterparts in other areas during the past several decades. Living in rural areas may impose barriers to cervical cancer control, including lack of transportation and medical care infrastructures. Population characteristics that place women at greater risk for developing and dying from cervical cancer, such as low income, lack of health insurance, and physician availability, are concentrated in rural areas. Published data, however, are insufficient to identify the key reasons for the observed mortality patterns. At this time, given the lack of definitive evidence in the published literature, decisions about priorities in areas with high-rates of cervical cancer mortality will depend on knowledge of current levels of screening, incidence, and stage distribution, and service delivery infrastructures, resources, and acceptability of interventions to the target population (Yabroff et al. 2005).

Most cancers of the uterine cervix are SCC. Although the incidence of such carcinomas of the uterine cervix has declined over time, that of cervical adenocarcinoma has risen in recent years. The extent to which HPV infection and co-factors may explain this differential trend is unclear. The adjusted overall OR for cervical adenocarcinoma in HPV-positive women compared with HPV-negative women is 81.3 (95% CI 42.0–157.1). HPV-16 and HPV-18 are the two most commonly detected HPV types in case patients and control subjects. These two types are present in 82%

of the patients. Co-factors that show clear statistically significant positive associations with cervical adenocarcinoma overall and among HPV-positive women include never schooling, poor hygiene, sexual behavior-related variables, and long-term use of hormonal contraception, high parity, and HSV-2 seropositivity. Parity has a weaker association with adenocarcinoma and only among HPV-positive women. Use of an intrauterine device (IUD) has a statistically significant inverse association with risk of adenocarcinoma (for ever use of an IUD compared with never use, OR 0.41 (95% CI 0.18–0.93)). Smoking and chlamydial seropositivity are not associated with disease. HPV appears to be the key risk factor for cervical adenocarcinoma. HPV testing in primary screening using current mixtures of HPV types and HPV vaccination against main HPV types should reduce the incidence of this cancer worldwide (Castellsagué et al. 2006; Trottier and Burchell 2009; Bruni et al. 2010; Regan et al. 2010; Stanley 2010; Li et al. 2011).

4 HPV Infection and Cervical Cancer in Pregnancy

Pregnancy may foster the development of infections, particularly HPV infections. The pathology may be latent or manifest. Possible methods of diagnosis are clinical, namely gynecological examinations, Pap tests, colposcopy or molecular, using viral DNA assay, Southern Blot, PCR, hybrid capture (HC), etc. The prevalence of HPV infection in pregnancy varies between 5.4% and 68.8%. The population with the highest risk, also among pregnant women, are those under the age of 26. A number of different opinions emerge from the literature regarding the possibility of maternal-fetal virus transmission. The data reported in the literature on the relationship between HPV and pregnancy is highly discordant. This discrepancy depends on the diagnostic techniques used, the clinical history of the pregnant woman, and the period of pregnancy when the sample is collected. Pregnancy enhances the development of the pathology which often recedes in the post-partum. The possible maternal-fetal transmission of the virus is an important aspect: the latter is the main factor responsible for juvenile laryngeal papillomatosis. A number of Authors report an initial presence of HPV in newborns which often disappears within 6 months after birth (Arena et al. 2002). However, HPV have been detected in asymptomatic women, infants, and children. Several studies have demonstrated that infants can acquire HR-HPV infections from their mothers at birth. Thus, the traditional view that cervical cancer-associated HPV infections are primarily STD needs to be reassessed. Accordingly, the role of mother-to-child transmission of cancer-associated HPV may need to be investigated further (Cason et al. 1998).

The prevalence of HPV infections is unchanged among pregnant women with infection by LR viruses. The viral load increases at the time of pregnancy, and decreases in the post-partum period. Cervical cytology is easily to perform with reliable results: among the 5% of pathological cervical smears, LG lesions predominate. The HG smears require colposcopic exploration, usefully completed by directed biopsies to rule out invasive lesions. Surveillance of HG-CIN is required during pregnancy with post-partum control: most regress. Non- specific clinical signs delay

clinical diagnosis and the histological aspects of the lesions that are identical to those observed in young women. The intrinsic outcome of cancer is not modified by pregnancy, and the cesarean section is often preferred (vaginal delivery likely facilitates vascular dissemination). For fetal reasons, a therapeutic delay can be proposed for small sized lesions with a favorable histological subtype and no progression after 20 weeks of gestation. Pregnancy offers the opportunity to perform cervical smears in women not regularly followed. A conservative attitude with a reevaluation in post-partum can be proposed in the event of diagnosis of CIN during pregnancy. Pregnancy has little influence on invasive cervical cancers. Management decisions must be made on a case-by-case basis (Zoundi-Ouango et al. 2006). An isolated report of atypical squamous cells on cervical cytology obtained at the initial prenatal visit does not warrant colposcopic evaluation during pregnancy, unless a repeated cytology suggests CIN. Repeated cytology and evaluation to exclude infections and inflammatory lesions is appropriate. However, if a subsequent cytology is abnormal, post-partum colposcopy and colposcopically directed biopsies seem appropriate, since the prevalence of HPV or CIN is 21% (Kaminski et al. 1992).

The rate of CIN discovered during pregnancy is around 1%. Pregnancy should be a period for the checking of Pap smear. So a Pap smear should be performed if the last one is more than 2 years old. If the Pap smear is less than 2 years old, a copy of its result should be obtained. Cervical cytology is valid during pregnancy, and usually pregnancy induces an opening of the junction zone that helps to have a good evaluation of the cervix. When atypical cells are found in the Pap smear performed during pregnancy, the management should include a colposcopy and biopsies, whatever the severity of the abnormal cells. The biopsy should be guided by the colposcopy. Biopsy is required for an optimal diagnosis. The management of the pregnant patient should be based on the results of cytology, colposcopy, and biopsies. Currently there is no indication for HPV-typing during pregnancy. If an invasive tumor is excluded after cytology, colposcopy, and biopsy, no treatment is performed during pregnancy, and the treatment is postponed after delivery following a complete cervical re-evaluation. If an invasion cannot be excluded with the biopsy, a diagnostic conization completed with a cerclage should be performed. This procedure should be exceptionally performed. During pregnancy, CIN lesions are usually stable or regressing, progression of CIN is rare. When managing an abnormal Pap smear during pregnancy, a micro-invasive cervical cancer may be found on a biopsy or conization specimen. Staging of these lesions requires always a conization and eventually a pelvic magnetic resonance imaging (MRI). Micro-invasive cervical cancers can be only followed until the end of the pregnancy (Selleret and Mathevet 2008).

5 HPV Infection in Childhood and Adolescence

Worldwide, cervical cancer is a major health concern for women of all ages. However, the epidemiology and biology of HPV infection differs in female adolescents and adults. HPV infections are known to affect predominantly adult, sexually active

age groups, whereas skin warts, at various anatomic sites, are usually associated with younger individuals. The modes of viral transmission in children remain controversial, including perinatal transmission, auto- and hetero-inoculation, sexual abuse, and, possibly, indirect transmission via fomites. Recent studies on perinatal infection with HPV have been inconclusive. It is still unclear how frequently perinatal infection progresses to clinical lesions, whether genital, laryngeal, or oral. Conflicting reports have been published on the prevalence of HPV infections in children. The current consensus is, however, that newborn babies can be exposed to cervical HPV infection of the mother. The detection rate of HPV DNA in oral swabs of newborn babies varies from 4% to 87%. The concordance of HPV types detected in newborn babies and their mothers is in the range of 57–69%, indicating that the infants might acquire HPV infection postnatally from a variety of sources. HPV antibodies have been detected in 10–57% of the children, and there is usually no correlation between seropositivity and detection of HPV DNA in either the oral or the genital mucosa. There is also evidence that transmission in utero or post-natal acquisition is possible. The mode of in utero transmission remains unknown, but theoretically the virus could be acquired hematogenously, by semen at fertilization, or as an ascending infection in the mother. The serologic response to HPV detected in different populations of young women or women at risk of cervical cancer might be due to genital infections, but the possibility that HPV infection has been acquired earlier in life through the oral mucosa or respiratory tract cannot be ruled out (Syrjänen and Puranen 2000). Major differences in estrogen and progesterone levels and in vaginal/cervical cell types exist among children, adolescents, and adults, which may help explain the observed differences in the prevalence of HPV among these groups. Although sexual transmission occurs in both children and adolescents, the persistence of genital HPV in the neonate from maternal transmission is highly questionable. The discordances in HPV types between neonatal transmission studies and the studies in older children have yet to be explained. Neonatal infections seem to be predominantly HPV types 16 and 18 and persist for short periods in the genital area. The majority of condylomatous lesions in children are HPV types 6 and 11. Acquisition of HPV during childhood and adolescence is not an immediate cause of severe morbidity. However, significant evidence suggests that early exposure not only hastens the development of anogenital cancers but may also increase the actual risk of cancer. Future studies should include large scale longitudinal designs to test and follow neonates with careful attention to sexual abuse issues during the prospective period so that we may confidently diagnose and appropriately treat children with HPV infections. The role of age and immunity remains confusing, supporting the need for studies to examine cervical immunity more closely (Moscicki 1996).

In the USA, 50% of adolescent and young women acquire HPV within 3 years after initiating sexual intercourse, resulting in relatively high prevalence rates. Most infections, however, are transient and clear within several months. Consequently, HPV infections detected in adolescents are likely to reflect benign disease, whereas infections detected in older women are likely to reflect persistent infections and a higher risk of advanced cervical intraepithelial lesions that can lead to invasive cervical cancer (Widdice and Moscicki 2008). Natural history

studies of HPV infection in healthy young women show that infection is quite prevalent, but is generally transient. New and sensitive technologies such as HPV DNA testing and liquid-bases cytology (LBC) are more likely to detect cytologic abnormalities in young women who are at LR for actual invasive cervical disease. This sensitivity potentially places adolescents at risk for increased anxiety, testing, and intervention. The multicenter atypical squamous cells of undetermined significance (ASC-US)-L-SIL Triage Study (ALTS) has shown that HPV DNA testing can be used safely to minimize intervention in many cases. HPV infection is common in young women, but rarely progresses to invasive cervical disease (Gray and Walzer 2004). The typical HPV infection will resolve in approximately 1 year. The American Cancer Society (ACS) has recommended that Pap test screening begin at 21, or 3 years after the onset of sexual activity. The American Society of Colposcopy and Cervical Pathology (ASCCP) guidelines for the management of CIN 1 conclude that observation is the preferred therapy (Guido 2004). Natural history studies of HPV, in fact, suggest there is little risk of a significant pre-cancerous lesion going undetected within the first 3–5 years after the onset of sexual activity. So, the ASCCP allows for follow-up of L-SIL with repeated cytology or HPV DNA testing rather than immediate referral to colposcopy (Moscicki 2005a). Because of the high-rate of regression of L-SIL in adolescents, the cytologic study should be repeated within 6–12 months. Colposcopy should be reserved for persistent L-SIL. Patients with H-SIL should be referred for colposcopy and biopsy. Confirmed H-SIL should be treated with cryotherapy, laser therapy, or loop electrosurgical excision procedure (LEEP) (Leung et al. 2005). The H-SIL detected, however, does not appear to progress rapidly to invasive cancer. Understanding the natural history of HPV in adolescents has shed light into optional treatment strategies that include watchful observation of ASC-US and L-SIL (Moscicki 2007). Current strategies for adolescents with abnormal cytology include conservative management, avoiding invasive procedures. For cytologic ASC-US or L-SIL, management can be obtaining cytology only at 1-year intervals for up to 2 years before referral for colposcopy is necessary. For biopsy-proven CIN 1, management is similar with yearly cytology indefinitely or until H-SIL or CIN 2–3 develops. CIN 2–3 in compliant adolescents can be managed with 6-month cytology and colposcopy up to 2 years (Moscicki 2008a, b).

The primary risk factors for acquiring HPV are generally associated with sexual activity. Evidence suggests that condoms provide some protection against infection and disease progression, but any genital contact is sufficient for HPV transmission. HPV is so common and transmissible that having just one sexual partner often results in infection. Indeed, cumulative prevalence rates are as high as 82% among adolescent women in selected populations. As such, nearly all sexually active adolescents are at HR for acquiring HPV. Persistent infection with HR-HPV types is considered necessary for the development of cervical cancer, whereas infection with LR-HPV types is associated with the development of genital warts and other LG genital abnormalities. Most infections are asymptomatic and are efficiently cleared by the immune system. Similarly, both LG and HG lesions caused by HPV can regress in adolescent and young adult women. Treatment guidelines allow for observation of adolescent

women who develop LG lesions rather than immediate colposcopy. Nonetheless, a small percentage of adolescents will develop precancerous lesions that may progress to invasive cervical cancer (Moscicki 2005b). Clinical cancer of the cervix is rare in adolescence. The treatment is radical surgery. Preclinical stages CIN 3 (severe dysplasia, CIS) and micro-invasive cancer (stages IA1, IA2) are important because of their frequency. Diagnosis is based on colposcopy, cytology, direct biopsy, histological examination, and conization. In addition, virology (HPV) and DNA cytometry may become prognostic factors. Treatment consists of conization with an exact histological examination in serial sections as a basis for preserving the uterus (Tscherne 1997). The association between age of first intercourse and invasive cancer cannot be ignored. Consequently, initiating screening at appropriate times in this vulnerable group is essential. In addition, with the advent of the HPV vaccine, vaccination prior to the onset of sexual activity is critical since most infections occur within a short time frame post initiation (Moscicki 2007).

Two prophylactic vaccines using virus-like particles (VLP) of L1 capside protein of HPV-16 and HPV-18 have been developed and have been approved by Food and Drug Administration (FDA), European Medicines Agency (EMEA), and Agenzia Italiana del Farmaco (AIFA) for use in 9–26 year-old females. Of these, one also contains VLP of HPV-6 and HPV-11. Large scale studies have shown that these vaccines are safe, well tolerated, elicit high levels of neutralizing antibodies, prevent chronic HPV infections due to genotypes present in the vaccine, and associated cervical lesions (and genital warts for the quadrivalent vaccine). To be effective, the vaccines should be given prior to sexual debut. In Italy, the vaccination is offered to 12 years-old girls (Bartolozzi et al. 2007). The pre-specified, end-of-study combined analysis of HPV vaccine efficacy studies for prevention of cervical cancer, and now also for prevention of vulvar and vaginal cancers, confirmed 98–100% vaccine efficacies. Post licensure surveillance identified a new association of vaccine administration with syncope, and provides assurance of the safety of inadvertent vaccination during pregnancy. Several cost-effectiveness analyses consistently demonstrated that HPV vaccination of 12-years-old girls and catch-up vaccination through 18 years of age, and possibly to 26 years of age, is cost-effective, although the thresholds of affordability vary by study (Jenson 2009).

Educational strategies are essential, given several new and highly effective technologies to prevent HPV and related diseases such as cervical cancer. Although little has been published regarding adolescent knowledge about HPV and HPV vaccines, studies conducted primarily in adult women demonstrate that knowledge generally is poor. Studies of adolescent attitudes about HPV vaccines have identified several modifiable factors associated with intention and confidence in one's ability to receive the vaccine, including higher perceived severity of cervical cancer and fewer barriers to vaccination. Studies of clinician attitudes about HPV vaccines have demonstrated that although clinicians generally support vaccination, some report concerns. For example, adolescents may practice riskier sexual behaviors after vaccination. Studies also show that clinicians believe that educational materials developed specifically for adolescents are essential. The recent literature on adolescent knowledge about HPV and attitudes about HPV vaccines supports the importance of

designing developmentally appropriate educational materials for adolescents about HPV and HPV vaccines, and provides guidance for the development of key educational messages (Kollar and Kahn 2008; Mammas et al. 2009).

6 Conclusions

HPV is a common viral infection of squamous epithelial tissues, but the medical community has only recently recognized its importance. HPV are now realized to consist of many genotypes and are associated with a diverse spectrum of clinical manifestations. Within the genital tract, some diseases have been recognized since antiquity, i.e. genital warts which are caused by HPV types distinct from those causing genital cancer. However, others (such as cervical cancer), although recognized centuries ago as linked to sexual activity, have only been associated with oncogenic HPV relatively recently, with the tools of molecular biology. We now understand that genital HPV infections are the most common STD, are largely transient, asymptomatic, and of no consequence. This virus manifests as more than just benign warts. Chronic carriage of with oncogenic genotypes (over years and in a minority of patients), together with other co-factors (host and/or exogenous) in complex pathways not totally understood, result in severe dysplasia or, ultimately, carcinogenesis. As it takes time for precursor lesions to develop and there are effective screening programs for their detection and treatment, HPV-related neoplastic disease of the cervix is largely a preventable reproductive health issue of women. Yet, on a global scale, cervical cancer is the second most common cancer of women, with the majority of cases occurring in developing countries. Although HPV is non-cultivatable by traditional diagnostic virological methods, successfully applied molecular biology techniques have underpinned development of vaccines. Successful vaccination ultimately has the greatest potential to impact upon the global burden of disease from genital HPV infection. However, the outcome from reduction in incidence of dysplasia and neoplasia will take years to eventuate. Consequently, various cervical cancer prevention strategies still need to be endorsed and maintained in the meantime (Garland 2002).

In the first half of the twentieth century, Peyton Rous and Colleagues demonstrated the joint action of tars and Shope Papillomavirus to consistently induce SCC in rabbits. Using the Rous model as a prototype, one might hypothesize that some cases of cervical cancer arise from an interaction between oncogenic viruses and cervical tar exposures. Cervical tar exposures include cigarette smoking, use of tar-based vaginal douches, and long years of inhaling smoke from wood and coal burning stoves in poorly ventilated kitchens (Haverkos 2005). More than 90 years have passed since Peyton Rous reported that a tumor was transmitted between chickens like an infection disease. Currently, viruses are considered the second most important cause of cancer in humans and contribute to 10–20% of all cancer cases in the world, some of them being very common, like cervical and hepatocellular carcinomas. Human recognized cancer viruses include HPV, Hepatitis B Virus

(HBV), Hepatitis C Virus (HCV), Epstein-Barr Virus (EBV), Herpes Hominis Virus 8 (HHV-8), and Human T-cell Lymphotropic Virus (HTLV)-1. The knowledge of how viruses participate in the etiopathogenesis of cancer will allow fighting the disease with similar strategies than those that we use to control those infective agents nowadays. Great efforts are being initiated to decrease incidence of the neoplasms by preventing the initial infection or by prophylactic vaccination (Delgado-Enciso et al. 2004). So, approximately 35 years ago a role of HPV in cervical cancer has been postulated. Today, it is well established that this very heterogeneous virus family harbors important human carcinogens, causing not only the vast majority of cervical, but also a substantial proportion of other anogenital and head and neck cancers. In addition, specific types have been linked to certain cutaneous cancers. In females, HPV infections on a global scale account for more than 50% of infection-linked cancers, in males for barely 5%. Vaccines against HR-HPV types 16 and 18 represent the first preventive vaccines directly developed to protect against a major human cancer (cervical carcinoma) (zur Hausen 2009).

Over the past several years, the majority of research on the pathology of the cervix has been focused on HPV and its role in the pathogenesis of cervical neoplasia. Several major points have emerged from these studies. First, the incidence of latent HPV infection in the general population is greater than previously thought. Up to 31% of a college-aged population has HPV DNA detected in cervical swabs. Second, in situ hybridization (ISH) to detect HPV has been found to be a useful quality control measure for laboratories diagnosing cervical lesions. Finally, it is now recognized that many different HPV types can infect the cervix and be associated with cervical neoplasia. With regard to treatment, the introduction of LEEP for treating CIN lesions promises to have a significant impact on the management of cervical disease (Richart and Wright 1991). More than 200 different types of HPV have been isolated. More than 40 of these types infect the epithelial lining of the anogenital tract and other mucosal areas. In the majority of individuals, HPV infections are transient and asymptomatic with most new infections resolving within 2 years. Epidemiological data from the USA National Health and Nutrition Examination Survey determined that the prevalence of HPV infection in a representative sample of women was highest in those aged 20–24 years (44.8%). HPV infection has been firmly established as the primary cause of cervical cancer. It is not clearly understood why HPV infections resolve in certain individuals and result in CIN in others, but several factors are thought to play a role, including individual susceptibility, immune status and nutrition, endogenous and exogenous hormones, tobacco smoking, parity, co-infection with other sexually transmitted agents such as HIV, HSV-2, and Chlamydia trachomatis as well as viral characteristics such as HPV type, concomitant infection with other types, viral load, HPV variant, and viral integration. Worldwide, pooled data from case–control studies indicated that HPV DNA could be detected in 99.7% of women with histologically confirmed cervical SCC compared with 13.4% of control women. Both HPV infection and cervical cancer are associated with a substantial economic burden. Pharmaco-economic data from the USA indicate that HPV infection and HIV were associated with similar total direct medical costs, and HPV infection was more costly than genital herpes and hepatitis B combined in

the 15–25 year age group. Furthermore, false-negative Pap smears from women with precancerous lesions are among the most frequent reasons for medical malpractice litigation in the USA (Steben and Duarte-Franco 2007).

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Chapter 3
The Immunobiology of Human Papillomavirus Associated Oncogenesis

Peter L. Stern and Mark H. Einstein

Infection of many types of anogenital epithelia by specific HPV types contributes to the development of anogenital cancer with HPV16 and HPV18 the most oncogenic and common types present in the carcinomas (Bosch et al. 2008). For anogenital infections the key target tissues include the cervix transformation zone or the anal dentate line. During the infectious cycle of the virus there is a reprogramming and hijacking of cellular functions that support virus replication and viral particle production. The latter process can be achieved without necessarily activating host immunity and occasionally this can allow for persistence of infection, which is the major risk factor for development of an intraepithelial neoplasia and subsequently invasive cancer (Walboomers et al. 1999). The immunobiological events accounting for natural control of HPV infection and development of neoplasia are difficult to investigate because of the extended times wherein following exposure, infection is established (weeks–months), intraepithelial neoplasia develops (months to years) or a cancer emerges (years to decades). Individuals with iatrogenic immunosuppression or primary immunodeficiencies are predisposed to persistent HPV infection and subsequent carcinoma, which clearly demonstrates the importance of natural immune control (Strickler et al. 2005). Other genetic factors, such as polymorphism, may also influence immune function and control (Einstein et al. 2009a; Wang et al. 2009; Yu et al. 2007). Avoiding the immune system is likely to be important in allowing for HPV persistence but malignant transformation also requires intracellular replication

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and expression of the HPV genome in addition to integration into the host genome. The recognition that oncogenic HPV is the cause of anogenital cancer has driven the quest for vaccines to protect against or treat persistent HPV infections. Preclinical studies have established that antibodies to animal papillomaviruses can be protective against infection and HPV oncogene products can be used as targets for inducing tumor immunity in animal models. This review describes the current understanding of natural immune control and identifies the unique hurdles in prophylactic and therapeutic vaccine design for HPV compared to other common viral pathogens which derive from its fundamentally stealthy lifecycle which occurs entirely within the target epithelium with no systemic response or viremia.

1 Cells and Molecular Biology of Virus Infection

HPV is a small virus of 55 nm in diameter and comprises a double-stranded circular DNA of nearly 8,000 bp. The HPV genome encodes eight proteins: early proteins E5, E6 and E7 are involved in cell proliferation and survival, and E6 and E7 play a key role in HPV-associated carcinogenesis; three other early proteins (E1, E2 and E4) are involved in control of viral gene transcription and viral DNA replication; two late proteins L1 and L2 are involved in assembly of new virus particles. The virus particle is composed of 72 capsomeres each composed of major L1 and minor L2 capsid proteins. The virus has evolved a replication cycle which is intimately linked to the life history of the differentiating epithelial cell where the small number of HPV genes coordinate in function with the host cell's own mechanisms to support viral replication and the production of large numbers of new infectious virions (Howley and Lowy 2007; Doorbar 2006).

The dependence on epithelial differentiation for virion production has limited the study of the early steps in infection by native oncogenic HPV. However, using HPV L1 pseudo-virion particles, self-assembling proteins that resemble the HPV L1 capsid, in both in vitro and in vivo animal models has established some of the key factors involved. Heparan sulphate proteoglycans (HSPG) are the primary attachment factors for most HPV types (Shafti-Keramat et al. 2003). These are ubiquitously expressed on mammalian cells and are integral components of the basement membrane (BM) and extracellular matrix that surrounds most tissues. It is thought that minor damage to the tissue exposes the BM allowing for a primary step of viral capsid binding with a subsequent adsorption to the basal surface of epithelial cells during their regrowth and repair, which enables virion uptake. On cell binding, there is a capsid conformation change which exposes an L2 furin consensus site which allows cleavage of N-terminal sequence from L2 and internalization which can occur rapidly (Richards et al. 2006). This is consistent with the requirement of the minor capsid protein L2 for production of fully infectious HPV virions. An elegant murine cervicovaginal challenge model showed that HPV 16 L1/L2 pseudovirion particles that resemble papillomavirus (Buck and Thompson 2007), can cause widespread infection of epithelia within 48–72 h (Roberts et al. 2007).

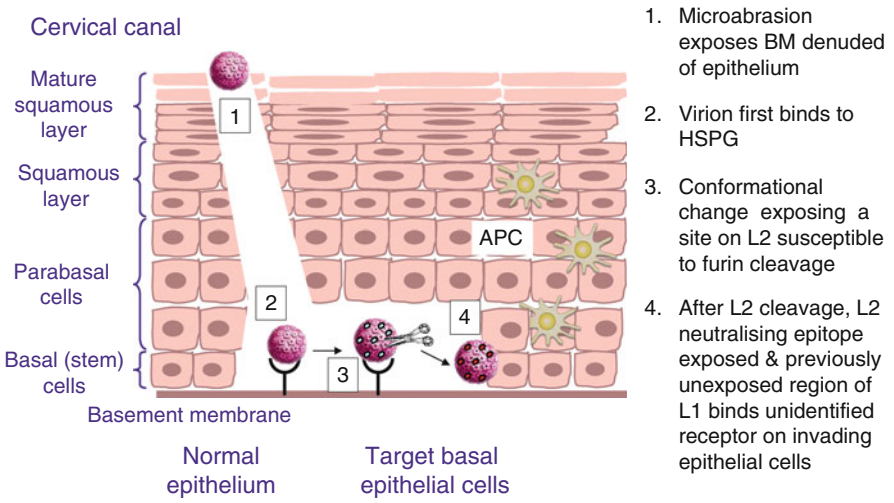

Fig. 3.1 Virus infection: Uptake and internalization. Based on the cervicovaginal challenge model the initial steps of HPV infection depend on exposure of the basement membrane, for example of the transformation zone of the cervix, through micro-abrasion, followed by binding through L1 protein to the heparan sulphate proteoglycans leading to conformational changes which expose L2 to a furin protease cleavage with exposure of L2 epitopes necessary for stable association with an unidentified receptor on epithelial target cell

Recent studies (Day et al. 2010; Kines et al. 2009) describe the use of the cervicovaginal challenge murine model of HPV infection to dissect distinct steps in the process and the nature of the vaccine-induced antibodies that can interfere. Importantly, the data emphasizes how cell culture methods are unable to mimic the in vivo conditions a thus give potentially misleading results. This work provides formal proof that antibodies mediate the protective capacity of HPV VLP vaccines and a means for further refinement of the specificities and mechanisms involved. The in vivo model delineates the initial steps of HPV infection as taking place at the basement membrane (BM) followed by transition of the L1/L2 capsids to a furin (-like) protease cleaved version with exposure of L2 epitopes necessary for stable association with the epithelial target cell (Fig. 3.1). L1 VLP vaccine induced protection was shown to include at least two distinct mechanisms mediated by polyclonal antibodies, which are concentration dependent. High antibody levels can block binding to HSPG on the BM but lower amounts can block the secondary L1 receptor on epithelial cells. In the latter case, the number of different epitopes recognized has not been determined. As previously described (Gambhira et al. 2007), anti-L2 antibody can also prevent binding and infection on the epithelial targets and importantly are not HPV type specific.

While providing insights into processes likely more relevant to real infection in the genital track than in vitro studies, the model is still a surrogate. It uses HPV L1/L2 pseudovirions for passive challenge and the multiplicity of conditions of "infection" are obviously not physiological. It also does not exclude other methods of infection

not dependent on exposure of the basement membrane and epithelial regrowth. For example, it is not clear whether native virions produced under physiologically relevant conditions of differentiating host tissue show the same dependence on cleavage by a cellular proprotein convertase to undergo a successful infection. Recent work (Cruz et al. 2010) has suggested that when human foreskin keratinocytes are infected with HPV16, HPV18, HPV31 or HPV45 and cultured to produce infectious virus that the HPV16 and -45 produced were not dependent on cleavage by furin. Preliminary studies suggest that the HPV16 native virion L2 N-terminus may be cleaved during virion morphogenesis in the differentiating tissue. Thus, these viruses appear to be independent of primary binding to a heparan sulfate attachment receptor. By contrast, the furin (-like) enzyme is required during infection with HPV18 and HPV31. Nevertheless the cervicovaginal challenge model does show VLP type specificity and cross protection similar to that documented in clinical efficacy studies (Day et al. 2010; Paavonen et al. 2009). These methods may be useful for further characterization of the capsid specific epitopes responsible for virus neutralizing antibodies including the evaluation of sera from immunized women.

After the virus has entered cells in the basal layer of the epithelium, the genome is uncoated and transferred to the cell's nucleus where it exists as a non-integrated circular episome of less than 100 copies per cell; it is possible that the target cells include the reserve, or stem cell, population. The molecular and cell biology underwriting the natural history of the virus infection are intimately entwined (Roberts and Young 2009) (Fig. 3.2). As these infected basal cells undergo cell division, the viral genome replicates and becomes equally segregated between the two daughter cells enabling maintenance of the HPV genome in this cell layer. New virus production is inhibited in the initial basal target cells and the productive infection process only begins with their migration upwards as suprabasal cells to generate the tissue architecture and maintain its constant renewal. In uninfected epithelium the upward migration of basal cells triggers their exit from the cell cycle and they enter the pathway of terminal differentiation. HPV relies upon the host cell for provision of key replication enzymes and other factors necessary to replicate its own genome and its HPV E6 and E7 proteins stimulate continued proliferation of the infected suprabasal cells (and therefore production of the cell's replication machinery) to potentiate survival long enough for the virus to replicate its own genome. Once the virus has amplified its genome, sometimes to levels exceeding many thousands of copies per cell, the HPV life cycle then switches to production of the coat proteins L1 and L2. This stage is controlled by the early E2 protein that down-regulates E6 and E7 protein expression by blocking the binding of transcription factors to the early virus promoter allowing the cell to continue the process of terminal differentiation. New virions are thus assembled in the uppermost cells of the lesion which undergo apoptosis and are sloughed off as the tissue is renewed from below (Conway and Meyers 2009). If all the progeny of an initially infected stem cell differentiate with no reinfection, then the HPV infection will be self-limiting. On the other hand, if an infected stem cell remains out of cycle but with viral episomes, this provides the basis of a latent infection that could be revealed at some later time when there is a call for epithelial differentiation.

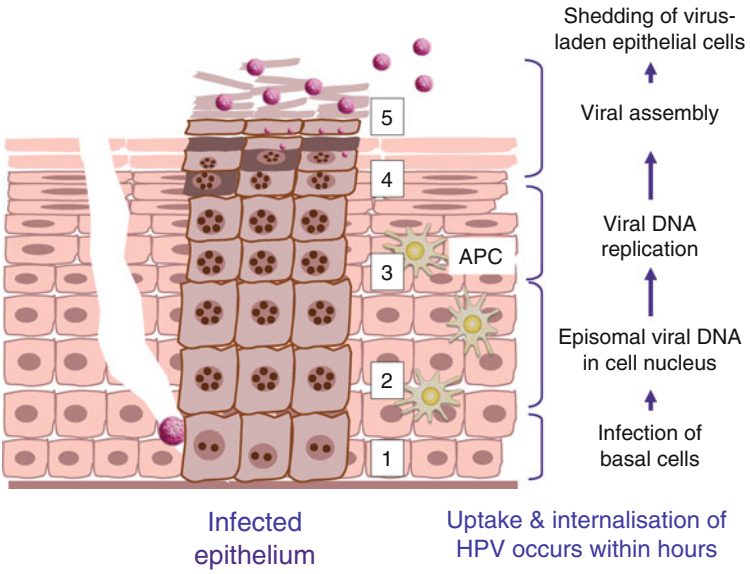

Fig. 3.2 Natural infection: HPV lifecycle in the cervix. The life cycle of productive HPV infection with no viremia or cell death in target. The HPV genome encodes eight genes: the 'early' or nonstructural proteins (E1, E2, E4, E5, E6, E7) and the 'late' or structural proteins (L1, L2). (1) Virus infects a primitive basal keratinocyte at low (<10) copies per cell (E1, E2, E5, E6, E7). (2) Virus and cell replicate together (E1, E2). (3) Viral DNA amplification in non-dividing cells. (4) Viral genomes at 1,000 s per cell (E6, E7, E1, E2, E5). (5) Virus-laden cells ready for desquamation and infection of naïve individual (L1, L2, E4). There is low abundance of viral target proteins in basal cells. This stealthy strategy may cause no activation of innate immunity

An anogenital cancer is a late and rare complication of a persistent oncogenic type HPV infection and is the end result of a chain of events that can take many years to unfold. Infection with HPV is a necessary initial event, but not a sufficient cause of cervical cancer. Indeed, HPV infection is extremely common, with cumulative incidence rates as high as 40% reported among sexually active college age women (Dunne et al. 2007; Ho et al. 1998). In immunocompetent women, though, most HPV infections clear, often within a matter of months. The persistence of HPV infection cumulatively increases the chance of integration of HPV genome into the host DNA (Einstein et al. 2002) and this most frequently functionally deletes the E2 gene which controls the expression of E6 and E7 oncoproteins. Such changes can prevent the terminal epithelial differentiation and thereby halts virion production. The HPV oncogenes E6 and E7 also modulate p53 and Rb mechanisms, respectively, which provide signals for exiting the cell cycle and not allowing time for repair of host DNA allowing for random mutations which increase the chance for the accumulation of genetic mutations that can lead to cellular immortalization and sometimes even malignant conversion (Duensing and Munger 2004; Huang et al. 2008). Immune control of persistent HPV infection is vital to reduce the development of anogenital cancers. This is a battle between immune attack and viral defence, the key features of which are shown in Figs. 3.3 and 3.4.

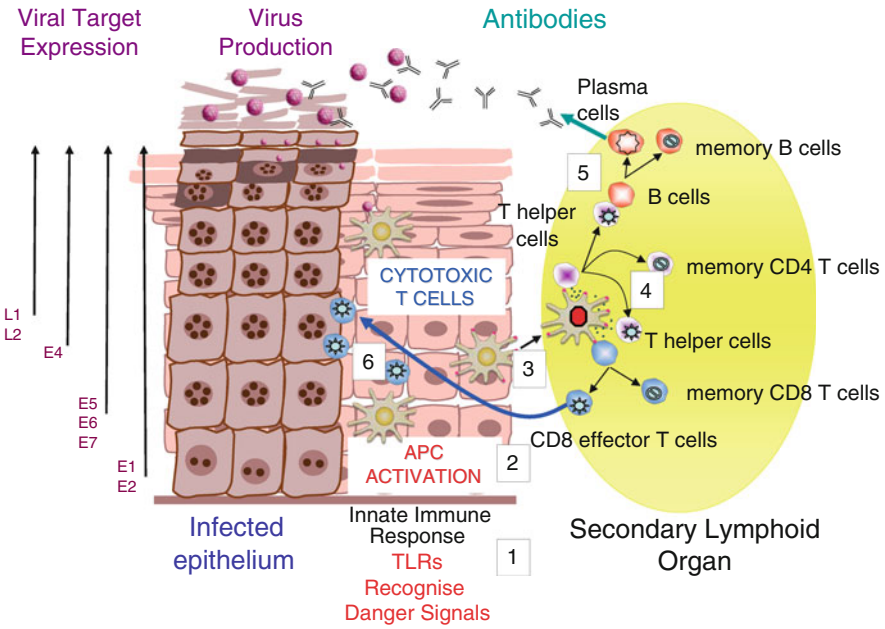

Fig. 3.3 Immune control of HPV infection. (1) Detection of damage through innate immune system. (2) Activation of immediate non-specific effectors; secretion of interferons. (3) Proinflammatory cytokines and chemokines support optimal activation of local antigen presenting Langerhans' cells, viral target antigen processing and migration to locoregional lymph nodes. (4) Optimal activation of adaptive immunity and generation of specific CD4 T helper 1 type immunity supporting development of effector and memory CD8 cytotoxic T cells against viral E2, E6 and E7. Cell mediated immunity is believed to be critical in clearance of virus in basal epithelial cells. (5) T helper cells also support optimal activation of B cells secreting HPV capsid type specific neutralizing antibodies. Long lived plasma cells providing high levels of antibodies can protect against a subsequent infection through transudation into the mucosal secretions or through serous exudation. (6) Multiple HLA restricted CTL specific for viral early antigens traffic to the lesion and target virus infected cells to provide immune control and viral clearance

2 Natural Immune Control

Infectious HPV particles are shed from mucosal surfaces as the result of production in the terminally differentiated cells which result from normal epithelial desquamation with this whole process causing little or no cellular damage (Stanley 2007; Frazer 2009; Einstein et al. 2009b; Stern 2009). This strategy enables the virus to avoid stimulating any inflammatory signals, which could potentially activate the innate immune response. Overall, natural immune control of an HPV infection is likely the result of the interplay of the innate (non-specific) and the adaptive mechanisms delivered by specific antibodies and cellular effectors (e.g. T cells).

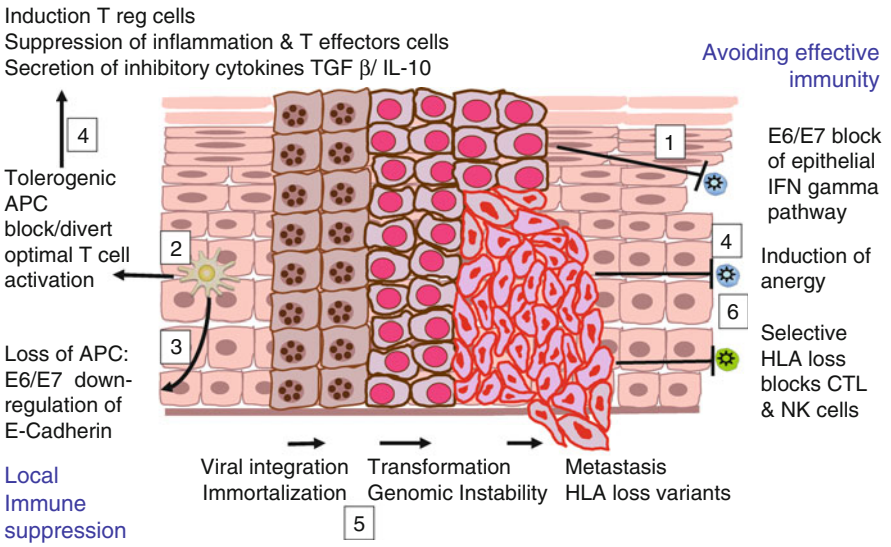

Fig. 3.4 Loss of immune control and escape. (1) E6 and E7 mediated protection against interferons. (2) Immature APC presentation of viral antigens inducing immune tolerance. (3) Reduction of number and activation of Langerhans' cells in intraepithelial lesions inhibiting activation of adaptive immune response. (4) Immune activation is skewed toward T helper 2 responses with cytokine balance supporting differentiation and infiltration of T regulatory cells that limit therapeutic T cell effector mechanisms. The balance of local immune infiltration in persistent infection and chronic stimulation of the immune system with viral antigens can anergize controlling effector responses. (5) Integration of the virus with the host cellular genome blocks the productive life cell cycle, encourages immortalization and generates opportunity for acquisition of mutants which may offer additional means to escape immune control. (6) Frequent mutational events in neoplasia include HLA loss of expression make the tumor cells invisible to the CTL restricted by particular allelic genes but still resistant to NK cells

The innate immune system includes monocytes, macrophages, polymorphic leucocytes, natural killer cells and the sentinel antigen presenting cells (APC). These cells have receptors such as Toll-like-receptors (TLR) or other signaling pathways which can detect evidence of extracellular molecules such as high mannose structures, heat shock proteins, DNA, or RNA which are released from damaged cells or derived from pathogens. When the innate immune effectors are activated they produce cytokines like interferons and/or direct cell lysis of the infected cells. It is possible that innate immunity can clear an HPV infection but often HPV genomes in basal cells will provide a continued reservoir of the infection. Innate immune activation is the critical step in recruitment of the adaptive immune response which subsequently acts like the cavalry coming to the rescue of the innate immune defenders which have held the line while waiting for reinforcements. Importantly the adaptive immune response brings new weaponry with specificity for the particular

threat and numbers to control and subsequently clear the infection. The ability to recognize a pathogen specifically, amplify this activity and retain the memory so that if the pathogen is encountered again it can be dealt with more efficiently are the hallmarks of the adaptive immune response. Importantly, adaptive immunity only gets activated with the correct and sufficient innate immune signals. The lack of any significant cytopathic effect in the process of virus infection and production, limits such innate immune activation which together with low levels of viral gene expression in the lower layers of the epithelium can prevent or significantly delay the adaptive immune response to the initial HPV infection, and thus favor viral persistence.

The principle components of natural immune control of an HPV infection are summarized in Fig. 3.3. The sentinel or antigen presenting cells that are dendritic cells found in the dermis and Langerhans' cells mostly within epidermis and superficial epithelial layers of the mucosa are the key messenger cells for activation of adaptive immunity through detection of HPV infection through their TLRs (Banchereau et al. 2000; Steinman and Hemmi 2006). These recognize specific patterns associated with infectious agents or tissue damage and, when activated, induce cellular expression of many proinflammatory products that combat the immediate threat and alert the adaptive immune response. The APCs are activated by, and integrate, the signals received on the nature of the infection and travel to their local lymph nodes where they direct activation of specific T cell subsets that are appropriate for the particular threat (Niedergang et al. 2004; Wang et al. 2004; Kalinski et al. 1999). Adaptive HPV specific cellular immunity is critical in the clearance of established infection, since tissue type HLA molecules on the surface of infected cells can present evidence of HPV infection. The T cell receptors recognize a complex of virally derived peptides and HLA molecules. APCs have efficient antigen processing mechanisms that generate the viral peptide-HLA complexes that can be presented in conjunction with particular co-stimulatory molecules to activate the appropriate HPV specific T cells in the lymph nodes. Optimally, this process provides for a balance of T helper one and two type responses, which are mediated by different cytokines, leading to the appropriate activation of HPV specific cytotoxic T cells (CTL) and support for production of virus neutralizing antibodies by B cells. In this process, the effector T cells receive direction to the infected site where they can eliminate the virus-infected cells. The life history of the virus only produces viral particles in the terminally differentiated and dead cells so the target virally infected cells, which are likely stem cells in the basal layer, can retain the viral genome as a latent infection. To fully clear any infection, virus specific killer T cells are required since they can recognize the intracellular viral early gene products that are displayed as HLA-peptide complexes on the cell surface and destroy them directly by cytotoxic mechanisms or indirectly through release of interferons or other cytokines. A successful cellular immune reaction will also generate memory T cells that can clear subsequent infections (Kaech et al. 2002).

The detection of T cells specific for HPV early gene products such as E2, E6 or E7 in the blood of patients with HPV associated anogental lesions is often very low. Clearly the functionally relevant T cells would need to be in the lesion to be effective

and any detection in the blood would vary in relation to the nature of lesion and it's the longevity of any persistence or time from clearance. Specific memory T cells are reactivated by subsequent infection and limit the consequences of the infection through clearance (de Gruijl et al. 1996, 1998; Bontkes et al. 2000; van der Burg et al. 2001). However, there is evidence that repertoires of T cell in patients with persistent HPV infections and high grade CIN are depleted of activity versus the HPV proteins E2 and E6 while E6 specific T cells are detectable in patients whose lesions have regressed (de Jong et al. 2004; Farhat et al. 2009).

Appropriately stimulated B cells can produce type specific antibodies to the capsid proteins that neutralize the particular HPV virions. While these may limit local reinfection, they offer reduced or no protection against other HPV types and cannot clear the virally infected cells. Memory B cells provide the ability to immediately produce more specific antibodies on subsequent infections, but local levels of such antibodies could be very important in preventing any infection. The required protection at the mucosal surface occurs through transudation of antibodies to the cervical vaginal mucus or by serous exudation following local trauma (Schwarz 2008). It seems likely that at least some women who fail to develop antibodies against HPV after infection must have cleared their infection using cell mediated innate and/or adaptive immune mechanisms (Viscidi et al. 2004). Analysis of HPV 16 specific antibodies detected in women from the control arm of HPV prophylactic vaccine efficacy studies have shown that only the upper quartile levels are associated with any protection against a reinfection; 80% of natural exposed women show no evidence of protection to the same serotype (Poppe 2010; Safaeian et al. 2010). Given the lack of any local immune tissues in the cervix, any memory B cells induced either naturally or in response to vaccination may be unable to respond quickly enough to produce sufficient levels of local antibodies to prevent the uptake, internalization and execution of the HPV lifecycle. More importantly, current thinking about mechanisms that determine the lifespan of the antibody producing plasma cells and thus the duration of humoral immunity, focus on the need for optimal B-cell signaling during the induction of an antigen-specific response (Amanna and Slifka 2010). This imprinting determines the lifespan of the plasma cells and given the observed natural humoral immunity to oncogenic HPV, this pathogen has clearly evolved to minimize its presentation to the immune response. Even when neutralizing antibodies are detected they have no influence on clearance of the current infection (Hildesheim et al. 2007). When neutralizing antibodies are generated in many individuals exposed to HPV at some level, which is probably the majority of the population, these may have developed as a consequence of effective activation of cellular immunity and the latter effector mechanisms may be pivotal in delivering viral clearance with subsequent antibody development. The lag time between infection with HPV and production of antibodies reinforces the role that innate immunity initially plays and also the effectiveness of viral immune evasion tactics. Indeed, a recent study of the role of sexual activity and HPV infection in adult women concluded that natural humoral immunity does not play a role in controlling reinfection (Trottier et al. 2010).

3 Immune Evasion

The relatively poor humoral immunity detected in many individuals naturally exposed to oncogenic HPV infection is a consequence of a multifaceted adaption of HPV to avoid or hide from effective immune control. In some cases this allows for persistent infection and subsequent transforming events driven by genomic instability derivative from viral integration and can ultimately lead to the emergence of metastatic tumor cells that are also able to avoid critical cellular immunity (Fig. 3.4). Thus, in some cases of HPV infection the sentinel or antigen presenting cells may fail to notice the infection. Without activation of the innate immunity no appropriate controlling proinflammatory response or adaptive immune recruitment occurs. Importantly if the APCs identify HPV infection but are not properly activated they can send negative signals to the adaptive immune mechanisms inducing immune tolerance, thereby energizing effectors which could otherwise combat the infection (Stanley 2007). The virus stealth strategy and only low expression of viral early gene targets within the basal and immediate suprabasal layers of the stratified squamous epithelium can fail to activate the local APCs. While the virions are highly immunogenic structures they are made only in the apical cell layers of the epithelium already committed to terminal differentiation and thus unavailable to engage with the local immune surveillance mechanisms. Langerhans' cells may in fact be resistant to direct activation by viral particle structures in contrast to dendritic cells (Fausch et al. 2002), and in cervical intraepithelial neoplasia (CIN) there is a reduction in numbers and a change in their phenotype (Giannini et al. 2002; Tay et al. 1987; Guess and McCance 2005; Connor et al. 1999; Hubert et al. 2005). The virus has involved additional strategies to limit immune activation including E6 and E7 modulation of the infected cells sensitivity to anti-viral responses of the tissue such as production of interferons (Nees et al. 2000; Chang and Laimins 2000; Barnard and McMillan 1999; Li et al. 1999), other inflammatory mediators (Cho et al. 2001), TLR9 activation (Hasan et al. 2007), and E5 mediated down-regulation of HLA expression (Zhang et al. 2003), all of which facilitate persistent HPV infection. With the latter, the viral and host genomes can become integrated, leading to failure to respond to apoptotic signals and failure of programmed cell death as well as cellular immortalization. The increased expression of viral E6 and E7 oncoproteins interferes with the tumor suppressor genes which guard the host genome by delaying DNA replication if genes are damaged and normally allows sufficient time for their repair (Duensing and Munger 2004). When such errors occur, mutant cells will be generated and the properties enabling unlimited growth, avoidance of immune surveillance and spread will be selected with time and recognized as an invasive cancer.

With persistent HPV infection, CIN or invasive cancer, the constitutive expression of the E6 and E7 oncogenes can provide for chronic antigenic stimulation of the specific T cells. However, the absence of the necessary co-stimulation provided but by optimal APC activation this can induce an anergic response (Kalinski et al. 1999). Further, T-regulatory cells (Treg), which are usually induced as a part of the homeostatic control of immune responses acting to reduce the levels of virus specific cellular immunity when the danger has passed (Wang et al. 2004), can also be induced

by these conditions and contribute to tolerance of viral antigens and the promotion of neoplasia (Belkaid and Tarbell 2009). There are several studies showing increased numbers of Treg in HPV-associated diseases as well as tumors of different types including those of the cervix (van der Burg et al. 2007; Fattorossi et al. 2004; Molling et al. 2007). These effectors can suppress the activity of cytotoxic cells either directly or by the production of cytokines like IL10 and tumor growth factor (TGF)-beta. In studies of recurrent respiratory papillomatosis (RRP), a disease caused by HPV-6 and -11 (two common but low oncogenic risk HPV types that also infect the cervix), there is a dramatic polarization of cell mediated immune responses that are biased towards Th2-like T-cells, cytokine and chemokine repertoires (Bonagura et al. 1999, 2004; DeVoti et al. 2004, 2008; Rosenthal et al. 2005, 2006, 2008), and increased number of $CD4^+$, Foxp3, regulatory T-cells (Treg) (Hatam et al. 2008) in diseased, but not in normal laryngeal tissues.

Thus, both innate and adaptive immune cells orchestrate an inflammatory environment that may function to either stimulate or inhibit lesion growth. Infiltrating cells such as particular types of macrophages or myeloid-derived suppressor cells can influence the T helper cell phenotype (Th2) producing an environment rich in IL-10, IL-4, IL-5, and IL-6 favoring help for B-cell responses. At the same time Tregs are elicited via these antigen-presenting cells presenting viral-antigens, and the resultant secretion of TGF-beta by Treg inhibits the generation of cytotoxic T lymphocytes. However, when Th1 T cells are stimulated by properly activated antigen presenting cell dendritic or Langerhans cells, an environment rich in IL-12, interferon-γ, tumor necrosis factor (TNF) -alpha, and IL-2 is produced where a functional CTL response can predominate (Disis 2010). Model studies using murine skin grafts expressing E6 or E7 proteins of HPV 16, have also implicated natural killer (NK) T cells, which preferentially target the glycolipid alpha-galactosylceramide, as having an immune modulatory role on CTL activity (Bhat et al. 2011).

The balance of lesion infiltrating immune cells is likely to be pivotal for the outcome of any HPV infection. One might speculate that the evolution of HPV has exploited a skew of cellular immunity to a Th2 and/or a Treg rather than a Th1 type response, which favors a sustained HPV infection. Indeed there is evidence that in cervical intraepithelial neoplasia, the in situ cytokine balance is associated with an environment that favors lesion persistence (Clerici et al. 1997; Saleh et al. 1998). Importantly, the continued assault of an HPV lesion by interferons produced by chronic inflammation can promote viral genome integration, a key step in the natural history of carcinogenesis (Herdman et al. 2006).

HPV specific T cells may also fail to gain access to the dysplastic epithelium. A recent study of T cell infiltration used a prospective observational cohort of patients with colposcopy directed, biopsy confirmed HPV 16+ CIN2/3, who were monitored for 15 weeks before standard excision treatment; CD8 T cell infiltration was assessed by immunohistochemistry in the epithelial and stroma areas (Trimble et al. 2010). At diagnosis, in normal cervical mucosa, CD8 T cells were sparsely distributed, perivascularly at the epithelial-stroma interface. Intralesional CD8 T cell infiltrate density was significantly higher in regressor compared to non-regressor lesions whereas the stroma infiltrates, which were higher in dysplastic lesions, did not predict

regression. Importantly, non-regressing lesions were associated with recruitment of activated memory T cells (CD45RO+CCR7+CD62L+) but these clearly failed to access the epithelial compartment. Nearly all the T cells isolated from the cervix were α4β7 integrin positive and the adhesion molecule that mediates homing, MAdCAM-1 was upregulated on the vascular endothelium and colocalized in the dysplastic epithelium but not the normal epithelium. Recent studies showing some success in influence of immune modulators like imiquimod in treating high grade HPV 16 lesions of the vulva might reflect affects on both vascular endothelial addressin expression as well as antigen presentation through TLR7 (van Seters et al. 2008). Whether other T cell subsets (e.g. Treg) have different factors favoring their infiltration which can act to thwart the potentially curative CD8 cells remains to be established.

As in many cancers avoidance of tumor specific T cells can occur because the target cells lose HLA expression. In HPV associated neoplasia, T cell effectors specific for the oncogenes will be unable to recognize the target cells because there is no cell surface HLA-HPV peptide complexes for the T cell receptor (TCR) to recognize (Bontkes et al. 1998; Brady et al. 2000; Keating et al. 1995; Koopman et al. 2000). Such HLA down regulation is often associated with increased sensitivity to NK cells, but the tumor phenotype reflects the selection of allelic and/or locus specific HLA expression loss which conspires to provide immune escape from specific T cells, Residual HLA expression for control of the NK cells occurs through killer inhibitory receptor engagement (Garrido et al. 1997; Orange et al. 2002). In some circumstances the balance of positive and negative immune factors may be changed so that the lesions can be cleared (Piersma et al. 2007) and therapeutic vaccine studies exploiting optimized cell-mediated targeted immune responses are in progress (Frazer 2009; Kadish and Einstein 2005). A key influence of Tregs is supported by studies showing that the therapeutic impact of local imiquimod treatment of HPV 16 driven high grade vulvar intraepithelial lesions followed either by photodynamic therapy or TA-CIN (E6E7L2 fusion protein) vaccination depends on the differential immune response of responders and non-responders correlating both locally with Treg density and systemically with E6/E7 T cell responses (Daayana et al. 2010; Winters et al. 2008). The balance of contributions and the kinetics of the various immune processes across the natural history of cervical neoplasia and in particular the reasons why immune control fails in some individuals increasing the likelihood of progression is still sparsely documented. The importance of immunogenetic factors in susceptibility and/or progression of oncogenic HPV infections also needs further study (Einstein et al. 2009a; Apple et al. 1995).

Persistent high risk HPV 16 and 18 infections carry the principle risk for developing cervical neoplasia. While HPV infection in young women is very frequent, most of the time it is cleared naturally. There are currently two licensed prophylactic vaccines available which include HPV 16 and 18 L1 virus like particles which can generate supra normal levels of neutralizing antibodies and are highly efficacious in protection of clinically-relevant neoplasia for those who receive the vaccine prior to exposure of the vaccine related types. Unfortunately, most women worldwide will not receive this type of vaccination, particularly those in low-resource regions

who carry the principle burden of cervical cancer as well as those who already have persistent HPV infections (Hildesheim et al. 2007; Garland et al. 2007). Furthermore, the vaccines will reduce, but not eliminate cervical cancer or HPV infection, since many HPV types are not included in the current vaccines and cross-protection is limited. Current treatment strategies for the management of precancerous cervical lesions are extirpative and deliver high rates of efficacy but do have clinical risks for future childbearing (Kyrgiou et al. 2006; Sadler et al. 2004). A better understanding of the immune mechanisms that govern the balance of T cell subsets as well as other immune cell infiltration and function locally within a HPV-infected anogenital neoplasia (clearance vs. persistence immune profiling) is required. This will be central to developing effective immune-based therapies, microbicides, and local or systemic therapeutic vaccines to treat oncogenic HPV infection, CIN, and cervical cancer, and other anogenital cancers.

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Chapter 4
The Epidemiology of Cervical Cancer

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1 Introduction

Cervical cancer, or cancer of the cervix (CC) is one of the few malignancies in the twenty-first century that can be considered a largely preventable disease through vaccination and/or adequate screening. However, CC remains a major problem in many societies, particularly for those regions in the world with limited economical resources. CC ranks highly among the most common cancers in women. The burden of disease not only affects the women themselves but also their children and extended family.

For more than a century, epidemiological observations had pointed out the parallels between sexual behavior and cervical cancer, leading to the hypothesis that one (or several) sexually transmittable agent(s) had to be a major cause of cervical cancer. Only in the last decade has the etiology of cervical cancer been established: it is now recognized that over 99% of the cases in all countries are related to certain types of Human Papillomaviruses (HPV) (Bosch et al. 2002; Walboomers et al. 1999). The best marker of exposure is currently the detection of type-specific HPV DNA (Deoxi-nucleic-acid) in cancer cells. These infections are common in the

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young age groups (i.e. prevalence as high as 30–50%) and resolve spontaneously in most instances (Bruni et al. 2010). The typical HPV DNA prevalence after the third decade lies between 5% and 20% among women in most populations and these probably form the true high-risk group for cervical cancer (Bruni et al. 2010). These findings offer new opportunities for improving screening and primary prevention of cervical cancer through HPV testing and vaccination. However, they also bring the troubles and emotional issues associated with the management of a potentially oncogenic sexually transmitted disease to the screening scenario.

2 Incidence and Mortality of Cervical Cancer. Worldwide Perspective

Based on crude incidence rates, cancer of the cervix is the third most common cancer in women worldwide after breast and colorectal cancer, excluding non-melanocytic skin cancers. The International Agency for Research on Cancer (IARC) estimated that the number of new cases of invasive cancer of the cervix in 2008 was 530,232 (about 9% of new cancer cases in women), with over 275,000 deaths from the disease (Ferlay et al. 2008). More than 85% of the global incidences occur in less developed regions, where it accounts for 13% of all female cancers (Fig. 4.1).

In developing regions, cervical cancer is the second most common after breast cancer. However, if age-standardized incidence rates are used, cervical cancer ranks as the second most common female cancer worldwide (Fig. 4.2) (Ferlay et al. 2008; United Nations Statistics Division 2011) and ranks third in mortality after breast and lung cancers (Ferlay et al. 2008; Arbyn et al. 2011a). Among all female cancers, cervical cancer ranks first in Sub-Saharan Africa and South Central Asia and Melanesia, second in Latin America and the Caribbean, and third in Central and Eastern Europe and in the Middle East and Northern Africa (MENA) (Fig. 4.2). Further examination of the data at a single country level can be obtained from the WHO/ICO Information Centre on HPV and Cervical Cancer (http://www.who.int/hpvcentre/en/).

Cervical cancer primarily affects young adult women, with overwhelming consequences not only individually but also socially in terms of child care and labor force. Figure 4.3 (Ferlay et al. 2008) shows maps with the ranking of cervical cancer incidences relative to other female cancers in women of all ages (*Panel A*) versus women aged 15–44 years (*Panel B*). Whereas cervical cancer incidence ranks among the three most common cancers in 61% of the countries among women of all ages, the corresponding percentage in younger women increases up to 85% (*Panel B*). In more developed regions, these differences are even greater.

Figure 4.4 shows maps with the age-adjusted cervical cancer incidence rates across the world and by region (Ferlay et al. 2008). There are marked differences within and between regions with a range of at least 30-fold. Higher rates of occurrence are observed in lower-income regions as compared to those observed in higher-income regions. Within the same population, the risk of cervical cancer is approximately

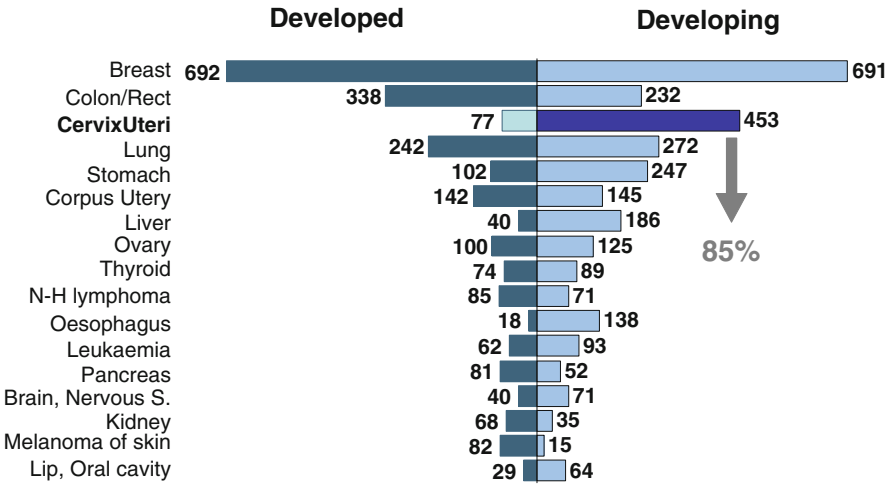


Fig. 4.1 The most frequent cancers in women worldwide (in thousands of new cases per year) (Data source: (Ferlay et al. 2008). Available at: HPV Information Centre. Human Papillomavirus and Related Cancers in World. Summary Report 2010. [Accessed: June 2011]. Available at www. who. int/hpvcentre)

two times higher in lower-income women than in more affluent women. High-risk regions include Eastern and Western Africa (ASIR greater than 30 per 100,000), Southern Africa (26.8 per 100,000), South-Central Asia (24.6 per 100,000), South America, Melanesia and Middle Africa (ASIRs 23.9, 23.7 and 23.0 per 100,000, respectively). Incidence rates are lower in Western Asia, North America and Australia/New Zealand (ASRs less than 6 per 100,000). The large regional variation in cervical cancer rates mostly reflects geographic differences in HPV prevalence and the availability of cervical cancer screening.

Mortality rates are substantially lower than incidence rates. Worldwide, the ratio of mortality to incidence is 52%. IARC estimated 275,000 deaths from cervical cancer in 2008, about 88% of which was in less developed regions: 53,000 in Africa, 31,700 in Latin America and the Caribbean, and 159,800 in Asia (Ferlay et al. 2008). 1.6% of women worldwide are expected to develop cervical cancer before the age of 75 years, and 0.9% to die from the disease. 1.1% of women from less developed regions are expected to die versus a 0.3% of women from more developed areas (Ferlay et al. 2008).

Data on survival rates is less available as compared to incidence or mortality data. Survival statistics are only available in limited world areas and on few occasions when the data is nationally based (as in the UK) as opposed to regionally based. Survival rates vary between regions. Best standards are observed in highly resourced societies (65.8% at 5 years in US registries and 60.4% in Europe (Verdecchia et al. 2007)). In most countries of sub-Saharan Africa survival is poor, with up to 5% of women dying of cervical cancer in countries such as Zambia o Guinea (Ferlay et al. 2008; Verdecchia et al. 2007; Sankaranarayanan et al. 2010).

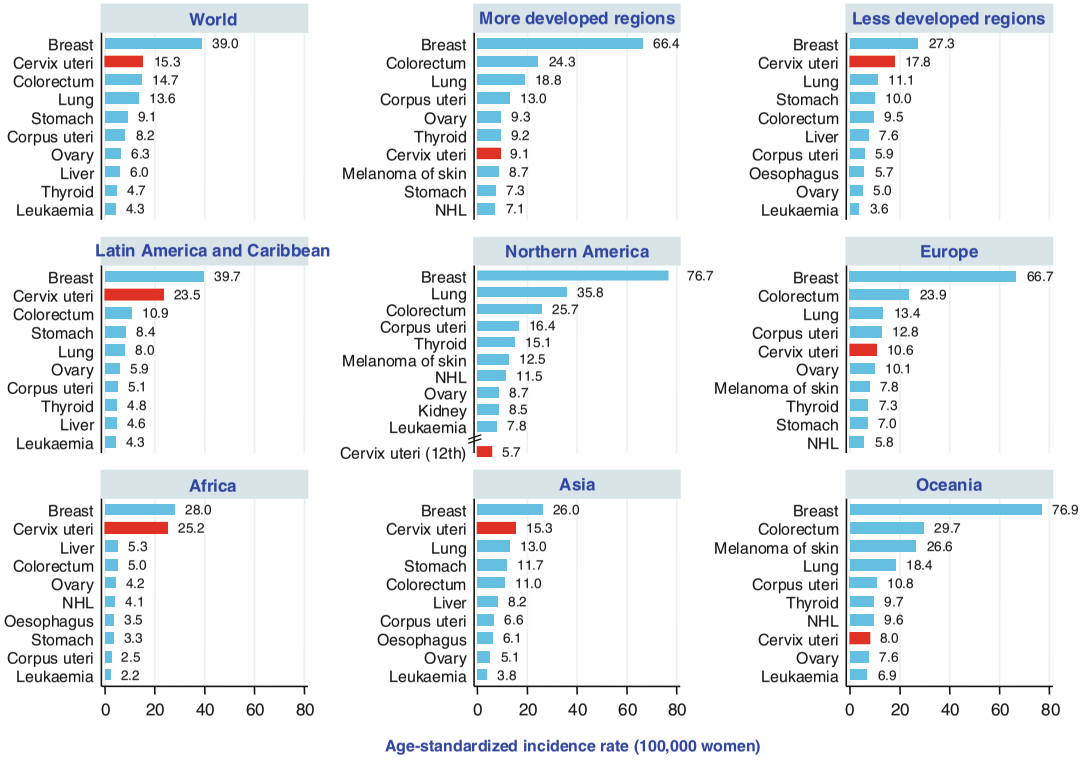
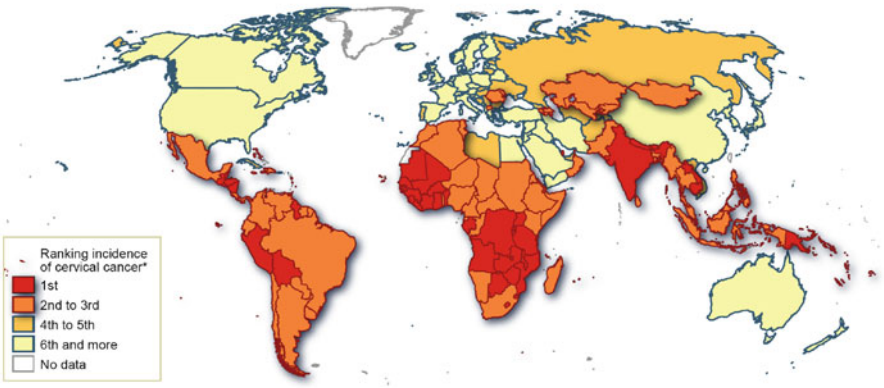


Fig. 4.2 Cervical cancer incidence rates relative to other female cancers by geographical region. NHL: Non-Hodgkin lymphoma. More developed regions: Northern America, Europe, Japan, Australia and New Zealand. Less developed regions: Africa, Latin America and Caribbean, Asia excluding Japan, Oceania excluding Australia and New Zealand (United Nations Statistics Division 2011). The rest of regions follow IARC Globocan classification (Data source (Ferlay et al. 2008))

a : women of all ages.

Percentage of countries in which cervical cancer incidence ranks among the three most common female cancers: 61%



b : women 18-44 years.

Percentage of countries in which cervical cancer incidence ranks among the three most common female cancers: 85%

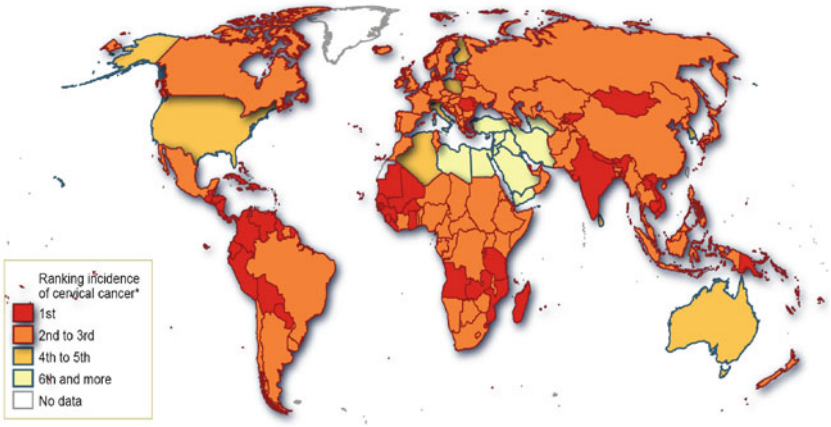


Fig. 4.3 Ranking of cervical cancer incidence relative to other female cancers in women of all ages (*Panel A*) and in women 15–44 years of age (*Panel B*) (Data source: (Ferlay et al. 2008). Available at: HPV Information Centre. Human Papillomavirus and Related Cancers in World. Summary Report 2010. [Accessed: June 2011]. Available at www. who. int/hpvcentre)

The fact that cervical cancer occurs at a relatively young age results in proportionally more years of life lost (YLLs), compared with other major cancers. Moreover, the burden of premature deaths, counted as YLLs, is inversely proportional to income. Cervical cancer was the single biggest cause of YLLs from cancer in low-income

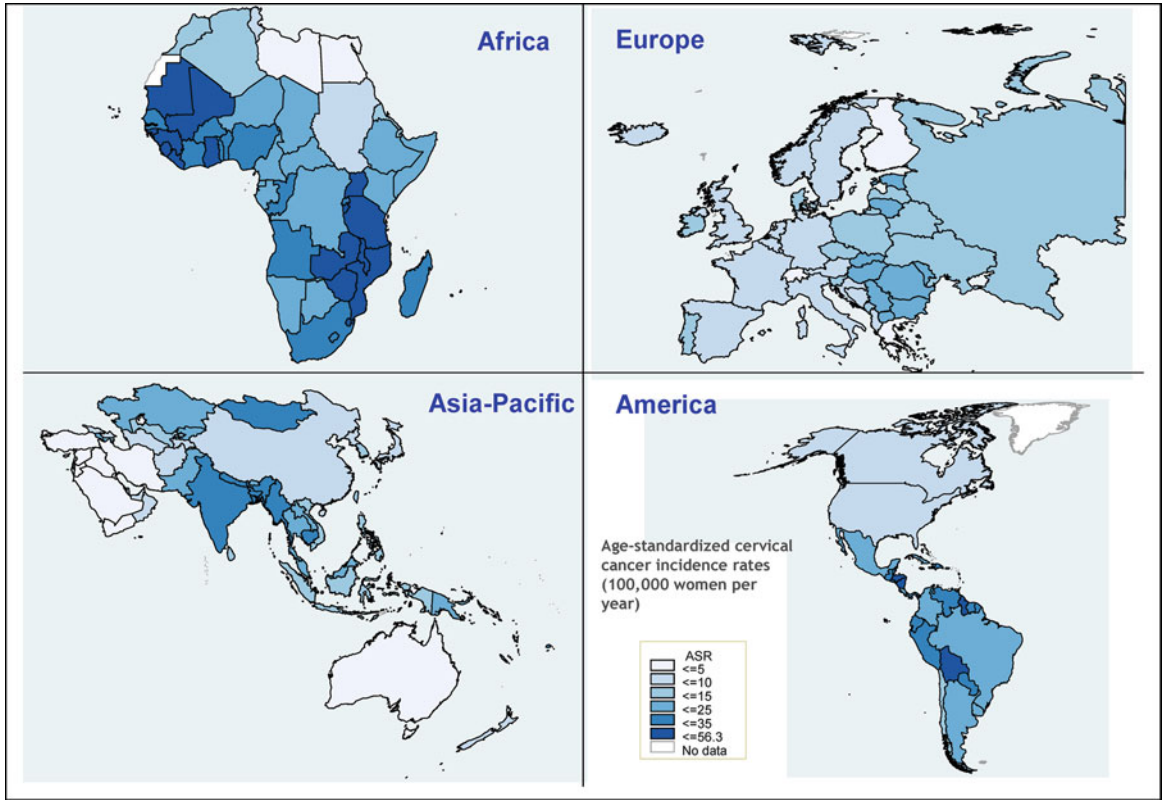


Fig. 4.4 Age-standardized incidence rates to the World standard population for cervical cancer in Africa, America, Asia-Pacific, and Europe (Data source: (Ferlay et al. 2008). Available at: HPV Information Centre. Human Papillomavirus and Related Cancers in World. Summary Report 2010. [Accessed: June 2011]. Available at www. who. int/hpvcentre)

countries in 2004, accounting for 3.4 million YLLs worldwide. It was responsible for the 20% of premature cancer deaths (22% in women aged 15–59 years) in 2004 (Mathers and World Health Organization 2008). Cervical cancer is a disease of inequality, a paradigm of global health disparity (Varughese and Richman 2010). Cervical cancer takes a toll on young women from the poorest countries and the most disadvantaged populations.

3 Time Trends in Cervical Cancer

Incidence and mortality due to cervical cancer have been declining in most developed countries since the 1960s. Part of the reduction is attributed to the widespread screening practices as well as the increasing level of obstetrical and gynecological care being offered to women. Correspondingly, in countries where such developments have not been implemented, the scarce available data suggests that incidence and mortality have remained constant or have even increased.

The declining trends in incidence and mortality in the Nordic countries are the best-known examples of the impact that organized programs can have (Ferlay et al. 2010; World Health Organization (WHO) 2010). For example, in Finland, cervical cancer incidence rates decreased from 16% in 1965 to 4.18% in 2007. Similar observations were also made in Canada and the USA (IARC Working Group on the Evaluation of Cancer-Preventive Strategies 2005).

In spite of the overall declines in crude or age-adjusted incidence and mortality in Western countries, increases have been reported among young women, presumably reflecting changes in sexual habits and increased transmission of HPV in younger generations. This phenomenon was first described in England and Wales (Hill and Adelstein 1967), where successive generations of women born since the mid-1930s have been at increasingly high-risk. Similar observations have been made elsewhere in Europe (IARC Working Group on the Evaluation of Cancer-Preventive Strategies 2005).

The majority of cervical cancer cases are squamous cell carcinomas (SCCs). Adenocarcinomas (ADCs) are less common. In general, the proportion of ADC cases is higher in areas with a low incidence of cervical cancer, and this histology may account for up to 20–30% of cervical cancer cases in many Western countries (Ferlay et al. 2010; Parkin and Bray 2006), having risen in recent decades (Bray et al. 2005). Cytology screening has been shown to effectively detect SCCs in early stages, but be less effective with ADCs. ADCs and their precursors occur within the cervical canal (from the glandular epithelium), and methods of Pap smear collection are relatively less efficient in sampling the endocervical canal compared to squamous cell lesions (DeMay 1996).

Less information is available regarding trends in less developed countries due to the lack of long term studies. In general, incidence and mortality rates have been relatively stable in many countries, or shown modest declines (IARC Working Group on the Evaluation of Cancer-Preventive Strategies 2005). The absence of an

overall decline, as observed in high resource populations, probably reflects the lack of screening implementation, or perhaps where programs have been introduced, their low population coverage and poor quality cytology prevents us from gathering accurate statistics (Parkin and Bray 2006).

Rates have decreased in China, Taiwan, Korea, and India, partly due to improved screening activities and socio-economic conditions, although the decreases in proportionate terms are much smaller compared with those in western countries (Jemal et al. 2010). In Shanghai, China, there have been dramatic declines in cervical cancer incidence; the rates fell from 26.7 to 2.5 per 100,000 women between 1972–74 and 1993–94. In Qigong, China, from a much lower base (4.2 per 100,000 in 1978–82), rates in the next 20 years declined at 4.7% annually. However, rates are increasing in women under the age of 45 years (Parkin et al. 2008a).

In Eastern Europe and some former Soviet Union countries rates have been rising rapidly since the 1990s, particularly among recently born generations (Ferlay et al. 2010; World Health Organization (WHO) 2010). These increases have been attributed to inefficiency or lack of screening, and have led to the enactment of organized programs in the 2000s in many of these countries (Arbyn et al. 2011b).

Despite the efforts to control cervical cancer in Latin America, this region continues to have one of the highest rates in the world. The marked reductions in incidence and mortality observed in most developed countries after the introduction of well-organized screening programs have not been observed in most Latin American countries. Pap smear reporting has been estimated at over 50% for most programs and evaluation studies in the region, especially in the South American countries. Only a few have been able to implement the necessary comprehensive infrastructure to provide wide coverage with high quality diagnostic and treatment services that is sustainable enough to achieve the levels of reduction in cervical cancer burden that resource-rich countries have experienced (Franco et al. 2008; Munoz et al. 2008). In Cali, Colombia, declines in incidence rates had been seen after the introduction of a screening program in 1967, but there has not been a direct translation in mortality. On the other hand, in Costa Rica, in spite of nationwide cytology services that have been available to women aged 15 years or older since 1970, mortality in young women (under age 50 years) remained almost unchanged, with a decline only since 1997 (Ferlay et al. 2010; World Health Organization (WHO) 2010; Parkin et al. 2008b).

4 Natural History of Human Papillomavirus Infection and Cervical Cancer

Cervical cancer is a rare and late outcome of a common sexually acquired infection with some genotypes of HPV. More than 100 genotypes with several subtypes of HPV have been described; and about 40 types are able to infect the genital tract (Bernard et al. 2010). HPV types are considered to be “high risk” or “low risk” taking into account the epidemiologic and mechanistic evidence of their association with

cervical cancer. Recently, the 2009 International Agency for Research on Cancer (IARC) review on human carcinogens concluded that there is sufficient evidence in humans for the carcinogenicity of 12 types in the cervix: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 (Bouvard et al. 2009). An intermediate risk group is also described (possibly carcinogenic HPV types), largely including types for which evidence is still limited and uncertain. HPV types 6 and 11 are considered "low risk types" not classified as cervical carcinogens, which are related to the development of external genital warts and are rarely found in cervical cancer specimens (i.e. <1 in 100). Large studies aimed at describing the distribution of HPV types in invasive cervical cancer show very strong results identifying as the first and second most common types, HPV 16 and 18, accounting for approximately 70% of all cervical cancers (Munoz et al. 2004; Li et al. 2011; de Sanjose et al. 2010).

Most HPV infections first occur in the young age groups after sexual activity begins and then it clears up spontaneously within 2 years. However, a fraction of HPV exposed women remain persistent carriers and constitute the high-risk group for developing pre-neoplastic lesions and cervical cancer. Some of the known determinants of HPV infection are thus the common risk factors for any other Sexually Transmitted Infections (STIs), namely the early age at sexual initiation, a high number of different sexual partners of the women and the husband or male partner, number of recent new partners, and history of other STIs. These factors also apply to the sexual behavior of the husband and other surrogates of HPV infection such as the presence of other STIs in his medical history.

HPV has been firmly established as a central and necessary cause of cervical cancer, however not all HPV infected women will develop invasive lesions, suggesting that factors other than HPV infection (co-factors) are in the pathway for progression from cervical HPV infection to cancer. Three groups of potential cofactors have been described: (1) viral determinants, such as type, load or perhaps genetic variants of the common HPV types, (2) host factors such as genetic and hormone factors related to the immune response, and (3) environmental factors such as smoking and hormonal treatments. These will be briefly discussed in the next section on risk factors for cervical cancer.

5 HPV and Cervical Cancer

5.1 HPV as the Necessary Cause for Cervical Cancer

The evidence relating HPV infections of some types to cervical cancer includes an impressive and consistent body of studies indicating a strong and specific role of the viral infection in all countries where investigations have taken place. The association has been recognized as causal in nature by a number of international review parties since the early 1990s (Walboomers et al. 1999; Munoz et al. 1992; Bosch et al. 1995). For illustration, we will briefly review some of the most relevant studies.

The International Biological Study on Cervical Cancer (IBSCC) is an IARC-coordinated survey that included over 1,000 biopsy specimens of invasive cervical cancer from 22 countries around the world. Frozen biopsies were analyzed using a Polymerase Chain Reaction (PCR) based system including 26 types specific and a generic probe. This large study identified HPV DNA in 93% of the specimens. Subsequently, the 7% of specimens that remained HPV DNA-negative were re-tested using a different HPV detection system and using a sandwich method (verifying in multiple histological cuts that recognizable neoplastic cells were used in the HPV testing procedure) to ensure the quality of the materials processed for HPV. As a result, the estimated prevalence of HPV DNA in cervical cancer biopsies increased from 93.0% to almost all of the cases: 99.8% (Walboomers et al. 1999; Bosch et al. 1995).

The IARC research program on HPV also organized a series of case control studies in different countries, mostly in areas at high risk for cervical cancer. Recently, the IARC reported a pooled analysis of these studies. The pooled data included about 2,500 women diagnosed with cervical cancer and about 2,500 control women without cervical cancer from 11 countries worldwide. The main advantage of these studies is that they followed a common protocol and the samples were analyzed in a central laboratory using a validated PCR assay for detection of 22 HPV types. Figure 4.5 summarizes the odds ratios (OR) for invasive cancer (the factor by which the risk of cervical cancer of a given woman is multiplied if HPV DNA is detected) associated with the 15 most common HPV types showing ORs ranging from 3.6 for HPV 6 (not statistically significant), to 573 for HPV 33, and 282 for HPV 16, and 223 for HPV 18 (Munoz et al. 2003; Castellsague et al. 2006; Munoz et al. 2006). The magnitude of the ORs, suggested that the association was one of the strongest identified for any human cancer and, along with the previous data on the IBSCC and consistent experimental and animal data, lead to the conclusion that HPV is a necessary cause of cervical cancer. However, in developed countries, invasive cervical cancer is a relatively rare disease and most diagnoses occur at earlier stages (i.e. high grade preneoplastic lesions). Several studies in both developed and developing countries have also shown that HPV is related to these precursor lesions with the same strength as the more advanced invasive cancers (Schiffman et al. 1993; Bosch et al. 1993; Olsen et al. 1995; Moreno et al. 1995; Liaw et al. 1995; Kjaer et al. 1996).

5.2 HPV Types in Cervical Cancer

Among viral co-factors, the HPV type is the strongest influence that affects the risk of viral persistence and progression to precancerous and invasive lesions. As previously mentioned, HPV types are classified into different groups according to their risk of progression. A recent IARC review on human carcinogens (volume 100b) concluded that there is sufficient evidence in humans for the carcinogenicity of 12 types in the cervix: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 (Bouvard et al. 2009). Some studies have evaluated the HPV type-specific risk of developing

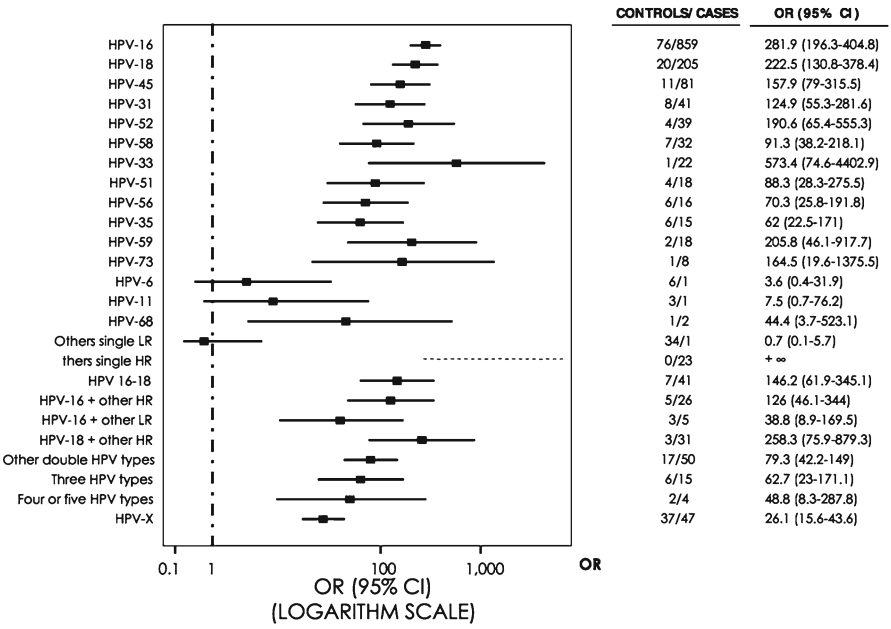


Fig. 4.5 Type-specific odds ratio (ORs) and 95% confidence intervals (CI) for cervical carcinoma. Subjects with HPV-DNA negative results were used as the reference category. ORs are adjusted by country and age-group. HR: High Risk; LR: Low Risk. HPV-X denotes undetermined type (Adapted from (Munoz et al. 2003; Castellsague et al. 2006; Munoz et al. 2006))

CIN-2/3 and they have consistently reported an increased risk for CIN 2/3 linked to a baseline detection of HPV 16. Fewer findings showed an increased risk associated with the presence of HPV type 18 and types phylogenetically related to HPV 16 and 18 (Khan et al. 2005). Regarding HPV types identified in cervical cancer specimens, the largest published studies that have evaluated the distribution of HPV types in cervical cancer worldwide are these three: (1) a pooled analysis of about 3,000 cervical cancer cases from the previous mentioned IARC studies (IBSCC and case–control studies) (Munoz et al. 2004); (2) a study performed at the Catalan Institute of Oncology that included more than 10,000 cervical cancer specimens preserved in paraffin that were processed following a centralized and standardized protocol of pathology and HPV evaluation using SPF-10/LiPA25 method (de Sanjose et al. 2010); and (3) a meta-analysis of more than 30,000 cases (Li et al. 2011). These reports show very robust results identifying as the first and second most common types, HPV 16 and 18, accounting together for approximately 70% of all cervical cancers. In addition, there is a consistent ranking of the eight most common types with slight variations across studies and regions, these being HPV 16, 18, 31, 33, 35, 45, 52, and 58 (Fig. 4.6) (Munoz et al. 2003; Castellsague et al. 2006; Munoz et al. 2006). These eight types are responsible for about 90% of all cervical cancers worldwide. Regarding histology, HPVs 18 and 45 are particularly common in ADCs of the cervix and the second most common histological diagnosis in cervical tumors

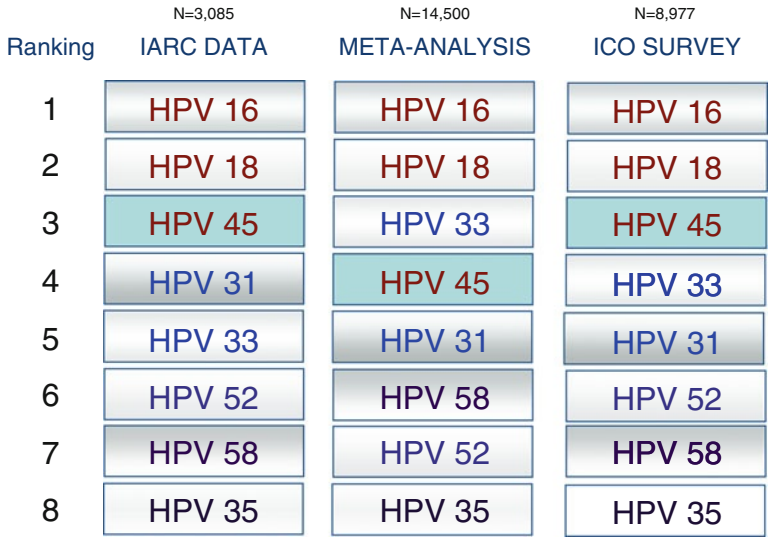


Fig. 4.6 Ranking of the eight most common HPV types among HPV-positive cervical cancer specimens worldwide, from three different analyses (Data sources: (Munoz et al. 2004; Li et al. 2011; de Sanjose et al. 2010))

after SCCs (Fig. 4.7) (de Sanjose et al. 2010). Together with HPV 16, they account for more than 90% of the ADCs that were found to be HPV-positive. In this study, the authors also observed that HPV 16, 18, and 45 cervical cancer cases were presented at younger ages than the rest of HPV types. These observations are of great importance for the development of preventive measures and strategies.

5.3 Other Viral Factors

Beyond HPV type, other viral factors related to persistency and progression have been also studied. During the last decade, several studies have found that certain HPV 16 and HPV 18 variants may be more strongly associated with an increased risk of persistent infections, and preneoplastic and invasive lesions. An intratypic variant of HPV is a type whose L1 coding regions differ by less than 2% when compared to the "prototypic" genomic sequence. For example, an elevated risk of cervical cancer associated with some variants of HPV 16 (non-European variants) has been suggested, although not all the studies have observed these findings. In this direction, several studies have pointed out that only those with large numbers of trials will provide enough statistical power to detect associations between low prevalent variants and disease risk (Almonte et al. 2008). Moreover, increased viral load, particularly for HPV 16, was found to be related to progression rate in some studies

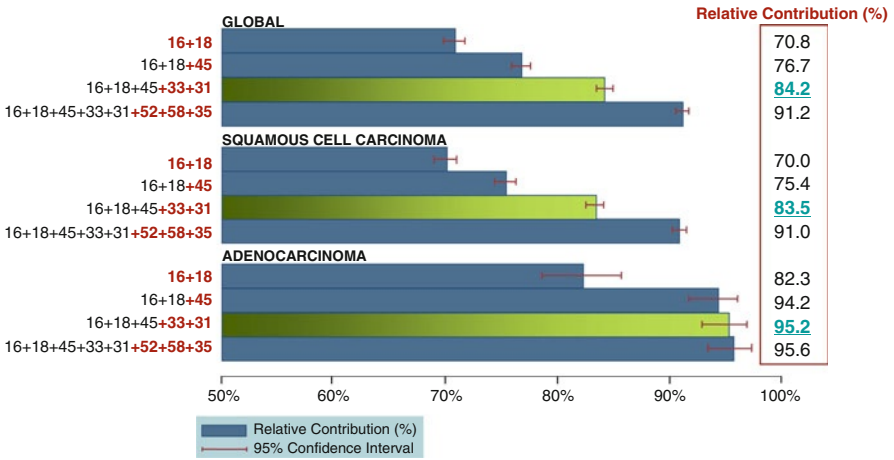


Fig. 4.7 HPV relative contribution and 95% confidence interval of the eight most common types among HPV-positive cervical cancer (Adapted from: (de Sanjose et al. 2010))

and these changes in viral load may predict persistence and progression of HPV 16 infections (Xi et al. 2011). Less consistently, the total risk of precancerous lesions for a woman infected with several HPV types (co-infection) may be increased compared with women infected with only one of those same HPV types (Herrero et al. 2005). However, full evaluation often depends on technical issues of the HPV assay used in the different studies.

6 Environmental Co-factors in Cervical Carcinogenesis

Before HPV was investigated, epidemiological studies identified a series of factors as being more prevalent in cases of cervical cancer than in their control groups. This was the case for different sexual and reproductive behavioral traits, use of hormonal contraception, smoking, and history of STI (typically Herpes Simplex virus type two {HSV-2} and *Chlamydia Trachomatis* {CT}). The ORs observed for such associations were in the range 1–3 (as compared to ORs in the hundreds for some HPV types) and the results were inconsistent across multiple studies. Having revealed very strong associations with HPV, all these putative additional factors required reevaluation. In general terms, what remained to be established is if and which additional environmental factors result in persistence of HPV infection and its progression to cervical cancer. Most investigators have attempted such evaluation by restricting the comparison of the relevant exposures in cases of cervical cancer, most of which were shown to be HPV-positive, with their HPV-positive controls (women in the same age groups from the same underlying population, with HPV infection but without cancer).

6.1 Other Sexually Transmitted Infections

The most studied STIs for which some evidence has been shown in relation to cervical cancer are HSV-2, CT, and HIV.

Individuals with HIV are at increased risk of HPV-associated ano-genital cancers compared with age-matched healthy individuals. This association appears to be stronger for women with a low CD4 T-lymphocyte count (Palefsky et al. 2006). The presence of cervical cancer was recommended in 1992 as an independent criterion to establish the diagnosis of AIDS in women with HIV infection. New data show that the incidence of cervical cancer has not declined since the introduction of anti-retroviral therapy. Several studies also highlight high rates of HPV infection and HPV-associated disease at sites other than the cervix and anus, including the penis and mouth (Palefsky 2009).

