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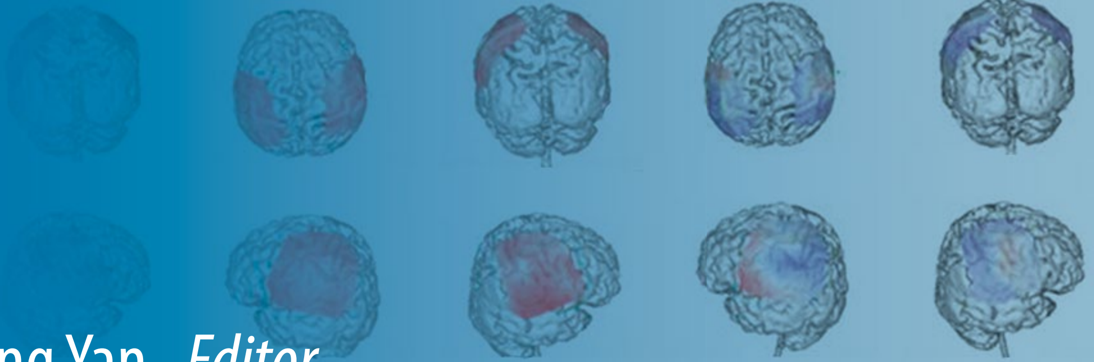

Qing Yan *Editor*

Pharmacogenomics in Drug Discovery and Development

Second Edition

METHODS IN MOLECULAR BIOLOGY

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Pharmacogenomics in Drug Discovery and Development

Second Edition

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Preface

Pharmacogenomics is considered the future of drug therapy. It is a rapidly growing area in the recognition of the necessity of personalized medicine, a medicine that deals with the complexity of the human body. Because of the diversity of patients' biological backgrounds, the same disease may be caused by genetic variations in different people, who will respond differently to the same drug. Such situations require individualized treatment that avoids adverse drug responses and ensures the best possible results. The development of pharmacogenomics represents the evolution of biomedicine from treating the disease itself to treating the malfunction of an individual person, the "root" of diseases. With the change of focus from disease-centered to human-centric medicine, pharmacogenomics brings hope for the transformation from simple disease treatment to accurate prediction and effective prevention. For the drug discovery and development industry, pharmacogenomics is useful in identifying drug targets to obtain the optimal drug efficacy for specific patient groups.

However, many challenges need to be resolved before pharmacogenomics can be applied in the clinic. Most importantly, the mechanisms inside the human bodies that control therapeutic responses are complex and multifactorial. It is necessary to elucidate the complexity in various spatial and temporal levels, such as the interactions among genes, drugs, as well as natural and psychosocial environments at various physiological and pathological stages. Accurate biomarkers and effective drug targets can be found only based on such understanding at system levels.

In this book, we approach these challenges from several angles. In the first part of the book, we introduce some novel concepts and important cutting-edge technologies that are useful for the development of system-based pharmacogenomics to solve the complexity (*see* Part I). A framework of systems and dynamical medicine is proposed on the basis of the understanding of the properties of complex adaptive systems (CASs) (*see* Chapter 1). Various "omics" technologies such as approaches in bioinformatics and transcriptomics are described to support the system analyses (*see* Chapters 2 and 3). These methods are useful for understanding the complex and dynamical interconnections and interactions among genes, drugs, diseases, and the environment. Network and dynamical models can be established for the identification of robust biomarkers to evaluate disease states, disease progression, and therapeutic responses (*see* Chapter 1).

For example, bioinformatics is essential in finding the spatiotemporal patterns in pharmacogenomics, including the time-series analyses for the elucidation of structure–function associations at various disease stages. Specific experimental methods are also introduced, such as the mutational analysis procedures on paraffin-embedded tumors for the prediction of individual responses to anticancer therapy (*see* Chapter 4). The combination of bioinformatics and experimental approaches is helpful for studying drug adverse effects such as those caused by statin, including genotyping, phenotyping, and statistical analysis strategies (*see* Chapter 5).

Another feature of this volume is the emphasis on the examinations of gene–drug interactions, that is, how drugs act and how they are processed in the human body, including drug absorption, distribution, metabolism, and excretion. Biomarkers and molecules

such as ion channels, membrane transporters, receptors, and enzymes are playing increasingly essential roles in drug design and pharmacogenomics studies (*see* Chapters 6, 7, 8, and 9). These biomarkers provide critical links between drug discovery and diagnostics efforts. Updated introductions and detailed methods about studies in these molecules are provided in this book. For example, membrane transporters are profoundly involved in drug disposition through transporting substrate drugs between organs and tissues. Investigations of genetic variations, genotyping methods, and substrate identification of membrane transporters are helpful for drug design and development (*see* Chapter 6). Methods for the clinical development of transporter markers can be meaningful for the practice of translational medicine.

In addition, studies of G protein-coupled receptors (GPCRs) may provide insight into disease pathways, such as the involvement of the regulator of G protein signaling (RGS) protein polymorphisms in hypertension. Pharmacogenomics of GPCR studies the involvement of genetic variations in structural and functional roles, such as GPCR activation and inactivation, their relationships with diseases, and their potential uses in defining optimized novel drug targets (*see* Chapters 7, 8, and 9). These investigations can be useful for refining drug discovery because GPCR disorders are associated with a wide variety of human diseases, including obesity, diabetes, cardiovascular diseases, cancer, asthma, and infectious diseases.

The second part of this book focuses on how to translate pharmacogenomics studies from the "bench side" to the "bedside" in clinical therapies of diseases to support the development of translational medicine (*see* Part II). These diseases include cardiovascular diseases, cancer, Alzheimer's disease, psychiatric disorders, rheumatoid arthritis, osteoporosis, and pediatric diseases. Comprehensive information for each disease system is discussed, including biomarkers involved in the diseases and the associations among genes, diseases, drug responses, and the environment. For example, genetic variations may play important roles in heart failure pharmacotherapy (*see* Chapter 10). Pharmacogenomics studies are making significant contributions toward the elucidation of pharmacological atheroprotection for finding novel therapeutic approaches for atherosclerosis, the condition that can result in stroke, myocardial infarction, and death (*see* Chapter 11). In cancer therapy, translational investigations in pharmacogenomics may also make genetic profiling effective for the analysis of chemotherapy-induced neurotoxicity (CIPN) (Chapter 12).

As a complex disorder with multifactorial clinical features, Alzheimer's disease (AD) needs to be studied in the context of diverse environmental impacts, cerebrovascular dysfunction, epigenetic phenomena, as well as various structural and functional genomic dysfunctions (*see* Chapter 13). This book provides a comprehensive and detailed discussion of the pharmacogenomics of AD, from functional genomics to therapeutic strategies, from the discovery of reliable biomarkers to the optimized drug development.

The identification of pharmacogenomic biomarkers for the prediction of drug efficacy and adverse reactions is a growing area of research in the studies of psychiatric disorders such as schizophrenia (*see* Chapter 14). Such methods have the potential to replace the current trial-and-error approach for the optimal treatment selections toward the personalized medicine. Pharmacogenomics investigations may also elucidate the roles of genetic, biological, social, and environmental components in the therapeutic responses of drug addiction (*see* Chapter 15).

For rheumatoid arthritis (RA), the pharmacogenomics of traditional disease-modifying antirheumatic drugs (DMARDs) as well as the newer biologic DMARDs are discussed in details for individualized therapy (*see* Chapter 16). In addition, with comprehensive examinations including genome-wide association studies, exciting opportunities are open to provide a better insight into the pharmacogenomics of osteoporosis and osteoporotic fractures

(*see* Chapter 17). In pediatrics, developmental changes may account for the variability in drug responses. Various "omics" approaches including genome-wide haplotype mapping, proteomics, epigenomics, as well as genetic epidemiological studies over years may expand the scope of personalized therapies in children (*see* Chapters 18 and 19).

By covering topics from individual molecules to systemic diseases, from basic concepts to advanced technologies, this book intends to provide a practical, state-of-the-art, and integrative view of the application of pharmacogenomics in drug discovery and development. A wide range of theoretical and experimental approaches are introduced to meet the problem-solving objectives for understanding the complexity in health and diseases, from laboratory tests to computational analysis. Written by leading experts in the field, this book intends to provide comprehensive resources and a holistic view for the translation of pharmacogenomics into better preventive and personalized medical care.

I would like to thank all of the authors for their valuable contributions to this exciting field. I also thank the series editor, Dr. John Walker, for his help with the editing.

Santa Clara, CA, USA ***Qing Yan, M.D., Ph.D.***

Contents

Preface v
Contributors xi

PART I SYSTEMS AND "OMICS" STUDIES IN PHARMACOGENOMICS

1 From Pharmacogenomics and Systems Biology to Personalized Care: A Framework of Systems and Dynamical Medicine 3
Qing Yan

2 Translational Bioinformatics Approaches for Systems and Dynamical Medicine 19
Qing Yan

3 Whole Blood Transcriptomic Analysis to Identify Clinical Biomarkers of Drug Response 35
Grant P. Parnell and David R. Booth

4 Diagnostic Procedures for Paraffin-Embedded Tissues Analysis in Pharmacogenomic Studies 45
Raffaele Palmirotta, Maria Laura De Marchis, Giorgia Ludovici, Patrizia Ferroni, Pasquale Abete, Fiorella Guadagni, and David Della-Morte

5 Approach to Clinical and Genetic Characterization of Statin-Induced Myopathy 67
QiPing Feng

6 Pharmacogenetics of Membrane Transporters: A Review of Current Approaches 91
Tristan M. Sissung, Andrew K.L. Goey, Ariel M. Ley, Jonathan D. Strope, and William D. Figg

7 G Protein-Coupled Receptor Accessory Proteins and Signaling: Pharmacogenomic Insights 121
Miles D. Thompson, David E.C. Cole, Pedro A. Jose, and Peter Chidiac

8 G Protein-Coupled Receptor Mutations and Human Genetic Disease 153
Miles D. Thompson, Geoffrey N. Hendy, Maire E. Percy, Daniel G. Bichet, and David E.C. Cole

9 Pharmacogenetics of the G Protein-Coupled Receptors 189
Miles D. Thompson, David E.C. Cole, Valerie Capra, Katherine A. Siminovitch, G. Enrico Rovati, W. McIntyre Burnham, and Brinda K. Rana

PART II CLINICAL APPLICATIONS OF PHARMACOGENOMICS

10 Pharmacogenomics of Heart Failure 245
Anastasios Lymperopoulos and Faren French

11 Pharmacogenomics in the Development and Characterization of Atheroprotective Drugs 259
Efi Valanti, Alexandros Tsompanidis, and Despina Sanoudou

12 Management of Side Effects in the Personalized Medicine Era: Chemotherapy-Induced Peripheral Neuropathy. 301
Paola Alberti and G. Cavaletti

13 Pharmacogenomics of Alzheimer's Disease: Novel Therapeutic Strategies for Drug Development 323
Ramón Cacabelos, Pablo Cacabelos, Clara Torrellas, Iván Tellado, and Juan C. Carril

14 Pharmacogenetics of Antipsychotic Treatment in Schizophrenia 557
Jennie G. Pouget and Daniel J. Müller

15 Pharmacogenetics of Addiction Therapy 589
David A. Nielsen, Ellen M. Nielsen, Teja Dasari, and Catherine J. Spellicy

16 Pharmacogenetics in Rheumatoid Arthritis 625
Deepali Sen, Jisna R. Paul, and Prabha Ranganathan

17 Pharmacogenomics of Osteoporotic Fractures. 661
José A. Riancho and Flor M. Pérez-Campo

18 Pharmacogenomics and Pharmacoepigenomics in Pediatric Medicine 671
Barkur S. Shastry

19 Pharmacogenomics in Children. 687
Michael Rieder

Index *709*

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Part I

Systems and “Omics” Studies in Pharmacogenomics

Chapter 1

From Pharmacogenomics and Systems Biology to Personalized Care: A Framework of Systems and Dynamical Medicine

Qing Yan

Abstract

With the integration of pharmacogenomics and systems biology, personalized medicine would be possible by switching the gear from the reductionism-based and disease-focused medical system toward a dynamical systems-based and human-centric health care. Comprehensive models are needed to represent the properties of complex adaptive systems (CASs) to elucidate the complexity in health and diseases, including the features of emergence, nonlinearity, self-organization, and adaptation. As all diseases have the dynamical elements, nonlinear time-series analyses are necessary to characterize the system dynamics at various levels to elucidate the physiological and pathological rhythms, oscillations, and feedback loops. Such analyses can help detect patterns across multiple scales in both the spatial (e.g., from molecules to cells, from organisms to psychosocial environments) and the temporal (e.g., from nanoseconds to hours, from years to decades) dimensions. Based on such understanding, systems and dynamical medicine can be developed with the emphasis on the whole systems that change over time to address the nonlinearity and interconnectivity toward a holistic and proactive care. Accurate and robust biomarkers with predictive values can be discovered to reflect the systemic conditions and disease stages. Network and dynamical models may support individualized risk analysis, presymptomatic diagnosis, precise prognosis, and integrative interventions. Systems and dynamical medicine may provide the root for the achievement of predictive, preventive, personalized, and participatory (P4) medicine.

Key words Biomarkers, Complex adaptive systems (CASs), Dynamical diseases, Dynamical medicine, Emergence, Nonlinearity, Personalized medicine, Pharmacogenomics, Systems biology, Systems medicine

1 Understanding the Complex Adaptive Systems (CASs): Strategies Addressing the Dynamical Features of Health and Diseases in Personalized Medicine

Pharmacogenomics and systems biology are rapidly developing disciplines that may bring fundamental progress toward personalized and systems medicine [1]. Pharmacogenomics studies the individual variations in treatment responses and may contribute to the prediction of disease development and the prevention of adverse events for optimal therapies and lower costs [2].

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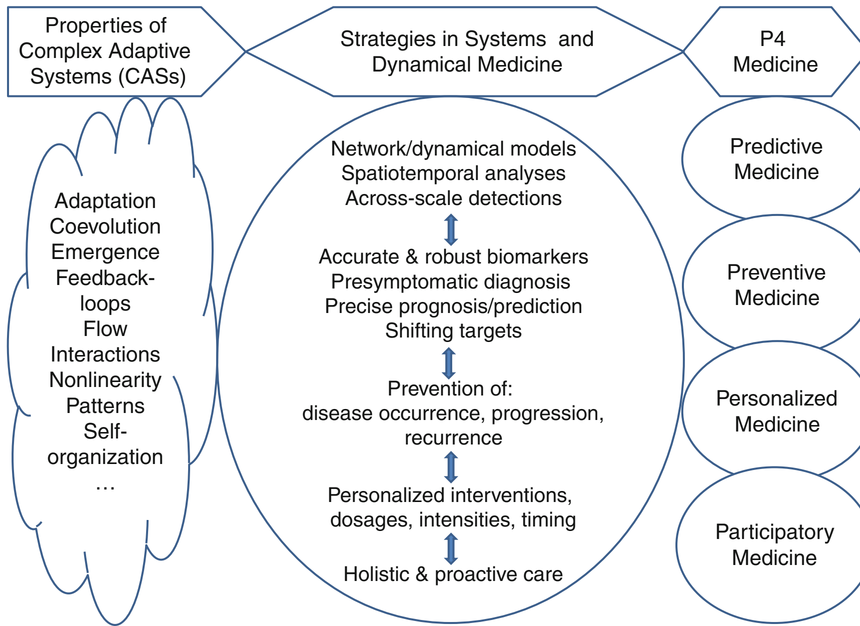

Fig. 1 A framework of systems and dynamical medicine: from addressing the properties of complex adaptive systems (CASs) to the achievement of P4 medicine

Systems biology focuses on the holistic view for the understanding of the complexity of biological systems with the analyses of various levels of information [1]. With the integration of pharmacogenomics and systems biology, personalized medicine would be possible by switching the gear from the reductionism-based and disease-focused medical system toward a dynamical systems-based and human-centric health care [3].

As the scientific basis of personalized medicine, both pharmacogenomics and systems biology have multidisciplinary features that require a global view crossing various domains [4]. These domains include biological sciences (e.g., genomics, proteomics, metabolomics, physiology, pharmacology, toxicology), medical branches (e.g., radiology, epidemiology, internal medicine), as well as quantitative sciences (e.g., mathematics, bioinformatics). Comprehensive models are needed to represent the flow of information in multiple domains, especially the properties of complex adaptive systems (CASs), to elucidate the complexity in health and diseases. Some of the important CASs characteristics include emergence, nonlinearity, self-organization, and adaptation [5]. As illustrated in Fig. 1, the recognition of the CASs properties may contribute to the development of effective preventive and therapeutic strategies in personalized medicine. With more studies on the complexity in humans, more connections between the CASs properties and personalized care can be revealed for better approaches.

1.1 Emergence, Interaction Patterns, and the "Whole Body System"

Emergence is the essential feature of complex systems and probably the most important concept in systems biology. The common understanding is that "the whole is greater than the sum of its parts" [1]. That is, the complex behaviors or properties are the collective results arising from the interconnectivity, interactions, and coevolution of the components or entities (also called agents) of the biological systems across different scales [5, 6]. Such properties cannot be predicted from single entities or isolated parts within the systems. For example, cell phenotypes are emergent forms arising from the collective nonlinear interactions among various cellular and microenvironmental components [7]. The phenotype of systemic inflammation is the overall result of multiple pathways and interactions among multiple cells and molecules, but not the feature of any single cytokines.

To illustrate the characteristics of emergence, the patterns of interactions need to be elucidated (*see* Fig. 1). For instance, the same outcomes can result from different interactions and combinations of agents, while the different outcomes may be caused by the similar combinations of agents [8]. The understanding of such patterns is especially meaningful for personalized medicine. For example, the same disease cancer can have different patterns of genetic alterations in different tissues and different patients, hence needs different treatments [9]. On the other hand, the same lifestyle such as unhealthy diets may lead to different disorders including obesity, diabetes, and cardiovascular diseases in different people. Chronic inflammation has been suggested as the common link among many different chronic diseases including cardiovascular diseases, chronic kidney disease, Alzheimer's disease, type 2 diabetes, and cancer [10]. In another example, diseases such as gout, type 2 diabetes, heart failure, and cancer are seemingly unrelated, but all of them have been found to be responsive to IL 1β neutralization [11].

On the basis of such understanding, personalized medicine should focus on the "whole body system" of the humans or patients as the root of the diseases so that the same disease often needs to be treated differently in different patients. On the other hand, in many cases different diseases in different patients can still be treated with the similar methods if they share the common causes and mechanisms (such as inflammation). Such methods can help transform the disease-centered medicine to human-centric care. Furthermore, expanded and novel applications may also be found for available drugs for the common therapies in different diseases with common mechanisms, allowing for more efficient usages of drugs for a cost-effective care.