Both HSV-2 and CT have been also associated with an increased risk of cervical cancer among HPV positive women. Two pooled analysis from IARC case–control studies were performed to assess the effect of HSV-2 (Smith et al. 2002) and CT (Smith et al. 2004) in the etiology of invasive cervical. Among HPV DNA-positive women, the risk of SCC was elevated in both, HSV-2 (OR 2.19; 95% CI 1.41–3.40) and CT seropositive women (OR 1.8; 95% CI 1.2–2.7). HSV-2 seropositivity was also associated with increased risk of adeno or adenosquamous-cell carcinoma (OR 3.37; 95% CI 1.47–7.74). It has been described that the mechanism for these associations are partially due to induction of inflammation associated with production of free radicals and development of DNA instability (Munoz et al. 2006).

6.2 Hormonal Factors: Oral Contraceptives and Parity

The IARC monograph on hormones and cancer classified combined contraceptives as carcinogenic to the cervix. A meta-analysis (Appleby et al. 2007), including data from 24 studies worldwide showed that among current users of oral contraceptives the risk of invasive cervical cancer increased with increasing duration of use (relative risk {RR} for 5 or more years' use versus never used, 1.90; 95% CI 1.69–2.13). The risk declined after the use ceased, and by 10 or more years had returned to that of those who never used. A similar pattern of risk was seen both for invasive and in situ cancer, and in women who were HPV DNA-positive. Mechanisms of action proposed may be an enhancing effect of HPV gene-expression in the cervix via hormone-response mechanisms (Munoz et al. 2006).

Regarding parity, The International Collaboration of Epidemiological Studies of Cervical Cancer (ICESCC) (Cervical carcinoma and reproductive factors 2006) brought together data on 11,161 women with invasive carcinoma, 5,402 women with CIN3/carcinoma in situ and 33,542 women without cervical carcinoma from 25 epidemiological studies. The analysis showed that a number of full-term pregnancies were associated with a risk of invasive cervical carcinoma for each additional pregnancy (RR 1.10; CI 95% 1.08–1.12). This study also indicated that younger women

who experienced a full-term pregnancy were at increased risk to get cervical cancer later in life than women who became pregnant at older ages. Results were similar in analyses restricted to high-risk HPV DNA-positive cases and controls. Mechanisms of action proposed may be hormone factors and maintenance of the transformation zone on the exocervix (Munoz et al. 2006).

6.3 Tobacco Smoking

The last IARC review on tobacco carcinogenesis stated that there is now sufficient evidence to establish a causal association of SCC with smoking. However, in the small number of studies available for adeno and adenosquamous-cell carcinoma, no consistent association was observed (IARC Monograph on the Evaluation of Carcinogenic Risks to Human 2004). The most recent studies have been for controlled infection with HPV. The effect of smoking was not diminished by the adjustment for HPV infection, or analysis restricted to cases and controls both positive for HPV (as ascertained by HPV DNA or HPV serological methods).

It seems that the risk of this malignancy increases significantly with intensity and duration of smoking, but there is still controversy regarding these associations. A pooled analysis of ten previously published case–control studies (eight of invasive cancer and two of carcinoma in situ), found an excess risk for smoking among HPV DNA-positive women (OR 2.17; 95% CI 1.46–3.22). In this analysis, there was no evidence of an increased risk of cancer with increasing number of cigarettes per day, duration and age of starting smoking (Plummer et al. 2003). Another study of the International Collaboration of Epidemiological Studies of Cervical Cancer (ICESCC) (Cervical carcinoma and reproductive factors 2006) brought together and combined individual data on 13,541 women with and 23,017 women without cervical carcinoma, from 23 epidemiological studies. This analysis showed that smokers are at an increased risk of squamous cell but not of ADC of the cervix. The risk of SCC increased in current smokers with the number of cigarettes smoked per day and with those who began smoking at a younger age, but not with duration. Eight of the studies had tested women for cervical HPV-DNA, and in analyses restricted to women who tested positive; there was also a significantly increased risk when compared to those who never smoked in the SCC study. Globally, there was no clear trend with time since stopping smoking. Regarding this last issue of the effects of smoke cessation in the decrease of risk and regression of cervical abnormalities, some studies have shown that tobacco smoking may interfere with regression of cervical precursor lesions, and show a higher percentage of regression of lesions in those who quit smoking (Matsumoto et al. 2010; Szarewski et al. 1996).

Previous reviews suggested that the mechanism for this association could be a reduction of immuno-response in the cervix, an effect of hormone metabolism, or direct genetic damage or epigenetic effects by tobacco-related carcinogens (Munoz et al. 2006; Ma et al. 2011).

6.4 Nutritional Factors

Dietary factors are related to epithelial cancers, and fresh fruits and vegetables are strong protective factors for some cancers. Several studies have evaluated food and nutrients as possible co-factors in cervical cancer. In 2005, a systematic literature review regarding the epidemiologic evidence about the role of diet and nutrition on the risk of HPV persistence and cervical neoplasia was published (Garcia-Closas et al. 2005). This systematic review included 33 observational studies and clinical trials published between 1991 and 2003. In this review, the scientific evidence was classified into four levels: convincing, probable, possible, or insufficient. The studies on HPV persistence showed a possible protective effect of fruits, vegetables, vitamins C and E, beta- and alpha-carotene, lycopene, luterin/zeaxanthin and cryptoxanthin. Evidence for a protective effect of cervical neoplasia was probable for folate, retinol and vitamin E and possible for vegetables, vitamins C and B12, alpha-carotene, beta-carotene, lycopene, lutein/zeaxanthin and cryptoxanthin. Evidence for an increased risk of cervical neoplasia associated with high blood homocysteine was probable. Results did not differ between studies looking at pre-neoplastic and invasive lesions. In general, associations between foods or nutrients with cervical cancer were yet not convincing.

After this review, a few studies have been published. Hernandez and colleagues reported results from a case–control study conducted in Hawaii that found that riboflavin and thiamin from food sources, vitamin B12 supplements, and total (food and supplements) folate displayed inverse, dose-responsive associations with high-grade pre-neoplastic lesions (Hernandez et al. 2003). This inverse association between levels of folate and risk of cancer has been also described elsewhere (Wang et al. 2006; Ghosh et al. 2008). Moreover, some recent case–control studies have described an inverse association between serum antioxidant micronutrient concentrations and the risk of cervical neoplasia (Tomita et al. 2010; Kim et al. 2010; Cho et al. 2009).

Recently, Gonzalez and colleagues described the first results of the effect of fruits and vegetables intake on cervical cancer from a cohort study design (Gonzalez et al. 2010). They examined the association between the intake of fruits and vegetables and selected nutrients and the incidence of carcinoma in situ and invasive squamous cervical cancer in a prospective study of 299,649 women participating in the European Prospective Investigation into Cancer and Nutrition study (EPIC study). They observed a statistically significant inverse association of invasive cervical cancer with a daily increase in intake of 100 g of total fruits (Hazard Ratio-HR 0.83; 95% CI 0.72–0.98) and a statistically non-significant inverse association with a daily increase in intake of 100 g of total vegetables (HR 0.85: 95% CI 0.65–1.10). Statistically, non-significant inverse associations were also observed for leafy vegetables, root vegetables, garlic and onions, citrus fruits, vitamin C, vitamin E, and retinol for invasive cancer. No association was found regarding beta-carotene, vitamin D and folic acid for invasive cancer. None of the dietary factors examined were associated with carcinoma in situ, suggesting a possible protective role of fruit intake and other dietary factors on invasive cancer that needs to be confirmed on a larger number of cases.

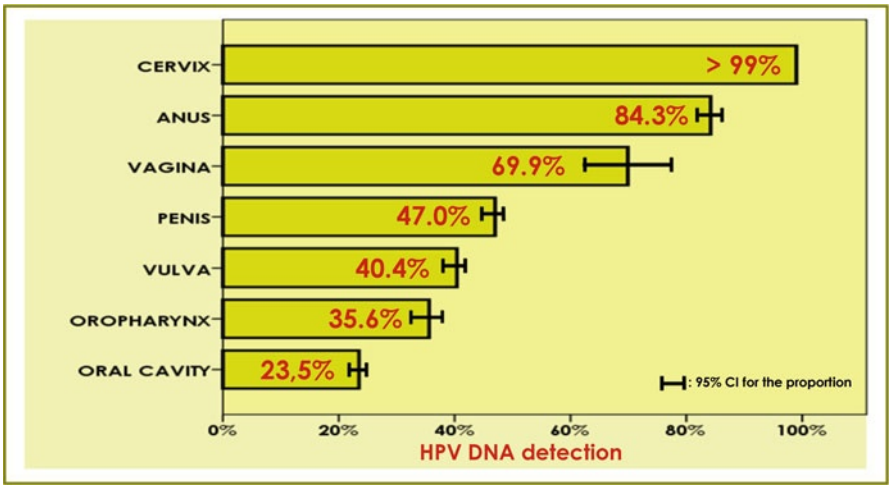


Fig. 4.8 Estimated prevalence of HPV for anogenital cancers and cancers of the upper aerodigestive tract, in both sexes (Data sources: (Walboomers et al. 1999; De Vuyst et al. 2009; Miralles-Guri et al. 2009; Kreimer et al. 2005))

7 The Spectrum of HPV-Related Diseases

Although it is out of the scope of this chapter, it is important to highlight that besides cervical cancer, it has been demonstrated that most cancers of the vagina and anus are linked to HPV, as are a fraction of cancers of the vulva, penis and other non-anogenital sites such as the oral cavity and pharynx (Fig. 4.8). Among these types of cancers, HPV 16 is the most frequently detected type, followed by HPV 18 (De Vuyst et al. 2009; Miralles-Guri et al. 2009; Kreimer et al. 2005).

HPV infection can cause benign lesions accounting for virtually all cases of genital warts and recurrent respiratory papillomatosis (RRP). Contrary to what is observed in neoplastic lesions, genital warts and RRP are predominantly caused by HPV 6 and 11 (Giuliano et al. 2008).

8 Conclusions

Several studies have convincingly shown that cervical cancer is long-term sequelae of certain unresolved HPV infections of the uterine cervix. As observed for other sexually transmitted diseases, HPV infections and cervical cancer are most common in the poorest countries and among the deprived social groups in affluent societies. The strongest known determinants of the geographical and social variation in the incidence of cervical cancer are related to the sexual behavior patterns of the population, to lack of screening, or low participation of the population in the available

screening programs. Cervical cancer continues to be a major public health problem that kills approximately a quarter of million women every year and affects developing countries and younger women in particular. The recognized problem of HPV-related disease is increasing worldwide and efforts to improve related data collection are strongly needed. The available options for cervical cancer prevention such as vaccines and improved screening tools should help us to accelerate the path to a reduction of cervical cancer outbreaks worldwide.

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Chapter 5
The Basic Elements of a Correct Diagnosis: From Cytohistopathology to Screening

Franco Borruto and Ciro Comparetto

1 Introduction

Laboratory diagnosis of skin warts is usually unnecessary as they can be distinguished morphologically. Large numbers of cutaneous warts may develop in patients with epidermodysplasia verruciformis (EV), a rare familial disorder. Exposure to sunlight sometimes causes these lesions to progress to skin cancer. Human Papillomavirus (HPV) can be transmitted from the vagina at birth, and may cause recurrent respiratory papillomas in childhood or adult life. Genital infection usually clears within a few months, but may persist in some individuals. The distribution of genital HPV types varies and is related to the degree of cervical dysplasia present. HPV types 6 and 11 are frequently found in sexually active adults, and are associated with low-grade (LG) squamous intraepithelial lesions (L-SIL). HPV types 16, 18, 31, and 45 are found less frequently, and are associated with progression to invasive cancer (Fig. 5.1). Commercial dot blot hybridization and deoxy-ribonucleic acid (DNA)-ribonucleic acid (RNA) hybrid capture (HC) assays are available for laboratory diagnosis of genital HPV infection. The polymerase chain reaction (PCR) is used for diagnosis and epidemiological surveys. Detection of particular HPV types could be useful in the diagnosis and management of cervical cancer in older women, and for resolving equivocal (borderline) cytology. HPV assays, which can distinguish between high-grade (HG) and LG disease, may also have a role in routine cervical screening (Swygart 1997).

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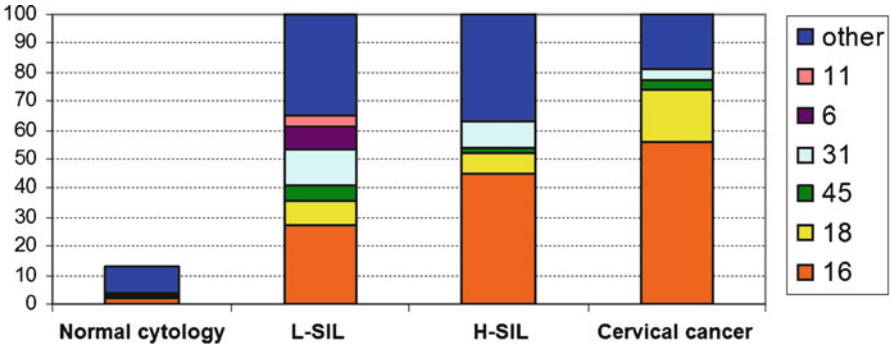

Fig. 5.1 Proportion of women infected with HPV types

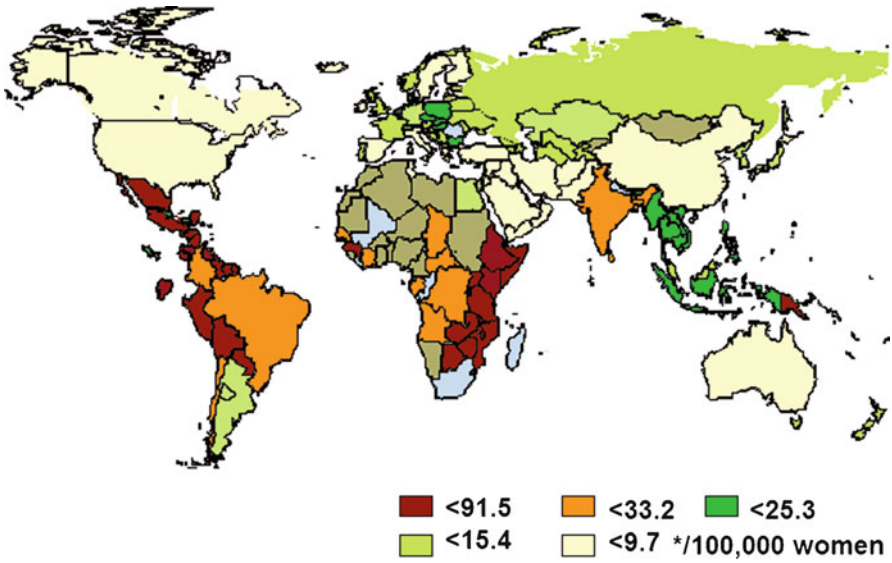

Fig. 5.2 Worldwide cervical cancer incidence

Worldwide, cervical cancer is diagnosed annually in more than 500,000 women and accounts for 270,000 deaths, making it the second leading cause of cancer in women (Fig. 5.2). In Europe, where many countries have set up screening program, cervical cancer ranks third among cancers in women. Since its introduction, Papanicolaou (Pap) smear screening has transformed cervical cancer from a fatal disease into a rare condition. Despite the considerable success of this cytologic screening, Pap smears have not, as it was first hoped, reduced incidence on a large scale. The principal reasons are related to the difficulties in ensuring optimum coverage of the population to be screened and in maximizing women's adherence: the success of screening depends on strict compliance with the calendar from 25 to

65 years of age. In one-third of cases, invasive cancers are found in women who undergo regular screening, because Pap smears are insufficiently sensitive. In 5% of cases, cancers are observed in women who were inappropriately managed after an abnormal Pap smear finding. The contribution of HPV test to primary screening opens up promising perspectives of optimum protection. The test's sensitivity for HG lesions exceeds 95% and its negative predictive value exceeds 99%. HPV test is the only test available for which a negative result provides instantaneous assurance that there is no risk of cervical cancer. The Pap smear alone, with its sensitivity of less than 70%, cannot provide this certainty. European and American guidelines recommend screening strategies based on a combined test using the Pap smear and HPV test after the age of 30 years. The availability of prophylactic HPV vaccines, which are expected to provide 70% protection against cervical cancer, does not affect the practice of screening, which must continue (Monsonego 2007).

2 Cytohistopathology

HPV infection causes cellular alterations that are detectable from a cervico-vaginal smear. Cells marked by these alterations are defined as koilocytes and diskeratocytes. Whereas koilocytes are pathognomonic of HPV infection, diskeratocytes, found in isolation on a smear, do not always play a reliable diagnostic role (Ventura et al. 1990). Currently used histological and cytological classification systems for cervical lesions suffer from poor inter and intra-observer reproducibility and do not allow accurate identification of which mild lesions will progress towards cancer. It is postulated that L-SIL and HG-SIL (H-SIL) might represent distinct entities with different potential for progression rather than necessary stages of a continuum leading to cervical cancer. Improved understanding of the etiological role of HPV types in cervical cancer and of the natural history of L-SIL and H-SIL might result in more suitable clinical treatment of LG lesions (Kiviat et al. 1992).

In December 1988, the National Cancer Institute (NCI) in Bethesda, Maryland, United States of America (USA), held a workshop that produced published guidelines for reporting the results of cervical and vaginal cytology. A second workshop was held in April 1991 to discuss the initial proposal and suggest modifications. The final recommendations were the following: the Papanicolaou classification for reporting consultation is not acceptable in the modern practice of diagnostic cytopathology, because it does not reflect current understanding of cervical/vaginal neoplasia, has no equivalent in diagnostic histopathologic terminology, does not provide diagnoses for non-cancerous entities, and as a result of numerous modifications, the specific Papanicolaou classes no longer reflect diagnostic interpretations uniformly. The cytologic report is a medical consultation in the same manner as is the histomorphologic report. Clinical information is absolutely essential for making the diagnosis. A cytopathologic diagnosis should be an interpretation of morphologic findings, but this interpretation is best made in the context of the patient's clinical situation. The cytopathologist should determine whether the specimen is adequate for diagnostic evaluation. If unsatisfactory or satisfactory for evaluation but limited

by, this should be noted in the report. The use of precise diagnostic terms facilitates unambiguous communication between cytopathologist and clinician. The terminology for SIL included:

1. Atypical squamous cells of undetermined significance (ASC-US)
2. SIL, which encompass the spectrum of squamous cells carcinoma (SCC) precursors, divided into L-SIL (HPV-associated cellular changes, mild dysplasia, and cervical intraepithelial neoplasia, CIN 1) and H-SIL (moderate dysplasia or CIN 2, severe dysplasia and carcinoma in situ, CIS, or CIN 3) (Broso and Buffetti 1993)

The Bethesda System and its 1991 revisions aimed to simplify Pap smear reporting and made it more reproducible. It redefined the Pap smear request as a medical consultation. The pathologist consultant is required not only to provide the smear reading but also its clinical recommendation. The Bethesda System insisted on a detailed Pap smear report assessing specimen adequacy and types of epithelial changes. Squamous cell abnormalities were grouped according to their biologic potential. Both CIN grade 1 (mild dysplasia) and HPV lesions were grouped together as L-SIL, while moderate and severe dysplasia (CIN 2 and 3) belonged to the H-SIL category. ASC-US and atypical glandular cells of undetermined significance (AGC-US) needed further qualification as to whether they favor either a reactive or neoplastic process (Nguyen and Nordqvist 1999). The criteria used for grading CIN were arbitrary and subjective with consequent considerable intra- and inter-observer variability. None of the used criteria made a clear-cut case for changing terminology. The combination of CIN 2 and CIN 3 in an HG lesion was not supported by biologic behavior or HPV typing and led to overtreatment. The various shifts in nomenclature over the last 50 years through the dysplasia, CIN, and Bethesda System, although intellectually stimulating, have neither improved diagnostic accuracy nor patient management. On the contrary, they often caused confusion and duplication, leading to the common and ironic practice that several terminologies were used in an additive fashion. New diagnostic markers are on the horizon as a result of the rapid development in the areas of genomics and proteomics. It seems likely that specific molecular biomarkers will become available, allowing the consistent and accurate discrimination between those intraepithelial lesions that will ultimately become invasive from the vast majority of lesions that will regress or persist. It is preferable at this time to maintain the current three-tier system, which is well entrenched and accepted around the world, until a novel approach places the classification of cervical precursor lesions on a new and solid footing. Ideally, we will then have a single-tier system identifying reliably those lesions that have the potential to become invasive (Schneider 2003).

Besides all the confusion and associated problems that the use of ASC has created, it has initiated substantial investigational interest that has resulted in a better understanding of SIL and the biology of cervical neoplasia. Although the category of ASC has created and will continue to create controversy in the diagnostic and management fields, it allows the pathologist to convey uncertainty that may be the result of poor sampling or difficulty in interpretation of a case. It is a valuable tool that the cytopathologist can use to make it known that the Pap test has its limitations and

may need and benefit from support of ancillary studies. Similar limitations are recognized in other areas of pathology and the use of immuno-histochemistry (IHC) or molecular studies is widely accepted as an aid to a more specific and definitive interpretation. HPV DNA testing may not be the perfect test for cervical cancer screening because of high prevalence of HPV infection in the general population. However, it is currently the best-studied ancillary test and has been proven to be cost-effective for the triage of Pap tests with equivocal squamous cells. It is important for the cytopathologist to have well-developed diagnostic skills in interpreting gynecologic preparations, and to classify cases as ASC only when deemed appropriate. Downgrading cytological findings that are diagnostic of a SIL to ASC with the hope of supporting it by an HPV test will only result in a devaluation of the Pap test. Such recourse may be acceptable in specific situations, such as with patients who have complex histories, atypical clinical presentations, or during pregnancy. Quality assurance measures to closely monitor the ASC/SIL ratio and the rate of HPV positivity in ASC cases will be essential to ensure the appropriate use of this interpretive category. The coordination of the 2001 Bethesda and the American Society for Colposcopy and Cervical Pathology (ASCCP) consensus meetings resulted in the new subcategories of ASC-US and atypical squamous cells cannot rule-out a HG lesion (ASC-H), along with well-defined management strategies for these interpretations. This new and clinically relevant terminology should lead to a reduction in difficulties at the clinical level and a more uniform management of patients, unlike the situation following Bethesda 1991 where the gynecologist was faced with a new "diagnosis" without specific management recommendations. The standardization of reporting and clinical management will also allow more reliable evaluation of patient outcomes and cost analysis. The 2002 American Cancer Society (ACS) guidelines did not make specific recommendations regarding HPV DNA testing for the triage of patients who have a cytology result of ASC-US. The Food and Drug Administration (FDA) approved the expanded use of HPV testing in conjunction with the Pap test for cervical cancer screening in March 2003. The future is likely to bring additional testing modalities that may be more specific for detecting squamous lesions that are more likely to persist or progress to carcinoma, than the currently available HPV tests. In addition, recently published data from HPV vaccine trials suggest that immunizing women who are negative for HPV-16 may eventually reduce the incidence of cervical cancer. At present, the most effective method to decrease the mortality of this disease process is to make sure that all women have access to and receive effective cervical cytologic screening (Nayar and Tabbara 2003).

3 Diagnosis

The accurate detection and diagnosis of cervical carcinoma and its malignant precursors (collectively referred to as HG cervical disease) represent one of the current challenges in clinical medicine and cytopathology. In recent years, the relationship

between HPV and genital neoplasia has been explored intensively and a molecular basis for the role of HPV in the genesis of these diseases has been convincingly demonstrated. These findings have provided justification for efforts to apply this molecular information to the early detection and possible prevention of HPV-related neoplasia. The technology of detecting viral nucleic acids in genital fluids brought with it initial hopes that it would serve to identify women at risk for having or developing precancers or cancers of the cervix. Subsequent studies, however, have demonstrated limitations of the technology for predicting future disease. Recently, molecular immunology has complemented these prior efforts with the intent to identify serological indices of exposure to HPV and perhaps delineate individuals at risk (Crum et al. 1991). HPV are formally described by isolation of their circular double-stranded DNA genomes and establishment and comparison of the nucleotide sequence of these genomes. Alternatives such as serological diagnosis and maintenance of HPV in culture are neither clinically useful nor consistently feasible. Novel HPV isolates have traditionally been described as "types". The analysis of specific HPV types is of medical importance, because HPV types typically induce type-specific lesions, i.e. they may be specific for cutaneous or mucosal epithelia, or give rise to benign warts or malignant carcinomas. Recently, it was formally decided that Papillomaviruses are a virus family separate from the Polyomaviruses. Within the Papillomavirus family, closely or remotely related types can form new species or genera. These formal agreements were important as they brought the taxonomy of Papillomaviruses in line with that of other viruses, bacteria, and higher organisms, although their impact on medical practice and terminology used in clinical studies is limited. Notably, HPV types that are closely related (i.e. form "species") are associated with similar lesions. Confusion of the terms "type" and "subtype" should be avoided, as the latter term refers to some specific but rare taxonomic assemblages. In contrast to many RNA viruses, HPV types evolve very slowly, and have diverged since the origin of humans only by about 2%. These divergent isolates are called "variants". HPV is an ancient disease which evolved alongside humans and consequently, we, as a species, have never been without warts and cervical cancer. Variants of the same HPV type may have different pathogenicity and account for part of the worldwide disparities in the occurrence of genital cancers (Bernard 2005).

Concerning the prediction of HPV-associated cervical disease, several importance issues are related both to the management of women with diagnosed CIN and those with cervical cancer. Oncogenic HPV are capable of contributing to the development of malignant phenotype by several different mechanisms, most of which seem to be closely interrelated. Because of the fact that these molecular interactions are mediated by proteins, the logical strategy is to study the functions of these proteins utilizing the capabilities of IHC, which offers practically unlimited possibilities to study any target molecules against which a monoclonal or polyclonal antibody. Apart from getting new insights into the molecular pathogenesis of HPV-associated cervical carcinogenesis, the disclosure of individual markers, a set of markers, or an expression profile of any such marker sets would be of clinical value as predictors of disease outcome in cervical carcinogenesis (Syrjänen 2005). The presence of HPV DNA in the genital tract and the genotype of the infecting HPV are now widely

employed as biochemical markers in epidemiological studies of cervical cancer. Additional HPV markers could be utilized in future investigations. The amount of HPV DNA is likely to be higher in case specimens than in control specimens. Viral genomes would be frequently integrated in certain cases but almost never in controls, and early region transcripts may be relatively more abundant in cases than in controls (Shah 1992). Tests for HPV using nucleic acid probes have been commercially available since the late 1980s, but early tests were cumbersome, involving the use of nucleic acid probes labeled with radioactive phosphorus (32P). These early HPV tests did not achieve widespread use because they did not detect all oncogenic HPV genotypes. The current commercial HPV detection kit, Digene's HC-2 kit (Digene Corporation of Gaithersburg, Maryland, USA), detects virtually all oncogenic HR-HPV types, as well as most non-oncogenic LR-HPV genotypes. The HC-2 test format is a proprietary nucleic acid hybridization signal amplification system owned by Digene Corporation. Virtually all test formats for DNA sequence analysis are amenable to applications intended to detect and perhaps quantify the various HPV genotypes. These methods can involve direct hybridization with complementary DNA probes, such as Southern blotting or in situ hybridization (ISH), signal amplification such as the HC-2 method or target nucleic acid amplification, most notably the PCR, which has been used for HPV detection, genotyping, and viral load determination. General or consensus primer-mediated PCR assays have enabled screening for a broad spectrum of HPV types in clinical specimens using a single PCR reaction. Following amplification using consensus primers, individual HPV genotypes are identified using a variety of methods. Using consensus primers in a test format known as real-time quantitative PCR (RQ-PCR), it is possible to generate viral load (concentration) data from reaction curves generated by monitoring PCR reaction kinetics in real time (Hubbard 2003). Tests of choice for detecting HPV from clinical specimens are based on nucleic acid probe technology. Until recently, most epidemiologic and molecular studies employed Southern blot, dot blot, and ISH. With the exception of ISH, which continues to have many uses and a strong following in the pathology community, Southern blot and dot blot have been essentially replaced by PCR and the HC System. These newer in vitro probe tests have proven to be accurate and robust workhorses for epidemiologic and clinical use (Lörincz 1996).

A number of validation experiments have compared the most commonly used HPV hybridization methods with the accepted gold standard, Southern blot hybridization. The methods are filter ISH (FISH), dot blot hybridization (ViraPap/ViraType; Life Technologies Inc., Gaithersburg, Maryland, USA), and PCR. FISH now appears to be too inaccurate for recommendation in future epidemiological studies. ViraPap/ViraType compares well to Southern blot, but is limited to the detection of seven genital HPV types. PCR-based methods may be more sensitive than Southern blot and are likewise capable of detecting most known genital HPV types. Currently, there is no perfect method for HPV testing, because Southern blot itself is prone to some errors in performance and interpretation. Given that the scientific and clinical usefulness of HPV tests depends on the repeatability and accuracy of the assays, more intra- and inter-assay comparisons should be done to

establish reference standards applicable to this area of molecular diagnostics (Schiffman 1992). With the recent development of PCR, it has become possible to detect small numbers of HPV genomes in clinical samples. The sensitivity and specificity of this technique, together with the possibility of performing the test on crude cervical scrapes, makes PCR the method of choice for screening. The question arises whether screening for diagnostic purposes must include all the HPV types associated with infections of the genital tract or only those which are strongly associated with cervical cancer (HPV-16 and HPV-18) (Melchers et al. 1991).

Research has focused on the potential role of HPV testing in three conditions: screening for cervical neoplasia, triage of women with LG lesions, and follow-up after conservative surgical treatment for CIN. Concerning the first condition, HPV testing does not seem to offer an obvious advantage over traditional cytology screening, mainly due to false positive results in younger women with transient HPV infections. A possible exemption to this is the case of middle-aged women and low-resource settings, where the excellent sensitivity of a HPV test is desirable. Although data is controversial regarding LG lesions, results from randomized studies indicate that HPV testing could be useful in a triage of women with an initial cytological diagnosis of ASC-US, where detection of DNA of a HR type should lead to colposcopy. Although there is a lack of randomized controlled trials (RCT) in this field, data from observational studies indicate that HPV DNA testing after conservative surgical treatment for CIN may be very sensitive and detect early residual and recurrent disease (Agnantis et al. 2003).

It is accepted that HPV DNA testing has a role in the management of cervical disease both in a pre and post-vaccination era. However, to improve the specificity of this approach, there is a requirement to develop and validate tools/assays that can identify women at risk for progressive disease. There is evidence to suggest that detection of viral gene expression both directly and indirectly may constitute a more specific approach for delineating clinically significant infection compared with HPV DNA-based assays. HPV oncogene expression and evidence of its deregulation can be monitored through direct detection of viral messenger-RNA (mRNA) transcripts or through detection of the cellular protein p16. For both approaches, commercial assays have been introduced and numerous studies have been conducted. Currently, there are promising data indicating that HPV mRNA and p16 might play an important role in future cervical cancer screening scenarios. Still, large randomized studies are necessary to confirm the preliminary data (Cuschieri and Wentzensen 2008). The advent of molecular diagnostics and the use of whole-genome profiling using DNA microarrays promises to yield improved understanding of the disease process with the subsequent development of more accurate diagnostic procedures based upon these discoveries. Recent reports describing a variety of experimental approaches have identified a series of candidate genes that are overexpressed in cervical carcinoma. An emerging class of markers, the mini-chromosome maintenance (MCM) protein family of DNA licensing factors (MCM-2, MCM-6, MCM-7), shows promise for the specific detection of HG cervical disease using simple antibody based immunochemistry formats. These proteins are overexpressed in cervical disease as a result of infection by oncogenic strains of HPV and subsequent uncontrolled activation of

gene transcription and aberrant S-phase induction, mediated through the E2F transcription factor pathway. This behavior appears to be a hallmark of HG cervical disease and provides the link between oncogenic HPV infections and the molecular behavior of CIN. The use of these molecular descriptors of CIN in simple immunochemistry formats compatible with conventional cytology preparations is anticipated to improve the screening and detection of cervical disease within the healthcare system (Malinowski 2005). However, because HPV are causative agents and alter the cell cycle in cervical neoplasms, host genes interacting directly or indirectly with HPV oncoproteins have been identified in vitro. Recent research has centered on identifying the host genes upregulated in association with HPV infection, determining their suitability as surrogate endpoint biomarkers (SEB) for HPV infection. Then using these markers, include proliferating cell nuclear antigen (PCNA), Ki-67, cyclin E, p16, MN antigen, carcinoembryonic antigen (CEA), and telomerase, to identify HPV-associated epithelial lesions in tissue or cytologic specimens. Based on current studies, the strongest candidates for diagnosis and screening are p16 and cyclin E (squamous) and MN (glandular) biomarkers. As new genes are identified and tested, the concept of biomarkers as tools in primary screening and lesion recognition will continue to mature (Keating et al. 2001).

In vitro studies have suggested that HPV E6 interacts more efficiently with the arginine (Arg) p53 variant at position 72, as it appears to be more susceptible to degradation through the ubiquitin proteasome pathway. However, few reports have corroborated this data, and the role of the p53 codon 72 polymorphism in the development of cervical cancer requires further elucidation. The p53 Arg/Arg genotype does not seem to represent a risk marker for the development of cervical lesions in the majority of the European countries analyzed. However, in countries with low incidence rates of cervical cancer, this polymorphism might represent a significant genetic marker (Sousa et al. 2007). As consequence of the expression of E7 a cellular marker protein (p16) is increasingly expressed in dysplastic cells. Monoclonal antibodies directed against p16 specifically identify dysplastic cells and derived invasive cancers in histological slides, but also cytological smears (CIN-tec assay; Roche mtm Laboratories, Heidelberg, Germany). In advanced pre-neoplastic lesions HPV genomes are often integrated into cellular chromosomes. This leads to enhanced expression of the viral oncogenes. The detection of specific viral mRNA transcripts derived from integrated HPV genomes allows to identify pre-neoplastic lesions with a particularly HR for progression to invasive cancers (Amplification of Papillomavirus Oncogene Transcripts, APOT, assay). These findings will allow to establish highly sensitive, but specific and cost efficient new cancer early detection assays (von Knebel 2001a; Kostopoulou et al. 2011). p16(INK4a), an indirect marker of cell cycle dysregulation, is commonly expressed in cervical dysplasias and carcinomas associated with HR-HPV infections. Although p16 (INK4a) immunohistology is routinely used as a cost-effective SEB, many of the published articles are confusing and contradictory. The discrepancies can be ascribed to a multitude of factors operating at the molecular, technical, and interpretative levels. In the first place, our simplistic model of viral mediated oncogenesis is speculative and fails to account for all the known biomolecular changes. Unresolved technical issues

include the variables of tissue fixation, antibody dilution, antibody isotype and clone, and the sensitivity of the particular detection method. Within any controlled staining method, strong diffuse or "block" immuno-reactivity in squamous cells may be found in moderate/severe dysplasia (CIN 2/3) and invasive squamous carcinoma. In contrast, focal or multifocal reactivity in squamous cells may be artefactual, and related to LR- or HR-HPV. p16(INK4a) is less reliable when dealing with glandular lesions since considerable overlap exists between reactive and dysplastic lesions. In addition, not all glandular dysplasias/carcinomas are HR-HPV-related, nor are all p16(INK4a) immuno-reactive lesions associated with HR-HPV. We can conclude that p16(INK4a) immuno-peroxidase shows greater specificity than sensitivity for squamous lesions. In comparison, glandular dysplasias/carcinomas show reduced specificity and sensitivity. Like all cell cycle regulatory proteins, the future diagnostic role of p16(INK4a) is limited. The ideal diagnostic molecular test for cervical dysplasias will detect a HR-HPV-related product after, but not before, cell transformation and will reliably predict those cases yet to experience disease progression (Mulvany et al. 2008). p16(INK4a) is a biomarker for transforming HPV infections that could act as an adjunct to current cytological and histological assessment of cervical smears and biopsies, allowing the identification of those women with ambiguous results that require referral to colposcopy and potentially treatment. The proportion of cervical smears overexpressing p16(INK4a) increases with the severity of cytological abnormality. Among normal smears, only 12% are positive for the biomarker, compared to 45% of ASC-US and L-SIL and 89% of H-SIL smears. In histology only 2% of normal biopsies and 38% of CIN 1 show diffuse staining for p16(INK4a), compared to 68% of CIN 2 and 82% of CIN 3. Although there is good evidence that p16(INK4a) immuno-staining correlates with the severity of cytological/histological abnormalities, the reproducibility is limited due to insufficiently standardized interpretation of the immuno-staining. Therefore, a consensus needs to be reached regarding the evaluation of p16(INK4a) staining and the biomarker needs to be assessed in various clinical settings addressing specific clinical questions (Tsoumpou et al. 2009).

Review of the available data indicates that telomerase is activated in the majority of cervical SCC as it is in most malignant neoplasms. Telomerase activity can also be detected in some pre-neoplastic cervical lesions, but the significance of this in unclear, because non-neoplastic, proliferating epithelial cells also can have telomerase activity. The bias introduced by cytologic sampling methods can complicate the interpretation of results. Quantitative telomerase assays may be useful in distinguishing nonmalignant, physiologic activation of telomerase from malignant activation. Studies evaluating telomerase component (hTR or hTERT) expression by evaluation of RNA, mRNA, or antigen have yielded conflicting results, but the observation that many nonmalignant, non-telomerase active cells have detectable hTR and hTERT suggests that many cells express telomerase RNA and catalytic components, but do not have active telomerase. The implication is that a regulatory overlay must exist that controls telomerase activation. Activation of the enzyme in carcinogenesis could conceivably be a physiologic activation that normally accompanies cellular proliferation, a direct appropriation of telomerase activity by the neoplastic

process, or both. The presence of inactive telomerase in many cells also raises the possibility of a non-catalytic function for the telomerase complex (Nowak 2000).

Activator protein-1 (AP1) is a dimeric protein, consisting either of homodimers between c-Jun, JunB, and JunD or by heterodimers with members of the Fos-family by physically interacting via a "leucine zipper" region. AP1 is an important transcription factor initially identified as a DNA binding protein that bound to enhancer sequences of the human metallothionein IIA gene. The protein components of AP1 are encoded by a set of genes known as "immediate-early" genes that can be activated by a variety of growth factors and mitogens through several different signaling pathways. Until recently, AP1 was considered a transcription factor expressed in most tissues to regulate cellular and viral genes, now it is becoming evident that AP1 can be involved in tissue-specific regulation of target genes due to the differential combination of the components of this important transcription factor. AP1 plays a crucial role during HPV early gene expression, in particular of the expression of E6 and E7 oncoproteins. During cellular transformation, HPV infect basal cells in stratified epithelium. Their DNA integrates into the host genome usually through the E2 gene. As these cells differentiate and migrate into the upper layer of the epithelium, viral oncogenes are expressed blocking their differentiation. Mutagenesis in AP1 sites belonging to the HPV promoter long control region (LCR) completely abolished the HPV promoter activity in different cell lines. Biochemistry assays on this AP1 transcription factor include protein–protein interactions between AP1 and another factor as E7 from HPV, and YY-1. The post-translational modification and the retinoic acid interaction suggest a role for this AP1 factor in tissue-specific transcription of HPV (Velazquez Torres and Gariglio Vidal 2002).

The polymorphism rs1042522 at codon 72 of the Tp53 tumor-suppressor gene has been investigated as a genetic co-factor. More than 80 studies were done between 1998 and 2006, after it was initially reported that women who are homozygous for the arginine allele had a risk for cervical cancer seven times higher than women who were heterozygous for the allele. However, results have been inconsistent. No association was found between cervical cancer and Tp53 codon 72 polymorphism when the analysis was restricted to methodologically sound studies (Klug et al. 2009).

Migration of cancer cells from the origin tissue to surrounding or distant organs is essential for tumor progression. Many studies of tumor invasion and metastases have focused on the degradation of the extracellular matrix where matrix metalloproteinases (MMP) play a central role. Two of these enzymes, MMP-2 and MMP-9, have been correlated with the processes of tumor cell invasion and metastasis in human cancers, including uterine neoplasms. It has been shown that the upregulation of MMP is associated with progression of cervical uterine neoplasms (Libra et al. 2009).

A variety of serological assays to detect antibodies to genital-type HPV have been developed. Bacterially expressed fusion proteins, synthetic peptides, and HPV-11 virus propagated in a xenograft system have been the most commonly used antigen targets in either Western blot assays or enzyme-linked immuno-sorbent assays (ELISA). HPV antibodies have been readily detected and most studies suggest that they are

type-specific. Primarily, antibodies appear to be directed against the capsid antigens. The presence or titer of antibodies to the HPV-16 E7 protein is strongly associated with cervical cancer in approximately 25% of cases (Galloway 1992). When valid serological markers for past HPV infection become available (very likely, antibodies to HPV capsid proteins), they will be useful to estimate lifetime exposure to HPV. Serological markers for HPV-associated neoplasia (very likely, antibodies to early proteins) may prove useful for surveillance and have prognostic value. A serological marker capable of detecting past Herpes Simplex Virus 2 (HSV-2) infection would permit an analysis of the role of this virus in cervical cancer, either as an independent risk factor or in interaction with HPV. Other possible biomarkers include activation of oncogenes and inactivation of tumor-suppressor genes, assays for serum micronutrients, and analysis of leukocytes for HLA antigens. These should provide insights into the sequence of events that lead to cervical cancer and help to explain the geographic distribution of the disease (Shah 1992).

The exact histologic diagnosis of CIN lesions is essential for a differentiated therapy. Data indicate that mild and moderate CIN lesions are detected more frequently by Pap smears and colposcopically directed punch biopsies than by cervical smears alone. Histological diagnosis and HPV-typing by ISH offers the possibility to establish a differentiated therapy, e.g. by way of close follow-up, local destruction, or cone biopsy. Main attention should be directed at early detection of dysplasia, at timely institution of follow-up programs and a possibly conservative therapeutic management (Breitenecker and Gitsch 1992). Screening colposcopy has the highest area under the receiver operating characteristic (ROC) curve (0.95), followed by screening cervicography (0.90), HPV testing (0.88), cervicoscopy (0.85), fluorescence spectroscopy (0.76), and Pap smear screening (0.70). In terms of screening for SIL, fluorescence spectroscopy performs better than the standard technique, Pap smear screening, and less well than screening colposcopy, cervicography, HPV testing, and cervicoscopy (Mitchell et al. 1999a). Cervicography is not a surrogate for colposcopy. Its easy availability and economy maximize the predictive potential of the existing screening test, the Pap smear. It can also be used as a triage tool for women "at risk" for cervical HPV infections, such as women with a history of vulvar condylomata. It is not colpophotography, as the magnification and focus are stable, and the operator cannot manipulate the position of the cervix and change focus and light settings. Cervicography cannot replace Pap smears in detection programs, but can augment the predictive value of screening when used in tandem. What cervicography does provide is a screening tool with great potential and a way to attack the troubling death rates from cervical cancer still facing us in the twenty-first century (Greenberg et al. 1993). Fluorescence spectroscopy using the neural net algorithm has the highest area under the ROC curve, followed by fluorescence spectroscopy using the Bayesian algorithm, and by colposcopy, the standard diagnostic technique. Cervicography, Pap smear screening, and HPV testing perform comparably with each other but not as well as fluorescence spectroscopy and colposcopy. Fluorescence spectroscopy performs better than colposcopy and other techniques in the diagnosis of SIL. It also permits real-time diagnosis and has the potential of being used by inexperienced health care personnel (Mitchell et al. 1999b).

In summary, invasive cervical cancer is the second most common female cancer worldwide with about 493,000 new cases per year. About 273,000 women die from cervical cancer each year, 85% of which take place in developing countries. Cervical cancer has a slow progress, from pre-invasive CIN to invasive phases, meaning that the disease can be diagnosed while in the phase of pre-invasive lesion, and treated successfully thanks to the regular screening of asymptomatic women (the Pap smear). Additional diagnostic procedures for pre-invasive lesions of the uterine cervix like DNA cytometry (flow cytometry) can point to dysplasia that can progress to severe stages, such as H-SIL. If the level of chromosomal disturbance is higher (aneuploidy), it is more probable that H-SIL will develop. Laser screening of cells extracted with modern cytologic screening liquid-based cytology (LBC) enables us to automatically measure ploidy (chromosome regularity or irregularity) and PCR provides analysis of HPV types. These methods are recommended for a routine check-up of borderline cervical lesions in order to anticipate the ones likely to regress or progress (Grubisić et al. 2009). HPV testing can identify women at risk of cervical cancer. Currently, molecular detection methods are the gold standard for identification of HPV. The three categories of molecular assays that are available are based on the detection of HPV DNA and include:

1. Non-amplified hybridization assays, such as Southern transfer hybridization, dot blot hybridization, and ISH
2. Signal amplified hybridization assays, such as HC-2 assays
3. Target amplification assays, such as PCR and in situ PCR

Southern transfer hybridization requires large amounts of DNA, is laborious and not reproducible, while ISH has only moderate sensitivity for HPV. The sensitivity of the HC-2 assay is similar to that of PCR-based assays, with high sensitivity being achieved by signal rather than target amplification. PCR-based detection is both highly sensitive and specific. Since PCR can be performed on very small amounts of DNA, it is ideal for use on specimens with low DNA content. In the future, with the advance of technology, viral DNA extraction and amplification systems will become more rapid, more sensitive, and more automated (Zaravinos et al. 2009). Minor cytologic abnormalities of the cervix, such as ASC-US, are vastly more common than H-SIL or invasive cancer. Current guidelines for the management of ASC-US include repeating the Pap smear at specific intervals, referring all patients for colposcopy, or using an adjunctive test such as HC-HPV testing or cervicography. The usefulness of the Pap smear is limited by its considerable false-negative rate and its dependence on clinician and laboratory performance. Colposcopy is a highly sensitive procedure, but many patients with ASC-US have normal colposcopic findings. The HC test not only measures quantitative HPV load but also detects both oncogenic and non-oncogenic HPV types, thereby increasing the probability that serious cervical disease is not missed. HC sampling is simple to perform, and positive results are strongly associated with cervical dysplasia. HPV testing in women with ASC-US can be used as an adjunctive test to identify those with HPV-associated disease. It can also serve as a quality assurance measure. Together, repeat Pap smears and HPV testing should identify most patients with underlying cervical dysplasia. Combined testing may

also minimize the number of unnecessary colposcopic examinations in women who have no disease (Apgar and Brotzman 1999). The ASC-H category accounts for less than 1% of cytology reports, and 33% to 84% will test positive for oncogenic HPV. The number of patients with CIN 2/3 and cancer on biopsy is quite variable, from about 12% to more than 70%, averaging about 40%. The variation reflects patient population as well as local laboratory practices, but older subgroups are more likely to have negative HPV results and negative follow-up. Both the sensitivity of HPV testing for CIN 2/3 detection and the negative predictive value for a patient with ASC-H and negative HPV testing average more than 95%. Additional studies evaluating other types of ancillary testing for the ASC-H category are needed. ASC-H is an uncommon cytology result, and HPV testing results and biopsy follow-up show variation according to patient age group and local laboratory practices. A negative HPV result in ASC-H offers a high negative predictive value and could be considered as a management strategy in mature women as well as women 30 years and older receiving combined cytology and HPV screening (Coutlée et al. 2009; García-Tamayo et al. 2009; Stillman et al. 2009; Davey et al. 2010; Kinney et al. 2010; Poljak and Kocjan 2010; Burger et al. 2011).

4 Colposcopy

HPV infection results can be clinically evident or be subclinical and in this latter case they are revealed by a highly-sensitive colposcopic integrated examination after acetic acid application at 3% followed by Schiller's test with Lugol solution in weak iodine concentration. Indeed, the distal districts of male and female uroano-genital tracts take advantage of the colposcopic diagnostics although complementary analysis like histology or DNA tests for HPV typing have sometimes to be performed to confirm the results or to evaluate the prognosis. HPV subclinical lesions, above all at cervical and vaginal level, are those mostly involved with the cancer genesis: the bright-white acidophilia often combined with irregular surface, atypical vascularization, and discrete iodine caption represents a colposcopic indication to complementary diagnostic analysis aiming at choosing the most suitable therapy for which colposcopy can show the lesion topography and its frequent plurilocalizations (Peroni and Visci 2000). The colposcopic criteria for abnormal epithelium are leukoplakia, a white area detected after the application of acetic acid, or iodine-negative areas not reacting to the acetic acid test. However, white epithelium and iodine-negative areas are not specific for abnormal tissue, condyloma or CIN. The lack of specificity of colposcopic findings has motivated the distinction between minor and major grade abnormalities in the recently proposed new colposcopic terminology. Metaplasia and dysplasia cannot be distinguished by colposcopy. All criteria proposed so far lack specificity and reproducibility. Thus, it is at present considered that colposcopy is not a diagnostic method, but an investigative technique that allows the evaluation of the extent of the lesion and localization of the squamo-columnar junction (SCJ) (Barrasso 1992).

When metaplastic cervical epithelium is exposed to the factors responsible for neoplasia, the transformation zone (TZ) becomes atypical. These changes, visible as acetowhite epithelium, altered vascular patterns, and irregular surface contour, are easily recognized through the colposcope in most patients. It is possible to grade the changes to determine the severity of the abnormality and so, in consultation with the patient, decide on the best management course. However, there will always be lesions where colposcopy is indeterminate, and biopsy of such lesions is mandatory. Combined colposcopy and histology are still the mainstays of clinical management. As yet, more refined investigations, such as HPV-subtype determination, have not demonstrated any advantage for these patients (Coppleson et al. 1993). As for the distinction between condyloma and HG-CIN, all criteria proposed so far also lack reproducibility. Moreover, condyloma is often found at the periphery of HG-CIN, rendering such a distinction meaningless. The only features specific of HPV infection without dysplasia are condyloma acuminatum and, to a lesser extent, non-acetowhite microcapillary surfaces. Finally, it has been shown that there is no colposcopic sign specific of HPV types (Barrasso 1992). HPV-induced flat condylomatosis is not only much more frequently observed on the hexocervix than the florid type, but it also induces colposcopic changes in the epithelium without completely altering its morphology. HPV-induced changes have now been largely identified and are recognizable in colposcopy. They can therefore be classified in the various patterns that characterize colposcopically the transformation epithelium, with undeniable advantages not only from the point of view of their correct classification, but also from that of their prognostic evaluation. This is important because viral modifications (abnormal TZ, ANTZ) have a different diagnostic and prognostic significance from those observable in the notice squamous epithelium (Mossetti et al. 1989).

Colposcopic practice continues to evolve. As its need has expanded, so has the role of training, audit, and continuing medical education. The recently published National Health Service Cervical Screening Programme clinical guidelines document covers almost every aspect of clinical practice in an evidence-based directory. Excision of the TZ may be a very minor or major entity. The recent TZ classification system of the International Federation of Cervical Pathology and Colposcopy (IFCPC) attempts to clarify and standardize nomenclature so that therapy can be realistically compared. For the evaluation of borderline nuclear abnormal smear and for post-treatment surveillance, HPV test is clinically useful and efficient. Other biological tumor markers are likely to become clinically useful as their predictive profiles emerge (Prendiville 2005). Current evidence supports the use of colposcopy for the detection of intraepithelial lesions as a second line tool. CIN treatment involves either excisional or destructive techniques, usually performed under local anesthesia. Although a debate exists about the most efficient approach, the currently available evidence reveals no differences in efficacy among the available conservative methods of treatment. New evidence supports treatment by destructive rather than excisional techniques, at least for LG lesions in women wishing future childbearing, as they appear to have no apparent pregnancy-related morbidity. Treatment failure rates might increase in cases of involved excision

margins, older age, or glandular involvement. There is no worldwide consensus on the optimal follow-up policy, interventions, or frequency in surveillance after treatment. HPV DNA test combined with either colposcopy or cytology is a promising combination for the early detection of treatment failures due to residual disease. Existing guidelines should probably be updated incorporating the new information emerged from recently published works (Kyrgiou et al. 2006).

5 Screening

The objective of cervical cancer screening is to reduce cervical cancer incidence and mortality by detecting and treating precancerous lesions. Conventional cytology is the most widely used cervical cancer screening test. Although cytology has been effective in reducing the incidence of and mortality from cervical cancer in developed countries in both opportunistic and (more dramatically) organized national programs, it has been less successful and largely ineffective in reducing disease burden in low-resource settings where it has been implemented (Sankaranarayanan et al. 2005). Screening with Pap smears should begin at age 18 or at the age of first sexual intercourse and should be repeated every 1–3 years, depending on individual risk factors, until age 65. Screening may be discontinued in women over age 65 who have had normal findings on two consecutive Pap smears. Use of a spatula and cytobrush for cervical sampling will improve the chances of collecting an adequate sample containing endocervical cells. In women with cervical or genital HPV infection and persistent inflammatory cervical changes unresponsive to appropriate therapy, colposcopy is necessary to screen for underlying dysplasia (Miller et al. 1992).

Population screening for cervical cancer resulted in significant reduction in the morbidity and mortality from cervical cancer. An increased understanding of the relationship of HPV infection with cervical cancer and the natural history of cervical cancer precursor lesions further solidifies and expands the biological basis for cervical cancer screening. Pap tests in asymptomatic women remain the cornerstone of cervical cancer screening. Clinicians should be cognizant of the significant false-negative rate of Pap smears. Meticulous attention to proper Pap smear technique is necessary to maximize the sensitivity of the test (Dewar et al. 1992). In the early days of cervical cytology there was a general assumption that mistakes would be a rare occurrence. These expectations were unrealistic given the fatiguing nature of the work and its dependence on human judgement. Media attention and the introduction of quality control have highlighted inadequacies in the service. The reasons for both sampling and screening errors that give rise to the issuing of false negative smear results need to be understood before measures can be taken to minimize them. An acceptable error rate should be recognized and, if possible, improved upon (Allen 1996). The high-rate of false negatives in cervical screening by Pap smear takes to the necessity of frequent testing. Because women do not like the sampling procedure, many avoid being screened. Testing for the causative HR-HPV types, by PCR or other technologies, on self-collected (tampon) samples

permits women to be monitored non-invasively. The high negative predictive value of HPV testing means a greater interval between tests, and thus reduces costs. HPV testing lends itself to primary screening. A kit for self-collection and return to a testing laboratory, followed by practitioner notification and follow-up if required, should result in wider participation. The higher accuracy of HPV testing should lead to improved cervical cancer prevention (Morris and Rose 2007).

A standardized system of reporting abnormal cervical cytology, the Bethesda System, has been developed and revised to reflect our current understanding of HPV-related pre-cancers. The Pap smear has been the backbone of cervical cancer screening programs over the past five decades (Holcomb and Runowicz 2005). The role of cytology in cervical cancer screening is rapidly evolving. Widespread implementation of the Bethesda System with its accompanying atlas has provided uniform terminology and criteria for reporting cervical pathology. The development of new methods for preparing cytologic specimens as well as many other screening techniques suggests that current practices may be modified in the near future. The implementation of these new approaches may permit more conservative management of women with self-limited lesions related to HPV exposure, improve detection of serious cancer precursors, and provide more cost-effective cervical cancer screening (Sherman and Kurman 1996).

As we have seen, although current cytomorphology-based cervical cancer screening has reduced the incidence of cervical cancer, Pap smears are associated with high false positive and false negative rates. This has spurred the search for new technologies to improve current screening. New methodologies are automation of Pap smear analysis, addition of new biological or molecular markers to traditional cytology or using these new markers to replace the current screening method (Nijhuis et al. 2006). The new types of Pap smear preparations, including the ThinPrep system (Hologic Inc., Bedford, Massachusetts, USA) and computer-assisted automated Pap test screening, have increased the sensitivity of this screening test and reduced the false-negative rate of Pap smears (Perlman 1999). New screening approaches, such as quantitative cytochemistry, detection of loss of heterozygosity (LOH), and hypermethylation analysis have the potential to replace or augment current screening. HPV DNA detection stands closest to implementation in nation-wide screening programs of all markers. However, specificity is low in women aged <35 years and the psychological effects of knowledge of HPV positivity in absence of cervical premalignant disease is an important drawback. New technologies based on molecular changes associated with cervical carcinogenesis might result in comparable sensitivity, but improved specificity. Hypermethylation analysis is likely to be more objective to identify patients with H-SIL or invasive cancer with a higher specificity than current cytomorphology based screening (Nijhuis et al. 2006).

The most promising methylation marker candidates for cervical cancer early detection across all stages of cervical carcinogenesis are 15 genes: DAPK1, RASSF1, CDH1, CDKN2A, MGMT, RARB, APC, FHIT, MLH1, TIMP3, GSTP1, CADM1, CDH13, HIC1, and TERT. The published data on these genes is highly heterogeneous: 7 genes (CDH1, FHIT, TERT, CDH13, MGMT, TIMP3, and HIC1) had a reported range of methylation frequencies in cervical cancers of greater than 60% between studies. Stratification by analysis method did not resolve the heterogeneity.

Three markers (DAPK1, CADM1, and RARB) showed elevated methylation in cervical cancers consistently across studies. There is currently no methylation marker that can be readily translated for use in cervical cancer screening or triage settings. Large, well-conducted methylation profiling studies of cervical carcinogenesis could yield new candidates that are more specific for HPV-related carcinogenesis. New candidate markers need to be thoroughly validated in highly standardized assays (Wentzensen et al. 2009).

Since the mid-1990s, there has been substantial interest in the use of HPV DNA testing in cervical cancer screening under the premise that the testing of cervical cells for the causative agent of cervical cancer could have acceptable screening performance, while being more reproducible in clinical practice than Pap cytology. There have been several studies assessing the utility of HPV testing compared with the Pap test as a screening tool. These studies varied widely in lesion-outcome definition and in methodology. All of the studies were based on concomitant testing for HPV and cytology or additional tests. HPV testing has greater sensitivity (average, 27%) but somewhat lower specificity (average, 8%) than Pap cytology for detecting HG lesions. Screening of women aged 30 years or older tends to improve test specificity, but it also does so for cytology. The combination of cytology and HPV attained high negative predictive values, which suggests that their joint use could allow screening intervals to be safely increased, thus lowering costs. HPV testing is clearly one of the most promising new technologies and has the potential to improve cervical cancer screening effectiveness in many settings (Franco 2003). The changes in cervical cytology characterization agreed on by the Bethesda committee meeting in 2001 created a category of atypical findings that has caused some management confusion. By description, the characterization of cervical cytology as only atypical implies a less worrisome prognosis. However, more than 40% of HG lesions (CIN 2 or 3 or cancer) will be discovered within this category. The development and FDA approval of the HC-2 for detecting HR-HPV subtypes and the subsequent level I evidence supporting use of this test in the triage of women with atypical cytology has revolutionized the management of this cytology. With this success has come numerous additional uses for HR-HPV testing in the treatment and follow-up of women with a variety of cytologic abnormalities (Moore and Walker 2004).

With reliable techniques (PCR and HC-2), HR-HPV types are found in a very high proportion of women with invasive cancer and HG pre-invasive lesions. On the other hand, prevalence is low in cytologically normal women, except younger women, who seem to have a high frequency of transient infections in the years following the beginning of sexual activity. Some studies found a role of the presence and persistence of HR-HPV types in the progression of LG pre-invasive lesions versus HG rather then versus spontaneous regression. For these reasons HPV testing has been suggested as a possible tool for primary screening. A few studies suggest that it could allow increasing sensitivity, although problems of extrapolability of results exist. It must, however, be considered that traditional cytological screening is already very protective and that simply adding a further test would lead to an unfavorable cost-benefit ratio. An appealing possibility is applying HPV testing

with long intervals between screening rounds. This would reduce the burden for women and plausibly allow reaching higher coverage at each round. A key element is the duration of infection before progression to pre-invasive lesion. A long duration would allow selecting women at LR of developing a lesion for years (those HPV-negative), who could have long-interval test, and others (those HPV-positive) at HR, to be followed more strictly (Ronco 1999).

HPV DNA testing is a more sensitive indicator for prevalent HG-CIN than either conventional or LBC. A combination of HPV DNA and Pap testing has almost 100% sensitivity and negative predictive value. The specificity of the combined tests is slightly lower than the specificity of the Pap test alone, but this decrease can potentially be offset by greater protection from neoplastic progression and cost savings available from extended screening intervals. One "double-negative" HPV DNA and Pap test indicates better prognostic assurance against risk of future CIN 3 than three subsequent negative conventional Pap tests and may safely allow 3-year screening intervals for such LR women (Lörincz and Richart 2003). HPV tests in combination with Pap tests are 96% to 100% sensitive for detection of CIN 2/3 and cancer. However, because HPV infection is common in young women and most commonly transient, HPV testing is not recommended as part of primary cervical screening for women younger than 30 years of age. HPV testing is recommended for women of any age for the clarification of ASC-US and as an option for follow-up of women with HPV-positive ASC-US, ASC-H, or L-SIL not found to have CIN 2/3. HPV testing is also recommended as an alternative to colposcopy and/or cytology for follow-up of treated cases. Proper use of HPV testing improves the management of women with cytologic abnormalities. In addition, a negative HPV test in combination with a normal Pap test result in women age 30 and older allows the safe extension of the interval between cervical screenings. Thus, when used properly, HPV testing may reduce morbidity and mortality and do so in a cost-effective manner (Cox 2006).

HPV DNA testing was then approved by the FDA for use as an adjunct to cytology for cervical cancer screening. To help provide guidance to clinicians and patients when using HPV DNA testing as an adjunct to cervical cytology for screening, a workshop was cosponsored by the National Institutes of Health (NIH)-NCI, ASCCP, and ACS. Consensus was reached based on a literature review, expert opinion, and unpublished results from large ongoing screening studies. The conclusions of the workshop were that HPV DNA testing might be added to cervical cytology for screening in women aged 30 years or more. Women whose HPV DNA test results and cytology were both negative did not need to be rescreened before 3 years. Women whose results are negative by cytology, but are HR-HPV DNA-positive, are at a relatively LR of having HG cervical neoplasia, and colposcopy should not be performed routinely in this setting. Instead, HPV DNA testing along with cervical cytology should be repeated in these women at 6–12 months. If test results of either are abnormal, colposcopy should then be performed. This guidance should assist clinicians in utilizing HPV DNA testing in an effective manner, while minimizing unnecessary evaluations and treatments (Wright et al. 2004). Replacement of cytology by HR-HPV testing altogether is considered, but for this to be cost-effective,

accurate information about the specificity of the HR-HPV test is required. Additional test systems that can be used to stratify women with a positive HR-HPV test are HPV genotyping, viral load analysis, and HR-HPV mRNA analysis. The need for HPV genotyping of cervical smears is illustrated by the increased risk for HG cervical lesions associated with HPV types 16 and 18. In particular, for women who have normal but persistently (>1 year) HPV-18-positive smears, endocervical curettage is suggested (evidently considering the age and possible future pregnancies of the respective woman) because HPV-18 is associated with glandular lesions in the cervix, which are difficult to detect by cytology (Brink et al. 2006). The classic model of cervical cancer prevention-primary screening with cytology, followed by diagnostic colposcopically directed biopsy, and finally treatment of cancer precursors, is undergoing dynamic change. The introduction of HPV DNA testing and other new modalities provides more options but increases complexity in the sequence of screening, triage, diagnosis, and patient management. The utility of HPV testing has been established for triage of cytologic findings of ASC-US but not for L-SIL or worse. Countries without established cytology services may consider alternative screening, triage, and treatment programs that may be more readily implemented than a resource-rich "cytology followed by colposcopy" paradigm requiring an infrastructure of highly trained personnel. The diagnostic step of colposcopy and directed biopsy is not completely sensitive in the detection of CIN 2 or 3 as is sometimes assumed. The partial insensitivity of this diagnostic step results in a population of women with negative colposcopically directed biopsy findings but at increased risk for missed prevalent disease: these women may require additional triage rather than resumption of routine screening. As more efficient screening, triage, and diagnosis increase the sensitivity of detection of even very small CIN 2 or CIN 3, overtreatment of lesions that might otherwise regress becomes a concern and highlights the need to identify accurate markers of risk of progression to cancer. Markers of molecular events further along the pathway from HPV infection to development of cancer may ultimately provide more specificity in triage and diagnosis (Solomon 2003).

Expression of two viral oncogenes, E6 and E7, in epithelial stem cells is required to initiate and maintain cervical carcinogenesis and results in significant overexpression of the cellular p16INK4a protein. Since this protein is not expressed in normal cervical squamous epithelia, screening for p16INK4a over-expressing cells allows to specifically identify dysplastic lesions, and significantly reduces the inter-observer disagreement of the conventional cytological or histological tests. Progression of pre-neoplastic lesions to invasive cancers is associated with extensive recombination of viral and cellular genomes that can be monitored by detection of Papillomavirus oncogene transcripts (APOT assay) derived from integrated viral genome copies. Detection of integrated type oncogene transcripts points to far advanced dysplasia or invasive cancers and thus represents a progression marker for cervical lesions. These new assays will help to improve current limitations in cervical cancer screening, diagnosis, and therapy control (von Knebel 2001b). PCR primers that target the L1 or E1 region can be unreliable and may miss more advanced disease, whereas those directed at the E6 or E7 regions, which encode oncogenic products, are preferable because:

1. The L1/E1 regions, but never the E6/E7 regions, are lost during integration of viral DNA into host genomic DNA, a process that can represent an integral component of progression from infection to tumorigenesis
2. The E6/E7 nucleotide sequence exhibits less nucleotide variation

The choice of region used for PCR has implications for HPV screening strategies in the clinical diagnosis and management of cervical cancer (Morris 2005).

In the next 30 years, cervical cancer screening will have evolved through four phases. The first was traditional screening, which has been associated with a two-thirds reduction in cancer incidence and death rates in the last 50 years and currently is ending. The second phase is HPV testing, for managing cytologic abnormalities and possibly for primary screening. A third phase, new in development, proposes the use of host biomarkers (or combinations thereof) as either surrogates of HPV infection or, potentially, indicators to assess cancer risk and concentrate available resources on a subset of women. The fourth, likely final phase will be screening in an era of vaccines. If HPV vaccines are successful, the pool of at-risk individuals and the prevalence of Papillomaviruses that place them at risk will gradually shrink. In this climate, screening strategies that target HPV alone (as opposed to cytologic testing) may become more economical. If so, previous strategies may become obsolete as the balance of cervical cancer prevention shifts from traditional screening to primary prevention coupled with HPV testing (Crum et al. 2003). Cervical cancer is the most common malignancy amongst females in developing countries, mainly due to a lack of precursor screening. This absence of screening is the result of inherent disadvantages of the Pap smear: high cost, low sensitivity, the need for a laboratory with high human expertise, and a complex screening program logistic system. The prerequisites for screening in a developing country include a screening method that is affordable, which can be effectively applied once in a lifetime at the age of 30–35 years, provide an immediate result, and thereby allowing for on-site treatment of positive cases. None of the current screening methods comply with these prerequisites. More research is necessary into different combinations of tests, which improve sensitivity. On-site HPV identification, alone or in combination with other tests, is promising (Cronjé 2004).

The reduction in cervical cancer incidence in developed countries is largely attributed to the introduction of cervical screening. Cervical screening currently depends on the identification by cytology of abnormalities in cells taken from the surface of the cervix. The standard Pap test was developed >50 years ago, and despite modifications, still forms the basis of the test currently in use in most routine screening laboratories. Advances in our understanding of the molecular mechanisms that lead to the development of cervical cancer have been slow to impact on screening, despite the relatively high false negative rates that can be associated with the conventional Pap smear. Improvements in screening strategies fall into a number of categories. Methods that improve cell presentation and attempt to eliminate artefacts/obscuring debris can be combined with image analysis systems in order to enhance diagnostic accuracy. Such approaches still rely on cytological evaluation and do not incorporate advances in our knowledge of how HPV causes

cancer. By contrast, markers of virus infection or cell cycle entry, particularly those that offer some degree of prognostic significance, may be able to highlight abnormal cells more reliably than cytology, and could be combined with cytology to improve the detection rate. Our understanding of the molecular biology of HPV infection and the organization of the HPV life cycle during cancer progression provides a rational basis for marker selection. The general assumption that persistent active infection by HR-HPV types is the true precursor of cervical cancer provides the rationale for HPV DNA testing in conjunction with enhanced cytology, while the development of RNA-based approaches should allow active infections to be distinguished from those that are latent. The detection in superficial cells of marker combinations at the level of RNA or protein has the potential to predict disease status more precisely than the detection of markers in isolation. There is also a need for better prognostic markers if the predictive value of screening is to be improved. The potential to control infection by vaccination should reduce the incidence of HPV-associated neoplasia in the population, and this may cause a change in the way that screening is carried out. Nevertheless, the lack of a therapeutic vaccine, and the difficulties associated with eliminating infection by multiple HR-HPV types, means that some form of screening will still be required as a preventive measure for the control of cervical cancer for the foreseeable future (Doorbar and Cubie 2005).

In conclusion, although cytology-based cervical cancer prevention programs have reduced its incidence in many industrialized countries, the limited sensitivity of cervical cytology makes these programs difficult and expensive to maintain. Therefore, over the next several years it is likely that we will begin to switch from cervical cytology-based screening programs to programs based on testing for HR-HPV. Multiple, large, and well-controlled screening trials have clearly demonstrated that HPV testing is considerably more sensitive than cytology (either conventional or LBC) and only slightly less specific when used in women 30 years of age and older. Initially, we will use a combination of cervical cytology and HPV testing to screen, but as more data from large screening studies become available, it is becoming clear that cytology provides little benefit over using HPV testing alone to screen. Therefore, in the future it is likely that we will use HPV testing alone to screen, and reserve cervical cytology as a way to determine which HPV-positive women require additional follow-up or colposcopy (Wright 2007; Castle 2009; Lynge and Rebolj 2009; Belinson and Belinson 2010; Syrjänen et al. 2010).

6 Conclusions

Screening for cervical cancer precursors by cytology has been very successful in countries where adequate resources exist to ensure high quality and good coverage of the population at risk. Mortality reductions in excess of 50% have been achieved in many developed countries. However, the procedure is generally inefficient and

unworkable in many parts of the world where the appropriate infrastructure is not achievable. Four possible clinical applications of HPV DNA testing are:

1. Triage of women with equivocal or LG cytological abnormalities
2. Follow-up of women with abnormal screening results who are negative at colposcopy/biopsy
3. Prediction of the therapeutic outcome after treatment of CIN
4. Primary screening HPV DNA test, solely or in combination with Pap smear, to detect cervical cancer precursors

There are clear benefits for the use of HPV DNA testing in the triage of equivocal smears, LG smears in older women, and in the post-treatment surveillance of women after treatment for CIN. However, there are still issues regarding how best to use HPV DNA testing in primary screening. Primary screening with HC-2 generally detects more than 90% of all CIN 2, CIN 3, or cancer cases, and is 25% (95% confidence interval, CI: 15–36%) relatively more sensitive than cytology at a cut-off of ASC-US (or L-SIL if ASC-US unavailable), but is 6% (95% CI: 4–7%) relatively less specific. Several approaches are currently under evaluation to deal with the lower specificity of HPV DNA testing as associated with transient infection. These include HPV typing for HPV-16 and HPV-18/45, markers of proliferative lesions such as p16 and mRNA coding for the viral E6 and/or E7 proteins, with a potential clinical use recommending more aggressive management in those who are positive. In countries where cytology is of good quality, the most attractive option for primary screening is to use HPV DNA testing as the sole screening modality with cytology reserved for triage of HPV-positive women. Established cytology-based programs should also be gradually moving towards a greater use of HPV DNA testing to improve their efficacy and safely lengthen the screening interval. The greater sensitivity of HPV DNA testing compared to cytology argues strongly for using HPV DNA testing as the primary screening test in newly implemented programs, except where resources are extremely limited and only programs based on visual inspection are affordable. In such countries, use of a simple HPV DNA test followed by immediate "screen and treat" algorithms based on visual inspection in those who are HPV-positive are needed to minimize the number of visits and make best use of limited resources. The fact that HPV infection is a sexually transmitted disease (STD) may lead to anxiety and concerns about sexual relationships (Cuzick et al. 2008).

Twenty years have passed since the first studies using HPV testing began in clinical settings. At that time, controversy regarding the role of HPV in cervical carcinogenesis still divided the scientific world. Epidemiological and natural history studies on HPV and cervical cancer in the ensuing two decades secured the necessary role of carcinogenic HR-HPV in the genesis of cervical cancer, providing the rationale for testing for its cause. Subsequently, cross sectional studies and large RCT have provided clinical validation for HR-HPV testing in triage of ASC-US, in post-colposcopy management of women referred for ASC-US, ASC-H, atypical glandular cells (AGC) not otherwise specified (NOS), and L-SIL and not found to have CIN 2+ or adenocarcinoma in situ (AIS) at initial colposcopy, in post-treatment of CIN 2+ surveillance, and in co-testing with the Pap test of women age

30 and over. This is the story of the road traveled that brought the clinical use of HPV testing from its genesis only a few years after Dr. zur Hausen's discovery to its present eminent role in both primary cervical cancer screening and abnormal Pap management (Cox 2009).

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Chapter 6
Human Papillomavirus DNA Testing: What, How, and When

Ciro Comparetto and Franco Borruto

1 Introduction

Human Papillomavirus (HPV) has been associated with the production of many skin and mucosal lesions, the development of squamous cell carcinoma (SCC) of the genital areas, skin and aerodigestive tracts, and adenocarcinoma of the uterine cervix. There are more than 200 known genotypes of HPV. Some genotypes have been associated with lesions that have none or minimal chances of malignant transformation, while other genotypes (especially types 16, 18, 31, 35, 45, and 51) have been found in mild, moderate, and severe dysplasia, carcinoma in situ (CIS) or frank invasive carcinoma (Amortegui and Meyer 1990). HPV infections of the female genital tract are common. HPV is the most common genital viral infection in healthy, sexually active subjects, and the presence of chronic or persistent HPV types in genital cells may constitute a prognostic marker of underlying or predict future HPV-associated diseases (Pagliusi and Garland 2007). The association of these viruses with anogenital neoplasia has stimulated efforts to devise practical methods of detection and typing of HPV. For a long time, although experimental diagnostic tests were available, they were, for the most part, complex, time consuming, and were limited to medical centers researching HPV. Methods for preparing probes with higher sensitivities for hybridization tests have allowed use of in situ methods on formalin-fixed tissues. Antigen detection systems are not available except for antisera directed against the common structural antigens. The most useful immunologic test is directed toward detection of nonstructural antigens in fixed tissues: such a system can also be useful for virus typing (Lancaster and Jenson 1987).

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A variety of novel tests for detecting the presence of oncogenic HPV types in biological specimens have been reported. These are based on the various stages of infection and viral life cycle. HPV infects squamous epithelium with expression of various gene products intimately linked to epithelial cell differentiation. Hence, there are basically three classes of detectable markers directly derived from HPV: molecular markers based on detection of nucleic acid sequences (this has become the method of choice), serological markers based on detection of antibodies against viral proteins, and cellular markers based on detection of proteins expressed intracellularly, upon either infection or carcinogenesis. There is an increasing demand to develop standard tools to assess the quality of HPV detection systems, for regulatory and clinical management purposes. International standard reagents for HPV will help defining the analytical sensitivity and specificity of various detection methods, and will allow assuring that laboratory services used to evaluate disease burden, HPV vaccines, and cancer prevention strategies are accurate and comparable worldwide. The advent of prophylactic vaccines against HPV infections and related diseases stresses the increasing importance of HPV assays in monitoring the impact of HPV vaccination on disease burden (Pagliusi and Garland 2007). The issue of determining which HPV is present in a clinical specimen (typing specimens for HPV) has received attention because HPV causes condylomata acuminata and are associated with the continuum of disease that ranges from dysplasia to invasive genital cancer. Morphological inspection of precancerous lesions is not sufficient to determine which lesions will progress and which will not. A number of research tools based primarily on deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) hybridization have been developed. These permit identification and typing of HPV in genital tract scrapings or biopsies. Some HPV types (e.g. HPV-16 and HPV-18) have been identified in high-grade (HG) dysplasias and carcinomas more commonly than other types (e.g. HPV-6 and HPV-11) and have been designated respectively “high risk” (HR) and “low-risk” (LR) types for cervical cancer. Thus, the question arose whether HPV typing would have improved patient management by providing increased sensitivity for detection of patients at risk or by providing a prognostic indicator. Analysis of the typing data indicates that while HPV types can be designated HR and LR, these designations are not absolute and thus the LR group should not be ignored. In addition, interpretation of the data is complicated by finding HR types in individuals with no indication of disease (Roman and Fife 1989).

Genital warts (Fig. 6.1) are easily diagnosed by clinical criteria. However, the more prevalent subclinical infections generally require laboratory diagnosis. Satisfactory methods for HPV culture do not exist, and molecular technology in cytologic and histopathologic prelevements are the most widely available diagnostic techniques. Detection of HPV DNA is a more sensitive method (Douglas and Werness 1989). In fact, clinically visible venereal warts are only one manifestation of the much larger problem of HPV infection, which may be the most common venereal disease if subclinical infections are considered. HPV infection goes undiagnosed in at least 80% of cases, because subclinical infection can be identified only by acetic acid application, magnification, Papanicolaou (Pap) smears, or molecular DNA hybridization techniques applied to cytology or biopsy specimens. Genital HPV is present in up to 35%

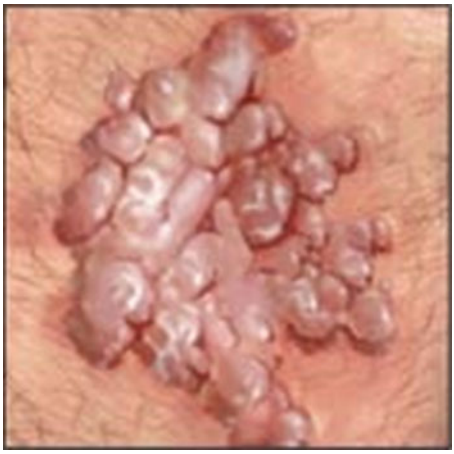

Fig. 6.1 Genital warts (modified from: Info about cervical cancer: Genital Human Papillomavirus (HPV). http://www.meowfm.net/2009/07/info-about-cervical-cancer-genital.html)

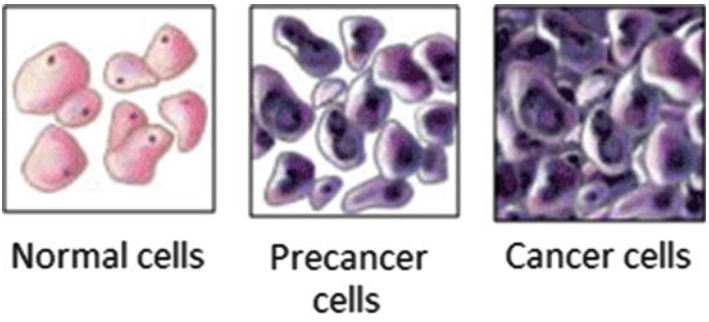

Fig. 6.2 HPV-induced cervical cells changes (modified from: Sexually Transmitted Diseases (STDs) – Diseases & Related Conditions – Human Papillomavirus (HPV). http://www.cdc.gov/std/hpv/pap/default.htm)

of some populations and in 6–11% of both men and women with clinically normal genitalia. Patients and their sexual partners should be monitored for cervical carcinoma and other epithelial malignancies associated with HPV infection. Although visible lesions can often be eradicated with the many available therapies, it is unlikely that all HPV can be destroyed where it has been integrated into the DNA of clinically normal epithelium. Therefore, excessively vigorous, painful therapy is probably unwarranted (Rapini 1990). Determining the viral genotype in the tissue involved will permit the separation of those lesions supposedly to be LR from those associated with the HR types. This knowledge may be helpful to determine the appropriate management of patients infected with HPV (Amortegui and Meyer 1990).

As we have said, genital HPV infections are associated with a spectrum of lesions ranging from benign condylomata to invasive cancer and its precursor lesions (Fig. 6.2). The transformation zone (TZ) of the cervix is the most frequent target of

the HR-HPV types. Depending on the nomenclature used, cancer precursors are subdivided on the basis of their morphologic presentation into dysplasias (mild, moderate, and severe), cervical intraepithelial neoplasias grade 1, 2, and 3 (CIN 1, 2, and 3), or low-grade (LG) and HG squamous intraepithelial lesions (L-SIL and H-SIL). H-SIL (i.e. moderate and severe dysplasias, CIN 2 and 3 lesions) are universally recognized as cancer precursors. L-SIL (i.e. very mild dysplasia and mild dysplasias, condylomata, and CIN 1 lesions), have shown that one of the most important denominators of their cancer potential is the presence of intermediate and particularly HR-HPV types. HPV typing provides the most rational basis for selecting women with L-SIL to be colposcoped and treated or given follow-up treatment with Pap smears (Ferenczy and Jenson 1996). In fact, early detection and treatment of precancerous lesions can prevent progression to cervical cancer. Identification of precancerous lesions has been primarily by cytologic screening of cervical cells. Cellular abnormalities however, may be missed or may not be sufficiently distinct, and a portion of patients with borderline or mildly dyskaryotic cytomorphology will have higher-grade disease identified by subsequent colposcopy and biopsy. Sensitive and specific molecular techniques that detect HPV DNA and distinguish HR-HPV types from LR-HPV types have been introduced as an adjunct to cytology. Earlier detection of HR-HPV types may improve triage, treatment, and follow-up in infected patients. Currently, the clearest role for HPV DNA testing is to improve diagnostic accuracy and limit unnecessary colposcopy in patients with borderline or mildly abnormal cytologic test results (Burd 2003). Thus, the subjectivity of morphologic methods contributes to a "swamping" of colposcopic services: excessive costs, overdiagnosis, and overtreatment. HPV DNA testing provides the objectivity required to clarify difficult patient management issues, including follow-up, LG cytologic abnormalities, noncorrelating (nonconfirmed) Pap smears, evaluation of nondiagnostic lower genital tract lesions, and cytology and histology laboratory quality assurance. Additionally, favorable data on HPV testing as a primary screen raise the exciting prospect of potentially lowering costs of cervical cancer screening programs, while further decreasing the incidence of cervical cancer (Cox 1996).

Minor cervical cytologic abnormalities of the cervix, such as atypical squamous cells of undetermined significance (ASC-US), are vastly more common than H-SIL or invasive cancer, but knowing which LG lesions will progress to cervical cancer and therefore deserve biopsy and excision, is difficult. Since some HPV types are strongly associated with cervical cancer, HPV typing may be a means of determining which patients with minor abnormalities require biopsy and treatment and which need only follow-up smears (Jin et al. 1999). So, testing of HPV DNA or RNA in cervical specimens offers a useful option in triaging women with equivocal Pap smear diagnosis such as ASC-US (Jin and Xu 2001). Current guidelines for the management of ASC-US include repeating the Pap smear at specific intervals, referring all patients for colposcopy, or using an adjunctive test such as HPV testing or cervicography. The usefulness of the Pap smear is limited by its considerable false-negative rate and its dependence on clinician and laboratory performance. Colposcopy is a highly sensitive procedure, but many patients with ASC-US have normal colposcopic findings. HPV test detects both oncogenic and nononcogenic HPV types

with high sensitivity, thereby increasing the probability that serious cervical disease is not missed. HPV DNA sampling is simple to perform, and persistence of HPV infection is strongly associated with cervical dysplasia. HPV testing in women with ASC-US can be used as an adjunctive test to identify those with HPV-associated disease and to limit the colposcopic examination, because HPV test has high negative predictive value. It can also serve as a quality assurance measure. Together, repeat Pap smears and HPV testing should identify most patients with underlying cervical dysplasia. Combined testing may also minimize the number of unnecessary colposcopic examinations in women who have no disease (Apgar and Brotzman 1999). In fact, the changes in cervical cytology characterization agreed on by the Bethesda committee meeting in 2001 created a category of atypical findings that has caused some management confusion. By description, the characterization of cervical cytology as only atypical implies a less worrisome prognosis. However, more than 40% of HG lesions (CIN 2 or 3 or cancer) will be discovered within this category. The development and Food and Drug Administration (FDA) approval of the Hybrid Capture second generation (HC-2; Digene Corporation, Gaithersburg, Maryland, United States of America, USA) for detecting HR-HPV subtypes and the subsequent level I evidence supporting use of this test in the triage of women with atypical cytology has revolutionized the management of this cytology. With this success numerous additional uses for HR-HPV testing in the treatment and follow-up of women with a variety of cytologic abnormalities have come (Moore and Walker 2004). HPV testing and repeat cytology are both proposed as methods to triage women with minor cytological cervical lesions. By triage, those women who need referral for diagnostic exploration with colposcopy and/or biopsy can be identified. HPV testing using the HC-2 test is more effective (more sensitive, equally specific) than cytology for the triage of patients with ASC-US Pap smears. Because of the high rate of HPV positivity, this is not the case for patients with L-SIL. Studies concerning post-treatment follow-up were heterogeneous. In general, HPV testing performed better than follow-up cytology to predict success or failure of treatment (significantly higher sensitivity, not significantly lower specificity). Overall, in comparison with follow-up cytology, HPV DNA testing is more sensitive and equally specific for triage of ASC-US cases and for predicting recurrence of CIN in women treated for HG-CIN or worse disease (CIN 2+) (Arbyn et al. 2005).

2 Historical Notes About HPV Testing

HPV types 16 and 18 have been identified in two different human cervical carcinomas. The viral DNA were molecularly cloned and used as probes to screen a large number of genital tumors by Southern blot analysis. HPV-16 and HPV-18 sequences were found in a high percentage of cervical carcinomas, but only in a small number of condylomata acuminata or flat condylomas. The majority of the latter lesions however, contained HPV-6 or HPV-11 sequences, which in contrast were detected only rarely in CIS or invasively growing carcinomas. A similar distribution of the

different papillomaviruses was observed when cell swabs taken from the cervix were tested by in situ hybridization (ISH) (Gissmann et al. 1984). In more recent years, the relationship between HPV and genital neoplasia has been explored intensively, and a molecular basis for the role of HPV in the genesis of these diseases has been convincingly demonstrated. These findings have provided justification for efforts to apply this molecular information to the early detection and possible prevention of HPV-related neoplasia. The technology of detecting viral nucleic acids in genital fluids brought with it initial hopes that it would serve to identify women at risk for having or developing precancers or cancers of the cervix. Subsequent studies, however, have demonstrated limitations of the technology for predicting future disease. Recently, molecular immunology has complemented these prior efforts with the intent to identify serological indices of exposure to HPV and perhaps delineate individuals at risk (Crum et al. 1991).

The identification of HPV DNA in the early 1980s generated interest in molecular classification of the virus and prompted studies regarding the oncogenic potential of genital HPV. Subsequent studies confirming the presence of HPV in greater than 90% of precancerous cervical lesions and close to 100% of cervical cancers has raised concerns regarding the adequacy of Pap smear testing for the detection of precancerous lesions/HPV infection. A variety of detection methods adjunctive to cytologic testing have been described, including detection at the macroscopic level, cervicography, colposcopy, and serologic and molecular-based HPV testing. Recently, there has been intense interest in molecular-based detection and typing of HPV-induced genital lesions. This has resulted in the development of a variety of molecular-based detection methods including Southern transfer, dot blotting, ISH, HC-2, and polymerase chain reaction (PCR)-based assays (Wick 2000). Testing for HPV relies exclusively on techniques of molecular biology using nucleic acid probes. Tests for HPV using nucleic acid probes have been commercially available since the late 1980s, but early tests were cumbersome and involved the use of nucleic acid probes labeled with radioactive phosphorus (32P). These early HPV tests did not achieve widespread use because they did not detect all oncogenic HPV genotypes. The current commercial HPV detection kit, Digene's HC-2 kit, detects virtually all HR oncogenic HPV types, as well as most LR nononcogenic HPV genotypes, but without determinate the genotype present in the prelevement. The HC-2 test format is a proprietary nucleic acid hybridization signal amplification system owned by Digene Corporation. Virtually all test formats for DNA sequence analysis are amenable to applications intended to detect and perhaps quantify the various HPV genotypes. These methods can involve direct hybridization of viral DNA with complementary RNA or DNA probes, such as Southern blotting or ISH, signal amplification, such as the HC-2 method or target nucleic acid amplification, most notably the PCR. PCR has been used for HPV detection, genotyping, and viral load determination. General or consensus primer-mediated PCR assays have enabled screening for a broad spectrum of HPV types in clinical specimens using a single PCR reaction. Following amplification using consensus primers, individual HPV genotypes are identified using a variety of hybridization methods. Using consensus primers in a test format known as real-time quantitative PCR (RQ-PCR), it is possible

to generate viral load (concentration) data from reaction curves generated by monitoring PCR reaction kinetics in real time (Hubbard 2003).

Despite its history of success in cancer screening, Pap cytology has important limitations, particularly its high false-negative rate, which carries important public health implications. Since the mid-1990s, there has been substantial interest in the use of HPV DNA testing in cervical cancer screening under the premise that the testing of cervical cells for the causative agent of cervical cancer could have acceptable screening performance, while being more reproducible in clinical practice than Pap cytology. There have been several studies assessing the utility of HPV testing compared with the Pap test as a screening tool. These studies varied widely in lesion-outcome definition and in methodology. All of the studies were based on concomitant testing for HPV and cytology or additional tests. HPV testing has greater sensitivity (average, 27%) but somewhat lower specificity (average, 8%) than Pap cytology for detecting HG lesions. Screening of women aged 30 years or older tends to improve test specificity, but also for cytology. The combination of cytology and HPV attained high negative predictive values, which suggests that their joint use could allow screening intervals to be safely increased, thus lowering costs (Franco 2003). Twenty years have passed since the first studies using HPV testing began in clinical settings. At that time controversy regarding the role of HPV in cervical carcinogenesis still divided the scientific world. Epidemiological and natural history studies on HPV and cervical cancer in the ensuing two decades secured the necessary role of HR (carcinogenic) HPV in the genesis of cervical cancer, providing the rationale for testing for its cause. Subsequently, cross sectional studies and large randomized trials have provided clinical validation for HR-HPV testing in triage of ASC-US, in postcolposcopy management of women referred for ASC-US, ASC "cannot rule out HG" (ASC-H), atypical glandular cells "not otherwise specified" (AGC NOS), and L-SIL. Some were not found to have CIN 2+ or adenocarcinoma in situ (AIS) at initial colposcopy, in post-treatment of CIN 2+ surveillance, and in co-testing with the Pap test of women age 30 and over. This is the story of the road traveled that brought the clinical use of HPV testing from its genesis only a few years after Dr. zur Hausen's discovery to its present eminent role in both primary cervical cancer screening and abnormal Pap management (Cox 2009).

As we have seen before, high quality cytology screening with good population coverage reduces the incidence and mortality of cervical cancer. Randomized controlled trials (RCT) have found HPV-testing to increase the detection rate of CIN 2+ compared with cytology. Two studies found a decreased detection rate of CIN 3+ in the HPV testing arm at the subsequent screening. RCT found that women not infected with vaccine HPV types at vaccination are well protected against CIN 2+ from these HPV types and protected against persistent infection with HPV 31, 33, and 45, but the vaccine does not protect against CIN 2+ from other HPV types and neither does it protect already HPV-infected women. The future of cervical cancer control may become a diversified strategy, one for non-vaccinated birth cohorts and another for vaccinated cohorts. It will take another 50 years before the non-vaccinated cohorts have passed the screening age. With the current uncertainty

concerning the long-term protection from HPV vaccination and because other HPV types are present in cervical cancer, it will furthermore be necessary to continue screening practice for the first cohorts of HPV-vaccinated women. Organized vaccination and screening programs with good record keeping are necessary to optimize the future control of cervical cancer (Lynge et al. 2009). Although cytology-based cervical cancer prevention programs have reduced the incidence of cervical cancer in many industrialized countries, the limited sensitivity of cervical cytology makes these programs difficult and expensive to maintain. Therefore, over the next several years it is likely that we will begin to switch from cervical cytology-based screening programs to programs based on testing for HR types of HPV. Multiple large, well-controlled screening trials have clearly demonstrated that HPV testing is considerably more sensitive than cytology (either conventional or liquid-based) and only slightly less specific when used in women 30 years of age and older. Initially, we will use a combination of cervical cytology and HPV testing to screen, but as more data from large screening studies become available, it is becoming clear that cytology provides little benefit over using HPV testing alone to screen. Therefore, in the future it is likely that we will use HPV testing alone to screen, and reserve cervical cytology as a way to determine which HPV-positive women will require additional follow-up or colposcopy (Wright 2007).