1.2 Adaptation and Coevolution

Another important property of CASs is adaptation ([5], also *see* Fig. 1). Complex systems can adapt to environmental stresses with the evolvement of the agents into new conditions. Because disease is

a dynamical process, different features can be shown at different stages during the evolvement while the "whole body system" can adapt to the changing environment. The variations at different stages represent the new dynamical interactions within the whole system and should not be measured with limited clinical factors [12].

The phenomena of adaptation can be observed in various diseases including coronary artery disease, chronic obstructive airway disease, and rheumatoid arthritis [8]. Coevolution can also be involved in such processes (*see* Fig. 1). As each agent is evolving in response to the changes, new dynamics and features may occur in subsystems. For example, the development of the resistance against multiple drugs is commonly seen in the treatment of many diseases including bacteria, viruses, parasites, and cancers [13]. On the basis of such concepts, another essential feature of personalized medicine is that the same disease in the same patient should be treated differently at different time points or stages. Because adaptation is a general phenomenon, this principle can be applied to not only chronic diseases such as cancer but also acute diseases such as influenza infections.

1.3 Self-Organization and Feedback Loops

Self-organization and robustness are the critical properties of CASs, as these systems contain regulatory loops, multiple interactions, and recursive positive and negative feedback mechanisms at various levels, without any external supervisory impacts or directions from levels above [5, 6]. Examples of such properties in biomedicine include the water balance and blood glucose levels. Similar organ phenotypes can have different levels of robustness while the disease state can develop its own robustness against treatments [14].

In the practice of personalized medicine, such properties of living systems can be used to adjust the pathological conditions back to normal. For example, in infectious diseases the purpose of treatment should be no longer the simple elimination of bacteria or viruses using antibiotics, which have caused many problems including drug resistance. Instead, integrative and dynamical interventions can be designed to address the relative stability from self-organization and robustness, the flexibility from adaptability, and the positive and negative feedback mechanisms in the humans (*see* Fig. 1). In this example, multiple inflammatory pathways (rather than the single pathogens) at different phases may become potential therapeutic targets. Therefore, the understanding of these properties is crucial for the transformation of disease-centered medicine to human-centric care.

Specifically, the feedback loops can link the interactive components including genes, drugs, and environment at various levels, forming the structure–function and genotype–phenotype correlations [4]. For instance, at the molecular level, the structural and functional alterations, polymorphisms, gene–gene interactions,

and gene–drug interactions can have profound impacts on the downstream pathways and networks [15]. In the meantime, the collective effects of such "emergent" property may be observed as clinical symptoms, behaviors, and therapeutic responses at the phenotypic level.

In addition, the higher levels can influence the lower levels (and vice versa). For example, environmental stress such as sunburn may cause DNA damages in skin cells at the molecular level. It is necessary to study the interactions not only within the same level (e.g., gene–gene interactions) but also across different levels (e.g., genes–cells–environment interactions) [16]. The elucidation of the cross-level feedback loops and interactions such as genotype–phenotype connections at various temporal and spatial scales may help switch the gear of medical practice from single targets to systems resolutions, from isolated symptoms to systemic biomarkers ([4]; also *see* Fig. 1).

1.4 Nonlinearity and Dynamical Pathophysiology

Nonlinearity refers to the property that the stimulus may not lead to proportional responses or results, while the system is sensitive to the initial status and massive changes may occur [5]. Many clinical observations have confirmed this feature. For example, having higher dosages of chemotherapy may not result in better treatment outcomes [17]. Although chemotherapy may help reduce the tumor size at the beginning, secondary tumors may also be induced [18]. Nonlinearities in cardiac electrophysiology have been found to affect normal and abnormal rhythms, and be involved in the cardiac arrhythmogenesis [19]. In individualized medicine, such effects of nonlinearity should be an important factor to be considered for the overall preventive and therapeutic plans including the dosages and intensities at various stages, as well as the timing and frequencies of the treatment. This is how the objectives of personalized care can be achieved, i.e., to bring the right interventions to the right people with the right dosages and intensities at the right time.

Because the human body is an open system interacting with the natural and social environments continuously, the nonlinear human–environment interactions may cause massive changes in a very short period of time [8]. For example, many infectious diseases such as influenza have seasonal features. Social events such as wars may cause psychological problems such as posttraumatic stress disorder (PTSD). The factors of such nonlinear psychosocial and environmental interactions should play a key role in the prevention of disease occurrence and progression (*see* Fig. 1).

In summary, one of the most prominent properties of CASs is probably the dynamical changes over time with the flow of matters, energy and information. As health and diseases are dynamical and adaptive processes but not static or equilibrium states, it is necessary to understand the properties of CASs at various levels in humans. While normal physiology is featured with nonlinear dynamics, such

dynamical features are altered in the pathophysiology of illnesses [20]. For example, sinus rhythm represents the regularly recurring dynamics in cardiology while atrial fibrillation represents the irregularly recurring dynamics [21]. Physiological and pathological parameters or variables are always changing. Such variability exists within the same patient over time, and between different patients at one specific time. The task of personalized medicine is to address such variability to achieve effective prevention and treatment. In the following section, the dynamics and complexity at both spatial and temporal dimensions will be discussed, leading to a framework of systems and dynamical medicine as the root of the personalized or P4 medicine (*see* Subheading 5).

2 Systems and Dynamical Medicine: Targeting the Spatial and Temporal Complexity

2.1 The Complexity in the Spatial Dimension

As discussed above, to have practical applications of personalized medicine in the clinic, pharmacogenomics and systems biology should address the essential aspects of the complexity in living systems. As shown in Fig. 2, such complexity can be analyzed in the spatial and temporal dimensions. In the spatial dimension, systemic understanding is needed across various levels, from molecules to cells, from tissues to organs, from organisms to psychosocial environments.

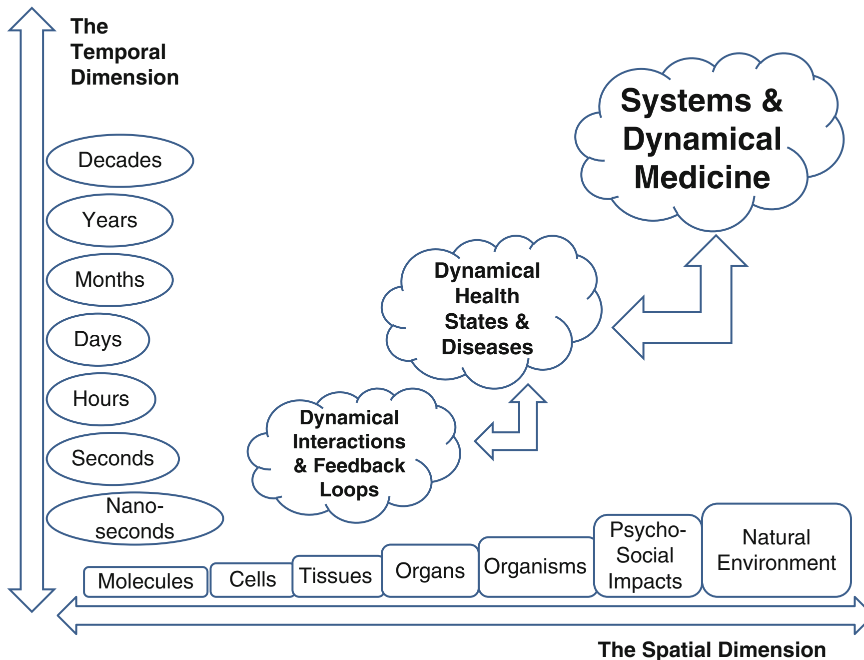

Fig. 2 Systems and dynamical medicine for holistic care: the integration of the spatial and temporal dimensions

Different tools and approaches can be used at different levels for dynamical and integrative investigations, from nanotechnology to high-throughput (HTP) analyses such as proteomic studies, from biophysics studies to physiological measurements.

Specifically, the analyses of protein–protein interactions and the real-time detections of expression profiles are needed at the molecular level for the understanding of the "molecular complexity" [22]. The detections of cellular compartmentalization and the cellular perceptions of dynamic protein complexes are necessary at the cellular level for the understanding of the cellular complexity. For instance, the disruption of redox organization has been identified as a common basis of illnesses. The description of the redox compartmentalization during cellular stress may elucidate the dynamic regulations of structure and function for disease prevention and treatment [23]. In terms of sizes, genes and proteins are studied at the nanometer scale, while cellular organelles are studied at the micrometer scale [22]. Tissues are measured at the millimeter scale, organs at the centimeter scale, and the systemic physiology and functions of the organisms can be measured at the meter scale. In addition, such systemic analyses need to integrate physical variables including temperature, motions, and currents.

More importantly, the gaps between different levels and scales need to be bridged. The connections across multiple levels, such as the genotype–phenotype correlations, need to be established to have an understanding of the collective and overall "emergent" properties of the hierarchical complexity for systems modeling. The integration of the information and dynamics from one scale to another is essential for the study of human physiology and the treatment of illnesses. The multiple scales and dynamical changes in cardiac excitation is an example [16]. A single ion channel opens and closes at the sub-millisecond or millisecond time scales. The actions of thousands of ion channels result in an action potential at the whole-cell level. At the whole-heart level, the electric impulses lead to synchronous contractions of the ventricles at the time scale of seconds, which can be detected by the electrocardiogram [16]. The stability of cardiac rhythm has been associated with the wave fronts propagate measured at the centimeter scale, while such functions are also related to the properties of the ion channels and gap junctions measured at the nanometer scale [24]. As shown in these examples, the scale-transcending understanding would be meaningful only with the integration of both the spatial and the temporal complexity.