In the next 20 years, cervical cancer screening will have evolved through four phases. The first was traditional screening, which has been associated with a two-thirds reduction in cancer incidence and death rates in the last 50 years and currently is ending. We are entering a second phase, HPV testing, for managing cytologic abnormalities and possibly for primary screening. A third phase, new in development, proposes the use of host biomarkers (or combinations thereof) as either surrogates of HPV infection or, potentially, indicators to assess cancer risk and concentrate available resources on a subset of women. The fourth and, likely, final phase will be screening in an era of vaccines. If HPV vaccines are successful, the pool of at risk individuals and the prevalence of Papillomaviruses that place them at risk will gradually shrink. In this climate, screening strategies that target HPV alone (as opposed to cytologic testing) may become more economical. If so, previous strategies may become obsolete as the balance of cervical cancer prevention shifts from traditional screening to primary prevention coupled with HPV testing (Crum et al. 2003).

3 Techniques of HPV Testing

In recent years, there has been a dramatic increase in the incidence of HPV infection of the female genital tract. Since the primary diagnosis of this condition is almost invariably based on cytologic observations, the diagnostic criteria are of paramount importance. The diagnosis is usually based on the presence of koilocytes, or balloon cells, and dyskeratotic cells. Although these cells remain fundamental to the diagnosis, it is important to recognize other possible cytologic manifestations of the

disease. Follow-up studies indicate clearly that any woman with cytologic evidence of HPV infection has a greatly increased risk of developing CIN. Morphologic attempts to quantify this risk are unreliable (Drake et al. 1987). The morphology of the lesion and the site in which the lesion is found are the initial clues in classifying Papillomavirus-induced neoplasia. HPV types have limited site-specificity and differ in their association with benign or malignant neoplastic development. Cytopathology, electron microscopy, antigen detection, and molecular hybridization all play a role in the armamentarium of diagnostic methods. Although nitrocellulose-blotting procedures provide an accurate and sensitive method for detecting and characterizing viral nucleic acid sequences, improvements in cytological hybridization methods allow for rapid detection of virus and analysis of HPV type directly in biopsied tissue and in cervical smears. In particular, these ISH procedures facilitate retrospective studies of stored specimens (McDougall et al. 1986). The cytologic diagnosis of cervical condylomata is based on criteria developed over the last 30 years. It has now become possible to document the presence of HPV DNA directly in cervical swabs by the highly sensitive technique of DNA filter ISH (FISH). "Classic" koilocytosis and dyskeratocytosis are not highly sensitive criteria for the presence of HPV infection, identifying only 15% of the HPV DNA-positive cases correctly. In an attempt to improve the sensitivity of the cytologic diagnosis of HPV infections, a panel of nine "non-classic" criteria was evaluated. The five most valuable signs were "mild koilocytosis", "mild dyskeratocytosis", hyperchromatic nuclei, bi- and multinucleation, and cleared cytoplasm. Using these criteria in combination, statistically discriminant analysis could correctly identify 84% of the HPV-positive group (Schneider et al. 1987).

A variety of ancillary tests useful in the diagnosis of HPV infection are currently at the clinician's disposal. Use of 5% acetic acid for detection of inapparent HPV infection is a simple procedure that can be performed in any office setting. Colposcopy is useful alone or in combination with the use of acetic acid but may not be as readily available. Use of laboratory-based tests is gaining popularity as an adjunctive measure, particularly in combination with Pap smears, for the detection of CIN or carcinoma. Although the most readily available ancillary laboratory tests were the immunologically based tests, they suffered from lack of sensitivity. The more sensitive and specific DNA hybridization tests, such as the dot blot and the Southern blot, have been adapted for general clinical use. These hybridization tests allow routine screening of patients for infection with potentially oncogenic HPV types. Single-stranded nucleic acid molecules that are complementary to each other will form hybrids under appropriate conditions. Hybridization tests make use of this phenomenon and employ labeled molecules called probes to detect specific complementary molecules called targets. Nucleic acid hybridization is the most sensitive method for detecting HPV in clinical specimens and the only one capable of identifying specific HPV types. The PCR is perhaps the most exciting development in hybridization technology. This technique is the most sensitive one available, allowing amplification of amounts of DNA as small as a single copy. With development and automation of this technique, the clinician has an almost unlimited ability to diagnose occult HPV infection (Sawchuk 1991). With the development of the

PCR, in fact, it has become possible to detect small numbers of HPV genomes in clinical samples. The sensitivity and specificity of this technique, together with the possibility of performing the test on crude cervical scrapes, makes PCR the method of choice for screening.

There are many alternative hybridization test formats: most of them use either filters or glass slides as solid supports. The question arises whether screening for diagnostic purposes must include all the HPV types associated with infections of the genital tract or only those which are strongly associated with cervical cancer (HPV-16 and HPV-18) (Melchers et al. 1991). DNA amplification methods allow the use of self-collected samples (including urine) from material collected away from the original disease site. For screening of cervical pathology, detection of HPV-DNA in urine would be useful only if it represents cervical HPV infection and/or HPV-related cervical pathology (Sehgal et al. 2009). (Lörincz 1987). The hybridization method of choice depends on the information desired and the availability of the proper diagnostic nucleic acid probes. As most of the HPV nucleic acid probes became readily available in the recent past, then the most sensitive test for screening clinical specimens, albeit the most laborious, has become the Southern blot procedure. As more information covering the involvement of HPV infections with the progression of lesions from benign to malignant was compiled, the need to know the particular subtype or status of HPV integration became more or less important in the screening of clinical samples. When this information became less important, or unnecessary for a simple screening procedure, then reverse dot blot hybridization proved to be a much easier method for obtaining the information desired. When the sensitivity of ISH using non-radioactive probes increased, then this method became the fastest, easiest, and cleanest technique for the screening of a large number of clinical samples where limited information was desired. The ideal test would be simple enough to permit automation. In order to automate a test for HPV infections, the test design must become much simpler, and in order to design a simpler test, more information will be needed concerning the biology of HPV infections, how they cause benign or malignant cellular proliferation, and how the host immune system responds to HPV infections (Rando 1990).

A number of validation experiments have compared the most commonly used HPV hybridization methods with the accepted gold standard, Southern blot hybridization. These methods are FISH, reverse dot blot hybridization (ViraPap/ViraType; Life Technologies Inc., Gaithersburg, Maryland, USA), HC, and different PCR. FISH now appears to be too inaccurate to be recommended for future epidemiological studies. ViraPap/ViraType compares well to Southern blot, but is limited to the detection of seven genital HPV types. HC and PCR-based methods may be more sensitive than Southern blot and are likewise capable of detecting most known genital HPV types, in general PCR plus hybridization in one or two steps. Currently, there is no perfect method for HPV testing, because Southern blot itself is prone to some errors in performance and interpretation. The scientific and clinical usefulness of HPV tests depends on the repeatability and accuracy of the assays (Schiffman 1992). More than ever, clinicians need regularly updated reviews given the continuously increasing amount of new information regarding innovative cervical cancer

prevention methods. A summary is given from recently published meta-analyses on three possible clinical applications of HPV DNA testing: triage of women with equivocal or LG cytological abnormalities, prediction of the therapeutic outcome after treatment of CIN lesions, and last not but not least, primary screening for cervical cancer and pre-cancer. Consistent evidence is available indicating that HPV triage with the HC-2 assay is more accurate (significantly higher sensitivity, similar specificity) than repeat cytology to triage women with equivocal Pap smear results. When triaging women with L-SIL, a reflex HC-2 test does not show a significantly higher sensitivity, but a significantly lower specificity compared to a repeat Pap smear. After treatment of cervical lesions, HPV testing easily detects (with higher sensitivity and not lower specificity) residual or recurrent CIN than follow-up cytology. Primary screening with HC-2 generally detects 23% (95% confidence interval, CI: 13–23%) more CIN 2, CIN 3, or cancer compared to cytology at cut-off ASC-US or L-SIL, but is 6% (95% CI: 4–8%) less specific. By combined HPV and cytology screening, a further 4% (95% CI: 3–5%) more CIN 3 lesions can be identified but at the expense of a 7% (95% CI: 5–9%) loss in specificity, in comparison with isolated HC-2 screening. Sufficient evidence exists to recommend HPV testing in triage of women with atypical cytology and in surveillance after treatment of CIN lesions. In the USA, recently reviewed knowledge has resulted in the approval of combined cytology and HC-2 primary screening in women older than 30 years. However, in Europe, cytology-based screening still remains the standard screening method (Arbyn et al. 2006).

The diagnostic group of dysplasias has been described as “a group of diagnostic impotence”. DNA aneuploidy detected by image cytometry of Feulgen stained Pap smears indicates a potentially progressive lesion, representing a HG lesion. DNA cytometry can identify cases of dysplasias that are likely to progress: DNA aneuploid dysplasias are H-SIL. DNA aneuploidy is the expression of an integral HPV infection. CIN lesions with episomal HPV infections are DNA diploid. With the newly developed laser scanning cytometer of ThinPrep (Hologic Inc., Bedford, Massachusetts, USA) preparation, an automatization of ploidy measurement is possible in combination with HPV test. DNA-Image cytometry has been recommended as a routine method for classification of uterine cervical borderline lesion into regressive and progressive (Bollmann 2001). Currently, HPV DNA tests validated in large trials and epidemiological studies are molecular hybridization methods such as the HC-2 HPV DNA assay and a variety of PCR protocols employing degenerate or consensus primers. Ideally, an HPV test should allow detection of multiple HPV types, identify individual types, and provide quantitative information about the viral load of each individual type found. Moreover, it should be easy to perform, be highly reproducible, with a high specificity and sensitivity, and amenable for high throughput analysis and automation. Any HPV test should be able to detect the clinically relevant HR types with a sufficient sensitivity of at least 10,000 genome copies per sample (Iftner and Villa 2003).

Research has focused on the potential role of HPV testing in three conditions: screening for cervical neoplasia, triage of women with LG lesions, and follow-up after conservative surgical treatment for CIN. Concerning the first condition,

HPV testing does not seem to offer an obvious advantage over traditional cytology screening, mainly due to false positive results in younger women with transient HPV infection. A possible exemption to this is the case of middle-aged women and low-resource settings, where the excellent sensitivity of a HPV test is desirable. Although data are controversial regarding LG lesions, results from randomized studies indicate that HPV testing could be useful in a triage of women with an initial cytological diagnosis of ASC-US, where detection of DNA of a HR type should lead to colposcopy. HPV DNA testing after conservative surgical treatment for CIN may be very sensitive and detect early residual and recurrent disease (Agnantis et al. 2003). Five main groups of commercial assays for the multiplex detection of alpha HPV are currently available. DNA-based screening assays, which test for the presence of 13–14 HPV without determination of HPV type, have been the standard for HPV detection in the last decade. Assays that combine testing for 14 HPV and HPV-16 and HPV-18 genotyping are a potential future standard for HPV detection. The clinical value of HPV genotyping assays has still not been finally determined. Recently, one of the messenger RNA (mRNA)-based assays showed equal clinical sensitivity but higher clinical specificity for CIN 2+/CIN 3+ in comparison with the validated DNA-based assay. Automation, price reduction, and improvement of clinical specificity are the main goals for the future development of HPV assays (Poljak and Kocjan 2010).

International reference materials such as International Standard reagents facilitate quality assurance of essential biopharmaceutical products and related in vitro diagnostic tests. Standardization of antibody and DNA measurements and harmonization of laboratory procedures is key to the success of cancer prevention strategies through screening methods as well as for development and implementation of vaccination against HPV. The World Health Organization (WHO) supported the preparation and initial analysis of a panel of candidate serological and DNA reference reagents aimed at facilitating inter-laboratory comparisons and detection of HPV worldwide. Two international collaborative studies assessed the performance of various HPV antibody and HPV DNA detection assays and examined the feasibility of generating HPV antibody and DNA standard reagents. These studies showed that improvement in performance and comparability of assays is urgently needed and that the use of the same International Standard reference reagent could significantly improve performance and comparability. It is hoped that the establishment of International Units and International Standards for HPV antibody and DNA analysis will be pursued with high priority (Pagliusi et al. 2006).

Concerning the prediction of HPV-associated cervical disease, several importance issues are related both to the management of women with diagnosed CIN and those with cervical cancer. Oncogenic HPV are capable of contributing to the development of malignant phenotype by several different mechanisms, most of which seem to be closely interrelated. Because of the fact that proteins mediate these molecular interactions, the logical strategy to dissect the complex molecular pathways is to study the functions of these proteins, utilizing the capabilities of immuno-histochemistry (IHC). IHC offers practically unlimited possibilities to study any target molecules, against which a monoclonal or polyclonal antibody can be raised. In the HPV-PathogenISS study, 13 different markers have been tested to evaluate

their predictive value in distinct viral events, e.g. persistence or clearance of HR-HPV in women treated for CIN. Apart from getting new insights into the molecular pathogenesis of HPV-associated cervical carcinogenesis, the disclosure of individual markers, a set of markers, or an expression profile of any such marker sets would be of clinical value as predictors of disease outcome in cervical carcinogenesis (Syrjänen 2005). The advent of molecular diagnostics and the use of whole-genome profiling using DNA microarrays yielded improved understanding of the disease process with the subsequent development of more accurate diagnostic procedures based upon these discoveries. Recent reports describing a variety of experimental approaches have identified a series of candidate genes that are overexpressed in cervical carcinoma. An emerging class of markers, the minichromosome maintenance protein family of DNA licensing factors (MCM-2, MCM-6, MCM-7), shows promise for the specific detection of HG cervical disease using simple antibody-based immuno-chemistry formats. These proteins are overexpressed in cervical disease as a result of infection by oncogenic strains of HPV and subsequent uncontrolled activation of gene transcription and aberrant S-phase induction, mediated through the E2F transcription factor pathway. This behavior appears to be a hallmark of HG cervical disease and provides the link between oncogenic HPV infections and the molecular behavior of cervical neoplasia. The use of these molecular descriptors of cervical neoplasia in simple immuno-chemistry formats compatible with conventional cytology preparations improved the screening and detection of cervical disease within the healthcare system (Malinowski 2005).

The HPV "early" proteins, E6 and E7, are the chief oncoproteins involved in cancer progression. These oncoproteins are more highly expressed in HG dysplasias and invasive cancer coincident with reduced viral DNA replication and reduced production of infective progeny virions. The E6 and E7 oncoproteins interact with several cellular proteins (classically Tp53 and retinoblastoma 1, Rb1, respectively) leading to the degradation of several of these proteins, although all interactions do not necessarily result in the degradation of a cellular protein. HPV infection is also associated with viral and host DNA methylation changes, many of which also occur in cancer types not associated with HPV infection. The E6 and E7 interactions with cellular proteins and DNA methylation changes are associated with changes in the integrity of key cellular pathways that regulate genomic integrity, cell adhesion, immune response, apoptosis, and cell cycle control. The alterations in key cellular pathways may provide useful biomarkers to improve the sensitivity of current cancer screening methods, such as the Pap test (Whiteside et al. 2008). The activity of the two viral oncogenes E6 and E7 initiates in a long-term process neoplastic transformation in few of the HPV harboring cells. As a consequence, a cellular marker protein (p16 INK4a) is increasingly expressed in dysplastic cells. Monoclonal antibodies directed against p16 allow therefore to specifically identify dysplastic cells and derived invasive cancers in histological slides but also cytological smears (CIN-tec assay; Roche mtm Laboratories, Heidelberg, Germany). In advanced preneoplastic lesions HPV genomes are often integrated into cellular chromosomes. This leads to enhanced expression of the viral oncogenes. The detection of specific viral mRNA transcripts derived from integrated HPV genomes allows to identify preneoplastic

lesions with a particularly HR for progression to invasive cancers (Amplification of Papillomavirus Oncogene Transcripts, APOT assay). These findings will allow to establish highly sensitive, but specific and cost efficient new cancer early detection assays (von Knebel Doeberitz 2001a). In fact, since p16 is not expressed in normal cervical squamous epithelia, screening for p16 over-expressing cells allows it to specifically identify dysplastic lesions, and significantly reduces the inter-observer disagreement of the conventional cytological or histological tests. Progression of preneoplastic lesions to invasive cancers is associated with extensive recombination of viral and cellular genomes which can be monitored by detection of Papillomavirus oncogene transcripts (APOT assay) derived from integrated viral genome copies. Detection of integrated type oncogene transcripts points to far advanced dysplasia or invasive cancers and thus represents a progression marker for cervical lesions. These new assays will help to improve current limitations in cervical cancer screening, diagnosis, and therapy control (von Knebel Doeberitz 2001b).

p16 is a cyclin-dependent kinase-4 inhibitor (p16INK4a) that is expressed in a limited range of normal tissues and tumors, being an indirect marker of cell cycle dysregulation. In recent years, IHC with p16INK4a antibodies has been used as a diagnostic aid in various scenarios in gynecologic pathology. Diffuse (as opposed to focal) positivity with p16INK4a in the cervix can be regarded as a surrogate marker of the presence of HR-HPV. In cervical squamous lesions, p16INK4a is positive in most HG-CIN and in some cases of LG-CIN, usually those associated with HR-HPV. p16INK4a may be useful to identify small focal HG-CIN lesions, to distinguish some cases of CIN involving immature metaplastic squamous epithelium from immature metaplastic squamous epithelium not involved by CIN and to distinguish HG-CIN from benign mimics. Most cervical carcinomas of squamous, glandular, and small cell-type are p16INK4a-positive. In cervical glandular lesions, p16INK4a is useful in the distinction between AIS (diffusely positive) and benign mimics, including tuboendometrial metaplasia and endometriosis, which are usually p16INK4a-negative or focally positive. p16INK4a may be used, in combination with other markers (Ki-67), to distinguish between a cervical adenocarcinoma (diffuse positivity) and an endometrioid-type endometrial adenocarcinoma (negative or focally positive). Some uterine serous carcinomas are diffusely positive (O'Neill and McCluggage 2006). Thus, p16INK4a has emerged as a valuable surrogate marker for HR-HPV infection and shows increased immuno-expression with worsening grades of CIN. Numerous studies have emerged in recent years supporting its role in the detection of HG dysplasia and have lead to the use of p16INK4a IHC in many laboratories (Kalof and Cooper 2006). Strong and full thickness staining of p16INK4a in the cervix epithelium is highly supportive of H-SIL, while weak and basal/rare staining favors L-SIL. All HPV-positive cases are also p16INK4a-positive, but no statistically significant relationship between HPV infection positivity and the intensity and distribution of p16INK4a has been found. HPV is not helpful in the grading of SIL, as an unignorable rate of HR-HPV positivity (26.6%) has been detected in L-SIL group (Yildiz et al. 2007).

Although p16INK4a immuno-histology is routinely used as a cost effective surrogate biomarker for transforming HPV infections, many of the published articles

are confusing and contradictory. The discrepancies can be ascribed to a multitude of factors operating at the molecular, technical, and interpretative levels. In the first place, the simplistic model of viral mediated oncogenesis is speculative and fails to account for all the known biomolecular changes. Unresolved technical issues include the variables of tissue fixation, antibody dilution, antibody isotype and clone, and the sensitivity of the particular detection method. Within any controlled staining method, strong diffuse or "block" immunoreactivity in squamous cells may be found in moderate/severe dysplasia (CIN 2/3) and invasive squamous carcinoma. In contrast, focal or multifocal reactivity in squamous cells may be artefactual, related to LR- or HR-HPV. p16INK4a is less reliable when dealing with glandular lesions since considerable overlap exists between reactive and dysplastic lesions. In addition not all glandular dysplasias/carcinomas are HR-HPV related, nor are all p16INK4a immunoreactive lesions associated with HR-HPV. p16INK4a immunoperoxidase shows greater specificity than sensitivity for squamous lesions. In comparison, glandular dysplasias/carcinomas show reduced specificity and sensitivity. Like all cell cycle regulatory proteins, the future diagnostic role of p16INK4a is limited. The ideal diagnostic molecular test for cervical dysplasias will detect a HR-HPV related product after, but not before, cell transformation and will reliably predict those cases yet to experience disease progression (Mulvany et al. 2008). p16INK4a could act as an adjunct to current cytological and histological assessment of cervical smears and biopsies, allowing the identification of those women with ambiguous results that require referral to colposcopy and potentially treatment. Although there is good evidence that p16INK4a immunostaining correlates with the severity of cytological/histological abnormalities, the reproducibility is limited due to insufficiently standardized interpretation of the immunostaining. Therefore, a consensus needs to be reached regarding the evaluation of p16INK4a staining and the biomarker needs to be assessed in various clinical settings addressing specific clinical questions (Tsoumpou et al. 2009). However, its routine application in the diagnosis of SIL of the uterine cervix may present difficulties for the general pathologist (Kostopoulou et al. 2011).

Review of the available data indicates that telomerase is activated in the majority of cervical SCC as it is in most malignant neoplasms. Telomerase activity can also be detected in some preneoplastic cervical lesions, but the significance of this is unclear, because nonneoplastic, proliferating epithelial cells also can have telomerase activity. The bias introduced by cytologic sampling methods can complicate the interpretation of results. Quantitative telomerase assays may be useful in distinguishing nonmalignant, physiologic activation of telomerase from malignant activation. Studies evaluating telomerase component (hTR or hTERT) expression by evaluation of mRNA or antigen have yielded conflicting results, but the observation that many nonmalignant, nontelomerase active cells have detectable hTR and hTERT suggests that many cells express telomerase RNA and catalytic components, but do not have active telomerase. The implication is that a regulatory overlay must exist that controls telomerase activation. Activation of the enzyme in carcinogenesis could conceivably be a physiologic activation that normally accompanies cellular proliferation, a direct appropriation of telomerase activity by the neoplastic process,

or both. The presence of inactive telomerase in many cells also raises the possibility of a noncatalytic function for the telomerase complex. An understanding of telomerase interaction with HPV infection in the pathogenesis of cervical neoplasia must await a further elaboration of telomerase regulation. Likewise, application of telomerase detection in cervical cancer screening programs must await a better integration of telomerase regulation in normal and specifically in HPV-infected squamous epithelial cells (Nowak 2000).

If the HPV genotype is HPV-16, HPV-16 RNA expression has been analyzed in premalignant cervical lesions of different severity and in SCC by RNA-RNA ISH in order to find differences in the topographic distribution of viral RNA, which might correlate with the severity of disease. In the basal layer of L-SIL only weak transcription of viral early genes was observed. Signal intensity increased strongly in the more differentiated cells accompanied by high levels of HPV DNA replication. This pattern of viral gene expression, together with the onset of viral late transcription in the upper differentiated layer of the epithelium, most likely reflects the productive phase of viral infection. In contrast, in H-SIL viral transcription was comparatively strong in basal cells and evenly distributed throughout the undifferentiated epithelium. This difference of viral transcription in the basal layer of the respective lesions points to an altered regulation of viral gene expression that may be causally linked to the progression of precursor lesions. Evidence for disrupted expression of 3′ early genes (E2, E4, and E5), analogous to the situation in HPV-DNA containing cervical carcinoma-derived cell lines, was not found in any of the HPV-16-positive premalignant lesions nor in the majority of cancers. The similarity of the viral transcription pattern of H-SIL and cancers suggests that additional host gene alterations are necessary for malignant progression (Dürst et al. 1992; Nindl et al. 1997).

4 HPV Testing and Cervical Cancer Screening

The objective of cervical cancer screening is to reduce cervical cancer incidence and mortality by detecting and treating precancerous lesions. Conventional cytology is the most widely used cervical cancer screening test. Although cytology has been effective in reducing the incidence of and mortality from cervical cancer in developed countries in both opportunistic and, more dramatically, organized national programs, it has been less successful and largely ineffective in reducing disease burden in low-resource settings where it has been implemented. Liquid-based cytology (LBC), testing for infection with oncogenic types of HPV, visual inspection with 3–5% acetic acid, magnified visual inspection with acetic acid, and visual inspection with Lugol's iodine have been evaluated as alternative tests (Sankaranarayanan et al. 2005). So, the role of cytology in cervical cancer screening is rapidly evolving. Widespread implementation of the Bethesda System with its accompanying atlas has provided uniform terminology and criteria for reporting cervical pathology. The development of new methods for preparing cytologic specimens as well as many other screening techniques suggests that

current practices may be modified in the near future. The implementation of these new approaches may permit more conservative management of women with self-limited lesions related to HPV exposure, improve detection of serious cancer precursors, and provide more cost-effective cervical cancer screening (Sherman and Kurman 1996).

Adjunctive diagnostic procedures for the detection of HPV infection could increase the sensitivity of primary and secondary screening of cervical cancer. HPV testing could also improve the specificity of screening programs resulting in avoidance of overtreatment and saving of costs for confirmatory procedures. Progression of HPV infection is associated with its persistence, involvement of HR-HPV types, high HPV viral load in specimens, integration of viral DNA, and possibly the presence of cofactors. The design of HPV diagnostic tests needs to take into account these parameters of disease progression. HPV DNA detection techniques based on signal-amplification are standardized, commercially available, and detect several HR-HPV types. They increase the sensitivity of screening for HG and LG lesions. Although they may yield false-negative results in the presence of significant HPV-related disease, new test formats could resolve this weakness. Amplification techniques are ideal instruments for epidemiologic purposes since they minimize misclassification of HPV infection status and allow for the detection of low viral burden infections (Coutlée et al. 1997). However, screening is still based upon cytology and for a long time further diagnostic investigation relied on colposcopy with punch biopsy. Exfoliative cytology is still widely considered as the method of choice for population screening. Primary HPV DNA screening proves equivalent or superior to cytology only in populations with a low prevalence of HPV infections (after 30 years). Data from serological HPV tests are less promising. Cytological diagnosis of HPV infection may be verified by HPV testing. Absence of HPV infection in ASC-US cytology allow to eliminate a precancerous lesion. Recognizing high oncogenic risk viruses in CIN 1 may help to reduce the control period from 24 to 12 months and may lead to immediate therapy for CIN 2 lesions (Hillemanns et al. 1997).

With reliable techniques (PCR and HC-2), HR-HPV types are found in a very high proportion of women with invasive cancer and HG pre-invasive lesions. On the other hand, prevalence is low in cytologically normal women, except in young women, who seem to have a high frequency of transient infections in the years following the beginning of sexual activity. Some studies found a role of the presence and persistence of HR-HPV types in the progression of LG pre-invasive lesions vs. HG rather then vs. spontaneous regression. For these reasons HPV testing has been suggested as a possible tool for primary screening. A few studies suggest that it could allow increasing sensitivity, although problems of extrapolability of results exist. It must, however, be considered that traditional cytological screening is already very protective and that simply adding a further test would lead to an unfavorable cost-benefit ratio. An appealing possibility is applying HPV testing with long intervals between screening rounds. This would reduce the burden for women and plausibly allow reaching higher coverage at each round. A key element is the duration of infection before progression to pre-invasive lesion. A long duration would allow selecting women at LR of developing a lesion for years (those HPV-negative), who

could have long-interval test, and others (those HPV-positive) at HR, to be followed more strictly (Ronco 1999). The current literature reflects three routes toward improving cervical cancer screening. The first is to improve the test qualities of cytology-based screening. The use of LBC and computerized analysis of Pap tests are examples of attempts at this approach. Secondly, through various combinations of parallel or sequential tests, either the sensitivity or the specificity of a given test could be improved depending on the tests chosen and the order in which they were performed (e.g. Pap test followed by HPV or vice versa). Several excellent studies have been published on the use of HPV DNA testing as a primary screening modality and as an adjunct to the triage of mildly abnormal cytologic findings. The recent literature also reflects increasing interest in visual inspection of the cervix and self-collected samples for HPV testing as an equally effective and viable alternative to cytology in low-resource settings. A third possibility is to make use of advances in digital and spectroscopic techniques. In these cost-conscious times, a significant number of articles address the cost-effectiveness of these technologies and the real value of cervical cancer screening (Soler and Blumenthal 2000).

Anyway, the classic model of cervical cancer prevention (primary screening with cytology, followed by diagnostic colposcopically directed biopsy, and finally treatment of cancer precursors) is undergoing dynamic change. The introduction of HPV DNA testing and other new modalities provides more options but increases complexity in the sequence of screening, triage, diagnosis, and patient management. The utility of HPV testing has been established for triage of cytologic findings of ASC-US but not for L-SIL or worse. Countries without established cytology services may consider alternative screening, triage, and treatment programs that may be more readily implemented than a resource-rich "cytology followed by colposcopy" paradigm requiring an infrastructure of highly trained personnel. The diagnostic step of colposcopy and directed biopsy is not completely sensitive in the detection of CIN 2 or 3 as is sometimes assumed. The partial insensitivity of this diagnostic step results in a population of women with negative colposcopically directed-biopsy findings but at increased risk for missed prevalent disease: these women may require additional triage rather than resumption of routine screening. As more efficient screening, triage, and diagnosis increase the sensitivity of detection of even very small CIN 2 or CIN 3, overtreatment of lesions that might otherwise regress becomes a concern and highlights the need to identify accurate markers of risk of progression to cancer. Markers of molecular events further along the pathway from HPV infection to development of cancer may ultimately provide more specificity in triage and diagnosis (Solomon 2003). That's because the currently mandated methods to measure the sensitivity of Pap smear interpretation (including the 5-year look back and review of 10% of negative smears) are misleading. They do not allow one to measure the true sensitivity of the test and allow only a small fraction of errors to be detected and corrected. Rapid prescreening and automated screening are the only methods that seem practically feasible, and will allow the sensitivity of the method to be measured on a routine basis, and, thereby allow a reduction in overall errors. Although HPV testing and other emerging technologies may allow alternative methods of measuring sensitivity, the most appropriate

way to use and interpret these data in this context are not yet fully developed. Unfortunately, there currently seems to be little desire to change the way things are done, and therefore to accurately measure sensitivity in cervical cytology. The first task that needs to be undertaken in fixing a problem is to admit that one exists. At present, most laboratory directors believe that their own laboratory is performing satisfactorily. They may well be, although the laboratories lack an analytic method to demonstrate this, and, therefore, the need for better methods does not seem to be acute. There is some educational value to the currently practiced and mandated performance measures, the 5-year look back and review of 10% of negative smears. Most laboratory directors seem to be happy with their methods and are not concerned that the data that are derived from the 10% review of negative slides does not reflect their actual sensitivity of screening in any meaningful way. Unfortunately, the forces that are currently in place in the USA ensure that accurate measures of the sensitivity of cervical cytology interpretation are unlikely to be implemented beyond the level of individual experimentation. As long as the expectation of cytologists is that the error rates are significantly less than they actually are, as long as there is significant legal and financial risk to actually measuring the true sensitivity, and as long as fictitious measures of performance are not only advocated but mandated, the confluence of incentives will ensure that the true sensitivity of the test will never be measured on a routine basis. Despite all of this, it is possible that cervical cytology screening may, in fact, already be performing at an optimal level. Being able to measure this operating performance may not effect any improvement to the overall process. The ultimate arbiter in this debate will always be the demonstration of a reduction of cervical cancer morbidity and mortality with any new measure implemented. At the present time, there is only one solution to the quality control issue: a force from outside the system must change the balance of the aforementioned incentives. The promise of data from European experiences with rapid rescreening may show that this method is effective and accurate. Such data might make the current methods that are in use in the USA more open to change. So yes, the answer is that "bad" data may be worse than no data at all. The bad data that we have been collecting for more than a decade is as effective a trap as anyone could have devised to ensure that actually measuring the performance of cervical smear interpretation does not happen (Renshaw 2003).

HPV DNA testing is a more sensitive indicator for prevalent HG-CIN than either conventional or liquid cytology. A combination of HPV DNA and Pap testing has almost 100% sensitivity and negative predictive value. The specificity of the combined tests is slightly lower than the specificity of the Pap test alone, but this decrease could potentially be offset by greater protection from neoplastic progression and cost savings available from extended screening intervals. One "double-negative" HPV DNA and Pap test indicated better prognostic assurance against risk of future CIN 3 than three subsequent negative conventional Pap tests and may safely allow 3-year screening intervals for such LR women (Lörincz and Richart 2003). Risk management efforts in the cytology laboratory must address the gap between what can be achieved with medical history's most effective cancer screening test, the Pap test, and even higher entrenched public expectations. Data from the ASC-US/L-SIL

Triage Study (ALTS) now provides level I clinical evidence from a large, randomized, controlled, multicenter clinical trial that shows HPV DNA testing of ASC-US cases is generally the preferred method for initial assessment of the most prevalent category of abnormal Pap interpretation. The proposed combination of HPV DNA testing with cytologic Pap testing, the DNA Pap test, further shows the potential to nearly eliminate false-negative screening results based on sensitivity and negative predictive values reported in available studies. HPV DNA testing also appears to represent a significant enhancement for detection of endocervical adenocarcinomas that are difficult to detect and prevent. HPV DNA testing, when used in conjunction with cervical cytology, can significantly reduce risk to both the patient and the laboratory (Austin 2003). The field of cytology automation, through long investigation, trial and error, and finally commercial success and failure, has arrived at the first levels of the "grail" of improvements in accuracy and productivity in cervical cytology screening. It remains to be seen how much further the road will lead toward so-called "diagnostic" instrumentation that would actually provide us with a fully automated system of "specimen in-diagnosis out" with little or no human input. Certainly the possibility of mass screening by HR-HPV DNA testing as a viable alternative is being discussed at present. Despite all of these uncertainties, the present (or nearly available) technology has the potential to improve the practice of cervical cytology. Improvements in accuracy that are necessary to provide the highest possible level of patient care and to protect practitioners from unreasonable levels of medico-legal risk are a reality. Improvements in productivity that are necessary to help in the impending labor shortage in the field of cytotechnology are also a reality. Automation is clearly the short-term solution to the most difficult of the challenges that we face (Wilbur 2003).

New guidelines for when to initiate cervical cancer screening have recently been revised. The American Cancer Society (ACS) now recommends that screening be initiated within 3 years of the onset of vaginal intercourse, but no later than 21 years of age and be continued at least until age 65 or 70. Natural history studies of HPV suggest that there is little risk of a significant precancerous lesion going undetected within the first 3–5 years after the onset of sexual activity. The new recommendations will assist in the over-referral and overtreatment of adolescents with HPV (Moscicki 2003). Annual screening is recommended also by the American College of Obstetricians and Gynecologists (ACOG), although in women aged ≥30 years with ≥3 negative Pap tests, screening may be conducted every 2–3 years. The USA FDA has approved HPV testing and most USA guidelines say that it is reasonable to consider HPV testing in combination with triennial cytology screening. Pharmacoeconomic analyses indicate that combined cytology and HPV testing every 3 years in women aged ≥30 years is comparable in sensitivity to annual LBC for the detection of cervical cancer precursors and is more cost-effective. Both surgical and nonsurgical therapies are commonly employed in patients with HPV lesions although papilloma recurrence is not uncommon. Treatment should be individualized based on the extent of disease and the needs of the patient. Current treatment of cervical cancer reflects the stage of the disease and should take into account patient- and tumor-related factors to ensure optimal patient outcomes (Spitzer 2007).

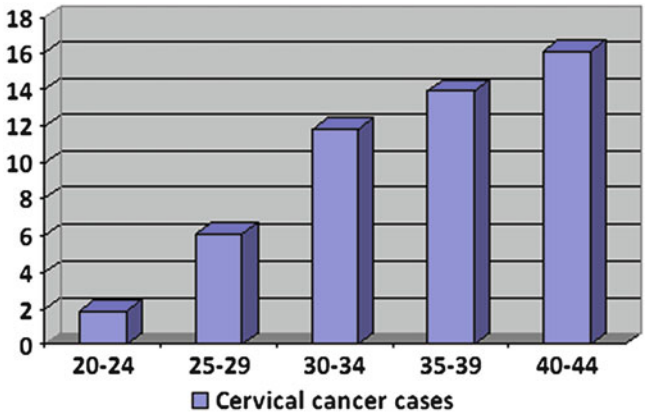

Fig. 6.3 Cervical cancer incidence (modified from: Sexually Transmitted Diseases (STDs) – Diseases & Related Conditions – Human Papillomavirus (HPV). http://www.cdc.gov/std/hpv/pap/default.htm)

HPV DNA testing has been approved by the FDA for use as an adjunct to cytology for cervical cancer screening. To help provide guidance to clinicians and patients when using HPV DNA testing as an adjunct to cervical cytology for screening, a workshop was co-sponsored by the National Institutes of Health (NIH)-National Cancer Institute (NCI), American Society of Colposcopy and Cervical Pathology (ASCCP), and ACS. Consensus was reached based on a literature review, expert opinion, and unpublished results from large ongoing screening studies. The conclusions of the workshop were that HPV DNA testing might be added to cervical cytology for screening in women aged 30 years or more. Women whose results are negative by both HPV DNA testing and cytology do not need to be rescreened before 3 years. Women whose results are negative by cytology, but are HR-HPV DNA positive are at a relatively LR of having HG-CIN, and colposcopy should not be performed routinely in this setting. Instead, HPV DNA testing along with cervical cytology should be repeated in these women at 6–12 months. If test results of either are abnormal, colposcopy should then be performed. This guidance should assist clinicians in utilizing HPV DNA testing in an effective manner, while minimizing unnecessary evaluations and treatments (Wright et al. 2004). The multicenter ALTS has shown that HPV DNA testing can be used safely to minimize intervention in many cases. HPV infection is common in young women, but rarely progresses to invasive cervical disease (Fig. 6.3). Providers need to inform themselves about new professional society guidelines that suggest delaying initiation of cervical cancer screening to within 3 years of onset of sexual activity. Given the idiosyncrasies of this population, some Authors counsel using clinical discretion when applying these guidelines to individual teenagers. In light of the extremely low likelihood of invasive disease in this age group, providers must separate the provision of contraceptive services

and sexually transmitted disease (STD) screening from requirements for cervical cancer screening (Gray and Walzer 2004).

Cervical cancer is the most common malignancy amongst females in developing countries, mainly due to a lack of precursor screening. This absence of screening is the result of inherent disadvantages of the Pap smear: high cost, low sensitivity, the need for a laboratory with high human expertise, and a complex screening program logistic system. The prerequisites for screening in a developing country include a screening method that is affordable, which can be effectively applied once in a lifetime at the age of 30–35 years, provide an immediate result and thereby allowing for on-site treatment of positive cases. None of the current screening methods comply with these prerequisites. More research is necessary into different combinations of tests, which improve sensitivity. On-site HPV identification, alone or in combination with other tests, is promising. Another promising development is immunization against HPV infection, either as a preventative measure or for stimulating immunity in infected women (Cronjé 2004). HPV testing is discussed in the context of primary screening, for triage, and as a test of cure of treatment and possible value in developing countries. The high negative predictive value of a "double negative" cytology and HPV result could allow considerable changes in policy such as increased intervals between screening rounds, adjustment of age ranges for testing, and schedule for return to routine screening post treatment. HPV testing for the triage of women to colposcopy with borderline or ASC-US cytology could be clinically effective, but may be limited in women with L-SIL or mild dyskaryosis by high HPV prevalence. Markers of HPV persistence harbor enormous potential to identify women at greatest risk of disease progression (Cuschieri and Cubie 2005). Testing for HR-HPV DNA is more sensitive than cytology in detecting pre-cancerous lesions. One of the main advantages will be the possibility of applying prolonged screening intervals. However adequate screening protocols (age of start and stop, screening intervals, management of HPV positive women) need to be applied in order to avoid over-referral to colposcopy and over-treatment and to maintain sustainable costs. Further follow-up of running trials and research on molecular markers will better define these parameters. The new situation will require organized screening programs with rigorous protocols and monitoring. This will be even more needed when women vaccinated for HPV-16 and HPV-18 will be screened. Research on how to best screen vaccinated women is a priority (Ronco and Giorgi Rossi 2008).

In conclusion, screening for cervical cancer precursors by cytology has been very successful in countries where adequate resources exist to ensure high quality and good coverage of the population at risk. Mortality reductions in excess of 50% have been achieved in many developed countries. However, the procedure is generally inefficient and unworkable in many parts of the world where the appropriate infrastructure is not achievable. Four possible clinical applications of HPV DNA testing are:

1. Triage of women with equivocal or LG cytological abnormalities
2. Follow-up of women with abnormal screening results who are negative at colposcopy/biopsy

3. Prediction of the therapeutic outcome after treatment of CIN
4. Primary screening HPV test, solely to detect cervical cancer precursors

There are clear benefits for the use of HPV DNA testing in the triage of equivocal smears, LG smears in older women, and in the post-treatment surveillance of women after treatment for CIN. However, there are still issues regarding how best to use HPV DNA testing in primary screening. Primary screening with HC-2 generally detects more than 90% of all CIN 2, CIN 3, or cancer cases, and is 25% (95% CI: 15–36%) relatively more sensitive than cytology at a cut-off of ASC-US (or L-SIL if ASC-US unavailable), but is 6% (95% CI: 4–7%) relatively less specific. Several approaches are currently under evaluation to deal with the lower specificity of HPV DNA testing as associated with transient infection. These include HPV typing for HPV-16 and HPV-18/45, markers of proliferative lesions such as p16 and mRNA coding for the viral E6 and/or E7 proteins, with a potential clinical use recommending more aggressive management in those who are positive. In countries where cytology is of good quality, the most attractive option for primary screening is to use HPV DNA testing as the sole screening modality with cytology reserved for triage of HPV-positive women. Established cytology-based programs should also be gradually moving towards a greater use of HPV DNA testing to improve their efficacy and safely lengthen the screening interval. The greater sensitivity of HPV DNA testing compared to cytology argues strongly for using HPV DNA testing as the primary screening test in newly implemented programs and in self prelevements, except where resources are extremely limited and only programs based on visual inspection are affordable. In such countries, use of a simple HPV DNA test followed by immediate "screen and treat" algorithms based on visual inspection in those who are HPV-positive are needed to minimize the number of visits and make best use of limited resources. The fact that HPV is a sexually transmitted infection may lead to anxiety and concerns about sexual relationships (Cuzick et al. 2008). Recently published baseline results of RCT, comparing cytology with HPV-based cervical cancer screening, consistently show increased detection of HG-CIN in the HPV-arm. These results are in line with the pooled estimates of the relative sensitivity derived from cross-sectional studies. From two randomized trials, also the longitudinal outcomes observed at the second screening round were reported. HPV-negative women had a relative risk (RR) of developing CIN 3 in the next 3–5 years, compared to cytology-negative women, of 0.53 (95% CI: 0.29–0.92) and 0.45 (95% CI: 0.28–0.67), respectively in the Swedish and Dutch trial. Consensus was reached at the Cochrane Workshop on Cervical Cancer Prevention, organized at the occasion of the 24th Conference of the International Papillomavirus Society, to join forces to conduct future meta-analyses of the HPV screening trials and to synthesize evidence on new methods for cervical cancer prevention (Arbyn and Cuzick 2009).

RCT that compare HPV testing with cytological testing for cervical screening are under way. The results published so far have been reviewed to compare the benefits and costs for participating women. At baseline screening, use of HPV testing increased the detection of CIN 2+. Detection of CIN 3+ was significantly increased in two trials (RR 1.70 and 2.26), but not in three other trials (RR 1.03, 1.09, and

1.31). In three trials, seven extra women had a false-positive test for each extra detected CIN 2+ case although, in another trial, this number was 49 in women ≥35 years of age. The outcome of HPV testing versus cytological testing depends not only on the relative accuracy of the primary test but also on how radical the different triage procedures are. In two trials with published outcomes from the subsequent screening round, an approximately 50% reduction in CIN 3+ detection was evident in the subsequent screening. However, in these two trials the screening modalities changed between the baseline and subsequent screening rounds, so the data doesn't show the effect of a switch from primary screening with cytological testing to primary screening with HPV testing (Lynge and Rebolj 2009). In the USA, federal and state behavioral surveillance systems routinely monitor self-reported sexual behavior and Pap test use to identify HR populations, trends, and disparities to guide and evaluate interventions for cervical cancer prevention and control. Clinical uptake of HPV vaccination and testing necessitates the expansion of behavioral surveillance systems. Cervical disease is the main focus of HPV-related behavioral surveillance because of greater cancer incidence and mortality relative to other susceptible organs, and the availability of effective technologies for prevention and control. Ongoing surveillance at the national, state, and local level is critical for monitoring the dissemination of HPV technologies and their impact on reducing disparities in the detection of precursor lesions, incidence of invasive cancer, and mortality (Tiro et al. 2008; Castle 2009; Franco et al. 2009a; Stillman et al. 2009; Wiwanitkit 2009; Huh et al. 2010; Scarinci et al. 2010).

HPV testing is more sensitive for the detection of cervical precancer and cancer than cervical cytology. The increased sensitivity of HPV testing and cytology combined ("co-testing") compared to cytology alone permitted professional societies to recommend 3-year screening intervals among the negative co-tested results. However, there is an increasing recognition that both clinical sensitivity and specificity of cervical cancer screening are important to patient safety and must be considered in the context of using current and future HPV DNA tests. Exquisite analytic sensitivity for HPV does not increase clinical sensitivity of an HPV test but does result in excessive test positivity and decreased clinical specificity. A recent USA FDA-approved HPV test, Cervista (Hologic Inc., Bedford, Massachusetts, USA), demonstrated excessive test positivity (two to four times more positive than the other FDA-approved HPV test) from its premarketing approval trial. The poor specificity of Cervista raises questions about the safety and applicability of using this test in routine cervical cancer screening. These data provide a didactic example of the potential dangers of mistaking excellent analytic sensitivity and even clinical sensitivity for good clinical performance (Kinney et al. 2010; Syrjänen et al. 2010). HPV vaccination is expected to reduce the burden of cervical cancer in most settings. However, it is also expected to interfere with the effectiveness of screening. In the future, maintaining Pap cytology as the primary cervical screening test may become too costly. As the prevalence of cervical dysplasias decreases, the positive predictive value of the Pap test will also decrease, and, as a result, more women will be referred for unnecessary diagnostic procedures and follow-up.

HPV DNA testing has recently emerged as the most likely candidate to replace cytology for primary screening. It is less prone to human error and much more sensitive than the Pap smear in detecting HG cervical lesions. Incorporating this test would improve the overall quality of screening programs and allow spacing out screening tests, while maintaining safety and lowering costs. Although HPV testing is less specific than Pap cytology, this issue could be resolved by reserving the latter for the more labor-efficient task of triaging HPV-positive cases. Because most HPV-positive smears would contain relevant abnormalities, Pap cytology would be expected to perform with sufficient accuracy under these circumstances. HPV Pap triage would also provide a low-cost strategy to monitor long-term vaccine efficacy. Although demonstration projects could start implementing HPV testing as a population screening tool, more research is needed to determine the optimal age to initiate screening, the role of HPV typing and other markers of disease progression, and appropriate follow-up algorithms for HPV-positive and Pap-negative women (Agorastos et al. 2010; Belinson and Belinson 2010; Tota et al. 2010; Burger et al. 2011; Carcopino et al. 2011).

Cervical cancer, the second most common cancer in young women, is still imperfectly screened even with the advent of primary prevention for this cancer in the form of prophylactic HPV vaccination. Indeed, the cervical Pap smear and its cytologic analysis, both operator and reader dependent, have limited sensitivities requiring repeated samplings and above all, producing a high rate of falsely negative tests. Although most cancers occur in women who are either not or insufficiently screened, the problem with cervical smears is the fact that cancers are also often diagnosed in young women having follow-ups in accordance with professional guidelines. The absence of an organized screening results in an inadequate female population coverage. Nowadays, it is unanimously recognized that HR-HPV represent the only independent risk factor for cervical cancer and that there cannot be any disease without this virus. It is therefore this strong association between a viral agent and the cervical cancer which opened the door firstly, to the notion of prophylactic vaccination and secondly, to the integration of HR-HPV testing in the screening for precancerous lesions. Molecular biological techniques based on the HR-HPV genome detection within the female genital tract have shown a very high sensitivity without any inter- and intraobserver variability and an excellent negative predictive value. Their integration in the primary screening for cervical cancer would improve the relevance of the latter and would suit the need for a wider population coverage and even for an organized screening thanks to the possibility for self-sampling. The specificity of these tests is inferior to that of the cervical smear, but the management of the falsely positive HPV tests has proved to be efficient by sorting residual cells obtained from LBC. What is urgent is the need for an organized screening program in order to improve population coverage and this does not go against a vaccination promotion or the integration of new technologies. Moreover, three randomized trials published in October 2007 have shown that it was quite safely possible to extend the time interval between two consecutive viral testing and thus improving the cost-effectiveness of cervical cancer screening (Riethmuller et al. 2008).

5 Pharmaco-Economics of HPV Testing

With the introduction of cervical screening programs, the incidence and mortality of cervical cancer has been drastically reduced. Techniques such as the traditional Pap test and the newer LBC allow for the early detection of cervical abnormalities prior to the development of invasive cervical cancer. As oncogenic HPV infection is necessary for cervical cancer, HPV DNA testing has also been proposed as a routine screening method for the general population. Screening limitations, such as adherence, test sensitivity and specificity, access, and cost-effectiveness are reflected in current screening guidelines. The development of prophylactic cervical cancer vaccines is a major milestone in cervical cancer prevention. These vaccines protect against the initial infection of certain oncogenic HPV types and therefore prevent the development of cervical dysplasia, precancerous lesions, and cervical cancer. Considering routine cervical cancer vaccination in adolescent girls, screening guidelines must adapt in order to retain efficient and cost-effective prevention measures. Although the true epidemiological and economic impact of cervical cancer vaccines cannot be immediately realized, mathematical models predict various scenarios in which vaccination, in addition to cervical screening, will be cost-effective and further reduce cervical cancer disease (Myers et al. 2008). The two main goals of modeling cancer screening are data analysis and evaluation. In data analysis, analytical-numerical statistical models are used to test hypotheses about preclinical disease, the screening test, and the association between early detection and risk of dying from the cancer. Evaluation in cancer screening is supported by model-based prediction of screening effects and cost-effectiveness. Simulation models are suitable for these tasks, and can also be used to identify efficient age-ranges and intervals between screening tests. Striking differences exist between screening models for cervical cancer and breast cancer, which are the two cancer types for which screening is common practice. The two main problems in cervical cancer screening are the proportion of progressive and regressive among screen-detected lesions, and the impact of screening on incidence and mortality. New or pending modeling issues include HPV-based screening in cervical cancer, screening models for colorectal cancer, the use of surrogate outcome measures and model-based meta-analysis of screening trials (van Oortmarssen et al. 1995).

Regardless of the research purpose, etiology or prevention, there are critical statistical and study design issues related to measurement of HPV infection status and its cervical lesion outcomes that need to be considered for the appropriate interpretation of the association of HPV infection and its determinants with cervical cancer in epidemiologic studies, and of screening effectiveness by HPV testing in clinical and intervention trials. These statistical issues may affect the validity of epidemiologic and screening studies and have led to inconsistent results in the literature. Meticulous attention to study design and laboratory detection issues helps to minimize the impact of such measurement errors and detection biases. Simple statistical analysis techniques are also available to correct or to control for these

biases if they were to be identified and additional information is known concerning the expected test performance (Franco 2000). Several factors are changing the landscape of cervical cancer control, including a better understanding of the natural history of HPV, reliable assays for detecting HR-HPV infections, and the available HPV-16/18 vaccine. There are important differences in the relevant policy questions for different settings. By synthesizing and integrating the best available data, the use of modeling in a decision analytic framework can identify those factors most likely to influence outcomes, guide the design of future clinical studies and operational research, provide insight into the cost-effectiveness of different strategies, and can assist in early decision-making when faced with criteria such as equity, public preferences, and political and cultural constraints (Goldie et al. 2006a).

In the last two decades, computer-based models of cervical cancer screening have been used to evaluate the cost-effectiveness of different secondary prevention policies. Analyses in countries with existing screening programs have focused on identifying the optimal screening interval, ages for starting and stopping screening, and consideration of enhancements to conventional cytology, such as HPV DNA testing as a triage for equivocal results or as a primary screening test for women over the age of 30. Analyses in poor-resource settings with infrequent or no screening have focused on strategies that enhance the linkage between screening and treatment, consider noncytologic alternatives such as HPV DNA testing, and target women between the ages of 35 and 45 for screening one, two, or three times per lifetime (Goldie et al. 2006b). The cost-effectiveness of HPV screening depends on the interval of the established Pap test screening strategy. In comparison with Pap screening every 2 years, only 25% of the HPV-based screening strategies were cost-effective. However, in comparison with Pap screening every 1, 3, or 5 years, 83%, 55%, and 92% of HPV screening strategies were cost-effective, respectively. Results for settings with annual Pap screening are based on models assuming 100% screening coverage. The introduction of HPV-based screening programs is cost-effective if the screening interval of the established Pap program exceeds 2 years. In settings with biennial Pap screening, introduction of HPV-based screening is unlikely to be cost-effective. Results also suggest cost-effectiveness of HPV-based screening in settings with annual Pap screening. However, this finding should be confirmed under realistic screening adherence assumptions (Mühlberger et al. 2008).

Modeling approaches have been used to estimate the impact of HPV vaccination on the performance of Pap cytology screening under different assumptions of lesion prevalence and expected changes in sensitivity and specificity likely to prevail post-vaccination. A major driver of the efficiency and costs of screening, the positive predictive value will be severely affected if Pap cytology continues to serve as the primary screening test in the post-vaccination era. Molecular-based screening with an HPV DNA test followed by Pap triage of HPV-positive cases has the potential for circumventing this problem. As a primary screening test, HPV testing can improve the overall quality of screening programs, thus allowing for increased testing intervals that would lower program costs with acceptable safety. Cytology should be reserved for the more labor-efficient task of triaging HPV-positive cases, a situation in which case loads would be “enriched” with

smears containing relevant abnormalities. HPV followed by Pap strategy can also serve a secondary role in post-vaccination surveillance (Franco et al. 2009b). Strong evidence now supports the adoption of cervical cancer prevention strategies that explicitly focus on persistent infection with the causal agent, HPV. New HPV infections acquired at any age are virtually always benign, but persistent infections with one of approximately 12 carcinogenic HPV types explain virtually all cases of cervical cancer. In the absence of an overtly persistent HPV infection, the risk of cervical cancer is extremely low. Thus, HPV test results predict the risk of cervical cancer and its precursors (CIN 3) better and longer than cytological or colposcopic abnormalities, which are signs of HPV infection. The logical and inevitable move to HPV-based cervical cancer prevention strategies will require longer screening intervals that will disrupt current gynecologic and cytology laboratory practices built on frequent screening. A major challenge will be implementing programs that do not overtreat HPV-positive women who do not have obvious long-term persistence of HPV or treatable lesions at the time of initial evaluation. The greatest potential for reduction in cervical cancer rates from HPV screening is in low-resource regions that can implement infrequent rounds of low-cost HPV testing and treatment (Schiffman et al. 2011).

6 Self-Collection of Cervical Samples for HPV Testing

Cervical screening by Pap smear involves a high rate of false negatives, necessitating frequent testing. Because women do not like the sampling procedure, many avoid being screened. Testing for the causative HR-HPV types, by PCR or other technologies, on self-collected (tampon) samples permits women to be monitored non-invasively. The high negative predictive value of HPV testing means a greater interval between tests, and thus reduces costs. HPV testing lends itself to primary screening. A kit for self-collection and return to a testing laboratory, followed by practitioner notification and follow-up if required, should result in wider participation. The higher accuracy and sensitivity of HPV testing should lead to improved cervical cancer prevention (Morris and Rose 2007).

Providing summary recommendations regarding self-collection of vaginal specimens for HPV testing is difficult owing to the wide range of published estimates for the diagnostic accuracy of this approach. Studies where patients used Dacron or cotton swabs or cytobrushes to obtain samples were pooled and had an overall sensitivity of 0.74 (95% CI: 0.61–0.84) and specificity of 0.88 (95% CI: 0.83–0.92), with diagnostic odds ratio (OR) of 22.3 and an area under the curve of 0.91. Self-specimens using Dacron or cotton swabs or cytobrushes collected by women enrolled at referral clinics had an overall sensitivity of 0.81 (95% CI: 0.65–0.91) and specificity of 0.90 (95% CI: 0.80–0.95). Sensitivity and specificity of tampons ranged from 0.67 to 0.94 and 0.80 to 0.85, respectively. These findings indicate that the combined sensitivity for HPV DNA is more than 70% when patients use Dacron swabs, cotton swabs, or cytobrushes to obtain their own vaginal

specimens for HPV DNA evaluation (Ogilvie et al. 2005). A high level of concordance of 0.87 (95% CI: 0.8–0.91) between self and physician sampling was obtained for detection of HPV DNA (kappa 0.66, 95% CI: 0.56–0.76). The prevalence difference of HPV DNA between sampling methods was −0.5 (95% CI: −2.8 to 1.8). Results were similar when restricting the analysis to HR-HPV but the prevalence of LR-HPV types was higher in self-collected samples. Self-sampling was as sensitive as physician-obtained sampling to detect HR-HPV or HPV DNA (Petignat et al. 2007).

The concordance between samples collected by patients and those obtained by clinicians was reasonably high in the majority of cases. Women in many countries across wide age ranges were successful in collecting samples for HPV DNA testing. In some studies, the quality of the cytology from patient samples was as good as clinician samples, with more than 95% of samples yielding HPV DNA results. The studies that examined acceptability found that women were generally very positive about collecting their own samples, although some concerns were noted. No study evaluated the effect of HPV DNA self-sampling on screening participation rates, early detection, survival, or quality of life. Self-sampling for HPV DNA testing is a viable screening option, but there is insufficient evidence to conclude that self-sampling for HPV DNA testing is an alternative to the Pap test. Although HPV DNA testing using self-collected samples holds promise for use in under-resourced areas or for women who are reluctant to participate in Pap testing programs, the evidence supporting it is limited. Further definitive research is needed to provide a solid evidence base to inform the use of self-sampling for HPV DNA testing for the purpose of increasing screening rates, especially in women who are never or seldom screened (Stewart et al. 2007; Schmeink et al. 2011).

7 HPV Testing and the Management of Cervical Cytological Abnormalities

The ASCCP National Consensus Conference for the Management of Women With Cervical Cytological Abnormalities and Cervical Cancer Precursors was held on the NIH campus in Bethesda, Maryland, USA, in September 6–8, 2001. 121 representatives attended the conference from 29 national organizations interested in cervical cancer screening issues. For the first time, guidelines for the management of women with abnormal cervical cytology, developed from evidence-based literature, were presented to delegates from the majority of organizations with interest in cervical cancer screening, voted on, and revised when necessary to achieve a majority two-thirds approval. This development of consensus-approved guidelines is likely to be considered one of the most important milestones to date in the management of women with abnormal cervical cytology. The timing of this Consensus Conference resulted from the convergence of many different factors, including new cytologic terminology developed at the Bethesda 2001 workshop and publication of the enrollment data from the NCI's ALTS. Additionally, new preliminary longitudinal

ALTS data provided much of the information on the natural history of abnormal Pap tests and CIN, as well as data on the performance of both new LBC and HPV DNA testing in the management of women following colposcopy. The result was a large database of new information that provided the foundation for the ASCCP Consensus Conference. The recommendations of the ASCCP Guidelines were based in large part on the results of the ALTS trial. Therefore, the focus is on the management of women with equivocal (ASC-US) and LG (L-SIL) cytologic abnormalities. Management of women with these cytologic abnormalities has been particularly problematic, because individually these women are at least risk for CIN 3 and cancer, yet their sheer numerical dominance ensures that they account for the majority of HG-CIN detected in the USA in the follow-up of abnormal cervical cytology. Data from ALTS confirmed that women with ASC-US could be safely managed by any of the conventional approaches (repeat Pap test, immediate colposcopy, or HPV testing), but that the preferred management approach for women having an ASC-US report from LBC was to assess the patient's risk by testing for HPV. Additionally, longitudinal ALTS data determined that repeat LBC at 6 and 12 months and an HPV test at 12 months were nearly equivalent options in the follow-up of women referred for HPV-positive ASC or L-SIL, yet not found to have CIN 2+ at initial colposcopy. Therefore, all follow-up recommendations for women with CIN 1 or lower postcolposcopy findings include these two options (Cox and American Society for Colposcopy and Cervical Pathology 2003).

The optimum management of women with minor SIL in Pap tests is controversial. With cytological surveillance after one Pap test showing ASC-US, a significant proportion of women will have undiagnosed SIL. On the other hand, using immediate colposcopy for ASC-US almost half of the women would not have needed the procedure. This number for referral for colposcopy can be reduced to only those women who test positive for HR-HPV DNA, however some women will have undiagnosed SIL. In addition, when surveillance with repeat Pap tests is used for the management of L-SIL a significant number of HG-CIN can be missed (Varras 2004). Management of women with ASC depends on whether the Pap test is subcategorized as of ASC-US or as cannot exclude H-SIL (ASC-H). Women with ASC-US should be managed using a program of two repeat cytology tests, immediate colposcopy, or DNA testing for HR types of HPV. Testing for HPV DNA is the preferred approach when LBC is used for screening. In most instances, women with ASC-H, L-SIL, H-SIL, and AGC should be referred for immediate colposcopic evaluation (Wright et al. 2002). The ASC-H category accounts for less than 1% of cytology reports, and 33–84% will test positive for oncogenic HPV. The number of patients with CIN 2/3 and cancer on biopsy is quite variable, from about 12% to more than 70%, averaging about 40%. The variation reflects patient population as well as local laboratory practices, but older subgroups are more likely to have negative HPV results and negative follow-up. Both the sensitivity of HPV testing for CIN 2/3 detection and the negative predictive value for a patient with ASC-H and negative HPV testing average more than 95%. Additional studies evaluating other types of ancillary testing for the ASC-H category are needed. ASC-H is an uncommon cytology result, and HPV testing results and biopsy follow-up show variation according

to patient age group and local laboratory practices. A negative HPV result in ASC-H offers a high negative predictive value and could be considered as a management strategy in mature women as well as women 30 years and older receiving combined cytology and HPV screening (Davey et al. 2010).

Besides all the confusion and associated problems that the use of ASC has created, it has initiated substantial investigational interest that resulted in a better understanding of SIL and the biology of cervical neoplasia. Although the category of ASC has created, and will continue to create controversy in the diagnostic and management fields, it allows the pathologist to convey uncertainty that may be the result of poor sampling or difficulty in interpretation of a case. It is a valuable tool that the cytopathologist can use to make it known that the Pap test has its limitations and may need and benefit, in some instances, from support from ancillary studies. Similar limitations are recognized in other areas of pathology and the use of IHC or molecular studies is widely accepted as an aid to a more specific and definitive interpretation. The time for the Pap test to be considered similarly has arrived. HPV DNA testing may not be the perfect test for cervical cancer screening because of high prevalence of HPV infection in the general population. However, it is currently the best-studied ancillary test and has been proven to be cost-effective for the triage of Pap tests with equivocal squamous cells. It is important for the cytopathologist to have well-developed diagnostic skills in interpreting gynecologic preparations and to classify cases as ASC only when deemed appropriate. Downgrading cytologic findings that are diagnostic from a SIL to ASC with the hope of supporting it by an HPV test will only result in a devaluation of the Pap test. Such recourse may however be acceptable in specific situations, such as in patients who have complex histories, atypical clinical presentations, or during pregnancy. Quality assurance measures to closely monitor the ASC/SIL ratio and the rate of HPV positivity in ASC cases will be essential to ensure the appropriate use of this interpretive category. The coordination of the 2001 Bethesda and ASCCP consensus meetings resulted in the new subcategories of ASC-US and ASC-H, along with well-defined management strategies for these interpretations. This new and clinically relevant terminology should lead to a reduction in difficulties at the clinical level and a more uniform management of patients, unlike the situation following Bethesda 1991 where the gynecologist was faced with a new "diagnosis" without specific management recommendations. The standardization of reporting and clinical management will also allow more reliable evaluation of patient outcomes and cost analysis. The 2002 ACS guidelines did not make specific recommendations regarding HPV DNA testing for the triage of patients who have a cytology result of ASC-US. The FDA approved the expanded use of HPV testing in conjunction with the Pap test for cervical cancer screening in March of 2003. The future is likely to bring additional testing modalities that may be more specific for detecting squamous lesions that are more likely to persist or progress to carcinoma, than the currently available HPV tests (Nayar and Tabbara 2003).

The indications for colposcopy have changed recently because of the new Bethesda terminology, the introduction of HPV testing in clinical practice, and the latest consensus guidelines on management of patients with an abnormal cervical

cytological test. Colposcopy remains the reference technique to assess patients with abnormal cytological test results, especially those with ASC-H, L-SIL, H-SIL, and AGC. In women with an ASC-US result, colposcopic examination of only those who test positive for HR-HPV increases the specificity of the technique. When LBC is used, HPV DNA testing is the preferred approach in these women. In primary screening using combined cytology and HPV DNA testing in women over the age of 30, colposcopy is indicated in patients with normal cytology and two positive HR-HPV DNA tests performed at 9 month intervals. For the follow-up of untreated patients with ASC-US/L-SIL and CIN 1, colposcopy carried out at 1 year after a single HR-HPV DNA positive test is as sensitive as colposcopy after two or three abnormal cytology tests. After excision or conization in patients with HG-CIN, colposcopy after a single HR-HPV DNA positive test is as sensitive as cytology testing and colposcopy at 6 months. Therapeutic decisions must not be based solely on the results of HPV DNA testing, except in specific cases (Monsonego 2004). Of the different strategies available for managing CIN 1, testing for HR-HPV DNA at 12 months has the highest sensitivity for predicting the development of CIN 2 or CIN 3 and leads to the lowest rate of referral to repeat colposcopy. If the HR-HPV DNA test result is negative at 12 months, then the patient may return to routine cytology screening. If the HR-HPV DNA test result is positive, the patient should undergo repeat colposcopy (Avrich et al. 2006). Consistent evidence underlines the utility of HPV DNA testing in the management of women with equivocal cervical cytological abnormalities, but not in case of LG lesions. The triage test-positivity rate reflects the colposcopy referral workload. Data were pooled on the HPV test positivity rate in women with ASC-US or L-SIL, derived from different cytological classification systems. In spite of considerable inter-study heterogeneity, the difference in HPV positivity between the two triage groups was large and highly significant: 32% (95% CI: 27–38%). HPV rates dropped tremendously as age and cutoffs of test positivity increased. Other factors (cytological classification system, country, continent, collection method, and year of publication) had no statistically significant impact except in L-SIL triage where HPV positivity was significantly lower in European compared to American studies. Women with L-SIL, especially younger women, have high HPV positivity rates suggesting limited utility of reflex HPV triaging these cases. Research is needed to identify more specific methods to triage women with L-SIL (Arbyn et al. 2009).

Management practices for L-SIL identified on screening swabs have been modified by research on the HPV. The appropriate approach for LG lesions is particularly difficult to determine due to the potential risk of malignant transformation coupled with the cost implications of treating lesions that will heal spontaneously in the majority of patients. Thin layer swabs that have a greater sensitivity than conventional swabs and can improve the diagnosis of these LG lesions. The current consensus is that swab results should be expressed according to the Bethesda classification. Routine tests for HPV (HC-2) should be reserved for patients with an ASC-US swab. Colposcopy is indicated if the swab is positive for a LG lesion followed by cytology if the colposcopy is normal. If there is no evidence of HPV,

search for oncogenes can lighten the treatment regimen due to the high specificity of the test. If an LG histological lesion (CIN 1) is proven, cytocolposcopic surveillance should be proposed; surgical resection undertaken if a colposcopy cannot be performed. A search for HPV oncogenes at 1 year is an interesting alternative if the examination is negative. These practices are applicable in adolescents and HIV-positive patients who are particularly exposed to HPV (Levêque et al. 2005). Again, a group of 146 experts representing 29 organizations and professional societies met on September 18–19, 2006 in Bethesda, Maryland, USA to develop revised evidence-based, consensus guidelines for managing women with abnormal cervical cancer screening tests. Recommendations for managing ASC-US and L-SIL are essentially unchanged. Changes were made for managing these conditions in adolescents for whom cytological follow-up for 2 years was approved. Recommendations for managing H-SIL and AGC also underwent only minor modifications. More emphasis is placed on immediate screen-and-treat approaches for H-SIL. HPV testing is incorporated into the management of AGC after their initial evaluation with colposcopy and endometrial sampling. The 2004 Interim Guidance for HPV testing as an adjunct to cervical cytology for screening in women 30 years of age and older was formally adopted with only very minor modifications (Wright et al. 2007).

Although Pap tests have enabled early detection of premalignant lesions, the introduction of new collecting devices has significantly improved the detection of lesions hidden in the endocervical canal, such as AIS. The term “atypical glandular cells of undetermined significance” (AGUS) was introduced at the 1988 Bethesda Conference and defined as morphologic changes in glandular cells beyond those that are suggestive of the benign reactive process, but insufficient for the diagnosis of AIS. In the new 2001 Bethesda System, the term has been eliminated and replaced with the term AGC, with the following subclassifications: NOS, favor neoplasia, endocervical AIS, and adenocarcinoma. The risks of premalignant or malignant disease associated with the AGC favor neoplasia category are substantially higher than in the AGC NOS category (96% versus 9–41%, respectively). Patients diagnosed with AGC NOS or AGC favor neoplasia will require colposcopy, endocervical sampling, and, for patients over 35 years of age, endometrial biopsy. If all of these tests are negative, the Pap test should be repeated in 4–6 month intervals until four consecutive normal tests are obtained. Positive results in one of the tests will require management according to ASCCP guidelines. The AGC favor neoplasia diagnosis also requires cervical conization and/or other testing, as the incidence of premalignant or malignant lesions in patients with this diagnosis is high (Levine et al. 2003). All women with AGC should undergo a comprehensive initial examination regardless of HPV status. The presence of HPV identifies a group of women at higher risk for cervical disease who should be followed closely. Women positive for HPV with AGC and concurrent ASC/SIL are at even higher risk. If, after a comprehensive initial examination, women with AGC NOS and positive HPV have no identifiable disease, a cervical conization may be considered (Sharpless et al. 2009; Kyrgiou et al. 2010).

8 HPV Testing and Follow-Up of HPV-Positive Women

Current evidence supports the use of colposcopy for the detection of intraepithelial lesions as a second line tool. CIN treatment involves either excisional or destructive techniques, usually performed under local anesthesia. Although a debate exists about the most efficient approach, the currently available evidence reveals no differences in efficacy among the available conservative methods of treatment. New evidence supports treatment by destructive rather than excisional techniques, at least for LG lesions in women wishing future childbearing, as they appear to have no apparent pregnancy-related morbidity. Treatment failures rates might increase in cases of involved excision margins, older age or glandular involvement. There is no worldwide consensus on the optimal follow-up policy, interventions or frequency in surveillance after treatment. HPV DNA test combined with either colposcopy or cytology is a promising combination for the early detection of treatment failures due to residual disease. Existing guidelines should probably be updated incorporating the new information emerged from recently published work (Kyrgiou et al. 2006). The women treated for a HG cervical lesion (CIN 2+) have a high and prolonged (beyond 25 years) risk of recurrence of cervical as well as extracervical lesion. The women treated for a CIN 2–3 are more likely to develop invasive cancer and the risk is two to five times greater than that of the general population. The main objective of the follow-up of patients treated for HG-CIN is in one hand, to detect and treat the recurrences and on the other hand, to determine a subpopulation presenting a HR of recurrence which should be followed-up more intensively. At present, frequent follow-up with cytology and colposcopic evaluation of the cervix is the preferred strategy recommended. The cytological and colposcopic protocols of follow-up raise the problem of their insufficient sensitivity, and the compliance of the patients to this prolonged follow-up is low. We suggest to test if HPV is seeking the presence of HR-HPV benefits from a high sensitivity and presents altogether a strong negative predictive value. Both the sensitivity and the negative predictive value of combined cytology and HPV testing in detecting a residual disease or recurrence are around 100%. The addition of this test to the cytological monitoring 3–6 months after the conization makes it possible to distinguish a group of patients with LR (with both tests negative) being able to profit from a traditional follow-up, from a HR group having at least one positive test, whose surveillance must be reinforced (with triage by colposcopy), prolonged in time and extended beyond the cervix. Like the primary screening of cervical lesions, the follow-up of the patients after conization must profit from the addition of HPV test and would deserve protocolization and organization (Mergui et al. 2008; Mergui and Levêque 2008).

According to the current guidelines in most Western countries, women treated for CIN 3 are followed for at least 2 years after treatment by cytology. HR-HPV infections are necessary for the development and maintenance of CIN 3. HR-HPV testing could be used to improve monitoring of women treated for CIN 3. This has prompted numerous studies for the implementation of HR-HPV testing in monitoring of women treated for CIN 3. Combined HR-HPV and cytology testing yielded the best test characteristics. It has been proposed to include HR-HPV testing in

conjunction with cytology for monitoring women treated for CIN 3. Some follow-up visits for women testing negative for both HR-HPV and cytology can be skipped. In Western countries, this could mean that for women double negative at 6 months, retesting at 12 months should be skipped while keeping the 24-month follow-up visit (Zielinski et al. 2004). There is an emerging interest concerning the role of HPV DNA testing in the follow-up period after conservative treatment for CIN. There is a marked heterogeneity in the design, population, intervention, and follow-up policy across different studies. The sensitivity of HPV DNA testing in detecting treatment failures was quite good in most studies, reaching 100% in some of them, whereas the specificity of the test differed across the studies, ranging from 44% to 95% depending of methods of HPV test. Among women in whom the treatment was considered to be successful, 84.2% had a negative postoperative HPV DNA test and 15.8% a positive one. The corresponding rates for cases with treatment failures were 17.2% and 82.8%. A systematic review of studies concerning HPV DNA testing in the follow-up period after conservative treatment for CIN indicates that a positive HPV test, even in the presence of normal cytology, may pick up early and accurately a treatment failure. Cytology and colposcopy may still be needed in order to rule out false positive and false negative results (Paraskevaidis et al. 2004).

9 HPV Testing and HPV Vaccination

The recognition that infection with certain HPV types is a necessary cause of cervical cancer has opened new fronts in the prevention of this disease. Primary prevention is now possible via immunization with highly efficacious HPV vaccines, and secondary prevention has gained impetus with the advent of sensitive HPV DNA testing to improve traditional Pap cytology screening programs. Although universal vaccination of teenagers and young women is a desirable policy, cost remains a key obstacle. To achieve cost-effective reductions in cervical cancer burden, prevention initiatives must consider screening and immunization as integrated and organized approaches that take advantage of HPV testing as a primary screening test followed by triage with Pap cytology. This strategy has the added benefit of providing epidemiological and immunological surveillance of vaccinated populations (Franco and Ferenczy 2007; Franco and Cuzick 2008). Screening for precancerous lesions cannot be discontinued because vaccination will not protect against HPV types not included in the first generation of vaccines. Moreover, protection for the target types, 16 and 18, which are responsible for most cases of cervical precancerous lesions and cancer, and 6 and 11, which are responsible for a substantial proportion of LG lesions, cannot be expected to be absolute, and the likely implementation of HPV vaccination in young women will not impact older groups initially. Cervical cancer control programs will need to be re-evaluated because the addition of HPV vaccination will make the existing approach of high-frequency screening by cytology too costly and inefficient for most public health budgets. Simply making cytology screening less frequent may

not be a viable strategy in light of potential problems that may plague cytology performance in conditions of low lesion prevalence. HPV testing has the performance characteristics that would make it an ideal primary screening test in such conditions. Cytology should be reserved for triage of HPV-positive cases because it is more likely to perform with sufficient accuracy in high-prevalence conditions. Another advantage of using HPV testing as a primary screening tool is the opportunity to create infection registries that can link test results from the same women over time, thus allowing an efficient and low-cost strategy to monitor long-term protection among vaccinated women (Franco et al. 2006).

HPV testing prior to HPV vaccination is not recommended unless HPV tests are part of the established local routines for cervical cancer screening. The reasoning is based upon the very low frequency of women who, at the time of vaccination, would show markers of prior/current exposure (HPV DNA or serological tests) to the HPV types included in the vaccine and because to vaccine women already infected with HPV 16 or 18 is not a problem. Thus, at least 1,000 women would need to be screened to find one that is HPV-16 and HPV-18 DNA positive. The increase in cost and the other barriers afforded by a prior to vaccination test requirement would result in a lower coverage, the key indicator of a successful vaccination program (Wright and Bosch 2008). Despite HPV vaccination, cervical cancer screening will remain the main preventive measure for both vaccinated and non-vaccinated women, but the nature of screening and management of women with cervical disease is being adapted to the new technologies. Although HPV DNA detection is more sensitive that cytology, its specificity is lower, since most HPV infections are transient. Therefore, other methods are considered to improve the management of women with cervical disease. Typing of HPV DNA and viral load measurements are still used for research purposes only. Detection of viral oncogene E6/E7 transcripts, which is the marker of the integrated infection, is a promising tool for follow-up of HPV DNA-positive women. The detection of p16INK4a overexpression as an indirect test of E6/E7 expression is used for confirmation of cervical neoplasia. Despite the lack of standardization, the detection of p16INK4a is useful in clinical settings, however, its reproducibility in the management of LG and borderline cases is low. Future perspectives include the determination of the methylation status of several cellular genes that could predict the progression of the disease (Grce et al. 2010).

10 Conclusions

HPV can be classified biologically or phylogenetically into cutaneous or mucosal types. Cutaneous Papillomaviruses produce benign skin tumors (warts) that occur commonly on the hands, face, and feet. They spread readily among children and young adults during recreational activities. Laboratory diagnosis of skin warts is usually unnecessary as they can be distinguished morphologically. Large numbers of cutaneous warts may develop in patients with epidermodysplasia verruciformis

(EV), a rare familial disorder. Exposure to sunlight sometimes causes these lesions to progress to skin cancer. HPV are the most common sexually transmitted viruses, infecting both men and women. They can be transmitted from the vagina at birth, and may cause recurrent respiratory papillomas in childhood or adult life. Genital infection usually clears within a few months, but may persist in some individuals. HPV has been firmly linked with cancer of the cervix, and is also associated with cancer at other mucosal sites. The distribution of genital HPV types varies and is related to the degree of cervical dysplasia present. HPV types 6 and 11 are frequently found in sexually active adults, and are associated with L-SIL. HPV types 16, 18, 31, and 45 are found less frequently, and are associated with progression to invasive cancer. Commercial dot blot hybridization and DNA-RNA HC assays are available for laboratory diagnosis of genital HPV infection. PCR is used for diagnosis and epidemiological surveys. Detection of particular HPV types could be useful in the diagnosis and management of cervical cancer in older women, and for resolving equivocal (borderline) cytology. HPV assays, which can distinguish between HG and LG disease, may also have a role in routine cervical screening (Swygart 1997).

Cervical SCC, adenocarcinomas, and their precursors are caused by HPV. Although HPV appears to be essential to the transformation of these epithelial cells, it is not sufficient and a variety of co-factors and molecular events must take place between when an HPV infection occurs and a cervical cancer or its precursor develops. Based on the available molecular, clinical, and epidemiologic data, a subset of HPV is unequivocally the etiologic agent for cervical cancers and their precursors. Different mucosotropic HPV have varying neoplastic potential. However, the great majority of cervical HPV have oncogenic potential. Since oncogenic HPV-induced epithelial transformation to a H-SIL or cancer is rare relative to the rate of infection, the term HR is discouraged. HPV's interaction with host cells has two principal biologic consequences:

1. All anogenital HPV induce L-SIL, which are the morphologic correlate of a productive infection
2. Rarely, HPV induces a proliferative epithelial phenotype that pathologists recognize as a H-SIL and is the proximate cytohistologic precursor of invasive cervical carcinoma

HPV biology and issues of practical clinical management should be reflected in the classification systems used for cytologic and histologic diagnosis. The molecular identification of HPV (HPV testing) potentially may be very useful for primary screening or secondary triage of patients with certain lesions. However, the technology available to the practicing clinician is still evolving. Optimization of type spectrum, sensitivity, specificity, and ease of use is under development (Richart et al. 1998). In the past years, new data have been published on the molecular biology of HPV infections and their relationship to cervical neoplasia. As molecular techniques have become more sophisticated and as the molecular knowledge of HPV infections has been pursued in greater depth, it is increasingly apparent that this human tumor DNA virus is similar to a number of other oncogenic DNA

viruses that have been described and well studied. These viruses appear to act through a common pathway of producing oncogenic proteins that interfere with key signaling elements that normally control the process of cell division. With a better mechanistic knowledge, it should be possible to design new therapeutic approaches to treating HPV-associated disease that are directed toward specific cellular events such as turning off the production of E6 and E7 proteins or restoring the activity of pRb or p53. Increased attention has also been turned to immunologic aspects of HPV infections, and a number of groups are eagerly pursuing the possibility of using simple office-based procedures to detect specific proteins encoded for by the HPV open reading frames in an attempt to determine who has been infected, is actively infected, and has proteins being produced that are indicative of neoplasia. From the clinical point of view, the use of outpatient excisional techniques such as the loop electrosurgical excision procedure (LEEP) is rapidly supplanting ablative techniques because of their superior ability to identify early invasive CIS and AIS that have not been detected by colposcopy (Richart and Wright 1992).

HPV is a significant health care burden in the USA. The majority of sexually active men and women will be infected with HPV at some point in their lives and are subject to developing HPV-associated disease. Current estimates suggest that 20 million Americans are currently infected, and more than 5 million new infections occur each year. The prevalence of HPV is highest in populations in their late teens and early twenties, with nearly half of all new HPV infections occurring within 3 years of first intercourse. HPV is the necessary cause of genital warts, CIN, and invasive cervical cancer. As such, HPV is responsible for significant medical morbidity and health care costs. Screening with cervical cytology has significantly reduced mortality rates. HPV DNA testing has shown a valid tool in identifying HG abnormalities as an adjunct to traditional cytology, and should be used according to guidelines established by the ACS and the ACOG (Cox 2006). Cervical screening currently depends on the identification by cytology of abnormalities in cells taken from the surface of the cervix. The standard Pap test was developed >50 years ago, and despite modifications, still forms the basis of the test currently in use in most routine screening laboratories. Advances in our understanding of the molecular mechanisms that lead to the development of cervical cancer have been slow to impact on screening, despite the relatively high false-negative rates that can be associated with the conventional Pap smear. Improvements in screening strategies fall into a number of categories. Methods that improve cell presentation and attempt to eliminate artefacts/obscuring debris can be combined with image analysis systems in order to enhance diagnostic accuracy. Such approaches still rely on cytological evaluation and do not incorporate advances in our knowledge of how HPV causes cancer. By contrast, markers of virus infection or cell cycle entry, particularly those that offer some degree of prognostic significance, may be able to highlight abnormal cells more reliably than cytology, and could be combined with cytology to improve the detection rate. Our understanding of the molecular biology of HPV infection and the organization of the HPV life-cycle during cancer progression provides a rational basis for marker selection. The general assumption that persistent active

infection by HR-HPV types is the true precursor of cervical cancer provides the rationale for HPV DNA testing in conjunction with enhanced cytology, while the development of RNA-based approaches should allow active infections to be distinguished from those that are latent. The detection in superficial cells of marker combinations at the level of RNA or protein has the potential to predict disease status more precisely than the detection of markers in isolation. There is also a need for better prognostic markers if the predictive value of screening is to be improved. The potential to control infection by vaccination should reduce the incidence of HPV-associated neoplasia in the population, and this may cause a change in the way that screening is carried out. Nevertheless, the lack of a therapeutic vaccine, and the difficulties associated with eliminating infection by multiple HR-HPV types, means that some form of screening will still be required as a preventive measure for the control of cervical cancer for the foreseeable future (Doorbar and Cubie 2005). Recent decades have witnessed a reduction in the incidence of cervical cancer in countries where screening programs have achieved broad coverage. The recognized importance of HR-HPV infection in the etiology of cervical cancer may introduce a role for HPV DNA testing in cervical screening programs. Positive HPV DNA tests indicate women at risk of cervical cancer with greater sensitivity, but reduced specificity, compared with exfoliative cytology. Combining HPV testing with cytology may be useful in the triage of minor cytological abnormalities into those requiring referral to colposcopy (HPV positive) compared with those who can be safely managed by cytological surveillance (HPV negative). With its high sensitivity and high-negative-predictive value, HPV testing may also be useful for predicting treatment failure, since residual disease is very unlikely in the event of a negative HPV test. Ultimately, prevention is better than cure, and the advent of HPV prophylactic vaccines may obviate the need for population-based cervical screening programs in the future. A multivalent vaccine administered to adolescents prior to the onset of sexual activity and boosted at regular intervals throughout their sexually active life may provide protection against type-specific HPV infection, malignant precursors, and invasive cervical disease. For those generations of women already exposed to HR-HPV infection, therapeutic vaccines may offer advantages over conventional treatment, although much work still needs to be done (Crosbie and Kitchener 2006).

In summary, worldwide cervical cancer is diagnosed annually in more than 500,000 women and accounts for 270,000 deaths, making it the second leading cause of cancer in women. In Europe, where many countries have set up screening program, cervical cancer ranks third among cancers in women. In France, cervical cancer is diagnosed in 3,400–4,500 women each year and kills 1,000–1,600. Since its introduction, Pap smear screening has transformed cervical cancer from a fatal disease into a rare condition. Despite the considerable success of this cytologic screening, Pap smears have not, as was first hoped, reduced incidence on a large scale. The principal reasons are related to the difficulties in ensuring optimum coverage of the population to be screened and in maximizing women's adherence: the success of screening depends on strict compliance with the calendar from 25 to 65 years of age. In one third of cases, invasive cancers are found in women who

undergo regular screening, because Pap smears are insufficiently sensitive. In 5% of cases, cancers are observed in women who were inappropriately managed after an abnormal Pap smear finding. The contribution of HPV test to primary screening opens up promising perspectives of optimum protection. The test's sensitivity for HG lesions exceeds 95% and its negative predictive value exceeds 99%. HPV test is the only test available for which a negative result provides instantaneous assurance that there is no risk of cervical cancer. The Pap smear alone, with its sensitivity of less than 70%, cannot provide this certainty. European and American guidelines recommend screening strategies based on a combined test using the Pap smear and HPV test after the age of 30 years. The availability of prophylactic HPV vaccines, which are expected to provide 70% protection against cervical cancer, does not affect the practice of screening, which must continue (Monsonego 2007). The major mechanisms through which HPV contributes to neoplastic initiation and progression include the activity of two viral oncoproteins, E6 and E7, which interfere with critical cell cycle tumor suppressive proteins, p53 and Rb protein. However, HPV infection alone is not sufficient to induce malignant transformation, and other significant co-factors contribute to the multi-step process of tumor formation, such as individual genetic variations as well as environmental factors. Moreover, these cofactors are not important in the absence of HPV. Pap smear and HPV DNA testing are tools used in the screening and diagnosis of cervical neoplastic lesions. Vaccination against HPV appears to be cost-effective in the prevention of HPV infection. A thorough understanding of the mechanisms that underline HPV carcinogenesis may result in the development of sophisticated targeted therapeutic approaches, such as antisense oligonucleotides against HPV oncoproteins (Subramanya and Grivas 2008).

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Chapter 7
Colposcopic Appearance of HPV Infection

Santiago Dexeus, Montserrat Cararach, and Damian Dexeus

1 Introduction

Human papilloma virus (HPV) infection is currently a very significant topic from a public health and social perspective. As a result of the remarkable impact on the mass media through which sensational opinion has been spread, the general public has been confused. The scientific community should be aware of the epidemiological and oncogenic aspects of HPV genotypes, the complexity of the clinical spectrum, and the oncogenic potential and treatment-related difficulties associated with some types of HPV.

Today, HPV is one of the most common sexually transmitted infections. More than 140 VPH types have been identified, of which about 40 are known to infect the anogenital tract. HPVs infecting the anogenital tract are divided into 15 high-risk and 12 low-risk types, based on their association with malignant or benign disease. High-risk types of HPV are responsible for almost all cases of cancer of the anogenital tract and precancerous lesions (cervical intraepithelial neoplasia [CIN], adenocarcinoma in situ [AIS], vulvar intraepithelial neoplasia [VIN], and vaginal intraepithelial neoplasia [VAIN]). Low-risk types are usually detected in benign cutaneous and mucosal growths, particularly HPV 16 and HPV 11; those mostly responsible for genital warts.

HPV is a DNA epidermotropic virus with high affinity for the genital tract and capacity to infect the squamous epithelium of skin and mucosa. HVP infection of the lower genital tract is typically transmitted through sexual contact and both men and women are equally susceptible to become infected. Genital HPV infection

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is very common among sexually active young women because it is easily acquired from their first sexual partner. More than 80% of sexually active women are infected with HPV at sometime in their lives. The majority of genital HPV infections (more than 90%) are transient and will be eliminated by the immune system, resolving within 1 and 2 years (Clifford et al. 2005).

The prevalence of HPV infection in asymptomatic women of the general population varies between 2% and 44% (adjusted overall prevalence rate 10.4%), with a considerable variability between different populations. The highest prevalence is found in young women, decreasing gradually with age. Generally, the prevalence is higher in ages immediately after the onset of sexual activity and reflects the pattern of sexual behaviour in the community. In populations where the number of different and occasional sexual partners is high, the prevalence of HPV infection may be as high as 30–40% in the 15–25 age year groups (Bosch and de Sanjose 2003; Trottier and Franco 2006; Dunne et al. 2007; Steben and Duarte-Franco 2007).

HPV exposure occurs primarily through sexual contact. To become infected, the virus must gain access to basal cells of the squamous epithelial surface, usually through minor traumas such as skin abrasion during sexual intercourse. Lesions are more likely to occur in the areas of highest friction during sexual activity. HPV may be considered a "field infection" of the anogenital tract involving any area of the female genital tract, including the cervix, vagina, vulva, perineum and anus.

2 Clinical Manifestations of HPV Infection

Once HPV has infected any region of the epithelium of the lower genital tract, in approximately 80% of the cases the organism will totally eliminate the infection, whilst in the rest of the cases the virus may remain latent, in a latent phase, or enter into a stage of active expression characterized by epithelial and capillary proliferation, which presents with clinical or subclinical manifestations (Alba et al. 2009).

HPV infection of the lower genital tract may be expressed as clinical, subclinical, or latent forms.

1. *Clinical infection*: lesions are evident with direct ocular examination.
2. *Subclinical infection*: lesions are only evident on cytological or histological studies and may be clinically detected on colposcopic examination and after applying 5% acetic acid.
3. *Latent infection*: it is only possible to diagnose through molecular hybridization techniques that detect the presence of viral DNA. Clinical and cytohistological images are lacking.

Condyloma acuminata (CA), or visible genital warts, are present in approximately 1% of sexually active adults in the United States. Anogenital warts are the "tip of the iceberg" of HPV genital infections because subclinical infections detected by colposcopic or cytological examination is present in 4% of the cases, and approximately 10–20% of the sexually active female population may be infected by HPV.

However, with newer sophisticated techniques for HPV detection, prevalence rates of over 50% are found (Koutsky 1997).

2.1 Clinical Lesions

Condylomata, also known as genital warts, are the most frequent clinical manifestation of HPV infection, and are characterized by the presence of fleshy excretions found generally in the skin and mucous membranes of the anogenital area. CA will appear in the area of the mucous membrane or skin when an infection has been found. CA generally presents an exophytic appearance, pediculated and papular with a common sessile base, distinctive cauliflower-like or cockscomb-like morphological appearance, and highly variable size and extension. In the mucous membranes, the CA are observed with the aspect of a hyperplastic lesion, fleshy and humid, with a pink or white color. These clinical signs vary when the condylomata are found in surrounding skin, with an aspect of dry and hyperkeratotic lesions or even as pigmented papules.

2.2 Subclinical Lesions

Subclinical lesions are epidemiologically important, as they are invisible to the human eye. Their clinical insignificance facilitates their spread. Subclinical lesions are only evident on colposcopic examination after applying 3–5% acetic acid. These lesions are found on the mucous membranes and are seen as flattened, multiple, white colored stains of variable extension and morphology. Acetowhite epitheliums in cervix and particularly in the vulva may be quite unspecific and for this reason, a specialist should evaluate an acetic acid test.

Clinical and subclinical lesions may be caused by any sexually transmitted HPV genotype. Generally, the most excrescent and largest lesions are due to low-risk genotypes (HPV 6 and 11), whereas subclinical or less apparent lesions are mainly related to high-risk genotypes, which are more likely to develop malignant lesions.

Although the clinical lesions are evident to the naked eye, broadened examination via colposcopy allows a more accurate evaluation of the extension of the disease and better assessment of its clinical aspects, on which a correct therapeutic plan will depend. These lesions may affect any area of the inferior genital tract and the simultaneous existence of mixed forms or clinical and subclinical manifestations is frequent. For this reason, in the presence of any clinical evidence or cytological suspicion of HPV infection or intraepithelial lesion, given the multicentricity and multifocality of the infection, an integral colposcopic examination of the lower genital tract (cervix, vagina, vulva, perineum and anus) should be performed.

Different authors in various atlas of colposcopy have extensively described colposcopic findings of HPV infection corresponding to the diverse clinical manifestations and anatomic sites (Dexeus et al. 1989; Gross and Barrasso 1997; Palacio 2000; Kesic 2005; Syrjänen 2005; De Palo et al. 2007).

3 Vulvar HPV Infection

HPV infection of the vulva may be manifested as: (1) clinical lesions, CA or genital warts evident with direct ocular examination with variable morphological appearance, color, size, and extension, and (2) subclinical lesions only visible with optical magnification systems.

3.1 Clinical Infection

The most well known clinical expression of HPV infection is CA or genital warts. These are benign lesions affecting the genital and anal zones of men and women, most frequently found during ages of higher sexual activity. HPV 6 and 11 types cause almost 100% of genital warts, although in 20–50% of cases, co-infection with high-risk HPV genotypes is also present (Lacey et al. 2006). Genital warts are primarily transmitted by sexual contact and the risk of contraction is strongly correlated with sexual behaviour. Genital warts are highly contagious, with a transmission rate of about 65% within sexual partners. The incubation period is usually in the range of 2–3 months but can vary between 3 weeks and 8 months.

Genital warts are the most common sexually transmitted infection. In the USA, It is estimated that 1% of sexually active men and women (between 18 and 49 years) have genital warts at any one time (Koutsky 1997), and one in every ten people will develop an episode of genital warts at some point in their life (Garland et al. 2007). In a study conducted in the UK among women age 16–44, a prevalence of 4.1% was reported (Fenton et al. 2001). Most HPV infections are acquired at a young age after the onset of sexual activity. The highest prevalence of genital warts is detected between 15 and 24 years of age (Monteiro et al. 2005), which accounts for an important psychological impact in these patients. The prevalence of genital warts has increased considerably in recent years, in both men and women. In the past 30 years in the UK (1971–2004), there has been a tenfold increase in the number of cases of genital warts (Health Protection 2008). This increase has been closely associated with changes in sexual behavior.

Lesions are detected by clinical inspection. Condylomata with cockscomb morphology are well known, although variable clinical forms may be observed, according to the epithelium affected and the duration of HPV infection. In mucous and hairless vulvar skin areas, CA appear as soft, pink of whitish masses, with multiple hypervascularized and translucent finger-like papillae, whereas in hair-bearing

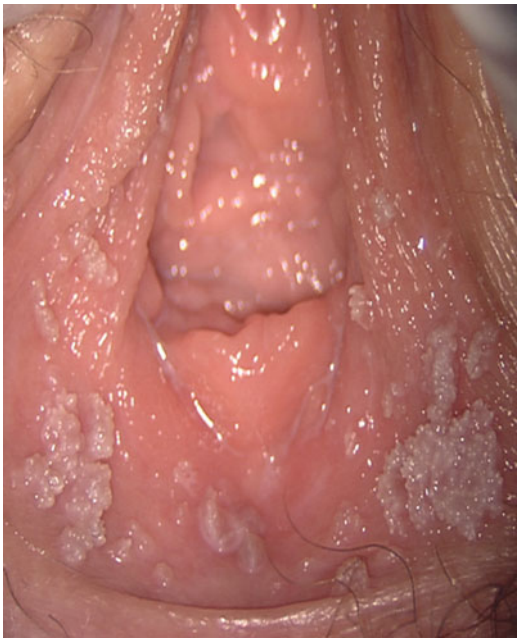

Fig. 7.1 HPV infection of the vulva. Typical condyloma acuminata on hairless skin

vulvar skin, CA are usually darker, sessile, or cauliflower-like appearing mass, more keratinized, and with less visible papillae (Figs. 7.1 and 7.2).

In relation to the duration of an HPV infection, recently-acquired condylomas are usually small, fleshy lesions forming groups of several papillae in the form of small crests, while chronic CA are generally flattened, irregular, of thick or hyperkeratotic papillae, with a rough surface and cauliflower-like appearance (Figs. 7.3 and 7.4).

Vulvar genital warts are most often found in the areas of highest friction during sexual activity (fourchette, labia majora, labia minora), although humidity conditions and possible associated infections may favor the spread of infection to the rest of the vulva, perineum, and perianal area (Fig. 7.5).

Less frequently, clinical infection may also manifest as papular lesions, appearing as small (3–7 mm) rounded papules, slightly raised and often multiple, sometimes forming coalescence plaques. Mucous and cutaneous lesions as well as pigmented lesions (Bowenoid papulosis) may be found, and usually appear in association with CA. Papular lesions are frequently associated with high-risk HPV types (HPV 16 and 18) and premalignant lesions (VIN II–III), so that a biopsy is mandatory (Obalek et al. 1986) (Fig. 7.6).

Although the clinical diagnosis of CA is easy to establish, the differential diagnosis may include anatomic conditions (Fordyce granules, vulvar vestibular papillomatosis), benign tumors (angiokeratoma, epithelial nevus, seborrheic keratosis) and even neoplasms (verrucous carcinoma).

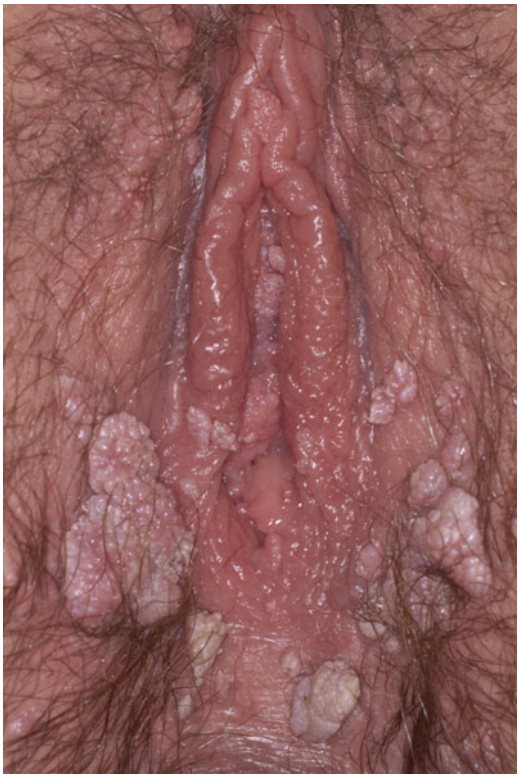

Fig. 7.2 HPV infection of the vulva. Typical condyloma acuminata on hair-bearing skin

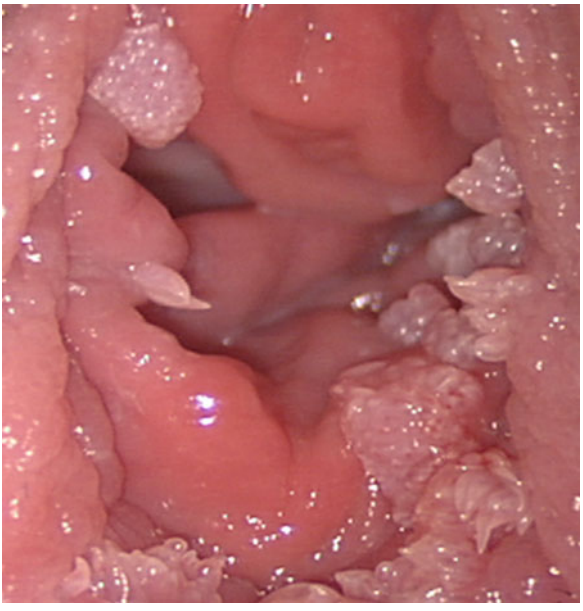

Fig. 7.3 Condylomata acuminata of the vulva. Recent lesions

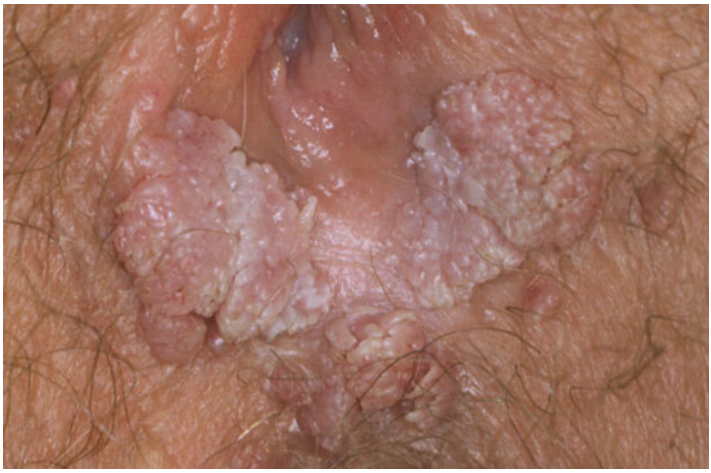

Fig. 7.4 Condyloma acuminata of the vulva. Old lesions

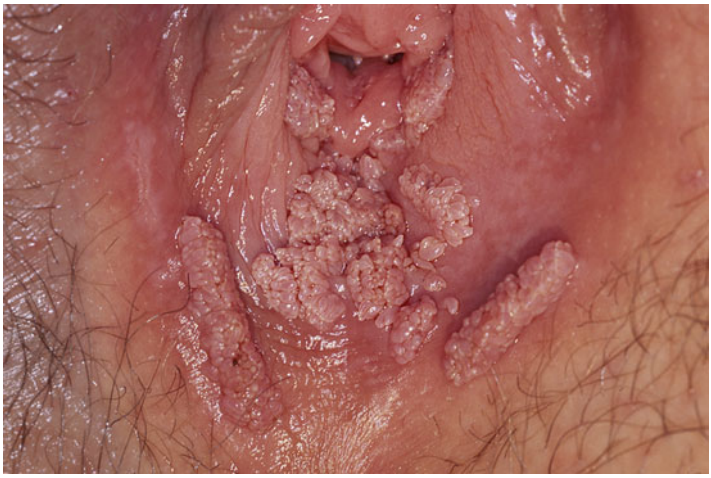

Fig. 7.5 Posterior fourchette, labia minora and the introitus are most frequent sites for condyloma

3.2 Subclinical Infection

Subclinical lesions of the vulva are only evident on colposcopic examination after applying acetic acid. Subclinical infections may present different forms (macular, papular and micropapillary) and are found in hairless areas.

Macular forms: flat or slightly raised acetowhite epithelium, either as small multifocal spots or confluent, mainly observed in mucosal sites (fourchette and inner

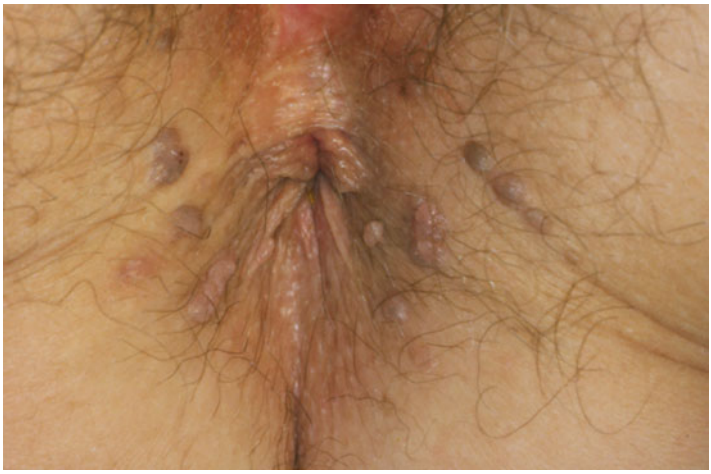

Fig. 7.6 Clinical HPV infection of the vulva. Pigmented papular lesions on the perianal area

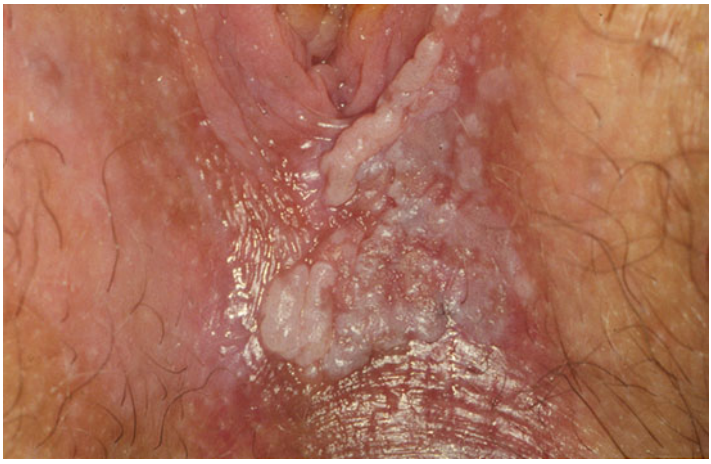

Fig. 7.7 Subclinical HPV infection of the vulva. Acetowhite lesions (flat condyloma)

surface of labia minora) (Fig. 7.7). Between 5% and 10% are accompanied by varying grades of VIN since in many cases are caused by high-risk genotypes. For this reason they should always be biopsied.

Papular forms: small and rounded lesions that react strongly to acetic acid, slightly prominent, multiple, and often with a shiny surface (Fig. 7.8).

Micropapillary forms: Small, multiple, acetowhite fibro-epithelial projections (spicules), surrounding a prominent central capillary. It is considered the minimal expression form. Localized in the vestibular mucosa especially in the inner surface of labia minora and introitus, conferring a granular aspect to the vulvar area (Fig. 7.9).

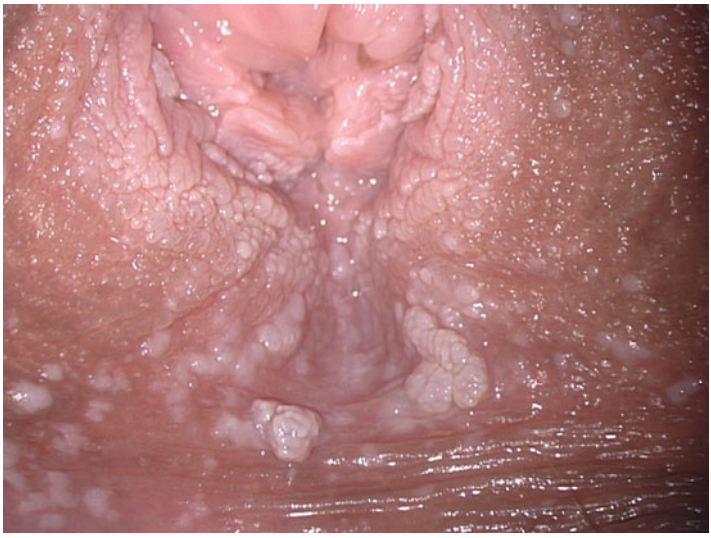

Fig. 7.8 Subclinical HPV infection of the vulva. Papular lesions

3.3 Differential Diagnosis

Sometimes it may be difficult to differentiate subclinical HPV infection from a VIN lesion, although the possibility of coexistence should be considered. Any macular or papular genital wart in the vulva should be biopsied to exclude the presence of a pre-invasive lesion. A positive acetic acid test is not necessarily a definite HPV infection. Any inflammatory process (e.g., candidiasis) may react to the acetic acid and confuse the diagnosis.

4 Vaginal HPV Infection

The vagina is less receptive to HPV infection, although it is an important reservoir in the sexual transmission of the virus. Vaginal condylomata can be detected in one third of women with vulvar genital warts and about 20% of them are associated with cervical infection. Vaginal HPV infection may express the following clinical forms.

4.1 Clinical Infection

The characteristic image is the classic condyloma which most often affects the fornices and the upper and lower thirds of the vagina. Lesions are often multiple, but not very large, appearing as pink-colored papillae that turn white following application of 5% acetic acid (Fig. 7.10). Warts may become large and exuberant in

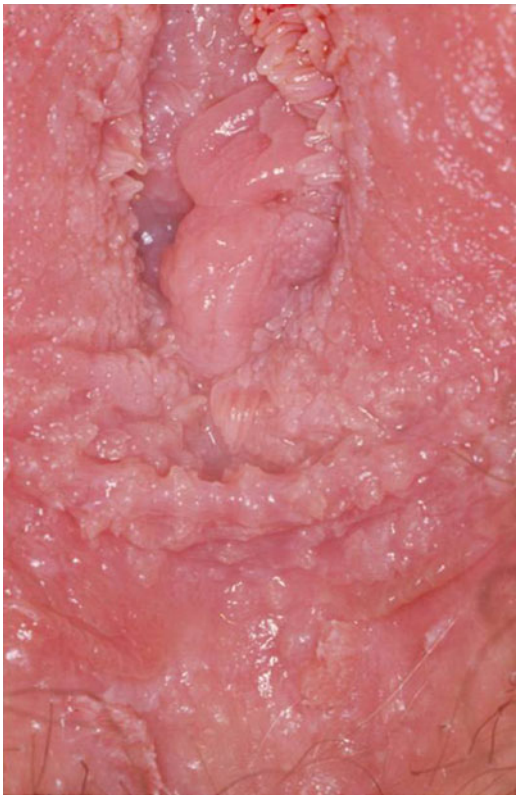

Fig. 7.9 Subclinical HPV infection of the vulva. Micropapillary lesions

pregnant and immuno-compromised women. Characteristic capillaries may be observed by magnification (Fig. 7.11). Sometimes they appear as whitish hyperkeratotic plaques with a rough surface (Fig. 7.12).

Vaginal condylomata, papillary warts in particular, are easily recognizable. However, small warts may remain undetectable if they are located within the folds of the vaginal mucosa or occult by the speculum valves (Fig. 7.13).

4.2 Subclinical Infection

Subclinical infections are more frequent. The colposcopic examination following application of acetic acid reveals a tiny whitish epithelium of smooth surface or one that is slightly raised, spiculated, or micropapillary, with sharp margins, and with or without capillary images, generally in the form of a fine punctation capillary pattern (Fig. 7.14). The Schiller test is a negative or partially iodine-colored one of well-defined limits (Fig. 7.15). Histological changes of VAIN I are sometimes documented. Occasionally, a dense white and opaque color may suggest its association with high-grade VAIN especially if irregular and thick punctate capillaries are observed. The coexistence of both lesions should be considered.

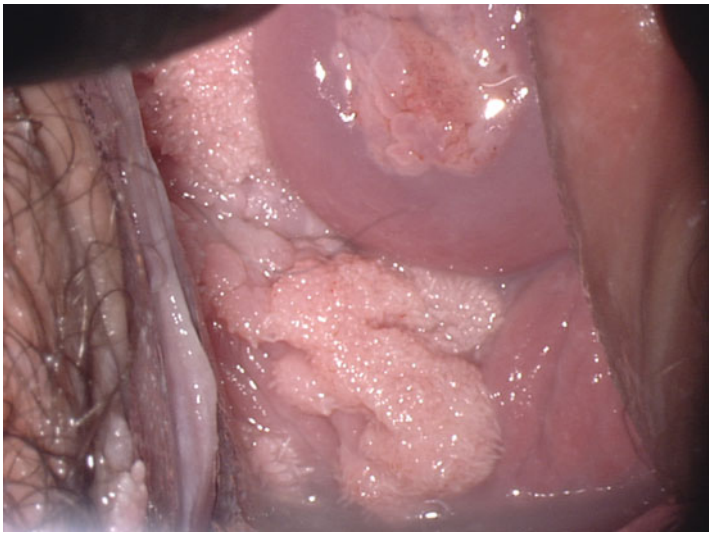

Fig. 7.10 HPV infection of the vagina. Condyloma acuminata in the fornix

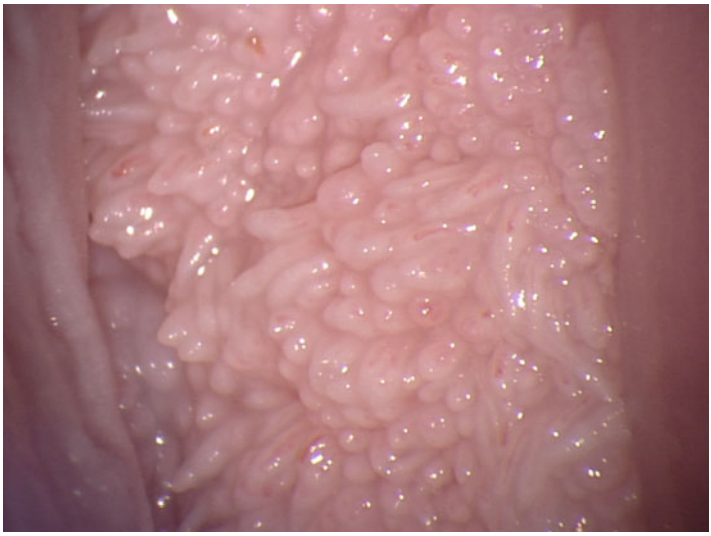

Fig. 7.11 High-power field of a vaginal condyloma acuminata showing papillary structures centered by regular vessels

The minimal expression form appears as iodine-negative whitish spiculae covering a prominent central capillary on an iodine-positive background. These subclinical forms may be colposcopically misdiagnosed with the diffuse white punctation pattern of mycotic or bacterial colpitis, although in these cases the pattern is less prominent.

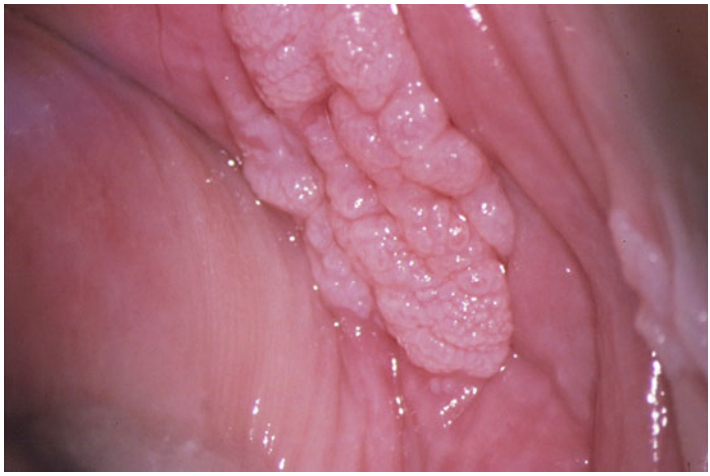

Fig. 7.12 Clinical HPV infection. Focal hyperkeratotic area in the lateral wall of the vagina

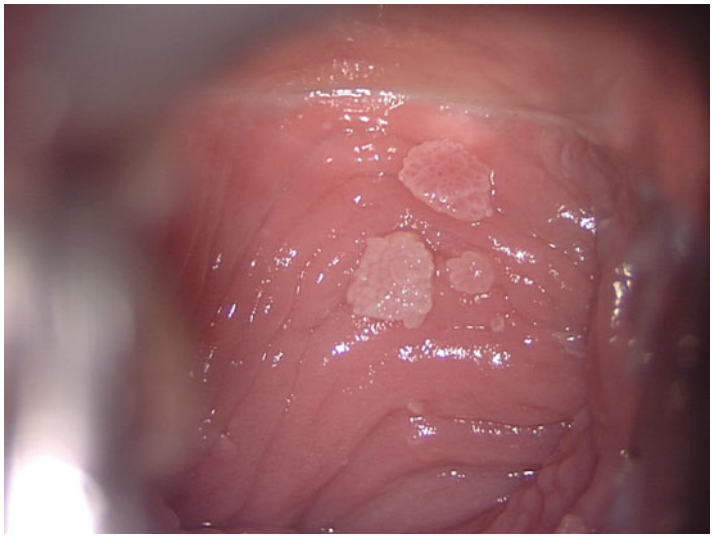

Fig. 7.13 Small condylomata masked in the mucosal folds of the anterior vaginal wall

5 Cervical HPV Infection

Cervical HPV infection is most frequently given the higher susceptibility of metaplastic epithelium in the transformation zone to viral colonization. HPV infection of the cervix is generally expressed as a subclinical form, whereas macroscopically evident lesions are uncommon. Similarly to vulvar and vaginal HPV infections, the following clinical forms can be recognized.

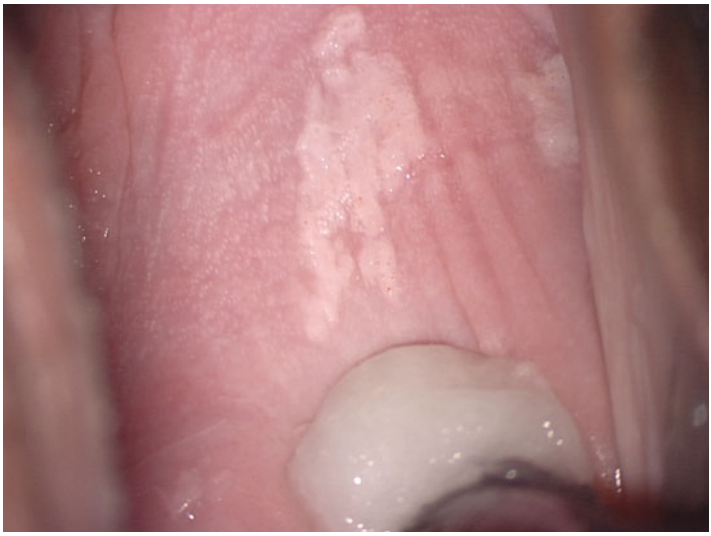

Fig. 7.14 Subclinical HPV infection of the vagina. Acetowhite lesions

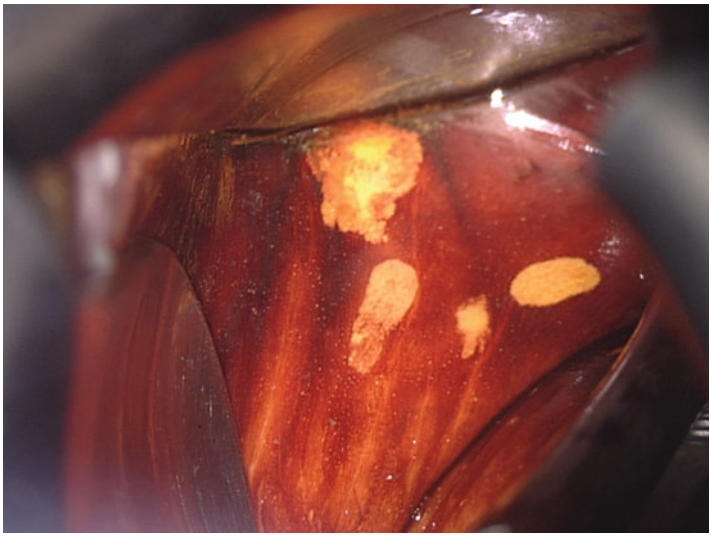

Fig. 7.15 Subclinical HPV infection of the vaginal. Iodine-negative areas

5.1 Clinical Infection

Cervical condylomata are relatively uncommon and frequently are associated with vaginal and/or vulvar genital warts. It is estimated that 6% of women with vulvar warts also have cervical condylomata.

Exophytic condylomata are easily diagnosed. They appear as epithelial papillary proliferations, with a central vascular loop, that become white after the application

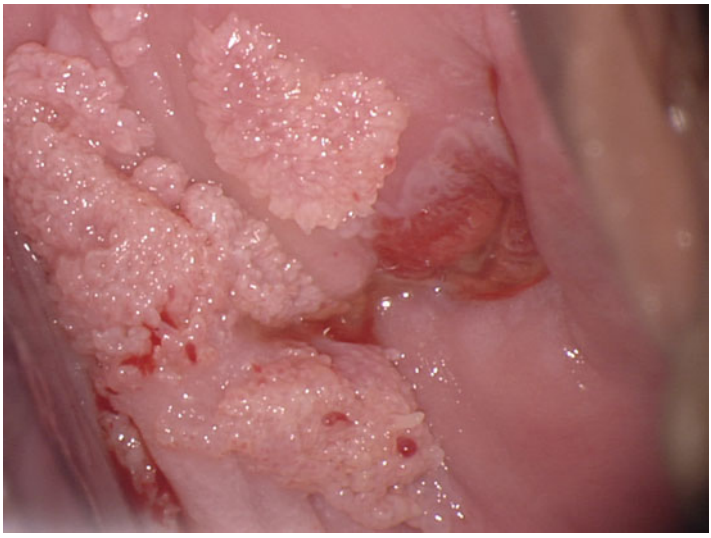

Fig. 7.16 HPV infection of the cervix. Multiple condylomata occupying a large area of the exocervix

of the acetic acid. Condylomata may be single or multiple, isolated or confluent, and pink-colored or whitish, depending on the epithelial thickness. They are usually found in the transformation zone, although lesions may also be seen in the original squamous epithelium or even spreading into the endocervical canal (Figs. 7.16 and 7.17). When epithelial keratinization increases, the surface may acquire an appearance that resembles the cerebral cortex, the so-called "cerebroid pattern" (Fig. 7.18). In some cases, exophytic condylomata show an abnormal vascular pattern. These lesions with atypical vessels may be misdiagnosed as an invasive lesion by the inexperienced colposcopist (Dexeus et al. 2005) (Fig. 7.19).

CA of the cervix is usually caused by low-risk types of HPV, such as 6 and 11. These lesions may be considered a marker of possible exposure to high-risk HPV genotypes. At least 20% of women with cervical CA have a comcomitant CIN. For this reason, colposcopic examination and biopsy of suspicious zones are mandatory in these patients.

5.2 Subclinical Infection

Subclinical infection is the most frequent form. Lesions are detected colposcopically following application of acetic acid and appear as acetowhite epithelium of variable morphology and size, both inside and outside the transformation zone.

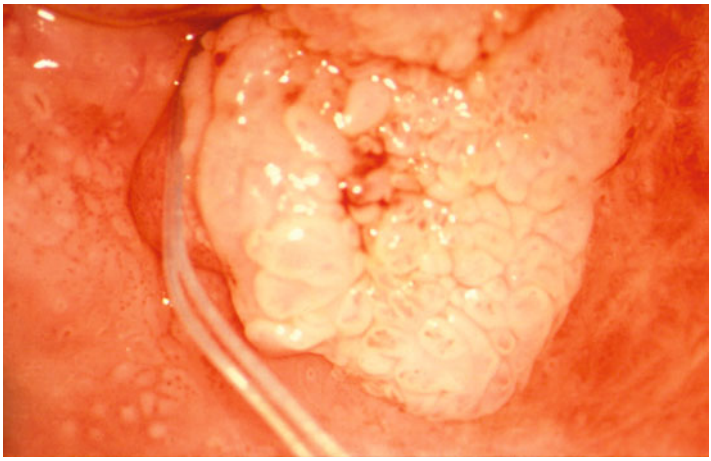

Fig. 7.17 Clinical HPV infection of the cervix. Exophytic condyloma on the posterior lip. The finger-like projections are irregular in size and shape. The smallest projections have a papillary structure and are centered by a capillary

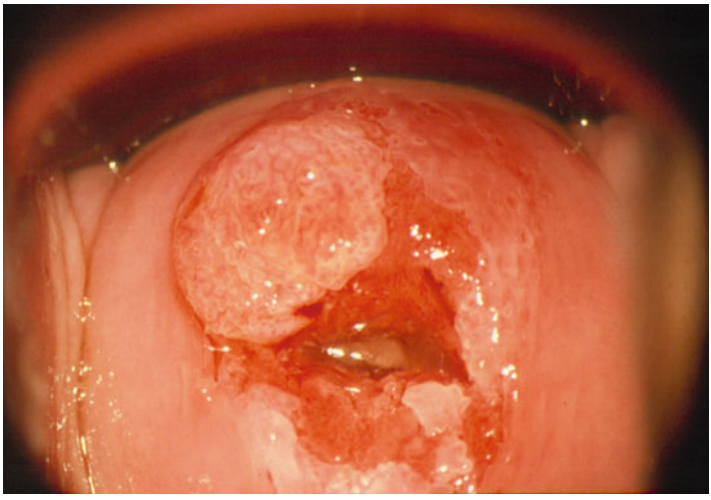

Fig. 7.18 Brain-like microconvoluted surface of an hyperkeratotic lesion on the anterior lip

In HPV subclinical infection, acetowhite epithelium is tiny or semi-translucent and of smooth, spiculated or micropapillary surface (Figs. 7.20 and 7.21). Occasionally, slightly raised leukoplastic plaques of rough (due to hyper and parakeratosis), spiculated or micropapillary surface are seen (Fig. 7.22). Acetowhite epithelium may exhibit punctation images and thin and regular mosaic-like pattern (Fig. 7.23). If the Schiller iodine test is applied, these lesions generally emerge as iodine-negative or partially positive (Fig. 7.24). Mixed forms of clinical and subclinical expression are frequent (Fig. 7.25).

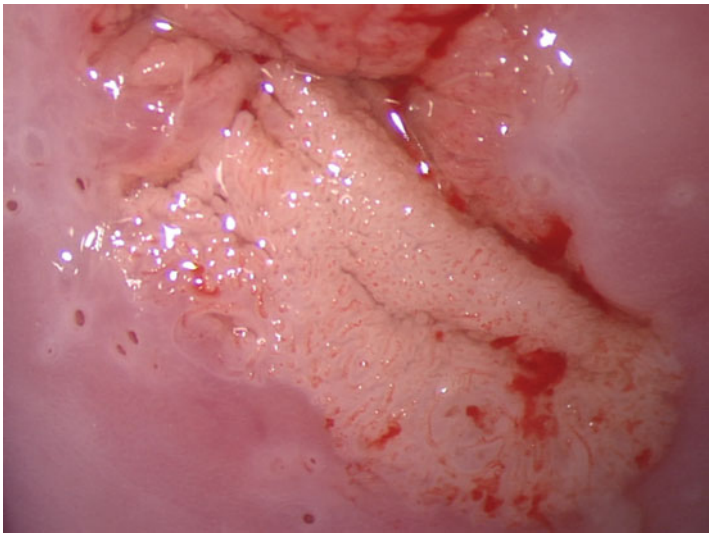

Fig. 7.19 Exophytic condyloma acuminatum with atypical vessels. This lesion may be confused with an early invasive carcinoma

Subclinical lesions due to HPV infection found in the transformation zone are difficult to differentiate from pre-invasive lesions as images, are very similar to CIN (Fig. 7.26). It should be remembered that both conditions (HPV and CIN) frequently occur simultaneously, and that subclinical forms may develop superimposed or adjacent to significant CIN areas. Similarly, individual colposcopic fields of the cervix may be infected simultaneously by different HPV genotypes, and in the same cervix low- and high-grade CIN may coexist (Agorastos et al. 2005).

In order to improve the accuracy of colposcopic findings in the prediction of histological diagnosis, different colposcopic indexes have been developed, the scores of which are related to different aspects of the lesions, including margins, contour, vascularization, and appearance following application of the acid acetic test and Lugol's iodine solution (Reid et al. 1984; Reid and Scalzi 1985). The use of scoring systems may improve the quality of colposcopic examinations but, in daily practice, there is a need to define practical selection criteria that would allow differentiating HPV infection from high-grade CIN lesions.

The International Federation for Cervical Pathology and Colposcopy (IFCPC) approved a revised colposcopic classification at its 11th World Congress in Barcelona, 2002, to be used for clinical diagnosis, treatment, and research in cervical cancer (Walker et al. 2003). In relation to abnormal colposcopic features, a distinction is made between features suggestive of metaplastic change, low-grade disease (subclinical HPV infection or CIN I) (minor change), and high-grade disease (major change) (Table 7.1). The colposcopic accuracy according to the IFCPC 2002 terminology was evaluated in a study of 3,040 women from the general population and showed

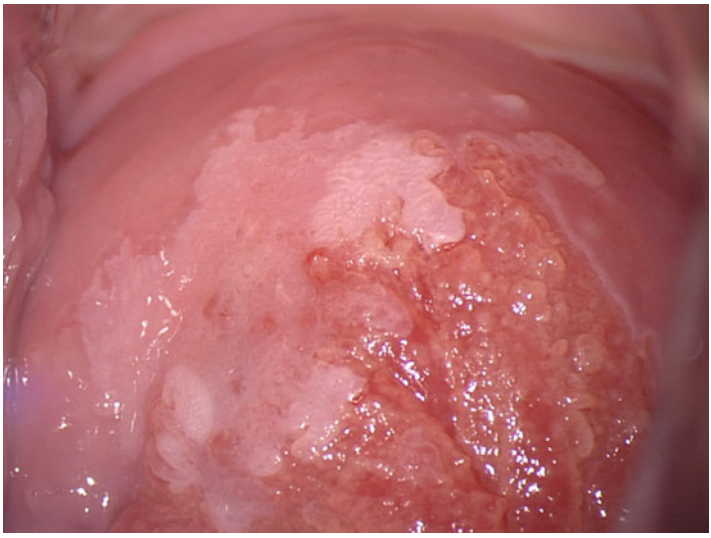

Fig. 7.20 Subclinical HPV infection of the cervix. Acetowhite epithelium with a flat and micropapillary surface

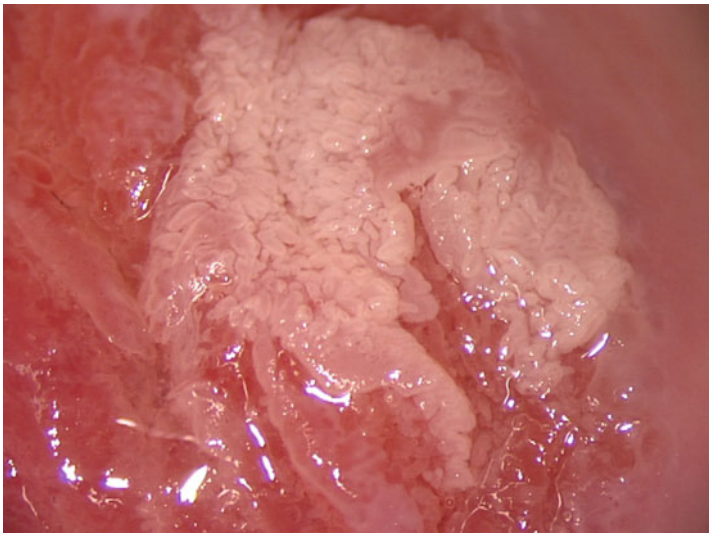

Fig. 7.21 Subclinical HPV infection of the cervix. Acetowhite lesion with a papillary surface

that the categorization of major changes and minor changes is appropriate, being important to describe the lesion localization in relation to the transformation zone and the lesion size because these characteristics are related to high-grade lesions (Hammes et al. 2007).

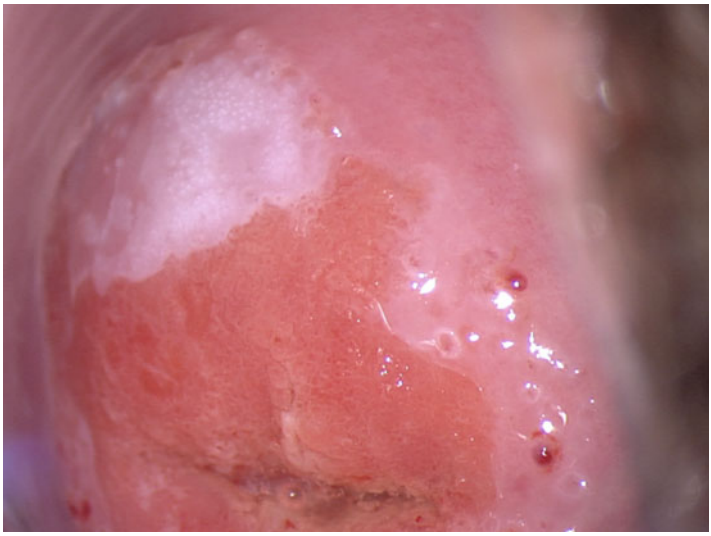

Fig. 7.22 Leukoplakia in the upper lip of the cervix

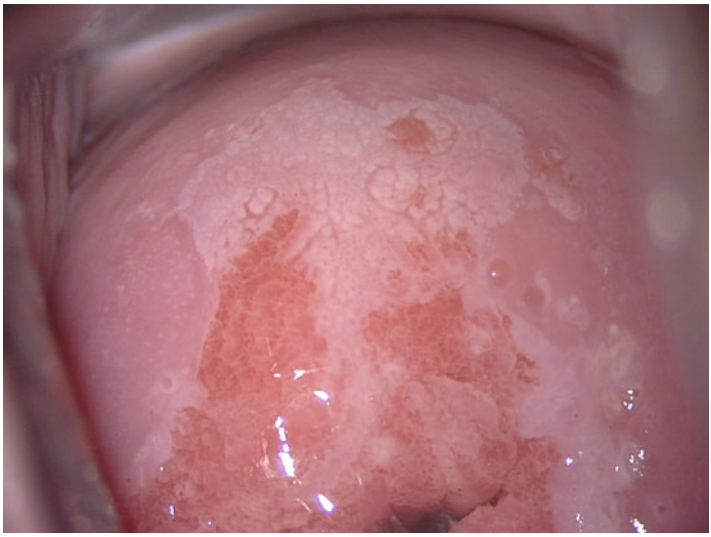

Fig. 7.23 Acetowhite lesion with a fine mosaic pattern and regular surface

6 Conclusions

HPV genital infection causes lesions of different appearance depending on which anatomical site of the lower genital tract is affected. Colposcopy is an indispensable procedure for the diagnosis of subclinical HPV infection. To take full

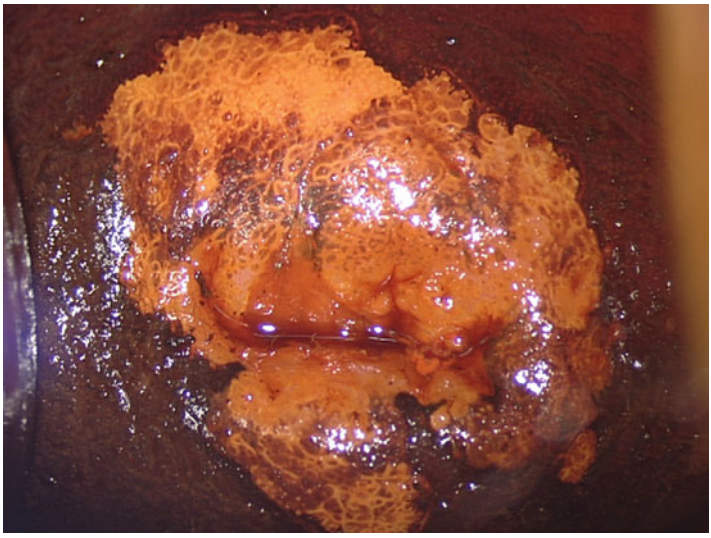

Fig. 7.24 Partial and irregular uptake of Lugol's solution

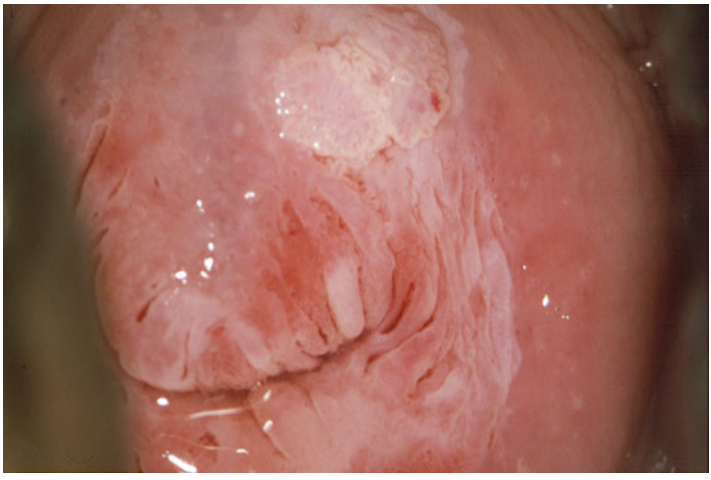

Fig. 7.25 A combined lesion with an exophytic and a flat condyloma in the cervix

advantage of colposcopy is essential to know the different normal and pathologic histological features as well as the etiopathogenic mechanisms to adequately interpret the colposcopic images. On the other hand, to avoid the risk of over diagnosis and/or overtreatment, the high sensitivity of colposcopy requires good training and experience, which has always been a cornerstone for a continued commitment to colposcopy excellence (Dexeus et al. 2002).

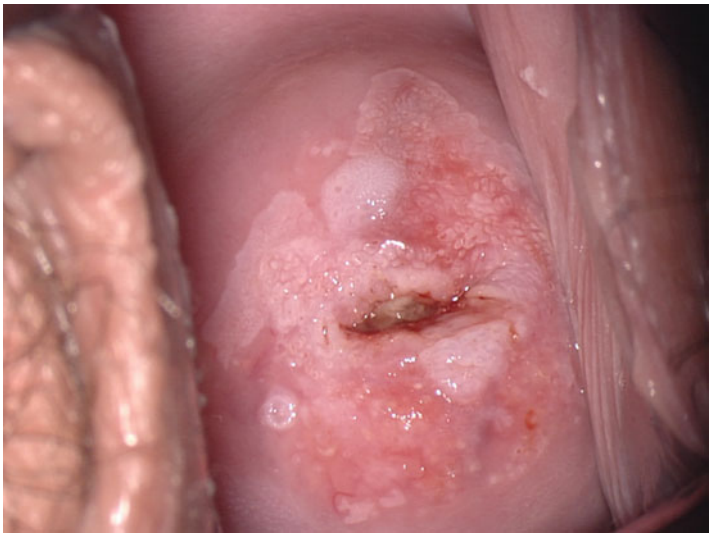

Fig. 7.26 Exophytic condyloma acuminata in the transformation zone with concomitant presence of CIN

Table 7.1 Abnormal colposcopic features graded according to the 2002 IFCPC classification

Colposcopic features suggestive of low grade disease (minor change)	– A smooth surface with an irregular outer border – Slight acetowhite change, slow to appear and quick to disappear – Mild, often speckled, iodine partial positivity – Fine punctation in fine regular mosaic
Colposcopic features suggestive of high-grade disease (major change)	– A generally smooth surface with a sharp outer border – Dense acetowhite change, that appears early and is slow to resolve; it may be oyster white – Iodine negativity, a yellow appearance in a previous densely white epithelium – Coarse punctation and wide irregular mosaics of different size – Dense acetowhite change within columnar epithelium may indicate glandular disease

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Chapter 8
Multiple Aspects of Human Papillomavirus Infections

Franco Borruto and Ciro Comparetto

1 Introduction

Cervical cancer is a sexually transmitted disease (STD) that results from infection with oncogenic types of Human Papillomavirus (HPV). Oncogenic HPV deoxyribonucleic acid (DNA) is found in over 95% of invasive cervical cancers worldwide. Cervical cancer is a leading cause of cancer deaths in developing countries because of high HPV infection rates and lack of comprehensive cervical Papanicolaou (Pap) smear testing of susceptible women. Vaccination against HPV prevents the acquisition of cervical dysplastic lesions among eligible women who have not already acquired the vaccine-specific HPV types. Enhanced understanding of HPV and population-based measures offer the best hope of limiting worldwide mortality due to cervical cancer. The development of therapeutic cervical cancer vaccines and/or virus-targeted drug therapies would be a giant step forward (Ibeanu 2011). HPV is the most common cause of cervical cancer. Cervical cancer, being the second most common cancer after lung cancer and affecting women of different age groups, has a prevalence of about 20% in young sexually active women. Among different types of HPV, HPV-16 is the major strain causing this cancer and is sexually transmitted and unnoticed for decades. Keeping in mind the multiple risk factors related with cervical cancer such as early age sexual activities, teenage pregnancies, smoking, use of oral contraceptives (OC), having multiple sex partners, hormone replacement therapies (HRT), and various other unknown factors lead to

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the onset of the disease. Awareness for various diagnostic procedures such as Pap smears screening proves to be an effective way in eradicating the oncogenic potential of HPV (Faridi et al. 2011).

Recent data indicate that persistent high-risk (HR) HPV infections represent a significantly increased risk of developing incident high-grade (HG) cervical intraepithelial neoplasia (CIN) and cervical cancer. Accordingly, 6-month (6 M+) or 12-month (12 M+) type-specific persistence of HR-HPV have been proposed as powerful surrogates of progressive disease. Because of substantial practical impact in future HPV vaccine trials using non-HPV-16/18 vaccines, studies on HR-HPV persistence as a surrogate endpoint biomarker (SEB) of progressive CIN have been subject to comprehensive meta-analyses recently. Based on a large number of relevant studies, there remains little doubt that persistence of HR-HPV for 6M+ or 12M+ is associated with a significantly increased risk of developing incident HG CIN. However, certain data also disclosed several important issues that need to be carefully considered and/or adequately resolved before adopting 6 M+ or 12 M+ HR-HPV persistence as a surrogate of progressive disease. These include:

1. Definitions of HPV persistence
2. HPV detection techniques
3. Testing intervals
4. Length of follow-up
5. Diagnosis of the SEB
6. Other study characteristics including the type of reference category used in calculating the risk estimates

The reference category used to calculate these risk estimates seems to be a major impact, as is evident from the New Independent States (NIS) of the Former Soviet Union-Latin American Screening (LAMS) cohort. Taken together, it is suggested that in all future studies using the 6 M+ or 12 M+ HR-HPV persistence as a SEB of progressive disease, a "gold standard" should be used in calculating the risk estimates. Also important in deciding is:

1. Whether to use 6 M+ or 12 M+ persistence criteria
2. Cytological, histological, or combined SEB
3. Exclusively use the HPV negative reference group in calculating the risk estimates for viral persistence endpoints

This is supported by the data from the recent meta-analysis as well as from the combined NIS-LAMS cohort, both implicating that the most consistent association to progressive disease is obtained when women with persistent HR-HPV are compared with HPV-negative women. It is the conviction of some Authors that the two other reference categories (HPV transient and HPV mixed outcome) are far too heterogeneous and subject to potential misclassifications to give consistent and reproducible risk estimates for HR-HPV persistence as a SEB of progressive CIN (Syrjänen 2011).

2 Molecular Biology

In the past years, new data has been published on the molecular biology of HPV infections and their relationship to cervical neoplasia. As molecular techniques have become more sophisticated and as the molecular knowledge of HPV infections has been pursued in greater depth, it is increasingly apparent that this human tumor DNA virus is similar to a number of other oncogenic DNA viruses that have been described and well-studied. These viruses appear to act through a common pathway of producing oncogenic proteins that interfere with key signaling elements that normally control the process of cell division. With a better mechanistic knowledge, it should be possible to design new therapeutic approaches to treating HPV-associated disease that are directed toward specific cellular events such as turning off the production of early (E6 and E7) proteins or restoring the activity of p53 or retinoblastoma (Rb) tumor-suppressor gene products. Increased attention has also been turned to immunologic aspects of HPV infections, and a number of groups are eagerly pursuing the possibility of using simple office-based procedures to detect specific proteins encoded for by the HPV open reading frames in an attempt to determine who has been infected, is actively infected, and has proteins being produced that are indicative of neoplasia. From the clinical point of view, the use of outpatient excisional techniques such as the loop electrosurgical excision procedure (LEEP) has rapidly supplanted ablative techniques because of their superior ability to identify early invasive carcinomas and adenocarcinomas in situ (AIS) that have not been detected by colposcopy (Richart and Wright 1992).

HPV types 16 and 18 have been identified in two different human cervical carcinomas. The viral DNA were molecularly cloned and used as probes to screen a large number of genital tumors by Southern blot analysis. HPV-16 or HPV-18 sequences were found in a high percentage of cervical carcinomas but only in a small number of condylomata acuminata or flat condylomas. The majority of the latter lesions, however, contained HPV-6 or HPV-11 sequences which, in contrast, were detected only rarely in carcinoma in situ (CIS) or invasive carcinomas. A similar distribution of the different Papillomaviruses was observed when cell scrapings taken from the cervix were tested by in situ hybridization (ISH) (Gissmann 1984; Gissmann et al. 1984). Infections with specific types of pathogenic HPV, most notably HPV-16 and HPV-18, appear to be necessary but not sufficient factors in the etiology of anogenital cancer. The expression of specific HPV genes (E6-E7) emerged as an important prerequisite for the proliferative phenotype of cervical carcinoma cells. Increasing evidence points to the existence of a host-mediated intracellular control that down-regulates these HPV genes in replicating normal cells. This control appears to be interrupted in HPV-positive carcinoma-cells, probably due to structural modifications of the respective host cell genes acquired during the period of viral DNA persistence. Factors affecting genes seem to be responsible for geographic differences in anogenital cancer incidence, since HPV infections appear to occur worldwide at similar frequency (zur Hausen 1989). The issue of determining which HPV is present in a clinical specimen (typing specimens for HPV) has received attention because HPV cause condylomata acuminata and are associated with the

continuum of disease that ranges from dysplasia to invasive genital cancer. Morphological inspection of precancerous lesions is not sufficient to determine which lesions will progress and which will not. A number of research tools based primarily on DNA hybridization have been developed. These permit identification and typing of HPV in genital tract scrapings or biopsies. Analysis of the typing data indicates that while HPV types can be designated HR and low-risk (LR), these designations are not absolute and thus the LR group should not be ignored. In addition, interpretation of the data is complicated by finding HR types in individuals with no indication of disease (Roman and Fife 1989).

HPV associated with anogenital cancers encode two oncoproteins, E6 and E7. Both E6 and E7 can form specific complexes with tumor suppressor gene products. The HR-HPV E6 proteins can associate with the p53 tumor suppressor protein. This interaction promotes the degradation of p53 in vitro, which presumably accounts for the very low levels of p53 in cervical carcinoma cell lines. The E7 protein binds to the Rb tumor suppressor gene product pRb, with a preference for the underphosphorylated, "active" form of pRb. The E7 proteins derived from the HR-HPV bind to pRb with a higher affinity than the E7 proteins from the LR-HPV. The functional inactivation of p53 and pRb by the HPV oncoproteins E6 and E7, respectively, are important steps in cervical carcinogenesis, since mutations in the p53 and Rb genes were detected in HPV-negative but not HPV-positive cervical carcinoma cell lines. Cytogenetic studies strongly suggest, however, that additional chromosomal changes may be necessary for carcinogenic progression of HPV-induced anogenital lesions (Münger et al. 1992). These viruses contain double stranded DNA that code for about eight gene products, some of which are oncogenic when introduced into cultured rodent or human cells. In particular, both the E6 and E7 gene products have different oncogenic capabilities and these genes are selectively retained within the genome of cervical carcinoma derived cells. The E7 gene product has immortalizing capabilities in primary cells and is able to cooperate with an activated ras oncogene to fully transform primary rodent cells. The E7 gene product from HPV type 16 is also capable of complexing in vitro to the anti-oncogene product, Rb. Similar complexes occur with Adenovirus E1A and Simian Virus 40 (SV40) with large T proteins that suggest a shared mechanism of transformation used by HPV type 16, Adenovirus, and SV40. Transformation studies using primary human cells and non-tumorigenic HeLa/fibroblast hybrid cells have also suggested that chromosome 11 is important in suppressing the HPV transformed phenotype. The transformed phenotype therefore also involves an impaired intracellular control of persisting HPV oncogenic sequences. There is evidence that both natural killer (NK) cells and activated macrophages preferentially kill HPV transformed cells in vitro (Matlashewski 1989).

Chromosomal localization of HPV-16 and HPV-18 on human cervical carcinomas and epithelial cell lines obtained after HPV transfection has uncovered a nonrandom association of viral integration and specific genome sites. Fragile sites appear to be preferential targets for viral integration because of their structural and functional characteristics through which chromosomal anomalies, alterations in protooncogene activity, and gene amplification can occur. Individually or in association, such changes lead to the acquisition of an unlimited cell growth potential but not tumorigenicity. Genetic instability and uncontrolled cell division resulting from

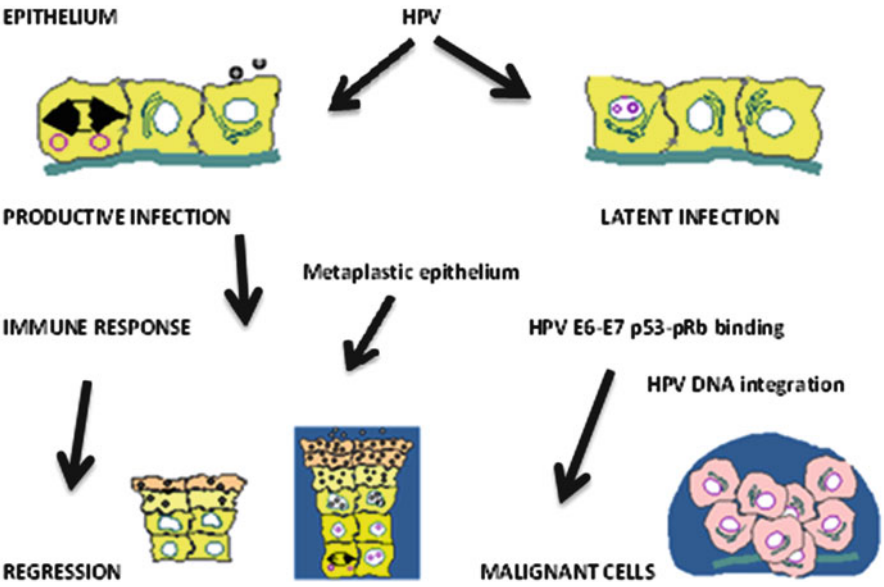

Fig. 8.1 Molecular biology of HPV infection (modified from: HPV and Cervical Cancer. http://web.uct.ac.za/depts/mmi/jmoodie/hpv.html. http://www.stanford.edu/group/virus/papilloma/2004goglincarnevale/Papilloma/Cancer.htm)

HPV integration increase the cell's susceptibility to other exogenous carcinogenic factors that may complete the process of neoplastic development (Popescu and DiPaolo 1989). Integration of the viral DNA into the cellular genome is an important step towards the development of malignancy. Two early genes of HPV (E6 and E7) are involved in cellular transformation. Another early gene (E2) participates in gene control by directly binding to conserved DNA motifs in the viral genome. Several protein factors of viral and cellular origin interact with the regulatory region of HPV and participate in the regulation transcription of oncogenes E6 and E7. Cellular factors, such as immune system and oncogene and anti-oncogene alterations, play an important role in Papillomavirus-associated cervical carcinogenesis (Fig. 8.1) (García-Carrancá and Gariglio 1993). A unique characteristic of the cancer-causing HPV types is the presence of a post-synaptic density protein 95 (PSD-95)-discs large-zona occludens tight junction protein (PDZ) recognition motif on the carboxy terminus of the E6 oncoproteins. Through this motif, E6 directs the proteasome-mediated degradation of cellular proteins involved in the regulation of cell polarity and in cell proliferation control. These include components of the Scrib and Par polarity complexes, as well as a number of other PDZ domain-containing substrates. Thus, Papillomaviruses are now providing novel insights into the functioning of many of these cellular proteins, and into which of these functions, in particular, are relevant for maintaining normal cellular homeostasis (Thomas et al. 2008). Productive infection by HR-HPV types is manifest as cervical flat warts or condylomata that shed infectious virions from their surface. Viral genomes are maintained as episomes in the basal layer, with viral gene expression being tightly controlled as

the infected cells move towards the epithelial surface. The pattern of viral gene expression in low-grade (LG) cervical lesions resembles that seen in productive warts caused by other HPV types. HG neoplasia represents an abortive infection in which viral gene expression becomes deregulated, and the normal life cycle of the virus cannot be completed. Most cervical cancers arise within the cervical transformation zone (TZ) at the squamo-columnar junction (SCJ), and it has been suggested that this is a site where productive infection may be inefficiently supported. The HR E6 and E7 proteins drive cell proliferation through their association with PDZ domain proteins and Rb, and contribute to neoplastic progression, whereas E6-mediated p53 degradation prevents the normal repair of chance mutations in the cellular genome. Cancers usually arise in individuals who fail to resolve their infection and who retain oncogene expression for years or decades. In most individuals, immune regression eventually leads to clearance of the virus, or to its maintenance in a latent or asymptomatic state in the basal cells (Doorbar 2006).

The development of HPV-immortalized cervical and foreskin cell lines represent a useful model for studying the role of HPV in cervical cancer. Studies with these cells show that HPV genes regulate epithelial cell growth and differentiation. Transfection of HPV types associated with invasive cervical cancer results in immortalization of human epithelial cells, whereas HPV not associated with cancer are ineffective. The combination of E6 and E7 genes, which are normally retained and expressed in cervical carcinomas, is sufficient for immortalization. However, the E7 gene alone induces immortality less efficiently. Although the immortalized cells actively express HPV oncoproteins observed in cervical cancer, after injection of immortal cells into nude mice, tumors are rare, having been reported only for HPV-18. Immortalized cells are resistant to terminal differentiation. In fact, HPV may contribute to the carcinogenic process by uncoupling the processes of cell growth and differentiation. Host regulation of viral genes also is important in the malignant process. Endogenous cytokines modify HPV gene expression and influence the pathogenesis of HPV infection in the cervix. Cellular transcriptional activators and repressors regulate HPV gene expression. This normal regulation is altered by viral integration. HPV become integrated preferentially at chromosomal regions near fragile sites and proto-oncogenes. In fact, immortality is associated with induction of structural rearrangements frequently affecting HPV integration sites. Structural and numerical alterations non-randomly involve chromosomes 1, 11, 19, and 20, with chromosome 1 alteration being the most predominant. Wild-type functions of Rb and p53 are necessary to control normal cell growth, and mutation or loss of these suppressor genes often contributes to cancer development. In HPV-containing carcinomas, pRb and p53 were wild- type. However, in carcinomas lacking HPV, both suppressor genes were mutated. Functional inactivation of these tumor suppressor genes by HPV oncoproteins E6 and E7 may explain this difference. Treatment of HPV-immortalized cells with ras or a sub-fragment of Herpes Simplex Virus (HSV) resulted in locally invasive carcinomas when the cells were implanted subcutaneously in nude mice. These experiments indicate that HPV integration and expression are insufficient for malignancy but that HPV participates in the multistep development of cancer (DiPaolo et al. 1993). HR-HPV are capable of immortalizing primary

human keratinocytes in tissue culture, but such cells become transformed only after certain chromosomal changes take place, possibly having to do with oncogene activation. The DNA of HR-HPV is frequently (if not always) integrated into the genome of cancer cells. It is normally episomal in premalignant lesions. Integration disrupts the E2 and E5 genes and viral gene regulation. Cells containing integrated viral DNA show excessively high levels of E6 and E7. While there is some conflicting evidence, it appears that the p53 and Rb tumor-suppressor genes are more frequently mutated in HPV-negative tumors than they are in HPV-positive tumors, suggesting that for tumor formation to proceed the p53 and Rb proteins must be inactivated either by interaction with the viral proteins or by mutation. The presence of an activated oncogene in a cell lacking functional p53 or Rb may then be sufficient to cause tumor progression (Swan et al. 1994).

Progression of an HPV-infected cell clone to invasive growth involves consecutive modifications of a set of host cell genes. Some of these modifications suppress viral oncogene functions post-transcriptionally, and others suppress transcription via a signaling pathway stimulated by activated macrophages and possibly by additional cells. A scheme tries to unify available data by postulating the existence of two intracellular signaling pathways in the control of latent HPV infections (zur Hausen 1994). We know that the products of the viral E6 and E7 oncogenes inactivate those from the p53 and Rb tumor suppressor genes. The p53 protein controls the entrance to the cell cycle. When DNA damage occurs, p53 levels are increased, resulting in cell arrest. This allows cells to repair the damage before replication occurs. Cells without p53 (either mutated or absent) will replicate their unrepaired DNA, increasing their genomic instability. In cells from cervical cancer the E6 protein will induce degradation of p53, so continuous expression of viral oncogenes will lead to genomic instability, that in turn will increase the risk of acquiring new mutations that probably contribute to cancer development (Toledo-Cuevas and García-Carrancá 1996).

In cervical cancer, E6-E7 gene control is deranged by mutations in viral control sequences and in integrated HPV fragments by the disruption of the viral repressor E2. Elimination of this sequence makes E6-E7 messenger-ribonucleic acid (mRNA) unstable, and deranges cellular regulation at the integration site. It is apparent that an intricate interplay of cellular and viral factors determines whether the outcome is active Papillomavirus infection, viral latency, or ultimately genital cancer (Turek and Smith 1996). The best-characterized E6 proteins are those of the genital HR-HPV, which function, at least in part, by inactivating the p53 tumor suppressor protein. Biochemical studies have shown that this occurs by targeted degradation of p53, dependent on the E6-associated protein (AP) ubiquitin-protein ligase. The model that has emerged from E6/E6-AP-dependent p53 degradation has provided insight into both HPV-associated carcinogenesis and the problem of substrate specificity of the ubiquitin system. Several observations suggest that the HR-HPV E6 proteins may also have activities in addition to inactivation of p53 (Huibregtse and Beaudenon 1996). The tumor suppressor p53 is a short-lived protein that under normal conditions is reduced to a barely detectable level. The stability of p53 protein is primarily regulated in normal non-transformed cells

by two interplayers: Mdm2 and p14 (ARF). Relocation of p53, Mdm2, and p14 (ARF) to the nucleolus seems to regulate, at least partially, the steady state of p53. Moreover, there are alternative pathways of the regulation of p53 stability in unstressed cells. Jun-N (amino)-terminal kinase (JNK) and poly-adenosine diphosphate (ADP)-ribose polymerase-1 (PARP-1) are involved in the regulation of the steady-state of wild-type (wt) p53 protein. However, in most human cervical carcinomas, which express the HR-HPV E6 protein, a complete switch from Mdm2 to HPV E6-mediated degradation of p53 occurs. Virally encoded E6 protein utilizes the cellular ubiquitin-protein ligase termed E6-AP to target p53 protein for proteolytic degradation. It has been recently addressed the question of whether p53 protein can be generally reactivated by chemotherapy in HeLa cells despite the E6 activity. It has been observed that an increase of cellular p53 after cisplatin (CP) treatment accumulated preferentially in the nucleoli. It has been checked the cellular level of E6 during CP therapy. Six hours after application of CP, the expression of E6 protein was markedly reduced. This coincided with the increase of cellular p53 level and preceded the nucleolar accumulation of p53 protein, thereby indicating that repression of virally coded E6 protein by CP contributes to the restoration of p53 expression (Wsierska-Gadek and Horky 2003). Additional agents such as sexual behavior, immunity deficiency, socio-demographic factors, and microbiological agents, are implicated in the multistage progression from viral infection to cancer. Inactivation of tumor suppressor gene products (p53, p105Rb), oncogene activation (c-myc, c-ras), aneuploidy, and karyotypic abnormalities are key events in the tumor progression (Mougin et al. 1998).

The integration of viral DNA in chromosomal regions is associated with well-known tumor phenotypes. Some of these viral integrations occur recurrently at specific chromosomal locations, such as 8q24 and 12q15, both harboring HPV-16 and HPV-18. There are other recurrent genetic alterations not linked to HPV. Recurrent losses of heterozygosity (LOH) have been detected in chromosome regions 3p14-22, 4p16, 5p15, 6p21-22, 11q23, and 17p13.3, but without effect on p53, 18q12-22, and 19q13, all of them suggesting the alteration of putative tumor suppressor genes. Recurrent amplification has been mapped to 3q+ arm, with the common region in 3q24-28 in 90% of invasive carcinomas. The mutator phenotype, microsatellite instability, plays a minor role and is detected in only 7% of cervical carcinomas. The development of cervical carcinoma requires the sequential occurrence and selection of several genetic alterations (Lazo 1999). So, the two viral early genes, E6 and E7, and an upstream regulatory region (URR) are preserved in cervical carcinoma cell lines as well as in clinical samples of cervical cancer, indicating that these regions are important in cancer development. E6 and E7 function as transforming genes. E6 protein binds to and promotes degradation of the tumor suppressor protein, p53, while E7 protein complexes and inactivates the Rb protein. Together, they disrupt cell cycle regulation. E6 and E7 are transcribed from a promoter, P97. P97 is regulated by complex interactions between multiple, positive and negative, cellular factors and the viral E2 product. E2 disruption caused by the integration into the cellular genome may induce overexpression of E6 and E7. The E6 and E7 proteins are thought to act as critical factors in cervical carcinogenesis

by inactivating the two tumor suppressor proteins, p53 and Rb, which are commonly mutated in other human cancers (Ishiji 2000). However, it is known that the carcinogenesis is a multistep process. Changes in the cytogenetic equilibrium, such as chromosomal imbalances, allelic loss, and structural aberrations happen during the transformation from normal epithelium to cervical cancer (Heilmann and Kreienberg 2002).

During an acute HPV infection, expression of viral genes, in particular the viral E6 and E7 oncogenes, is restricted to differentiated epithelial cells, which lost the capability to replicate their genomes and are therefore at no further risk for acquiring functionally relevant mutations upon genotoxic damage. HG cervical dysplasia, however, is initiated by deregulated expression of viral oncogenes in replicating epithelial stem cells. Here, the E6-E7 gene products submerge control of the cell cycle and mitotic spindle pole formation through complex interactions with various cellular protein complexes and induce severe chromosomal instability. The detailed molecular analysis of these interactions is allowed to define new biomarkers for dysplastic cervical cells. E7 for example, induces increasing expression of the cyclin dependent kinase inhibitor p16(INK4a) in dysplastic cells. This can be used to identify dysplastic cells in histological slides, cytological smears, or samples taken for biochemical analyses with a yet unmet fidelity. Detection of specific viral mRNA derived from integrated HPV genomes in advanced pre-cancers can be used to identify lesions with a particularly HR for progression into invasive carcinomas (amplification of Papillomavirus oncogene transcripts, APOT, assay) (von Knebel 2002). HPV DNA integration into the basal cells of cervical squamous epithelium precedes the transformation of LG into HG lesions. The HPV DNA tyramide-based ISH system may prove to be a powerful diagnostic/prognostic tool in this regard. The presence of mitoses (especially atypical forms) in the upper layers may be a discriminatory hallmark in the morphologic distinction between LG and HG lesions. Further, since the biologic changes manifested between these two lesions are reflected in their respective phenotype, it appears plausible to adopt the Bethesda System of two-tiered/binary classification of LG and HG squamous intraepithelial lesion (L-SIL and H-SIL) for histopathologic diagnoses (Cooper et al. 2003).

HR-HPV-associated carcinogenesis of the uterine cervix is a particularly useful model to study basic mechanisms of genomic instability in cancer. Cervical carcinogenesis is associated with the expression of two HR-HPV-encoded oncoproteins, E6 and E7. Aneuploidy, the most frequent form of genomic instability in human carcinomas, develops as early as in non-malignant cervical precursor lesions. In addition, cervical neoplasia is frequently associated with abnormal multipolar mitotic figures, suggesting disturbances of the cell-division process as a mechanism for chromosome segregation defects. Centrosomes and the HR-HPV E6 form spindle poles and E7 oncoproteins can each induce abnormal centrosome numbers. These two HPV oncoproteins, however, induce centrosome abnormalities through fundamentally different mechanisms and presumably, with different functional consequences. Cells expressing the HPV E6 oncoprotein, which inactivates p53, accumulate abnormal numbers of centrosomes in parallel with multinucleation and nuclear atypia. On the contrary, HR-HPV E7, which targets the pRb tumor suppressor pathway,

can provoke abnormal centrosome duplication in phenotypically normal cells. These two pathways are not mutually exclusive, since co-expression of HPV E6 and E7 has synergistic effects on centrosome abnormalities and chromosomal instability. Taken together, these findings support the general model in which chromosomal instability arises as a direct consequence of oncogenic insults and can develop at early stages of tumor progression (Duensing and Münger 2003a). Many HR-HPV-positive lesions are genomically unstable and show chromosomal gains and losses already at early stages of carcinogenic progression. Whereas E6 cooperates with E7 presumably by relaxing critical checkpoint control mechanisms, E7 drives genomic instability by inducing abnormal centrosome numbers. The ability of E7 to induce centrosome duplication errors (CDE) may be linked to the re-programming of the host cell cycle machinery, including deregulation of cyclin/cyclin-dependent kinase 2 (cdk2) activity. Given the role of cdk2 as a regulatory node not only for cell cycle progression but also for centrosome duplication, inhibition of cdk2 may not only retard cellular proliferation but also decrease CDE and centrosome-related mitotic defects. Compared to some conventional cytotoxic agents, which exclusively target DNA replication, modulation of cdk2 activity may hold the promise of diminishing the development of genomically unstable, aneuploid tumor cells that are frequently the source of chemotherapy resistance in malignant tumors (Duensing and Münger 2003b). The identification and functional verification of host proteins associated with HPV E6 and E7 oncoproteins may provide useful information for understanding cervical carcinogenesis and the development of cervical cancer-specific markers. In addition, proteomic profiling of altered proteins by anticancer drugs on cervical cancer cells may contribute to providing the fundamental resources for investigation of disease-specific target proteins, elucidation of the novel mechanisms of action, and development of new drugs. The advent of proteomics has provided the hope of discovering novel biological markers for use in the screening, early diagnosis, and prediction of response to therapy (Yim and Park 2006).

The microscopic phenotype of CIN reflects a fine balance between factors that promote or reduce CIN development. A shortcoming of the current grading system is its reliance on static morphology and microscopic hematoxylin-eosin features of the epithelium alone. In reality, CIN is a dynamic process, and the epithelium may exhibit differing results over time. Functional biomarkers p16, Ki-67, p53, and Rb protein cytokeratin 14 and 13, help in the assessment of an individual CIN lesion's potential for progression and regression. The aggregate information provided by these biomarkers exceeds the value of the classic grading system. Consequently, many more CIN that will either regress or progress can be accurately identified. These findings agree with known molecular interactions between HPV and the host. For accurate interpretation of a CIN, it is essential that these biomarkers be determined quantitatively and separately in the superficial, middle, and deeper layers of the epithelium. Such geography-specific epithelial evaluations of quantitative biomarkers emphasize the dynamic nature of a particular CIN lesion, thereby changing the art of static morphology grading into dynamic interpretation of the diseased tissue, with a strong prognostic effect (Baak et al. 2006). Papillomaviruses infect keratinocytes in the basal layer of stratified squamous epithelia and replicate in the nucleus

of infected keratinocytes in a differentiation-dependent manner. Viral gene expression in infected cells depends on cell differentiation and is tightly regulated at the transcriptional and post-transcriptional levels. A noteworthy feature of all Papillomavirus transcripts is that they are transcribed as a bicistronic or polycistronic form containing two or more open reading frames (ORF) and are polyadenylated at either an early or late poly (A) site. In the past 15 years, remarkable progress has been made in understanding how this complex viral gene expression is regulated at the level of transcription (such as via DNA methylation) and particularly post-transcription (including RNA splicing, polyadenylation, and translation). Current knowledge of Papillomavirus mRNA structure and RNA processing has provided some clues on how to control viral oncogene expression. However, we still have little knowledge about which mRNA are used to translate each viral protein. Continuing research on post-transcriptional regulation of Papillomavirus infection will remain as a future focus to provide more insights into Papillomavirus-host interactions, the virus life-cycle, and viral oncogenesis (Zheng and Baker 2006). p16INK4a has emerged as a valuable SEB for HR-HPV infection and shows increased immune-expression with worsening grades of CIN. Numerous studies have emerged in recent years supporting its role in the detection of HG dysplasia and have led to the use of p16INK4a immunohistochemistry (IHC) in many laboratories (Kalof and Cooper 2006).

Although many HPV genomes have been sequenced, knowledge of virus gene expression and its regulation is still incomplete. This is due in part to the lack, until recently, of suitable systems for virus propagation in the laboratory. HPV gene expression is polycistronic, initiating from multiple promoters. Gene regulation occurs at transcriptional, but particularly post-transcriptional levels, including RNA processing, nuclear export, mRNA stability and translation. A close association between the virus replication cycle and epithelial differentiation adds a further layer of complexity (Graham 2010). RNA interference (RNAi) is a natural process by which eukaryotic cells silence gene expression through small interference RNA (siRNA) that are complementary to mRNA. In this process, the siRNA that are 21–25 nucleotides long and are known as microRNA (miRNA), either associate with the RNA-induced silencing complex (RISC), which targets and cleaves the complementary mRNA by the endonucleolytic pathway, or repress the translation. It is also possible to silence exogenous gene expression during viral infections by using DNA templates to transcribe siRNA with properties that are identical to those of bioactive miRNA. HPV E6 and E7 oncogenes, which induce cellular transformation and immortalization, represent strategic targets to be silenced with siRNA. In several in vitro and in vivo studies, it has been demonstrated that the introduction of siRNA directed against the E6 and E7 oncogenes in human tumoral cervical cells transformed by HPV, leads to the efficient silencing of HPV E6 and E7 oncogene expression, which induces the accumulation of the products of the p53 and pRb tumor suppressor genes and activates the mechanism of programmed cell death by apoptosis. Thus, the progression of the tumoral growth process may be prevented. Understanding HPV mRNA expression and its regulation in the different diseases associated with infection may lead to development of novel diagnostic approaches and will reveal key viral and cellular targets for development of novel antiviral therapies (Peralta-Zaragoza et al. 2010).

As the primary etiological agents of cervical cancer, HPV must deliver their genetic material into the nucleus of the target cell. The viral capsid has evolved to fulfill various roles that are critical to establish viral infection. The particle interacts with the cell surface via interaction of the major capsid protein (late, L1) with heparin sulfate proteoglycans. Moreover, accumulating evidence suggests the involvement of a secondary receptor and a possible role for the minor capsid protein, L2, in cell surface interactions. The entry of HPV in vitro is initiated by binding to a cell surface receptor in contrast to the in vivo situation where the basement membrane has recently been identified as the primary site of virus binding. Binding of HPV triggers conformational changes, which affect both capsid proteins L1 and L2, and such changes are a prerequisite for interaction with the elusive uptake receptor. Most HPV types that have been examined appear to enter the cell via a clathrin-dependent endocytic mechanism, although many data is inconclusive and inconsistent. Furthermore, the productive entry of HPV is a process that occurs slowly and asynchronously and it is characterized by an unusually extended residence on the cell surface. Despite the significant advances and the emergence of a general picture of the infectious HPV entry pathway, many details remain to be clarified. The impressive technological progress in HPV virion analysis achieved over the past decade, in addition to the improvements in general methodologies for studying viral infections, provide reasons to be optimistic about further advancement of this field (Horvath et al. 2010).

3 Immunology

Studies of cervical secretions as well as cells composing the endocervix have provided evidence for a functional and potentially important immunological system in the mucosa of that organ. The availability of the tools of cell biology as well as three agents that may be used as probes to infect cervical mucosa experimentally has made possible a detailed approach to define the structural and functional characteristics of local cervical immunity. A long-term goal of these studies is to determine how the cervical immune response may be regulated to reduce local viral replication and virus-associated diseases. With Langerhans cells (LC) for antigen presentation, cervical immune responses generally remain detectable for more than 30 days, are predominantly of the immunoglobulin A (IgA) isotype, can be influenced by estrogen or progesterone, and are best elicited by local, rather than systemic exposure to antigen. Cervical immune responses to HPV are of particular importance in this regard. Responses in serum to HPV-16 proteins L1, E4, and E7 have been found in up to 78% of persons with HPV-associated cervical neoplasms (Roche and Crum 1991). NK lymphocytes, an important defense against viral diseases, are present in most HPV-associated lesions and CIN. HPV-positive cervical cancer cells and HPV-immortalized human cervical epithelial cells which possess properties similar to cervical dysplasia, however, are resistant to NK but are sensitive to lymphokine-activated killer (LAK) lymphocyte lysis. Sensitivity can be enhanced by treatment of cervical cells with leukoregulin, a cytokine secreted by lymphocytes.

Combination treatment with leukoregulin and a chemotherapeutic drug further enhances sensitivity of HPV-infected cells to LAK lymphocyte lysis. In contrast, gamma-interferon (gamma-IFN) treatment of cervical cells can result in decreased sensitivity to LAK lysis, illustrating the potential balance that cytokines can exert in the immunologic control of cervical cancer (Evans et al. 1993).

The clinical manifestation of HPV infection depends also on the host's immune status. Both innate and adaptive immunity play a role in controlling HPV infection. In untransformed HPV-infected keratinocytes, the innate immunity is induced to eliminate the invading HPV pathogen through sensitization to HPV-related proteins by epithelial-residing LC, macrophages, and other immune cells. Once the HPV infection escapes from initial patrolling by innate immunity, cellular immunity becomes in charge of killing the HPV-infected keratinocytes of the uterine cervix through systemic immune response developing by dendritic cells (DC) in the regional lymphoid organs or through local immune response developing by LC in the cervix. Thereby, DC/LC ratio plays a critical role in eliciting innate and adaptive cellular immune responses against HPV infection (Manickam et al. 2007). Cervical epithelium provides a protective niche to the virus to subvert the immune responses. The absence of an inflammatory milieu in the cervix makes the resident DC and LC tolerogenic to HPV antigens. Helper (CD4+) lymphocytes predominate in regressing CIN lesions, whereas cytotoxic (CD8+) lymphocytes are dominant in invasive carcinoma. A reduced expression of T-cell signaling molecule T-cell receptor zeta chain is observed in CD8+ cells. Decreased numbers of NKG2D expressing NK and T cells are present in patients with cervical cancer and CIN. Increased frequencies of CD4+ CD25+ FoxP3+ T regulatory (Treg) cells are observed in patients with cervical cancer. The neuropilin-1 Treg (Nrp-1+Treg) cells shows greater suppressive activity. A network of Treg and indoleamine 2,3-dioxygenase expressed in tumor cells facilitates immune escape of tumor cells. HPV uses different strategies to evade immune recognition. Understanding the immune evasion mechanisms used by HPV will help in designing newer therapeutic strategies for cervical cancer (de Araujo Souza et al. 2009; Patel and Chiplunkar 2009).

The first line of defense against HPV is the innate immune system, which provides non-specific protection against a variety of pathogens and also enhances the adaptive immune response. However, HPV-infected cells often evade innate immune recognition and elimination. HPV gene expression and release of virus occur in superficial squamous cells where virus antigens are not readily detected, and keratinocytes are not lysed during HPV infection, so there is no inflammatory response. In addition, HPV early proteins inhibit specific components of the innate immune system. E6 and E7 inhibit signaling by type I IFN and decrease expression of multiple IFN-inducible genes. E5 and E7 inhibit expression of major histocompatibility complex (MHC) class I proteins on the cell surface. HPV-infected cells are resistant to lysis by NK cells but are sensitive to cytokine-activated NK cells. Activated macrophages also kill HPV-infected cells and control malignant development. Innate immunity is important for prevention of HPV infections, but HPV often persists due to evasion or inactivation of innate defenses (Woodworth 2002). The development of HPV-associated cervical cancer has been closely linked to the expression of the viral oncogenes E6 and E7

in the tumor cells. The major viral oncoproteins, E6 and E7, target the cellular tumor suppressor gene products p53 and Rb, respectively. These interactions result in the stimulation of proliferation and the inhibition of apoptosis, thus representing major oncogenic insults to the infected cell. In addition to mediating transformation, the E6 and E7 genes also play significant roles in altering the immune response against infected cells by suppressing IFN expression and signaling. At the clinical level, IFN have been used in the treatment of HPV-associated CIN or cervical cancers with mixed results. The success of the treatment is largely dependent on the subtype of HPV and the immune response of the patients. Despite this inefficiency, the increasing knowledge about the regulation of IFN signaling pathways at molecular level may hold a promise for the use of new therapeutic strategies against HPV infection. Studies on the regulation of the function of IFN-inducible gene products by the E6 and E7 may lead to the development of new therapeutic approaches based on strategies that modify the function of the HPV oncoproteins and restore IFN-signaling pathways through endogenous control mechanisms (Koromilas et al. 2001). Cellular immunity is likely to be of major importance for the clearance of inapparent or overt infections caused by HPV. The highly polymorphic class I or class II HLA molecules present HPV-derived peptides to CD4+ or CD8+ T lymphocytes bearing specific receptors and condition the immune responsiveness to HPV infections. Recent data point to a role of an altered expression of HLA molecules in the persistence of HPV-induced cervical premalignant lesions and their progression towards invasive carcinoma. Furthermore, positive or negative associations of certain HLA alleles or haplotypes with cutaneous or cervical neoplasias have been found (Breitburd et al. 1996). Natural genital HPV infection seems to be poorly immunogenic because of its non-productive and non-inflammatory characteristics, but also because of the different mechanisms developed by the virus to counteract the immune response. The immune system organization and its regulation in the human female genital tract are mostly "programmed" to ensure a humoral response. Nevertheless, secretory IgA, which are particularly efficient for anti-infectious mucosal immunity, are poorly present in physiological vaginal secretions. These distinctive features could explain part of the relative immune deficiency against HPV. Moreover, reduction or loss of MHC class I molecules and a defect in antigen presentation to cytotoxic T lymphocytes (CTL) could partly explain the cytotoxicity deficiency. There is clear evidence that cellular immune response plays a major role in the control and course of HPV infection. This response varies according to the grade of the lesion and to the oncogenic potential of the infecting HPV. A deficiency in induction of cellular cytotoxicity mechanisms seems to be involved in the persistence of HPV infection and so in carcinogenesis (Riethmuller and Seilles 2000).

Both cellular and humoral immune responses are essential for the clearance of HPV-associated cervical lesions. There is increasing evidence that the immune system plays a pivotal role in determining the outcome of HPV infection. Viruses and associated neoplastic cells are proposed to have evolved mechanisms to avoid immune attack. T-cell-mediated immune responses against oncogenic HPV are believed to play a central role in cervical carcinogenesis. The presence of HPV-specific CTL in a majority of human cervical cancer patients provides an approach for further study of their functional role in modulating this malignancy. Tumor-infiltrating lymphocytes

(TIL) develop as manifestations of the recognition and defense against malignant cells by the host immune system. Cancer cells may overcome immune surveillance, either by down-regulating the proliferation of HPV-specific CTL, or altering the effector compositions of immune cells against HPV infections. TIL in the tumor micro-environment can be functionally inhibited and lose the ability of clonal proliferation as a result of depressed expression of interleukin (IL)-2Ralpha. The upregulation of inhibitory signaling relates to the modulation of the virus- and/or tumor-specific immune responses. Alteration of host genetic susceptibility may also lead to abnormal immune response as a general genomic instability resulting from virus persistence. Induction of HPV-specific immune responses is anticipated as an intimate point for the treatment of cervical neoplasia (Sheu et al. 2007).

The antibody response to the HPV particle is dominated by a neutralizing antibody response to a type-specific, conformationally dependent immune-dominant epitope. Vaccines based on virus-like particles (VLP) lacking the viral genome have been highly successful in preventing HPV infection. In humans, the serum antibody response to VLP is stable over time, also after the HPV infection has been cleared, resulting in HPV serology being used as a marker of cumulative HPV exposure, in spite of the fact that a significant proportion of HPV-exposed subjects fail to seroconvert. More than 90% of HPV infections will clear spontaneously. The factors that determine whether an HPV infection is cleared or persists and increases the risk for cancer are not known, but cellular immunity is implicated. Several HLA class II haplotypes are associated with cervical cancer:

1. DQw3 increases and DR13 decreases the risk for cervical cancer in general: odds ratios (OR) and 95% confidence interval (CI): 1.25 (1.15–1.37) and 0.69 (0.56–0.85)
2. DR15 increases the risk for HPV-16-carrying cancer (OR: 1.47, CI: 1.20–1.81)
3. DR7 may be either protective or increase the risk

Most cervical cancers have downregulated the expression of at least one HLA class I antigen, whereas class II expression is increased in infected epithelium. A T-helper 2 (Th2) cytokine profile is associated with progression to cervical cancer. HPV-antigen-specific proliferative responses have been detected in many studies. Specific CTL responses were originally reported in only a minority of infected subjects, typically cancer patients, but with advancing technology, specific CTL can be stimulated from about half of the women with HPV-carrying disease. In animal model systems, CTL responses can mediate clearance. The antibody response to HPV is a mediator of type-specific protective immunity, which forms the basis for prophylactic vaccine. The cellular immunity to HPV is implicated as an important factor in cervical carcinogenesis (Konya and Dillner 2001). Variability in host immunogenetic background is important in determining the overall cellular immune response to HPV infections. There is an association of the region containing the HLA-DQ and HLA-DR genes with the risk of developing squamous cells cancer (SCC). An increased risk was observed for carriers of allele 184 at the MHC class I chain-related gene A (MICA) locus, in particular for homozygotes, suggesting a recessive effect (Zoodsma et al. 2005). Polymorphisms in HLA genes have been implicated in the risk for cervical cancer. Association of certain class II HLA

alleles with cervical cancer has been documented in various ethnic populations. The implications of such an association, however, are controversial. A meta-analysis confirms the apparent association between certain HLA-DRB1 allele families and alleles and cervical SCC, suggesting that oncogenesis in this disease may be related to defects in immuno-regulation. Larger studies may be needed, particularly in various ethnic groups to further substantiate these associations (Yang et al. 2006).

HPV-infected individuals who are immuno-compromised in addition to having acquired (e.g. Human Immunodeficiency Virus, HIV) or iatrogenic (e.g. systemic lupus erythematosus, SLE patients and organ and hematopoietic stem cell transplant recipients undergoing immuno-suppressive therapy) immune deficiency are particularly at risk for HPV-initiated cervical neoplasia and cancer. Psycho and neuro-immunologic (PNI) research has demonstrated that psychosocial factors such as stress, pessimism, and sleep quality may play a role in the promotion of HPV-mediated cervical neoplasia in HIV-positive women. The PNI mechanisms may underlie the promotion of HPV-mediated cervical neoplasia and HPV-infected women who are iatrogenically immuno-suppressed are an understudied population at risk for cervical cancer. Female transplant recipients, one such group, may provide a unique paradigm in which to explore further PNI mechanisms of HPV-mediated cervical neoplasia (Jensen et al. 2007). Psychosocial associations have been observed with level of cervical dysplasia or "pre-cancer" and invasive cervical cancer (related to HPV infection). PNI relationships have been observed in HIV-1 infection, which is being described in an increasing number of women. Relationships of psychosocial factors and level of cervical dysplasia were similarly observed with reference to immunological and health status in asymptomatic and early symptomatic HIV-1 infected homosexual men, suggesting that a potentiating effect may occur in HIV-1 and HPV co-infected women. Consistency of relationships across studies appeared to be enhanced by the use of a bio-psychosocial model integrating the effects of life stressors, social support, and coping style as well as psychiatric disorders. Research is indicated on the relationships between psychosocial factors, immunological status, and clinical health status in this group of women. Because of the high prevalence of psychosocial risk factors for chronic psychological distress in these women and the known immunological and health status decrements occurring with progression of these two infections, a clinical screening program based on the bio-psychosocial model is recommended as a means of secondary prevention. If effective in generating treatment referrals, such a program would likely improve quality of life and could aid in the determination of relationships with immunological and health status as well (Goodkin et al. 1993).