2.2 The Complexity in the Temporal Dimension

As pharmacogenomics focuses on the "variations" of human responses, such variations come from not only individual differences but also temporal and periodic differences. However, the temporal dimension and the factor of time have often been neglected in biomedical studies. For example, most epidemiologic

studies about disease risks have been focusing on the "average" spatial patterns, while the approach to analyze the spatiotemporal disease risks has been used only recently [25]. Human organs, especially the central nervous system (CNS), are highly influenced by time at various temporal scales and spatial levels in terms of structural and functional interactive networks ([6, 26, 27]; also *see* Fig. 2). Both physical and chemical processes are influenced by chronological factors. The structural and functional states at one time-point can be very different from those at another time-point. For example, aging is an evolutionary process over time with progressive alterations of physiologic complexity, cell cycles, cell motility, and gene expression patterns [28–30].

As illustrated in Fig. 2, nonlinear time-series analyses are necessary to precisely represent the system dynamics at various spatial levels. The time scales can span from nanoseconds to seconds, from hours to days, years, and decades. For example, the ion channel gating events can be measured at the microsecond scale, the depolarization of the heart can be measured at the millisecond scale, and the stability of the cardiac cycle can be measured at the second scale [22]. In addition, the longevity and aging of the organism can be measured at the gigasecond scale.

Various rhythms and cycles in the physiological and pathological contexts can be examined. Specifically, circadian rhythms last about 24 h. Rhythms that are longer than 24 h are called infradian rhythms, such as the menstrual cycle [31, 32]. Cycles that are shorter than 24 h are called ultradian rhythms, such as the firing rate of neurons and the rhythm of heartbeats. Currently in biomedicine, the circadian rhythm is the one that has been studied the most extensively [32]. More studies on other patterns are still needed. In addition to biological and environmental rhythms, social rhythms and cycles such as school years, as well as the frequencies of biological, environmental, and social events should also be considered.

Temporal patterns and oscillations have been observed in a very broad spectrum across various spatial scales. For example, periodic dynamics have been studied in gene expression patterns in the cell division cycle and the cellular redox state alterations, while the genome-wide oscillations at the transcript and protein levels indicate the cell cycle as a developmental process [33]. Spatiotemporal oscillations have been observed in mitochondria, transmembrane potentials, heart excitation waves, neural activities and brain dynamics, cognition and verbal working memory, and even bacteria [16, 34–37].

Similar to the spatial features, the temporal patterns such as the oscillation behaviors should also be understood with the collective or "emergent" properties across multiple time scales in connection with multiple spatial levels. Across-scale detections in all scopes are necessary to achieve a thorough understanding (*see* Fig. 2).

For example, the spatial and temporal profiles of different growth factor (GF) signaling during memory formation indicate that the GF signaling in the behavioral and structural plasticity should be studied as interactive components in a complex network [26]. Only with the comprehensive profiling methods crossing multiple levels could such networks be elucidated accurately.

3 From Dynamical Diseases to Dynamical Medicine

In summary of the discussion above, systemic analyses are needed to detect patterns across multiple scales in both the spatial (e.g., molecules to cells to tissues) and temporal (e.g., nanoseconds to hours to years) dimensions. Based on such understanding, systems and dynamical medicine can be developed with the emphasis on the whole systems that change over time to address the nonlinearity and interconnectivity toward a holistic and proactive care. Here, the word "systems" underscores the concept of holism, and the term of "dynamical medicine" highlights the variations with the interwoven and integration of the spatial and temporal scopes (*see* Fig. 2).

For instance, at the organ level, the studies of the nonlinear dynamics of heart rates have identified the critical roles of multifaceted factors including circadian profiles, age, and the involvement of autonomic nervous system [38]. At the cellular level, the nonlinear dynamical behavior of mitochondria is involved in the regulation of energy metabolism in liver cells, while the alteration of such behavior has been related to the aging process and organ dysfunctions [39]. As the human nervous system contains "a hierarchy of oscillatory processes" [40], other organs and systems in the human body are also influenced by these oscillatory mechanisms. With more studies of nonlinear dynamics in biomedicine, more diseases can be investigated in terms of their dynamical features.

The "dynamical diseases" with altered dynamical complexity and rhythms in comparison with those during the normal states have been identified in many illnesses including depression, schizophrenia, epilepsy, substance abuse, Parkinson's disease, age-related diseases, osteoporosis, and hyperparathyroidism [41–48]. For instance, affective problems such as depressive disorders have shown fluctuating state variables at both biological and psychological levels, and the complex phenomena in such diseases may be represented with the nonlinear interactions of these variables [49]. In another example, a nonlinear relationship has been established between obesity and diurnal cortisol secretion [50]. In addition, chronic lymphocytic leukemia (CLL) has been found to be a dynamical disease as it is closely associated with B cell cycles [51]. Recent studies of microRNAs (miRNAs), their associations with cell cycle regulations and their roles in complex diseases such as

cancer [52] have made the dynamical properties of health and diseases even more significant.

Based on these discoveries, because "everything oscillates" [33] from mitochondria to cognition, all diseases have the dynamical features and need to be investigated dynamically from diagnosis to treatment. The analysis of bifurcations in dynamics, together with nonlinear time-series analysis may contribute to the elucidation of physiological and pathological rhythms, oscillations, and feedback loops for improving personalized interventions [53]. For example, temporal complexity and variability associated with biomarkers, functions, and symptoms have been explored in various disorders including lung diseases such as asthma [54]. Dynamical systems models have been suggested to capture systematic fluctuations in psychiatric symptom patterns [55]. In the case of cancers, certain molecular factors in the tumor cells mediating therapeutic sensitivity and resistance have been associated with the specific states and stages of prostate cancer during its evolvement and progression [56].

The dynamical features of the diseases require the identification of shifting targets (such as molecules and pathways) at different time points or phases for more effective treatments. Furthermore, the understanding of the oscillatory properties of the diseases suggests the needs for follow-ups to prevent recurrence, and potential interventions can be designed for recurrent patients such as in the case of depression [41]. In the next section, the identification of systems and dynamical biomarkers will be discussed for these purposes.

4 Identification of Accurate and Robust Biomarkers for Systems and Dynamical Medicine

As indicators of physiological and pathological states, biomarkers are crucial for accurate diagnosis and prognosis, prediction of diseases and therapeutic responses, and prevention of disease occurrence and progression. They have significant roles in the profiling and classification of diseases and in the effective discovery of new drugs.

However, the complexity and dynamical changes in various diseases make it difficult to precisely identify and validate biomarkers. Traditional approaches such as those using symptom checklists are very limited in discovering useful biomarkers to characterize the multifaceted pathophysiological states and stages for effective clinical care. In addition, single biomolecules are not enough to describe the composite biological functions because multiple pathways may be involved in the same disease phenotype. The identification of biomarkers in traditional ways faces serious limitations from both the theoretical aspect and the technical part.

For example, the pathology-type immunohistochemical markers (IHCM) have been considered inadequate for the applications in clinical pathology [57].

To overcome the difficulties, approaches based on systems analyses and dynamical explorations are necessary for the discovery of accurate and robust biomarkers. Systems biology and "omics"-based biomarkers can be identified for the grouping of disease subtypes to represent the diversified pathways and the disease heterogeneity for personalized medicine. Various factors need to be considered, including genomic variations, epigenetic variability, functional alterations, the structure–function associations, etiologic heterogeneity, as well as environmental impacts [58]. For example, specific cellular changes can be quantitatively examined using high content phenotypic tests, such as the profiling of alterations in cells' entire transcriptome or proteome [59]. Libraries of molecules, peptides, and polynucleotides including siRNA can be examined to detect the perturbing factors that influence the transcriptomic, proteomic, and cellular markers, as well as their roles in molecular pathways and networks.

More importantly, biomarkers with predictive values are needed to reflect the conditions and stages of diseases in both the temporal and the spatial dimensions. For instance, biomarkers with the feature of temporal evolution have been proposed for the study of the onset and progression of clinical symptoms in Alzheimer's disease (AD) [60]. Such dynamical models have suggested that time rather than clinical symptom severity is more appropriate for representing the disease progression in individual patients.

To achieve the across-scale analyses, HTP technologies may have a valuable role in the dynamical evaluations of systems-wide genotype–phenotype associations with time-series detections [61]. The usage of such technologies in analyzing genetic networks can help overcome the limitation of individual molecular markers that simply differentiate disease and normal samples in static states with narrow coverage and inaccurate clinical applications. Because the deteriorations of complex diseases often occur abruptly at a tipping point showing an imminent bifurcation, methods can be developed for identifying early-warning signals during this critical stage. Specifically, the dynamical network biomarkers (DNBs) based on the HTP gene expression data can be discovered [61]. Tissue-specific molecules in DNBs can be examined from the normal state to the disease state of the whole system [62]. Such methods may be especially useful for early diagnosis and prognosis.

For example, in the case of cancer, it is important to elucidate the alteration tendencies of carcinogenesis and the dynamic patterns of protein expressions during different stages. In addition to primary prevention, early detection has been deemed as the most effective way to reduce the mortality of solid cancers [63]. Conventional cancer screening models have been focusing on the

categorization of at-risk populations into three groups of normal, cancer without symptoms, and cancer with symptoms. However, such approach has not been very successful in decreasing cancer mortality. A model of dynamic clonal evolution of biomarkers has been suggested to promote the accurate prediction of progression and early detection of cancers for risk management in the clinic [63]. By using a set of robust biomarkers, such methods may enable the prediction of cancer progression with the stratification of various risk groups among patients for timely interventions and personalized management toward the optimized outcomes.

5 Conclusion and Future Perspectives: Systems and Dynamical Medicine as the Root of the P4 Medicine

Systems and dynamical medicine can provide the root for the achievement of predictive, preventive, personalized and participatory (P4) medicine [64]. By addressing the CASs properties in humans, network and dynamical models can be developed to meet the theoretical and technological challenges, with the approaches targeting the disease complexity of multiple types at multiple stages [65].

Specifically, the identification of systems and dynamical biomarkers with predictive values would help reduce the risks of developing diseases and disability, enabling the practice of preventive medicine among at-risk populations [64]. Early warnings of diseases at various stages can be detected timely for presymptomatic diagnosis and precise prognosis, allowing for integrative interventions for the prevention of disease occurrence, progression, and recurrence ([66]; also *see* Fig. 1). Such approaches would be especially helpful for the prediction and preclusion of disease development in chronic and complex disorders such as depression, cancers, and Alzheimer's disease.

Furthermore, the recognition of variations across the different scales of spatial and temporal dimensions would enable the identification of shifting therapeutic targets to address both of the individual and the time variances in personalized medicine (*see* Fig. 1). Accurate and robust biomarkers can also be useful for the stratification of diseases and classification of patient subgroups for more effective prevention and therapy. The prediction of drug responses would in turn help avoid adverse events for better clinical outcomes. In addition, the construction of dynamic disease predictive networks derived from the analyses of "omics" data would allow for the transition from reactive treatments to holistic and proactive care. With the transformation from disease-centered to human-based care, the systems and dynamical models would provide patient-centric information to enhance the participation of individuals, the goal of participatory medicine.

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Chapter 2

Translational Bioinformatics Approaches for Systems and Dynamical Medicine

Qing Yan

Abstract

The exponential growth of experimental and clinical data generated from systematic studies, the complexity in health and diseases, and the request for the establishment of systems models are bringing bioinformatics to the center stage of pharmacogenomics and systems biology. Bioinformatics plays an essential role in bridging the gap among different knowledge domains for the translation of the voluminous data into better diagnosis, prognosis, prevention, and treatment. Bioinformatics is essential in finding the spatiotemporal patterns in pharmacogenomics, including the time-series analyses of the associations between genetic structural variations and functional alterations such as drug responses. The elucidation of the cross talks among different systems levels and time scales can contribute to the discovery of accurate and robust biomarkers at various diseases stages for the development of systems and dynamical medicine. Various resources are available for such purposes, including databases and tools supporting "omics" studies such as genomics, proteomics, epigenomics, transcriptomics, metabolomics, lipidomics, pharmacogenomics, and chronomics. The combination of bioinformatics and health informatics methods would provide powerful decision support in both scientific and clinical environments. Data integration, data mining, and knowledge discovery (KD) methods would enable the simulation of complex systems and dynamical networks to establish predictive models for achieving predictive, preventive, and personalized medicine.

Key words Bioinformatics, Biomarkers, Data mining, Decision support, Dynamical medicine, Health informatics, Personalized medicine, Pharmacogenomics, Systems biology, Systems medicine

1 Introduction

The exponential growth of experimental and clinical data generated from systematic studies, the complexity in health and diseases, and the request for the establishment of systems models are bringing bioinformatics to the center stage of pharmacogenomics and systems biology. Bioinformatics plays an essential role in bridging the gap among different knowledge domains for the translation of the voluminous data into predictive, preventive, and personalized medicine ([1]; also *see* Chapter 1).

Specifically, bioinformatics can be used together with the "omics" and high-throughput (HTP) studies for the development

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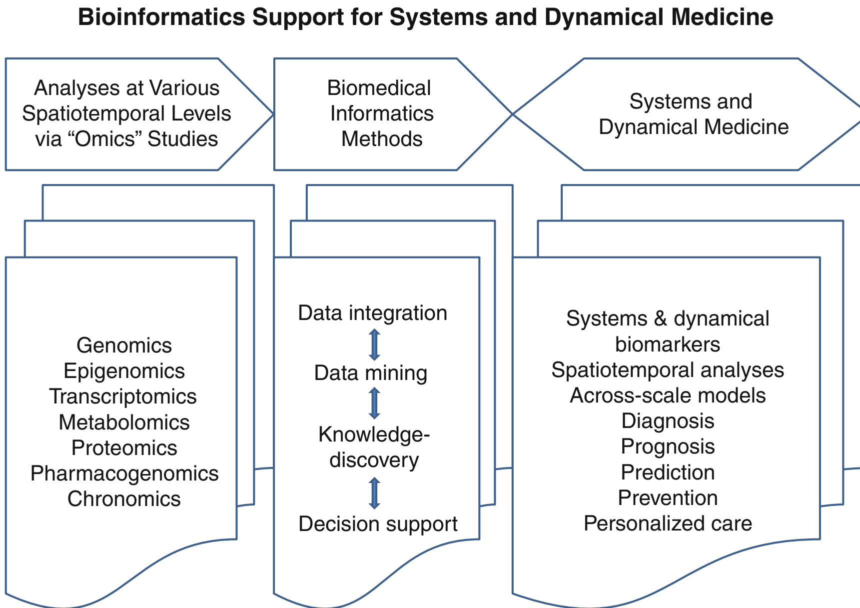

Fig. 1 Bioinformatics support for systems and dynamical medicine

of patients' unique profiles and subgroups (2, also *see* Fig. 1). Data integration, data mining, and knowledge discovery (KD) methods would enable the simulation of the complex systems and dynamical networks to establish the predictive models for better preventive and treatment strategies. Such approaches are critical for understanding the spatiotemporal relationships at various levels to have insight into the complex mechanisms of health and diseases (*see* Chapter 1). On the basis of such understanding, accurate and robust biomarkers can be discovered for better diagnosis, prognosis, prevention, and interventions to enable the transformation from disease-centered medicine and single drug targets to human-centric systems and dynamical medicine (*see* Fig. 1, also *see* Chapter 1).

Furthermore, the integration of genomic analysis in bioinformatics and clinical data analysis in health informatics may help elucidate the connections between different systems levels such as the establishment of genotype–phenotype correlations ([1]; also *see* Chapter 1). Data integration methods are needed for linking experimental and clinical data streams to achieve more efficient workflow and better multidisciplinary collaborations among scientists and clinicians [3]. Techniques such as data mining and electronic health records (EHRs) would enable better decision support for better drug discovery, as well as better diagnosis and treatment approaches to bring the right preventive and therapeutic strategies to the right people at the right time [1]. These biomedical informatics methods

may help achieve the goals of personalized medicine, including the optimal outcomes with reduced costs, errors, risks, drug resistance, and adverse events.

2 Bioinformatics Approaches for the Analyses at Various Levels Toward Systems Medicine

2.1 Bioinformatics Support for "Omics" Studies

With the understanding of the explicit role of bioinformatics, it is necessary to have detailed approaches to achieve these goals. Various resources are available for the development of systems and dynamical medicine (*see* Chapter 1), including those supporting "omics" studies such as genomics, proteomics, epigenomics, transcriptomics, metabolomics, lipidomics, pharmacogenomics, and chronomics ([4], also *see* Fig. 1). Some of the databases and tools are listed in Tables 1, 2, 3, 4, and 5. Lists like these are constantly growing with the fast development in the fields.

Table 1
Bioinformatics databases and tools for "Omics" studies[a]

Sources	URL	Features
Entrez	http://www.ncbi.nlm.nih.gov/sites/gquery	A cross-database search engine
Ensembl Genome Browser	http://uswest.ensembl.org/index.html	Genomic resources
ExPASy	http://expasy.org/	Bioinformatics resources
UniProt	http://www.uniprot.org/	Protein features
BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	Sequence alignments and similarity searches
PROSITE	http://www.expasy.org/prosite/	Protein families, functional searches
CLUSTAL	http://www.clustal.org/	Multiple sequence alignments
PredictProtein	http://www.predictprotein.org/	Protein structure predictions
PDB	http://www.pdb.org	Protein structures
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/	A database of SNPs
International HapMap Project	http://hapmap.ncbi.nlm.nih.gov/	Sequence variation resources
Human Variome Project	http://www.humanvariomeproject.org/	Genetic variations and effects on human health

(continued)

Table 1
(continued)

Sources	URL	Features
KEGG	http://www.genome.jp/kegg/pathway.html	A pathway database
Reactome	www.reactome.org/	A pathway database
GenMAPP2	http://www.genmapp.org	Pathways
IntAct	http://www.ebi.ac.uk/intact/	Molecular interactions
Cytoscape	http://www.cytoscape.org	Visualization of complex networks
MIPS	http://mips.helmholtz-muenchen.de/proj/ppi	Mammalian protein–protein interactions
HPRD	http://www.hprd.org/	Pathways and proteins
GEO	http://www.ncbi.nlm.nih.gov/geo/	Gene expression and arrays
ArrayExpress	http://www.ebi.ac.uk/arrayexpress/	About gene expressions
RNA-Seq Atlas	http://medicalgenomics.org/rna_seq_atlas	Gene expression profiling
HPA	http://www.proteinatlas.org/	Protein expression profiles
HMDB	http://www.hmdb.ca/	Human small molecule metabolites
LIPID MAPS	http://www.lipidmaps.org/	Lipidomics gateway
Biomarkers	http://pharmtao.com/health/category/systems-medicine/biomarkers-systems-medicine/	Resources and links for the discovery of biomarkers

[a]Resources accessed 15 Dec 2013

As genomics plays a critical role in personalized medicine, it is necessary to analyze both structural and functional features as well as the relationships between them. Nucleotide and protein information can be found at comprehensive platforms including NCBI's Entrez, the Ensembl Genome Browser, ExPASy, and Uniprot (*see* Table 1). Based on the analyses of sequence similarities, structural motifs, and patterns in gene families, functional changes can be investigated. Phylogenetic trees are helpful for understanding functional and evolutionary changes. As listed in Table 1, bioinformatics tools including BLAST [5], PROSITE [6], and CLUSTAL [7] are frequently used for such investigations. The two and three dimensional structural modeling using tools such as PredictProtein [8] and PDB [9] would be needed to elucidate the structure–function and protein–protein interactions (*see* Table 1).