4 Relationships Between HPV, HIV Infection, and Cervical Cancer

In acquired immuno-deficiency syndrome (AIDS), opportunistic infections are important causes of morbidity and mortality, but non-infectious conditions frequently make substantial contributions to the disease course. Patients with HIV infection

may be at increased risk for neoplastic disease. They do not however, have an increased incidence of the most common tumors affecting the general population, such as breast, colon, and prostate carcinoma. Immuno-deficiency results in increased susceptibility to malignant neoplasms, both by decreased immunologic response to abnormal cells and increased susceptibility to infection by viruses. All of the malignant neoplastic diseases that are Center for Disease Control (CDC) and Prevention AIDS indicator conditions have been shown to have an association with a virus: Kaposi sarcoma (KS) with Herpes Hominis Virus 8 (HHV-8), malignant lymphoma with Epstein-Barr Virus (EBV), and cervical carcinoma with HPV. Patients with HIV can also develop reactive processes that are attributable to direct effects of HIV or immune system alterations. Such conditions include salivary gland cystic lympho-epithelial lesions, lymphadenopathy, lymphocytic interstitial pneumonitis, encephalopathy, enteropathy, nephropathy, hepatic conditions, dermatologic conditions, and anemia (Klassen et al. 1997). Cell-mediated immunity likely plays an important role in prevention of HPV-associated disease, and HPV-associated SCC has been shown to occur with increased frequency among iatrogenically immuno-suppressed individuals. Similarly, individuals with HIV-associated immuno-deficiency have been shown to have a high prevalence of anogenital HPV infection as well as a high prevalence of HPV-associated lesions thought to be cancer precursors. Thus, HIV-positive women have a high prevalence of CIN, and HIV-positive men have a high prevalence of anal intraepithelial neoplasia (AIN). The risk of disease in these populations appears to increase as the degree of immuno-suppression increases, and these individuals are likely at risk for development of invasive SCC. Because these diseases are preventable, women should be screened on a regular basis with cervical Pap smears, followed by colposcopy if the Pap smear is abnormal. Lesions that are detected should be biopsied for histopathologic assessment. Thorough assessment of the entire anogenital region should be performed because of the multicentric nature of HPV-associated diseases. Following treatment, rigorous follow-up should be maintained because of the high recurrence rate of HPV-associated disease in these populations (Palefsky 1991).

HIV infection appears to alter the natural history of HPV-associated oncogenesis, but its impact on gynecology has only recently been defined. Taking this data into account, the CDC designated moderate and severe cervical dysplasia as a category B defining condition, and invasive cervical cancer as a category C defining condition of AIDS in 1993. Anal HPV infection and AIN have been found to be highly prevalent among HIV-positive homosexual men, and recent preliminary data suggest a relatively high prevalence among HIV-positive women as well. Moreover, HPV infection and associated lesions are also observed in body sites other than the anogenital area, particularly the skin and the oral cavity (Del Mistro and Chieco Bianchi 2001). Once cervical cancer develops in HIV-positive women, the disease may be aggressive and less responsive to treatment. A primary means by which HIV infection may influence the pathogenesis of HPV-associated cervical pathology is by molecular interaction between HIV and HPV genes. Although these have not been well defined, an upregulation of HPV E6 and E7 genes expression by HIV proteins (such as tat) has been postulated by some of the contributing Authors.

Cervical cytology appears to be an adequate screening tool for CIN in HIV-positive women, but the high recurrence rate and multi-focality of this disease reinforces the need for careful evaluation and follow-up of the entire anogenital tract in these women. Probably in the next few years, cervical tumors will represent one of the most frequent complications of HIV infection, as part of the progression through AIDS. This points to a need for greater interdisciplinary cooperation for a best disease definition and for the development of effective prevention measures (Boccalon et al. 1996).

An increasing body of information permits certain conclusions to be drawn about the nature and magnitude of the interactions between HPV and HIV infections and their influence on the genesis of intraepithelial neoplasia and, to a lesser extent, cancer. Importantly, findings tend to be consistent across a number of independent studies. While HPV infection probably does not significantly alter the course of HIV infection, HIV-induced immuno-suppression does increase the severity and duration of anogenital warts, increase their infectiousness, and reduce treatment efficacy. However, in developed countries, the countervailing effects of enhanced HPV infectiousness and declining rates of unsafe sexual behavior have resulted in stable or declining incidence rates of anogenital warts. Advanced immuno-suppression due to HIV infections results in highly significant increases in rates of HPV-associated CIN and AIN (Judson 1992). Prolonged severe immuno-deficiency provides the necessary milieu for the emergence of anogenital neoplasia caused by HPV. Cervical and anal neoplasia are likely to become more common manifestations of HIV disease as patients with profound immunodeficiency, who would have succumbed to opportunistic infections earlier in the epidemic, are now surviving for extended periods of time because of increasingly effective antiretroviral, prophylactic, and antimicrobial therapies. Cervical cancer in the setting of HIV infection appears to be a more aggressive disease, less likely to be successfully treated by standard therapies, and consequently associated with a poorer prognosis than in comparable non-HIV-infected women. Strategies need to be developed for earlier detection and treatment of neoplasia and anogenital cancer in the setting of HIV-induced immuno-deficiency (Northfelt 1994).

Although many basic questions about the relationship between HIV and HPV infections remain unresolved, epidemiological studies have consistently shown a strong association between HIV infection and the development of HPV-related squamous intraepithelial neoplasia. HIV infection may promote the clinical manifestation of subclinical or latent HPV infection. Technical advances localizing virus DNA and gene products in situ will provide new avenues for investigation, allowing us to go beyond correlations and to clarify the mechanisms of interaction between the two viruses in individual patients. With improved antiretroviral therapy and prophylaxis for HIV-associated opportunistic infection and prolonged survival of women with HIV, HPV infection and its most serious consequence, cervical cancer, are likely to assume greater significance in the clinical management of HIV-infected women throughout the world. A better understanding of the role of HIV in promoting the clinical manifestation of HPV infection will be essential to the control of this disease (Braun 1994). Management of women with HIV infection or AIDS

should follow the established guidelines for antiretroviral therapy and prevention and treatment of opportunistic complications of HIV infection. Gynecological manifestations of HIV are primarily cervical dysplasia and cancer associated with HPV infection and vaginal and muco-cutaneous candidiasis. HPV-associated cervical dysplasia/neoplasia is more common in women with advanced rather than early HIV disease, and monitoring with Pap smears should probably increase to every 6 months in patients with CD4+ cell counts <500 cells/microl (and certainly when this value falls below 200), with positive Pap smears confirmed by colposcopy and biopsy. For patients with CD4+ cell counts >350 cells/microl, cryotherapy is probably adequate, but therapy should be increasingly aggressive at lower CD4+ cell counts. Women with childbearing potential should be treated as medically indicated for other HIV-infected patients, including during pregnancy. In fact, preliminary results of AIDS Clinical Trial Group (ACTG) Study 076 indicated that zidovudine (ZVD) or azidothymidine (AZT) therapy during pregnancy reduces vertical transmission of HIV about threefold (Baker 1994). The recent therapeutic advances that may affect the management of neoplastic anogenital HPV-related lesions in HIV patients are highly active antiretroviral therapy (HAART) and HPV vaccines. HAART shows limited benefit on the incidence of H-SIL and cancer in the cervix, vulva, and anus. On the other hand, it seems to raise the spontaneous regression rates of cervical LG lesions (CIN 1) and condylomata, as well as the regression of it after treatment of CIN 2-3. The benefit of HAART in SIL seems to be modest, mostly due to the improvement of the immune status. The usefulness of HPV DNA in HIV is not well established. HPV vaccines represent a mid-term new possibility of prevention for these lesions according to the high effectiveness shown, although the lack of data about effectiveness and security in HIV patients restrict its application. Second generation vaccines, still to be developed, might better adapt the specific needs of these patients (Fusté et al. 2008).

The association between cervical cancer and HPV is well known, but its association with HIV is controversial. Co-infection with HPV and HIV is to be expected and recent epidemiological data from Africa show that cervical cancer is the most common AIDS defining neoplasm in women. Unlike other AIDS defining neoplasms, the occurrence of cervical cancer is not dependent on immune compromise. HIV alters the natural history of HPV infection, with decreased regression rates and more rapid progression to HG and invasive lesions, which are refractory to treatment, requiring more stringent intervention and monitoring. A different molecular pathway mirrors the more aggressive behavior. HIV-associated cervical cancers are thought to progress through the microsatellite instability pathway, whereas HIV-negative ones progress through LOH. Interaction is probably via viral proteins, with HIV proteins enhancing effectiveness of HPV proteins, and perhaps contributing to cell cycle disruption. Deregulation of the cellular and humoral arms of the local and systemic immune systems may ensure disease progression. Furthermore, HPV infection may predispose to HIV infection and facilitate its progression (Clarke and Chetty 2002). As we have said, all HIV-infected women should be screened at least annually for cervical cancer with cervical cytology. All patients with any abnormality in the Pap smear should be referred for colposcopic evaluation. Patients with documented HG

dysplasia should be treated. All patients must be observed closely after treatment for recurrence. Treatment with agents such as imiquimod or 5-fluorouracil (5-FU) may help to decrease recurrence rates. Patients who are diagnosed with cervical cancer should be treated with surgery or concurrent chemotherapy and radiation as determined by extent of disease and health status. It appears that HAART may have a positive impact on cervical dysplasia and cancer. Thus, all HIV-infected patients should be managed optimally on HAART. Hopefully, as patients live longer and healthier on HAART, they will be more compliant with cervical cancer screening, and cervical cancer will become an increasingly rare disease (Stier 2003). Recent reports cast doubt on the efficacy of HAART therapy for HPV-induced anogenital neoplasms, despite efficacy in improving disease caused by other infectious agents. VLP serology has been reported to be associated with outcome of cervical cancer. VLP seropositivity has been reported to be a favorable prognostic sign in women with HPV-16-positive cervical carcinoma. Several investigators have questioned the immunogenicity of the oncogenic HPV type 16 compared with other HPV types. It has recently been found that in HIV-infected patients, lymphoproliferative cellular immune responses (CMI) to HPV-16 peptides are not associated with CD4+ counts, whereas responses to recall antigens and mitogens are associated with CD4+ counts. CD4 + T cells may not be responsible for protective cellular immune responses to HPV. VLP serology and CMI responses may be the future SEB for HPV-associated anogenital neoplasia trials. Most anogenital neoplasms occurring with increased frequency in patients with HIV/AIDS are associated with HPV infections. Current treatment strategies are not effective in clearing anogenital HPV infection and need improvement (Einstein and Kadish 2004). HIV-infected men who have sex with men remain at HR of developing anal cancer despite the widespread use of HAART. In HIV-infected women, however, there is some evidence that HAART may be associated with regression of HPV-related cervical disease. So far, epidemiologic data provided by cancer registries have shown no reduction in the incidence of cervical and anal cancer in patients with HIV infection since the initiation of HAART in 1996. Recent data suggest that HPV infection occurs in the anal canal of immuno-compromised patients as an opportunistic infection in the absence of receptive anal intercourse. Taken together, these lines of evidence support the need for developing anal and cervical cancer screening programs for patients with HIV, whether untreated or on HAART (Piketty and Kazatchkine 2005).

The question is whether cervical screening, which helped to dramatically reduce cervical cancer rates through the detection of precancerous lesions in the general population, is as efficient in the setting of HIV. The risk for cervical cancer remained high and stable during the last decade in HIV-infected women, and incidence did not decrease with improving CD4+ cell counts in women receiving antiviral therapy. The use of HPV DNA tests in primary screening endorsed in the general population may be less specific in immuno-compromised women and might thus not be as efficient on screening. HPV vaccines, recently available, have no therapeutic effect and might thus not be very useful in preventing cervical cancer in a population highly infected with multiple and persistent HPV. Cervical cancer prevention remains an important goal in HIV-infected women and specific guidelines are warranted for this increasing population (Heard 2009).

HIV-infected women in different geographic regions (such as Zambia, Brazil, etc.) appear to be infected with less prevalent types of HR-HPV as compared to the general population who, across all continents, are more commonly infected with types 16 and 18. Secondly, integration of HPV DNA into the host genome is no longer thought to be a necessary cause of malignant transformation of cervical cells. However, rate of integration appears to differ by the type of HPV. In fact, the types of HPV which appear to be more common in cervical dysplasia of HIV-infected women are the same types which are more likely to require integration for malignant transformation. Finally, HPV types found in HIV-infected women are relatively common and likely to persist. The most common among these types belong to the alpha-9 and -7 species, which are the most carcinogenic. Given that current vaccines target HR-HPV-16/18, the findings from the above mentioned studies might have important implications for the design of HPV vaccines that target the types of HPV associated with disease risk in HIV-infected women. HPV typing and assessment of the physical state (whether it is integrated or episomal) appear to be two valuable parameters for the prognostic evaluation of dysplastic lesions of the uterine cervix. However, this has not yet been assessed in HIV-infected women. Recent data about the immune response in HPV/HIV co-infection may lead to understanding potential mechanisms for less virulent HPV causing malignant transformation in HIV-infected women (McKenzie et al. 2010).

HIV and HPV infections are both STD with many risk factors in common. Studies have found that HIV-seropositive women are at least five times as likely to be infected with HPV as seronegative controls. In immuno-compromised HIV-seropositive women, the risk of CIN is almost as high as in women with SIL on their Pap smear. Some studies have shown the false-negative rate of cervical cytology in HIV-seropositive women to be very high, although others have shown it to be comparable with the rate in seronegative controls. However, given the prevalence of CIN in this population, even a "normal" false-negative rate may result in many missed CIN lesions. Among HIV-seropositive women and especially among those who are immuno-compromised, CIN is more likely to progress and recur after treatment. Recurrence rates may reach 87% 36 months after treatment in markedly immuno-suppressed women. Cryotherapy is especially ineffective in these patients. Vulvar condyloma and VIN are much more prevalent in HIV-seropositive women and especially in those who are markedly immunosuppressed or who have been immunosuppressed for a prolonged period of time. It is recommended that all HIV-seropositive women undergo periodic evaluation at intervals no less than every 6 months. Immuno-compromised women should be followed with cytology and colposcopy of the cervix and vulva, although those with normal immune systems may be followed with cytology alone. Because the rates of recurrence and progression are so high after treatment of these women, they should be followed with colposcopy and cytology every 6 months. New approaches to treatment need to be explored in this population (Spitzer 1999). The existence of cervical neoplasia in women with HIV represents one of the most serious challenges in the oncologic care of immuno-suppressed patients. While the development of most cancers in the immuno-suppressed patient can be attributed solely to immune deficiency, the relationship

between SCC of the cervix and HIV is quite unique because of common sexual behavioral risk factors. Screening strategies in HIV-positive women must take into account the high prevalence of cervical dysplasia in this subgroup as well as the limitations of cytologic screening. Cervical dysplasia in HIV-positive women may be of higher grade than in HIV-negative patients, with more extensive involvement of the lower genital tract with HPV-associated lesions. The presence and severity of cervical neoplasia in HIV-positive women correlate with both quantitative and qualitative T-cell function. Standard therapies for pre-invasive cervical disease have yielded suboptimal results with high recurrent rates. While poor treatment results of standard ablative and excisional therapies warrant unique therapeutic strategies, one must recognize that close surveillance and repetitive treatment have been successful in preventing progressive neoplasia and invasive cervical carcinoma. The disease characteristics of invasive cervical carcinoma may take a more aggressive clinical course in HIV-infected women. HIV-positive women with cervical cancer have higher recurrence and death rates with shorter intervals to recurrence and death than do HIV-negative control subjects. CD4+ status does influence subsequent outcome. In general, the same principles that guide the oncologic management of cervical cancer in immune-competent patients should be applied. However, extremely close monitoring for both therapeutic efficacy and unusual toxicity must be instituted (Maiman 1998).

5 Endocrinology of HPV Infection and Cervical Cancer

Persistent infection with HR-HPV is involved in cervical cancer, a major cause of cancer mortality worldwide. Infection occurs primarily at the TZ, the most estrogen and retinoid-sensitive region of the cervix. Development of cervical cancer affects a small percentage of HR-HPV-infected women and often takes decades after infection, suggesting that HR-HPV is a necessary but not sufficient cause of cervical cancer. Thus, other co-factors are necessary for progression from cervical HR-HPV infection to cancer such as long-term use of hormonal contraceptives, multiparity, smoking, as well as micronutrient depletion, and particularly retinoid deficiency, which alters epithelial differentiation, cellular growth, and apoptosis of malignant cells. Therefore, early detection of HR-HPV and management of precancerous lesions together with a profound understanding of additional risk factors could be a strategy to avoid this disease. These risk factors may act in concert to induce neoplastic transformation in squamous epithelium of the cervix, setting the stage for secondary genetic or epigenetic events leading to cervical cancer (Gariglio et al. 2009). Among the cofactors involved in the malignant transformation of cells infected by HPV, sex hormones may facilitate the cervical carcinogenesis by different mechanisms, including the induction of squamous metaplasia in the TZ of the cervix, interactions between steroid hormones and HPV gene expression, and alterations of the local immune microenvironment (Delvenne et al. 2007). Steroids can interact

with hormone-response elements in the viral long control region, enhancing HPV transcription and resulting in transformation of cervical cells. Subsequent malignant progression may involve virus-induced chromosomal instability, facilitating viral DNA integration and deregulation of gene expression (Pater et al. 1994).

Sex hormones influence susceptibility and disease predisposition for many genital tract infections. Sex steroids affect mucosal immunity. Estrogen's role is important in the early stages of several infections as it stimulates antibody and cell-mediated immune responses. There is increased expression of some cytokines in peripheral blood and vaginal fluids during the follicular phase of the menstrual cycle and with use of hormonal contraception. Whether estrogen exerts a protective or deleterious influence depends on the infecting organism and stage of infection or disease. Estrogen apparently reduces susceptibility to primary HPV infection but in the event of persistent HPV infection, sex steroid hormones (estrogen and/or progesterone) are associated with progression to cervical cancer (Brabin 2002). Steroid contraception has been postulated to be one mechanism whereby HPV exerts its tumorigenic effect on cervical tissue. Steroids are thought to bind to specific DNA sequences within transcriptional regulatory regions on the HPV DNA to either increase or suppress transcription of various genes. Although some earlier studies were reassuring as no increased incidence of cervical cancer was observed, subsequent research has shown a causative association, especially among long-term users. The role of steroids was further enhanced by the discovery of hormone receptors in cervical tissue. Some earlier studies of OC steroids found no increased risk, even after controlling for other risk factors, including smoking and number of partners. However, prospective studies have shown a greater progression of dysplasia to CIS with more than 6 years of steroid OC use. Similar findings were also evident from other works, including the Royal College of General Practitioners Oral Contraception Study. The World Health Organization (WHO) Collaborative Study of Neoplasia and Steroid Contraceptives showed a relative risk (RR) of 1.2 for invasive cancer in users of the long-acting progestational contraceptive depo-medroxyprogesterone acetate (depo-MAP). However, in users of more than 5 years duration, an estimated risk of 2.4 was reported. The URR of the HPV-16 viral genome mediates transcriptional control of the HPV genome and is thought to contain enhancer elements that are activated by steroid hormones. It has been shown that steroid hormones bind to specific glucocorticoid response elements within HPV DNA. Experimental evidence has revealed that HR-HPV type 16 are able to stimulate the development of vaginal and cervical SCC in transgenic mice exposed to slow-release pellets of 17 beta-estradiol (17 beta-E2) in the presence of human keratin-14 promoter. SCC developed in a multi-stage pathway only in transgenic mice and not in non-transgenic mice. The E6 oncoprotein of HPV-16 has been shown to bind to the p53 tumor suppressor gene and stimulate its degradation by a ubiquitin-dependent protease system. Steroid hormones are thought to increase the expression of the E6 and E7 HPV-16 oncogenes, which in turn bind to and degrade the p53 gene product, leading to apoptotic failure and carcinogenesis (Moodley et al. 2003). Results from published studies were combined to examine the relationship between CIS and invasive cervical cancer and duration and recently of use of hormonal contraceptives, with particular

attention to HPV infection. Compared with never users of OC, the RR of cervical cancer increased with increasing duration of use for durations of approximately less than 5 years, 5–9 years, and 10 or more years, respectively; the summary RR were 1.1 (95% CI 1.1–1.2), 1.6 (1.4–1.7), and 2.2 (1.9–2.4) for all women, and 0.9 (0.7–1.2), 1.3 (1.0–1.9), and 2.5 (1.6–3.9) for HPV positive women. The results were broadly similar for CIS and invasive cervical cancers, for SCC and adenocarcinoma, and in studies that adjusted for HPV status, number of sexual partners, cervical screening, smoking, or use of barrier contraceptives. The limited available data suggest that the RR of cervical cancer may decrease after use of OC ceases. However, study designs varied and there was some heterogeneity between study results. Although long duration use of hormonal contraceptives is associated with an increased risk of cervical cancer, the public health implications of these findings depend largely on the extent to which the observed associations remain long after use of hormonal contraceptives has ceased (Smith et al. 2003).

6 Conclusions

Cervical cancer is a complex disease that, by its association with HPV, has elicited research in a broad range of areas pertaining to its basic diagnostic and clinical aspects. The complexity of this association lies not only in the fundamental relationship between virus and cancer but also in its translation to pathologic diagnosis and clinical management. Offshoots from the relationship of virus to pathology include studies targeting the link between Papillomavirus infection and cervical epithelial abnormalities, the molecular epidemiology of Papillomavirus infection, and the potential use of HPV testing as either a screening technique or a tool for managing women who have Pap smear abnormalities. A second variable that is critical to the pathogenesis of cervical neoplasia is the cervical TZ. The wide range of invasive and non-invasive lesion phenotypes associated with HPV infection in this region indicates that not only the virus, but also specific host-target epithelial cells in the TZ play an important part in the development of cervical neoplasia. Further understanding of this relationship between the virus and the host epithelium will hinge on determining the subtypes of epithelial cells in the TZ and their phenotypic response to infection. New technologies, such as expression arrays, promise to clarify, if not resolve, the complexity of molecular interactions leading to the multiplicity of tumor phenotypes associated with HPV infection of the uterine cervix (Crum 2000; Burk et al. 2009; Hamid et al. 2009; Lehoux et al. 2009; McLaughlin-Drubin and Münger 2009; Szalmás and Kónya 2009; Thierry 2009; Yugawa and Kiyono 2009; Lagunas-Martínez et al. 2010; Moody and Laimins 2010; You 2010).

Although most women clear the infection within a few months, the virus induces a shift towards immune tolerance that can facilitate persistence and permit tumorigenesis. Mechanisms used by HPV to avoid immune surveillance and control include

infecting only the basal layer of the cervical epithelium, limiting expression of viral proteins until later stages of epithelial differentiation, undergoing non-lytic replication, and downregulating the expression of important receptors on cells of the innate immune system. Furthermore, HPV suppresses the expression of several pro-inflammatory proteins that are crucial in clearing infection and activating the CTL involved in killing virus-infected cells. Interestingly, neutralizing antibodies, although of uncertain effectiveness in preventing infection or re-infection after natural exposure (prior infection), are highly protective after immunization with HPV VLP-based vaccines. Understanding what is known and unknown about the interaction between the immune system and HPV is important in the assessment of the potential contribution of prophylactic vaccination in reducing the incidence of cervical cancer. However, despite our growing understanding, many aspects of the interactions between HPV and the host immune system remain unknown (Einstein et al. 2009).

Since the first reports between the association of HIV infection and neoplasia, there has been a dramatic change in the incidence and epidemiology of AIDS-related malignancies. KS, non-Hodgkin's lymphomas (NHL), and cervical cancer are classified by the CDC as AIDS-defining malignancies. However, since the availability of HAART, especially protease inhibitors, there has been a steady increase in non-AIDS defining malignancies, such as Hodgkin's lymphoma (HL), lung cancer, hepatocellular cancer, anal cancer and others, along with a decline in AIDS-defining neoplasias. Although the emergence of non-AIDS defining cancers could be a result of longer life expectancy and due to a better control of HIV, toxic habits and co-infection with other viruses such as Hepatitis B and Hepatitis C Viruses (HBV and HCV) and HPV could play an important role. The interactions of HAART and incomplete immune reconstitution could be other factors explaining the increase in non-AIDS defining cancers. These emerging non-AIDS defining malignancies present a new challenge in the care of patients with HIV infection, and require optimal treatment protocols that take into consideration the interaction between HAART and systemic chemotherapy (Palefsky 2009; Cáceres et al. 2010; Pantanowitz and Michelow 2011).

Since OC use for long durations is associated with an increased risk of cervical cancer, it is important to know whether HPV infection is more common in OC users. There is no evidence for a strong positive or negative association between HPV positivity and ever use or long duration use of OC. The limited data available, the presence of heterogeneity between studies, and the possibility of bias and confounding means that these results must be interpreted cautiously (Green et al. 2003). In many studies, combined OC have been associated with an increased risk of cervical abnormalities and cervical cancer, but there might be alternative explanations for these epidemiological associations (combined OC users can start having sexual intercourse at an earlier age, they have more sexual partners, and they rarely use barrier methods of contraception), so OC act as a promoter for HPV-induced carcinogenesis (Deligeoroglou et al. 2003; Sepkovic and Bradlow 2009; Chung et al. 2010).

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Chapter 9
Prevention of HPV-Associated Diseases in the United States

Diane M. Harper

1 Introduction

Prior to 1940, cervical cancer killed more women than any other cancer with US mortality rates exceeding 36/100,000 women (Wingo et al. 2003). The incidence for cervical cancer prior to Pap testing among white women collated from the Second National Cancer Survey in 1947 was reported at 33/100,000 women (Gardner and Lyon 1977; Kessler 1974) while non-white women were reported to have an incidence of 78/100,000 women (Dorn and Cutler 1955). George Papanicolaou published the first evidence that a vaginal cytology screening test could predict cervical cancer (Papanicolaou 1928; Papanicolaou and Traut 1941) and ushered in the next six decades of cervical cancer prevention throughout much of the developed world. Since 2000, the secondary prevention method of repeated primary cytology screening over a woman's lifetime has reduced the incidence of cervical cancer by 75% (Anttila et al. 1999; Ries et al. 2002).

Human papillomavirus (HPV) has been classified as a human carcinogen (ROC 2004) after its biological properties were fully described for cervical oncogenesis (zur Hausen 1976; zur Hausen 2009). Epidemiologic studies have verified that HPV is a necessary infection for the development of cervical cancer (Bosch et al. 2002; Castellsagué et al. 2006; Muñoz et al. 2006). Prophylactic vaccination against two oncogenic HPV types has been established (Harper and Williams 2010b). HPV vaccination will not reduce the incidence of cervical cancer in countries with organized

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secondary prevention any lower than cytology has accomplished (Barnabas et al. 2006), but does offer the opportunity to reduce, to some degree, the morbidity inherent in primary cytology screening (Schiffman 2007).

2 Secondary Prevention: Screening Program for Early Detection, Diagnosis and Treatment

Cytology-based screening has been the mainstay of US cervical cancer prevention screening programs. The success of Pap testing in reducing the incidence of cervical cancer has shifted the emphasis away from the less frequent cervical cancer detections whose incidence in the US is 8.3/100,000 (US Cancer Statistics Working Group 2010) to the early detection and treatment of precancerous lesions whose incidence peaks around 800/100,000 among women 25–29 years old (Saslow et al. 2002; Insinga et al. 2004). Both conventional cytology and liquid based cytology are equivalent methods for the detection of precancerous lesions, commonly called either cervical intraepithelial neoplasia grade 3 (CIN 3), or carcinoma in-situ (CIS) (Siebers et al. 2009). The intermediate histologic classification of CIN 2 has little true prognostic significance for cervical cancer being reproducible and validated for oncogenic potential in less than a third of the interpretations (Carreon et al. 2007), yet is often classified with CIN 3/CIS as a cancer precursor. True pre-cancer endpoints only consider CIN3, but international and US medical regulatory authorities accommodate a CIN 2/3 endpoint as a cervical cancer surrogate (Pagliusi and Teresa Aguado 2004). In the US the average incidence of CIN 3 is 150/100,000 women per year (Insinga et al. 2004).

Cytology is the primary screening methodology for all women ages 21 years and older and is recommended for those screening "negative for intraepithelial lesion or malignancy (NILM)" every 2–3 years (ACOG 2009). Co-testing for the presence of a cocktail of oncogenic HPV types has been FDA approved for women 30 years and older at an interval of every 3 years when both the cytology is NILM and HPV testing negative (ACOG 2009). This ongoing screening program offers 17 screens in an average NILM woman's lifetime, ceasing at the age of 65 years. The more specific testing combination of primary HPV testing followed by cytology triage is being adopted instead of HPV co-testing elsewhere in the world (Arbyn et al. 2006; httph 2011).

2.1 Benefits of the Cytology Screening Program

While historical evidence shows that annual repeated Pap smears starting as young as the age of sexual debut have reduced the average incidence of cervical cancer from 40 to 80/100,000 women (Dorn and Cutler 1955) in the US by 75% to an average of 8.3/100,000 women of all races and ethnicities (US Cancer Statistics Working

Group 2010), it is not the frequency or age of screening that accomplished this reduction. Population benefit for population reduction of cervical cancer only occurred when at least 70% of the women in the country participated in the routine screening program (Quinn et al. 1999; Peto et al. 2004). When less than 70% of the population participated, there was individual woman benefit for those who did participate, but the incidence of cervical cancer remained high on a population basis. Hence, the most important parameter to population health is recruiting and retaining at least 70% of the population in the screening program (Harper et al. 2010a). In the US, health economics has influenced the screening program recommendations such that the screening interval is now increased to every 3 years and screening begins at the older age of 21 years (Cuzick et al. 2008; Sasieni et al. 2009).

Within the US there are racial and ethnic populations who do not participate in the screening program leading to a wide range of cervical cancer incidences by race: 6.4/100,000 for American Indian/Alaska Natives, 7.5/100,000 for Asian/Pacific Islanders, 7.9/100,000 for caucasians, 11.1/100,000 for blacks and 12.8/100,000 for Hispanic women (US Cancer Statistics Working Group 2010), all rates still less than the incidence of cervical cancer without any screening and all rates less than the best possible reduction achievable with Gardasil at 14/100,000 (Harper 2009b).

2.2 Harms of the Cytology Screening Program

There are five risks or harms associated with the cervical cytology screening program (Herbert et al. 2010). As the incidence of cervical cancer has decreased, the personal connection with friends and relatives dying of cervical cancer has dramatically fallen. While women often have friends and relatives with abnormal Pap tests, they do not see the devastating effect of the death process from cervical cancer. Instead women hear of the pain and psychological anguish of women having to undergo colposcopy for an abnormal Pap test, which in turn scares women away from screening. The first risk of the Pap screening program is noncompliance with screening due to the perceived non-risk of cancer or the perceived agony of the work up if the screen is abnormal.

The second risk of the cervical cancer screening program is the false negative rate of screening. The lowest incidence that repeated Pap screening could accomplish is 2–3/100,000 (Sawaya and Grimes 1999); this represents the 30% of women who develop cancer yet participated in the program and had consistent NILM reports. This group of women is failed by the system.

The third harm of the cervical cancer screening program is the decreased quality of life associated with screening. Because a Pap test includes an uncomfortable speculum exam that invades an intimate personal space, acquiescence to the exam creates a strong feeling of vulnerability. Should the results of this exam be abnormal, 40–50% of women do not follow up for diagnostic colposcopy due to a complex interaction of psychosocial, demographic, and institutional factors which include potential relationship dissolution based on an acquired sexually transmitted disease, HPV

(Eggleston et al. 2007). These sequelae of being told that the screening exam is abnormal are worsened by the fact that there is a high false positive rate, manifested by the high ratio of abnormal cytology incidences to CIN 3 incidence (7,800/100,000 respectively) (Eversole et al. 2010; Insinga et al. 2004).

The opposite of not detecting a cancer that is truly present is detecting disease that is not there: the false positive rate. Effects of the false positive rate include anxiety, depression, and lifetime changes in body perception (Rogstad 2002; Blomberg et al. 2009). As discussions evolve to include HPV testing in the cytology program, the false positive rate can increase to nearly a quarter of all screened women with a tripling of the rate of colposcopies that currently occur (Kitchener et al. 2009; Mayrand et al. 2007).

The fourth harm inherent in screening and early detection is the treatment that prevents the development of cancer from a precancerous state. While the purpose of the screening program is to provide early detection and treatment, the most common treatment itself results in reproductive morbidity. Several meta-analyses show that with cervical conizations and electrosurgical excisions there is a 70–300% increase in preterm births, low birth weight infants and Cesarean sections, which is not present with cryotherapy or laser ablation treatments (Kyrgiou et al. 2006).

The fifth is that the screening programs are secondary prevention for the present disease state only. There is no lifetime protection against new same or different type HPV infections from natural HPV infection induced antibodies (Trottier et al. 2010; Olsson et al. 2009b). Within 10 years of treatment for any CIN lesion there is a 3–12-fold increase in the development of cervical, vaginal, anal and vulvar cancers (Kalliala et al. 2005) and a threefold increased risk of death from any HPV associated ano-genital cancer excluding cervical cancer (Kalliala et al. 2010). From initial CIN treatment, the risk of cervical and vaginal cancer development over the subsequent 25 years continues to be double the general population incidence (Strander et al. 2007).

The balance of benefit and harm from the current screening program has resulted in very good cervical cancer control in the US. Current prophylactic HPV vaccination cannot reduce the incidence of cervical cancer in the US below the levels that screening has already achieved on a population basis, but can reduce the number of HPV infections and abnormal cytology tests which result in reduction in the number of excisional therapies that would be performed.

3 Primary Prevention: HPV Vaccination

Gardasil and Cervarix were approved by the Food and Drug Administration (FDA) in 2006 and 2009, respectively, as prophylactic vaccines for cervical cancer containing antigens derived from the two most common HPV types associated with cervical cancer, HPV 16 and 18 (Paavonen et al. 2009; Koutsky 2007). Gardasil has the dominant market share in the US, thus further discussion is limited to Gardasil.

Merck aggressively advertised to parents, adolescents and professional medical associations in such a way to maximize the risk of cervical cancer in adolescents, which does not exist, and to minimize the information that HPV was sexually transmitted. Professional medical organizations including the American College of Obstetrics and Gynecology, the American College Health Association, and the American Society of Colposcopy and Cervical Pathology were paid hundreds of thousands of dollars to create educational campaigns to promote Gardasil use among their physician and provider members (Rothman and Rothman 2009). Physician leaders within these societies influenced vaccine policy development through their voice on the Advisory Council on Immunization Practices (ACIP) where the decision to federally fund the vaccine for children through 18 years of age was made.

A public policy push to mandate Gardasil by brand name was attempted in 2007 through Women in Government who were actively assisted in drafting each state's legislation by industry (Charo 2007). The public response to pressures from the direct to consumer (DTC) marketing, from pediatricians and primary care physicians, and to the mandate proposal was outrage. To date all legislation regarding mandates has been withdrawn except for the District of Columbia (Washington DC) and the state of Virginia that have laws that mandate HPV vaccination for girls before the age of 13 or before entering sixth grade, respectively, with liberal opt-out opportunities (NCSL 2011).

Public outrage has limited the uptake of Gardasil in the US. In the states with aggressive legislation there is only 17% uptake of at least one dose of Gardasil among eligible girls in Virginia and among 23% of eligible sixth graders in Washington DC (httpe 2011). On a national level the CDC estimates that 44% of girls 13–17 years of age have received at least one dose and 27% have received three doses irrespective of timing (CDC MMWR 2010a, b). Among adult women 18–26 years of age, 9.9% have received at least one Gardasil dose (Euler et al. 2008). Only three doses of Gardasil given within a 1 year time span can provide the efficacy reached in the FUTURE II trials; prolonged dosing intervals, or less than three doses are significantly inferior (Neuzil et al. 2011; Krajden et al. 2011) and are considered a costly waste of health care resources. Recent evidence shows that Cervarix is 100% effective with only one dose (Kreimer et al. 2011) and may be more influential in cervical cancer incidence reduction because, in part, of its simplicity of implementation.

3.1 Gardasil Composition

Gardasil contains 120 μg of antigenic protein consisting of virus like particles (VLPs) to the L1 protein of HPV 16 and 18 as well as HPV 6 and 11. The adjuvant, whose general purpose is to maximize duration of vaccine efficacy, is a proprietary aluminum mixture. There are additional manufacturing components used along with a yeast expression system, for which Gardasil is contraindicated, if allergic, to result in the final 0.5 ml dose of Gardasil (Table 9.1) (Package Insert 2009).

Table 9.1 Gardasil composition (Package Insert 2009)

	Concentration (μg)
Protein subunit component	
HPV 16 L1 VLP	40
HPV 18 L1 VLP	20
HPV 6 L1 VLP	20
HPV 11 L1 VLP	40
Adjuvant	
Amorphous aluminum hydroxyphosphate sulfate	225 mg
Manufacturing components	
Sodium chloride	9.56 mg
L-Histidine	0.78 mg
Polysorbate 80	50 mg
Sodium borate	35 μg
Expression system	
Yeast expression system in recombinant *Saccharomyces cerevisiae*	

3.2 Gardasil Efficacies for Women

3.2.1 Efficacies for Infection and CIN in Young Women

In young women 16–26 years of age, the per protocol for efficacy cohort of women: sero-negative for relevant vaccine HPV type at baseline, PCR negative for relevant vaccine HPV type through Month 7, without protocol violations, with positive or negative Pap tests, with cases counted 1 day after Month 7 after completing three doses had a 98% vaccine efficacy (95% CI: 94, 100) for CIN 2+ caused by HPV 16/18 and a 52% (95% CI: 41, 61) efficacy in women included regardless of baseline HPV status, regardless of baseline Pap test, and cases counted after 1 day after Month 1 after an average of 3.6 years of follow up after first injection (Muñoz et al. 2010; UCM111274 2008). There are similarly high efficacies in preventing HPV 16/18 persistent infections for 5 years in phase II studies among the former population of women (Villa et al. 2006). For CIN 2+ regardless of associated HPV type, Gardasil provided 18% efficacy (95% CI: 7, 28) for an average of 3.6 years in this latter population of women (UCM111274 2008; Dillner et al. 2010).

There is some evidence that Gardasil has cross protection for CIN 2+ lesions caused by HPV 31. But these lesions included those co-infected with HPV 16/18 (Brown et al. 2009) and this cross protection was not seen in other population definitions such as the per protocol population, the modified intent to treat population or those women with only HPV 31 infections. Hence, the FDA denied Gardasil the claim of cross protection for any other oncogenic type beyond HPV 16 and 18.

It is very clear that Gardasil can only prevent HPV type-specific infections if the young woman is not currently infected with that type-specific infection. Women who were DNA positive for HPV 16/18/6/11 regardless of serostatus at the time of first vaccination were not protected from CIN development (Garland et al. 2007).

Women with a prior type specific vaccine relevant HPV infection, seropositive for HPV 16/18/6/11, but DNA negative for HPV 16/18/6/11 prior to vaccination likewise had no protection from CIN 2+ disease caused by HPV 16/18 (Olsson et al. 2009b; Haupt et al. 2011) This is not true for Cervarix which does protect against CIN 2+ disease caused by HPV 16/18 in women already seropositive for HPV 16/18 (Szarewski 2011). Trials for both vaccines showed that vaccination does not potentiate dysplastic progression when given to women already infected with HPV 16/18 or 6/11 (UCM251763 2010; Hildesheim et al. 2007).

3.2.2 Efficacies for Infection and CIN in Mid-Adult Women

The prevalence of HPV infections increases in older women in the US as well as most of the world (de Sanjosé et al. 2007), preceding a second peak incidence of CIN 3 and a second peak incidence of cervical cancer in older women (Bosch and de Sanjosé 2003; Schiffman and Kjaer 2003). Women older than 42 years of age develop new incident high risk HPV infections at the same rate or faster than re-activated past high risk HPV infections (González et al. 2010; Trottier et al. 2010); and these infections progress to CIN 3 at the same rate of progression as do high-risk HPV infections in young women (Rodríguez et al. 2010). The incidence of new high-risk HPV infections is 83/1,000 women older than 40 years (Trottier et al. 2010) and the incidence of CIN 2+ in women older than 45 years is 385/100,000 (Porras et al. 2009), representing a quarter of all CIN 2+ development in all ages of women. If women develop and are treated for CIN 3 at the age of 50 or older, they remain at two to eightfold higher risk for cervical and vaginal cancers for another 25 years than those women developing CIN 3 at 30–39 years of age (Strander et al. 2007). Hence, prevention of HPV 16/18 could offer older women a substantial health benefit.

In the cohort of mid-adult women 24–45 years old who were seronegative to the relevant vaccine HPV type at day 1 and PCR negative to that type in cervico-vaginal swabs or biopsy samples, or both, from day 1 to month 7; and additionally, had received all three vaccinations within 1 year, and had one or more follow-up visits after month 7 with cases being counted starting at month 7 (per protocol population), Gardasil was effective at preventing HPV 16/18/6/11 persistent infections (89%, 95% CI: 79, 95) and HPV 16/18/6/11 associated CIN 1+ (92% (95%CI: 49, 100) for an average of 3.8 years (Castellsagué et al. 2011), but not at preventing CIN 2+ caused by HPV 16/18 (UCM251763 2010; Castellsagué et al. 2011).

The vaccine failure rate evaluated in the per protocol population of mid-adult women for CIN 2+ caused by HPV 16/18 is nearly 14 times higher than the vaccine failure rate in the same population of young women (UCM251763 2010). The full analysis set which includes women with prior exposure to HPV, showed no efficacy in mid-adult women for CIN2+, related or unrelated to HPV 16/18 (UCM251763 2010). This is overwhelming evidence that universally vaccinating all women through 45 years of age will not decrease the population incidence of cervical cancer in the US.

Despite the inappropriate recommendation for universal Gardasil vaccination of older women, there may be evidence for offering vaccination to some women in their mid 40s who have already had prior HPV exposure. In the population of 34–45 year old women who were seropositive and DNA negative for the relevant HPV type at baseline, vaccine efficacy for persistent infection caused by HPV 16/18/6/11was 81% with broad confidence intervals (95% CI: 14, 98) (Castellsagué et al. 2011). This evidence though, is not supported by further disease progression evidence of protection, as there were no cases of CIN 2+ in either the vaccine or placebo arms of this seropositive/DNA negative analysis cohort.

The lack of efficacy for the specific CIN 2+ endpoint associated with HPV 16/18, the lack of efficacy in the broader pertinent population and the substantially poor antibody response were primary reasons why Gardasil was not approved by the FDA for use in women 24–45 years of age in the US (UCM251763 2010; Castellsagué et al. 2011). Additionally, this lack of efficacy was also seen in the trial of mid-adult women who were seropositive, but DNA negative prior to vaccination for HPV 16/18/6/11 associated CIN (UCM251763 2010).

3.2.3 Adenocarcinoma In-Situ (AIS) Efficacies

While adenocarcinomas constitute only a quarter of all cervical cancers, adenocarcinoma is the most difficult cervical cancer to identify by screening or in diagnostic work up and has the worst survival rate (Smith et al. 2000; Gunnell et al. 2007). Long-lasting HPV vaccination to prevent adenocarcinoma would be an advance in cervical cancer prevention that could change US cervical cancer statistics.

Vaccine efficacy for adenocarcinoma in-situ, the precursor to adenocarcinoma, associated with HPV 16/18 was 100% (95% CI: 31–100) (UCM111274 2008) with seven cases developing in the control arm at an average 3.6 years follow up for young women 16–26 years old who were seronegative and DNA negative for HPV 16/18 at baseline, remaining HPV 16/18 DNA negative through the vaccination series, month 7, with cases counted starting at day one of month 7.

Gardasil does not induce any cross protection against other HPV types associated with AIS, and Gardasil does not prevent AIS associated with HPV 16/18 in women already seropositive for HPV 16/18 at baseline.

No efficacy against AIS associated with HPV 16/18 is evident in mid-adult aged women (UCM251763 2010).

3.2.4 Efficacies to Prevent Abnormal Cytology and Excisional Procedures

The vast majority of morbidity comes from the high absolute numbers of US women who screen positive for squamous cell cancer precursors. Reducing the rate of abnormal cytology and cervical excisional procedures are the most important potential outcomes of HPV vaccination in the US. Table 9.2 shows that Gardasil is able to reduce the abnormal cytology rate by about 20% within 3 years after vaccination in women 16–26 years regardless of serostatus or DNA status at study entry.

Table 9.2 Prevention of abnormal screens, colposcopies, and treatments in young women (Muñoz et al. 2010; Olsson and Paavonen 2009a)

	Population	Vaccine efficacy (95% confidence intervals)
Abnormal cytology screens		
ASCUS	USP-N	22% (9, 36)
LSIL	USP-N	17% (9, 24)
HSIL	USP-N	45% (4, 69)
All abnormal cytology	USP-N	17% (10, 24)
Reduction in colposcopies	USP-N	20% (12, 27)
Reduction in excisional therapies	USP-N	42% (28, 54)

ASCUS means atypical squamous cells of undetermined significance irrespective of high risk HPV type association
All abnormal cytology means ASCUS HR positive or worse
LSIL means low grade squamous intraepithelial lesion irrespective of HPV type association
HSIL means high-grade squamous intraepithelial lesion irrespective of HPV type association
USP-N means unrestricted susceptible population approximating the sexually naive female population where women 16–26 years old at baseline had normal cytology and were seronegative and DNA negative for 6, 11, 16, 18; and were DNA negative, regardless of serostatus, for 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59; received at least one injection and had at least one follow up datum; case counting started after day 1; subjects were followed for a mean of 3.6 years (43.2 months) after first injection

Gardasil is unable to reduce the incidence of abnormal cytology in women 24–45 years of age (UCM251763 2010). In women who were seronegative and PCR negative at enrollment to HPV 6, 11, 16 and 18, who were PCR-negative at enrollment to HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, who had NILM on Pap at enrollment, who received at least one dose of vaccine, and whose cases were counted after day 1 (the generally HPV naïve {GHN} population) the vaccine efficacy was not significant at 12% (95% CI −18, 35) (UCM251763 2010).

To the heart of cervical cancer prevention in the US, Table 9.2 also shows Gardasil's efficacy in preventing the need for 20% of the colposcopic examinations for abnormal cytology and its follow up algorithms, as well as 40% of the excisional procedures necessary to treat CIN 2, CIN 3 and adenocarcinoma in-situ lesions in young women.

In mid-adult aged women of the GHN population, Gardasil provides no efficacy at preventing colposcopic examinations (13% (95% CI: −14, 33), cervical biopsies (15% (95% CI: −12, 36) or excisional procedures at 36% (95% CI: −15, 65) (Castellsagué et al. 2011; UCM251763 2010).

3.2.5 Vaginal and Vulvar Lesion Efficacies

The incidence of vaginal and vulvar cancers in the US is 2.2/100,000 and 3.7/100,000, respectively [www.cancer.gov/cancertopics/types/vaginal, [www.cancer.gov/cancertopics/types/vulvar]. While pathologists have separated CIN into three

distinct categories with CIN 2 being the least reliable and uncertain in its oncogenicity, vaginal and vulvar intraepithelial neoplastic (VaIN, VIN, respectively) categories are less clear. VaIN resembles the pathophysiology of CIN more closely than does VIN (Srodon et al. 2006), with the VaIN 2 diagnosis the least accurate and reproducible at 10% concordance rate among pathologists (Sherman and Paull 1993). Likewise, VIN 1 and VIN 2 diagnoses have a 5% and 6% concordance, respectively, between pathology readers compared to 57% concordance for VIN 3 (Prati et al. 2000).

Both VaIN and VIN classifications are improved when grade 2 and grade 3 neoplasias are combined into one category of 'high grade dysplasia' with concordance rates exceeding 70% for VaIN and VIN (Sherman and Paull 1993; Prati et al. 2000). While this combination of categories produces better concordance among pathologists, it is not necessarily related to the oncogenicity of the lesion. Both VIN 3 and VaIN 3 are definitive cancer precursors, while VIN 2 lesions do not appear to be part of a biological continuum that progresses to vulvar cancer (Prati et al. 2000; van de Nieuwenhof et al. 2008) and VaIN 2 lesions are too difficult to histologically distinguish from VaIN 3 to understand their oncogenic potential (Insinga et al. 2008).

HPV associated VaIN 3 and VIN 3 are associated with high risk HPV types, with over 90% associated with HPV 16 alone (Srodon et al. 2006). The grade 3 lesions are also often found immediately adjacent to their respective carcinomas concluding that these intraepithelial lesions are the precursors of vaginal and vulvar carcinomas (Srodon et al. 2006). The respective cancers are attributed to HPV in 91% of the vaginal cancers and in 50% of the vulvar cancers with HPV 16 as the dominant high-risk type (WHO/ICO 2010).

The uncertainty of vaginal and vulvar intraepithelial pathologic diagnoses impacts the validity of the combined respective grade 2 and grade 3 intraepithelial neoplasia endpoints chosen for the Gardasil trials. While this composite is superior for pathologic agreement of lesion severity, it does not address the importance of only the oncogenic grade 3 neoplasia. Since the study endpoint was a combined grade 2/grade 3 lesions, the association with oncogenic HPV 16 and 18 becomes the important decisive factor for determining, the degree of public health benefit Gardasil will provide for vaginal and vulvar cancers.

The efficacy in young women who were HPV DNA negative by PCR and seronegative to the relevant vaccine-HPV-type at enrolment, remained PCR negative to the same vaccine-HPV-type through 1 month after dose three, received three doses of vaccine or placebo within 1 year, and did not violate the protocol (per protocol population) was only significant for HPV 16 associated VaIN 2/3 and VIN 2/3 lesions (100% 95% CI: 31, 100 and 100% 95% CI 42, 100, respectively) (Joura et al. 2007; UCM216352 2008). This significance held for all populations inspected: intention to treat and unrestricted susceptible population. Unfortunately, Gardasil showed no efficacy for HPV 18 associated VaIN 2/3 and VIN 2/3 lesions, potentially due to the low attack rate of HPV 18 during the 3.7 year trial.

The efficacy, regardless of HPV causation, was only significant for VIN 2/3 lesions in the intent to treat population at 51% (95% CI: 9, 75), and not for VaIN 2/3 lesions due to any HPV (12 cases in the Gardasil arm, 21 cases in the placebo arm). This evidence along with the evidence that there was no cross protection efficacy against other high risk HPV types seen for VIN 2/3 or VaIN 2/3 means that the population

benefit of vulvar cancer prevention from Gardasil vaccination will be small, if detectable (UCM111274 2008). The small possible population benefit of vaginal cancer prevention from Gardasil may be measurable several decades from now due to the protection against HPV 16 associated VaIN 2/3, if booster vaccines are able to maintain efficacy over this time frame.

In young women already exposed to HPV 16 and 18, seropositive at baseline, Gardasil has no demonstrated efficacy against VIN 2/3 or VaIN 2/3 associated with HPV 16/18 over 3.7 years of follow up (UCM111274 2008).

In the mid-adult aged women, no cases of VIN 2/3 or VaIN 2/3 were reported in either the vaccinated or placebo groups, providing no evidence for protection for the older 24–45 year old women (Castellsagué et al. 2011; UCM251763 2010).

3.2.6 Anal Intraepithelial Neoplasia Efficacies in Women

The incidence of anal cancer is 0.6/100,000 in men and 1.6/100,000 in women in the US (Harper and Vierthaler 2011). Anal cancer is a predominantly female cancer occurring twice as commonly in women as in men, but with the same attributable fraction due to oncogenic HPV between genders. About 10% of women with cervical HPV associated lesions also have anal HPV infections (Santoso et al. 2010; Koppe et al. 2011). Anal cancers are attributed 87% of the time to HPV for women and men, but are nearly 100% attributed to HPV in men who have sex with men (MSM). Frequent receptive anal intercourse and immunosuppression are independent risk factors for anal cancer (Brewster and Bhatti 2006; Shiels et al. 2011); hence most of the studies of AIN have been documented in men with HIV disease or receiving transplants (WHO/ICO 2010; Palefsky et al. 1998; Brewster and Bhatti 2006). HPV 16 and 18 account for about 70% of the HPV associated anal cancers (WHO/ICO 2010; De Vuyst et al. 2009).

While much of the biology of the anal canal and cervical epithelium appears to be similar regarding HPV infection and cytologic and histologic morphologic pathology, it is an oversimplification to state that the prevention of AIN 2 and AIN 3 disease will prevent anal cancer in both men and women of any sexual identity. As seen in cervical, vulvar and vaginal lesions, the distinction of a grade 2 AIN category is highly unreliable among pathologists and unpredictable for cancer progression (Colquhoun et al. 2003). AIN 3 lesions, on the other hand, have a nearly complete association with HPV infection (Varnai et al. 2006), but have a relatively low potential for malignant transformation in the immuno-competent patient (Scholefield et al. 2005). In immuno-suppressed patients, or those with HIV infection, high grade AIN (AIN 2/3) has a high HPV infectivity rate and is considered a precursor to anal cancer in men (Hoots et al. 2009; Devaraj and Cosman 2006; Watson et al. 2006; Kreuter et al. 2010). There are no data for the natural history of AIN in women. This distinction is important for understanding the value of Gardasil protection as determined by efficacy against an AIN 2/3 endpoint in an immuno-competent population. Most importantly, it is unknown what proportion of AIN 3 lesions progress to invasive squamous cell anal carcinoma and over what time frame (Darragh and Winkler 2011). No data supports the malignant transformation rate

of AIN 3 to be the same as seen in immuno-competent CIN 3 women where 40% develop invasive cancer over 30 years (McCredie et al. 2008).

Gardasil has not been tested in women for prevention against AIN or anal cancer. The FDA awarded Gardasil the labeling use in women for the prevention of anal cancer and AIN precursors based on the evidence presented for AIN prevention in MSM, the histologic morphologic similarities between AIN 3 and CIN 3 (httpc 2010; UCM231522 2010) and because receptive anal intercourse is increasing in frequency with women who report a tenfold greater incidence than men (Brewster and Bhatti 2006). Whether this indication is warranted remains to be seen.

3.2.7 Head and Neck Cancer Efficacies in Women

The incidence of HPV associated oropharyngeal cancers is approximated at 15/100,000 and 3/100,000 unscreened men and women, respectively, in the US (Marur et al 2010). Oropharyngeal cancers vary in their HPV association from 20% to 90%. The HPV associated oropharyngeal cancers are 20–60% attributed to HPV 16 (Marur et al. 2010; Sturgis and Dahlstrom 2009). Oropharyngeal cancer is more common in men than women and has a better prognosis if HPV associated than those related to alcohol and tobacco use (Herrero et al. 2003; Marur et al. 2010; Ramqvist and Dalianis 2010). The prevalence of oral high risk HPV infection in the US mirrors the bimodal distribution of cervical high risk HPV infection with peaks at 25 years and 55 years (Gillison et al. 2012). While there is much excitement in the US about the possible prevention of oropharyngeal cancer with HPV vaccines, there is no evidence that Gardasil will provide any protection for a long enough duration to reduce the incidence of HPV associated oropharyngeal cancers, as the natural history of oropharyngeal cancer has not been sufficiently documented.

3.2.8 Benign HPV-Associated Disease Efficacies

Condyloma

Over a study period of 3.7 years, condyloma caused by HPV 6/11 was prevented by Gardasil 96% of the time (95% CI: 93, 98) in 16–23 year old women who were in the unrestricted susceptible population. They received at least one vaccination, were seronegative, and DNA negative for HPV 6 and 11 at the onset and had cases counted after day 1 (Dillner et al. 2010). In the generally HPV- naïve population (GHN definition above) efficacy against any condyloma was nearly as good at 83% (95% CI: 74, 89) (Dillner et al. 2010).

In women 24–45 years old who were seronegative/DNA negative at baseline, in the per protocol analysis, there was 100% efficacy (95% CI: 31, 100) for the combined endpoint of HPV 16/18/6/11 associated condyloma. The full analysis set shows no Gardasil efficacy for the more specific HPV 6/11 associated condyloma or condyloma caused by any HPV type, contributing to the denial of the FDA licensing of Gardasil for older women (Castellsagué et al. 2011; UCM251763 2010).

Vulvar/Vaginal External Genital Lesions (EGL) Efficacies

Among young women 16–23 years old in the unrestricted susceptible population, Gardasil prevented VIN 1 caused by HPV 6/11 over a period of 3.7 years with an 89% efficacy (95% CI: 53, 99) (Dillner et al. 2010). In the GHN population, efficacy against VIN 1 regardless of HPV type had broad confidence intervals around the 75% efficacy estimate (95% CI: 22, 94), probably due to the lower attribution rate of HPV with all VIN in general (Dillner et al. 2010).

VaIN 1 caused by HPV 6/11 was also prevented 100% of the time over a period of 3.7 years by Gardasil (95% CI: 30, 100) in young women with unexpected wide confidence intervals (Dillner et al. 2010). In the GHN population, efficacy against VaIN 1 regardless of HPV type, was greater than 0% but with wide confidence intervals (95% CI: 10, 71) (Dillner et al. 2010).

Juvenile and Adult Onset Respiratory Papillomatosis

Other HPV associated noncancerous diseases affecting both genders include juvenile and adult onset respiratory papillomatosis (JRP, AORP). The prevalence of JRP is about 1/100,000 in children usually between 0 and 4 years, and is primarily caused by HPV 6 and 11 (Larson and Derkay 2010; Campisi et al. 2010). The prevalence of AORP is about 2/100,000 and is slightly more common in men than in women (Larson and Derkay 2010). It is usually detected between 21 and 30 years of age (Verguts et al. 2009) and is more likely to be related to increased lifetime numbers of sexual partners and oral sex (Kashima et al. 1992).

Gardasil does not have an effect on the incidence of JRP as the disease occurs in those too young to receive Gardasil vaccination; and there is no indication that the maternal antibody titers induced by Gardasil are significantly high for newborn protection. In young women, there may be a decrease in AORP, but because the prevalence of AORP is so low, this decrease may not be clinically significant nor detectable at a population level.

3.3 Immunogenicity

3.3.1 Immunogenicity in Young Women

Duration of efficacy is paramount to the success of Gardasil. Without at least 15 years of efficacy, no cancer will be prevented, they will only be postponed (Barnabas et al. 2006). The meaning of the immune responses is highly debated, but without long-term trials purposely committed to routine human sampling and immunologic testing for at least 15 years, the best surrogates for duration of efficacy are immunologic responses.

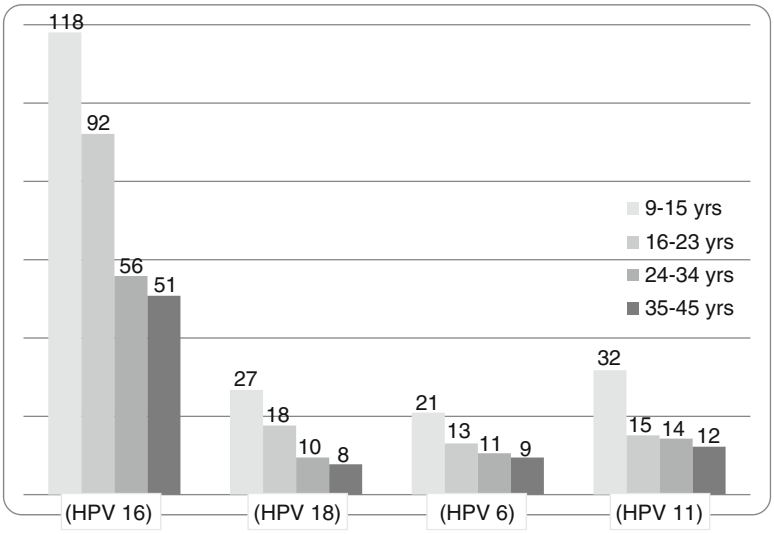
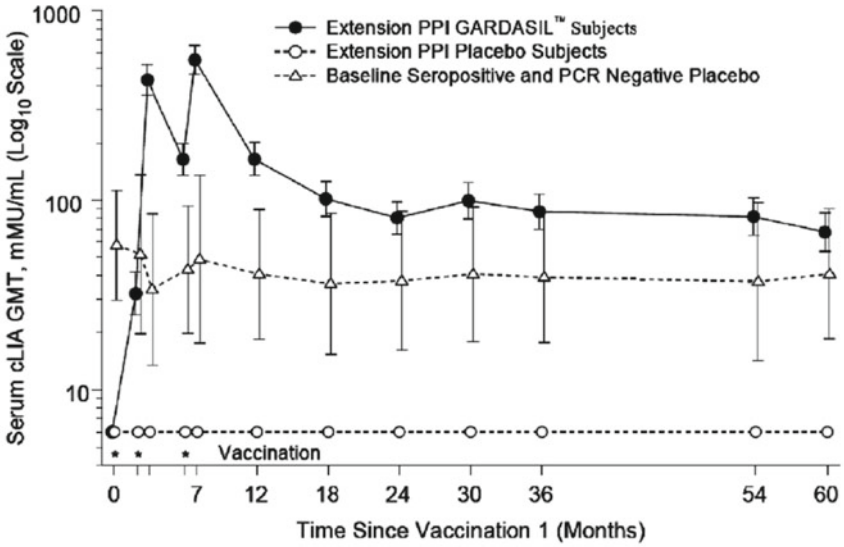


Fig. 9.1 Factor of antibody titer increase over natural infection titers for each age group of females for peak response after vaccination at month 7 (Muñoz et al. 2009; Castellsagué et al. 2011; Villa et al. 2006; UCM251763 2010). Per protocol immunogenicity population that includes all participants who were not general protocol violators, received all three vaccinations within acceptable day ranges, and was seronegative at day 1 and PCR negative from day 1 to month 7 for the relevant HPV type or types, and had a month 7 serum sample collected within an acceptable day range

Antibody responses are classic indicators of protection against non-viremic infections. Neutralizing type specific antibody responses have been measured in Gardasil trials by competitive Luminex immunoassay (cLIA) in milliMerck units/ml version 1 and version 2, yet no correlate of protection has been identified. Antibody titers are expressed as geometric mean titers (GMTs) or geometric mean concentrations (GMCs).

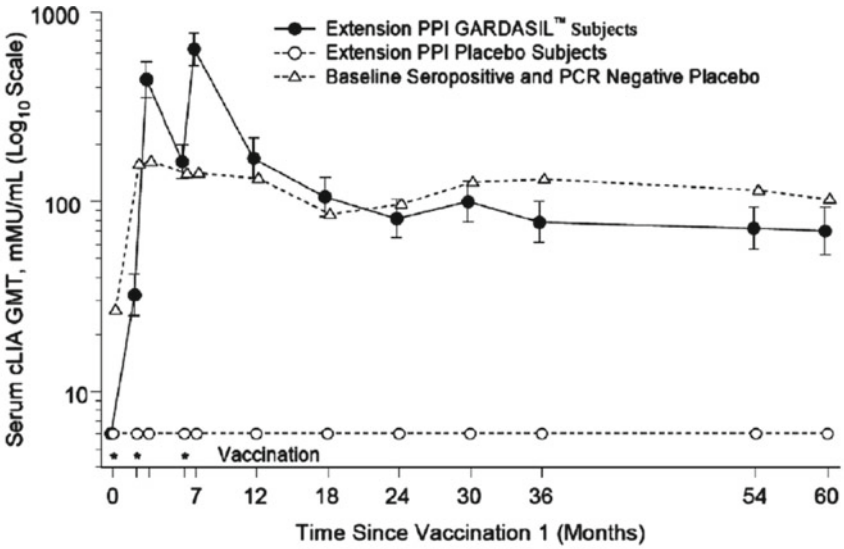
Vaccine induced peak antibody titers in 16–23 year old seronegative women at baseline exceed natural infection titers by multiple folds. The peaks are higher in younger girls 9–15 years and lower in mid-adult women 24–45 years (Fig. 9.1) (Olsson et al. 2007; Rowhani-Rahbar et al. 2009; UCM251763 2010). Within 5 years the antibody titers drop to natural infection titers for three of the four vaccine relevant types (Muñoz et al. 2009; Villa et al. 2006; UCM111274 2008) (Figs. 9.2, 9.3, 9.4, and 9.5). While some naturally induced infection titers are high enough to prevent future infection with the same HPV type (Safaeian et al. 2010) most natural infection titers do not prevent future type specific infections (Olsson et al. 2009b), leaving the woman at risk for new same type infections. Some view this as evidence of the need for booster shots after initial vaccination. Others assume lifetime Gardasil protection until boosters are clinically proven necessary.

Compounding the question of the need for Gardasil boosters is the fact that within 5 years after vaccination 35% of women lost all antibody detection for anti-HPV 18, and 10% of women lost all anti-HPV 6 and anti-HPV 11 detection; after



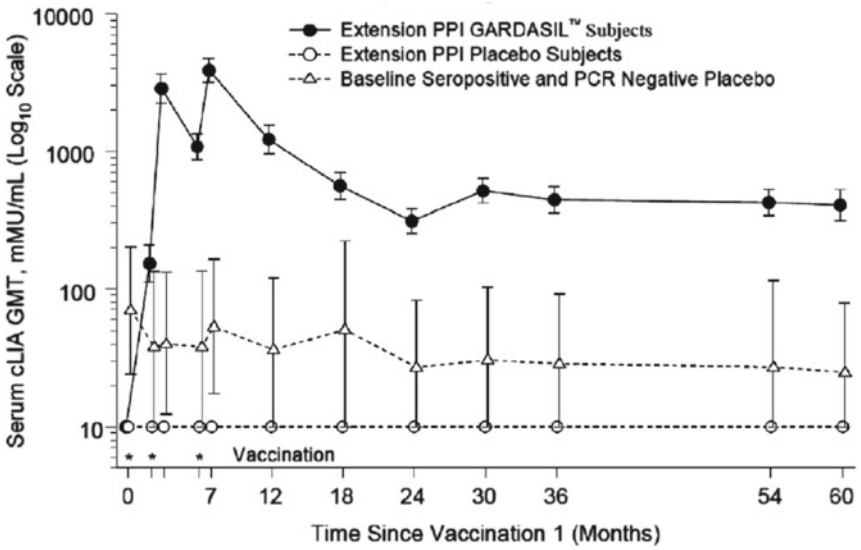
HPV = Human papillomavirus; cLIA = Competitive Luminex immunoassay; GMT = Geometric mean titers; mMU = Milli Merck unit; PPI = Per-protocol immunogenicity; PCR = Polymerase chain reaction.

Fig. 9.2 Longitudinal plot of anti-HPV 6 serum cLIA GMTs (95% confidence intervals) from day 1 through month 60 (UCM111274 2008)



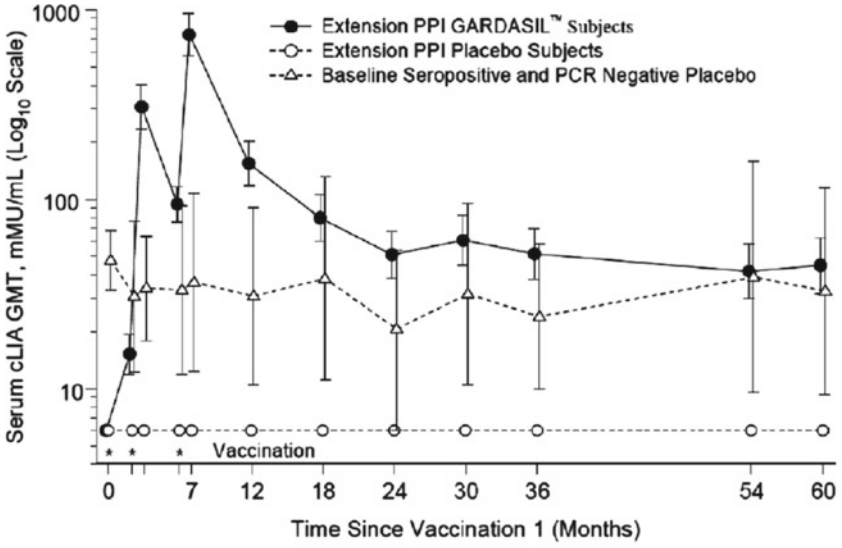
HPV = Human papillomavirus; cLIA = Competitive Luminex immunoassay; GMT = Geometric mean titers; mMU = Milli Merck unit; PPI = Per-protocol immunogenicity; PCR = Polymerase chain reaction.

Fig. 9.3 Longitudinal plot of anti-HPV 11 serum cLIA GMTs (95% confidence intervals) from day 1 through month 60 (UCM111274 2008)



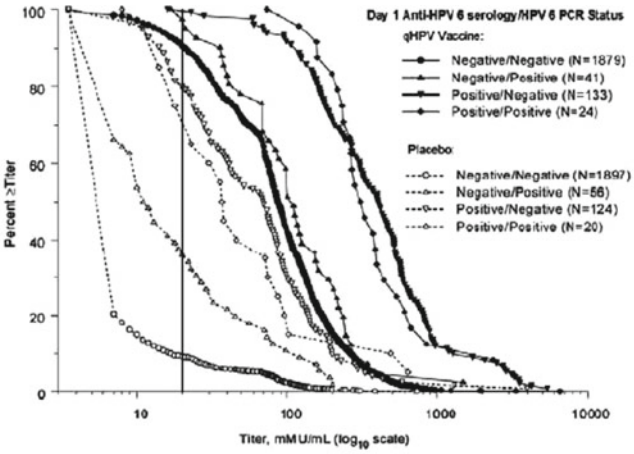
HPV = Human papillomavirus; cLIA = Competitive Luminex immunoassay; GMT = Geometric mean titers; mMU = Milli Merck unit; PPI = Per-protocol immunogenicity; PCR = Polymerase chain reaction.

Fig. 9.4 Longitudinal plot of anti-HPV 16 serum cLIA GMTs (95% confidence intervals) from day 1 through month 60 (UCM111274 2008)



HPV = Human papillomavirus; cLIA = Competitive Luminex immunoassay; GMT = Geometric mean titers; mMU = Milli Merck unit; PPI = Per-protocol immunogenicity; PCR = Polymerase chain reaction.

Fig. 9.5 Longitudinal plot of anti-HPV 18 serum cLIA GMTs (95% confidence intervals) from day 1 through month 60 (UCM111274 2008)



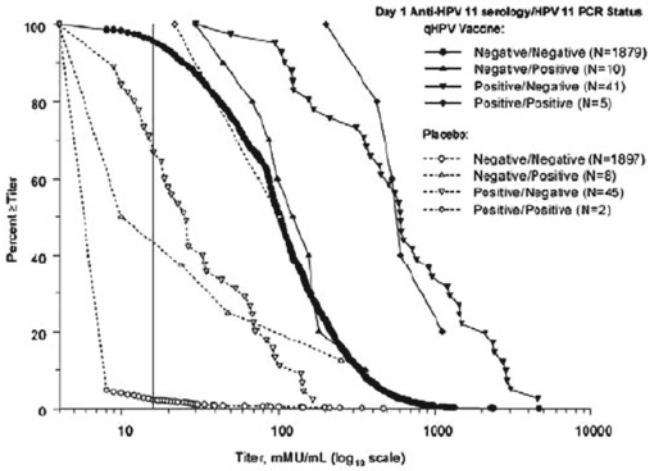
Note: The vertical line corresponds to the anti-HPV 6 cLIA cutoff value for being seropositive to HPV 6.
cLIA = Competitive luminex immunoassay; HPV = Human papillomavirus; mMU = Milli Merck units; PCR = Polymerase chain reaction; End-of-study visits were generally scheduled earlier than Month 48. This timepoint includes all visits occurring within six months of the approximate mean interval of 44 months

Fig. 9.6 Reverse cumulative distribution plot of anti-HPV 6 cLIA titers at end of study (UCM111274 2008)

8.5 years 15% of women no longer have detectable anti-HPV 16 titers (Muñoz et al. 2009; Rowhani-Rahbar et al. 2009; Villa et al. 2006; UCM111274 2008). Some debate the meaning of the partial and complete antibody titer losses to be a laboratory artifact (Opalka et al. 2003), but until long term studies correlate titers with efficacy, it is important to note the waning titers.

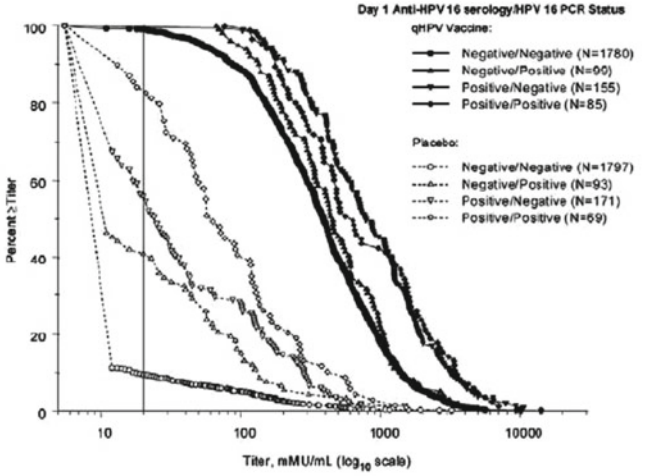
The reverse cumulative distribution plots of anti-16, 18, 6 and 11 titers (Figs. 9.6, 9.7, 9.8, and 9.9) (UCM111274 2008) show the wide range of overlapping antibody titers present among Gardasil recipients and those placebo recipients naturally infected during the 3.7 year study. Specifically for anti-HPV 18 titers, those women in the placebo arm who were seronegative and DNA negative at baseline and who became naturally infected had titers that exceeded the titers induced in 80% of women vaccinated with Gardasil. One would have expected much higher titers in the Gardasil vaccinated group. The attack rate of HPV 18, while similar to natural history, was very low during the course of the 3.7 year study, making the lack of breakthrough CIN 2+ attributed lesions expected. Future longitudinal studies may indicate the immune correlate of protection.

Around 5–10% of young adolescents are already seropositive for HPV 16 prior to sexual exposure (Dunne et al. 2005; Stone et al. 2002; Cubie et al. 1998). The cumulative incidence of cervical HPV infections among virgins prior to penetrative sexual intercourse is reported at 15% (Winer et al. 2003). This is important because the age at which girls are vaccinated should maximize protection for the most sexually active 5 years of her life and not be based solely on age. While Gardasil will be most effective in preventing those who are seronegative and DNA negative from HPV 16/18/6/11 associated CIN 2+ for at least 5 years, the



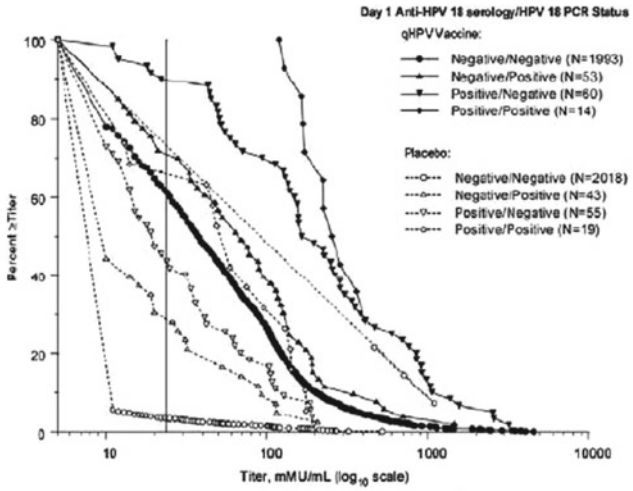
Note: The vertical line corresponds to the anti-HPV 6 cLIA cutoff value for being seropositive to HPV 6.
cLIA = Competitive luminex immunoassay; HPV = Human papillomavirus; mMU = Milli Merck units; PCR = Polymerase chain reaction; End-of-study visits were generally scheduled earlier than Month 48. This timepoint includes all visits occurring within six months of the approximate mean interval of 44 months

Fig. 9.7 Reverse cumulative distribution plot of anti-HPV 11 cLIA titers at end of study (UCM111274 2008)



Note: The vertical line corresponds to the anti-HPV 6 cLIA cutoff value for being seropositive to HPV 6.
cLIA = Competitive luminex immunoassay; HPV = Human papillomavirus; mMU = Milli Merck units; PCR = Polymerase chain reaction; End-of-study visits were generally scheduled earlier than Month 48. This timepoint includes all visits occurring within six months of the approximate mean interval of 44 months

Fig. 9.8 Reverse cumulative distribution plot of anti-HPV 16 cLIA titers at end of study (UCM111274 2008)



Note: The vertical line corresponds to the anti-HPV 6 cLIA cutoff value for being seropositive to HPV 6.
cLIA = Competitive luminex immunoassay; HPV = Human papillomavirus; mMU = Milli Merck units; PCR = Polymerase chain reaction; End-of-study visits were generally scheduled earlier than Month 48. This timepoint includes all visits occurring within six months of the approximate mean interval of 44 months

Fig. 9.9 Reverse cumulative distribution plot of anti-HPV 16 cLIA titers at end of study (UCM111274 2008)

opposition to Gardasil vaccination in the US is the very young age at which it is first recommended. CDC estimates that 3% of girls 13 years of age or younger have had penetrative sexual intercourse and less than 30% of young women 16 years of age or younger are currently sexually active (CDC MMWR 2010a). The highest uptake of Gardasil in the US is in women 16 years of age, the time at which parents and physicians are most comfortable with initiating discussions about the vaccination series for Gardasil (CDC MMWR 2010a).

Gardasil induced antibody titers correlate well with the efficacy seen in women 16–26 years old already exposed to HPV prior to vaccination (Olsson et al. 2009b). While Gardasil demonstrates no CIN 2+ disease efficacy in women who were seropositive for one of the vaccine relevant HPV types at the time of vaccination, Gardasil induces significantly higher antibody titers, which are sustained above titers induced in seronegative/DNA negative females through at least 3.7 years (Fig. 9.10) (Block et al. 2006; Harper 2009a; Villa et al. 2006). The question this poses is, given that Cervarix has both CIN 2+ protection in seropositive/DNA negative women and induces the same antibody titers in women both seronegative and seropositive prior to vaccination, what proportion of the titers induced by Gardasil in seropositive women are actually effective as neutralizing antibodies?

In addition to the higher antibody titers induced in seropositive/DNA negative young women, Gardasil also evokes a memory B cell response among vaccinated seronegative young women when given a booster injection at 5 years (Olsson et al. 2007). This is documented by an increase in the respective vaccine relevant HPV type GMTs within 1 month after the booster injection. Among those women who had lost anti-HPV 6, 11

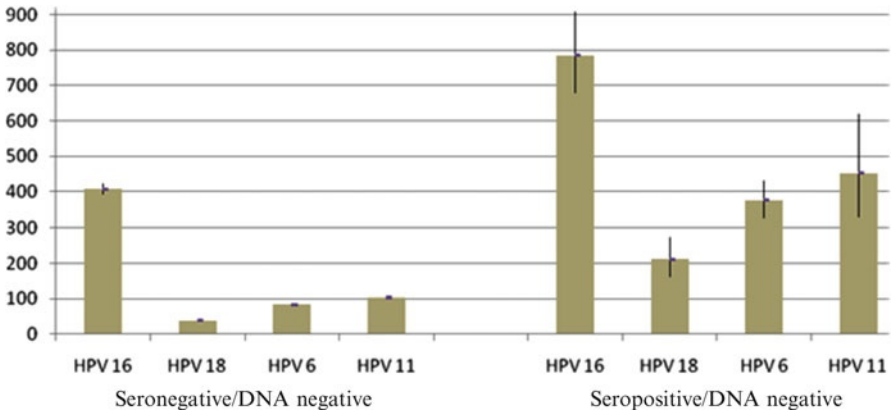


Fig. 9.10 End of study geometric mean concentrations (*GMC*) of antibodies by baseline serostatus among women 16–23 years at entry (Block et al. 2006). End of study was 38–50 months after study enrollment. *Column* represents GMC; vertical lines represent 95% confidence intervals. Seronegative/DNA negative refers to women seronegative to the vaccine relevant HPV type and HPV DNA negative from cervical samples to the same vaccine relevant HPV type at baseline. Seropositive/DNA negative refers to women who had detectable titers to the HPV relevant type but were HPV DNA negative from cervical samples to the same vaccine relevant HPV type at baseline. *Columns* represent the GMC in milliMerck Units/milliliter with the 95% confidence intervals denoted by the *line*

and 18 titers by 5 years, though, only 38%, 71% and 73% of the women, respectively, responded to the booster by mounting antibody titers to levels as high as induced after the original three vaccine doses (Olsson et al. 2007). There is no information on how the continued waning of antibody titers after boostering will affect vaccine efficacy among those women who received Gardasil boosters at 5 years.

3.3.2 Immunogenicity in Mid-Adult Women

Gardasil does not induce as great of an antibody response in women 24–45 years old for any of the four HPV types: 6/11/16/18 as it does for women 16–26 years old. Hence, it is not surprising that these titers waned to undetectable levels in greater proportions and over a shorter time period than in younger women for three of the four HPV types. Anti-HPV 18 titers were undetectable in 52% of 24–45 year old women after only 3.8 years compared to 35% of 16–26 year old women after 5 years (Castellsagué et al. 2011). A similar 10% loss of detectable antibodies to HPV 6 and 11 is documented for 24–45 year old women after 3.8 years as is for 16–26 year olds after 5 years.

The kinetics of antibody decay was similar in older women as to younger women with titers at or below natural infection titer levels for HPV 11 within a year, and HPV 6 and HPV 18 before 24 months (Villa et al. 2006; Castellsagué et al. 2011). The maintenance of HPV 16 titers in the older women, while substantially less than in younger women, remained tenfold higher than natural infection titers through the 3.8 years of the trial (Villa et al. 2006; Castellsagué et al. 2011).

3.3.3 Breakthroughs after Gardasil Vaccination

There were seven 24–45 year old women, seronegative/DNA negative at baseline, who seroconverted after vaccination but developed persistent HPV 16 infections detectable on multiple visits starting at the month 12 study visit, but never developed a CIN lesion. One woman who was seronegative/DNA negative to HPV 16 at baseline developed a HPV 16-associated CIN 2 lesion 12 months after the first vaccination. Two women developed persistent HPV 6 infections detected multiple times starting at 12 months after first vaccination (Castellsagué et al. 2011). An immune correlate of protection against HPV 16 and 6 infections might be calculated from these women's data, but has not been published by Merck scientists.

3.3.4 Evaluation of Gardasil in Compromised Populations

Table 9.3 enumerates the registered clinical trials for Gardasil in compromised populations such as those with systemic lupus erythematosus, inflammatory bowel disease, juvenile idiopathic arthritis, juvenile dermatomyositis, solid organ transplants, HIV/AIDS, and chronic illnesses.

Antibody response has become the surrogate measure of Gardasil efficacy in these trials: the primary outcome measure is the type specific antibody titer response and seroconversion rate at month 7. Only two trials will be collecting genital samples for short-term efficacy against HPV infection outcomes, not disease endpoints. Future studies will need to address whether these antibodies function as effective neutralizing antibodies, whether antibody decay accelerates in immuno-compromised states and whether there is an accelerated need for boosters in these vulnerable populations.

3.4 Safety in Women

At this time, both vaccines are considered safe for most women. However, there is no medicine or vaccine that is completely harmless. International regulatory bodies designate a safety threshold above which they must be concerned for population protection. The threshold of very rare side effects occurs at a frequency of 1 in 10,000 or lower (httpa 2011), but the side effect is not considered significant until it occurs at twice the threshold frequency, hence 2/10,000 (UCM183640 2009). These very rare side effects deserve to be mentioned even while regulatory bodies deem Gardasil as safe. Case reports are the weakest link of causality, yet case reports are the first opportunity to identify a problem that controlled trials are incapable of detecting. Recognition of very unusual rare side effects usually comes to light by multiple identical observations by independent physicians. Observational case studies are suitable for detecting very unusual rare or late adverse effects of vaccines.

Independent scientists have reported additional side effects after Gardasil administration including lipoatrophy (Ojaimi et al. 2009), aluminum granuloma

Table 9.3 Registered clinical trials for Gardasil in compromised populations (httpg 2011)

Trial number	Location	Compromised health condition(s) being studied	Primary outcome measure	Time point at which primary outcome measure occurs	Number to be recruited/enrolled	Length of trial
Active trials recruiting (R) or not recruiting (NR)						
NCT00505063 R	Memorial Sloan Kettering, New York, NY USA	Childhood cancer survivors	Type specific antibody titer response	7 months	75 11–18 year old male and female	12 months
NCT00677677 NR	Edmonton, Alberta, Canada	Solid organ transplant recipients	Type specific antibody titer response	7 months	50 (males and females combined)	36 months
NCT01298869 R	Chulalongkorn, Thailand	Chronic kidney disease	Type specific antibody titer response	7 months	60	7 months
NCT00911521 R	Hong Kong	Systemic lupus erythematosus	Type specific antibody titer response	7 months	100	12 months
NCT01101750 R	Washington DC USA	Kidney and liver transplant	Seroconversion rates	7 months	9–17 years 60 male and female	36 months
NCT00666107 NR	Long Beach, CA USA	HIV infected men	Type specific antibody titer response anal HPV infection	9 months	18 and older 150 males	36 months
NCT00573651 R	Cleveland, OH, USA	Juvenile idiopathic arthritis (JIA)/ seronegative arthritis	Type specific antibody titer response	7 months	72 females	24 months
NCT00806676	Baltimore, MD, USA	Chronic kidney disease	Type specific antibody titer response	7 months/24 months	9–21 years 60 females	36 months

NCT01092195 R	NIH, Washington DC USA	Post stem cell transplantation	Type specific antibody titer response	7 months	18–45 years 72 women	12 months
NCT00786409 NR	Chicago, IL, USA	Systemic lupus erythematosus	Type specific antibody titer response	7 months	9–26 years 30 females	7 months
NCT00604175 R	NIAID, USA	HIV	Type specific antibody titer response	7 months	282 females	72 months
NCT01034358 R	Rochester, MN, USA	Inflammatory bowel disease	Type specific antibody titer response	7 months	195 females	12 months
Completed trials with no results published to date						
NCT00727636	Boston, MA, USA	Inflammatory bowel disease	Type specific antibody titer response	7 months	100 females	7 months
NCT00964210	Australia	1. Inflammatory bowel disease 2. Paediatric rheumatological diseases 3. Acute lymphoblastic leukaemia 4. Solid organ transplant recipients (kidney and liver) 5. Chronic renal disease 6. Bone marrow transplant	Type specific antibody titer response	7 months	240 total among all six diseases	7 months

(Marsee et al. 2008), supraclavicular adenopathy (Studdiford et al. 2008), anaphylaxis (Brotherton et al. 2008), pancreatitis (Das et al. 2008), immune thrombocytopenia purpura (Pugnet et al. 2009), and erythema multiforme (Katoulis et al. 2010), all potentially related to an immunologic reaction caused by Gardasil.

Gardasil has been associated with cases of autoimmune demyelinating diseases, blindness, and paralysis. It is important for women and decision makers about cervical cancer prevention to be aware of the very rare incapacitating adverse events occurring after Gardasil injection. Ampiginous choroiditis, a painless blindness, is reported after Gardasil administration (Khalifa et al. 2010). Opsoclonus myoclonus syndrome (OMS) is a rare neurological disorder of unknown causes that may be the result of an autoimmune process involving the nervous system. OMS has been reported after Gardasil injection (McCarthy and Filiano 2009). Multiple evanescent white dot syndrome is a rare chorioretinopathy causing transitory vision loss; this temporary blindness occurred after Gardasil injection (Cohen 2009). Complete blindness associated with chiasmal neuritis proven by biopsy to be a tumefactive demyelinating lesion occurred after Gardasil injection (DiMario et al. 2010).

A severe demyelinating autoimmune syndrome occurred after Gardasil injection, with a poor clinical outcome (Wildemann et al. 2009). Multiple sclerosis is a demyelinating autoimmune disease whose clinical course can be exacerbated by external factors. A case series of multiple sclerosis exacerbations after Gardasil injection is reported (Sutton et al. 2009) and presented at the 134th American Neurological Association Conference. At this same conference concerns about Gardasil exacerbating autoimmune demyelinating diseases such as amyotrophic lateral sclerosis (ALS) were being independently investigated in Greece, Australia and Germany (httpb 2009).

Guillain-Barré Syndrome (GBS) can result in paralysis and/or death. GBS, a reported serious adverse event after vaccination, occurs at a baseline rate in the US of 0.6–2.6 cases per week per ten million persons (McGrogan et al. 2009). This rate is much lower than the one in 10,000 threshold being used to decide if Gardasil is associated with GBS by regulatory bodies (httpa 2011). While statisticians debate the manipulation of VAERS data, the average weekly rate of GBS, often with paralysis, within the first 2 weeks after Gardasil vaccination was 14.5 cases per week per ten million subjects, much higher than the general population (Souayah et al. 2010; Slade et al. 2011). The Institute of Medicine reviewed the adverse events of Gardasil and concluded that anaphylaxis was related to Gardasil vaccination; and, that there was insufficient data to accept or reject other adverse events' association with Gardasil (IOM 2011). As of April 2010, ten claims filed with the US National Vaccine Injury Compensation Program against Gardasil for damages caused by the vaccine, including GBS, have already been awarded.

Additionally, fetal malformations and death were evaluated over various time intervals between conception and injection. Analysis of pregnancy events within 30 days of injection has evolved over two publications (Koutsky 2007; Garland et al. 2009), indicating initial significant fetal anomalies within 30 days of Gardasil; however, upon the discovery of one additional fetal anomaly in the placebo group, differences were no longer statistically significant. The prevalence

of schizencephaly and anencephaly in the general population is 1.5/100,000 and 1.1/10,000, respectively, with both events happening to fetuses of women who received Gardasil at a significantly higher prevalence rate (Dana et al. 2009). Teratogenic adverse events continue to be monitored via self-report pregnancy surveillance databases to monitor this potential harm in a focused manner (Garland et al. 2009; Dana et al. 2009; McCune-Smith and Sawaya 2009; Descamps et al. 2009; Wacholder et al. 2010). At this time, Gardasil is not recommended during pregnancy.

3.5 Co-administration of Other Vaccines with Gardasil in Women

Significantly lower antibody titers for HPV 16/18/6/11 were induced when Gardasil was co-administered with a meningococcal and Tdap vaccine in 11–18 year old subjects than when administered 1 month after each of the other vaccines (Arguedas et al. 2010). Higher pain, erythema and induration were also seen within 15 days when Gardasil was co-administered with a meningococcal and Tdap vaccines than when injected alone (Arguedas et al. 2010; Reisinger et al. 2010). The three vaccines together nearly doubled the proportion of girls experiencing injection site reactions. No long-term follow up was reported.

3.6 Gardasil Efficacies for Men

Heterosexual men, men who have sex with men (MSM) and HIV infected men experience different HPV associated diseases in different prevalence (Wilkin et al. 2010; UCM190978 2009; UCM184997 2009; UCM190977 2009; UCM183842r 2009). These three populations provided the opportunity to evaluate different disease outcomes, as there are higher prevalent rates for these diseases according to the susceptibility of each population for a particular cancer.

3.6.1 Persistent Infection Efficacies

The benefit of preventing persistent infections in males is much less certain than for females (UCM190977 2009). Unlike cervical cancer, which is nearly 100% attributable to oncogenic HPV and whose epidemiology clearly indicates persistent infection is a risk factor for cancer progression, penile cancers have not been adequately studied to clarify the risk status that persistent penile HPV infection confers towards cancer progression.

While the vaccine studies used uniform protocols to sample men and women on a routine basis for HPV detection, the acquisition and clearance of male infections

is much faster than in women (Giuliano et al. 2008; Trottier et al. 2008; Giuliano et al. 2011b) confounding the meaning of the collected data. Male oncogenic genital HPV infections clear in almost 6 months, the interval used for sample procurement. Because sampling could vary within a 1-month window, an infection about to clear might still be detectable or vice versa. This potential bias can affect both the placebo and vaccinated groups of men. For these several reasons, the FDA clinical reviewers have stated that prevention of 6-month persistent infection in men is not an endpoint of clinical significance (UCM190977 2009).

While the Gardasil studies did show efficacy against HPV 16 and 18 6-month persistent infection taken from a composite source of penile, scrotal/perineal and perianal swabs in heterosexual men (UCM190978 2009; UCM184997 2009; UCM190977 2009), there are no studies that Gardasil can prevent HPV 16/18 transmission from males to females, a most persuasive argument for vaccinating both genders, if it could be proven true.

Likewise, there is no evidence that there is cross protection with other non-vaccine related HPV types for men (Haupt 2010; UCM190977 2009).