Table 2
Bioinformatics databases and tools for epigenomics and microRNA studies[a]

Sources	URL	Features
MENT	http://mgrc.kribb.re.kr:8080/MENT/	DNA methylation and gene expression database of cancers
DBCAT	http://dbcat.cgm.ntu.edu.tw/	Methylation profiles in cancers
CMS	http://cbbiweb.uthscsa.edu/KMethylomes/	Cancer methylome datasets
MethylomeDB	http://www.neuroepigenomics.org/methylomedb/	Brain DNA methylation profiles
NGSmethDB	http://bioinfo2.ugr.es/NGSmethDB/index.php	DNA methylation data from NGS
EPITRANS	http://epitrans.org/EPITRANS/Service	Epigenetic and transcriptomic data
EpiExplorer	http://epiexplorer.mpi-inf.mpg.de/	For the analysis of large epigenomic datasets
miRBase	http://www.mirbase.org	MicroRNA database
microRNA.org	http://www.microrna.org	MicroRNA targets and expressions
miRNAMap 2.0	http://mirnamap.mbc.nctu.edu.tw	MicroRNA, genomic maps
mirGen 2.0	http://diana.cslab.ece.ntua.gr/mirgen	MicroRNA, genomic information
miRTarBase	http://mirtarbase.mbc.nctu.edu.tw	MicroRNA-target interactions
miRDB	http://mirdb.org/miRDB	MicroRNA target predictions, functional annotations

[a]Resources accessed 15 Dec 2013

To understand the individual differences at the molecular level, the analysis of genetic variations including single nucleotide polymorphisms (SNPs) is especially important. Such analyses have specific meanings for personalized medicine. Bioinformatics resources such as dbSNP [10] and the International HapMap Project can be applied especially for the genetic association studies [11] (*see* Table 1). For example, bioinformatics segmentation algorithm was used for analyzing SNP array data of esophageal cancer [12]. The analysis detected genomic abnormalities with similar and different frequencies from a large cohort data of esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). On the basis of such analysis, histology-specific therapeutic agents can be developed for esophageal cancer [12].

Table 3
Bioinformatics databases and tools for the studies of disease phenotypes and drug responses[a]

Sources	URL	Features
OMIM	http://www.ncbi.nlm.nih.gov/omim	Human genes, diseases
Gentrepid	http://www.gentrepid.org/	Genetic disorders
dbGaP	http://www.ncbi.nlm.nih.gov/gap	Genotype–phenotype interactions
Clinical Trials.gov	http://clinicaltrials.gov/	Clinical trials
NHANES	http://www.cdc.gov/nchs/nhanes.htm	Health and nutritional status
DrugBank	http://www.drugbank.ca	Drugs and targets
DIDB	http://www.druginteractioninfo.org/	Drug interactions
Drugs@FDA Database	https://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm	A database of drugs
ChEMBL	https://www.ebi.ac.uk/chembl/	Drug-like small molecules
AERS (FDA)	http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/AdverseDrugEffects/default.htm	Adverse events and medication error reports
MedWatch	http://www.fda.gov/Safety/MedWatch/	FDA safety information
SIDER (EMBL)	http://sideeffects.embl.de	Adverse drug reactions
HuGENet	http://www.cdc.gov/genomics/hugenet/default.htm	Genetic variations in health and diseases

[a]Resources accessed 15 Dec 2013

With the study of the structure–function relationships, further analyses are needed to elucidate the networks and pathways among various molecules to enable a systemic understanding. Bioinformatics sources including Kyoto Encyclopedia of Genes and Genomes (KEGG) [13], Reactome [14], and HPRD [15] are often used for analyzing cellular pathways and biochemical interactions (*see* Table 1). The genome-wide association studies (GWAS) and HTP technologies including microarray techniques are helpful for pharmacogenomics analyses to support better diagnosis, outcome assessments, and biomarker identifications. Resources including gene expression omnibus (GEO) [16] and ArrayExpress are the commonly applied platforms for the HTP analyses (*see* Table 1).

Table 4
Bioinformatics databases and tools for molecular dynamics and spatiotemporal studies[a]

Sources	URL	Features
DNAtraffic	http://dnatraffic.ibb.waw.pl/	Genome dynamics
CDDB	http://www.cdyn.org/	Conformational dynamics of proteins and assemblies
Dynameomics	http://www.dynameomics.org	Protein dynamics
Dynamic Proteomics	http://www.weizmann.ac.il/mcb/UriAlon/DynamProt/	Dynamics of proteins in living human cells
CHARMM-GUI	http://www.charmm-gui.org/	Macromolecular dynamics and mechanics
CircaDB	http://circadb.org	Mammalian circadian gene expression profiles
Allen Brain Atlas	http://www.brain-map.org	A spatiotemporal platform for studying the CNS
CDT-DB	http://www.cdtdb.neuroinf.jp/CDT/Top.jsp	Spatiotemporal gene expressions in mouse brains
GEMS	http://bio-imaging.liacs.nl/gems/	Spatiotemporal gene expressions in zebrafish
Eurexpress atlas	http://www.eurexpress.org	Transcriptome in the mouse embryo
MitoGenesisDB	http://www.dsimb.inserm.fr/dsimb_tools/mitgene/	Spatiotemporal dynamics of mitochondrial biogenesis
CELDA	http://cellfinder.org	Cell types in complex systems
Arena3D	http://arena3d.org	Visualization of time-driven phenotypic differences
EpiScanGIS	http://www.episcangis.org	Visualization of spatiotemporal clusters of diseases
STSE	http://www.stse-software.org/	Spatiotemporal simulations of microscopy images
EUCLIS	http://www.bioinfo.mpg.de/euclis/	For circadian systems biology

[a]Resources accessed 15 Dec 2013

Sources for specific "omics" studies are also available. For instance, tools such as the RNA-Seq Atlas [17], Human Protein Atlas (HPA) [18], and the Human Metabolome Database (HMDB) [19] are useful for systemic genomic, proteomic, and metabolomics studies of human beings. LIPID Metabolites and Pathways Strategy (LIPID MAPS) [20] is a lipidomics gateway, an integrative platform for studies in lipid biology. More resources and updated links can be found at the Biomarkers portal (*see* Table 1).

Table 5
Bioinformatics and health informatics standards and resources[a]

Sources	URL	Features
GO	http://www.geneontology.org/	Gene ontology
HGNC	http://www.genenames.org/	Gene nomenclature
caBIG	https://cabig.nci.nih.gov/	For cancer studies
SNOMED-CT	http://www.nlm.nih.gov/research/umls/Snomed/snomed_main.html	Clinical terminology
ICD	http://www.who.int/classifications/icd/en/	Disease classifications
LOINC	http://loinc.org/	For laboratory observations
DICOM	http://medical.nema.org/	For medical imaging data
UMLS	http://www.nlm.nih.gov/research/umls/	For medical terminology integration
HL7	http://www.hl7.org/	For health information systems interoperability
SBML	http://sbml.org/Main_Page	For models of biological processes
MedDRA	http://www.meddramsso.com/	A medical terminology for adverse events
RxNorm Resource	https://www.nlm.nih.gov/research/umls/rxnorm/	Drug vocabularies
GINA	http://www.genome.gov/24519851	Genetic information nondiscrimination act
HIPAA and PSQIA	http://www.hhs.gov/ocr/privacy/	Patient privacy, safety, and security rules
eMERGE	https://www.mc.vanderbilt.edu/victr/dcc/projects/acc/index.php/Main_Page	Electronic medical records and genomics
Personal Genome Project	http://www.personalgenomes.org/	About the personal genome project

[a]Resources accessed 15 Dec 2013

With the applications of the HTP technologies, a great number of variation data will become available for further analyses using data integration and mining methods (*see* Subheading 4).

2.2 Bioinformatics Support for Epigenomics and microRNA Studies

The discoveries in recent years have indicated that epigenetic patterns such as DNA methylation can be very useful for understanding the mechanisms of complex diseases including cancer [21]. For instance, abnormal DNA methylation alterations may be involved in many neurodevelopmental and neuropsychiatric

diseases such as schizophrenia and depression [22]. Many resources and databases have been developed to support such disease-associated analyses. For example, MENT (Methylation and Expression database of Normal and Tumor tissues) is a database about DNA methylation and gene expression in normal and tumor tissues ([21], also *see* Table 2). DBCAT (database of CpG islands and analytical tools) is a platform for studying DNA methylation profiles in cancers [23]. Cancer Methylome System (CMS) is a database for the visualization and statistical analysis of DNA methylation data of human cancers [24]. MethylomeDB is a database of genome-wide brain DNA methylation profiles that can be used for the investigation of neuropsychiatric disorders including schizophrenia and depression ([22], also *see* Table 2).