3.6.2 Condyloma and External Perineal, Perianal and Penile Neoplasia Efficacy at 2.9 Years

Heterosexual Men

The Gardasil efficacy study surveyed 3,464 heterosexual males who were followed for 2.9 years. They were between 16 and 23 years old at enrollment and had more than one sexual partner, but less than seven lifetime partners. The sexual partners were only female and sex was defined as penetrative vaginal intercourse (UCM190977 2009). While the primary endpoint of this trial was a composite endpoint of HPV 16/18/6/11 attributed external genital warts (EGW), as well as HPV 16/18/6/11 penile/perianal/perineal intraepithelial neoplasias and penile/perianal/perineal cancers (PIN+), the clinical endpoints of interest are condyloma caused by HPV 6 and 11 and PIN+ caused by the oncogenic HPV types 16 and 18.

Unfortunately, the studies showed that only HPV 16/18/6/11 associated condyloma occurred in sufficient frequency over 2.9 years to show its own distinct efficacy (separate from the composite endpoint) at (89% 95% CI: 66, 98). The study enrollment was not large enough to show efficacy against HPV 16/18 associated high grade penile or perianal or perineal intraepithelial neoplasia (UCM190978 2009). There is no evidence that Gardasil can provide any efficacy for perianal, perineal or penile cancer precursors or cancers.

Closer inspection of the data showed that Gardasil only prevented condyloma caused by HPV 6 and 11 (not 16/18) for at least 2.9 years in heterosexual males 16–26 years of age who received three doses of Gardasil and were both negative for DNA from HPV types 6, 11, 16, 18, 31, 33, 35, 39, 41, 51, 52, 56, 58, and 59 and were seronegative to HPV types 6, 11, 16, and 18 at enrollment (the per protocol population) (UCM190978 2009; UCM190977 2009). No data has been

presented on the efficacy against condyloma in heterosexual men regardless of HPV type.

Unlike females, where clinical efficacy is seen for HPV 16/18 associated CIN 2+ and for HPV 6/11 associated condyloma (Olsson et al. 2009b; Koutsky 2007; Garland et al. 2007), clinical efficacy in heterosexual males is only seen for HPV 6/11 associated condyloma and not in HPV 16/18 associated penile, perianal or perineal precancers. This lack of efficacy may be due to lack of study power, or may be related to the men's circumcision status, an independent risk factor for penile in-situ and invasive carcinomas (Backes et al. 2011) that was not disclosed in the studies.

MSM

A subset of the Gardasil efficacy study in men followed 602 MSM for 32 months who were HIV negative, 16–26 years old, and had less than seven lifetime sexual partners. Sex was defined as penetrative anal intercourse (receptive or insertive) or oral sex (Giuliano et al. 2011a; UCM247722 2010). Unlike the vaccine efficacy in heterosexual men against condyloma, the vaccine efficacy in MSM against condyloma caused by HPV 6/11/16/18 was not significant in the per protocol population as defined above (Giuliano et al. 2011a; UCM247722 2010). This may be speculated to be an indicator that frequency of anal exposure to HPV 6 and 11 in MSM significantly overrides the immunologic protection Gardasil induces to HPV 6 and 11 in those with less frequent HPV exposure (UCM190977 2009; UCM247722 2010). No data has been presented on the efficacy against condyloma associated with any HPV type in non-HIV MSM.

No data has been presented for Gardasil efficacy against perianal, perineal and penile lesions caused by HPV 16/18 or caused by HPV 16/18/6/11 in non-HIV infected MSM. While this population of MSM were not immunosuppressed, an independent risk factor for HPV associated penile in-situ and invasive cancers (Chaturvedi et al. 2009), the other independent risk factor, circumcision (Backes et al. 2011), was not disclosed in the study population descriptions, making it possible that this population was at very low risk for HPV associated penile lesions, if circumcised.

3.6.3 Anal Cancer

Heterosexual Men

Heterosexual men did not provide anal samples in any Gardasil study; hence there is no direct evidence to support any protection in heterosexual men against anal intraepithelial lesions or cancers despite similarity arguments of analogous HPV carcinogenicity in all ano-genital tissue. The limited data about anal cancers in heterosexual men emphasize the very stable AIN 3 precursor that might take decades, if ever, before malignant transformation (Fox 2006).

MSM

In the MSM Gardasil sub-study, no efficacy was documented for HPV 16/18/6/11 associated anal cancer prevention, as there were no cases of anal cancer detected (UCM247722 2010; UCM239865 2010).

Only in the per-protocol population was there efficacy for AIN 2+ caused by HPV 16/18/6/11 (75%, 95% CI: 9, 95) with all of the efficacy coming from HPV 6/11 associated AIN 2+ disease, not the oncogenic HPV 16/18 related AIN 2+ disease. No efficacy against the firm cancer precursor of AIN 3 caused by HPV 16/18 or 16/18/6/11 was documented in any analysis population. Gardasil recipients developed about half the number of cases of AIN 3 as seen in the placebo group (UCM247722 2010). The lack of efficacy against AIN 3 could have been due to the short time of follow up and the small number of enrolled subjects, or could be the truth.

There was no efficacy seen against AIN 2+, regardless of HPV-type in any analysis population of MSM (UCM247710 2010). Likewise, there is no cross protection against AIN 2+ caused by any other HPV anal-type evident in any of the MSM analysis populations (UCM247722 2010).

Efficacy against AIN 1 caused by HPV 6/11/16/18 in the per-protocol MSM population is 73% (95% CI: 16, 93) and remains significant in all population analyses. While this disease state is important to prevent in the MSM or immuno-compromised at risk person, the natural regression rates of AIN 1 in the general population are very high, especially in immuno-competent men and women (Shepherd 2007).

Recent evidence of a 5% increase in the rate of HPV associated AIN 2/3 and anal cancer in Denmark over 30 years shows an absolute increase in incidence of AIN 2/3 to 0.58/100,000 in women and to 0.43/100,000 in men, and an increase in incidence of anal cancer to 1.48/100,000 in women and 0.8/100,000 among men (Nielsen et al. 2011). This increase was felt to be due to the increase in receptive anal intercourse, more prevalent in females than males, rather than immunosuppression or an increase in MSM population. Despite increasing rates, the low absolute rates of anal cancer do not support universal Gardasil vaccination in men in the US.

3.7 Immunogenicity in Males

While the meaning of waning antibody titers after Gardasil vaccination remains undefined, peak antibody response after vaccination has become the surrogate for efficacy for Gardasil trials. Disease endpoints differ depending on the male population studied. Condylomata are prevalent in boys and heterosexual young men 9–15 and 16–26 years, and hence these defined male populations were appropriate to study for efficacy and immunogenicity after Gardasil vaccination. Anal cancer precursors are prevalent in those who practice anal receptive intercourse and are immuno-compromised; hence the HIV-negative MSM population and the HIV

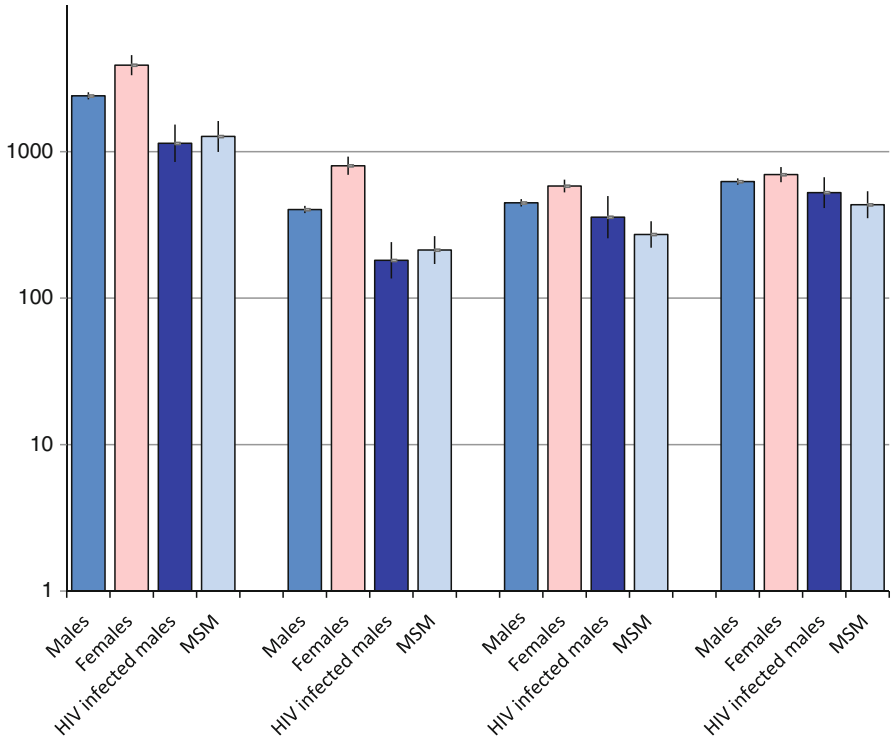


Fig. 9.11 Month 7 peak antibody titers after three doses of Gardasil in men and women seronegative/DNA negative for study relevant HPV types at enrolment (Wilkin et al. 2010; UCM190977 2009; Haupt 2010; Villa et al. 2005). Males and MSM were 16–23 years old, HIV infected males were 22–61 years old, females were 15–25 years, HIV infected men had CD4 counts >200 if on HART, or >350 if not on HART

positive population were appropriate to study. The MSM 16–26 years old population provided data on efficacy and immunogenicity after Gardasil vaccination, and the HIV positive male population of 22–61 years old provided only immunogenicity profiles.

In the 9–15 year old and 16–26 year old heterosexual males and the MSM population the GMTs measured 1 month after the third dose of Gardasil (month 7) produced peak antibody titers that were several fold higher than natural infection titers for HPV 16 and 18, as well as HPV 6 and 11, (UCM190978 2009; UCM184997 2009; Haupt 2010). Peak anti-HPV GMTs for all four HPV types were significantly lower in heterosexual males than in females of the same age (Fig. 9.11) (UCM184997 2009; UCM190977 2009), while MSM induced lower GMTs for all four vaccines related HPV types (Haupt 2010).

HIV infected men aged 22–61 years, seronegative to HPV 16/18/6/11 and DNA negative for HPV 16/18/6/11 from an anal canal sampling at study baseline mounted similar titers for HPV 6 and 11, but significantly lower antibody titer for

HPV 16/18 even after accounting for age, as seen in the younger heterosexual cohort (Wilkin et al. 2010). For those with preexisting anti-HPV antibodies, Gardasil induced a marked increase in antibody concentrations over that induced in the seronegative men approaching titers seen in the heterosexual male cohort similar to the response seen in seropositive women after Gardasil injection. Whether low CD4 counts will inhibit seroconversion initially or facilitate waning and loss of detectable antibodies after vaccination, or whether more frequent boosters will be needed, as have been described for other vaccines in this population (Sutcliffe 2011), remains to be seen.

The pattern of decline in titers after peak induction mimics the fall seen in data for females. Of those men with measurable antibody titers for HPV 18 at 24 months, the GMT was 39 mMU/ml, the same anti-HPV 18 titer reached for females 16–26 years at 44 months (Harper 2009a); this anti-HPV 18 titer in males at 24 months is equivalent to male natural infection titers (UCM190978 2009). Natural infection induced titers in women may not be protective against new infections of the same HPV type (Olsson et al. 2009b); we do not know if natural infection titers induced in men will provide a similar protection.

Seroconversion at month 7 for HPV 16/18/6/11 was over 99% for 9–15 year old boys and between 96% and 99% for 16–26 year old immuno-competent males (UCM190978 2009). Males have a more rapid loss of detectable antibody titers than females for unexplained reasons. 38% of the male population lost detectable antibodies for HPV 18 by 2 years, while it took 5 years for a comparable loss in females (Fig. 9.12). Likewise, men lost antibodies against HPV 6 and 11 at least as fast as or faster than women (Harper 2011). At 24 months, 9%, and 4% had lost antibodies to HPV 6 and 11, respectively. Whether the accelerated antibody loss continues and has meaning for efficacy remains to be seen.

3.8 Safety in Males

Safety both in terms of local site reactions and systemic adverse events was similar to the trial reports of safety in females. Injection site pain was most frequent. Nine out of 3,100 Gardasil recipients experienced one or more severe adverse events compared to one out of 2,300 placebo recipients (UCM184997 2009). In general, Gardasil appears safe for most males.

Similarly to the 44,000 females under surveillance, health maintenance organization databases will be used to follow 27,000 men who received at least one dose of Gardasil. The endpoints to be evaluated are emergency room visits or hospitalizations (UCM183842 2009). The vaccine adverse events reporting system (VAERS) has five serious adverse events reported among males receiving Gardasil with one report of verified Guillain Barre Syndrome (Brisson et al. 2009; httpf 2011; Gee et al. 2010). Until post marketing surveillance has data to report, no data to refute or support safety from rare events exists in males.

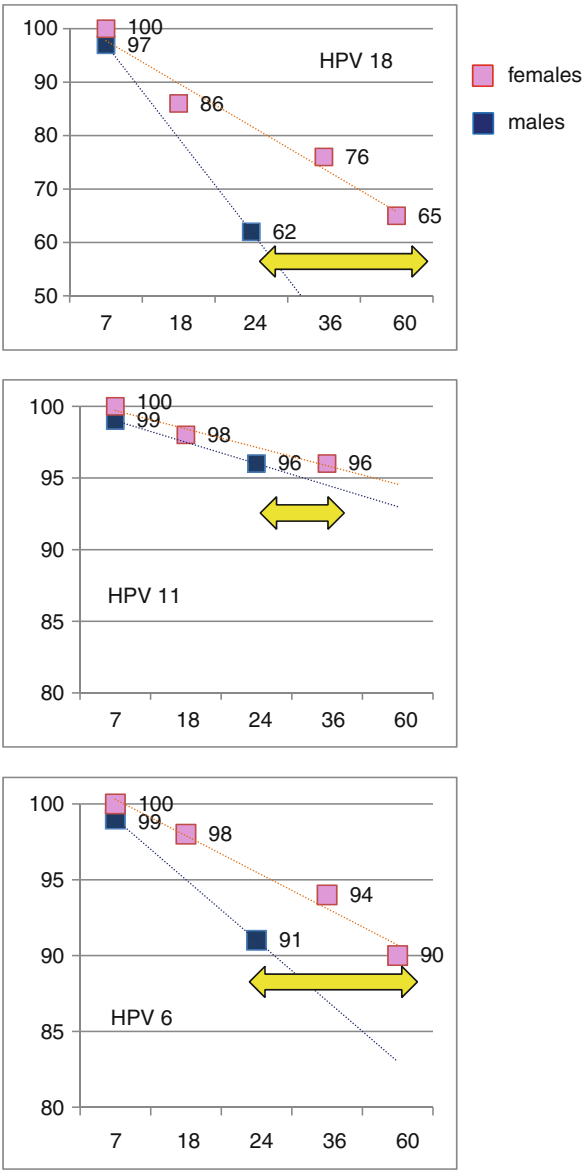


Fig. 9.12 Loss of detectable antibody titers quicker in males than females (Olsson et al. 2007; Villa et al. 2006; Villa et al. 2005; UCM190977 2009)Months after first of three Gardasil injections. Males lose detectable antibodies 2–3 years before a similar proportion of females lose detectable antibodies after Gardasil vaccination

3.9 Co-administration of Gardasil with Other Vaccines in Males

Similar reactions were seen for males as in females when Gardasil was administered concomitantly with Repevax (diphtheria, tetanus, pertussis (acellular), and poliomyelitis (inactivated) vaccine (Sanofi Pasteur Inc, Lyon, France)) at the first vaccination (Vesikari et al. 2010). As in the trials in females, the 15 day follow up after the concomitant administration of Repevax and Gardasil showed significantly more injection site and systemic reports of adverse events than if the two vaccines had been given independently. No follow up data beyond 15 days were reported.

3.10 Policy Implications for Vaccinating Males

Perspective of benefit determines whether one views the cost effectiveness of vaccinating males to be favorable or not. If the cost effectiveness analyses are modeled for maximal cervical cancer prevention, then Gardasil offers merely marginal incremental cost effectiveness ratios (ICER) when given to males, and is not viewed favorably by policy makers of countries of any gross domestic product (GDP) (Brisson et al. 2009; Zechmeister et al. 2009; Kim and Goldie 2009; Kim et al. 2007; Marra et al. 2009; Hibbitts 2009; Jit et al. 2008; Cuschieri 2009). Cost models show that there may be a temporary cost effectiveness point at which vaccinating 12 year old boys is cost effective for a short time when the vaccination coverage of females is low (Brisson et al. 2010; Kim 2010).

No cost effectiveness studies have used penile, anal, or oropharyngeal cancer prevention outcomes to determine the ICER for extensive programmatic male Gardasil vaccination. It is possible that the low incidence rates of these cancers would preclude a favorable ICER. Likewise, no cost effectiveness studies have used external genital wart prevention as a sole outcome to determine the ICER for extensive male vaccination programs.

While the highest gain of male Gardasil vaccination is in the prevention of anal cancer in the unscreened MSM population, if protection wanes at or before 20 years, Gardasil vaccination of MSM at any age is not cost-effective for the MSM population in the US (Kim 2010). This does not mean that individual vaccination will not offer individual protection for the limited time of Gardasil efficacy; it only means that a publicly subsidized vaccination program of MSM is not cost effective given the potential need for future boosters.

A summary of the proven efficacies of Gardasil in men and women at different anatomic sites in shown in Table 9.4.

4 Conclusion

The 2006 commercial arrival of Gardasil in the US coincided with the active change of CDC recommendations, in concert with the American Academy of Pediatrics, to create an ‘adolescent platform’ of vaccinations that was targeted to the 11–12 year old

age range (httpd 2007). This platform consisted of the tetanus, diphtheria, acellular pertussis (Tdap) booster, the primary meningitis vaccine (Menactra™) and the primary HPV vaccine. This change in vaccine recommendations was based, in large part, on the paltry 38% visit rate young adolescents made to their pediatricians' offices (Committee on Adolescence 2008). Recognizing that anticipatory guidance, screening and counseling to reduce the health risks of alcohol and substance abuse, drunk driving, sexual activity, depression, suicide, smoking, violence and guns are the centerpiece of adolescent preventive care, the vaccination platform was created to encourage adolescent patient participation above the current level (Committee on Adolescence 2008; httpd 2007). Hence, there has been great pressure from industry and from the CDC, on behalf of pediatricians in the US, to promote HPV vaccination.

In the US market, Gardasil dominates HPV vaccination. There is already an aggressive marketing campaign underway in the US to vaccinate both young girls and boys, facilitated by the funding of Gardasil in the Vaccine For Children program for all 18 years of age and younger.

Surveys of decision makers for Gardasil show most are dissatisfied with their decision to be vaccinated or to have their child vaccinated. The main reason given is the overly optimistic benefits physicians provided without appropriate discussion of alternatives for cervical cancer prevention. Public mistrust and abstinence from HPV vaccination continues (Cooper et al. 2008; Downs and Larson 2007; Larson et al. 2010).

Decreasing condyloma rates, the first expected population benefit from Gardasil vaccination, is not yet documented in the US for young women as it has been in Australia (Donovan et al. 2011). The duration of decreased condyloma rates has not been shown for more than 5 years in any population.

Given that there will be a 10 year gap between the 11 year old receiving the vaccine and the 21 year old starting her Pap screening program, the duration of Gardasil's efficacy will determine whether there is a population decrease in the incidence of absolute numbers of abnormal Pap tests, colposcopies and excisional treatments for CIN 2+ as promised from the trial data (Paavonen et al. 2010; Olsson and Paavonen 2009a).

The next generation nonovalent Gardasil is in clinical testing in the US and worldwide as a primary and booster vaccine containing two condyloma HPV types 6 and 11; and seven oncogenic types: 16, 18, 31, 33, 45, 52, and 58. The pentavalent vaccine with five non-16/18 oncogenic types is being tested in those who have already received Gardasil (http://clinicaltrials.gov/ 2011). Whether adjuvant design considerations for increasing the duration of Gardasil efficacy have been included in the second-generation vaccine plan is unknown.

Cervical cancer is the only HPV-associated cancer for which there is a screening program in the US. The ongoing participation of repeated screens over a woman's lifetime is more important than Gardasil vaccination in controlling the incidence of cervical cancer in the US. All other HPV associated cancers have no formal screening program for early detection and treatment and hence could potentially be reduced on an individual basis if Gardasil can be shown to maintain efficacy for more than 15 years. The population benefit of Gardasil will be to reduce the incidence of abnormal cytology screens, colposcopies and excisional procedures in the US not the reduction of HPV associated cancers.

Table 9.4 Summary of HPV-associated diseases and documented Gardasil efficacy for ≤5 years

	Young women	Mid-adult women	Heterosexual men	MSM
HPV 6/11/16/18 persistent cervical infection	Yes (UCM111274 2009)	Yes (UCM251763 2010)	–	–
Abnormal cytology	Yes (UCM251763 2010)	No (UCM251763 2010)	–	–
Colposcopy	Yes (Olsson and Paavonen 2009a)	No (Castellsagué et al. 2011)	–	–
HPV 16/18 CIN 2+	Yes (UCM111274 2009)	No (Castellsagué et al. 2011)	–	–
HPV 16/18 adenocarcinoma in situ	Yes (UCM111274 2009)	No (Castellsagué et al. 2011)	–	–
Cross protection to non-vaccine types	No (Brown et al. 2009)	No (UCM251763 2010)	No (UCM190977 2009)	No (UCM247722 2010)
Cervical excisional procedures	Yes (Olsson and Paavonen 2009a)	No (Castellsagué et al. 2011)	–	–
HPV 16/18/6/11 external genital lesions	Only 6/11 (Dillner et al. 2010)	Yes (UCM251763 2010)	–	–
HPV 16/18 VaIN 2/3	Only HPV 16 (Joura et al. 2007)	–	–	–
HPV 16/18 VIN 2/3	Only HPV 16 (Joura et al. 2007)	–	–	–
Cervical cancer	–	–	–	–
Vulvar cancer	One non-HPV associated case detected in Gardasil arm	–	–	–

Vaginal cancer	–	–	–	–
HPV 6/11 Condyloma	Yes (UCM111274 2009)	Only 16/18/6/11 (UCM251763 2010)	Yes (UCM190978 2009)	Yes (UCM231522 2010)
Any HPV type Condyloma	Yes (Dillner et al. 2010)	No (UCM251763 2010)		
HPV 16/18/6/11 perineal, perianal, penile infection	–	–	Yes (Giuliano 2011a)	No (Giuliano 2011a)
HPV 6/11 persistent anal infection	–	–	–	Only HPV 6 (UCM231522 2010)
HPV 16/18 persistent anal infection	–	–	–	Yes (UCM231522 2010)
HPV 6/11/16/18 perineal, perianal, penile intraepithelial neoplasias	–	–	No (UCM190977 2009)	No (UCM190977 2009)
HPV 6/11 AIN 2+	–	–	–	Yes (UCM247722 2010)
HPV 16/18 AIN 2+	–	–	–	No (UCM247722 2010)
JRP	–	–	–	–
AORP	–	–	–	–
Oropharyngeal cancer	–	–	–	–
Penile cancer	–	–	–	–

Shaded areas indicate that no trials were conducted with this endpoint or no endpoints were collected within the trial

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Chapter 10
Immunogenicity, Efficacy, Effectiveness and Overall Impact of HPV Vaccines

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1 Natural History of Genital Human Papillomavirus Infection with Special Reference to Immunity

Practically all genital infections with human papillomavirus (HPV) stem from sexual transmission following sexual debut, after which half of the young women are infected within 3 years (Collins et al. 2005; Winer et al. 2003). HPV transmission does not necessarily require mucosal contact but can also happen through contact of the skin and the genital mucosa (Moscicki et al. 2006). This is why both condom use and circumcision are protective against HPV transmission only partially (Winer et al. 2006; Wawer et al. 2011). It is noteworthy that HPV prevalence increases almost linearly with the increase of sexual partner numbers (Dillner et al. 1996; Koutsky 1997). Eventually, 70–80% of the sexually active population will acquire HPV infection. One third of young adult females and males are positive for HPV DNA, meaning they are able to transfer the virus (Auvinen et al. 2005; Kjaer et al. 2005; Giuliano et al. 2007).

HPV causes superficial infections in the genital area (Doorbar 2005, 2006). Following micro-trauma, epithelial basal cells get exposed to the infectious virus, which then expresses its E1 and E2 genes that direct replication. Depending on HPV type, viral replication goes hand in hand with differentiation of the host cells. Strict control on the expression of oncogenic viral E6 and E7 genes is maintained, and the cells

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undergoing productive virus cycle are not permissive to malignant transformation. Finally, phenotypically (due to the viral E4 protein) altered koilocytes are exfoliated from the top of the epithelium, and intact virions are released. The new virions are able to form secondary foci on the host mucosa/skin and to infect new sexual partners a minimum of 2 weeks post primary infection (Stanley 2010).

The approximately 40 different HPV types causing genital infections are divided into low-risk (lr) and high-risk (hr) types according to their oncogenic potential. The most important lrHPV types are HPV6 and 11, which belong to the clade A10. Out of the hrHPV types HPV16, 31, 33, 35, 52, and 58 belong to clade A9, HPV18, 39, 45, 59, 68 belong to clade A7, HPV56 belongs to clade A6, and HPV51 and 69 belong to clade A5. Altogether, these hrHPV types comprise more than 95% of HPV types found in cervical cancer (Schiffmann et al. 2005, 2009). For infections caused by lrHPV types, regression takes about 4 months; for hrHPV types the corresponding time is 14–18 months (Stanley 2010). The fact that columnar epithelial cells are usually infected and possibly preferred over squamous epithelial cells by both HPV types 18 and 45 (Namujju et al. 2010) is noteworthy.

In the epithelium, viral antigens, e.g., the regulatory E2 and E6 proteins and structural viral proteins L1 and L2, are recognized by antigen-presenting Langerhans cells and by antibody producing B-cells. Interaction of the Langerhans cells and T-cells results in restriction of the infection at the primary foci by effector (CD8+) T-cells, and enhancement of the HPV-specific antibody response by helper (CD4+) T-cells. Following primary infection the cell mediated response becomes detectable within weeks and the antibody response within months (Stanley 2010) but some individuals not produce antibodies following HPV infection (Kjellberg et al. 1999). Eighty to 90% of infections caused by both lr and hrHPV types heal spontaneously in non-immunocompromized individuals (Stanley 2010). However, natural immune response to HPV infection does not confer permanent immunity, and re-infections, as well as infections with other HPV types occur (Palmroth et al. 2010; Stanley 2010; Trottier et al. 2010).

HrHPV types are able to cause persistent infections in epithelial basal cells and/or dividing epithelial cells. This is partially due to integration of HPV DNA into host cell DNA, but persistence of episomal HPV DNA also occurs. Over time the likelihood that the control of viral E6 and E7 oncogenes deteriorates, partially due to the DNA integration increases, and the resulting overexpression of E6 and E7 proteins release the host cell from growth control by inactivating cellular Rb and p55 proteins, also apoptosis of the transformed cells does not occur. Another function of the E6 and E7 proteins is to weaken immune surveillance of the infected cells by inhibition of interferon production (Pett et al. 2006; Konodia et al. 2007), and down-regulation of cellular genes necessary for the recognition of the infected cells by T-cells (Hasan et al. 2007).

Most important modifiers of the natural history of genital HPV infection are smoking and *Chlamydia trachomatis*. Infection with *C. trachomatis* is common, 25% of the population acquire it. It prolongs duration of HPV-infection (Ylä-Outinen et al. 1990; Silins et al. 2005). According to recent cohort studies, *C. trachomatis* infection obtained either before or after HPV infection increases the risk for development of cervical intraepithelial neoplasia grade 2 (CIN2), or worse (Lehtinen et al. 2011a).

In longitudinal studies, the increased risk of cervical neoplasia, most notably cervical cancer, has been associated with early *C. trachomatis* infection (Koskela et al. 2000; Lehtinen et al. 2009) and/or specific *C.trachomatis* subtypes (Anttila et al. 2001). On the other hand, early smoking increases the likelihood of acquiring genital HPV infection (Syrjänen et al. 2007), and weakens HPV-specific antibody responses (Kapeu et al. 2008). The probability of an HPV infection progressing into CIN may not be increased by smoking (Syrjänen et al. 2007), but smoking impairs the healing of cervical HPV lesions (Szarewski et al. 1996). Most importantly, in cervical carcinogenesis, where HPV is the only necessary factor, both smoking and *C. trachomatis* are proven co-factors independently increasing the neoplasia risk (Wallin et al. 2002; Lehtinen et al. 2011; Plummer et al. 2003; Kapeu et al. 2009).

Infections with multiple HPV-types are common in the young. Twenty-five to 50% of individuals who have acquired genital HPV infection will acquire (at least) another HPV infection with a different HPV type in a few years (Trottier et al. 2006). HrHPV infections cluster in the same individuals, as it seems, irrespectively of the immune response. The risk of acquiring another hrHPV infection for a hrHPV seropositive woman is 3–6 times higher than for a hrHPV seronegative woman (Kaasila et al. 2009; Lehtinen et al. 2006; Merikukka et al. 2011). Compared to single hrHPV infections, infections with multiple hrHPV types are associated with considerably higher risk of developing HSIL, possibly due to the longer duration (persistence) of multiple HPV infections (Trottier et al. 2006, 2008).

2 Immunogenicity of the Prophylactic HPV Vaccines

The two licensed prophylactic HPV-vaccines (Gardasil™ and Cervarix™) are comprised of virus-like particles (VLP) that are produced by expressing the major structural L1 gene of HPV types 6/11/16/18 or HPV16/18 in yeast or insect cells, respectively. The L1 proteins assemble into VLPs, which as such are excellent immunogenes, and have been shown to induce antibody response following a three shot regimen (0, 1/2, 6 months) in virtually all vaccinated female and male participants of sizeable phase II/III studies (Future II study Group 2007; Paavonen et al. 2007; Petaja et al. 2009; Hillman et al. 2012).

Adjuvants used in the two vaccines: aluminiumhydroxy-diphoshosulphate (Gardasil™) and aluminium hydroxside supplemented with monophosphoryl lipid AS04 (Cervarix™) enhance the immune reaction. Due to the aluminium derivatives, antigen-presenting cells able to stimulate T-helper cells migrate to the injection site (Calabro et al. 2011). Th2-type cytokine response of the T-helper cells (Serre et al. 2010) promotes antibody production, which without the adjuvant(s) might remain sub optimal (Dauner et al. 2010). The monophosphoryl lipid AS04 in the HPV16/18 (Cervarix™) vaccine further amplifies the vaccine induced antibody production and promotes generation of memory B-cells (Giannini et al. 2006).

Although the antibody response induced by the HPV VLP vaccines is very good in all ages, it is somewhat age-dependent. Before puberty, or in early adolescents (<15 years of age), the prophylactic HPV vaccines induce remarkably higher antibody

responses than after the age of 15 (Pedersen et al. 2007; Perez et al. 2008). Gender, smoking, or a combination of the HPV vaccine with another VLP vaccine or other vaccines does not seem to have a significant effect on the induced HPV antibody responses (Wheeler et al. 2008; Kapeu 2009; Petaja et al. 2009; Vesikari et al. 2010; Giuliano et al. 2011), but there is too little data on the effect of (various stages of) HIV infection.

Immediately after the third dose, HPV VLP vaccine-induced antibody levels are ten to a hundred times higher than HPV antibody levels detected in HPV infected women (Villa et al. 2005; Harper et al. 2006). Following vaccination of young women, antibody levels induced by the HPV16/18 vaccine (Cervarix™) remain at a high level for at least 8 years (Harper et al. 2006, GlaxoSmithKline Vaccine HPV-007 Study Group 2009). During the same follow-up time, the HPV6/11/16/18 vaccine (Gardasil™) induced antibody levels, especially HPV18 antibody levels, decreased to the level of natural infection-derived HPV18 antibodies (Olsson et al. 2007). It is important to note that no relationship has been observed between the protection from HPV infections and associated lesions conferred by the HPV6/11/16/18 (Gardasil™) vaccine and (decrease of) the antibody levels (Joura et al. 2008). One booster dose of this vaccine, 5 years after initial immunization, readily provoked a very efficient secondary response after which the HPV6/11/16/18 antibody levels were even higher than after the initial three dose immunization (Olsson et al. 2007).

Mucosal immunity induced by both the HPV VLP vaccines in the females is due to leakage of humoral antibodies to the mucosal surfaces, probably following, e.g., a coital microtrauma. There may be higher levels of serum neutralizing antibodies following vaccination with the HPV16/18 (Cervarix™) vaccine, but the more generally used ELISA method found no significant difference in the mucosal antibody levels following vaccination with the HPV16/18 (Cervarix™) or the HPV6/11/16/18 (Gardasil™) vaccines (Einstein et al. 2010). Mucosal antibodies are detectable 4 years following the HPV16/18 vaccination in two thirds of the vaccine recipients, especially in individuals with initially high bivalent vaccine induced humoral HPV antibody levels (Petaja et al. 2011).

3 Efficacy of the Prophylactic HPV Vaccines Against Genital Infections in Women

Genital HPV infection is usually symptomless and hence defined by laboratory measurements. Because HPV infections are common and most heal spontaneously, it is difficult to say on the basis of a single, highly sensitive (e.g. PCR) laboratory measurement whether the identified (sign of) HPV is an indicator of a true infection, or due to contamination. Quantifying the amount of the detected HPV DNA is possible and an association between increased risk of developing cervical carcinoma and increased viral load has been described (Ylitalo et al. 2000). Hence HPV DNA screening tests make use of selected/optimal threshold levels. In HPV vaccine

efficacy studies threshold levels have, however, not been applied, and one infection end-point in all studies has been persistent (6 months) HPV vaccine type DNA positivity against which both vaccines have shown 90–100% efficacies among the baseline negatives, and 50–60% efficacy in the intention-to-treat analyses (Koutsky et al. 2002, Castellsague et al. 2011; Lehtinen et al. 2012a).

Overall, one has to be careful, when comparing results reported for the two vaccines against different HPV infection end-points: vaccine efficacies (VE) against persistent 6 month HPV DNA positivity for the five most common hrHPV types (31, 33, 45, 52, 58) and against CIN grades 1+, 2+ and 3 are summarized below (Tables 10.1 and 10.2). Differences in PCR methodologies/stratification (negative for HPV6/11/16/18 vs. negative for all hrHPVs) defining baseline negatives and pathology panels defining end-points warrants caution when may exist differences (Paavonen et al. 2009).

Vaccine efficacy against persistent (6 month) PCR positivity has been reported against the five most common non-vaccine HPV types 31/33/45/52/58 (Brown et al. 2009; Wheeler et al. 2009, Lehtinen et al. 2012b), again with materially similar results (Table 10.1). Vaccine efficacies against 6-month persistence with one of the five above-mentioned non-vaccine hrHPV types have been: 25% (FUTURE II; Brown et al. 2009) and 30% (PATRICIA; Paavonen et al. 2009) among baseline HPV negative participants, and 18% (FUTURE II; Wheeler et al. 2009) and 15% (PATRICIA; Wheeler et al. 2012) among all participants ("intention to treat" cohorts).

Results from the large phase III trials on the efficacy of the HPV6/11/16/18 and the HPV16/18 vaccines (FUTURE I&II and PATRICIA) against CIN1+ lesions, irrespectively of HPV type, have been 30% (FUTURE II; Munoz et al. 2010) and 50% (PATRICIA; Lehtinen et al. 2012a) among baseline HPV negative participants, and 19% (FUTURE II; Munoz et al. 2010) and 18% (PATRICIA; Lehtinen et al. 2012a) among all participants ("intention to treat" cohorts) (Table 10.2). Corresponding vaccine efficacies against CIN1 and CIN1+ associated with HPV-types 16/18 were materially similar: 97% (FUTURE II; Munoz et al. 2010) and 97% (PATRICIA; Lehtinen et al. 2012a) among baseline HPV negative participants, and 68% (FUTURE II; Munoz et al. 2010) and 63% (PATRICIA; Lehtinen et al. 2012a) among all participants ("intention-to-treat" cohorts), even if the end-points were CIN1 for the quadrivalent vaccine and CIN1+ for the bivalent vaccine.

Cytological end-points have not been reported for both vaccines consistently, and efficacy against external genital lesions (EGL) or condyloma accuminatum has been reported for the HPV6/11/16/18 vaccine. Vaccine efficacy estimates against low grade EGL associated with all HPV types have been higher than the above CIN1 estimates both among baseline HPV negative participants and all participants: 83% and 62% (FUTURE I; Dillner et al. 2010). Corresponding results for HPV-type 6/11/16/18 associated EGLs were: 99% and 79% (FUTURE I; Dillner et al. 2010).

Data on breakthrough infections in HPV-vaccinated individuals are scarce, and in the few reported cases concomitant (multiple) infections with other, non-vaccine HPV types have been found. This leaves open whether a vaccine type or a non-vaccine HPV type acquired post vaccination had caused the CIN and/or EGL (Paavonen et al. 2007, 2009; Dillner et al. 2010).

Table 10.1 Reported efficacies of two licensed human papillomavirus (HPV) vaccines against 6 month persistent high-risk HPV infection in young women negative for HPV PCR at study entry (*Baseline negative*) or in all participating women (*Intention-to-treat)* – data from 3 to 4 year analyses

End-point: persistent infection/vaccine	Vaccinated/infected	Control vaccinated/ infected	VE (95% CI)	References
Baseline negative				
HPV31/33/45/52/58//Cervarix™	7,664/534	7,640/755	30.2% (21.5–38.1)	Paavonen et al. (2009)
HPV31/33/45/52/58/Gardasil™	1,036/127	1,032/167	25.0% (5.0–40.9)	Brown et al. (2009)
Intention-to-treat				
HPV31/33/45/52/58//Cervarix™	8,863/1,179	8,870/1,364	14.5% (7.5–20.9)	Wheeler et al. (2012)
HPV31/33/45/52/58/Gardasil™	1,732/359	1,727/424	17.7% (5.1–28.7)	Wheeler et al. (2009)

Table 10.2 Reported efficacies of two licensed human papillomavirus (HPV) vaccines against HPV16/18 associated and all cervical intraepithelial neoplasia (CIN) grades 1–3 in women negative for HPV PCR at study entry (*Baseline negative*) or all participants (*Intention-to-treat)*

End-point: CIN/vaccine	Vaccinated/infected	Controls infected	VE (95% CI)	References
Baseline negative				
HPV16/18 CIN1+/Cervarix™	5,466/5	5,452/141	96.5% (91.6–98.9)	Lehtinen et al. (2012a)
HPV16/18 CIN1/Gardasil™	4,616/3	4,680/107	97.2% (91.5–99.4)	Munoz et al. (2010)
CIN1+/Cervarix™	5,466/174	5,452/346	50.3% (40.2–58.8)	Lehtinen et al. (2012a)
CIN1+/Gardasil™	4,616/272	4,680/390	29.7% (17.7–40.0)	Munoz et al. (2010)
Intention-to-treat				
HPV16/18 CIN1+/Cervarix™	8,694/121	8,708/324	62.9% (54.1–70.1)	Lehtinen et al. (2012a)
HPV16/18 CIN1/Gardasil™	8,562/99	8,598/304	67.5% (59.1–74.4)	Munoz et al. (2010)
CIN1+/Cervarix™	8,694/579	8,708/798	27.7% (19.5–35.2)	Lehtinen et al. (2012a)
CIN1+/Gardasil™	8,562/975	8,598/1,199	19.1% (11.9–25.7)	Munoz et al. (2010)
Baseline negative				
HPV16/18 CIN2+/Cervarix™	5,466/1	5,452/97	99.0% (94.2–100)	Lehtinen et al. (2012a)
HPV16/18 CIN2/Gardasil™	4,616/0	4,680/45	100% (88.7.–100)	Munoz et al. (2010)
CIN2+/Cervarix™	5,466/61	5,452/172	64.9% (52.7–74.2)	Lehtinen et al. (2012a)
CIN2+/Gardasil™	4,616/77	4,680/136	42.7% (23.7–57.3)	Munoz et al. (2010)
Intention-to-treat				
HPV16/18 CIN2+/Cervarix™	8,694/90	8,708/228	60.7% (49.6–69.5)	Lehtinen et al. (2012a)
HPV16/18 CIN2/Gardasil™	8,562/79	8,598/168	53.0% (35.5–59.9)	Munoz et al. (2010)
CIN2+/Cervarix™	8,694/287	8,708/428	33.1% (22.2–42.6)	Lehtinen et al. (2012a)
CIN2+/Gardasil™	8,562/296	8,598/367	19.3% (5.7–31.0)	Munoz et al. (2009)
Baseline negative				
HPV16HPV16/18 CIN3/Cervarix™	5,466/0	5,452/27	100% (85.5–100)	Lehtinen et al. (2012a)
HPV16/18 CIN3/Gardasil™	4,616/0	4,680/34	97.2% (91.5–99.4)	Munoz et al. (2010)
CIN3/Cervarix™	5,466/3	5,452/44	93.2% (78.9–98.7)	Lehtinen et al. (2012a)
CIN3/Gardasil™	4,616/36	4,680/64	43.0% (13.0–63.2)	Munoz et al. (2010)

(continued)

Table 10.2 (continued)

End-point: CIN/vaccine	Vaccinated/infected	Controls infected	VE (95% CI)	References
Intention-to-treat				
HPV16HPV16/18 CIN3/*Cervarix*™	8,694/51	8,708/94	45.7% (22.9–62.2)	Lehtinen et al. (2012a)
HPV16/18 CIN3/Gardasil™	8,562/100	8,598/177	43.5% (27.3–56.2)	Munoz et al. (2010)
CIN3/Cervarix™	8,694/86	8,708/158	45.6% (28.8–58.7)	Lehtinen et al. (2012a)
CIN3/Gardasil™	8,562/237	8,598/284	16.4% (0.4–30.0)	Munoz et al. (2010)
End of study analyses				

4 Efficacy of the Prophylactic HPV Vaccines Against Precursors of Cervical and Vaginal/Vulvar Cancers

Persistent HPV infection in the cervix or vagina usually is symptomless, and usualy diagnosed by cervical cytology. Depending on the cytological findings (repeated low grade squamous intraepithelial lesion, LSIL or high grade squamous intraepithelial lesion, HSIL), colposcopy directed biopsies are taken for histology, and the histological end-points: CIN1 and CIN2/3 correspond to the above-mentioned cytological findings. CIN2/3 (with different variations: CIN2, CIN2, CIN3, CIN3+) has been the most important end-point of the phase III HPV vaccine efficacy trials. Due to ethical reasons, it has not been possible to actively follow participants after the diagnosis of CIN2 (sometimes CIN3 has developed so rapidly between study visits that a small proportion of these have been diagnosed). As a surrogate end-point, CIN2 is complex because of the high regression probability (among women <35 years of age nearly equal to progression likelihood, [Myers et al. 2000]). Because there is no possibility to distinguish lesions that would have progressed from lesions which would have then regressed again, it remains open whether the vaccine is equally efficacious against both. Another issue worth noting below is the difference between the FUTURE and PATRICIA trials that include baseline HPV negative women, who are negative for all vaccine HPV types 6/11/16/18 + 10 oncogenic non-vaccine HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59) (FUTURE) or women, who are negative for 14 oncogenic HPV types (in addition HPV types 66 and 68) (PATRICIA). Given the above restrictions, comparable results on vaccine efficacy against stringent cervical cancer end-points are summarized below (Table 10.2).

The largest clinical phase III trials on the efficacy of the HPV6/11/16/18 and HPV16/18 vaccines against all CIN2+, CIN3 and adenocarcinoma in situ (AIS) lesions have reported their 4-year, end of study results: 19%, 16% and 60% (FUTUREI&II; Munoz et al. 2010), and 33%, 46% and 100% (PATRICIA; Lehtinen et al. 2012a) among all trial participants ("intention-to-treat" cohorts) (Table 10.2). As for baseline HPV negative participants, corresponding CIN2+ and CIN3 VEs were 43% and 43% (FUTURE II), and 65% and 93% (PATRICIA; Lehtinen et al. 2012a). Corresponding results against CIN2 or CIN2+, CIN3 and AIS associated with vaccine HPV-types 16/18 have been identical: 100%, 97% and 100% (FUTURE II; Munoz et al. 2010), and 99%, 100% and 100% (PATRICIA; Lehtinen et al. 2012a) among baseline negative participants (Table 10.2).

It should be noted, that even if the 95% confidence intervals of the compared VE estimates against all CIN3 among baseline HPV negative women for the two vaccines do not overlap, there are a number of plausible differences between the trials that may explain this: starting from different baseline characteristics (naïve for the vaccine: HPV6/11/16/18, FUTURE, or for all tested hrHPV types, PATRICIA), short follow-up time/small numbers, different criteria forcolposcopy biopsy, and the fact that the histopathological end-point definitions may have varied. For instance, 4-year passive, cancer registry based follow-up of the 1749 Finnish FUTURE II cohort

and a concomitant cohort of 15,444 unvaccinated females 2007–2011, with a stringent CIN3+ end-point, has yielded 100% VE against CIN3+ for the quadrivalent vaccine (Paavonen et al. 2011). On the other hand, differences in the cross-protective efficacies of the two vaccines have been reported (Wheeler et al. 2009, 2012), and eventually it may be possible to note different efficacies of the two vaccines against the most stringent cervical neoplasia end-points. The population-based cancer registry follow-up of Finnish phase III trial participants has 80% power to assess VE against CIN3+ in the next six (2018, FUTURE II) and 3 years (2015, PATRICIA) (Lehtinen et al. 2006; Paavonen et al. 2011; Rana et al. 2011).

Vaccine efficacies against precursors of vaginal or vulvar cancers (VAIN2/3 and VIN2/3) have only been published for the HPV6/11/16/18 vaccine (FUTURE; Kjaer et al. 2009), due to the fact that appropriate samples were not collected in the PATRICIA trial. Efficacy of the HPV6/11/16/18 vaccine against VAIN2/3 and VIN2/3 lesions among all trial participants were 86% and 73%, and among baseline HPV-negative participants, 100% (Kjaer et al. 2009).

5 Implementation: Effectiveness and Ethics of Alternative Vaccination Strategies

Effectiveness of implementing a new vaccine into national vaccination program is crucial. Other points of view that are needed to rank different vaccination strategies are feasibility, justice, and equality. The following vaccination strategies should be considered: (1) vaccinating girls and boys before sexual debut, (2) vaccinating persons in risk-groups, and (3) subsidized vaccination at will.

These strategies are effective and feasible in theory, but in practice the feasibility of the 2nd strategy is jeopardized by the common (almost ubiquitous) nature of genital HPV infections (70–80% of the population gets infected during lifetime), and the fact that there is not an easily defined risk group for genital HPV infections (Kibur et al. 2000). Furthermore, it is impossible to identify early adolescents who may or may not belong to a risk group in the future. The 3rd strategy would be effective among girls/women who would get vaccinated. However, according to the existing examples from Germany and US which have applied the subsidized vaccination strategy the vaccine coverage has remained much too low to generate any herd immunity (Barnabas et al. 2006; Lehtinen and French 2007), and this option has little if any impact on public health.

Evaluating the effectiveness of different vaccination strategies both directly and indirectly (herd-immunity derived) means vaccine efficacy needs to be considered. The direct efficacy of HPV vaccines in a number of controlled studies has been 90% or more against HPV-infections or their sequelae in individuals receiving the vaccine before sexual debut. In addition to this, however, according to the best dynamic transmission models, vaccinating both girls and boys with 50–80% coverage remarkably prevents (up to three times) more HPV infections than vaccinating girls alone (Barnabas et al. 2006; Regan et al. 2007).

Fifty percent or higher HPV mass vaccination coverage is feasible for vaccination of 12/13-year olds at junior high schools by school health care personnel. In the largest randomized effectiveness trial (with parental informed consent) involving 80,000 early adolescents, vaccine coverage among girls was 50%, and among boys 20–30% (Lehtinen et al. 2012c). Previous experience from vaccinating 13-year old girls against Rubella and current school-based HPV vaccination programs suggest that it is possible to reach 70% vaccine coverage in girls (Peltola et al. 2000; Brabin et al. 2008; Donovan et al. 2011), but going over 80% HPV vaccine coverage will be very difficult (Bonannini et al. 2011). Thus, clustering of marginalization among the unvaccinated, future unscreened girls is an issue (Lehtinen and Paavonen 2009) and it remains to be seen to what extent it can be tackled with herd immunity.

The aspect of justice is important for all vaccination strategies (Natunen et al. 2011). Poor (≤50%) vaccine coverage in Rubella vaccination has resulted in increased incidence of congenital Rubella syndrome in Greece, due to transfer of the epidemic from small and school aged children to young adults (Panagiotopoulos et al. 1999). Poor vaccination coverage may have similar consequences (Tookey and Peckham 1999). It is important to note that the subsidized HPV vaccination programs have received only 30–40% coverage (Rouzier and Giordanella 2010; Taylor et al. 2010), and worsening the situation of the lower income population, which is already marginalized from vaccination and screening is imminent and not just (Malmqvist et al. 2011).

6 Future Impact

Judging between the vaccination strategies, equality between genders should be considered now that the first report on the very good (>90%) efficacy of the prophylactic HPV6/11/16/18 vaccine against genital HPV infection and associated lesions in males have been published (Giuliano et al. 2011). Also the impact of herd-immunity in reducing HPV associated lesions in both males and females appears to be notable (Brotherton et al. 2011; Donovan et al. 2011). These findings together with numerous reports on the role of hrHPVs in increasingly common head and neck cancer (most notably oropharyngeal/tonsillar cancer), (Mork et al. 2001; Shiboski et al. 2005; Näsman et al. 2009; Blomberg et al. 2011) and increasing incidence of anal cancer (Brewster and Bhatti 2006; Grulich et al. 2010) underline the fact that a significantly larger proportion of human cancers than previously anticipated could probably be prevented both in females and males by HPV vaccination. Finally, the implicit/explicit message to early adolescent boys and girls about common effort for vaccination-gained protection against the most common sexually transmitted infection and its multiple sequelae is worth pursuing.

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Chapter 11
Treatment, Follow-up, and Prevention of Papillomavirus Infection and Cervical Cancer

Franco Borruto and Ciro Comparetto

1 Introduction

Cervical and other anogenital cancers are initiated by infection with one of a small group of Human Papillomaviruses (HPV). Virus-like particle (VLP)-based vaccines have recently been developed to prevent infection with two cancer-associated HPV genotypes (HPV-16, HPV-18) and have been ~95% effective at preventing HPV-associated disease caused by these genotypes in virus-naive subjects. Although immunization induces virus-neutralizing antibody sufficient to prevent infection, persistence of antibody as measured by current assays does not appear necessary to maintain protection over time. Investigators have not identified a reliable surrogate immunological marker of protection against disease following immunization. HPV prophylactic vaccines are the first vaccines designed to prevent a human cancer and are the practical outcome of a global collaborative effort between basic and applied scientists, clinicians, and industry. The prophylactic vaccines are not therapeutic for existing infection. Trials of HPV-specific immuno-therapy have shown some efficacy for existing disease, although animal modeling suggests that a combination of immunization and local enhancement of innate immunity may be necessary for optimal therapeutic outcome (Frazer et al. 2011). Reversing advanced pre-invasive and invasive cervical neoplasia with immuno-therapeutics is then a more difficult challenge, because little or no evidence for natural immune-mediated regression of these diseases exists. Nonetheless, recent controlled trials have shown some

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success in inducing precursor regression with vaccines targeting viral oncoproteins. Anecdotal reports of therapies that augment cellular immunity raise hopes that therapeutics targeting multiple pathways of anti-viral or anti-tumor immunity will be beneficial to women with established cervical cancer. However, success will require identifying and circumventing the mechanisms by which tumor cells evade the immune system (Crum and Rivera 2003).

2 Management

Although much has been learned about the biology of HPV during the last decades, little progress has been made in the development of rational and effective patient management strategies. Papanicolaou (Pap) smear screening for detection of early precancerous changes of the cervix and referral for colposcopy and biopsy of areas of epithelium that are suspicious for cervical, vaginal, vulvar, penile, or anal intraepithelial neoplasia (CIN, VAIN, VIN, PIN, or AIN) remain the cornerstones of genital tract and anal cancer prevention. For this reason, and because none of the available therapies are curative, treatment of large areas of normal-appearing genital tract epithelium also cannot be recommended at this time. It is hoped that with the growing research focus on therapies that have the potential for virologic cure, someday, effective treatment for subclinical infection will be available. Until that time, patients with recalcitrant or recurring genital warts may benefit most by the sequential application of different treatment modalities (Koutsky and Wølner-Hanssen 1989). Many therapies are available for the treatment of HPV-associated disease, particularly external genital warts. However, at present, these therapies aim to remove the lesion rather than specifically target HPV infection. When disease and infection are local, as in CIN, excisional therapies removing lesion and transformation-susceptible cells are highly effective. However, when infection is regional, as is usually the case for the anogenital warts, VIN, AIN, PIN, and VAIN, then current treatments are generally inadequate, with high recurrence rates. Future therapies will be directly or indirectly antiviral, targeting HPV protein functions or enhancing the ability of the immune system to resolve infection or inducing apoptosis indirectly in HPV-infected cells. In the short to the medium term, immunotherapies for low-grade (LG) disease are the most likely to be in the clinic. Vaccines targeting the E1 and E2 early proteins combined with immuno-modulators or conventional adjuvants that induce a strong cell-mediated HPV antigen-specific response and good immune memory are the predicted combination. Vaccines designed to target high-grade (HG) intraepithelial disease, even when used in combination with immuno-modulators, are unlikely to affect lesion clearance in more than a fraction of the cases. However, they may have a role as adjunct therapy after cervical conization to prevent the recurrence of CIN or HPV reinfection. They certainly appear to have a role in multifocal disease, such as VIN and AIN, where partial clearance may be affected and lesion size reduced enough for effective ablative or excisional therapy. However, agents such as indole-3-carbinol have shown efficacy in small clinical

trials, and if these effects are confirmed in larger, placebo-randomized controlled trials (RCT), they could be clinically useful (Stanley 2003).

Before the initiation of screening and treatment for cervical cancer precursors, approximately 3–4% of women were destined to eventually develop cervical cancer. During the last 50 years, the rate of cervical cancer incidence and mortality has decreased by more than 75%, primarily because of the widespread availability of cervical cytological screening and of treatment for documented cervical pre-cancer. Successful screening of the entire population and appropriate treatment of lesions could theoretically reduce this risk to one tenth of the risk of an unscreened population. The relatively recent understanding of the etiology of cervical cancer precursor lesions and of the immune response to them has given new direction to management options that incorporate healthy habits and dietary measures as part of traditional ablative or excisional treatment options. As we look to the future we can expect that new markers that more specifically identify individuals at-risk for cervical pre-cancer and cancer will be developed and take precedence in cervical screening. At the same time, treating the cause of these lesions, rather than the result, should provide less traumatic and more successful therapies. To this end, harnessing the immune system through immune response modifiers and HPV vaccines are now a reality, as do new chemo-preventative approaches. Of all human cancers, only cervical cancer, once the second most common cancer among women, stands on the threshold of being virtually eliminated (Cox 2002a). An understanding of the natural history of HPV-induced pre-cancer and cancer, and of the immune response to HPV and to these lesions, has significantly changed the management of lower genital tract neoplasia. New management guidelines incorporate this understanding, providing a more rational approach to diagnosis and treatment. Understanding that LG-HPV-induced lesions are not true cervical cancer precursor has fostered expectant management of women with these lesions. However, management approaches are still hampered by the inability to better predict who is at risk for HG-CIN and cancer and who is not. This is particularly problematic in the expectant management of CIN 1. In addition, the decision whether or not to treat these LG lesions may depend on a number of complex factors that take into account the woman's preferences and reliability for follow-up, as well as a host of issues related to costs and the reliability of the original diagnosis and the tests used for follow-up. Management options for HG cervical cancer precursor lesions are much more definitive, because the option of expectant management is given except in pregnancy and for adolescents with CIN 2. New markers that better predict which women with high-risk (HR)-HPV are at highest risk for subsequent development of cervical cancer precursor lesions makes the management of LG lesions as clear as present guidelines for their HG cousins (Cox 2002b).

The optimum management of women with minor squamous intraepithelial lesions (SIL) in Pap tests is controversial. With cytological surveillance after one Pap test showing atypical squamous cells of undetermined significance (ASC-US), a significant proportion of women will have undiagnosed SIL. On the other hand, using immediate colposcopy for ASC-US, almost half of the women would not have needed the procedure. This number for referral for colposcopy can be reduced to

only those women who test positive for HR-HPV deoxy-ribonucleic acid (DNA), however some women will have undiagnosed SIL. In addition, when surveillance with repeated Pap tests is used for the management of LG-SIL (L-SIL) a significant number of HG-SIL (H-SIL) can be missed (Varras 2004). A CIN 1 is a lesion of basal cells consisting in an architecture disorganization and cytological atypia limited to the lower third of the cervical epithelium. It is considered as a precancerous lesion for uterine cervix carcinoma while they spontaneously regress in more than 60% of cases in 2 years. The problems related to the management of CIN 1 as defined by the recommendations established in 2002 are the over-treatment and the great variability of clinical practices. Moreover, the potential of new tests has been investigated since 2002. One of the problems encountered when facing a CIN 1 is to misdiagnose a more severe lesion, firstly because of the intra- and inter-observer variability, and secondly because the colposcopy-directed biopsy is not mandatorily representative of the more severe lesion. Nevertheless, because the risk of cancer is extremely low, a conization is not necessary in an asymptomatic woman with a L-SIL/ASC-US pap smear in case of CIN 1 even if the squamo-columnar junction (SCJ) is not entirely visualized (level of evidence, LE 2-3). The endocervical curettage cannot be recommended in this case because its efficacy is globally poor and unknown in case of CIN 1. Concerning the natural history of CIN 1, the recent studies, which included more than 1,200 women and more than 700 for two of them, confirm that the rate of progression of a CIN 1 to a CIN 3 or more severe lesion is less than 9% in the 2 years following the initial diagnosis (LE 2). A CIN 1 is associated with a risk of evolution to a CIN 2 or more severe lesion that is not markedly more important than the risk associated with a L-SIL/ASC-US Pap smear while the colposcopy is normal or the biopsy is negative (between 8% and 13% whatever the results of the initial colposcopy) (LE 2-3). The recommendation in case of CIN 1 is a strict follow-up. A colposcopy and a treatment are necessary in case of persistence or progression of the abnormalities (LE 2). Data from trials studying the contribution of HPV testing in case of CIN 1 shows that its sensitivity is similar to repeated cytology with less referral to colposcopy to detect CIN 2 or more severe lesion. These data have been considered to establish follow-up recommendations to manage CIN 1: if the exams (cytology and/or HPV testing) at 12 months are negative, patients can be followed by an annual cytology. In case of aggravation of the cytology, a colposcopy is necessary. In case of positive HPV testing or persisting ASC-US/L-SIL at 12 months, a repeated control is necessary at 18 months and a treatment is proposed according to colposcopy findings (Rouzier 2008). Of the different strategies available for managing CIN 1, testing for HR-HPV DNA at 12 months has the highest sensitivity for predicting the development of CIN 2 or CIN 3 and leads to the lowest rate of referral to repeat colposcopy. If the HR HPV DNA test result is negative at 12 months, then the patient may return to routine cytology screening. If the HR-HPV DNA test result is positive, the patient should undergo repeated colposcopy (Avrich et al. 2006).

The knowledge that the persistent infection with HR-HPV is the etiological factor in the development of cervical cancer has led to the development of the HPV DNA detection methods as well as the prophylactic vaccine against the most common HR-HPV types, HPV-16 and HPV-18. Despite HPV vaccination, cervical

cancer screening will remain the main preventive measure for both vaccinated and non-vaccinated women, but the nature of screening and management of women with cervical disease is being adapted to the new technologies. Although HPV DNA detection is more sensitive that cytology, its specificity is lower, since most HPV infections are transient. Therefore, other methods are considered to improve the management of women with cervical disease. Typing of HPV DNA and viral load measurements are still used for research purposes only. Detection of viral oncogene E6/E7 transcripts, which is the marker of the productive infection, is a promising tool for follow-up of HPV DNA-positive women. The detection of p16INK4a over-expression, as an indirect test of E6/E7 expression, is used for confirmation of cervical neoplasia. Despite the lack of standardization, the detection of p16INK4a is useful in clinical settings, however its reproducibility in the management of LG and borderline cases is low. Future perspectives include the determination of the methylation status of several cellular genes that could predict the progression of the disease (Grce et al. 2010; Kyrgiou et al. 2010).

3 Medical Treatment

At present, there are no antiviral agents for the treatment of genital HPV infections, with many lesions requiring surgical intervention. Although other approaches are available for the treatment of genital warts, HPV infection cannot usually be cured and lesion recurrence is often a problem. A growing understanding of the molecular biology of HPV infection has identified several viral protein functions that may serve as drug targets. Among these are the HPV E1 and E2 proteins, which are necessary for viral genome replication and partitioning, and the E6 and E7 proteins, which are necessary for cell proliferation and apoptotic inhibition. With the exception of E1, these proteins lack enzymatic activity and achieve their effects by interacting with cellular proteins. Protein–protein interactions are in general quite difficult to inhibit using conventional small molecule drugs, but are amenable to inhibition using intracellular antibodies or intrabodies, which bind the viral proteins and sterically inhibit their association with cellular partners. The lack of homology between viral and cellular proteins, and the fact that HPV infections can be treated topically, makes them particularly well suited to the intrabody approach (Doorbar and Griffin 2007).

The cervix is an ideal organ for chemoprevention studies and the study of squamous carcinogenesis. The cervix is accessible and can be safely followed with Pap smears and colposcopy. Suitable agents include those likely to work in squamous lesions, including retinoids, difluoromethylornithine, beta-carotene, and others. Surrogate endpoint biomarkers (SEB) include quantitative histopathology, biologic measures of proliferation, regulation, differentiation, genetic instability, and fluorescence emission. Quantitative histopathologic markers include nuclear grading (i.e. shape, area, optical density, texture), nuclear pleomorphism, ploidy, and nucleolar size and position. Biomarkers under study at the present time in the cervix include

proliferation markers (PCNA), regulation markers (epidermal growth factor receptor, EGFR, ras, myc, p53, retinoic acid receptors, ornithine decarboxylase, ODC, spermidine/spermine ratios), differentiation markers (involucrin, cornifin, keratins), and markers of genetic instability (chromosome polysomy). Fluorescent spectroscopy uses light to probe the biochemical properties of tissue. This technique provides an automated diagnosis in real time with comparable sensitivity and specificity to colposcopy and can be used to monitor lesions in chemoprevention trials (Mitchell et al. 1995). CIN represents a spectrum of epithelial changes that provide an excellent model for developing chemo-preventive interventions for cervical cancer. Possible drug effects are dependent on the agent under investigation. Published and preliminary clinical reports suggest retinoids and carotenoids are effective chemo-preventive agents for CIN. Determination of plasma and tissue pharmacology of these agents and their metabolites could serve as drug effect intermediate endpoints. In addition, retinoic acid receptors could serve as both drug and biological effect intermediate endpoints. Given the demonstrated causality of HPV for cervical cancer, establishing the relationship to HPV will be an essential component of any biological intermediate endpoint biomarker. The pathologic effect SEB for cervical cancer is CIN, used clinically for years. The desired effect for chemo-preventive trials is complete regression or progression prevention. In planning chemoprevention trials, investigators need to consider spontaneous regression rates, the subjective nature of detecting CIN, and the impact of biopsy on regression. If intermediate endpoint biomarkers that met the above criteria were available for cervical cancer, then new chemo-preventive agents could be rapidly explored. The efficacy of these new agents could be determined with a moderate number of subjects exposed to minimal risk over an acceptable amount of time. The impacts on health care for women would be significant (Ruffin et al. 1995).

Cervical cancer chemoprevention agents include diet and micronutrients (particularly beta-carotene, folate, and vitamins A, C, and E), medications such as retinoids (retinyl acetate gel, all-trans-retinoic acid, and 4-hydroxyphenylretinamide) that are chemically related to micronutrients, and other chemo-preventatives meant to affect the carcinogenic process at the cellular level, including such polyamine synthesis inhibitors as alpha-difluoromethylornithine. Agents become reasonable candidates for study when they have a biologic rationale, they are of low toxicity, and they can be taken for a long period of time. Since HPV is the major etiologic agent, the medication should show activity against HPV-positive pre-invasive and invasive cell lines. The medication needs to be of low toxicity because it may be taken for long periods of time and less toxicity is tolerated in the precancerous setting. Until 1995, none of the studies used SEB, relying instead on histologic and colposcopic regression as end points. All studies typically included subjects with CIN. Conclusions to be drawn from these studies include the following:

1. Though micronutrients are logical candidates for chemoprevention, they haven't worked consistently and the reasons remain unclear
2. SEB need to be validated in phase I trials

3. A better understanding of the role of HPV needs elucidation, including an understanding of the relationship of the medication to HPV status and of the immuno-biology of HPV throughout the trial (Vlastos et al. 2003)

Some studies highlight the importance of dietary vitamin A (retinol) and other retinoids in maintaining normal cervical cell function and in inhibiting the growth of cervical tumors. Based on these results it can be concluded that:

1. HPV-16-immortalization enhances cervical cell sensitivity to retinoids
2. Cytokeratin expression may be useful as a marker for evaluating the success of retinoid therapy in vivo
3. Retinoids do not necessarily act to inhibit proliferation of HPV-immortalized cervical cells via effects on HPV E6 and E7 ribonucleic acid (RNA) levels
4. Retinoids may act to inhibit cervical proliferation by "suppressing" the activity of EGF and insulin-like growth factor (IGF) signaling pathways

Based on these and other results, it is worth considering the possibility that vitamin A or related retinoids could be administered therapeutically, early in the neoplastic process (either systemically or locally), to inhibit the progress of the disease. These results also suggest that combined interferon (IFN)/retinoid therapy may provide an enhanced beneficial effect to reduce cervical tumor size due to the fact that each agent is inhibiting cervical cell proliferation via distinct, but reinforcing, pathways (i.e. IFN gamma reduces E6/E7 expression, retinoid acid inhibits the function of EGF and IGF1 signaling pathways) (Eckert et al. 1995).

IFN objectively regresses HPV-induced warty disease and affects the re-growth of the transformed epithelium. IFN effectively controls the most serious and potentially life-threatening Papillomavirus associated diseases, respiratory papillomatosis, and genital warts, but not without the anticipated side effects associated with this potent biological weapon. It is entirely possible and even likely that individual species of IFN act preferentially on certain cells, tissues, or organs in response to particular viruses. Combined therapies, such as surgery in combination with IFN, or podophyllin in conjunction with IFN alfa-n1, may prove most efficacious (Weck et al. 1986). IFN treatment of existing genital HPV lesions has had mixed results. While HPV proteins down-regulate the expression of IFN-inducible genes, IFN treatment ultimately induces their high-level transcription after a delay. Cells containing complete HPV genomes that are able to undergo productive replication upon differentiation are sensitive to IFN-induced growth arrest, while cells from HG cancers that only express E6 and E7 are resistant. Recent studies indicate this sensitivity is dependent upon the binding of the IFN-inducible factor, p56, to the E1 replication protein. The response to IFN by HPV proteins is complex and results from the action of multiple viral proteins (Beglin et al. 2009).

HPV, found in 99.7% of cervical cancers, represents an attractive immuno-therapeutic target for novel adjuvant dendritic cells (DC) immuno-therapy. DC primed with HPV antigens have been shown to be capable of inducing cytotoxic T lymphocytes (CTL) responses powerful enough to eradicate established murine tumors expressing HPV-16 antigen. The use of tumor lysate has been found to be an effective

means of priming DC with tumor-associated antigens in animal models and in clinical trials leading to significant anti-tumor responses. Autologous DC primed with sonicated HPV expressing tumor lysate have been shown to be capable of inducing HPV specific classes I and II T-cell immunity in a pilot clinical study. Synthetic double-stranded polyribonucleotides are effective in vitro activation/maturation agents capable of inducing a stable mature DC phenotype producing high levels of interleukin (IL)-12. However, the prototype polymer poly [I]: poly [G] has proved to be clinically toxic. Preliminary in vitro data have demonstrated that a novel clinically non-toxic analogue polymer poly [I]: poly [C (12) U] (Ampligen R) can effectively induce in vitro maturation of human monocyte-derived DC with sustained bioactive IL-12 production. Human monocyte-derived DC primed with tumor lysate and matured with synthetic double-stranded RNA (dsRNA) may therefore offer an effective way of optimizing T-helper 1 (Th1) specific anti-cancer T-cell responses in cancer patients (Adams et al. 2003).

Five of the more than 50 known genital HPV types, HPV-6, 11, 16, 18, and 31 have become the models to study gene expression. The comparison of the studies of these five viruses and analyses of the genomic sequences of those genital HPV types that have not been transcriptionally studied make it likely that genital HPV share most strategies for regulating their transcription. These strategies are quite different from those of unrelated human and animal Papillomaviruses. Among these common properties are:

1. A specific promoter structure allowing for fine-tuned negative feedback
2. A transcriptional enhancer that is specific for epithelial cells
3. Regulation by progesterone and glucocorticoid hormones
4. Silencers, whose principal function appears to be transcriptional repression in the basal layer of infected epithelia
5. Specifically positioned nucleosomes that mediate the functions of some enhancer and the silencer factors
6. Nuclear matrix attachment regions that can, under different conditions, repress or stimulate transcription
7. Poorly understood late promoters that are positioned remotely from the late genes

Cellular proteins that, due to their simultaneous importance for cellular processes, may not be useful as HPV-specific drug targets control most of these properties. It should be possible, however, to target complex cis-responsive elements unique to these HPV genomes by nucleotide sequence-specific molecules, such as antisense RNA, polyamides, and artificial transcription factors. The application of small molecule-based drugs may be restricted to target proteins encoded by the HPV DNA, such as the replication factor E1 and the transcription/replication factor E2 (Bernard 2002). The E2 proteins from some HPV types induce growth arrest and apoptosis, and these proteins could be used as therapeutics for HPV-induced disease. A major obstacle to this approach concerns the delivery of the protein to HPV-transformed cells and/or HPV-infected cells in vivo. One possible solution is to use recombinant viruses to deliver E2. Another possible solution is to use purified E2 proteins or E2 fusion proteins. The Herpes Simplex Virus (HSV) virus protein

(VP) 22 is one of a small number of proteins that have been shown to cross the cell membrane with high efficiency. VP22-E2 fusion proteins produced in bacterial cells are able to enter mammalian cells and induce apoptosis. This suggests that VP22-E2 fusion proteins could be topically applied as a treatment for HPV-induced diseases, most probably post-surgery (Green and Gaston 2006).

Two novel approaches for inactivating gene expression involve ribozymes (RZ) and oligonucleotides. Methods for identification of target genes involved in neoplastic transformation and tumor growth have been established, and these will lead to therapeutic approaches without any damage to normal cellular RNA molecules, which is often associated with conventional therapeutics. RZ and oligonucleotides represent rational antiviral approaches for inhibiting the growth of cervical lesions and carcinomas by interfering with E6/E7 RNA production. The E6 and E7 genes of HR-HPV cooperate to immortalize primary epithelial cells and because they are found in cervical cancer and considered the hallmark of cervical cancer. The use and modification of RZ and antisense oligodeoxynucleotides (AS-ODN) can inhibit the growth of HPV-16 and HPV-18 immortalized cells, and tumor cells by eliminating E6/E7 transcript. Hammerhead and hairpin RZ have been widely studied because of their potential use for gene therapy and their place as therapeutic tools for cervical cancer is being evaluated. Although antiviral RZ and antisense molecules have been effective as in vitro or in vivo inhibitors of HR-HPV-positive cells, none is currently in clinical trial (Alvarez-Salas et al. 2003). The multi-step cervical carcinogenesis process is amendable to molecular therapeutics such as therapeutic nucleic acids (TNA). As seen, TNA-based therapies for cervical carcinoma include RZ, AS-ODN, and small interfering RNA (siRNA). In vitro experiments with TNA successfully inhibited E6/E7 expression and caused induction of apoptosis and/or senescence in cervical carcinoma cells. Early RZ and AS-ODN approaches showed promise as therapeutic moieties for cervical cancer. Despite the very high in vitro efficiency of siRNA-based therapies they present the same issues that burdened clinical development of RZ and AS-ODN. These issues include intracellular target accessibility, specificity, and delivery. RZ are useful for functional genomic studies including diagnosis. Moreover, AS-ODN appear better suited for clinical protocols because recent advances in nucleic acid chemistry allow higher cell uptake with very low off-target effects leading to actual AS-ODN clinical applications. By using combined treatments with multiple targets it will be possible to apply TNA directly to the cancerous cervix to destroy viral RNA and obliterate the tumor (Alvarez-Salas and DiPaolo 2007). Basic, clinical, and epidemiological analyses indicate that expression of HR-HPV E6/E7 genes is the primary cause of cervical cancer and represent ideal targets for the application of TNA. AS-ODN and RZ are the most effective TNA able to inhibit in vivo tumor growth by eliminating HPV-16 and HPV-18 E6/E7 transcripts. Expression of multiple RZ directed against alternative target sites by triplex expression systems may result in the abrogation of highly variable HPV. More recently, RNA interference (RNAi) gene knockdown phenomenon, induced by siRNA, has demonstrated its potential value as an effective TNA for cervical cancer. siRNA and aptamers as TNA will have a place in the armament for cervical cancer. TNA against cervical cancer is in a dynamic state, and clinical trials will define the TNA in preventive

and therapeutic roles to control tumor growth, debulk tumor mass, prevent metastasis, and facilitate immune interaction (DiPaolo and Alvarez-Salas 2004). Cervical cancer is an attractive model in which to test gene-specific therapies, because elimination of the HPV oncogenes E6 and E7 may result in cancer cell senescence. Oligonucleotide-based therapies tested over the years include, as we have said, AS-ODN, RZ, and, more recently, siRNA-based treatments. siRNA-based therapies have been touted as potential treatments for cancers, genetic disorders, and viral infections and have a number of advantages over antisense and RZ technologies. As with the older technologies, in vitro testing of siRNA against cervical cancer has shown promising results, however, the issues that held up the clinical development of RZ and antisense are currently also challenging the siRNA field: these are target selection, specificity, and delivery. If these issues can be overcome, a range of new and potent therapies for cervical cancer could become available (Gu et al. 2007). The newly discovered phenomenon of RNAi offers the dual facility of selective viral gene silencing coupled with ease of tailoring to meet genetic variation within the viral genome. Such promise identifies RNAi as an exciting new approach to treat viral-induced diseases, including viral-induced cancers (e.g. cervical carcinoma, hepatocellular carcinomas, and hematopoietic and lymphoid malignancies). Silencing of HPV gene expression by RNAi induces apoptosis of cervical carcinoma cells in culture, and the effect is apparent within 3 days. The RNAi process is triggered by dsRNA, and a single dose is sufficient to maintain RNAi for several days in vitro (cell culture) and in vivo (experimental animal models). Thus, the stage is set for the development of novel antiviral and anticancer therapies based upon selective gene silencing by RNAi (Milner 2003).

Estrogen interacts with HPV at a cellular level causing cell growth and inhibition of apoptosis. Indole derivatives, formed during digestion of cruciferous vegetables, have been shown to have chemo-preventative properties inhibiting HPV transcription and influencing estrogen metabolism. Further clinical research is required to evaluate the chemo-preventative properties of these agents (Rieck and Fiander 2008). Potential molecular targets for therapeutic interventions in human HPV-related carcinomas, with emphasis on cervical cancer, based on the alterations observed in the signaling transduction pathways caused by HPV infection, with a special attention to tyrosine kinase receptors (TKR), and other kinases involved in signal transduction and angiogenesis, are evaluated as novel targets for anticancer therapies in HPV-related carcinomas (Kim et al. 2010; Saito Ramalho et al. 2010).

4 Surgical Treatment

The main goal of the therapy of HPV infection is the management of the virus. There is no cure for HPV infections. The treatment goal for the female patient is to destroy lesions that are malignant or premalignant. The male patient is treated because he is a carrier of HPV who can infect previously uninfected women and possibly

re-infect an already treated partner with a potentially oncogenic virus (Kling 1992). In current practice, the most cost-effective diagnostic and therapeutic means for H-SIL/CIN lesions is loop electrosurgical excision procedure (LEEP). Judgment must be exercised, however, to tailor the depth of electro-excision of cervical cancer precursors, particularly with respect to the LG variants, according to the position of their endocervical lesion margin. About half of the LG lesions also carry intermediate- to HR oncogenic viruses: unfortunately, morphology cannot identify them unless abnormal mitotic figures are present on the histologic sections. When sophisticated, low-cost viral probing tests and perhaps cervicography become available, it may be pertinent to determine whether a LG lesion contains low-risk (LR) virus or is associated with a high- to intermediate-risk virus group. Indeed, the former are unlikely to progress to invasion and may, in fact, regress without therapy other than multiple biopsies, whereas the latter carry HR carcinoma potential. Treating long-term monogamous men with asymptomatic, subclinical lesions who are partners of HPV-infected women is not considered cost-effective, for they do not seem to re-infect each other. Management strategies for patients with HPV infections should not only be therapeutic but also educative, encouraging patients to practice sexual monogamy (Ferenczy 1993).

Since the introduction of the Pap test by George Papanicolaou, the incidence of cervical cancer has fallen and mortality has decreased, in parallel with effective treatment of the precancerous and in situ stages of the disease. However, women who are not diagnosed through screening usually present with advanced disease. Early invasive disease can be treated successfully with radical abdominal hysterectomy and pelvic lymphadenectomy or with radiotherapy. A surgical alternative is radical trachelectomy, which can preserve fertility in young women. Advances in the techniques of laparoscopy facilitate procedures, such as radical vaginal hysterectomy with laparoscopic or extra peritoneal lymphadenectomy, that decrease intra-abdominal scarring and length of hospital stays (Savino et al. 2001; Herzog 2003). The most effective treatments to date are surgery in the form of large loop excision of the transformation zone (LLETZ) for pre-invasive disease, LLETZ or simple hysterectomy with laparoscopic pelvic lymphadenectomy for International Federation of Gynecology and Obstetrics (FIGO) Stages IA1 and IA2 micro-invasive carcinomas, and Wertheim's hysterectomy or celio-Schauta for FIGO Stage IB disease along with concurrent chemo-radiotherapy in patents with at least FIGO Stage IB disease. However, radical trachelectomy, which involves a radical excision of the cervix with simultaneous laparoscopic or extraperitoneal lymphadenectomy, may be used selectively in patients with up to FIGO Stage IB1 cancers, as this may preserve fertility in younger women (Jordan and Monaghan 2004). The presence of lymph node metastases alters the type of therapy. A range of methods is available to assess lymph nodes, including positron emission tomography (PET). Radiotherapy alone may not be successful in women with locally advanced disease and adding chemotherapy may eradicate systemic micro-metastases that are not affected by radiation. Single-agent cisplatin (CP) is the current drug of choice for adding to radiotherapy but new agents are being evaluated (Herzog 2003).

5 Follow-up

Recurrences of CIN as well as invasive cervical carcinoma have been reported to arise following ablative or excisional treatment for CIN. Cervical cytology, colposcopy, endocervical curettage, and HPV typing have been advocated for use as tools for follow-up protocols for women treated by LEEP for CIN. Involvement of the surgical margins and the presence of HPV DNA are associated with HR of recurrence and should be taken into consideration. The psychological impact of undergoing colposcopy may affect compliance with follow-up visits and should be dealt with appropriately (Bornstein et al. 2004). The women treated for a HG cervical lesion (CIN 2+) have a high and prolonged (beyond 25 years) risk of recurrence (fivefold compared to the general population) of cervical as well as extra cervical lesions. The main objective of the follow-up of patients treated for HG-CIN is, on one hand, to detect and treat the recurrences and, on the other hand, to determine a subpopulation presenting a HR of recurrence which should be followed-up more intensively. At present, frequent follow-up with cytology and colposcopic evaluation of the cervix is the preferred strategy. The cytological and colposcopic protocols of follow-up raise the problem of their insufficient sensitivity, and the compliance of the patients to this prolonged follow-up is low. HPV test seeking the presence of HR-HPV benefits from a high sensitivity and presents altogether a very strong negative predictive value. Both the sensitivity and the negative predictive value of combined cytology and HPV testing in detecting a residual disease or recurrence are around 100%. The addition of this test to the cytological monitoring 3–6 months after the conization makes it possible to distinguish a group of patients with LR (with both tests negative) being able to profit from a traditional follow-up, from a HR group having at least one positive test, whose surveillance must be reinforced (with triage by colposcopy), prolonged in time, and extended beyond the cervix. Women presenting negative results of both tests could, then, be considered at a LR of recurrence and their surveillance should be the same as that of the screening of the general population. Like as the primary screening of cervical lesions, the follow-up of the patients after conization must profit from the addition of HPV test and would deserve protocolization and organization (Mergui et al. 2008; Mergui and Levêque 2008).

According to the current guidelines in most Western countries, women treated for CIN 3 are followed for at least 2 years after treatment by cytology. HR-HPV infections are necessary for the development and maintenance of CIN 3. HR-HPV testing could be used to improve monitoring of women treated for CIN 3. This has prompted numerous studies for the implementation of HR-HPV testing in monitoring of women treated for CIN 3. Combined HR-HPV and cytology testing yielded the best test characteristics. It has been proposed to include HR-HPV testing in conjunction with cytology for monitoring women treated for CIN 3. Some follow-up visits for women testing negative for both HR-HPV and cytology can be skipped. In Western countries, this could mean that for women double negative at 6 months, retesting at 12 months should be skipped while keeping the 24-month follow-up

visit (Zielinski et al. 2004). There is an emerging interest concerning the role of HPV DNA testing in the follow-up period after conservative treatment for CIN. There is a marked heterogeneity in the design, population, intervention, and follow-up policy across different studies. It seems that a positive HPV test, even in the presence of normal cytology, may pick-up early and accurately a treatment failure. Cytology and colposcopy may still be needed in order to rule out false-positive and false-negative results. A systematic review of studies concerning HPV DNA testing in the follow-up period after conservative treatment for CIN indicates that a positive HPV test, even in the presence of normal cytology, may pick-up early and accurately a treatment failure. Cytology and colposcopy may still be needed in order to rule out false-positive and false-negative results (Paraskevaidis et al. 2004).

6 Prognosis

Cervical carcinoma is a leading cause of mortality from cancer among women worldwide, accounting for approximately 160,000 deaths annually. Prognosis in patients with this disease is dependent on several well-established clinical features (stage of disease and age of patient) and pathologic features (lymph node status, grade of tumor, and depth of invasion). Although the features associated with poor clinical outcome have been well studied, molecular markers such as HPV type may reflect the underlying biologic basis for clinical behavior. HPV-18 DNA type is an independent prognostic factor in patients with cervical carcinomas treated with radical hysterectomy and pelvic lymphadenectomy. The use of molecular markers such as HPV DNA type may allow the identification of patients with early stage cervical cancer at HR for disease recurrence (Burger et al. 1996). Some studies have suggested that the presence in tumors of nucleic acids from HPV constitutes a prognostic marker of disease severity in cervical cancer. There are two conflicting lines of evidence in this regard. First, the presence of HPV-18 is equated to rapid progression through early disease stages, possibly resulting in a more aggressive clinical course. Although fragmentary, in terms of clinical and epidemiological basis, this line of evidence has some experimental support. Second, the absence of HPV from the tumor would confer a worse prognosis than if any viral types were present. Unlike the former, the latter line of evidence is not bolstered by experimental data but emerged from persuasive clinical studies, which had adequate sample sizes, used survival end points, and controlled for confounders. The absence of HPV in some tumors could indicate that they originated through different oncogenic mechanisms, perhaps resulting in different cell proliferation rates and, consequently, distinct clinical behavior. On the other hand, HPV detectability could simply be a correlate of other genuine prognostic characteristics, which would explain its association with survival (Franco 1992).

Conflicting evidence on the prognostic influence of some of the clinical and histopathological variables in cervical cancer of the HPV status and type and chemotherapeutic response prompted a number of reviews from nearly 40 years experience.

The collection and analyses of these data with those from recent literature allow some proposals to be made. The disease is more prevalent in the young women in whom, in many centers, the mortality is also higher. The latter may be related to the reported increase in both small cell types and adeno- and adeno-squamous carcinoma, a finding more marked in the young. Lymph node metastases, related to increasing grade, size, stage, and lymph space invasion, are unequivocally associated with a worse prognosis. Resolution of the exact nature of the intimate association of this disease with HPV remains to be resolved as does the influence on prognosis of the tumor HPV status and that of the different oncogenic types. Reports on the efficiency of neoadjuvant platinum-based combination chemotherapy are generally promising but vary considerably depending on the regimen used (Elliott 1995).

7 Prevention

Advances in screening and diagnosis make it increasingly possible to prevent cervical cancer. However, if misused or poorly understood, these new tools will only increase costs and potentially harm patients without benefit. As a framework for standardized care that maximizes patient safety and well being, it has been proposed that a risk model be adopted to guide clinical management now and in the future. The model would use thresholds of increasing risk for cervical precancer and treatable cancer to guide clinical decision making for screening intensity, diagnostic evaluation, or treatment. Experts would decide on these risk thresholds and stratum based on the patient risk to benefit, independent of current (e.g. cytology, carcinogenic HPV testing, and colposcopy) and future methods of measuring risk. A risk management model for cervical cancer prevention, based on appropriate clinical actions that correspond to risk stratum, can result in better allocation of resources to and increased safety for women at the greatest risk and increased well-being for women at the lowest risk (Castle et al. 2007). Prevention falls into two main categories: primary and secondary. Primary prevention is characterized by vaccination and health promotion to promote lifestyles and behaviors minimizing risk of cervical cancer. Interventions to promote the use of condoms for sexual intercourse (especially early intercourse amongst young women), sexual partner reduction, and negotiated safer sex strategies has been recommended as one approach to limit the spread of HPV, the main risk factor for cervical cancer. Studies showed a statistically significant positive effect on sexual risk reduction, typically with increased use of condoms for vaginal intercourse. This positive effect was generally sustained up to 3 months after intervention. Educational interventions targeting socially and economically disadvantaged women in which information provision is complemented by sexual negotiation skill development can encourage at least short-term sexual risk reduction behavior. This has the potential to reduce the transmission of HPV, thus possibly reducing the incidence of cervical carcinoma (Shepherd et al. 2000).

Continuing high-rates of HPV and other sexually transmitted diseases (STD) in young people demonstrate the need for effective behavioral interventions.

The majority of interventions provided information about STD and taught safer sex skills (e.g. communication), occasionally supplemented with provision of resources (e.g. free sexual health services). They were heterogeneous in duration, contact time, provider, behavioral aims and outcomes. A variety of STD were addressed including Human Immunodeficiency Virus (HIV) and Chlamydia. None of the trials explicitly mentioned HPV or cervical cancer prevention. Statistically significant effects for behavioral outcomes (e.g. increasing condom use) were common, though not universal and varied according to the type of outcome. There were no statistically significant effects of abstaining from or reducing sexual activity. There were few statistically significant effects on biological (STD) outcomes. Considerable uncertainty exists in the risk of bias due to incomplete or ambiguous reporting. Behavioral interventions for young women which aim to promote sexual behaviors protective of STD transmission can be effective, primarily at encouraging condom use. Future evaluations should include a greater focus on HPV and its link to cervical cancer, with long-term follow-up to assess impact on behavior change, rates of HPV infection, and progression to cervical cancer. Studies should use a RCT design where possible with integral process evaluation and cost-effectiveness analysis where appropriate (Shepherd et al. 2011). So, there are evolving primary and secondary preventive strategies that could further reduce the burden from cervical carcinoma. The primary preventive strategies include risk reduction, diet or dietary supplements, HPV vaccines, and other chemo-preventive agents. The possible advances in secondary preventive strategies include new technologies for Pap smears, HPV typing triage, and other adjuvant screening procedures. The impact of these strategies will depend upon evidence to support their use along with the characteristics of the population and environment in which they are used (Rock et al. 2000).

Although cervical cancer screening, primarily with the Pap smear, has reduced the incidence of this cancer in industrialized countries, cervical cancer remains the second most common cause of death from cancer in women worldwide, because the developing world has lacked the resources for widespread, high-quality screening. In addition to advances in Pap smear technology, the identification of HPV as the etiologic agent has produced two recent advances that may have a major impact on approaches to reduce the incidence of this disease. The first is the development of a preventive vaccine, the current versions of which appear to prevent close to 100% of persistent genital infection and disease caused by HPV-16 and HPV-18. Future second-generation vaccines may be able to protect against oncogenic infections by a broader array of HPV types. The second is the incorporation of HPV testing into screening programs. In women aged >30 years, HPV testing can identify HG-CIN earlier than Pap smears with acceptable rates of specificity. These results, together with the high sensitivity of HPV testing, suggest that such testing could permit increased intervals for screening. An inexpensive HPV test in development may be incorporated as part of an economically viable "screen-and-treat" approach in the developing world (Lowy et al. 2008). Unlike cervical cytology, HPV testing provides an objective assessment of neoplasia risk. The major advantages of this technology are the potential for "reflex testing" (when used with liquid-based cytology, LBC),

efficient exclusion of HPV-negative women, who can be triaged to yearly follow-up, and identification of HPV-positive women, who require colposcopic triage. However, practitioners should be aware that highly sensitive HPV tests will also identify many women with little or no immediate risk of significant neoplasia, may impose a psychosocial burden on the patient and may be used or interpreted inappropriately by both practitioners and patients. However, these caveats are similar to those inherent in any screening program involving a STD, and the disadvantages of HPV testing will be minimized by attention to patient concerns and a keen awareness of the limitations of this technology. Ultimately, control of cervical cancer and its precursors rests with active prevention via vaccination programs targeting HPV (Crum and Berkowitz 2002). These ongoing secondary prevention efforts considerably complicate the question of whether vaccination against HPV types 16 and 18 should be introduced. Policy questions focused primarily on the target ages of vaccination, appropriate ages for a temporary "catch-up" program, possible revisions in screening policies to optimize synergies with vaccination, including the increased used of HPV DNA testing, and the inclusion of boys in the vaccination program. Decision-analytic models are increasingly being developed to simulate disease burden and interventions in different settings in order to evaluate the benefits and cost-effectiveness of primary and secondary interventions for informed decision-making. Mathematical models have been used to evaluate HPV vaccination in the context of developed countries with existing screening programs. Despite variations in model assumptions and uncertainty in existing data, pre-adolescent vaccination of girls has been consistently found to be attractive in the context of current screening practices, provided there is complete and lifelong vaccine protection and widespread vaccination coverage. Questions related to catch-up vaccination programs, potential benefits of other non-cervical cancer outcomes, and inclusion of boys are subject to far more uncertainty, and results from these analyses have reached conflicting conclusions. Most analyses find that some catch-up vaccination is warranted but becomes increasingly unattractive as the catch-up age is extended, and vaccination of boys is unlikely to be cost-effective if reasonable levels of coverage are achieved in girls or coverage among girls can be improved (Kim et al. 2008).

Testing for the DNA of HR-HPV is more sensitive than cytology in detecting pre-cancerous lesions. One of the main advantages will be the possibility of applying prolonged screening intervals. However, adequate screening protocols (age of start and stop, screening intervals, management of HPV positive women) need to be applied in order to avoid over-referral to colposcopy and over-treatment and to maintain sustainable costs. Further follow-up of running trials and research on molecular markers will better define these parameters. The new situation will require organized screening programs with rigorous protocols and monitoring. This will be even more needed when women vaccinated for HPV-16 and HPV-18 will be screened. Research on how to best screen vaccinated women is a priority (Ronco and Giorgi Rossi 2008). Thus, HPV test results predict the risk of cervical cancer and its precursors (CIN 3) better and longer than cytological or colposcopic abnormalities, which are signs of HPV infection. The logical and inevitable move to HPV-based cervical cancer prevention strategies will require longer screening intervals that will disrupt current gynecologic and cytology laboratory practices built on frequent

screening. A major challenge will be implementing programs that do not over treat HPV-positive women who do not have obvious long-term persistence of HPV or treatable lesions at the time of initial evaluation. The greatest potential for reduction in cervical cancer rates from HPV screening is in low-resource regions that can implement infrequent rounds of low-cost HPV testing and treatment (Schiffman et al. 2011).

Cervical cancer prevention programs should include education (of health care providers and women), stressing the benefits of screening, the age of the peak cervical cancer incidence, and the signs and symptoms of precancerous lesions and invasive disease. The aim of screening actions is to detect precancerous lesions that may lead to cancer if left untreated. Screening can only be effective if there is a well-organized system of follow-up, diagnosis, and treatment. Cervical cytology, or Pap testing, has for decades been a cornerstone of cervical cancer screening. According to recent guidelines issued by the World Health Organization (WHO) Regional Office for Europe, the primary task of the public health system is the introduction of secondary prevention through properly organized screening programs. Launching the national immunization program is only possible in the countries with well-organized secondary prevention programs and in those that can afford it (Corusić et al. 2010). The European Research Organization on Genital Infection and Neoplasia (EUROGIN) 2008 Roadmap represents a continuing effort to provide updated information on primary and secondary prevention of cervical cancer. The report addresses several areas including the progress made toward global implementation of currently licensed HPV vaccines, the possibilities and value of future-generation HPV vaccines, endpoints under consideration for evaluation of candidate HPV vaccines, and monitoring impact of HPV vaccination programs that can be implemented within developed and less-developed countries (Arbyn and Cuzick 2009; Franceschi et al. 2009; Cuzick 2010; Scarinci et al. 2010).

8 Vaccine

As a result of several advances in molecular biology, the causal association between HPV infection and cervical cancer has been firmly established and the oncogenic potential of certain HPV types has been clearly demonstrated. In recognition of the causal association of cervical cancer with this STD, substantial interest has arisen to develop effective prophylactic and therapeutic vaccines. Several lines of evidence suggest the importance of the host's immune response, especially cellular immune response, in the pathogenesis of HPV-associated cervical lesions. These observations formed a compelling rationale for the development of vaccine therapy to combat HPV infection. Because there is no effective culturing system to propagate HPV, traditional approaches for studying HPV and developing vaccines have been hampered. However, studies using recombinant subunit preparations in animals have yielded promising results and encouraged their investigation in human trials. Prophylactic strategies focused on the induction of effective humoral and cellular immune responses that are potentially protective against subsequent HPV infection. In this

respect, impressive immune-prophylactic effects have been demonstrated using VLP, which are antigenic and protective, but are devoid of any viral DNA that may be carcinogenic to the host. Using recombinant techniques to express the L1 major capsid protein, VLP have been synthesized in order to induce neutralizing antibody responses in both animals and humans. For the treatment of existing HPV infections, techniques to improve cell-mediated immunity by enhancing viral antigen recognition were studied. For this purpose, vaccines targeting the oncogenic proteins E6 and E7 of HPV-16 and HPV-18 are the focus of current clinical trials for cervical cancer patients (Steller and Schiller 1996; Murakami et al. 1999; Steller 2002a, b).

The interaction of HPV with the immune system has been studied, but the results are still inconclusive for several reasons. Until now, we have not been able to understand the mechanisms of immune system regulation in the uterine cervix. HPV infection does not unleash an inflammatory response, and consequently an efficient and specific immune response against the virus. Moreover, an understanding of HPV infection and local immune response is indispensable for the development of new bioactive drugs and therapies for patients with both non invasive and invasive tumors, mainly for patients that do not present regression with radiotherapy or chemotherapy or in whom the tumors are surgically irremovable (Michelin and Murta 2008). Oncogenic HPV types infecting the anogenital tract have the potential to induce natural immunity, but at present we do not clearly understand the natural history of infection in humans and the mechanisms by which the virus can evade the host immune response. Natural acquired immune responses against HPV may be involved in the clearance of infection, but persistent infection with oncogenic virus types leads to the development of precancerous lesions and cancer. B-cell responses are important for viral neutralization, but antibody responses in patients with cervical cancer are poor (Einstein 2008). Genital HPV infection with both LR and HR types is common, but most infections resolve as a result of a cell-mediated immune response. Failure to induce an effective immune response is related to inefficient activation of innate immunity and ineffective priming of the adaptive immune response. This defective immune response facilitates viral persistence, a key feature of HR-HPV infection. This milieu becomes operationally HPV-antigen tolerant, and the host's defenses become irrevocably compromised. HPV antigen-specific effector cells are poorly recruited to the infected focus and their activity is down-regulated. Neoplastic HPV containing cervical keratinocytes expressing high levels of E6 and E7 oncoproteins are not killed in this immuno-suppressive, tolerant milieu, and progression to HG disease and cancer can result. Highly efficacious prophylactic HPV L1 VLP vaccines circumvent viral epithelial evasion strategies since they are delivered by intramuscular injection. The stromal DC of the muscle that encounter the highly immunogenic repeat structure of the VLP then migrate with their cargo to the lymph node, initiating an immune cascade that results in a robust T-cell dependent B-cell response, which generates high levels of L1-specific serum neutralizing antibodies and immune memory (Stanley 2008a). For prophylactic vaccines there is clinical evidence of efficacy (i.e. 100% protection from HPV infection and dysplasia by VLP vaccine-induced neutralizing antibodies). Also, therapeutic vaccines have entered clinical evaluation. While prophylactic VLP vaccines are

immunogenic per se, therapeutic vaccines will need further adjuvants to guide T-cell differentiation, expansion, survival, and homing to tumor sites. To enhance clinical outcome of successful T-cell induction in patients, the susceptibility of the tumor cells for lysis must be addressed in the future, since tumor immune evasion is a severe problem in cervical cancer. While successful prophylactic HPV vaccines have entered large clinical use, therapeutic HPV vaccines, in spite of T-cell induction, lack clinical responses due to the problem of tumor immune evasion. Adjuvants for systemic and local immune modulation will be mandatory for effective therapy (Schreckenberger and Kaufmann 2004).

Since the early 1990s, it has been prospected that despite current technical difficulties, it could soon be possible to treat cervical cancer and pre-malignant CIN with anti-HPV-16 vaccines. A prophylactic vaccine could be developed to induce neutralizing antibodies to HPV-16 virions in genital secretions, and a therapeutic vaccine to elicit CTL responses against HPV-16 early proteins in established lesions. Although significant advances had been achieved, problems remained before such vaccines could be used routinely (Cason et al. 1993; Khan 1993). Over the past decade, several vaccines that target common HPV types have entered clinical trials. These vaccines are classified as prophylactic or therapeutic. The goal of prophylactic vaccines is to prevent primary or persistent HPV infections, and thus prevent cervical cancer and/or genital warts. Recent evidence indicates that prophylactic vaccines are well tolerated, highly immunogenic, and effective in preventing persistent HPV infection and CIN. Questions remain, however, concerning vaccine efficacy against HPV-related diseases other than cervical cancer, the duration of protection, vaccine acceptability, and feasibility of vaccine delivery in the developing world. The goal of therapeutic vaccines is to prevent progression of HPV infection, induce regression of CIN or condylomata, or eradicate residual cervical cancer. Although therapeutic vaccines appear to induce both humoral and cell-mediated immunity, they have not consistently demonstrated clinical efficacy. HPV vaccines in development have the potential to reduce the substantial morbidity and mortality associated with cervical cancer and other HPV-associated diseases. Current and future efficacy studies will provide additional information about vaccine tolerance and its effectiveness (Kahn and Bernstein 2005).

As we have seen, the technical problems of developing cheap, effective vaccines against HPV-associated tumors were formidable, but they were by no means insuperable. Experiments in cows with Bovine Papillomavirus type 2 (BPV-2) showed that both therapeutic and prophylactic vaccines worked to some extent and the immunogens used were by no means the best that could be envisaged. There were both practical and ethical problems, as with any STD, but the main problem was one of support. Pharmaceutical companies saw no immediate profit in vaccines of this type, preferring to invest in drugs for treatment or diagnostic kits for detection. Vaccines against HPV were unlikely to be forthcoming, and indeed, the people most in need of protection against cervical cancer were the least able to afford any sort of treatment, especially a preventive one. This left the cancer charities, and these in their financial difficulties were understandably reluctant to commit substantial resources to the long term programs that were needed to tackle the problems of

developing and evaluating candidate vaccines. It seemed certain that intervention against HPV and cervical cancer would come in time, but with that level of commitment, progress was inevitably going to be less rapid than one would like (Crawford 1993). It could then be feasible to develop prophylactic vaccines to prevent HPV infection using the L1 and L2 capsid proteins or therapeutic vaccines to modulate the development or recurrence of disease based on the E6 and E7 oncoproteins or other viral proteins. In favor of success was:

1. The relative simplicity of the HPV genome (only two proteins in the viral coat, and a small number of other genes)
2. The lack of genetic variability within types and stability of the genome
3. The encouraging results with vaccines against animal Papillomaviruses

It has been difficult to provide evidence of the efficacy of HPV vaccines because of the inability to propagate the virus in culture or in animal models and because of the incomplete understanding of the natural history of HPV infection (Galloway 1994). The natural history of cervical cancer and its precursors (CIN) as well as animal experiments strongly suggest that the immune system controls both the primary infection (by neutralizing antibodies directed against the major structural protein L1) and the progression of the disease (via CTL specific for the viral oncoproteins expressed in transformed cells, e.g. E7). By the expression of an HPV-16 L1 E7 fusion protein, there have been generated chimeric VLP. Immunization of mice with chimeric VLP induces neutralizing antibodies directed against L1 VLP (devoid of the E7 portion) and E7-specific T-cells as measured in vitro. Vaccinated animals are protected against tumor growth following inoculation of syngenic HPV-16-transformed cells. In addition, it has been observed a therapeutic effect of vaccination on pre-existing tumors. This data allowed us to conclude that chimeric VLP are suitable for prevention and therapy of HPV infection (Jochmus et al. 1999).

VLP could be obtained by expression of the major capsid protein L1 alone or by co-expression with the minor capsid protein L2 in various systems. VLP and virions have very similar capsid structures. Immunization with VLP yielded antibodies neutralizing virions in vitro. Vaccination of animals with VLP had been shown to protect against viral challenge. VLP of HPV were therefore the most promising vaccine candidate to prevent infections with HPV associated with cervical cancer, the most frequent carcinoma in women worldwide (Sapp et al. 1996). VLP were attractive subunit vaccine candidates since they lack potentially oncogenic Papillomavirus DNA and express the conformationally dependent epitopes necessary to induce high-titer neutralizing antibodies. Prophylactic VLP vaccination had achieved a high degree of protection in animal studies. Thus, VLP were considered the immunogen of choice for human vaccine trials to prevent genital HPV infection. VLP of different HPV had been developed to study the serologic relationship between HPV types. VLP-based enzyme-linked immune-sorbent assay (ELISA) were able to detect antibodies in human sera and were widely used in epidemiologic studies of the natural history of HPV infection and the associated risk of developing neoplasia (Kirnbauer 1996).

Prophylactic vaccines for genital HPV infection had been shown to be feasible in animal models, and suitable vaccine material based on VLP could be produced in bulk at reasonable cost. Initiation of phase III clinical trials followed definition of trial outcome measures through further epidemiological studies, and development of assays of host protective immunity. Vaccines could in principle eliminate HPV-related disease, as the human race is the only natural host for the relevant Papillomaviruses. Therapeutic vaccines for genital HPV infection are also possible, but have not yet been demonstrated as feasible in practice because the choice of vaccine antigens is difficult, the method of their optimal delivery is uncertain, and the nature of the relevant antiviral immunity is unknown. Papillomavirus species specificity will require trials to be conducted in man, which will slow definition of an ideal vaccine (Frazer 1996). Three vaccine strategies that target HPV were likely to be effective in the control of HPV-associated pre-neoplastic and neoplastic lesions of the uterine cervix:

1. Immuno-therapy for HPV-associated cervical cancer targeted at two non-structural Papillomavirus proteins expressed in cancer cells (E6 and E7)
2. Vaccines against existing HPV infection and early premalignant lesions targeted at early viral proteins expressed in supra-basal stem cells of infected anogenital epithelium
3. Prophylactic vaccines to prevent HPV infection involving immunization with genetically engineered VLP to elicit neutralizing antibody

Strategies 1 and 2 will need to evoke CTL mediated responses (Tindle 1997). The design of such vaccines has evolved from an understanding of the nature of HPV infections and their consequences, together with evaluation of the efficacy of different approaches to vaccination in animal models. These studies have culminated in the production of several different vaccine preparations. The usefulness of therapeutic HPV vaccines will require evidence that they can substantially augment or substitute for the effectiveness of currently available treatments (Duggan-Keen et al. 1998). In addition, the strongly immunogenic characteristics of VLP raise the possibility that they could also serve as vehicles for inducing therapeutic responses against HPV-induced neoplasia and other diseases (Schiller 1999). The perfect HPV vaccine, in fact, will have both preventive and therapeutic capabilities, and because it is likely to be used worldwide, especially in developing countries, it must also have low production costs (van Driel et al. 1999). Prophylactic vaccines against infection with HPV are based on alum adjuvanted VLP. The genotype-specific neutralizing antibody directed at conformational epitopes of the L1 major capsid protein is likely to mediate protection. Therapeutic vaccines for persisting HPV infection can eliminate transplantable tumors in animal models, but are of limited efficacy in mice grafted with skin that expresses HPV antigens or in humans. This paradox has been partially resolved by data clarifying the immune-regulatory role of skin cytokines (e.g. transforming growth factor-beta, TGF-beta, and IL-10) and the consequences of antigen presentation by subsets of skin-associated antigen-presenting cells (Leggatt and Frazer 2007).

In 2003, the WHO convened a gathering of experts including scientists, national regulatory authorities, industry representatives, epidemiologists, and government officials from both developed and developing countries to discuss appropriate endpoint measurements for HPV vaccine efficacy and effectiveness trials. The consultation also considered the regulatory requirements and public health issues that vaccine candidates should address before deployment, particularly in developing countries. The general consensus of the consultation was that it would be desirable to have a globally agreed, measurable efficacy endpoint for considering deployment of HPV vaccines in public health settings. After hearing from experts about virological and clinical endpoints to be considered, requirements of regulatory authorities of various countries and endpoints used to measure efficacy and effectiveness for another known cancer vaccine (hepatitis B), the experts agreed that ethical and time considerations make it necessary to use a SEB, and not invasive cervical cancer, to define efficacy of HPV vaccines. While regulatory authorities of each country ultimately will determine the endpoints required for licensure, the consultation recommended that the endpoint for efficacy in population-based studies be, based on current knowledge, histologically-classified CIN of moderate or HG, as well as cancer. Since persistent infection with the same HR type is considered a predictor for moderate or HG cervical dysplasia and cancer, they might represent a useful endpoint in future vaccine efficacy studies. Indeed, if vaccines prove to be effective against transient or persistent HPV infections, it is likely that they will protect women against cervical cancer. The consultation recognized that in the context of many developing countries, efficacy alone might not provide enough information for countries to decide whether or not to adopt HPV vaccines as a public health prevention tool against cervical cancer. The consultation unanimously agreed that additional clinical bridging studies as well as studies to clarify local epidemiology should be conducted in certain developing countries to determine the potential impact of vaccination. Such countries should also undertake targeted interventions to ensure acceptability and programmatic feasibility of the vaccination. Recognizing that upon vaccine introduction it will be some years before a reduction in cervical cancer is detectable at the population level, the consultation stressed the importance of maintaining existing cervical screening programs while such long-term studies are conducted (Pagliusi and Teresa Aguado 2004).

HPV major capsid protein L1 self-assembles into VLP. Immunization after parenteral vaccination with it provided very good protection against experimental infection in different animal models. The first clinical trials revealed the satisfactory tolerance and excellent immunogenicity of these vaccines. Two vaccine approaches were selected: one based on protection against cervical cancer from a bivalent VLP L1 vaccine containing the two genotypes most frequently involved in cervical cancer (types 16 and 18) and the other, protecting against warts as well as cervical cancer, with a quadrivalent HPV VLP L1 vaccine containing genotypes 6, 11, 16, and 18. Results with these vaccines show an efficacy of more than 90% against infection and 100% against the onset of dysplastic lesions (Hantz et al. 2005). Following preclinical research by laboratories in the non-profit sector, Merck and GlaxoSmithKline have developed commercial versions of the vaccine. The two

prophylactic HPV vaccines are: Gardasil (Merck & Co., Inc., Whitehouse Station, New Jersey, United States of America, USA), a quadrivalent vaccine containing L1 VLP of types 6, 11, 16, and 18, and Cervarix (GlaxoSmithKline Biologicals, Rixensart, Belgium), a bivalent vaccine containing VLP of types 16 and 18. Both vaccines target HPV-16 and HPV-18, which account for approximately 70% of cervical cancer. The Merck vaccine also targets HPV-6 and HPV-11, which account for approximately 90% of external genital warts. The vaccines have an excellent safety profile, are highly immunogenic, and have conferred complete type-specific protection against persistent infection and associated lesions in fully vaccinated women. Unresolved issues include the most critical groups to vaccinate and when the vaccine's cost may be low enough for widespread implementation in the developing world, where 80% of cervical cancer occurs (Lowy and Schiller 2006). Proof-of-principle trials of monovalent vaccines that protect against HR-HPV types have confirmed that intramuscular injection with VLP induces the production of HPV type-specific neutralizing antibodies. The bivalent vaccine incorporating oncogenic HPV types was shown to be safe, well tolerated, and 100% efficacious in preventing persistent HPV infection. The quadrivalent vaccine that protects against genital wart-causing HPV types and oncogenic HPV types demonstrated 100% efficacy in preventing clinical disease. Because VLP vaccines are prophylactic, vaccination before exposure to HPV will result in the greatest public health benefit. Therefore, a successful vaccination program should target preadolescents and stress the importance of vaccination before sexual debut (Ault 2006). HPV quadrivalent recombinant vaccine is administered intramuscularly in a three-dose regimen, with the initial injection followed by subsequent doses at months 2 and 6. The vaccine is indicated for use in the prevention of cervical cancer, vulvar and vaginal precancer and cancers, precancerous lesions, and genital warts in adolescents and young women. The quadrivalent vaccine has demonstrated good immunogenicity in young women (16–26 years) and male and female adolescents (aged 9–15 years), inducing high and persistent anti-HPV antibody titers. In a randomized phase III trial designed to bridge efficacy in young women to adolescents (using immunogenicity as a surrogate), the quadrivalent HPV vaccine in adolescents was at least as immunogenic as that in young women. In double-blind, placebo-RCT in >20,000 young women (aged 16–26 years), the vaccine was highly effective in preventing cervical dysplasia of any grade and external genital lesions. These women were followed-up for an average of 2 years. The vaccine was well tolerated, with injection-site reactions and fever being the most common vaccine-related adverse events (Siddiqui and Perry 2006).

In June 2006, the USA Food and Drug Administration (FDA) approved the quadrivalent HPV vaccine for use among females 9–26 years of age. The Center for Disease Control (CDC) and Prevention's Advisory Committee on Immunization Practices (ACIP) has recommended the three-dose series for girls 11–12 years of age, catch-up vaccination for girls and women 13–26 years of age, and permissive use as early as age 9 (Mahoney 2006). The licensed HPV vaccine is composed of the HPV L1 protein, the major capsid protein of HPV. Expression of the L1 protein in yeast using recombinant DNA technology produces non-infectious VLP that resemble HPV virions. The quadrivalent HPV vaccine is a mixture of four HPV

type-specific VLP prepared from the L1 proteins of HPV-6, 11, 16, and 18 combined with an aluminium adjuvant. Clinical trials indicate that the vaccine has high efficacy in preventing persistent HPV infection, cervical cancer precursor lesions, vaginal and vulvar cancer precursor lesions, and genital warts caused by HPV types 6, 11, 16, or 18 among females who have not already been infected with the respective HPV type. No evidence exists of protection against disease caused by HPV types with which females are infected at the time of vaccination. However, females infected with one or more vaccine HPV types before vaccination would be protected against disease caused by the other vaccine HPV types. The vaccine is administered by intramuscular injection, and the recommended schedule is a three-dose series with the second and third doses administered 2 and 6 months after the first dose. Vaccination is not a substitute for routine cervical cancer screening, and vaccinated females should have cervical cancer screening as recommended (Markowitz et al. 2007).

Cervarix is a prophylactic vaccine comprised of a mixture of VLP derived from the L1 capsid proteins of HPV types 16 and 18 formulated with the adjuvant system (AS) 04. It is administered by intramuscular injection as a three-dose vaccine regimen at 0, 1, and 6 months. The vaccine is indicated for the prevention of HG-CIN 2 and CIN 3 and cervical cancer causally related to HPV types 16 and 18. In double-blind, phase II or III RCT in>19,000 women aged 15–25 years, the HPV-16/18 vaccine showed high efficacy in preventing CIN 2+ associated with HPV-16/18. Cross-protection against new incident or 6-month persistent HPV-31 or HPV-45 infection was also evident. In a bridging study, in adolescent girls aged 10–14 years, the HPV-16/18 vaccine induced twice the already high antibody titers as it did in young women (aged 15–25 years). The immune response in older women (aged 26–55 years) at 24 months in another study was ≥8-fold higher than antibody levels reported in younger age groups. Anti-HPV-16/18 antibody responses were greater with an AS04-adjuvated HPV-16/18 vaccine than with an aluminum salt-adjuvated formulation. The HPV-16/18 vaccine was generally well tolerated and injection-site reactions were the most common vaccine-related adverse events reported (Keam and Harper 2008). The AS04-adjuvated vaccine Cervarix was developed focusing on preventing cervical cancer by inducing durable protection against new infection. In clinical trials, it shows evidence of cross-protection against other important oncogenic HPV types using a range of clinic-pathological and virological endpoints. The current evidence suggesting the cross-protective effect comes from its overall impact on precancerous lesions and on 12-month or more persistent oncogenic HPV infection, together with specific evidence of protection against incident and new persistent infection lasting 6 months or more with individual HPV types. The use of virological endpoints for such studies, in particular for cross-protection evaluation, in view of the lower frequency of many important oncogenic HPV types other than HPV-16 or HPV-18 in precancerous lesions and the frequent presence of multiple HPV infections, complicate the interpretation of type-specific, vaccine-induced protection against CIN lesions, in which other HPV DNA types are found along with HPV-16 and HPV-18. The observed high level of overall protection against clinic-pathological lesions, including CIN 2+ in the vaccinated subjects (regardless of their HPV DNA status), predicts a potentially broader impact of

the vaccine in the prevention of HPV-related pre-cancers that goes beyond HPV-16 and HPV-18. The prevention of persistent infections by individual types such as HPV-45 provides specific information on the protection against that type, using an alternative endpoint that relates to both precancer and cancer development. Together with sustained protection against HPV-16 and HPV-18, protection against HPV-45 could offer an additional effect on invasive cervical cancer and may have an important impact on endocervical adenocarcinoma, which is not effectively prevented by screening and is becoming increasingly important in young women (Jenkins 2008).

Studies of the HPV vaccines, Cervarix and Gardasil, provide strong evidence for the recommendation that HPV vaccines may minimize the incidence of cervical cancer over time. Both Cervarix and Gardasil provided more than 90% efficacy in preventing CIN 2+ disease caused by HPV-16 and HPV-18 in women 16–26 years who were seronegative and PCR-negative for HPV-16 and HPV-18 at baseline. Cervarix provides more than 75% efficacy in independent cross-protection against persistent HPV-31 and HPV-45, and 47% efficacy against HPV-33, whereas Gardasil offers 50% efficacy only against persistent HPV-31. A reduction in excisional therapies for CIN 2+ is nearly 70% for Cervarix, and 40% for Gardasil. Immunologically, Cervarix induces threefold to ninefold higher peak-neutralizing antibody titers to HPV-16/18 than Gardasil, has significantly higher cervico-vaginal mucus-neutralizing antibody presence than Gardasil, and significantly higher B-memory-cell response than Gardasil (Harper 2009a). HPV-16/18 AS04-adjuvated vaccine has demonstrated type-specific protection against the five most frequent cancer-causing types (16, 18, 31, 33, and 45) that are responsible for 82% of invasive cervical cancers globally. Cervarix has demonstrated efficacy against HPV-45, which is the third most common HPV type in cervical cancer and adenocarcinoma. Final results of a large phase III trial recently showed Cervarix substantially reduced the overall burden of cervical precancerous lesions (CIN 2+) by 70.2% in an HPV-naïve population approximating young girls prior to sexual debut, the target of most current vaccination programs. Protection offered by Cervarix against non-vaccine types (mainly 31, 33, and 45) might potentially allow for 11–16% additional protection against cervical cancers, compared to a vaccine only offering protection against HPV-16/18. Another recent study directly compared the antibody response of Cervarix to that of quadrivalent HPV-6/11/16/18 vaccine. Cervarix induced significantly superior neutralizing antibody levels as compared with Gardasil for HPV-16 and HPV-18 in all age groups studied. This may translate into more women having detectable (neutralizing) antibodies in cervico-vaginal secretions for HPV-16 and HPV-18 after vaccination with Cervarix when compared with Gardasil. Cervarix induced significantly higher frequencies of antigen-specific memory B-cells and T-cells in responders for HPV-16 and HPV-18 as compared with Gardasil. Cervarix continues to show sustained high levels of total and neutralizing antibodies for HPV-16 and HPV-18, associated with high efficacy and no breakthrough cases in the HPV-naïve population, and is the longest duration follow-up for safety, immunogenicity, and efficacy for any licensed HPV vaccine to date (Schwarz 2009). Safety reports indicate injection site reactions for both Cervarix and Gardasil. Rare

serious adverse events have been reported. Although Guillain-Barré syndrome and death have been reported in women who received the vaccine, an analysis of available data by the USA FDA found no association between the vaccine and these adverse events. Since post-vaccination syncope is common among young women, providers should ensure that patients remain seated when vaccinated and under observation for at least 15 min following vaccination (Tovar and Bazaldua 2008).

In well-designed clinical trials in young women aged 15–25 years who were HPV-16/18 seronegative and DNA negative to 14 HR-HPV types, high levels of immunogenicity and protection were sustained for follow-up periods of up to 8.4 years. High and persistent immunogenicity against infection with HPV-16/18 has also been demonstrated in older and younger females (aged 10–55 years) who were seronegative for vaccine HPV types. The AS04-adjuvanted HPV-16/18 vaccine elicited a greater immunogenic response than the quadrivalent HPV vaccine in women aged 18–45 years who were seronegative and DNA negative for HPV-16/18. The AS04-adjuvanted HPV-16/18 vaccine confers cross protection against certain non-vaccine, HR-HPV types. A rapid and strong anamnestic humoral immune response was elicited following a fourth dose of the vaccine. The AS04-adjuvanted HPV-16/18 vaccine is generally well tolerated, and pharmacoeconomic analyses have demonstrated the potential for public health benefits and cost effectiveness when vaccination programmes are run in conjunction with screening programmes. Thus, the AS04-adjuvanted HPV-16/18 vaccine prevents cervical disease associated with certain oncogenic HPV types, thereby reducing the burden of premalignant cervical lesions and, very likely, cervical cancer (Chiriva-Internati, Kast and Bot 2012; Einstein et al. 2011a; Einstein et al. 2011b; McKeage and Romanowski, 2011; No et al. 2011; Romanowski et al. 2011; Frederiksen, Lynge and Rebolj 2012; Shi et al. 2012).

In October 2009, the ACIP approved the Cervarix vaccine. The ACIP also expanded its recommendations against HPV by giving permission to physicians to vaccinate males aged 9–26 years with the previously licensed vaccine, Gardasil, to prevent genital warts, in addition to its previous recommendation for females aged 9–26 years to prevent cervical cancer and genital warts. The marketing, expense, safety, and reactivity of Gardasil and Cervarix continue to be the subject of controversy (Broomall et al. 2010). In efficacy trials involving young women, both vaccines produced outstanding efficacy against primary and secondary endpoints associated with the vaccine type HPV and were highly and consistently immunogenic. Both had excellent safety records and, as expected, the most frequent vaccine-related adverse effects were mild to moderate injection site sequelae. No evidence of waning protection was observed after 4 years for endpoints examined ranging from incident infection to CIN 3 associated with the vaccine type HPV. Gardasil was also highly efficacious at preventing vaginal/vulvar lesions and genital warts. However, neither vaccine demonstrated therapeutic efficacy against prevalent infections or lesions, regardless of the associated HPV type. Cervarix has shown limited cross-protection against infection with specific closely related types while preliminary results of limited cross-protection have been presented for Gardasil. As expected, more limited efficacy was noted for both vaccines when women with prevalent infection were included or endpoints associated with any HPV type were evaluated. Immunological

bridging trials involving adolescent girls and boys were also recently published. For both vaccines, serum VLP antibody levels in girls were non-inferior to those generated in young women and antibody response to Gardasil was also non-inferior in boys. The results of these studies have led to the approval of Gardasil and Cervarix by national regulatory agencies in a number of countries (Schiller et al. 2008). Both vaccines are very immunogenic and well tolerated. They have been shown in the various RCT to be very effective at preventing infection and premalignant disease related to the vaccine HPV genotypes in women who were DNA negative and seronegative for the vaccine HPV types at base line. HPV vaccines containing HPV-6/11 will reduce the incidence of genital warts by 80–90% in the medium term. The vaccines will reduce but not eliminate the risk of cervical cancer since at the present they only target two of the oncogenic genital types. Cervical cancer screening programs will remain as important secondary interventions for cervical cancer even in vaccinated populations. The duration of protection remains unknown but there is evidence of good immune memory, it is possible that protection will be long lasting. The primary target group for cost effective immunization with HPV vaccines are peripubertal females. There may be benefit in vaccinating other groups (men, sexually active women of all ages) but the cost-effectiveness of these interventions will need to be evaluated. In societies in which organized screening programs are not available, HPV vaccines are probably the most realistic intervention against HPV-associated disease. Second generation vaccines that offer protection against additional types are term stable, and delivered by non-injection methods are an important area of investigation (Stanley 2008b). However, many controversial issues still remain regarding routine administration and widespread acceptance. These include appropriate age at time of vaccination, parental concerns, vaccination of men or women age 26 years or older, inadequate long-term efficacy and safety data, and potential for non-vaccine-related strains to emerge as prominent oncogenic serotypes. HPV vaccines provide a high level of protection for seronegative women against persistent infection and precancerous cervical lesions associated with vaccine-specific HPV types. However, many controversial issues still remain regarding the vaccines' routine administration and widespread acceptance (Hutchinson and Klein 2008). In particular, the following issues are still debated:

1. The public health benefit of mandated HPV vaccination is not sufficient to warrant the intrusion on parental autonomy
2. A vaccine that prevents a non-casually transmitted infection should not be mandated
3. Opt-out provisions are inherently unfair to parents who oppose HPV vaccination
4. Limited health care dollars should not be directed toward cervical cancer prevention
5. The vaccine is expensive and potential problems with supply suggest that mandates should not be implemented until insurance coverage and supply issues are resolved
6. Giving girls HPV vaccine implies tacit consent to engage in sexual activity
7. Giving girls this vaccine will confer a false sense of protection from STD and will lead to sexual uninhibition

8. Children already have too many vaccinations on the immunization schedule
9. Long-term side effects of HPV vaccine are unknown
10. The vaccine's enduring effectiveness is unknown and booster shots may be required
11. It is wrong to only target girls with HPV vaccine: boys should be vaccinated as well (Haber et al. 2007)

The success of HPV vaccination programs will depend on individuals' willingness to accept vaccination, parents' willingness to have their pre-adolescent and early adolescent children vaccinated, and health care providers' willingness to recommend HPV vaccination. The research published to date suggests that there is a good deal of misunderstanding about HPV infection, cervical cancer screening, and the sequelae of HPV infection. However, the majority of research studies to date indicate that young women, parents, and health care providers are interested in vaccines that prevent HPV and other STD. Of particular note are the consistent findings that providers are less comfortable vaccinating younger versus older adolescents and that endorsement of vaccination by a professional organization is of great importance. Furthermore, research suggests that most parents are interested in having their preadolescent and adolescent children vaccinated against HPV. Parents value the information and recommendations provided by their children's health care providers. To the extent that providers are concerned about potential negative reactions of parents to a recommendation of HPV vaccination, these findings should provide reassurance. At the same time, health care providers will need to be prepared to provide patients and parents with information about HPV and HPV immunization and to respond productively to the rare parent who expresses opposition to HPV vaccine or any other vaccine (Zimet 2005). Furthermore, there may be unique issues related to the acceptance of a vaccine designed to prevent a STD that is poorly understood by many women. Among the acceptance issues are: individual comfort with a STD vaccine, parental comfort with vaccination of their preadolescent/early adolescent daughters, physician comfort with recommending a HPV vaccine to women and parents of preadolescents, and physician communication skills related to talking with women and parents about the vaccine. Potentially difficult as it might be to implement a vaccination program, vaccination and prevention of HPV-associated disease are still infinitely preferable to observation and treatment (Zimet 2006). Recent studies document stark knowledge gaps about HPV at all levels (among policy makers, healthcare providers, parents, and teens) in both the industrialized and developing worlds. Pharmaceutical companies, public health advocates, medical trainers, and health educators need to understand their diverse audiences and respond appropriately to the needs of each. They also must use research-based communication strategies and materials to most effectively, and accurately, convey the need for an HPV vaccine and to manage expectations about how the vaccine can, and cannot, protect women and men (Sherris et al. 2006).

Given the absence of data on the long-term effectiveness of HPV vaccination, a number of mathematical models have been developed to provide insight to policy makers by projecting the long-term epidemiologic and economic consequences

of vaccination and evaluate alternative vaccination policies. Three types of HPV mathematical models have been reported in the literature: cohort, population dynamic, and hybrid. All have demonstrated that vaccination can significantly reduce the incidence of cervical cancer in the long term. However, only the cohort and hybrid models have evaluated the cost-effectiveness of vaccination strategies for preventing cervical cancer. These models have generally shown that vaccinating females can be cost-effective. None has accounted for the potential benefits of vaccinating the population to reduce the burden of recurrent respiratory papillomatosis and cancers of the vagina, vulva, anus, penis, and head/neck. Given that only the population dynamic model can account for both the direct and indirect (i.e. herd immunity effects) benefits of vaccination in the population, future research should focus on further development of dynamic models by expanding the range of epidemiologic outcomes tracked and including the ability to assess the cost-effectiveness of alternative vaccination policies (Dasbach et al. 2006). More than 80% of cervical cancer cases occur in developing countries, and this is expected to increase to 90% by the year 2020. The 5-year survival rate of patients in developing countries is less than 50%, compared to 66% in developed nations. A worldwide HPV vaccine program would significantly reduce the spread of HPV-16 and HPV-18 and lower the incidence of cervical cancer. Mathematical models have determined that vaccinating 66% of the population will decrease the incidence of cervical cancer by 80% over the next 40–60 years. For every 5-year delay in a cervical cancer prevention/detection program, there will be an additional 1.5–2.0 million deaths. The introduction of a vaccination program will be a challenge due to high costs, unknown durability of the vaccine, and the potential for new oncogenic strains to emerge. A global effort will be required to eliminate cervical cancer from developing countries (Kling and Zeichner 2010). Studies on the distribution of HPV types indicate that the marketed vaccines could lower the incidence of cancer and CIN 2–3 by about 67% and 53%, respectively, after taking into account reported cross-protection. About 65% of women below 25 years of age had lesions related to the non-vaccine types and after the last normal smear these cases accumulated at the same frequency as cases with vaccine-included types. Cases with combined vaccine and non-vaccine types accumulated at a slower rate. Screening should continue to start at age 20 years, with invitations at 2-year intervals up to age 39 years and thereafter at 4-year intervals up to age 65–69 years. Current data support the conclusion that the optimal age for catch-up HPV vaccination should be considered in the context of sexual practices and the data do not support changes in the lower age limit or screening intervals for the vaccinated women (Sigurdsson 2010). Cost-effectiveness models that combine routine vaccination with the HPV vaccine with temporary catch-up programs are variable. Results were dependent on differences between models used, their design, and input data. Modeling aspects and assumptions were not always sufficiently described, making comparison difficult. Despite this, several differences between models likely to impact results were identified. All models used dynamic transmission modeling techniques except for one, which did not incorporate the effect of herd immunity. Catch-up strategies varied between models and comparator strategies were not necessarily the same. Cervical diseases outcomes were considered in all

base cases, but the impact of genital warts was not always considered. A conclusion on cost-effectiveness should be based on a fully transparent model including all possible benefits of vaccination (de Peuter et al. 2010). Although long-term protection is a key-point in evaluating HPV-vaccine over time, there is currently inadequate information on the duration of HPV vaccine-induced immunity and on the mechanisms related to the activation of immune-memory. Longer-term surveillance in a vaccinated population is needed to identify waning immunity, evaluating any requirements for booster immunizations to assess vaccine efficacy against HPV-diseases. Current prophylactic vaccines have the primary end-points to protect against HPV-16 and HPV-18, the genotypes more associated to cervical cancer worldwide. Nevertheless, data from many countries demonstrate the presence, at significant levels, of HPV that are not included in the currently available vaccine preparations, indicating that these vaccines could be less effective in a particular area of the world. The development of vaccines covering a larger number of HPV represents the most complex challenge for the future (Mariani and Venuti 2010).

HPV vaccines will likely have an impact as a preventive strategy for cervical cancer. Screening for precancerous lesions cannot be discontinued, because vaccination will not protect against HPV types not included in the first generation of vaccines. Moreover, protection for the target types cannot be expected to be absolute, and the likely implementation of HPV vaccination in young women will not impact older groups initially. Cervical cancer control programs will need to be re-evaluated because the addition of HPV vaccination will make the existing approach of high frequency screening by cytology too costly and inefficient for most public health budgets. Simply making cytology screening less frequent may not be a viable strategy in light of potential problems that may plague cytology performance in conditions of low lesion prevalence. HPV testing has the performance characteristics that would make it an ideal primary screening test in such conditions. Cytology should be reserved for triage of HPV-positive cases, because it is more likely to perform with sufficient accuracy in high prevalence conditions. Another advantage of using HPV testing as a primary screening tool is the opportunity to create infection registries that can link test results from the same women over time, thus allowing an efficient and low-cost strategy to monitor long-term protection among vaccinated women (Franco et al. 2006). In some developed countries cervical screening programs have reduced the incidence of invasive cervical cancer by up to 80%, although this decline has now reached a plateau with current cancers occurring in patients who have failed to attend for screening or where the sensitivity of the tests have proved inadequate. Cervical screening is inevitably associated with significant anxiety for the many women who require investigation and treatment following abnormal cervical cytology. However, it is vitally important to stress the need for continued cervical screening to complement vaccination, in order to optimize prevention in vaccinated women and prevent cervical cancer in older women where the value of vaccination is currently unclear. It is likely that vaccination will ultimately change the natural history of HPV disease by reducing the influence of the highly oncogenic types HPV-16 and HPV-18. In the long term, this is likely to lead to an increase in recommended screening intervals. HPV vaccination may also reduce the positive

predictive value of cervical cytology by reducing the number of truly positive abnormal smears. Careful consideration is required to ensure vaccination occurs at an age when the vaccine is most effective immunologically and when uptake is likely to be high. Antibody titers following vaccination in girls 12–16 years have been shown to be significantly higher than in older women, favoring vaccination in early adolescence prior contact with the virus. Highest prevalence rates for HPV infection are seen following the onset of sexual activity and therefore vaccination would need to be given prior to sexual debut. Since 20% of adolescents are sexually active at the age of 14 years, vaccination has been suggested at 10–12 years. However, parental concerns over the sexual implications of HPV vaccination may reduce uptake of vaccination thereby reducing the efficacy of an HPV vaccination program. Concerns have already been raised over the acceptability of a vaccine preventing a STD in young adolescents, particularly amongst parents or communities who consider their children to be at LR of infection. This may be a particularly sensitive issue for ethnic minority groups (Adams et al. 2007).

HPV testing prior to HPV vaccination is not recommended, unless HPV tests are part of the established local routines for cervical cancer screening. The reasoning is based upon the very low frequency of women who, at the time of vaccination, would show markers of prior/current exposure (HPV DNA or serological tests) to the HPV types included in the vaccine. Thus, at least 1,000 women would need to be screened to find one that is HPV-16 and HPV-18 DNA positive. The increase in cost and the other barriers afforded by a prior to vaccination test requirement would result in a lower coverage, the key indicator of a successful vaccination program (Wright and Bosch 2008). The availability of two prophylactic HPV vaccines will require thorough considerations about monitoring and surveillance of those vaccinated and the general population, respectively. Vaccinated populations should be followed-up for long-term safety, sustained immune responses, and vaccine efficacy. Effective monitoring will benefit from linkage of vaccination history and screening history, as well as precise measurement of HPV exposure, both DNA and serological testing. Lack of record linkage in many settings is one of the main obstacles for an effective surveillance program, though other surveillance activities can make contributions to assessing HPV vaccine effectiveness, including information from organized screening programs and phase IV studies (Stanley and Villa 2008). Anti-Papillomavirus vaccination of young girls before their first sexual encounter is now a common practice. However, this prophylactive measure could also be extended to older patients. HPV infection is indeed not limited to teenagers: even if the highest incidence rate is noticed between the age of 18 and 30, it can also be found in older women. These show a sustained prevalence due to the longer persistence of the infection. This is clearly illustrated by the incidence of cervical cancer after the age of 50. Moreover, phase III studies in large unselected populations have shown the effectiveness of HPV vaccination in patients who had previously been infected by HPV (and got cured of it) as well as with patients who had never had any Papillomavirus contact. Actually, less than 1% of women who present simultaneously a HPV-16 and a HPV-18 infection will not derive any benefit from a HPV vaccination. Therefore, it seems logical to positively consider a HPV prophylaxis in

patients who are above the age of 25 and who do not present any Papillomavirus-induced cervical lesion (Simon and Poppe 2008).

The costs of developing and producing new-generation vaccines have increased compared to many of the older, "traditional" vaccines because of new technologies and regulatory requirements. While the public sector often supports basic research costs, private manufacturers are usually responsible for the investments in product development and production scale-up. When considering investments, firms evaluate the probability of a market. Unfortunately, the developing country vaccine market is small (in revenue terms) and often unpredictable, particularly given inaccurate forecasting in the past. Low-income developing countries expect low prices. Demand (actual decisions to pay for and introduce the vaccine) is almost always lower than need (estimates of requirements to achieve optimal public health outcomes), a distinction that may be even more significant for HPV vaccines given the number of new vaccines against priority diseases that will become available over the coming 10 years. One new mechanism under consideration to address some of these challenges is Advanced Market Commitments (AMC). By providing an assured price subsidy for developing country purchase of a future vaccine meeting predefined standards, an AMC would provide industry with greater assurances of earning a reasonable return on their investment to serve the poorest developing countries. The AMC mechanism could provide critical motivation for increased industry (private) investment that would otherwise not occur. HPV vaccines are one of six vaccines being considered for a possible AMC pilot (Batson et al. 2006). Considerable variation was observed across HPV vaccine cost-effectiveness models in a number of influential assumptions. Holding constant factors for which current data are lacking, the combined impact of assumptions made for the remaining parameters examined would appear to tend toward underestimation of the cost-effectiveness of HPV vaccination within existing studies. However, uncertainty concerning parameters, such as the duration of vaccine protection and acquired immunity following HPV infection, and the relationship between age and HPV virulence, complicates precise estimation of the cost-effectiveness of HPV vaccination and rigorous evaluation of the validity of existing modeling results (Insinga et al. 2008).

There are large study variations in estimates of the cost per quality-adjusted year of life (QALY) gained. The most influential source of uncertainty is the duration of the vaccine protection. Between-study variations are mainly due to three causes: methodological differences, assumptions, and local conditions in the application area. Economic evaluation models are increasingly sophisticated, but scientific treatment of epidemiological and market uncertainty does not compensate for the lack of basic information. The large disparities in cost per QALY estimates of massive vaccination programs around the world may be attributed to several critical sources (unavoidable and avoidable) of uncertainty. An asset of economic evaluation is the ability to highlight the areas of research that could be undertaken to reduce uncertainty (Puig-Junoy and Lopez-Valcarcel 2009). The total cost of HPV-related diseases accounts for euro 200–250 million of which euro 210 million is absorbed by the prevention and treatment of precancerous lesions and cervical cancer. Although both available HPV vaccines are below the threshold value for economic convenience

(euro 9,569 and euro 26,361 per QALY gained for the quadrivalent and bivalent vaccines, respectively), at this point in time, long-term economic models developed for Italy seem to indicate the quadrivalent vaccine as the most cost-effective option. Recent publications by official bodies including the WHO and the Supervisory Authority for Public Contracts in Italy, recommend that the decision-making process be based on both the quality of goods and services as well as the best achievable price (Mennini et al. 2009). However, the current cost of these vaccines precludes sustained global delivery, and they target only two of the approximately 15 known oncogenic HPV types, although approximately 70% of cervical cancer cases are attributed to these two types and there is evidence for some degree of cross-protection against other closely related types. A possible approach to broader immunity at lower cost is to consider vaccination against L2. L2 vaccines can be produced inexpensively and they also have the promise of conferring much broader cross-type protective immunity than that observed with L1 VLP immunization. However, L2 vaccine development lags behind L1 VLP vaccines and several technical hurdles remain (Karanam et al. 2009).

Modeling approaches have been used to estimate the impact of HPV vaccination on the performance of Pap cytology screening under different assumptions of lesion prevalence and expected changes in sensitivity and specificity likely to prevail post-vaccination. A major driver of the efficiency and costs of screening, the positive predictive value will be severely affected if Pap cytology continues to serve as the primary screening test in the post-vaccination era. Molecular-based screening with an HPV DNA test followed by Pap triage of HPV-positive cases has the potential for circumventing this problem. As a primary screening test, HPV testing can improve the overall quality of screening programs, thus allowing for increased testing intervals that would lower program costs with acceptable safety. Cytology should be reserved for the more labor-efficient task of triaging HPV-positive cases, a situation in which case loads would be "enriched" with smears containing relevant abnormalities. HPV followed by Pap strategy can also serve a secondary role in post-vaccination surveillance (Franco et al. 2009b). When the first preventive HPV vaccine became available in 2006, it drew both enthusiasm and multiple ethical problems. In the case of HPV vaccination, there is a clear conflict between the scientific data that claim a definitive advantage for preventing HPV infection in the exposed population and the ethical and moral issues resulting from a compulsory program. Despite the evident success of routine and compulsory vaccination in young women, there is increasing concern about safety, efficacy, and equity of the vaccine and to close the knowledge "gaps" about HPV infection and consequent health outcomes. Some of these fears are expressed particularly in conservative groups that link these arguments to those of religious and moral issues contending that HPV vaccination is an indirect licence for liberal sexual activity in youths, resulting in promiscuity and/or less participation in cervical cancer screening. It has been well demonstrated that HPV infection can lead to harm through the induction of premalignant and cancerous lesions. Therefore, any proven method for preventing infection, such as HPV vaccines, should be used in persons at risk. These policies, however, should be strictly linked to cervical cancer screening programs (Benítez-Bribiesca 2009).

Approaches for cervical cancer prevention are changing. Screening still remains the most effective method for cervical cancer prevention. Guidelines are moving to an older group of women to be screened less frequently with combinations of technologies that include biomarkers and cytology. HPV vaccination is an appropriate option for this older group of women as well, should the woman not wish to make her decision about vaccination until 21 years of age, the age of screening. Parents making decisions about HPV vaccination for their young adolescent daughters need to be fully informed that only continued screening prevents cervical cancer. HPV vaccination reduces the possibility of their daughter having an abnormal Pap test by 10% if the vaccines have not waned by the time the young adolescent becomes sexually active. HPV vaccine efficacy must last at least 15 years to contribute to the prevention of cervical cancers. At this time, protection against CIN 2/3 is 5 years for Gardasil and 8.4 years for Cervarix. Different women will view the value of the current protection HPV vaccines offered in various ways. Physicians' ethical duties are to provide full explanation of the risks and benefits of adding HPV vaccination to the ongoing screening programs, and to support women in their personal choice for cervical cancer prevention (Harper and Williams 2010). HPV vaccination is expected to reduce the burden of cervical cancer in most settings, however it is also expected to interfere with the effectiveness of screening. In the future, maintaining Pap cytology as the primary cervical screening test may become too costly. As the prevalence of cervical dysplasia decreases, the positive predictive value of the Pap test will also decrease and, as a result, more women will be referred for unnecessary diagnostic procedures and follow-up. HPV DNA testing has recently emerged as the most likely candidate to replace cytology for primary screening. It is less prone to human error and much more sensitive than the Pap smear in detecting HG cervical lesions. Incorporating this test would improve the overall quality of screening programs and allow spacing out screening tests, while maintaining safety and lowering costs. Although HPV testing is less specific than Pap cytology, this issue could be resolved by reserving the latter for the more labor-efficient task of triaging HPV-positive cases. Because most HPV-positive smears would contain relevant abnormalities, Pap cytology would be expected to perform with sufficient accuracy under these circumstances. HPV Pap triage would also provide a low-cost strategy to monitor long-term vaccine efficacy. Although demonstration projects could start implementing HPV testing as a population screening tool, more research is needed to determine the optimal age to initiate screening, the role of HPV typing and other markers of disease progression, and appropriate follow-up algorithms for HPV-positive and Pap-negative women (Tota et al. 2010).

As we have said, several lines of evidence suggest that cell-mediated immune responses are important in controlling both HPV infections and HPV-associated neoplasia. Since HPV E6 and E7 oncoproteins are constantly expressed in these lesions and are necessary for the maintenance of the malignant phenotype, these foreign proteins might represent potential ideal tumor-specific target antigens for immuno-therapy of cervical cancer. The gold standard treatment for locally advanced cervical cancer is primary radiation therapy and chemotherapy combined with radical surgery. Although these represent effective modalities of treatment for invasive

cervical cancer, up to 35% of these patients overall will develop recurrent/metastatic disease for which treatment results remain poor. Novel therapeutic strategies that are effective in reducing the risk of recurrence/metastatic disease are still needed desperately. A potential drawback of this potentially curative treatment is a profound and long lasting negative effect on the immune system. Treatment-induced immunosuppression combined with tumor-induced subversion of the immune system may therefore impose severe limitations on the efficacy of conventional vaccination strategies in late stage cervical cancer patients. Recently, the recognition of DC as powerful antigen-presenting cells capable of inducing primary T-cell responses in vitro and in vivo has recently generated widespread interest in DC-based immunotherapy of several human malignancies (Santin et al. 2005; Bellone et al. 2007). Transmission of Papillomavirus may be prevented by the generation of antibodies to capsid proteins L1 and L2 that neutralize viral infection. Since the capsid proteins are not expressed at detectable levels by infected basal keratinocytes or in HPV-transformed cells, therapeutic vaccines generally target the nonstructural early viral antigens. Two HPV oncogenic proteins, E6 and E7, are critical to the induction and maintenance of cellular transformation and are co-expressed in the majority of HPV-containing carcinomas. Although other early viral antigens show promise for vaccination against Papillomaviruses, therapeutic vaccines targeting E6 and E7 may provide the best opportunity to control HPV-associated malignancies. Various candidate therapeutic HPV vaccines are currently being tested whereby E6 and/or E7 are administered in live vectors, as peptides or proteins, in nucleic acid form, as components of chimeric VLP, or in cell-based vaccines (Roden and Wu 2003; Roden et al. 2004; Tomson et al. 2004; Mahdavi and Monk 2005; Lin et al. 2007). Two types of second-generation VLP-based subunit vaccines with therapeutic implications, both related and unrelated to Papillomavirus infection, are in preclinical development. One type seeks to induce cell-mediated immune responses, especially CTL, against non-structural Papillomavirus proteins, proteins of other viruses, or tumor associated antigens. The target antigen is incorporated into the VLP as a fusion protein of L1 or the L2 minor capsid protein. In mouse models, this approach has generated potent CTL responses after low-dose vaccination in the absence of the adjuvant. The second type of therapeutic VLP-based vaccine seeks to generate autoantibodies to self-antigens. The display of self-polypeptides in the context of the highly ordered array of repetitive elements on the Papillomavirus VLP surface abrogates the ability of the humoral immune system to functionally distinguish between foreign and self. High titer and high avidity auto-reactive IgG antibodies have been induced to both soluble (tumor necrosis factor-alpha, TNF-alpha) and cell surface (chemokine receptor 5, CCR5) central self-antigens. Vaccines based on this approach could potentially be effective alternatives to monoclonal antibody (mAb)-based therapies for a variety of disease targets (Schiller and Lowy 2001).

Polynucleotide and recombinant viral vaccines encoding non-structural viral proteins show therapeutic and prophylactic efficacy in animal models and are candidate immunotherapies for established LG benign genital infections. Recombinant virus, peptide, protein, polynucleotide, and DC vaccines designed to illicit CTL specific for the HPV oncoproteins E6 and E7 show immunogenicity and efficacy in transplantable

tumor models in rodents. Immunogenicity, but no efficacy, has been demonstrated in small clinical trials with some of these approaches (Stanley 2002). These E6/E7 oncoproteins are the only HPV-coded proteins expressed in cervical cancer. They are involved in malignant transformation of HPV-infected cells, their presence is necessary for the maintenance of the malignant phenotype of the cells, and their expression correlates with the transforming potential of HPV. Therefore, the E6/E7 oncoproteins are used for the construction of therapeutic vaccines against HPV-associated neoplasms (Bubeník 2002). Using either proteins or peptides based on E6 and E7 oncoproteins of HPV-16 and HPV-18, clinical trials of therapeutic vaccines against HPV-associated cervical cancers have recently been reported. Although the effectiveness of these vaccines cannot be evaluated in such studies, they constitute an important step toward the development of therapeutic uterine cervix cancer vaccines. A polytope DNA vaccination approach combined with immune-modulatory cytokines may offer an excellent strategy to reduce the risk of relapse and metastasis following conventional therapies (Gariglio et al. 1998). A variety of approaches are being tested in therapeutic vaccine clinical trials and in various preclinical animal Papillomavirus models for efficacy. Approaches include genetic vaccines, recombinant virus vaccines, DC-based strategies, immune-modulatory strategies, and various combination strategies to maximize cell-mediated immunity to Papillomavirus proteins present in HPV infections and cancers. The success of preventive HPV VLP vaccines is clear. However, current therapeutic vaccine trials are less effective with respect to disease clearance. Nevertheless, a series of combination approaches have shown significant therapeutic enhancement in preclinical Papillomavirus models and await testing in patient populations to determine the most effective strategy. There is much encouragement that HPV vaccines will be the most effective approach to prevention and cure of infections caused by this group of viruses, which represent a significant human pathogen (Christensen 2005).

In summary, although a prophylactic vaccine is available, millions of women, already infected, will continue to suffer from HPV-related disease, emphasizing the need to develop therapeutic vaccination strategies. A majority of clinical trials examining therapeutic vaccination have shown limited efficacy due to examining patients with more advanced-stage cancer who tend to have decreased immune function. Current trends in clinical trials with therapeutic agents examine patients with pre-invasive lesions in order to prevent invasive cervical cancer. However, longer follow-up is necessary to correlate immune responses to lesion regression. Meanwhile, preclinical studies in this field include further exploration of peptide or protein vaccination, and the delivery of HPV antigens in DNA-based vaccines or in viral vectors. As long as pre-clinical studies continue to advance, the prospects of therapeutic vaccination to treat existing lesions seem good in the near future. Positive consequences of therapeutic vaccination would include less disfiguring treatment options and fewer instances of recurrent or progressive lesions leading to a reduction in cervical cancer incidence (Brinkman et al. 2007). Two HPV oncogenic proteins, E6 and E7, are consistently co-expressed in HPV-expressing cervical cancers and are important in the induction and maintenance of cellular transformation. Therefore, immuno-therapy targeting E6 and/or E7 proteins may provide an opportunity to

prevent and treat HPV-associated cervical malignancies. It has been established that T cell-mediated immunity is one of the most crucial components to defend against HPV infections and HPV-associated lesions. Therefore, effective therapeutic HPV vaccines should generate strong E6/E7-specific T-cell-mediated immune responses. DNA vaccines have emerged as an attractive approach for antigen-specific T-cell-mediated immuno-therapy to combat cancers. Intradermal administration of DNA vaccines via a gene gun represents an efficient way to deliver DNA vaccines into professional antigen-presenting cells in vivo. Professional antigen-presenting cells, such as DC, are the most effective cells for priming antigen-specific T-cells. Using the gene gun delivery system, there have been tested several DNA vaccines that employ intracellular targeting strategies for enhancing major histocompatibility complex (MHC) class I and class II presentation of encoded model antigen HPV-16 E7. Furthermore, it has been developed a strategy to prolong the life of DC to enhance DNA vaccine potency. More recently, it has been developed a strategy to generate antigen-specific helper (CD4+) and cytotoxic (CD8+) T-cell immune responses to further enhance DNA vaccine potency. The impressive pre-clinical data generated from these studies have led to several HPV DNA vaccine clinical trials (Hung et al. 2007). Therapeutic vaccines however, are expected to have an impact on cervical cancer or its precursor lesions, by taking advantage of the fact that the regulatory proteins (E6 and E7) of HPV are expressed constantly in HPV-associated cervical cancer cells. Vaccine types targeting these regulatory proteins include the recombinant protein and DNA vaccines, peptide vaccines, DC vaccines, and viral and bacterial vector deliveries of vaccines, and these may provide an opportunity to control cervical cancer. Further approaches incorporating these vaccine types with either conventional therapy modalities or the modulation of CD4+ and CD8+ T-regulatory (Treg) cells appear to be more promising in achieving increased therapeutic efficacy (Sin 2006). A report describes a therapeutic HPV DNA vaccination strategy using the HPV-16 E7 antigen fused to the invariant chain to enhance the E7-specific CD4+ and CD8+ T-cell immune responses, resulting in a potent anti-tumor effect against E7-expressing tumors. Continued exploration of HPV therapeutic DNA vaccines may lead to eventual clinical application (Wu 2007).

Therapeutic strategies based on the insertion of cytokine or other immune-stimulatory genes into the genome of tumor cells followed by vaccination with the resulting, genetically modified, cytokine-producing vaccines represent a new potential prospect for the treatment of cancer patients. During the last decade, animal models have substantially contributed to the development of the therapeutic vaccines against HPV-16-associated tumors. It has been demonstrated that the HPV-16 E6/E7 oncoproteins can serve as tumor rejection antigens (TRA) and that the HPV-16-associated tumor cells can be genetically modified with DNA encoding immune-stimulatory cytokines (IL-2, IL-12, granulocyte-macrophage colony-stimulating factor, GM-CSF) or other immune-stimulatory molecules, used for vaccination, and inhibit tumor growth. To improve the HPV-16 antigen presentation in tumor-bearing individuals, DC-based vaccines loaded with HPV-16 E6/E7 DNA or hybrids of DC and tumor cells have also been successfully employed. Unfortunately, when these encouraging approaches used in animal models were translated into

clinical trials, the results were less optimistic (Bubenik 2008). The reason for this drawback is the lack of knowledge about the immune mechanisms that control the growth of HPV-infected or transformed cells in vivo. It became evident that the preclinical models in rodents provide only limited information about the performance of a candidate vaccine in humans. In particular, the immune correlate for a clinical response remains to be determined. On the other hand, HPV-related malignancies provide an excellent model for cancer immune therapies in general. There is hope that the continuous efforts of academic research combined with corporate involvement will finally present an efficient product (Gissmann and Nieto 2009). Therapeutic vaccines are quite different from preventive vaccines in that they require the generation of cell-mediated immunity, particularly T-cell-mediated immunity, instead of the generation of neutralizing antibodies. Unfortunately, therapeutic vaccination has been limited by inadequate antigen-specific immune responses. Different therapeutic strategies have been developed including peptide immunization-based therapies, DNA vector-based therapies, viral/bacterial vector-based therapies, immune response modifiers, photodynamic therapy (PDT), and T-cell receptor based therapy. At present, the design of therapeutic vaccines to control the growth of HPV-induced tumors has focused on utilization of E6 and E7 proteins or peptides as vaccine antigens. Human trials are the most important test for the efficacy of HPV-16/18 E6 and E7 proteins as immuno-therapy for cervical cancer (Bolhassani et al. 2009). In fact, the HPV-encoded early proteins (E6 and E7 oncoproteins) form ideal targets for therapeutic HPV vaccines, since they are consistently expressed in HPV-associated cervical cancer and its precursor lesions. Thus, they play crucial roles in the generation and maintenance of HPV-associated disease (Su et al. 2010). Though their development has been challenging, many therapeutic HPV vaccines have been shown to induce HPV-specific antitumor immune responses in preclinical animal models and several promising strategies have been applied in clinical trials. With continued progress in the field of vaccine development, HPV therapeutic vaccines may provide a potentially promising approach for the control of lethal HPV-associated malignancies (Hung et al. 2008). Therefore, it is necessary to develop therapeutic HPV vaccines to facilitate the control of HPV-associated malignancies and their precursor lesions. Among the various forms of therapeutic HPV vaccines, DNA vaccines have emerged as a potentially promising approach for vaccine development due to their safety profile, ease of preparation, and stability. However, since DNA does not have the intrinsic ability to amplify or spread in transfected cells like viral vectors, DNA vaccines can have limited immunogenicity. Therefore, it is important to develop innovative strategies to improve DNA vaccine potency. Since DC are key players in the generation of antigen-specific immune responses, it is important to develop innovative strategies to modify the properties of the DNA-transfected DC. These strategies include increasing the number of antigen-expressing/antigen-loaded DC, improving antigen processing and presentation in DC, and enhancing the interaction between DC and T-cells. Results from impressive preclinical studies have led to several clinical trials (Monie et al. 2009).

In conclusion, cervical cancer remains a critical public health problem that is second only to breast cancer in overall disease burden for women throughout the

world. In spite of the success of cervical cancer screening, Pap cytology screening is yet to be effectively implemented or has failed to reduce cervical cancer rates to an appreciable extent. Screening appears to benefit only a small fraction of women although a much larger percentage endures the inconvenience of the Pap test in order to avoid cervical cancer. The establishment of HPV infection as the necessary cause of cervical pre-cancers and cancers provides a tremendous opportunity for cervical cancer prevention through vaccination against HPV-16 and HPV-18, which cause 70% of cervical cancers worldwide. Thus, a prophylactic vaccine to prevent HPV-related precancerous lesions and cancers would save lives, reduce the need for costly medical procedures, and provide both women and communities throughout the world with substantial benefits. Based on the induction of neutralizing antibodies by non-infectious VLP of L1 capside protein, prophylactic HPV vaccines have consistently induced high titer of neutralizing antibodies with minimal side effects and induce more than 90% protection from persistent HPV-16-18 infection and HPV-16 and HPV-18 associated high-grade CIN in proof of concept efficacy trials. HPV-16-18 vaccination will prevent HPV-16-18 incident infection, and subsequently decrease in 90% the frequency of abnormal Pap attributable to these types and in about 50% overall abnormal Pap. HPV vaccination will reduce the number of women who require colposcopy, biopsy, and cervical treatment for precancerous cervical lesions. The level of protection from death due to cervical cancer could exceed 95%. HPV vaccination targeting young female adolescents aged 11–16 years with a catch-up of those aged 17–25 years would be a good beginning strategy. Cervical cancer screening strategies that will be cost-effective for the proper surveillance of women protected by HPV vaccination are under analysis (Monsonégo 2006). Prophylactic vaccines against HPV are on the market and will certainly reduce the incidence of genital warts and the risk of developing cervical cancer. In addition, they will contribute to reducing anal, as well as head and neck cancers. However, effort should be made in the short term in order for these vaccines to have a real impact in the developing world where almost 80% of cervical cancer cases occur. Since the available vaccines include only two of the HPV types found in cancers (approximately 70%), improvements in current mass screening programs with the use of molecular techniques must be made, particularly in developing countries. Therapeutic vaccines have been designed to control advanced lesions and residual illness and although success has usually been obtained in animal models, clinical studies have not yet provided the anticipated results. Finally, the next generations of prophylactic HPV vaccines will probably include subunit vaccines, transgenic bacteria, and plants among others, and could represent useful and cheaper alternatives for reducing cervical cancer, particularly in the developing world (García Carrancá and Galván 2007).

The first prophylactic quadrivalent HPV VLP vaccine against HPV types 6/11/16/18 was licensed in 2006 for girls and women aged 9–26 years in several countries following the determination that it has an acceptable benefit/risk profile. The second prophylactic bivalent HPV vaccine against HPV types 16 and 18 has also been licensed in 2008. Both vaccines are prepared from non-infectious, DNA-free VLP produced by recombinant technology and combined with an adjuvant.

These vaccines are almost 100% effective in preventing infection and moderate and severe precancerous cervical lesions associated with types 16 or 18 included in the vaccine among women with no previous infection with these types. With three doses administered, they induce high levels of serum antibodies in virtually all vaccinated individuals. Vaccinating at an age before females are exposed to HPV would have the greatest impact. Since HPV vaccines do not eliminate the risk of cervical cancer, cervical screening will still be required to minimize cancer incidence. Tiered pricing for HPV vaccines, innovative financing mechanisms, and multidisciplinary partnerships will be essential in order for the vaccines to reach populations in greatest need (Cutts et al. 2007). The vaccines are well tolerated, safe, and highly immunogenic when given in three doses within 6 months. Efficacy of the vaccine against external vulvar and HPV-related vaginal lesions is also high. Even though the vaccine is highly effective against HG cervical, vaginal, or vulvar pre-cancers, this only applies to women unexposed to these HPV types and only to H-SIL caused by these HPV types. Therefore, it is important to understand that the population impact of the vaccines will be much lower than vaccinating naïve populations. Implementing HPV vaccine is a great opportunity but also a great challenge. However, mandatory HPV vaccination may raise many questions, and more answers are needed (Paavonen and Lehtinen 2008). Achieving long-term protection following vaccination is crucial to ensuring that high levels of immunity are maintained within a population while eliminating the need to introduce booster vaccinations. Based on an analysis of the Hepatitis B Virus (HBV) vaccine, several factors have been shown to contribute to long-term protection, namely specific lympho-proliferation, the in vivo humoral response, and immune memory. To ensure protection against persistent HPV infection and the subsequent development of cervical lesions, an effective HPV vaccine must be able to induce strong humoral immune responses. Mathematical modeling analyses based on a three-dose regimen of HPV-16 prophylactic vaccine indicated that 99% of 16–23-years-old women would have almost life-long detectable anti-HPV-16 levels. Available data on the quadrivalent HPV vaccine demonstrated that long-term immune memory was induced, with anti-HPV geometric mean titers at or above those observed with natural infection. Vaccination also resulted in a substantial reduction in the combined incidence of HPV-6/11/16/18-related persistent infection or disease, and there were no cases of precancerous cervical dysplasia compared with six cases in women receiving placebo. Similarly, the bivalent HPV vaccine has been shown to induce long-term immunity with >98% seropositivity maintained and geometric mean titers at this time point remaining substantially higher than those noted with naturally acquired infection (Ault 2007).

In the recent period following the approval of the first vaccine targeted against HPV, it is imperative that we reevaluate the past, present, and future of cervical cancer prevention. It is clear that the subject of vaccinating young women against a STD has incited a candid debate among all groups involved. Therefore, we must make the most appropriate and accurate information available to the public and medical communities. The development of vaccines targeted against oncogenic HPV types has made it possible to eliminate approximately 70% of all invasive cervical cancers in women worldwide. However, knowledge about HPV infection

and cervical cancer, as well as the need to vaccinate against oncogenic HPV infection, is still lacking among women and physicians. This deficiency could be a key reason why some parents continue to have reservations about vaccinating their daughters. In order for HPV vaccination programs to be highly successful, multiple barriers must be overcome. Review of lessons learned to date has demonstrated that continued tailored and targeted educational and awareness initiatives are required for healthcare professionals, media, patients, and parents (Herzog et al. 2008; Agorastos et al. 2009; Albers and Kaufmann 2009; Astbury and Turner 2009; Bornstein 2009; Brisson et al. 2009; Chang et al. 2009; Descamps et al. 2009; Flaherty and Alkhateeb 2009; Franco et al. 2009a; Galani and Christodoulou 2009; Gissmann 2009; Hakim and Dinh 2009; Harper 2009b, c; Hershey and Velez 2009; Hsueh 2009; Klosky et al. 2009; Koutsky 2009; Luciani et al. 2009; Lynge et al. 2009; Madrid-Marina et al. 2009; Marquez-Calderon et al. 2009; Marra et al. 2009; Massad et al. 2009; Medeiros et al. 2009; Mougin et al. 2009; Palmer et al. 2009; Prymula et al. 2009; Saleem et al. 2009; Satyaprakash et al. 2009; Trimble and Frazer 2009; Wentzensen and Klug 2009; Armstrong 2010; Bermúdez-Humarán and Langella 2010; Bonanni et al. 2010; D'Andrilli et al. 2010; Downs et al. 2010; Dubensky and Reed 2010; Fernández et al. 2010; Garland and Smith 2010; Herzog et al. 2010; Huh et al. 2010; Kane 2010; Katz et al. 2010; Liddon et al. 2010; Lin et al. 2010; Markowitz et al. 2010; McCormack and Joura 2010; Monsonego et al. 2010; Syrjänen 2010; Szarewski 2010; Wain 2010; Buonaguro et al. 2011; Dillner et al. 2011; Laurent-Ledru et al. 2011; Lu et al. 2011; Syrjänen 2011).

9 Counseling

The potential for HPV DNA testing in cervical cancer prevention programs has been a topic at the forefront of cervical cancer policy discussions in recent years. To prevent some of the anxiety and psychological distress often experienced on HPV diagnosis and during the period of management, mass patient education must accompany the incorporation of HPV DNA testing into screening protocols (Anhang et al. 2004). There are no RCT to review regarding information for women about HPV but a number of well-conducted qualitative studies explore the issues that are faced both by the women undergoing cervical screening and their healthcare providers (Tristram 2006). Public awareness of HPV is generally very low, particularly with respect to its relation to abnormal smears and cervical cancer although knowledge levels vary to some extent according to socio-demographic characteristics. There is also much confusion around which types cause warts and the types that can cause cancer. The sexually transmissible nature of the infection is of major concern and confusion to women. Due to the lack of current awareness of HPV, significant education initiatives will be necessary should HPV vaccination and/or HPV testing be introduced. Organized edification of health-care workers and the media, who constitute the two most preferred sources of information, will be crucial (Cuschieri et al. 2006). Women are more susceptible to the oncogenic effect

of HPV, mostly at the genital site on the uterine cervix. Psychosexual vulnerability increases with number of recurrences of HPV infections. Depression, anxiety, and anger are the emotions most frequently reported. However, to date, there is no conclusive evidence of a specific correlation between HPV infection and a specific female sexual disorder. The relationship between HPV and vulvar vestibulitis/vulvodynia-related dyspareunia seems not to be direct. The evidence of psychosexual consequences of HPV-related genital warts and intraepithelial lesions is limited. Specific research on the sexual impact of genital warts and intraepithelial HPV-related lesion in women is urgently needed (Graziottin and Serafini 2009; Verhoeven et al. 2009).

10 Guidelines

More than 20 years ago, a relationship between HPV infection and cervical cancer was recognized. Since then, important strides in understanding the virus have been made, particularly in the following areas: modes of transmission and risk factors associated with it, the oncogenic potential of specific viral types and the mechanism by which they cause cancer, and the spectrum of infection, ranging from asymptomatic carrier states to overt warts, pre-neoplastic lesions, and invasive cancer. Sophisticated new tests for the detection of HPV have been developed and hold promise for improved screening of cervical cancer precursors, invasive cancer, and for the triage of abnormal cervical cytology. Understanding the immunology of HPV has allowed the development of new and more effective treatment modalities for HPV infection and the preliminary development of primary prevention modalities, including HPV vaccines (American College of Obstetricians and Gynecologists 2005). The American Cancer Society (ACS) has developed guidelines for the use of the prophylactic HPV vaccine for the prevention of CIN and cervical cancer. These recommendations are based on a formal review of the available evidence. They address the use of prophylactic HPV vaccines, including who should be vaccinated and at what age, as well as a summary of policy and implementation issues (Saslow et al. 2007).

The focus of the American Society of Colposcopy and Cervical Pathology (ASCCP) National Consensus Conference for the Management of Women With Cervical Cytological Abnormalities and Cervical Cancer Precursors held on the National Institutes of Health (NIH) campus in Bethesda, Maryland, USA, September 6–8, 2001, is on the management of women with equivocal (ASC-US) and LG (L-SIL) cytological abnormalities. Management of women with these cytological abnormalities has been particularly problematic, because individually these women are at least risk for CIN 3 and cancer, yet their sheer numerical dominance ensures that they account for the majority of HG-CIN detected in the USA in the follow-up of abnormal cervical cytology. Data from ASC-US/L-SIL Triage Study, otherwise known as ALTS, confirmed that women with ASC-US could be safely managed by any of the conventional approaches (repeated Pap test, immediate colposcopy, or HPV testing), but that the preferred management approach for women having an

ASC-US report from LBC was to assess the patient's risk by testing for HPV. Additionally, longitudinal ALTS data determined that repeated LBC at 6 and 12 months and an HPV test at 12 months were nearly equivalent options in the follow-up of women referred for HPV-positive ASC or L-SIL, yet not found to have CIN 2+ at initial colposcopy. Therefore, all follow-up recommendations for women with CIN 1 or lower post-colposcopy findings include these two options (Cox and American Society for Colposcopy and Cervical Pathology 2003). Management of women with ASC depends on whether the Pap test is subcategorized as ASC-US or ASC cannot rule out a high-grade lesion (ASC-H). Women with ASC-US should be managed using a program of two repeated cytology tests, immediate colposcopy, or DNA testing for HR-HPV. Testing for HPV DNA is the preferred approach when LBC is used for screening. In most instances, women with ASC-H, L-SIL, H-SIL, and atypical glandular cells (AGC) should be referred for immediate colposcopic evaluation (Wright et al. 2002). Recommendations for managing ASC-US and L-SIL are essentially unchanged. Changes were made for managing these conditions in adolescents for whom cytological follow-up for 2 years was approved. Recommendations for managing H-SIL and AGC also underwent only minor modifications. More emphasis is placed on immediate screen-and-treat approaches for H-SIL. HPV testing is incorporated into the management of AGC after their initial evaluation with colposcopy and endometrial sampling. The 2004 Interim Guidance for HPV testing as an adjunct to cervical cytology for screening in women 30 years of age and older was formally adopted with only very minor modifications (Wright et al. 2007).

Although Pap tests have enabled early detection of premalignant lesions, the introduction of new collecting devices has significantly improved the detection of lesions hidden in the endocervical canal, such as adenocarcinoma in situ (AIS). The term "AGC of undetermined significance" (AGC-US) was introduced at the 1988 Bethesda Conference and defined as morphologic changes in glandular cells beyond those that are suggestive of the benign reactive process, but insufficient for the diagnosis of AIS. In the new 2001 Bethesda System, the term has been eliminated and replaced with the term AGC, with the following sub-classifications: not otherwise specified (NOS), favor neoplasia, endocervical AIS, and adenocarcinoma. The risks of premalignant or malignant disease associated with the AGC favor neoplasia category are substantially higher than in the AGC NOS category (96% vs 9–41%, respectively). Patients diagnosed with AGC NOS or AGC favor neoplasia will require colposcopy or endocervical sampling, and patients over 35 years of age, endometrial biopsy. If all of these tests are negative, the Pap test should be repeated in 4–6 month intervals until four consecutive normal tests are obtained. Positive results in one of the tests will require management according to ASCCP guidelines. The AGC favor neoplasia diagnosis also requires cervical conization and/or other testing, as the incidence of premalignant or malignant lesions in patients with this diagnosis is high (Levine et al. 2003).

In conclusion, carcinoma of the cervix is causally related to infection with the HPV. Reducing the rate of HPV infection by changes in sexual behaviors in young people and/or through the development of an effective HPV vaccine would reduce

the incidence of this disease. Pap smear screening remains the best available method of reducing the incidence of and mortality from invasive cervical cancer. Persons with stage IA1 disease have a high cure rate with either simple hysterectomy, or where fertility preservation is an issue, by cone biopsy with clear margins. For patients with either stage I or stage IIA disease, radical surgery and radiation are equally effective treatments. These patients should be carefully selected to receive one treatment or the other, but not both, as their combined use substantially increases the cost and morbidity. Women with more advanced, non-metastatic disease should be treated with radiation. Recurrent cervical cancer confined to the pelvis should be treated with the modality not previously received. Radiation therapy is recommended to palliate symptoms in patients with metastatic disease (Cervical Cancer NIH Consens Statement 1996; National Institutes of Health Consensus Development Conference Statement: cervical cancer 1996; National Institutes of Health Consensus Development Conference Statement on cervical cancer 1997). Recent evidence has shown that the risk of malignant and premalignant cervical disease and HPV infections varies significantly with age. Furthermore, evidence now shows that treatment for cervical disease carries significant risk for future pregnancies. These factors have led to a re-evaluation of the guidelines for the management of premalignant cervical disease (American College of Obstetricians and Gynecologists 2008).

The management of abnormal cervical cytology in adolescents differs from that of the adult population. Cervical cancer is almost non-existent in adolescents, yet HPV infection is very common in this population. In the past 5 years there has been significant advancement in the management of HPV-related diseases in adolescents. The publication of the ASCCP 2006 consensus guidelines has led to major changes in the prevention and management of cervical disease in adolescents. With the availability of the HPV vaccination (since 2006), it is expected that these guidelines will continue to change. The ASCCP guidelines now advise against HPV testing and recommend against treatment of L-SIL or CIN 1. In addition, among adherent adolescents, treatment of CIN 2 also should be deferred. These new guidelines were established to minimize the potential negative impact that treatment can have on future pregnancy outcomes, while taking advantage of the natural history of HPV in young women (Committee on Adolescent Health Care 2009).

11 Conclusions

Screening for cervical cancer is accomplished utilizing a Pap smear and pelvic exam. While this technology is widely available and has reduced cervical cancer incidence in industrialized nations, it is not readily available in third world countries in which cervical cancer incidence and mortality is high (Brinkman et al. 2005). Despite the considerable success of early screening for prevention of cervical cancer, Pap smears have not fulfilled the hopes that it would lead to a large-scale reduction of this cancer's incidence. Screening appears to be useful for a tiny portion of the world population, although a relatively large portion must put up with its

limitations and disadvantages. HPV-16 and HPV-18 are responsible for two thirds of all cervical cancers worldwide. The condylomata (condyloma acuminatum), or genital warts, induced by HPV-6 and HPV-11 are frequent among the young and difficult to manage. The extent and burden of HPV infection are considerable, as is the psychological and emotional impact of the diseases associated with it. Because cancer of the cervix is the final consequence of chronic HPV infection, it can be prevented by vaccination (Monsonego 2007). Development of cervical cancer, in fact, is associated with infection with HR-HPV types, creating a unique opportunity to prevent or treat cervical cancer through anti-viral vaccination strategies. Several strategies have been examined in clinical trials for both the prevention of HPV infection and the treatment of pre-existing HPV-related disease. Clinical trials utilizing prophylactic vaccines containing VLP indicate good vaccine efficacy. But preclinical research in this area continues in order to deal with issues such as cost of vaccination in underserved third world populations (Brinkman et al. 2005). A prophylactic vaccine to protect against the precancerous and cancerous lesions associated with HPV should save lives, reduce expensive diagnostic and therapeutic interventions, and have substantial individual and collective benefits. Clinical trials of anti-HPV vaccines for the prevention of cervical cancer and condyloma have shown remarkable results and an efficacy unequaled in the history of vaccination against infectious diseases. Vaccine efficacy has been shown only in young girls never exposed to the virus and only for the lesions associated with the specific viral types in the vaccine. Data indicate that the vaccination is effective in women who have previously eliminated naturally the virus. It has no therapeutic effects on existing lesions or in healthy virus carriers. Practical questions remain to be resolved. If the vaccination is left to individual initiative and vaccination coverage is insufficient, there will be no perceptible reduction in the frequency of cervical cancer. Vaccination policies will not be identical in poor countries, where the disease represents one of the leading causes of mortality among women, and in the rich countries, where screening programs have considerably reduced the frequency of this cancer. Current planning calls for the introduction of systematic vaccination of young girls aged 9–15 years, with progressive "catch-up" vaccination of the cohorts of young women aged 16–26 years. Nonetheless, mathematical models and immunogenicity results indicate a possible benefit for individual vaccination of adults. This approach must still be assessed in the clinical trials underway. Because the vaccine does not protect against all types of HPV associated with cervical cancer, screening must be continued according to the conditions currently set. Vaccination and screening, which are complementary and synergistic, now constitute the new standards for prevention of this disease (Monsonego 2007). A majority of clinical trials using therapeutic agents which aim to prevent the progression of pre-existing HPV associated lesions or cancers have shown limited efficacy in eradicating established tumors in humans possibly due to examining patients with more advanced-stage cancer who tend to have decreased immune function. Future trends in clinical trials with therapeutic agents will examine patients with early stage cancers or pre-invasive lesions in order to prevent invasive cervical cancer. Meanwhile, preclinical studies in this field continue and include the further exploration of peptide or protein vaccination,

and the delivery of HPV antigens in DNA-based vaccines or in viral vectors. The consequences for clinical management may include a significant reduction in the frequency of Pap smear screening in the case of prophylactic vaccines, and the availability of less invasive and disfiguring treatment options for women with pre-existing HPV associated lesions in the case of therapeutic vaccines. Implementation of both prophylactic and therapeutic vaccine regimens could result in a significant reduction of health care costs and reduction of worldwide cervical cancer incidence (Brinkman et al. 2005).

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Chapter 12
Therapeutic Vaccines for HPV Infection

Margaret Stanley

1 Introduction

1.1 Ano-Genital HPV Associated Disease

Approximately 30–40 human papillomaviruses (HPVs) sporadically or regularly infect the ano-genital mucosae and are the most commonly sexually transmitted infections worldwide. HPV infection of the ano-genital skin and mucosae results in lesions with two morphologies – ano-genital warts (condyloma acuminata) and squamous intra-epithelial lesions. Condylomata are associated predominantly, but not exclusively, with infection by the low risk HPVs 6 and 11 and are frank, polypoid growths that generate infectious virus and have a low to negligible risk of malignant progression (Lacey et al. 2006). Squamous intra-epithelial lesions are classified histologically and form a distinct spectrum of histological atypia- mild to severe. In Europe three grades of cervical intra-epithelial neoplasia (CIN) are recognised: CIN 1- mild, CIN 2- moderate and CIN 3- severe. In the vagina, vulva, anus and penis a similar but not identical spectrum of changes can be identified, VAIN, VIN, AIN and PIN, but risk of progression of these lesions to frank malignancies is not as well defined as for the cervix. It is probable, but not unequivocally proven, that the majority of these intra-epithelial lesions are a result of HPV infection (Moscicki et al. 2006). To ensure clarity in this review high grade intra-epithelial neoplasia (HGIN) will include CIN 2/3, AIN 3, PIN 3, VIN 3 and VAIN 3: low grade intra-epithelial neoplasia LGIN encompasses CIN1 and the equivalent lesions in other sites.

LGIN at any site can be associated with both high and low risk HPV types although low risk types predominate. The majority of lesions maintain the virus as

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an episome, support a complete virus replication cycle and viral gene expression is tightly regulated. Late genes are expressed and virus particles generated. HGIN are associated almost exclusively with high-risk types particularly HPV 16 and 18. In general because of the defects in cellular differentiation that characterise these lesions, they do not support a complete viral infectious cycle (Doorbar 2006). Viral gene expression is deregulated with expression of the E6 and E7 oncogenes in dividing cells throughout the epithelium. These lesions are characterised by chromosomal aneuploidy and genetic instability with a high risk of progression to invasive carcinoma. High grade cervical disease arises almost exclusively at the squamo-columnar junction. Disease therefore is localised even though infection may be regional and ablative therapies are highly effective for disease but do not treat the field infection. However AIN, PIN, VAIN and VIN are often multi- focal, both disease and infection are regional and ablation may not be feasible or, if attempted, ineffective.

1.2 Effective Therapies for Infection and Disease Are a Priority

HPV associated disease is a major cause of morbidity and mortality. The estimated prevalence of genital warts is 2.4/1,000 of the population per year (Lacey et al. 2006) and HPV associated cancers are estimated to represent 5% of the global burden of cancer in men and women (Arbyn et al. 2011). Effective therapies for infection and disease are therefore major priorities. Therapeutic vaccination is an obvious strategy. In both benign and malignant lesions viral antigens are consistently expressed, and lesion regression of warts and LGIN correlates with a local Th1 type cell mediated immune response (Stanley 2006). The rationale for therapeutic immunisation therefore is to activate or reactivate a strong cell mediated cytotoxic immune response against viral antigens expressed by the infected and/or neoplastic cells. However this apparently straightforward objective, at least for high grade disease (HGIN and cancer), has proved very difficult to achieve. It is now recognised that both viral immune evasion and tumour immune evasion strategies must be overcome and understanding these therefore is a pre-requisite for successful therapeutic immunisation.

2 HPV a Successful Pathogen That Evades Host Defences

HPVs are very successful infectious agents: they induce chronic infections lasting months or years that have no apparent systemic sequellae and rarely kill the host, but periodically shed large amounts of infectious virus for transmission to naïve individuals. To achieve this successful lifestyle, HPVs must either avoid or negotiate the powerful innate and adaptive immune defence systems of the host for extended time periods.

2.1 HPV Immune Evasion Strategies

The exclusively intra-epithelial virus life cycle is in itself an immune evasion mechanism with key features that impact on the recognition and response of the host immune system to the infection (Stanley 2006).

- Firstly no inflammation accompanies viral infection and thus there is no danger signal to alert the innate immune sensors. HPVs are not lytic viruses the life cycle is played out in the keratinocyte a cell destined for death from natural causes as an enucleate squame. High level viral replication and viral assembly occur in terminally differentiated keratinocytes, cells that have already undergone a regulated death programme.
- Secondly although HPVs appear to be able to bind to, and enter, cells other than keratinocytes viral gene expression and viral protein synthesis are confined to keratinocytes. There is no synthesis of viral protein in antigen presenting cells (APC) such as dendritic cells Langerhans cells, macrophages.
- Finally there is either no or very little viraemia. Virus infects via epithelial micro-abrasions that leave the epithelial basal lamina intact (Roberts et al. 2007) and is shed from mucosal or cutaneous surfaces far from vascular channels. Thus there is poor access to the draining lymph nodes where adaptive immune responses are initiated.

2.1.1 Immune Ignorance and HPV

In this intra-epithelial infectious cycle, the virus is basically a hitchhiker joining the keratinocyte at the start of its journey as a primitive basal cell in the epithelium through to its end as a terminally differentiated squame. For most of the duration of this journey, there is little or no release into the local milieu of pro-inflammatory cytokines, important for APC activation and migration. The central signals to kick start the immune responses in squamous epithelia are absent and minimal amounts of viral proteins and infectious virus are exposed to immune defences; in effect, the virus is practically invisible to the host who remains ignorant of the pathogen for long periods of time.

2.1.2 HPV Downregulates Innate Immunity

Central to this achievement is the ability of HPV, particularly the high risk HPVs, to compromise the role of keratinocytes as innate immune sentinels. Keratinocytes can respond to cell injury, cell stress and sense pathogens via pathogen recognition receptors such as Toll like receptors (TLR) and Nucleotide binding oligomerisation domain like receptors (NLR) the sentinels of innate immunity that kick start the cascades of effector immune responses (Nestle et al. 2009). Recent evidence shows

that in HPV infected keratinocytes these crucial innate responses are dampened almost from the start of the infectious cycle with the consequence that the key cytokines of squamous epithelia Il-1β and IL-6 are not released and important signaling pathways not activated (Karim et al. 2011). As a result pro-inflammatory cytokines such as TNF-α and the type I interferons are not released (Pett et al. 2006). The abrogation of the interferon response a powerful generic, anti-viral defence is particularly important. HPV16 infection downregulates interferon-α inducible gene expression and the HPV 16, E6 and E7 oncoproteins directly interact with components of the interferon signalling pathway altering the expression of genes that enable host resistance to infection and immune function. In effect HPVs evade the innate immune response and delay activation of antigen specific adaptive immune responses (Stanley et al. 2007).

2.2 Immune Response to HPV in Natural Infections

Despite the best efforts of the virus to evade host defences at least 80–90% of genital HPV infections will resolve with time (Moscicki et al. 2006). Anogenital warts and CIN1 lesions regress as a result of a successful local cell mediated immune response directed against early viral proteins specifically E2 and E6 (de Jong et al. 2002; Welters et al. 2003; Woo et al. 2010). However despite the intense local cellular response described in immunohistological studies of regressing lesions, systemic antigen specific T cell responses are weak and often transient (Jain et al. 2006). The cellular effectors in these local responses are still not unequivocally identified but regression of CIN 1 in longitudinal studies has been shown to be correlated with the presence at study entry of functional cytotoxic CD8+ cells producing Granzyme B (Woo et al. 2008). Intra-epithelial CD8+ T cells express the intra-epithelial homing receptor α4/β7 and importantly in a retrospective analysis, lesion regression related to the expression on the stromal microvascular endothelium in dysplastic lesions of the mucosal addressin cell adhesion molecule 1 (MadCAM1), the ligand for α4/β7 implying that with increasing disease severity cytotoxic effector lymphocytes could not exit the lymphatics and vessels to enter the dysplastic epithelium (Trimble et al. 2010).

2.2.1 Immune Responses Are Deregulated During HPV Associated Neoplastic Progression

A more complete picture of events leading to progression of HPV infected lesions in the cervix is now emerging. About 10–20% of individuals develop persistent cervical HPV infection and remain HPV DNA positive; it is this group that are at high risk for progression for CIN2/3 (Bosch et al. 2008). In these persistent HPV infections the absence of cell death means that the inflammatory signals that would

activate intra-epithelial APC such as LC, recruit stromal DC and macrophages to the epithelium and plasmacytoid DC (major sources of type I interferons) to the infected focus are absent. Furthermore, HPVs downregulate innate sensing signalling pathways in the infected keratinocyte, pro-inflammatory cytokines, particularly the type 1 interferons are not released and again the signals for Langerhans cell activation and migration and the recruitment into the epithelium of stromal dendritic cells and macrophages are either not present or inadequate.

In this scenario there are long periods of uninterrupted virus replication in the epithelium during which the host is ignorant of virus. This is a high risk strategy for the host when the infection is with an oncogenic genital HPV as it increases the risk of "accidents" in the virus infectious cycle that result in the deregulated expression of viral E6 and E7 oncoproteins, the bypassing of cell cycle checkpoints and neoplastic transformation (Bodily and Laimins 2010).

2.2.2 Low Grade to High Grade Disease Progression Is Accompanied by a Local Immunosuppressive Milieu

The transition from low grade to high grade disease in the cervix (and probably other anogenital sites) is characterised by an increasingly immunosuppressive *local* milieu. There is a loss in locally present IFN-γ and increase in IL-10 (Kobayashi et al. 2008; Scott et al. 2009; Syrjanen et al. 2009). The local T cell infiltrate of activated CD4+ and CD8+ T cells express PD-1 (Karim et al. 2009), a marker of the exhausted T cell, and few CD8+ produce Granzyme B (Bontkes et al. 1997; Woo et al. 2008) suggesting that they are not functionally cytotoxic. Importantly there is a steady increase in the number of tissue infiltrating macrophages with the M2 phenotype, IDO cells and Foxp3+ regulatory T cells (T Regs) all of which suppress Th1 mediated cytotoxic responses promoting a milieu characterised by Il-10 and TGFβ and a switch to a more Th type 2 response or non responsiveness (Hammes et al. 2007; Kobayashi et al. 2004; Lepique et al. 2009).

The systemic T cell response is either weak or absent. The evidence is that viral persistence and disease progression corresponds to lack of demonstrable HPV specific T cell immunity (Trimble et al. 2009a) whereas systemic HPV antigen specific Th1 responses correlate in some studies with a favourable outcome in vulval and cervical high grade disease (Bourgault Villada et al. 2009; Seresini et al. 2007). Overall with neoplastic transformation and genomic instability the expression of key cytokines, adhesion molecules, chemokines and chemokine receptors on the infected epithelium and on the underlying microvascular endothelium of the stroma are deregulated, resulting in the downregulation of the receptors essential for the ingress of antigen specific killer T cells and other cytotoxic cells into the epithelium (Trimble et al. 2010). Thus even if HPV antigen specific cytotoxic cells have been generated their ingress into the epithelium is poor, regulatory T cells increasingly dominate the lesions and abrogate the killer effector response (Jaafar et al. 2009; van der Burg et al. 2007). Therapeutic vaccines must reverse or overcome this.