Other resources for general epigenomic studies include NGSmethDB, a database containing methylation data derived from next-generation sequencing (NGS) of human, mouse, and Arabidopsis ([25], also *see* Table 2). EPITRANS is a database containing epigenetic and transcriptomic data for the analyses of gene expression and epigenetic modifications [26]. EpiExplorer provides a Web tool for analyzing genomic and epigenomic data [27].

MicroRNAs (miRNAs) are small noncoding RNAs that are critical in many biological processes including the complex metabolic mechanisms such as energy and lipid metabolism. They play important roles in understanding complex diseases including cancer, diabetes, and obesity [28]. As mRNA regulators, miRNAs have been studied extensively in recent years as potential biomarkers because of their distinctive tissue and disease expression signature profiles [29]. Many miRNA resources have become available recently for miRNA studies. For example, the miRBase database is a microRNA sequence repository containing annotations and deep sequencing data ([30], also *see* Table 2). The microRNA.org site is a resource of miRNA target predictions and expression profiles for the analyses of various tissues and diseases [31]. The miRNAMap 2.0 provides genomic maps of miRNAs and target genes in human, mouse, and other metazoan genomes [32]. More resources relevant to miRNAs can be found in Table 2.

2.3 Bioinformatics Support for the Studies of Disease Phenotypes and Drug Responses

To connect the genotype features with phenotypes such as disease symptoms and drug responses, resources such as OMIM [33] and Gentrepid [34] are needed (*see* Table 3). The database of Genotypes and Phenotypes (dbGaP) represents the effort for understanding the genotype–phenotype interactions. Clinical trial resources are also useful to associate genomic data with clinical data, such as the site of Clinical Trials.gov from NIH (Table 3).

Moreover, environmental factors such as nutrients and drugs interact with human genomes and play key roles in personalized medicine. The National Health and Nutrition Examination Survey (NHANES) site contains information on how the nutritional

status affects health (*see* Table 3). Drug databases such as DrugBank and Drug Interaction DataBase (DIDB) can be used for analyzing drug targets, actions, and interactions. The Drugs@FDA Database contains the information of FDA-approved drugs. ChEMBL collects information about bioactive drug-like small molecules. The platform of Adverse Event Reporting System (AERS) (FDA) and SIDER (EMBL) are useful resources for studying drug safety and adverse reactions, a critical area in personalized medicine. The Human Genome Epidemiology Network (HuGENet) focuses on connecting genetic studies with public health and preventive medicine (*see* Table 3).

3 Bioinformatics Support for the Spatiotemporal Studies Toward Dynamical Medicine

As across-scale dynamical studies in the spatial and temporal dimensions provide the root for personalized, predictive, and preventive medicine, bioinformatics is indispensable for the development of systems and dynamical medicine (*see* Chapter 1). At the molecular and cellular levels, tools and databases are available for the studies of genomic and proteomic dynamics. For example, DNAtraffic is an annotated database for analyzing genome dynamics and the complexity of DNA network during the cell life, including information about chromatin remodeling, DNA damage, and DNA repair ([35], also *see* Table 4). The Conformational Dynamics Data Bank (CDDB) is a database about the conformational dynamics of proteins and protein assemblies, including the elastic strain energy distributions and correlations in molecular motions [36]. Dynameomics is a database about protein dynamics including information about protein folding and structure building [37]. Dynamic Proteomics is a database about the dynamics of proteins in living human cells including fluorescent images and movies obtained by the time-lapse microscopy [38]. The Membrane Builder in the CHARMM-GUI Web site is a tool for molecular dynamics simulations of protein and membrane complexes ([39], also *see* Table 4).

Resources for the spatiotemporal analyses across different levels from gene expression to microscopic images are emerging in the recent years. For example, CircaDB is a database about mammalian circadian gene expression profiles from time course expression experiments ([40], also *see* Table 4). The Allen Brain Atlas is an integrated spatiotemporal platform for studying the central nervous system (CNS) including both gene expression data and neuroanatomical information [41]. The Cerebellar Development Transcriptome Database (CDT-DB) supports the profiling of spatiotemporal gene expression patterns in the developing mouse brains [42]. The Gene Expression Management System (GEMS) is a platform for the spatiotemporal analysis of gene expression

patterns in zebrafish [43]. The Eurexpress atlas provides an anatomical map of the transcriptome in the mouse embryo for the examination of spatiotemporal gene expression profiles to study functional relationships among genes, development, and diseases ([44], also *see* Table 4). The MitoGenesisDB provides data mining tools for studying the spatiotemporal dynamics of mitochondrial biogenesis including the time-course of mRNA production, microarray analyses of mRNA localization, and mRNA transcription rates and stability [45].

At the cellular level, CELDA (Cell: Expression, Localization, Development, Anatomy) is an ontological system for the categorization of cells in complex systems including subcellular structures and developmental stages for both spatial and temporal analyses ([46], also *see* Table 4). At the phenotypic and disease level, Arena3D provides a visualization tool for time-driven phenotypic differences in biological systems for exploring temporal patterns and dynamic processes in morphological layers [47]. EpiScanGIS is a geographic surveillance system for the visualization of spatiotemporal clusters of meningococcal disease with real time data and demographic information [48]. In addition, The Spatio-Temporal Simulation Environment (STSE) provides a set of open-source tools for spatiotemporal simulations and analyses of microscopy images [49]. The EUCLOCK Information System (EUCLIS) is a platform for circadian systems biology studies including modules for models and experimental data for the investigations in chronobiology ([50], also *see* Table 4).

4 Bioinformatics and Health Informatics Methods for Decision Support in Personalized Medicine

As discussed above, the advances in genomics, pharmacogenomics, and systems biology have made large amounts of data available on individuals. However, it is still challenging to provide analyses across various spatial levels and time scales (*see* Chapter 1), such as the integration of genomic data (e.g., genotypes) with patients' medical records (e.g., phenotypes). A critical role of bioinformatics is to assist such integrative analyses across multiple levels to provide decision support for more accurate diagnosis and prognosis, and for making patient-specific suggestions with better preventive and therapeutic options [51]. The effective decision support should be based on better communication and workflow processes, more convenient resource retrieval, and efficient data mining and knowledge discovery tools. Such approaches would empower health practitioners to bring the "right knowledge to the right people in the right form at the right time" [1].

An essential element in successful decision support is data integration [51]. Data integration provides the crucial link between

laboratory and clinical settings for more efficient management of the workflow to save time and costs. To promote patient-specific medicine in clinical settings, it is necessary to integrate the information at various levels including genes, proteins, cells, drugs, interactions, and functional annotations with the phenotypic information in EHRs. Furthermore, the process of data integration can help pave the way for data mining and knowledge discovery [52]. For instance, a Web-based database called RNA-Seq Atlas was established for gene expression profiling with data mining and querying tools ([17], also *see* Table 1). The integrative system provides linkages to other functional databases, microarray profiles and gene expression data, as well as signaling pathways and gene ontologies. Such comprehensive tools can be useful for the comparison of tissue-specific expression profiles to find patterns, and for connecting tissue functions with genetic alterations toward the functional understanding at various systems levels to support further decision making processes [17].

An important step in data integration is the collection of different data sources that satisfy the domain and requirement analyses. For studies in pharmacogenomics and personalized medicine, scientific resources such as dbSNP (*see* Table 1) and clinical data from EHRs can be included. Many of these data are unorganized with errors, and need to be corrected, cleaned, updated, and organized [1]. Redundancies need to be removed, and inconsistencies need to be resolved. For example, one gene may have several different names as multiple entries. Such problems need to be solved to bring the common values together by using the genetic nomenclature references of Gene Ontology (GO) or The HUGO Gene Nomenclature Committee (HGNC) (*see* Table 5). Other resources including the Cancer Biomedical Informatics Grid (caBIG) can also be useful for solving such disparate problems (Table 5).

To facilitate decision support in various clinical and laboratory settings in different knowledge domains, standardization is a critical process to solve the interoperability issues. Various informatics resources are available for standardization including semantic mapping, as some of the examples shown in Table 5. These biomedical standards can be integrated into systems such as EHRs and clinical decision support systems (CDSSs). For instance, the comprehensive clinical terminology Systematized Nomenclature of Medicine Clinical Terms (SNOMED CT) and the classifications of diseases (ICD) are frequently used for clinical data and billing processes (*see* Table 5). The universal codes to identify laboratory observations (LOINC) are often applied for organizing laboratory data. The standards for processing imaging data in the clinic include the Digital Imaging and Communications in Medicine (DICOM). The Unified Medical Language System (UMLS) is a reference system integrating various standards. The platforms such as Health Level Seven International (HL7) intend to solve the interoperability issues among various health information systems and platforms.

For systems biology studies such as annotating genetic pathways, the Systems Biology Markup Language (SBML) is available. The Medical Dictionary for Regulatory Activities (MedDRA) supports the terminology to classify information of adverse events (*see* Table 5). The RxNorm Resource provides links between drug terminologies.

Data integration and data mining techniques can be used in an iterative and interactive process for knowledge discovery (KD), a critical component in decision support ([1], also *see* Fig. 1). When data have been organized and integrated, data mining is the next step for knowledge discovery to identify meaningful patterns and correlations, to build predictive models, and to validate potential biomarkers. These steps are needed for finding effective drug targets and applying personalized therapeutics.

Data mining is essential for systems studies and dynamical analyses such as the investigations in chronobiology [53]. Using data mining approaches, spatiotemporal patterns can be identified to build clustering, association, and dependency models. Many data mining techniques can be used for such purposes, including artificial neural networks (ANN), Bayesian network, decision trees, text mining, and genetic algorithms [2]. In addition, agent-based modeling is a useful approach for modeling nonlinear complex systems across various biological scales from cells to societies [54].