3 Therapeutic Vaccine Approaches

3.1 HPV Associated Cancers

In HPV associated cancers and HGIN, oncogenic viral gene expression is deregulated and the E6 and E7 genes are constitutively expressed. The continued expression of these oncogenes is essential for progression to, and maintenance of, the malignant phenotype. In effect therefore there are only two possible antigenic targets, E6 and E7, since these are the only viral proteins that will be expressed in all cancers and HGIN. The approach of deliberate immunisation with E6 and/or E7 of HPV 16 and HPV 18 predominantly, and the generation of antigen specific CTL as an immunotherapy for HPV associated cancer has been tested with a wide array of potential vaccine delivery systems in transplantable rodent tumour models. These systems include DNA vaccines, recombinant viral and bacterial vectors, HPV antigens targeted to the endosome of antigen presenting cells, fused to heat shock proteins, delivered with adjuvants that bias to a Th1 response, combined in prime boost protocols and many more [reviewed in (Stanley 2003; Su et al. 2010)].

It turns out from these studies that HPV expressing cancers in mice, are relatively easy to cure but human HPV induced cancers have been, to date, largely refractory to the approaches successful in rodents. All the vaccines tested in clinical trials in humans have been safe and well tolerated, they have induced vaccine specific T cell responses of varying magnitude but these responses did not necessarily correlate with clinical responses (Borysiewicz et al. 1996; Ferrara et al. 2003; Kenter et al. 2008; Santin et al. 2002, 2008; Trimble and Frazer 2009; van Driel et al. 1999). Most clinical trials of cervical cancers, Table 12.1, have been done in subjects with late stage malignant disease who, realistically, are poor candidates for immunotherapy since they are likely to be immunocompromised as a consequence of previous therapy and they have a large disease burden.

3.2 Immunotherapies for High Grade Intra-Epithelial Neoplasia

HGIN represents a more malleable target since only a proportion of these lesions progress to invasive carcinoma, although the size of the progressive fraction for each ano-genital site is not clear. Some cervical HGIN regress (McCredie et al. 2010), presumably by immune mechanisms (but there is no unequivocal evidence for this) indicating that immune escape strategies are not invariably successful in HGIN (Ovestad et al. 2010). In view of this it is distinctly possible that there could be a spectrum of responses to therapeutic vaccination of high grade precancers ranging from complete through partial to no clearance of the clinical disease and this notion is supported by the results from several clinical studies (Baldwin et al. 2003; Davidson et al. 2003; Frazer et al. 2004; Garcia et al. 2004; Goldstone et al. 2002; Kaufmann et al. 2007; Klencke et al. 2002; Muderspach et al. 2000; Sheets et al. 2003; Trimble et al. 2009b) (Table 12.2).

Table 12.1 Clinical trials of HPV specific immunotherapy in cervical cancer

Delivery system	Antigen	Disease group	Clinical outcome
Vaccina virus TA – HPV	E6 – E7 fusion protein	Late stage CaCx n = 8	Complete 1 remission of disease (Borysiewicz et al. 1996)
Peptide oil/water adjuvant	E7 peptides	Refractory CaCx HPV16+ HLA A201 n = 19	2/19 stable disease (van Driel et al. 1999)
Protein/algamunulin adjuvant	E7-GST fusion protein	CaCx n = 24	No change in disease natural history [quoted in (Trimble and Frazer 2009)]
Dendritic cells	HPV16 E7 HPV18 E7	CaCx stage IV n = 15	No objective clinical response (Ferrara et al. 2003)
SLP Montanide ISA-51	HPV16 E6 HPV16 E7	End stage CaCx n = 35	Not reported (Kenter et al. 2008)

CaCx carcinoma cervix, SLP specific overlapping long peptides

Table 12.2 Clinical trials of HPV specific immunotherapy in genital warts, anogenital intra-epithelial neoplasia

Delivery system	Antigen	Disease group	Clinical outcome
Heat shock protein (HSP-E7)	HPV16 E7 peptide	Genital warts n = 22	3/14 complete regression (Goldstone et al. 2002)
		Not HPV16	10/14 partial regression
Encapsulated polynucleotide ZYC101	HPV16 E7 peptide	CIN2/3 any HPV type n = 127	Lesion resolution higher in treated versus control group
			Not restricted to HPV16 (Garcia et al. 2004)
Protein/Isosometrix adjuvant	HPV16 E6-E7 fusion protein	CIN n = 31	No clinical regression. Reduction in HPV infection (Frazer et al. 2004)
Vaccinia virus TA HPV	E6-E7 fusion protein	VIN n = 18	50% reduction in disease 8/18 (Davidson et al. 2003)
Vaccinia virus TA HPV	E6-E7 fusion protein	VIN3 n = 12	83% reduction in disease 9/12 (Baldwin et al. 2003)
Peptide + incomplete Freunds adjuvant	E7 A0201 peptie	VIN/CIN HPV16+ HLA A2 n = 18	3/18 complete remission. 6/18 partial remission (Muderspach et al. 2000)
Chaemeric virus like particles	HPV16 E7 protein	CIN2/3 HPV16+ve n = 39	Histological improvement greater in treated compared to placebo (Kaufmann et al. 2007)
DNA vaccine	SigE7 (detox) HSP-70 fusion protein	CIN2 HPV16+ve n = 15	Complete histological regression 3/9 (Trimble et al. 2009b)
Peptide + incomplete Freunds adjuvant	E6-E7 HPV16 long peptides	VIN3 n = 20	9/19 complete response at 24 months post immunisation (Kenter et al. 2009)

CIN cervical intra epithelial neoplasia, *VIN* vulval intra-epithelial neoplasia

3.2.1 Therapeutic Vaccines for HPV Associated Disease Must Be Highly Immunogenic

Recent studies however provide reasons for cautious optimism that the blueprint for successful therapeutic immunisation of HPV associated neoplasms is becoming clearer. Firstly vaccines must be highly immunogenic. Adjuvants will be central to enhancing the immunogenicity of HPV therapeutic vaccines. The nature of the adjuvant in the vaccine formulation can determine the intensity of the response and the particular type of immune response skewing to a Th1 versus Th2 for example. Early innate immune responses shape subsequent immune responses and the development of adjuvants that exploit innate signals specifically focusing and biasing the adaptive response to cytotoxic effector Th1 type will be critical for effective HPV therapeutic vaccines.

The importance of robust immunogenicity has been demonstrated in a Phase II trial in which patients with longstanding and refractory VIN3 were immunised with a highly immunogenic vaccine comprising HPV 16 E6 and E7 long synthetic overlapping peptides (HPV16 SLP): 47% showed a durable and complete regression histologically and clinically (Kenter et al. 2009). Importantly responders had small lesions and regression was associated with a strong and broad HPV specific Th1 type CD4+ and CD8+ systemic T cell response, that peaked after the first vaccination; non responders on average had larger lesions and mounted a vaccine induced HPV specific regulatory T cell response (Welters et al. 2010).

3.2.2 The Local Lesional Immune Microenvironment Must Be Manipulated

Secondly the local immune milieu must be manipulated. The many observations in the literature that systemic T cell responses to HPV infections are weak and transient even though the regressing lesions have an intense T cell infiltrate implies that modulating the local immune micro-environment will be critical for successful immunotherapy. Generating large numbers of circulating vaccine induced HPV specific cytotoxic T cells will be ineffective if their ingress into the lesion is prevented and/or they are disabled by regulatory T cells when they arrive. Immune trafficking and modulation of the local milieu could be modified by mucosal immunisation. The route of administration significantly affects immunisation outcome and a mucosal route would be desirable for both practical and target driven reasons but immune induction at different mucosal surfaces requires specific signals. Vaccines delivered to mucosae will almost certainly require specific adjuvant formulations to efficiently target the mucosal inductive sites and induce strong cell mediated Th1 type responses at these sites.

Local immunomodulation with topical therapies is known to be effective against HPV associated benign and neoplastic anogenital disease (Stanley 2002). The immunomodulator Imiquimod (formulated as Aldara™) an agonist of TLR3 induces local secretion of interferon-α, interferon-β and other pro-inflammatory cytokines biasing to a local Th1 cell mediated response and, topically applied, Imiquimod has been shown to be effective against genital warts (Sauder et al. 2003), VIN (van Seters et al. 2008) and AIN of all grades (Fox et al. 2010).

TLR agonists such as Imiquimod are potentially powerful adjuvants and a combination of topical Imiquimod and systemic vaccination would be predicted to be synergistic. In a Phase II trial in patients with VIN 3 combining topical Imiquimod with systemic immunisation with an HPV 16/18 E6/E7 protein delivered via vaccinia virus (TA-CIN) 32% of patients showed complete regression of lesions (Daayana et al. 2010). Complete responders showed an enhanced local infiltrate of CD4+ and CD8+ T cells whereas non responders with histologically confirmed persistence of VIN showed an increased density of regulatory T cells.

Modulation of the tumour microenvironment can also be achieved by the chemoradiation modalities used routinely in the treatment of squamous cell carcinomas (Hoffmann et al. 2010; Sin et al. 2009). HPV+oropharyngeal squamous cell carcinomas (OPSCC) have enhanced sensitivity to chemoradiation compared to HPV−ve OPSCC and significantly enhanced survival even with late presentation (Dahlstrand et al. 2008; Spanos et al. 2009). Combinations of vaccination preceded by cisplatin or radiation in mouse models result in enhanced HPV specific T cell responses, greater T cell infiltration and enhanced cytotoxic T cell killing (Tseng et al. 2008; Tseng et al. 2009). If vaccination is used in combination with chemotherapy and radiation then it seems likely that lower, less toxic, doses of the latter may be used with enhanced therapeutic effect.

3.2.3 Strategies That Regulate the Regulators Are Needed

Therapeutic vaccines cannot cure large established malignancies or large persistent precancerous lesions and the evidence is that this is due at least in large part to the dominance of T regs over cytotoxic effectors (van der Burg et al. 2007; Welters et al. 2010). If therapeutic vaccine success is to be improved then either T regs should be depleted or the pool of cytotoxic effectors increased or both. T regs probably because of their lower intracellular ATP levels are more sensitive to cyclophosphamide then other T cell subsets (Zhao et al. 2010). Low dose cyclophosphamide treatment has been used to modify the immune milieu in giant condyloma acuminata after laser surgery resulting in a reduction in Foxp3+T cells and absence of recurrence (Cao et al. 2010). Potentially one could target the receptors and their ligands that regulate T cell function either expanding the pool of effector T cells (Curran et al. 2010) or reversing the T cell anergy and immunosuppression induced by T regs (Sharma et al. 2009). All of these strategies have been or are being tested in mouse models and have shown their potential but transfer to the clinic remains to be achieved.

4 Summary

Persistent infection with oncogenic HPV types is a prerequisite for the development of anogenital intra-epithelial lesions a proportion of which progress to invasive squamous cell carcinoma. Since the HPV genome encodes two potent oncoproteins

E6 and E7 and these are consistently expressed in HPV associated cancers and precancers they are obvious immune targets for therapeutic immunisation. The approach of deliberate immunisation with oncogenic HPV E6 and/or E7 and the generation of antigen specific CTL as an immunotherapy for HPV associated cancer and their high grade precancers has been tested with a wide array of potential vaccine delivery systems in Phase I/II trials with varying success. Advances in our understanding of viral and tumour immune evasion strategies has emphasised that modulation of the local tumour immune microenvironment combined with the induction of strong systemic HPV E6 and E7 antigen specific cytotoxic effector responses are prerequisites for successful therapeutic immunisation. The clinical trials to date have been reasonably successful in eliciting cell mediated immune responses to HPV antigens but these responses do not necessarily correlate with the clinical outcome. The identification of the immune parameters that faithfully reflect therapeutic vaccine responses, particularly those that measure responses in the local mucosal immune milieu, and the development of immunological assays that measure these is of crucial importance. In any event therapeutic vaccines are unlikely to be stand alone therapies but used in combination with other modalities could make a major contribution to treatment of HPV associated neoplasias.

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Chapter 13
Perspectives on Therapeutic HPV Vaccines: Where Are We Now?

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1 Introduction

Cervical cancer is the second most common gynecological malignancy worldwide, with an estimated 493,000 new cases resulting in approximately 274,000 deaths annually (Parkin et al. 2005). The identification of human papillomavirus (HPV) as the etiologic agent of cervical cancer and other HPV-associated malignancies, including anogenital cancers and a subset of head and neck cancers (Parkin et al. 2005), has created the opportunity to control HPV-associated disease through vaccines that target HPV. There are more than 100 types of HPV identified and can be classified

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by their malignant potential. While low-risk subtypes (i.e. HPV-6, HPV-11) are associated with low grade benign lesions, high-risk subtypes (HPV-16, 18, 45, 31) can lead to the development of high grade lesions and malignant tumors (de Villiers et al. 2004). The persistent infection with high-risk type HPV followed by the development of premalignant squamous intraepithelial lesions (SIL), also known as cervical intraepithelial lesions (CIN), is a necessary trigger of cervical cancer (Walboomers et al. 1999).

HPV is a non-enveloped, double-stranded, circular DNA virus with an icosahedral capsid (for review see Hoory et al. 2008). Its genome is approximately 8,000 base pairs long and is composed of early proteins (E1, E2, E4, E5, E6, E7) and late proteins (L1, L2). The early protes regulate the viral life cycle with E1 and E2 controlling viral DNA replication, E2 directing viral RNA transcription, E4 involved in cytoskeletal reorganization, and E5, E6, and E7 involved in cellular transformation. The L1 major capsid protein and L2 minor capsid protein are structural proteins that comprise the viral capsid. In some persistent high-risk HPV infections, HPV DNA may integrate into the host genome, resulting in the deletion of certain HPV viral genes, including E2, E4, E5, L1, and L2. Since E2 also serves as the transcriptional repressor of E6 and E7, the loss of E2 leaves E6 and E7 as the principal proteins expressed within the infected cell. HPV E6 and E7 inactivate tumor suppressors, p53 and retinoblastoma (Rb), which lead to the breakdown of cell cycle regulation. Hence, cells infected with high-risk HPV develop genomic instability, that result in the progression of cancer (for review, see zur Hausen 2002). Since HPV-16 and HPV-18 are the high-risk types most commonly associated with cervical cancer and together, account for up to 75% of all cervical cancers, they have been the focus of preventive and therapeutic HPV vaccine development.

Preventive HPV vaccines aim to block viral infection through the generation of neutralizing antibodies against L1 and/or L2 HPV viral capsid proteins. The current commercially available preventive HPV vaccines – Gardasil and Cervarix – capitalize on the use of HPV L1 virus-like particles (VLPs), which are morphologically similar to native HPV virions (Kirnbauer et al. 1992, 1993), to induce virus-specific protective humoral immune responses (Roden and Wu 2006; Roden et al. 2007). Merck's Gardasil is a quadrivalent L1 VLP recombinant vaccine that protects against four of the most medically relevant HPV genotypes: HPV-6 and HPV-11 for benign genital warts, and HPV-16 and HPV-18 for cervical cancer. GlaxoSmithKline's Cervarix is a bivalent L1 VLP recombinant vaccine derived from HPV types 16 and 18. They have been shown to be well tolerated, highly immunogenic, and able to induce the production of neutralizing antibodies to effectively prevent HPV-associated infection (Harper 2009; Villa et al. 2005, 2006). In addition, these vaccines, particularly Cervarix, have demonstrated partial cross-protection with other HPV types not included in the vaccine (HPV-31 and HPV-45). Thus, the commercial preventive vaccines may protect up to 80% of cervical cancers (Harper et al. 2006).

Nevertheless, there remains an urgent need for therapeutic HPV vaccines. Since some of the HPV-associated tumor cells with viral integration do not express

detectable levels of capsid antigens (L1 and/or L2), preventive HPV vaccines are unlikely to effectively eliminate these HPV-associated lesions. The high cost and need for appropriate storage of the current preventive HPV vaccines may also restrict their use in developing countries, which account for >80% of cervical cancers and have limited medical resources. Furthermore, it is estimated that it would take approximately 20 years from the implementation of mass vaccination for preventive vaccines to impact the cervical cancer rates due to the significant global burden of existing HPV infections and the slow process of carcinogenesis. Therefore the continued clinical development of therapeutic HPV vaccines is important for reducing the mortality and morbidity of HPV-associated malignancies.

2 Therapeutic HPV Vaccines

Therapeutic HPV vaccine differs from preventive HPV vaccines in that therapeutic HPV vaccines focus on the promotion of HPV antigen-specific cell-mediated immunity through the delivery of antigens to professional antigen-presenting cells such as dendritic cells (DCs), whereas preventive HPV vaccines focus on eliciting viral capsid-specific neutralizing antibody responses. The major function of dendritic cells is to present processed antigens through major histocompatibility complex (MHC) class I and/or II in order to prime antigen-specific cytotoxic CD8+ T and helper CD4+ cells respectively. The antigen-specific cellular immune responses elicited by therapeutic HPV vaccines can selectively kill HPV-infected cells and/or HPV-associated tumor cells at sites all over the body without damaging non-infected tissues. The development of therapeutic HPV vaccines has focused on the activation and promotion of T cells that recognize and target infected cells expressing HPV E6 and E7. Several factors make HPV-encoded antigens, E6 and E7, potentially ideal targets for the development of therapeutic HPV vaccine. For example, HPV E6 and E7 are constitutively expressed at every level of the epithelium of HPV-infected cells, whereas L1 and L2 are only expressed in terminally differentiated keratinocytes. E6 and E7 are critical for the induction of carcinogenesis in HPV-infected cells and therefore make it unlikely that HPV-infected tumor cells can escape the immune system through antigenic loss. Furthermore, since E6 and E7 are foreign proteins, the issue of immune tolerance that plagues cancer vaccines can be circumvented when the vaccine is targeting HPV-E6/E7 antigens. Herein, we examine different therapeutic HPV vaccine approaches that target E6 and/or E7, such as the use of live vector-based vaccines, peptide-based vaccines, protein-based vaccines, nucleic acid-based vaccines, cell-based vaccines, and approaches that are combinational. Table 13.1 is a summary of the pros and cons of each approach. Table 13.2 is a summary of ongoing therapeutic HPV vaccine clinical trials. This chapter will cover the clinical development of several therapeutic HPV vaccines for the control of HPV-associated malignancies and their precursor lesions.

Table 13.1 Characteristics of therapeutic HPV vaccine approaches

Vaccine approach	Advantages	Disadvantages	Future directions
Live vector-based	• High immunogenicity • Can facilitate intercellular antigen spreading • Wide variety of vectors available	• Safety concerns in using live vectors • Limited ability for repeated administration due to potential pre-existing immunity	• Increasing vector immunogenicity • Limiting vector-associated toxicity • Combining with other vaccines
Peptide-based	• Easy to produce, Stable, Safe • Can combine multiple epitopes • Can engineer peptides to enhance binding affinity for MHC	• Low immunogenicity • Need to define epitopes • Difficult to have one-fits-all peptide (unless using overlapping peptide) • Requires adjuvants	• Overlapping peptides • Optimize adjuvants
Protein-based	• Stable, Safe • No HLA restriction	• Low immunogenicity; requires adjuvant • Usually better induction of antibody response than CTL response	• Combine with strategies to increase antigen uptake and MHC class I presentation • Optimize adjuvants
DNA-based	• Safe, simple, stable for storage and transportation • Capacity for repeated administration • Easy to prepare at high purity on a large scale • Sustained antigen expression • Can be engineered to add targeting and/or co-stimulatory genes	• Inability to amplify and spread *in vivo*, leading to weak immunogenicity • Small risk of integration into genome or cellular transformation	• Optimize adjuvants • Optimize delivery methods

RNA replicon-based	• Non-infectious • No risk of chromosomal integration or cellular transformation • Capable of repeated immunization • Enhanced antigen expression • Multiple vectors available	• Unstable compared to DNA • Difficulty in long-term storage • Labor-intensive preparation • Difficult to prepare large amounts	• Optimize adjuvants
Dendritic cell-based	• High immunogenicity; uses the most potent APC • Efficient antigen presentation • Multiple methods of Ag loading available	• Labor intensive, costly, ex vivo, individualized cell processing • Variable quality control and a lack of standard criteria for quality of vaccines due to autologous nature • Difficult to produce on a large scale	• Optimize adjuvants
Tumor cell-based	• Useful if tumor antigen unknown • Likely to express relevant tumor antigens • Potency can be enhanced by cytokine treatment	• Safety concerns • Labor-intensive procedure • Costly, difficult to produce on a large scale	

2.1 Live Vector-Based Therapeutic HPV Vaccines

The use of live vector for therapeutic HPV vaccine development is an attractive option since these live vectors are engineered to express HPV E6 and/or E7 to provoke a strong immune response. Live vectors are able to replicate within host cells and permit cell-to-cell antigenic spread. Additionally, there is a wide variety of vectors available and therefore allows for flexibility when choosing a vector to engineer for the desired outcome. Live viral vectors are able to induce antigen-specific cytotoxic T cell immune responses. However, in spite of the great promises that live vector vaccines hold, their use is also associated with safety concerns. In addition, their capacity for repeated administration is limited since neutralizing antibodies are generated against the live vector. Currently, what is known as live vector-based vaccines include two categories: bacterial vectors and viral vectors.

2.1.1 Bacterial Vectors

Of the numerous bacterial vectors that have been considered as a potential candidate for bacterial vector-based therapeutic HPV vaccines, *Listeria monocytogenes* (*Lm*) stands out as a potentially promising vector (Lin et al. 2002; Sewell et al. 2004a, 2008). *Lm*, a gram-positive bacterium, escapes phagosomal lysis by secreting listeriolysin O (LLO) and replicating within the cytosol of an antigen-presenting cell. The antigens secreted by *Lm* can be presented through both major histocompatibility complex (MHC) classes I and II pathways to induce antigen-specific $CD8^+$ and $CD4^+$ T-cell immune responses. In addition, *Lm* is antibiotic-sensitive and consequently, can be eliminated with antibiotics should the patient respond negatively. Preclinical studies of *Lm* encoding a fusion protein comprised of HPV-16 E7 with either Acting assembly-inducing protein (ActA) (Lm-ActA-E7) or a fragment of LLO (Lm-LLO-E7) was able to induce potent E7-specific $CD8^+$ T-cell responses, resulting in the control of established tumors (Gunn et al. 2001). Furthermore, the PEST protein sequence at the amino end of the LLO is determined to be important for the observed enhancement of immunogenicity that results from linking HPV E7 to LLO (Sewell et al. 2004b). A therapeutic HPV vaccine based on live attenuated *Lm* expressing HPV-16 E7 fused with LLO (ADXS11-001) was tested in 15 cervical cancer patients who had failed to respond to previous modes of therapy (Maciag et al. 2009). Of the three dose levels of ADXS11-001 (1×10^9 cfu, 3.3×10^9 cfu, or 1×10^{10} cfu) that were intravenously administered to patients, the highest dosage caused three patients to present with severe fever and dose-limiting hypotension. In this phase I study, all the patients exhibited some degree of self-limiting flu-like symptoms that were thought to be the result of vaccine-induced potent innate immune responses. Of the 15 patients, six had (grade 3) severe adverse events. This study concluded with seven patients reporting stable disease, five with progressive disease, and of the 13 evaluable patients, one patient qualified as a partial responder while four patients reported tumor reduction (Maciag et al. 2009). However, it is unclear

how much of the tumor reduction is attributed to the vaccination since the patients have had previous chemo-radiation. In comparison to other therapeutic HPV vaccines, ADXS11-001 induces adverse effects of greater severity in patients; however, these adverse effects are still considerably milder than those caused by chemotherapy. ADXS11-001 is currently being planned for Phase II trials (www.advaxis.com). Several bacterial vectors, such as *Lactococcus lactis* (Bermudez-Humaran et al. 2005), *Lactobacillus plantarum* (Cortes-Perez et al. 2005), and *Salmonella enterica* (Echchannaoui et al. 2008) are also being explored for the development of therapeutic HPV vaccine. For bacterial vector-based HPV vaccine to become a viable treatment option, it is necessary to improve the immunogenicity of the vector while simultaneously limiting the vector-associated toxicity.

2.1.2 Viral Vectors

Presently, several viral vectors have been explored for therapeutic HPV vaccine development, such as vaccinia viruses (Zurkova et al. 2009; Hsieh et al. 2004), adenoviruses (Gomez-Gutierrez et al. 2007; Lee et al. 2008; Zhou et al. 2010), vesicular stomatitis viruses (VSV) (Liao et al. 2008; Brandsma et al. 2010), and alpha viruses (Cheng et al. 2002). Among these viruses, the vaccinia virus is considered especially promising and frequently studied in clinical trials due to its high infection efficiency and large genome. One viral vector-based therapeutic HPV vaccine candidate is TA-HPV, which is a recombinant vaccinia virus that expresses HPV-16/18 E6 and E7. The first TA-HPV evaluation occurred in an open label Phase I/II trial that consisted of eight patients with therapy-unresponsive late-stage cervical cancer. The patients were given a single dose (2×10^6 pfu) of TA-HPV by a scarification technique. Of the eight patients, three developed HPV-18 E7-specific antibodies while one of three evaluable patients had detectable HPV-specific CD8+ T cells, which demonstrated the immunogenicity of TA-HPV (Borysiewicz et al. 1996). In another Phase I safety and immunogenicity study, 29 patients with either stage Ib or IIa cervical cancer were vaccinated with a scarification technique estimated to deliver approximately 2.5×10^5 pfu of TA-HPV (Kaufmann et al. 2002). The patients exhibited no significant clinical side effects and a serological response to vaccinia was observed in 18 of 29 patients. Four patients had HPV-specific CTL responses, confirming the safety and immunogenicity of TA-HPV (Kaufmann et al. 2002). Other than the aforementioned cervical cancer trials, two other Phase II trials were conducted to examine the use of TA-HPV in treating vulvar intraepithelial neoplasia (VIN) patients. One of the Phase II trials involved administering 2.5×10^5 pfu of TA-HPV to 11 women with VIN 3 and one woman with vaginal intraepithelial lesions (VAIN) grade 2. Results showed an increase of antibody and T-cell responses in 11 out of 12 patients and therapeutic effect was noticed in 10 of the 12 patients where lesion reduction was observed. After vaccination, the lesions of the one VAIN patient had cleared completely and HPV-16 was no longer present at the previous site of the lesion (Baldwin et al. 2003). A separate trial evaluating the same regimen in 18 patients with HPV-16-positive

VIN 3 reported similar findings; eight patients presented reduced lesion diameter, viral load or viral clearance, and increased antigen-specific immune responses (Davidson et al. 2003).

Clinical trials have evaluated another viral vector-based therapeutic HPV vaccine known as MVA E2, a recombinant vaccinia virus derived from Modified Vaccinia Ankara (MVA) that encodes the E2 protein of bovine papillomavirus (Corona Gutierrez et al. 2004; Garcia-Hernandez et al. 2006; Albarran et al. 2007). MVA E2 is the transcriptional repressor of E6 and E7 oncogenic proteins. When there is HPV viral integration in the CIN lesion, the E2 region of cell genome tends to be the site at which HPV integrates. This leads to the disruption and loss of E2 expression. Thus MVA E2 can serve to provide intact E2 to suppress the expression of oncogenic E6 and E7 and potentially function as a form of gene therapy. MVA E2 may also function as a vaccine to induce E2-specific immune responses. In the case when CIN lesion is without HPV viral integration, E2 is not disrupted and therefore is expressed normally. As a result, the E2-expressing CIN lesion can be targeted by MVA E2 vaccine-induced E2-specific CD8+ T cell immunity. However, since E2 is derived from bovine papillomavirus, it is unclear if there is enough homology to induce HPV-16 E2-specific CD8+ T cell response. The efficacy of MVA E2 has been demonstrated in a Phase II trial that consisted of 34 patients with CIN2/3 that. In this trial, the patients were intrauterinely injected once a week (10^7 virus particles/dose of MVA E2) for a period of at least 6 weeks. Treatment with MVA E2 was shown to generate encouraging therapeutic effect in patients, with 20 out of 34 patients showing regression of high-grade lesion. MVA E2 was also well tolerated by patients and increased the antibody levels against both MVA virus and E2 protein (Garcia-Hernandez et al. 2006). The MVA E2 has exhibited similar safety and immunogenicity profiles in a Phase I/II study of 50 men with intraurethral flat condyloma (Albarran et al. 2007). In this study, 30 men were treated with MVA E2 while the control group of 20 men received five-fluorouracil. The experimental group of 30 men who received weekly intraurethral injections of MVA E2 (10^6 virus particles/dose) over a 4-week period developed antibodies against MVA virus and E2 protein. After the 4-week treatment with MVA E2, 28 out of 30 patients had no lesions or detectable viral DNA. In addition, the patients did not have lesion recurrence 1 year after treatment, whereas three of the control patients had lesion recurrence after 3 months (Albarran et al. 2007). Although MVA E2 is able to control HPV-associated lesions, how much of the therapeutic effect can be attributed to immunotherapy is unclear.

A third viral-based therapeutic vaccine, TG4001 (MVA-HPV-IL2), is made of a modified vaccinia using Ankara viral vector. TG4001 contains DNA that encodes HPV-16 E6 and E7, as well as IL-2, an immuno-regulatory cytokine. Early Phase I trials established the safety profile of TG4001, which allowed Phase II trials for the evaluation of vaccine efficacy to occur. In a Phase IIa trial that lasted 6 months, 21 patients with HPV-16-related CIN2/3 received three subcutaneous injections of MVA-HPV-IL2 (5×10^7 pfu/dose). After 6 months, 10 of the 21 patients were classified as clinical responders, thereby indicating the clinical efficacy rate was 48%. Additionally, 8 of the 10 clinical responders tested negative for HPV 16 DNA (Brun et al. 2011).

There are attempts to improve the immunogenicity of various viral vectors. Some of these efforts include the co-expression of soluble cytokines, such as the hFlt3 ligand that induces DC maturation (Zurkova et al. 2009), linkage with calreticulin-encoding gene to enhance antigen-presentation by major histocompatibility complex (MHC) class I (Hsieh et al. 2004), or a fusion protein composed of HPV-16/18 E7 joined with heat shock protein to enhance immune responses (Zhou et al. 2010). A major limitation is that the use of vector-based therapeutic HPV vaccines continues to show preexisting immunity to the viral vector. In research, there have been attempts to try prime-boost regimens using recombinant viral vectors in conjunction with other vaccines. Furthermore, it has been shown that Cox-2 inhibitors may enable the repeated administration of vaccinia virus (Chang et al. 2009). In short, recombinant viral vectors are administered with other forms of vaccines, such as inactivated viruses, proteins, peptides, DNA, or RNA-based vaccines, to synergistically enhance pathogen-specific immune responses, while simultaneously reduce vector-specific immunity.

2.2 Peptide-Based Therapeutic HPV Vaccines

The purpose of peptide-based therapeutic HPV vaccination is to directly provide HPV derived antigenic peptides for uptake by the DCs. The pros of peptide vaccines include easy production, stability, and safety, however their efficacy is greatly diminished by low immunogenicity. Furthermore, the polymorphism of human leukocyte antigen (HLA) molecules is a major impediment to the development of a peptide vaccine that is effective in all patients. It is impractical to produce a large-scale vaccination treatment using peptide-based vaccines since it would require the identification of specific immunogenic epitopes of HPV antigens for numerous HLA haplotypes. However, once the epitopes are defined, the peptide epitopes included in the vaccine can be controlled. This allows for the engineering of peptide vaccines to contain particularly immunogenic peptides or peptides that direct CD4+ T-helper or CD8+ cytotoxic immune responses.

The development of peptide vaccines for cervical cancer is made possible by the identification of various MHC-restricted CD4+ and CD8+ T cell epitopes of HPV early proteins, such as murine (H-2Db) and human (HLA-A2) CTL epitopes for HPV-16 (Feltkamp et al. 1993; Liu et al. 2007). Preclinical studies have attempted to boost the potency and immunogenicity of peptide vaccines with adjuvants such as 4-1BB ligand (Sharma et al. 2009), CpG oligodeoxynucelotide (Chen et al. 2004; Daftarian et al. 2006; Daftarian et al. 2007), and mutant cholera toxin (Manuri et al. 2007) (for review, see Roden et al. 2007). Additional strategies that have been employed to stimulate peptide vaccine potency include enhancement of the epitope to increase peptide affinity for MHC molecule and the linkage of peptides to lipids (lipopeptides) (Chen et al. 2008).

Early phase clinical trials have identified multiple peptide-based therapeutic HPV vaccines as safe and well tolerated (Steller et al. 1998; van Driel et al. 1999; Ressing et al. 2000; Muderspach et al. 2000). The peptide-based vaccine containing

a lipopeptide consisting of HLA-A*0201-restricted lipidated HPV-16 E7 peptide (aa 86–93) linked to PADRE, a non-specific helper peptide, was evaluated in a non-randomized Phase I dose escalating study. In five of seven patients who received two injections and in two of three patients who received all four injections, the lipopeptide-based vaccine was able to generate E7-specific cytotoxic T lymphocyte immune responses with no adverse side effects (Steller et al. 1998). HLA-A*0201-restricted HPV-16 E7 peptide (aa 11–20 and aa 86–93) and PADRE emulsified in Montanide ISA 51 adjuvant have also been tested in Phase I/II trials with HLA-A*0201-positive patients with HPV-16 positive recurrent or refractory cervical cancer (van Driel et al. 1999; Ressing et al. 2000). In both trials, that the patients had low lymphocyte counts before and after vaccination, which suggests the patients with advanced cervical cancer were immuno-incompetent. In the Phase I trial that tested HLA-A*0201-restricted, lipidated HPV-16 E7 peptide (aa 12–20 ± 86–93) linked to PADRE emulsified in Montanide ISA 51 adjuvant in 18 HLA-A2-positive patients with HPV-16-positive high-grade CIN or VIN, the lipopeptide generated higher HPV E7-specific T cell immunity in 10 of 16 patients tested as well as partial or complete regression of CIN lesions in 9 out of 17 evaluable patients. Generally, these peptide-based vaccines are well tolerated by patients but are only effective in immuno-competent individuals with pre-invasive diseases (Muderspach et al. 2000).

The developmental direction of peptide vaccines might be the creation of long overlapping peptides that can broaden the range of antigenic epitopes and therefore reduce the issue of MHC restriction. In preclinical animal models, overlapping peptides have effectively induced antigen-specific T cell immune responses in both mice (Zwaveling et al. 2002) and rabbits (Vambutas et al. 2005). One particular overlapping peptide-based vaccine containing 13 peptides (9 E6 and 4 E7 epitopes 25–35 aa long with an overlap of 10–14 aa) that represent HPV-16 E6 and E7 is of particular interest in several clinical trials. A phase I trial subcutaneously injected this overlapping peptide-based vaccine in 11 patients with HPV-16-positive VIN 3 for four times at a 3-week interval. This vaccine elicited HPV-16 E6 and E7-specific CD4+ and CD8+ T cells and in 4 out of 11 patients, resulted in complete lesion clearance (Melief et al. 2007). This very vaccine was examined in a Phase I trial conducted by Kenter et al., in which, it was shown that among the 43 end-stage cervical cancer patients, the vaccine was safe, well tolerated, and produced broad IFN-γ-associated T cell responses (Kenter et al. 2008). Moreover, when this vaccine regimen was examined in six patients with stage 1B1 HPV-16+ cervical cancer, enhanced HPV-16-specific CD4+ and CD8+ T cell responses to a broad array of epitopes was produced (Welters et al. 2008). Recently, this overlapping peptide vaccine was administered to 20 HPV-16+ grade 3 VIN patients during a Phase II clinical trial. In this trial, T cell responses were generated in all patients with objective clinical responses in 15 of 19 evaluable patients at the 12 month follow-up, with nine patients who continued to be disease-free at the 24 month follow-up (Kenter et al. 2009). The promising results of overlapping peptides have heightened the interest in the development of therapeutic HPV E6/E7 peptide-based vaccines.

2.3 Protein-Based Therapeutic HPV Vaccines

In many respects, protein-based vaccines have many advantages compared to other forms of vaccines such as those that are live vector- and peptide-based. Protein-based therapeutic HPV vaccines rank higher in safety than live vector-based vaccines, particularly for immuno-compromised patients. The advantage of protein-based vaccines is that their MHC specificity is not limited. However, just like peptide-based vaccines, they are not very immunogenic. Since proteins have all the possible HLA epitopes and are broken down by the patient's own cells into peptide epitopes, identifying the patient's HLA type and the immunogenic epitopes of the antigen is unnecessary. Regrettably, protein-based vaccines may be inadequate for the induction of cytotoxic T lymphocyte (CTL) responses since exogenously administered proteins usually trigger greater antibody responses.

In preclinical trials, greater CTL responses have been produced through the use of adjuvants and the fusion of proteins with other immunostimulatory molecules. Common adjuvants include liposome-polycation-DNA (LPD) (Cui and Huang 2005) and saponin-based ISCOMATRIX (Stewart et al. 2004). Immunostimulatory molecules are used to enhance the uptake and presentation of antigens since they target antigens to antigen-presenting cells (APCs). To create a fusion protein, HPV-16 E7 can be fused with the bacterial protein, *Bordetella pertussis* adenylate cyclase (CyaA), which will interact with $\alpha_M ß_2$ integrin to target DCs and induce E7-specific CTL responses (Preville et al. 2005). Another bacterial adjuvant protein that HPV-16 E7 can be fused with is *Mycobacteria*-derived heat shock proteins (Hsp) (Chu et al. 2000; Liu et al. 2008), which have been shown to augment CTL responses. When HPV-16 E7 is fused with truncated *Pseudomonas aeruginosa* exotoxin A, protein translocation is improved and as a result, so is MHC class I presentation, which leads to greater E7-specific T-cells as well as antibody responses (Liao et al. 2005).

There have been clinical trials that test protein-based therapeutic HPV vaccines for safety and efficacy (see Table 13.2) (Thompson et al. 1999; Lacey et al. 1999; de Jong et al. 2002; Hallez et al. 2004; Frazer et al. 2004; Goldstone et al. 2002; Derkay et al. 2005; Palefsky et al. 2006; Roman et al. 2007; Einstein et al. 2007; Van Doorslaer et al. 2010; NCI 2009a; NCI 2009b). From these clinical trials, one particular vaccine candidate has emerged. TA-CIN is a fusion of HPV-16 L2, E6, and E7 proteins. TA-CIN has demonstrated immunogenicity in a Phase I clinical trial, in which, L2-specific antibodies was induced in all patients, with 8 out of 11 patients who were administered the highest dose demonstrating T cell activity against HPV-16 E6 and E7 (de Jong et al. 2002). There are also phase II trials that examined TA-CIN's candidacy for use in a prime-boost regimen with TA-HPV (Smyth et al. 2004; Fiander et al. 2006; Davidson et al. 2004) since preclinical studies have indicated prime-boost has greater efficacy than single vaccination (van der Burg et al. 2001). TA-CIN was used in a Phase II clinical trial for priming while the recombinant vaccinia virus, TA-HPV, which encodes HPV-16/18 E6/E7 fusion protein, was the boost in treating 29 patients with anogenital intraepithelial neoplasia. Five of 29 patients had higher HPV-16-specific T cell specific immune responses

Table 13.2 Ongoing therapeutic HPV vaccine clinical trials

Type	Vaccine	Construct	Target antigen(s)	Sponsor	Phase	Patient population	Regimen	Ref.
Live vector	ADXS11-001 (Lovaxin C)	live *Listeria monocytogenes* secreting HPV-16 E7 fused to listeriolysin O	HPV-16 E7	Advaxis Inc.	I	15 patients with refractory or recurrent cervical cancer	Intravenous infusion of one of three dose levels (1×10^9 cfu, 3.3×10^9 or 1×10^{10} cfu), followed by second dose 3 weeks later	Maciag et al. (20090
	TA-HPV	Live recombinant vaccinia virus expressing the E6 and E7 proteins of HPV-16 and HPV-18	HPV-16/18 E6/E7	Xenova/Cantab (now acquired by Celtic Pharma)	I/II	8 patients with late-stage cervical cancer	Single dermal scarification of TA-HPV (2.5×10^5 pfu)	Borysiewicz et al. (1996)
					I	29 patients with Stage Ib or IIa cervical cancer	Two vaccinations at least 4 weeks apart, using dermal scarification to deliver approximately 2.5×10^5 pfu	Kaufmann et al. (2002)
					II	12 patients with high-grade VIN or VAIN: (11 VIN 3, 1 VAIN 2)	Single dermal scarification of TA-HPV (2.5×10^5 pfu)	Baldwin et al. (2003)

				II	18 patients with HPV-16-positive VIN 3	Single dermal scarification of TA-HPV (2.5×10^5 pfu)	Davidson et al. (2003)
			European Organization for Research and Treatment of Cancer	Ongoing, II	Patients with Stage Ib or IIa cervical cancer	Two vaccinations of TA-HPV, with 1st vaccination at least 2 weeks before surgery and 2nd vaccination at 8 weeks after 1st vaccination	NCI (2009d)
MVA E2	Recombinant vaccinia derived from Modified Vaccinia Ankara encoding E2 of bovine papillomavirus	HPV-16 E2	Instituto Mexicano del Seguro Social (IMSS)	I/II	36 patients with CIN 1–3	Intrauterine injection once a week over a 6 week period (10^7 virus particles/dose)	Corona Gutierrez et al. (2004)
				II	34 patients with high-grade CIN	Intrauterine injection once a week over a 6 week period (10^7 virus particles/dose)	Garcia-Hernandez et al. (2006)
				I/II	50 men with flat condyloma lesions	Intraurethral injection once a week over a 4 week period (10^6 virus particles/dose)	Albarran et al. (2007)

(continued)

Table 13.2 (continued)

Type	Vaccine	Construct	Target antigen(s)	Sponsor	Phase	Patient population	Regimen	Ref.
	TG4001 (MVA-HPV-IL2)	Recombinant vaccinia virus derived from Modified Vaccinia Ankara expressing HPV-16 modified E6 and E7 proteins and IL-2	HPV-16 E6/E7	Transgene/Roche	IIa	21 women with CIN 2/3	Three subcutaneous injections of MVA-HPV-IL2 at 5×10^7 pfu/dose	TG 4001 Brun et al. (2011)
					Plans for Phase IIb	200 women with CIN 2/3	Currently in planning	TG 4001 http://clinicaltrials.gov/show/NCT01022346
Peptide	Lipopeptide	Lipidated E7 (HLA-A*0201-restricted epitope, aa 86 – 93 lipopeptide) linked to PADRE helper peptide	HPV-16 E7	Cytel Corporation (later Epimmune, then aquired by IDM Pharma)	I	12 HLA-A2-positive patients with HPV-16-positive recurrent or refractory cervical cancer	Four subcutaneous injections at one of four dose levels (0.1, 0.3, 1.0 and 2.0 mg) of lipopeptide at 3-week intervals	Steller et al. (1998)

Peptide and Montanide ISA 51 adjuvant	HLA-A*0201-restricted HPV-16 E7 peptide (aa 11–20 and 86–93) and PADRE helper peptide, emulsified in Montanide ISA 51 adjuvant	HPV-16 E7	Cytel Corporation (later Epimmune, then aquired by IDM Pharma), Dutch Cancer Society	I/II	19 HLA-A*0201-positive patients with recurrent or residual cervical cancer	Four subcutaneous injections at one of three dose levels (100 ug, 300 ug, 1000 ug) at 3-week intervals	van Driel et al. (1999)
				I/II	15 HLA-A*0201-positive patients with recurrent or residual cervical cancer	Four subcutaneous injections at one of three dose levels (100 ug, 300 ug, 1000 ug) at 3-week intervals	Ressing et al. (2000)
Peptide and Montanide ISA-51 adjuvant	HLA-A*0201-restricted HPV-16 E7 epitopes (aa 12–20 ± aa 86–93) linked to PADRE, emulsified in Montanide ISA 51 adjuvant	HPV-16 E7	NCI	I	18 patients with high-grade CIN or VIN: (16 with CIN 2/3, 2 with VIN 2/3)	Four subcutaneous injections (10 mg/vial of lipopeptide) at 3-week intervals	Muderspach et al. (2000)

(continued)

Table 13.2 (continued)

Type	Vaccine	Construct	Target antigen(s)	Sponsor	Phase	Patient population	Regimen	Ref.
	Overlapping long peptide and Montanide ISA-51 adjuvant	13 peptides together (nine E6 and four E7 peptides of 25–35 aa long with an overlap of 10–14 aa) representing the entire sequence of HPV-16 E6 and E7, formulated in Montanide ISA 51 adjuvant	HPV-16 E6/E7	Dutch Cancer Society, ISA Pharma-ceuticals	I	11 patients with HPV-16-positive VIN 3	Four subcutaneous injections at a 3 week interval (0.3 mg/each of 13 peptides)	Melief et al. (2007)
					I	43 end-stage cervical cancer patients	Four subcutaneous injections at a 3 week interval (0.3 mg/each of 13 peptides)	Kenter et al. (2008)
					II	6 patients with resected HPV-16-positive stage 1B1 cervical cancer	Four subcutaneous injections at a 3 week interval after surgical dissection (0.3 mg/each of 13 peptides)	Welters et al. (2008)

					II	20 patients with HPV-16-positive VIN 3	Four subcutaneous injections at 3 week intervals, each time in a different arm or leg (0.3 mg/each of 13 peptides)	Kenter et al. (2009)
Protein	Therapeutic Antigen-Genital Warts (TA-GW)	Recombinant HPV-6 L2/E7 fusion protein adjuvanted with 2% Alhydrogel	HPV-6 L2/E7	Xenova/Cantab (now acquired by Celtic Pharma)	I	42 healthy male volunteers (32 immunized with TA-GW, 6 injected with placebo)	Three intramuscular injections at one of four dose levels (0, 3, 30 or 300 ug TA-GW) in accelerated scheme (weeks 0, 1, 4) or classical scheme (weeks 0, 4, 8)	Thompson et al. (1999)
					IIa	27 patients with genital warts	Three intramuscular injections at 4 week intervals	Lacey et al. (1999)
	TA-CIN	Recombinant HPV-16 L2/E6/E7 fusion protein	HPV-16 L2/E6/E7	Xenova/Cantab (now acquired by Celtic Pharma)	I	40 healthy volunteers	Three intramuscular injections into same upper arm at one of three dose levels (26, 128, 533 ug) at 4 week intervals	de Jong et al. (2002)

(continued)

Table 13.2 (continued)

Type	Vaccine	Construct	Target antigen(s)	Sponsor	Phase	Patient population	Regimen	Ref.
	PD-E7	Fusion protein comprising mutated HPV-16 E7 linked to first 108 aa of *Haemophilus influenza* protein D, formulated in AS02B adjuvant	HPV-16 E7	GlaxoSmith-Kline	I/II	Seven patients with CIN 1 or CIN 3: (5 with CIN3 and 2 with CIN 1)	Three intramuscular injections at 2 week intervals with 200 ug of PD-E7 formulated in 500 ul of AS02B adjuvant	Hallez et al. (2004)
	HPV16 Immunothe-rapeutic	Recombinant HPV-16 E6/E7 fusion protein adjuvanted with ISCOMATRIX	HPV-16 E6/E7	CSL Limited	I	31 patients with CIN 1–3	Three intramuscular injections at 3 weekly interval	Frazer et al. (2004)
	SGN-00101 (HspE7)	Fusion protein of HPV-16 E7 with *Mycobacterium bovis* variant bacille Calmette-Geurin heat shock protein Hsp65	HPV-16 E7	Nventa Biopharma-ceuticals (formerly Stressgen, recently bought by Akela Pharma)	I	22 patients with anal HSIL	Three subcutaneous injections of either 100 ug of HspE7 or placebo at monthly intervals	Goldstone et al. (2002)

II	14 patients with anal HSIL	Three subcutaneous injections of 500 ug of HspE7 at monthly intervals	Goldstone et al. (2002)
II	27 pediatric patients with recurrent respiratory papillomatosis	After baseline debulking, patients received Three subcutaneous injections of HspE7 (500 ug) at monthly intervals	Derkay et al. (2005)
I/II	15 HIV patients with high-grade AIN	Three subcutaneous injections in alternating thighs at one of three dose levels (100, 500 or 1000 ug) at 4-week intervals	Palefsky et al. (2006)
II	20 women with high-grade CIN	Four subcutaneous injections at a dose of 500 ug at a 3 week interval followed by LLETZ	Roman et al. (2007)

(continued)

Table 13.2 (continued)

Type	Vaccine	Construct	Target antigen(s)	Sponsor	Phase	Patient population	Regimen	Ref.
					II	58 patients with CIN 3	Three monthly subcutaneous vaccinations with 500 ug of HspE7 followed by colposcopic follow-up and cone biopsy of cervix	Einstein et al. (2007) and Van Doorslaer et al. (2010)
					II	Women with CIN 3	Three subcutaneous injections at 4 week intervals	NCI (2009a)
					Ongoing, II	Women with ASCUS/ LSIL	Three subcutaneous injections at 4 week intervals	NCI (2009b)
	SGN-00101 (HspE7) and Poly ICLC adjuvant	SGN-00101 adjuvanted in Poly ICLC	HPV-16 E7	Nventa Biopharma-ceuticals (formerly Stressgen, recently bought by Akela Pharma)	Ongoing, I	Women with CIN 1,2, or 3	Three subcutaneous vaccinations at 4 week intervals	Nventa (2008)

DNA	ZYC101	Plasmid DNA encoding HLA-A2-restricted epitopes derived from HPV-16 E7 protein (aa 83–95), encapsulated in 1–2 um biodegradable poly(lactide-co-glycolide) microparticles	HPV-16 E7	Eisai (formerly MGI Pharma, formerly Zycos)	I	12 HLA-A2-positive men with HPV-16-positive high grade AIN	Three intramuscular injections at a 3 week interval at one of four dose levels (50, 100, 200, 400 ug)	Klencke et al. (2002)
					I	15 patients with CIN 2/3	Three subcutaneous injections or intramuscular injections (depending on randomized group) every 3 weeks followed by surgical excision 4 weeks later	Sheets et al. (2003)

(continued)

Table 13.2 (continued)

Type	Vaccine	Construct	Target antigen(s)	Sponsor	Phase	Patient population	Regimen	Ref.
	ZYC101a (Amolimogene bepiplasmid)	Plasmid DNA encoding fragments derived from HPV-16 and 18 E6 and E7 proteins, encapsulated in 1–2 um biodegradable poly(D,L-lactide-co-glycolide) microparticles	HPV-16/18 E6/E7	Eisai (formerly MGI Pharma, formerly Zycos)	II	127 patients with CIN 2/3	Three intramuscular injections every 3 weeks followed by cervical conization 6 months after 1st injection	Garcia et al. (2004)
					Ongoing,II/III	251 patients with CIN 2/3	Three intramuscular injections at 100 ug/injection every 3 weeks	Eisai (2009)
	pNGVL4a-Sig/E7(detox)/Hsp70	Plasmid DNA expressing HPV-16 E7 mutated to abolish Rb binding site linked to sequences coding for Sig and for heat shock protein 70	HPV-16 E7	NCI	I	15 patients with CIN 2/3	Three intramuscular injections at one of three dose levels (0.5, 1.0 or 3.0 mg/dose at 4 week interval	Trimble et al. (2009)

				I	Patients with advanced HNSCC	Four intramuscular injections at one of four dose levels (500 ug, 1 mg, 2 mg, 4 mg) at weeks 1, 3, 5, 17	[a]
pNGVL4a-CRT/E7(detox)	Plasmid DNA expressing HPV-16 E7 mutated to abolish Rb binding site linked to sequence coding for calreticulin	HPV-16 E7	NCI	Ongoing, I	Patients with CIN 2/3	Dose-escalating gene gun administration with ND10 gene gun device	[b]
VGX-3100	Plasmid DNA expressing HPV-16 and HPV-18 E6 and E7 proteins	HPV-16/18 E6/E7	Inovio Biomedical Corp/VGX Pharma-ceuticals	Ongoing, I	Adult females, post-surgical or ablative treatment of CIN 2/3	Three dose series (0.6, 2 or 6 mg of DNA/dose) administered by intramuscular injection with electroporation with CELLECTRA device	VGX (2009)

(continued)

Table 13.2 (continued)

Type	Vaccine	Construct	Target antigen(s)	Sponsor	Phase	Patient population	Regimen	Ref.
DC	DC+IL2	Autologous mature, monocyte-derived DCs pulsed with HPV-18 E7 protein	HPV-18 E7		Case report	One patient with Stage IB2 cervical cancer	Subcutaneous injection 14 times with DCs (3 to 5×10^6/injection) combined with adoptive transfer of *in vitro* DC-primed and expanded HPV-17 E7-specific T cells and low-dose IL-2 treatment	Santin et al. (2002)
	DC	Autologous, mature, monocyte-derived DCs loaded with recombinant HPV-16 E7 or HPV-18 E7	HPV-16 E7 or HPV-18 E7	Deutsche Forschungs-gemeinschaft, BMBF	Pilot study	15 patients with progressive or recurrent cervical cancer	Subcutaneous injection (2×10^4 or 2×10^6 DCs/injection) into upper arm one to four times	Ferrara et al. (2003)

DC + IL2	Autologous, mature, monocyte-derived DCs pulsed with recombinant HPV-16 E7 or HPV-18 E7	HPV-16 E7 or HPV-18 E7	Italian Institute of Health (ISS)	Case series	Four cervical cancer patients with recurrent disease refractory to standard treatment	Subcutaneous injection (1×10^7 DCs/dose) into anterior mid-thigh. Vaccinations 1–5: 2 week intervals. Vaccinations 6–10: 30 day intervals. Vaccinations 11–13: 60 day intervals. All injections followed by twice daily subcutaneous injections of IL-2 from day 3 to day 7 postvaccination	Santin et al. (2006)
DC + KLH	Autologous, mature, monocyte-derived DCs pulsed with recombinant HPV-16/18 E7 antigens and keyhole limpet hemocyanin	HPV-16/18 E7	NIH	I	Ten patients with Stage Ib or IIa cervical cancer	Escalating doses (5, 10, 15×10^6 cells for injection) of 5 subcutaneous injections at 21-day intervals	Santin et al. (2008)

(continued)

Table 13.2 (continued)

Type	Vaccine	Construct	Target antigen(s)	Sponsor	Phase	Patient population	Regimen	Ref.
	DC	Autologous mature, monocyte-derived DCs pulsed with HPV-16 E7 peptide	HPV-16 E7	National Taiwan University Hospital	Ongoing, I	12 patients with recurrent cervical cancer	Four injections of DCs into inguinal lymph nodes under guidance of real-time sonography	Hospital (2009)
Prime-boost	Prime with TA-CIN, boost with TA-HPV	TA-CIN: (HPV-16 L2 E6/E7 fusion protein)	HPV-16/18 E6/E7 + HPV-16 L2/E6/E7	Xenova/Cantab (now acquired by Celtic Pharma)	II	29 patients with high-grade AGIN	Three intramuscular doses of TA-CIN at 4 week intervals followed by a single dermal scarification of TA-HPV	Smyth et al. (2004)
		TA-HPV: (vaccinia expressing HPV-16 and −18 E6 and E7)			II	29 patients with AGIN 3	Three intramuscular doses of TA-CIN on days 0, 28 and 52 followed by single dermal scarification of TA-HPV on day 72	Fiander et al. (2006)

Prime with TA-HPV, boost with TA-CIN	TA-HPV: (vaccinia expressing HPV-16 and −18 E6 and E7)	HPV-16 L2/E6/E7 + HPV-16/18 E6/E7	Xenova/Cantab (now acquired by Celtic Pharma)	I/II	Ten patients with high-grade AGIN	Single dermal scarification of TA-HPV followed by three intramuscular injections of TA-CIN 1 month apart at 24 weeks after TA-HPV vaccination	Davidson et al. (2004)
	TA-CIN: (HPV-16 L2 E6/E7 fusion protein)						
Prime with pNGVL4a-Sig/E7 (detox)/Hsp70, boost with TA-HPV ± imiquimod	pNGVL4a-Sig/E7(detox)Hsp70 plasmid DNA + recombinant vaccinia ± topical agent	HPV-16 E7 + HPV-16/18 E6/E7	NCI	I	Patients with HPV infection and CIN3	Intramuscular injection of DNA vaccine on days 1 and 29 and intramuscular TA-HPV on day 57 (with or without topical imiquimod)	NCI (2009c)

aa amino acid, *ASCUS* atypical squamous cells of undetermined significance, *AGIN* anogenital intraepithelial neoplasia, *CIN* cervical intraepithelial neoplasia, *HLA* human leukocyte antigen, *HNSCC* head and neck squamous cell carcinoma, *HPV* Human papillomavirus, *HSIL* high-grade squamous intraepithelial lesions, *Hsp* heat shock protein, *IL* interleukin, *KLH* keyhole limpet hemocyanin, *LLETZ* loop electrosurgical excision of the transformation zone, *LSIL* low-grade squamous intraepithelial lesions, *NCI* National Cancer Institute, *NIH* National Institutes of Health, *PADRE* Pan-DR binding T helper epitope, *Rb* Retinoblastoma, *VAIN* Vaginal intraepithelial neoplasia, *VIN* Vulvar intraepithelial neoplasia

[a]M Gillison, personal communication

[b]C Trimble and W Huh, personal communication

and none of the patients presented with serious adverse effects. However, this trial does not demonstrate a significant improvement over single TA-HPV vaccination (Fiander et al. 2006). In a subsequent prime-boost regimen, ten HPV-16+ high-grade vulvar intraepithelial neoplasia patients were primed with TA-HPV followed by TA-CIN boost. Of the ten patients, nine had HPV-16-specific T cell responses, and three had significant lesion regression. Unfortunately, the clinical results are without direct correlation with the prime-boost regimen (Davidson et al. 2004).

SGN-00101, otherwise known as HspE7, is the product of a fusion between *Mycobacterium bovis* variant, bacille Calmette-Guerin heat shock protein (Hsp65), and HPV-16 E7. HspE7 was efficacious in Phase I and II clinical trials, in which it induced the regression of HPV-associated lesions, such as genital warts (Goldstone et al. 2002), recurrent respiratory papillomatosis (Derkay et al. 2005), anal intraepithelial neoplasia (AIN) (Palefsky et al. 2006), and high-grade cervical intraepithelial neoplasia (CIN) (Roman et al. 2007; Einstein et al. 2007; Van Doorslaer et al. 2010). HspE7 was tested in a Phase II trial that consisted of 58 patients with CIN 3 who were subcutaneously injected with 500 μg of HspE7 three times per month, followed by a colposcopic checkup before undergoing cervical cone biopsy (Einstein et al. 2007). Thirteen out of 58 patients had complete response (post-vaccination biopsy specimen tested negative for CIN) while 32 had a partial response (> 50% reduction in lesion size), and stable disease was detected in 11 of the patients (persistent CIN 3 with less than 50% reduction of lesion size). However two patients continued to have progressive disease. Serological assessment has indicated that positive therapeutic outcome is correlated with higher HPV-16 E7-specific IgG levels (Van Doorslaer et al. 2010).

2.4 DNA-Based Therapeutic HPV Vaccines

The use of DNA-based therapeutics is an especially attractive approach for therapeutic HPV vaccines since they do not generate anti-vector immune responses in patients. DNA vaccines are generally simple, safe, and stable in comparison to other kinds of vaccines. In addition, it is also simple to do large-scale manufacturing of DNA vaccines. DNA vaccines can also improve immunological memory by continuously providing the release of antigenic proteins. Among the benefits of DNA vaccines is that they can be engineered to encode HPV antigenic proteins or peptides with many different delivery methods, allowing DNA vaccine to be delivered to APCs to induce both CD4+ and CD8+ antigen-specific T cell responses in vivo. Unfortunately, DNA vaccines are not very immunogenic because they lack intrinsic specificity for APCs and the ability to amplify and spread intercellularly in vivo. Consequently, new strategies for the enhancement of the immune responses induced by therapeutic HPV DNA vaccines have focused on delivery of the DNA encoding antigens to DCs.

2.4.1 Strategies for the Enhancement of DNA Vaccine Potency

Dendritic cells (DCs) are essential for the instigation of the adaptive immune response. Consequently, dendritic cells are the focus in the research for the enhancement of DNA vaccine immunogenicity. The three strategies for enhancing DNA vaccine potency discussed here are: (1) increasing the quantity of antigen-expressing/-loaded DCs, (2) enhancing HPV antigen expression, processing, and presentation by DCs and (3) improving the capacity of DNA transfected DCs to prime E6/E7-specific T cells to produce therapeutic action against preexisting HPV infections and HPV-associated lesions.

Increase the Number of HPV Antigen-Expressing/HPV Antigen-Loaded DCs

By increasing the efficiency of direct administration of DNA to dendritic cells, greater numbers of cells expressing the chosen antigen may be produced. Gene gun technology can efficiently transfect DCs by propelling DNA-coated gold particles into the skin, where immature DCs known as Langerhan cells reside. When the DCs mature, they migrate to lymphoid tissues where they produce antigen-specific immune responses through the priming of T cells. In comparison to regular intramuscular or biojector injection, gene gun technology is the most dose-efficient method of vaccine delivery (Trimble et al. 2003). Gene gun technology is also capable of delivering non-carrier naked DNA under a low-pressure system to produce antitumor effects that is similar to that of gold particle-mediated gene gun delivery (Chen et al. 2009).

Electroporation is an alternative method of delivery. Electric current is used to permeate the cell membrane so that cellular uptake of intramuscularly injected DNA is boosted. In addition, electroporation also causes local inflammation and cytokine recruitment, thereby making the environment ideal for the generation of vaccine-induced immune responses. Preclinical models that compared the use of electroporation to intramuscular injection or gene gun delivery determined that electroporation produced the greatest number of E7-specific cytotoxic CD8+ T cells, and therefore, also the strongest antitumor immune response against E7-expressing tumors (Best et al. 2009). Animal preclinical studies have also demonstrated electroporation enhanced antigen expression and DNA vaccine-induced antigen-specific immune responses (Seo et al. 2009; Yan et al. 2008). Lately, research has discovered that co-administration of DNA vaccine with a viral fusogenic membrane glycoprotein can enhance potency by way of intensified antigen transfer to DCs, improved antigen uptake, and acute local inflammatory responses (Mao et al. 2010). Vesicular Stomatitis Virus G protein (VSV-G) has been shown to induce widespread cell fusion and cell death that is connected with the release of antigen. The DNA encoding VSV-G combined with CRT/E7 DNA vaccine administered intramuscularly by electroporation induced vigorous and intensified E7-specific CD8+ T cell responses and in addition,

was able to control E7-expressing tumors in mice (Mao et al. 2010). A Phase I trial examining the DNA vaccine, VGX-3100, which targets the E6 and E7 of HPV-16 and HPV-18 for the treatment of patients with CIN 2 or 3 lesions (VGX 2009) has determined that the vaccine is generally safe. Moreover, VGX-3100 was able to induce antigen-specific cytotoxic T-lymphocyte action against all the targeted antigens, and succeeded in producing significant cellular immune response in three of six patients as well as humoral immune responses in five of six patients (Phase I clinical trial results to test the safety and immunogenicity of VGX-3100 released 2009).

Additional possibilities for improving the transfection efficiency of HPV DNA vaccines include intradermal vaccination followed by laser pulses (Zeira et al. 2007; Tsen et al. 2009), intramuscular administration of plasmid DNA that is shielded from nucleases by a biopolymer (Hedley et al. 1998; Storrie and Mooney 2006; Klencke et al. 2002), intradermal tattoo administration (van den Berg et al. 2009; Pokorna et al. 2008), skin patch delivery (Su et al. 2009), and microneedle administration (Prausnitz et al. 2009). One DNA-based therapeutic vaccine known as Amolimogene bepiplasmid (ZYC101a) encodes epitopes derived from HPV-16 and HPV-18 E6 and E7 that is encapsulated into microparticles of poly-lactic-co-glycolic acid to protect the DNA from nuclease degradation (Alvarez-Salas 2008). The precursor of amolimogene bepiplasmid, ZYC101, was examined in early phase clinical trials. ZYC101 encodes numerous HLA-A2-restricted HPV-16 E7 epitopes. The Phase I/II clinical trial of ZYC101 had 12 men with high-grade anal dysplasia intramuscularly injected with 4 doses (50, 100, 200, or 400ug) of the vaccine at 3-week intervals. In this trial, three patients partially responded with disease regression to low-grade disease (one at 200 ug and 2 at 400 ug dose levels). Furthermore, in ten patients, the vaccine-induced antigen-specific activated T cells lasted up to 6 months (Klencke et al. 2002). However, there was no correlation between the clinical responses and the immune responses. ZYC101 was examined in another dose-escalating Phase I trial, in which 15 patients with CIN 2/3 were subcutaneously injected or intramuscularly administered three doses (50 ug, 100 ug, or 200 ug) at 3-week intervals. The intramuscular injection of the highest dose generated four out of the five complete responses, as defined by the absence of cytologic or histologic indication of remaining pre-invasive disease. From the results, intramuscular injection of ZYC101 was deemed to have greater therapeutic action than subcutaneous administration. Moreover, in 11 of the 15 patients, detectable HPV-16-specific CTL responses were produced (Sheets et al. 2003). The Phase I trials of ZYC101a encouraged the initiation of Phase II trials. In one Phase II trial, amolimogene bepiplasmid vaccine was able to resolve CIN 2/3 in most patients below the age of 25 years in comparison to the placebo group of the same age (Garcia et al. 2004). Additionally, a Phase II/III randomized clinical trial studying the intramuscular administration of amolimogene (ZYC101a) in patients between the ages of 13 to 25 with HPV-associated CIN 2/3 has just recently concluded (Eisai 2009).

Antigen-loaded/antigen-expressing cell count can be increased through intercellular spreading of the HPV antigen encoded by DNA vaccine. In order to facilitate the intercellular spreading of E7 encoded by the DNA vaccine, E7 is fused to either

HSV-1 VP22 (Hung et al. 2001a) or a homologue, such as Marek's disease virus VP22 (Hung et al. 2002), which are able to transport antigens to neighboring cell. Vaccination with VP22/E7 DNA resulted in more E7-specific CD8+ T cells (Kim et al. 2004a) and greater antitumor effect than wild-type E7 DNA (Hung et al. 2001a) in mice. Similarly strong E7-specific immune responses have been produced by vaccination with MVP22/E7 DNA in mice (Hung et al. 2002).

DNA encoding antigens can also be engineered to link onto molecules that will selectively bind to dendritic cells and therefore augment the number of antigen-expressing DCs. The molecules that are commonly linked to antigens are ligands for DC receptors, for example, the ligand for DC Flt3 receptors, which are FMS-like tyrosine kinase 3 (Flt3) ligands, and heat shock proteins (Hsp), which is the ligand to CD91 scavenger receptor on DCs. When Flt3 ligand was linked to HPV-16 E7 in a HPV DNA vaccine, elevated levels of E7-specific cytotoxic immunity against E7-expressing tumors was detected along with the size reduction of established pulmonary metastases (Hung et al. 2001b). An additional example of using DC ligands is the case where Hsp70 was linked to HPV antigen and as a result, the antigen presented by MHC I was able to induce antigen-specific CD8+ T cell immune responses (Chen et al. 2000a; Zong et al. 2009). Furthermore, Hsp70 is known to have innate immunity activating abilities and therefore can induce DC maturation to enhance antigen cross-presentation.

Improve HPV Antigen Expression, Processing and Presentation in DCs

Increasing the efficiency of antigen expression, processing, and presentation in dendritic cells can trigger stronger antigen-specific cell-mediated immune responses by enhanced antigen-specific T cells priming. Increasing antigen expression can be accomplished by codon optimization and with demethylating agents. Codon optimization enhances gene translation, thus increases viral protein expression. The strategy that is applied to HPV DNA vaccines works by replacing rarely used codons with ones commonly recognized in target species. In mouse models, vaccination with codon optimized E6 (Lin et al. 2006) and/or E7 DNA vaccines (Seo et al. 2009; Cid-Arregui et al. 2003; Yan et al. 2009) strongly enhanced E6 and/or E7 protein expression and improved antigen-specific $CD8^+$ T cell immune responses. It is well known that methylated CpG motifs can suppress gene expression (Hirasawa et al. 2006), whereas the demethylation of specific CpG motifs can enhance DNA vaccine-encoded antigen expression levels. For example, nucleoside analogue 5-aza-2′-deoxycystidine (DAC) is a demethylating agent that has been experimentally determined to enhance E7-specific CD8+ T cell immune responses in mouse model when applied with an HPV DNA vaccine encoding calreticulin linked to HPV 16-E7 (CRT/E7) (Lu et al. 2009).

In order to enhance the immunogenicity of DNA vaccines, research has focused on the MHC class I and II antigen processing and presentation pathways. In the MHC class I pathway, peptide fragments in the cytosol derived from proteasome-degraded antigenic proteins are transported to the endoplasmic reticulum.

Therefore, protein antigens linked to proteasome-targeting (Hung et al. 2003) or endoplasmic reticulum-targeting (Cheng et al. 2001; Peng et al. 2005a; Kim et al. 2008a) may lead to enhanced MHC class I antigen presentation. Calreticulin (CRT) is an ER-based chaperone molecule that facilitates the antigenic peptide loading to MHC class I. Recent studies have shown that vaccination with CRT/E7 DNA significantly increases E7-specific $CD8^+$ T cell precursors and exhibits impressive antitumor effects in the E7-expressing tumors of the mouse model compared to vaccination with wild-type E7 DNA or CRT DNA (Cheng et al. 2001). According to a direct comparison study of HPV-16 E7 DNA vaccines, DNA vaccine encoding CRT/E7 generated the strongest E7-specific $CD8^+$ T cell responses and E7-specific antitumor effects in mice (Hsieh et al. 2004). The results of a Phase I clinical trial showed that DNA vaccines that encode an endoplasmic reticulum-targeting signal sequence (Sig) linked to an attenuated form of HPV-16 E7 (with a mutation that eliminates the Rb binding site) and fused to Hsp70 (Sig/E7(detox)/Hsp70) was found to be well tolerated in patients with CIN 2 and 3 lesions (Trimble et al. 2009). Three injections (0.5, 1.0 or 3.0 mg/dose) at 4-week intervals were applied intramuscularly to 15 patients, which resulted in complete histologic regression in three of nine individuals at the highest dose. Although HPV-16 E7-specific T cell responses were detected, the frequency and magnitude were low and did not correlate with clinical outcome. Phase I trials employing Sig/E7(detox)/Hsp70 for treating patients with HPV-16-associated advanced head and neck squamous cell carcinoma were completed (M Gillison, personal communication). There is another on-going clinical trial administering the same Sig/E7(detox)/HSP70 DNA vaccine boosted with recombinant vaccinia virus encoding HPV-16/18 E6/E7 fusion protein (TA-HPV) in the absence or the presence of imiquimod in patients with CIN 2/3 lesions (NCI 2009c). Additionally, a Phase I trial applying a DNA vaccine encoding CRT linked to modified HPV-16 E7 (CRT/E7(detox)) using a clinical-grade gene gun device in patients with high grade intraepithelial cervical lesion has recently begun (Trimble 2009). To prime CD4+ T helper cells, it is important to process and present antigens through the MHC class II pathway. The MHC class II pathway involves the endocytosis and degradation of extracellular pathogens and proteins by proteases, after which, the proteins are bound to MHC class II molecules and transported to the cell surface. In order to improve the MHC class II processing of antigens, Ji et al. created a DNA vaccine that encoded a fusion protein of HPV-16 E7 protein linked to the sorting signal of lysosomal-associated membrane protein type 1 (LAMP-1) (Wu et al. 1995). Cells transfected by this method were observed to redirect E7 antigen from the cytoplasm/nucleus to the endosomal/lysosomal compartments, which leads to improved MHC class II antigen presentation. In mice, those vaccinated with E7/LAMP-1 were observed to have increased E7-specific CD4+ and CD8+ effector cells in comparison to the mice that only received DNA vaccine encoding wild-type E7 (Ji et al. 1999).

In addition, other studies have also employed MHC class II-associated invariant chain (Ii) for the enhancement of MHC class II antigen presentation (Hung et al. 2007). Under normal circumstances, the CLIP (Class II-associated peptide) region of the invariant chain inhabits the MHC II peptide-binding groove in order to avoid

the antigenic peptide from binding prematurely. When CLIP is substituted with Pan-DR helper T lymphocyte epitope (PADRE) in the invariant chain (Ii-PADRE), antigen presentation efficiency is enhanced and the strong activation of PADRE-specific CD4+ T cell immune response can be induced. The intradermal vaccination by gene gun of Ii-PADRE DNA in conjunction with HPV-16 E7 DNA have been shown in mice to generate a significantly stronger E7-specific CD8+ T cell response than the control that only received E7 DNA in combination with DNA encoding unmodified Ii (Hung et al. 2007). Moreover, the PADRE activated CD4+ T cells will secrete IL-2, a T cell proliferation cytokine, which leads to improved CD4+ effects (Kim et al. 2008b). In order to advance the HPV DNA vaccine induction of antigen-specific CD8+ T cell immune responses even more, Ii-PADRE DNA has been combined with other forms of intracellular targeting strategies to produce synergistic effects (Kim et al. 2007). Furthermore, it has been shown that the co-delivery of Ii-PADRE DNA with CRT/E7 DNA in mice pretreated with doxorubicin was able to reverse the doxorubicin-mediated immunosuppression of antigen-specific immune responses (Kim et al. 2008c).

Several strategies have been used to improve cross-presentation, in which extracellular protein is processed through MHC class I pathway. For example, HSP70 (Chen et al. 2000a) GP96 (Bolhassani et al. 2008), or domain II of *P. Aeruginosa* (Hung et al. 2001c) have been employed to be linked to the HPV E7 in DNA vaccine to promote E7-specific CD8+ T cell immune responses. Moreover, when DNA vaccines are intramuscularly injected, antigen-specific immune responses were induced predominantly through cross-priming mechanisms. Recent research by Kang et al. have demonstrated that by intramuscularly administering a HPV E7 DNA vaccine in conjunction with DNA that encodes xenogenic MHC class I molecules, E7-specific CD8+ T cell immune responses as well as anti-tumoral action against E7-expressing tumors were significantly enhanced in tumor-bearing mice. This same regimen also increased the number of tumor infiltrating CD8+ T lymphocytes and activated APCs at the site of injection, which resulted in a greater release of antigen in local muscle tissue for cross-priming to promote MHC class I antigen processing and presentation (Kang et al. 2009).

On the other hand, the use of single chain trimer (SCT) technology can circumvent MHC processing altogether. The genes for E6 antigenic peptide can be linked to ß2 microglobulin in addition to MHC I heavy chain. This produces a single-chain construct that encodes the fusion of the antigenic peptide to an MHC class I molecule. In mouse studies, those that were intradermally vaccinated by gene gun with the DNA vaccine consisting of the SCT of MHC class I linked to HPV-16 E6 CTL epitope were completely protected from a lethal challenge of E6-expressing tumor cells. In comparison, the mice that received either control plasmid or wild-type E6 DNA all developed tumors (Huang et al. 2005).

The master regulator for MHC II expression, known as MHC CIITA, is a transcriptional activator that can upregulate the expression of MHC I molecules on DCs. Therefore, more T cells can be activated by increased MHC class I and II expression when the HPV DNA vaccine was used with the DNA encoding MHC CIITA. Kim et al. have shown that this strategy was capable of potentiating the HPV-16

E7-specific CD4+ and CD8+ T cell immune responses, which led to the prolonged survival of mice in contrast to those that only received the therapeutic HPV DNA vaccine without CIITA (Kim et al. 2008d).

Enhance DC Function and Interaction with T Cells

DCs must have direct cell-to-cell contact with T cells after the antigens are processed and presented in order to activate T-cells. Since DCs are vulnerable to T cell mediated apoptosis once T cells are primed, extending DC survival will prolong the duration of time that DCs can prime T cells. To achieve this, therapeutic DNA vaccines have been intradermally co-administered with DNA encoding different anti-apoptotic proteins such as Bcl-xL, Bcl-2, X-linked inhibitor of apoptosis protein, and dominant negative mutants (dn) of caspase-9, dn caspase-8 (Kim et al. 2003) or connective tissue growth factor (Cheng et al. 2008) to generate improved HPV antigen-specific CD8+ T cell immune responses. An issue raised by this combination is the risk of cellular transformations which limits the clinical usefulness of this approach. The employment of small, interfering RNA (siRNA) targeting key pro-apoptotic proteins may lessen concerns of oncogenicity by direct administration of DNA encoding antiapoptotic protein. By using gene gun to intradermally co-administer DNA vaccine encoding HPV-16 E7 with siRNA targeting pro-apoptotic proteins Bak and Bax, the survival of DCs in the draining lymph nodes are extended to stimulate stronger E7-specific CD8+ T cell responses and produce greater antitumor effects than HPV-16 E7 DNA alone (Kim et al. 2005).

Another method for improving the interaction between DCs and T cells is to increase the number of activated T cells by interfering with the apoptotic signaling to T cells. DCs express Fas Ligand (FasL), which upon binding to Fas, the death receptor on T cells, will initiate T cell apoptosis. The apoptosis of E7-specific CD8+ T cells can be abridged through intradermal administration of therapeutic HPV DNA vaccine containing the DNA encoding short hairpin RNA (shRNA) that targets the FasL on the HPV-16 E7 peptide-loaded DCs to induce a stronger E7-specific CD8+ T cell response in mice versus the use of HPV DNA vaccine alone (Song et al. 2006). This results in a more potent cytotoxic response against E7-expressing tumors (Huang et al. 2008). As a result, the potency of HPV DNA vaccine can be enhanced via anti-apoptotic signals to APCs in addition to the inhibition of apoptosis in T cells.

Yet another method by which T cell priming can be increased is through the activation of DC expansion. Antigen-specific cytotoxic T cell-mediated immune responses can be improved by co-administrating HPV DNA vaccines with DNA encoding immunostimulatory cytokines such as GM-CSF (Leachman et al. 2000), Il-2 (Chen and Wu 1998), IL-12 (Kim and Sin 2005), codon-optimized IL-2 and IL-12 (Ohlschlager et al. 2009). An alternate method for promoting DC activation is to inhibit the negative regulators of DC activation, such as SOCS-1, which inhibits signaling via the Jak-STAT pathway to suppress DC activity. Consequently, through the use of siRNA, SOCS-1 in DCs can be selectively inactivated to potentially enhance the CD8+ immune responses induced by therapeutic HPV DNA vaccines (Song et al. 2006).

To boost vaccine-induced immune responses, one strategy focuses on interfering with the negative regulators of the immune system. The primary role of regulatory T cells (T_{reg}) is to inhibit DC and CD8+ T cell responses to preserve immune tolerance. By depleting T_{reg}, the function of DCs and T cells may be enhanced. Agents such as anti-CD25 monoclonal antibodies have been used to selectively deplete T_{reg} cells and promote antitumor effects through HPV antigen-specific CD8+ T cell responses (Beyer and Schultze 2006; Nair et al. 2007; Chuang et al. 2009a).

2.5 RNA Replicon-Based Therapeutic HPV Vaccines

RNA replicon-based vaccines utilize modified naked RNA vector (termed RNA replicons) that can replicate within the transfected cell in a self-regulating fashion but are altered to lack necessary structural sequences for forming viral particles. These vaccines can be administered as RNA or DNA, which is transcribed in the cytoplasm into RNA replicons. RNA replicons may be derived from alphaviruses, such as Venezuelan Equine Encephalitis (Pushko et al. 1997; Cassetti et al. 2004), Semliki Forest virus (Berglund et al. 1997; Berglund et al. 1998) and Sindbis virus (Cheng et al. 2002; Hariharan et al. 1998) and are able to replicate within various cells, allowing them to increase the production of proteins-of-interest compared to conventional DNA vaccines. Repeated administration of these vaccines is attainable because the lack of structural genes prevents viral particle formation and antibody neutralization against the viral capsid. Moreover, the risks of integration of RNA replicons with host genome and cellular transformation associated with DNA vaccines can be aborted. However, clinical translation of the vaccine is difficult due to the chemical instability of RNA compared to that of DNA.

Researchers have attempted to overcome this disadvantage by integrating the stability of DNA with the high antigen expression encoded by RNA replicons. The combination resulted in the development of 'suicidal' DNA vector, which is a DNA-based self-replicating RNA replicon. Since the suicidal DNA eventually led to the apoptosis of transfected cells, it reduces the concerns for genomic integration or cellular transformation. Hsu et al. demonstrated that the use of a 'suicidal' DNA vector encoding HPV-16 E7 antigen in a mouse model generated a significant HPV E7 antigen-specific $CD8^+$ T-cell immune response and antitumor effects (Hsu et al. 2001). However, intradermal administration of suicidal DNA vector may render transfected DCs to undergo apoptosis which may cause poor immunogenicity. By adding a gene encoding an antiapoptotic protein to the suicidal DNA vector, the survival rate of antigen-presenting cells could be increased, which improves the immunogenicity. For example, it was reported that fusion of BCL-xL with E7 encoded by pSCA1, a suicidal DNA vector, generates stronger E7-specific $CD8^+$ T-cell immune responses and antitumor effects than the pSCA1 encoding wild type E7 (Kim et al. 2004b).

So far, no clinical trial has been conducted for naked RNA replicon vaccines to date, despite their preclinical successes. There are several reasons for the limited

clinical translation of RNA replicon vaccines, such as difficult long-term storage, unstable structure, and labor-intensive mass production.

2.6 Dendritic Cell-Based Therapeutic HPV Vaccines

Dendritic cell-based vaccines have been used for the development of therapeutic HPV vaccines in a preclinical model (Tillman et al. 2000; Wang et al. 2000; Peng et al. 2005b; Kim et al. 2009). DC-based vaccine can be prepared by loading viral antigens along with appropriate maturation stimuli in an *ex vivo* fashion. A clinical trial affirmed the potency of DC-based therapeutic vaccines to target HPV-related malignancies (for review, see Bellone et al. 2007). Current preparation methods include culturing DCs with tumor-derived proteins or transfecting DCs with viral-derived DNA or RNA. Direct delivery of mature DCs depletes the immuno-suppressive effects commonly found in cancer patients, and more effective antigen presentation generated stronger immunostimulatory effects (Santin et al. 2005). Despite the success DC-based vaccines have received, the preparation is labor-intense due to their autologous nature. In addition, standard preparation methods and evaluations have yet to be established for the quality control of DC-based vaccines. Furthermore, since effective priming occurs in the lymphoid organs where the naïve T cells are located, delivery methods are crucial in order to maximize DC-based vaccine efficacy.

DC-based vaccines have been in clinical trials for patients with advanced cervical cancer (Santin et al. 2002, 2006, 2008; Ferrara et al. 2003; Hospital 2009). For example, in a Phase I trial in ten cervical cancer patients with Stage IB or IIA, subcutaneous injections of autologous DCs pulsed with HPV-16/18 E7 recombinant protein and keyhole limpet hemocyanin (KLH) showed detectable cell-mediated immunity. Increases in E7-specific $CD4^+$ T cell were recorded in all ten vaccinated patients and E7-specific $CD8^+$ T cell in six out of ten patients (Santin et al. 2008). Despite the increase in HPV antigen-specific T-cell population, the trial did not lead to objective clinical responses in terms of tumor regression or metastatic lesion regression, possibly due to the advanced stage of disease. Still, the safety feature and the immunogenicity of DC-based vaccines ensure a bright future for further development. However, mass production of the vaccine is prevented by their labor-intensive procedure and autologous nature. Hence, future improvements on the vaccine should focus on efficient loading of antigen to DC, prolonging antigen presentation, and augmenting immunogenicity by adjuvants.

2.7 Tumor Cell-Based Therapeutic HPV Vaccines

Genetically modified tumor cells can trigger stronger immune responses by secreting immunomodulatory proteins, especially cytokines such as IL-2 (Bubenik 2008; Mikyskova et al. 2004), IL-12 (Hallez et al. 1999), and GM-CSF (Mikyskova et al.

2004; Chang et al. 2000). Since the effects of immunomodulatory proteins are not antigen specific, tumor-cell-based vaccines do not require a clear identification of tumor antigens. This characteristic made these vaccines more applicable for treating cancers without well-defined tumor-specific antigens than cervical cancer, which has well-defined antigens.

Several preclinical models have been established to test the efficacy of tumor-cell-based HPV vaccines. Irradiated E6/E7-positive tumor cells expressing IL-12 were used to vaccinate tumor-bearing mice, resulting in a significant regression in E6/E7-expressing tumors (Indrova et al. 2006). While autologous and allogeneic tumor-cell-based vaccines have been tested in clinical trials of other cancers such as colon cancer, melanoma, and prostate cancer (de Gruijl et al. 2008), clinical trials against HPV-associated cancers has yet to be established. In addition, the tumor-based vaccine most likely will not be used to treat early-stage HPV-related lesions due to the risk of introducing new cancers via tumor cell-based vaccine for relatively healthy HPV patients with mild neoplasia. Furthermore, production of individual autologous vaccines are impractical due to the high production cost, labor intensive procedures, and variations in purity and efficacy (de Gruijl et al. 2008). As a result, future development for tumor-cell-based vaccines is limited.

3 Combined Approaches

3.1 Prime-Boost Regimens

To increase the efficacy of cancer immunotherapy, combinatorial vaccination that includes a prime-boost regimen is a promising option. This approach includes the priming the immune system, then augmenting and maintaining a prolonged immune response through the administration of a booster vaccine. One preclinical trial indicated that priming with a therapeutic HPV-16 E7 DNA vaccine followed by a boost with a vaccinia encoding HPV-16 E7 antigen greatly enhances the E7-specific T-cell responses versus that induced by DNA vaccination alone (Chen et al. 2000b). Other methods to boost DNA vaccines include the use of recombinant adenovirus (Wlazlo et al. 2004) or tumor cells expressing HPV 16 E6/E7 (Rittich et al. 2005), both of which can increase HPV antigen-specific CTL response in vaccinated mice to a greater degree than DNA vaccination alone. Using the Sindbis virus RNA replicon expressing HPV-16 E7 linked to *M. tuberculosis* HSP70 (E7/HSP70) as the prime-boost regimen that precedes the recombinant vaccinia virus encoding E7/HSP70, the E7-specific CTL responses generated are greater than other combinations, such as DNA prime-vaccinia boost regimens (Lin et al. 2003). Two examples of additional prime-boost preclinical studies that generated promising results are HPV-16 E7 protein priming followed by E7 vaccinia boost (Mackova et al. 2006) and priming with HPV E6/E7 expressing Venezuelan equine encephalitis virus replicon particles (VRP) followed by E6/E7-expressing recombinant vesicular stomatitis virus (VSV) boost (Kast 2008).

The promising results from the preclinical studies of prime-boost regimens have led to the evaluation of the safety and efficacy of these heterologous strategies in several clinical trials (Smyth et al. 2004; Fiander et al. 2006; Davidson et al. 2004). One Phase II clinical trial involving patients with high-grade anogenital intraepithelial neoplasia (AGIN) examined the efficacy of priming with HPV-16 L2/E6/E7 protein (TA-CIN) vaccine followed by a boost with the HPV-16/18 E6/E7 (TA-HPV) vaccinia. In this clinical trial, E6-specific T-cell responses were elicited in 11 out of 25 patients; however, the relationship between the efficacy of the vaccine and the clinical outcome is considered inconclusive (Smyth et al. 2004). Another clinical study involving patients with high-grade, HPV-16 positive, vulvar intraepithelial neoplasia (VIN) used the regimen that consisted of priming with TA-HPV vaccinia and boosting with three TA-CIN proteins. This prime-boost regimen produced no significant CTL response but was able to enhance antigen-specific antibody response in nine out of ten patients and also caused regression of the lesions in two out of ten patients (Davidson et al. 2004). Currently, there is a Phase I trial in patients with HPV-16+ high-grade cervical intraepithelial neoplasia lesions using a topical adjuvant, imiquimod, in addition to prime-boosting with pNGVL4a/Sig/E7(detox)/HSP70 DNA and TA-HPV vaccinia (NCI 2009c).

3.2 Immunomodulatory Therapy

One of the major obstacles to the successful development of therapeutic vaccine is the immunosuppressive tumor microenvironment. Therefore, negative regulators of T-cell activation, such as CTLA-4 and PD-1, are desirable targets to improve vaccine efficacy (for reviews, see Blank and Mackensen 2007; Peggs et al. 2006). An approach combining HPV therapeutic vaccine with immunomodulatory agents that down-regulate immunosuppressive factors can potentially improve therapeutic effects against HPV-related cervical cancer. Agents that inhibit immunosuppressive factors which include immunosuppressive cytokines such as IL-10 (Yue et al. 1997) and TGF-β (Gorelik and Flavell 2001), T-cell expression of PD-1 (Goldberg et al. 2007), T regulatory cells (Curiel et al. 2004), myeloid-derived suppressor cells (Nagaraj et al. 2007), constitutive STAT3 activation (Yu et al. 2007), and tumor cell expression of MIC-A and B (Groh et al. 2002), indoleamine 2,3-dioxygenase (IDO) (Munn and Mellor 2004), and galectin-1 (Rubinstein et al. 2004) can potentially enhance the therapeutic effects of individual HPV vaccines.

3.3 Combination with Other Therapeutic Modalities

The performance of therapeutic vaccines may be strengthened by a combination with chemotherapeutic agents, topical agents, and/or radiation. For example, apigenin is a naturally abundant chemotherapeutic agent found in fruits and vegetables that

possess anti-carcinogenic properties (for review, see Patel et al. 2007). Administering apigenin with therapeutic HPV DNA vaccine improves vaccine potency against E7-specific tumor cells by enhancing tumor cell apoptosis and increased E7-specific cytotoxic T cell response (Chuang et al. 2009b). Another study has also shown epigallocatechin-3-Gallate (EGCG), a green tea derivative, as an effective chemotherapeutic agent capable of promoting apoptotic tumor cell death. A combined approach of EGCG and HPV-16 E7 DNA vaccine elicited stronger E7-specific T-cell activities and antitumor effects than either modality alone (Kang et al. 2007). Furthermore, it has been shown that the combination of cisplatin with therapeutic HPV DNA vaccine results in enhanced therapeutic antitumor effect *in vivo* (Tseng et al. 2008a).

A potential method for the enhancement of therapeutic vaccine potency is through the application of radiotherapy and/or targeted therapy. Preclinical results have indicated that low-dose radiation increased tumor cell susceptibility to E7-specific $CD8^+$ T-cell activity and promoted the antitumor action of the combined HPV DNA vaccine (Tseng et al. 2009). Subsequent studies on the clinical efficacy of death receptor five-specific antibodies (Tseng et al. 2008b) or proteasome inhibitor such as bortezomib (Tseng et al. 2008c) have also yielded results that indicate therapeutic vaccine efficacy is enhanced by combinatorial therapy. By combining various existing individual cancer treatments with individual therapeutic vaccines, scientists have recorded better therapeutic results than monotherapy. Thus, this multiple-treatment approach for treating HPV-related malignancy renders a promising future.

4 Perspectives

The goal that must be achieved for the current development of therapeutic vaccines is to facilitate the pace of clinical translation. The studies in preclinical models have resulted in great progress. In spite of promising preclinical trials, advanced phases of clinical trials must be conducted to evaluate the actual effectiveness of these vaccines. There is great difficulty in treating advanced cervical cancer with immunotherapy since the patient is typically immuno-compromised. Because of this, the best approach for therapeutic vaccination against cervical cancer is to employ combinatorial strategies such as prime-boost regimens, immunomodulatory agents, or therapeutic modalities such as chemo-radiotherapy and surgical debulking. The success of therapeutic HPV vaccines depends on whether or not research continues onto advanced phases of clinical trials. Only with continuous advances will therapeutic HPV vaccines eventually become available for the control of existing HPV-infection and HPV-associated lesions.

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