For example, various bioinformatics and data mining approaches were used to analyze the light–dark circadian-like cycle patterns of gene expression in the lung transcriptome, including the methods of BLAST, MATLAB, and clustering algorithms [55]. The cyclic oscillations and rhythms identified from such techniques may have important implications in lung diseases and drug actions. Using artificial neural networks (ANN), the dynamic thermal analysis (DTA) was found useful for the detection of breast cancer [56]. For the elucidation of the complexity in breast cancer, an agent-based model of mammary ductal epithelium dynamics was applied for the examination of the pathogenesis to address the multifactorial nature including both cellular and molecular mechanisms [57]. Moreover, semantic Web technologies have been suggested helpful for organizing and representing pharmacogenomics knowledge associated with drug development and medical decision making in various domains [58]. Such informatics methods can become the key elements in CDSSs.

5 Conclusions and Future Perspectives

In summary, bioinformatics plays an indispensable role in the development of systems and dynamical medicine by translating systems biology and pharmacogenomics studies into patient-centric therapeutics. Bioinformatics is essential in finding the spatiotemporal patterns in pharmacogenomics, including the time-series

analyses of the associations between genetic structural variations and functional alterations such as drug responses. The elucidation of the cross talks among different systems levels and time scales such as the genotype–phenotype associations can contribute to the discovery of systems-based biomarkers and the identification of patient subgroups during various disease stages (*see* Chapter 1). The systemic and dynamic profiling would also allow the finding of prognostic signatures and better preventive methods. The combination of bioinformatics and health informatics methods based on data integration and data mining techniques would provide powerful decision support in both scientific and clinical environments. These methods would facilitate the decision making processes by enabling more efficient communication and information retrieval, better knowledge discovery, and more accurate predictive modeling for better diagnosis and treatments toward personalized medicine (*see* Fig. 1).

Moreover, with the integration of bioinformatics and clinical decision support systems, all kinds of ethical, legal, privacy, and societal issues need to be considered. Such challenges request a closer collaboration between the communities of bioinformatics and those of health informatics. Many resources are emerging regarding these aspects. For instance, the Genetic Information Nondiscrimination Act (GINA) (*see* Table 5) was established to protect people from genetic discrimination. The Health Insurance Portability and Accountability Act of 1996 (HIPAA) Privacy and Security Rules and The Patient Safety and Quality Improvement Act of 2005 (PSQIA) Patient Safety Rule (*see* Table 5) also need to be followed when designing biomedical informatics systems such as EHRs. Furthermore, projects such as the eMERGE (Electronic Medical Records and Genomics) and the Personal Genome Project (Table 5) have been developed to associate genotypic information such as those from GWAS with clinical and individual phenotypes including diseases. With the rapid growth of both genomic and clinical phenotypic data, biomedical informatics would play even a more important role in every step of the development of systems and dynamical medicine, from diagnosis to treatment, from prognosis to prevention.

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Chapter 3

Whole Blood Transcriptomic Analysis to Identify Clinical Biomarkers of Drug Response

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Abstract

Since most immunological and hematological conditions might be expected to alter whole blood gene expression, its examination can lead to insights into disease processes, and biomarkers to assess molecular phenotypes, disease states, progression and response to therapy. In this chapter we describe collection and storage of RNA from whole blood, techniques to measure gene expression, and analytical approaches to identify the dysregulated gene expression using pathway and clustering analysis, gene set enrichment, heat map approaches, and cell subset deconvolution.

Key words Transcriptome, Gene expression, Pathways, Heat maps, Deconvolution, Gene set enrichment, Gene clustering

1 Introduction

Given that disease-causing and disease-protective immune cells need to transit from lymphoid and secondary lymphoid organs to their site of action in disease via the peripheral circulation, it should be possible to identify such cells and their effects through identification of dysregulation of whole blood gene expression. The nature of the dysregulation might be identified by examining the transcriptome of the blood as a whole, or by isolation of cell subsets and focusing on their ex vivo transcriptomes, or how gene expression is altered by response to pertinent stimuli in vitro. The transcriptome includes all RNA species produced in cells of the blood, including mRNA, miRNA, and lncRNA, all of which contribute to the regulation of cell and blood phenotype. Interrogation of the whole blood transcriptome can identify disease-specific altered gene expression, gene expression pathways, and cell subset representation. It might also be used to assess response to therapeutic intervention, to monitor disease progression, and to define molecular phenotypes that might respond to particular clinical management and pharmacological agents.

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Measurement of gene expression in whole blood is already used widely in clinical management. AlloMap is used to identify the absence of heart transplant rejection [1]. The Corus CAD Gene Expression Test is used to predict the likelihood of obstructive coronary artery disease (CAD) [2]. Following the identification of an interferon signature in the autoimmune disease systemic lupus erythematosis, anti-interferon therapy has been introduced for treatment [3]. The development of neutralizing antibodies to interferon beta is monitored by measuring interferon response in whole blood [4]. Transcriptomic biomarkers have been identified to stratify causative agents of sepsis [5], triage severity of influenza infection [6], and even identify risk factors for neurological conditions such as suicide behavior [7]. These biomarkers also indicate directions for drug development and assessment of drug response.

Assaying whole blood does have several limitations. It does not give any insight into transcriptomic changes of immune cells specific to the site of infection or a particular organ. The heterogeneous cell population collected from a whole blood sample may confound data interpretation. This can be limited to a certain extent by collecting full blood count data for each sample collected and including this information in downstream analyses [8]. Specific immune cell subsets can be isolated from blood samples using various techniques such as magnetic bead separation or fluorescence-activated cell sorting.

The following procedures are designed to enable discovery of whole blood dysregulation that is robust to replication, providing findings suitable for further testing for clinical applications.

2 Materials

1. A major consideration in transcriptomics studies is the source of the RNA used. Peripheral blood mononucleocytes (PBMCs) are widely used, but the Ficoll separation to remove neutrophils and red blood cells also introduces errors related to cellular response to this manipulation and time ex vivo [9]. An advantage of PBMCs is that they can be cryopreserved for latter examinations (*see* **Note 1**). PAXgene blood tubes collect the blood directly from the patient into fixative, are widely used in clinical settings, and provide reproducible results [10].
2. As circulating immune cells have diurnal [11] and seasonal rhythms [12], its vital that controls and samples be matched for these parameters, as well as standard criteria such as age, gender, and use of any immunomodulatory therapy.
3. Sample handling and storage of PAXgene tubes can greatly affect gene expression profiles (Fig. 1) [13]. Cryopreservation of PBMCs in liquid nitrogen has been used in many clini-

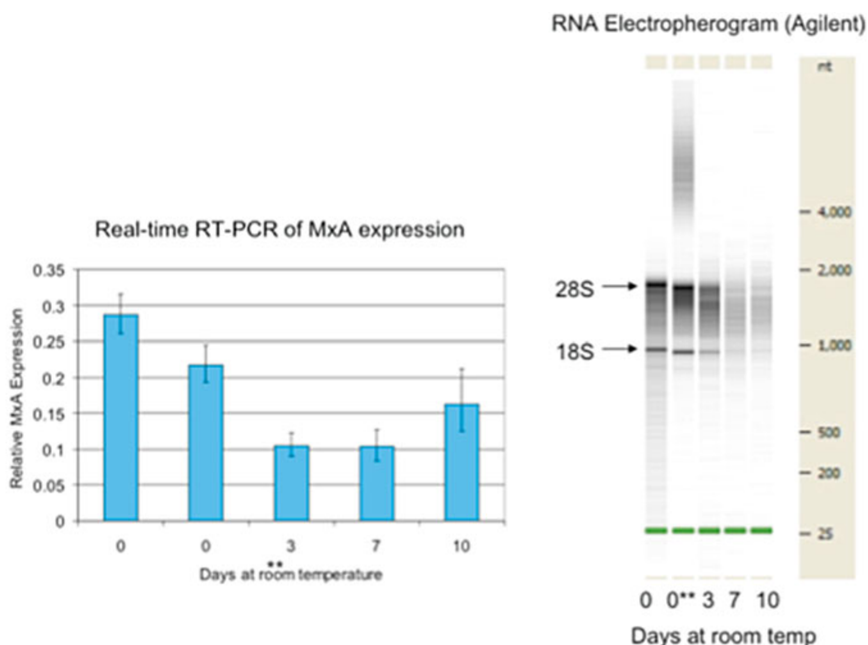

Fig. 1 Storage of Paxgene tubes affects RNA stability and measured gene expression. (*asterisk*) Days at room temperature before RNA extraction. (*double asterisk*) Immediate freezing (no lysis period)

cal trials [14]; however, it is known to induce significant changes in both the immunophenotype [15] and gene expression profile [13] of cells. For instance, dimethyl sulfoxide, one of the important components of the cryopreservation medium, is known to induce neutrophil differentiation [16]. It has been shown that whole blood samples collected in tubes containing an RNA stabilizing agent, such as PAXgene RNA tubes are robust to long term freezing without significantly affecting stability of gene expression profiles [9]. PAXgene tubes can be left at room temperature for several hours after collection, but need to be stored frozen at −80 °C thereafter (*see* **Notes 2** and **3**).

4. Additional advantages of RNA stabilization using PAXgene tubes include the ability to collect samples from multiple centers and transportation to a central site before standardized RNA extraction and downstream procedures are performed (*see* **Note 4**).
5. Source of materials used in Subheading 3:
   PAXgene Blood RNA Kit (Qiagen, Germany); Globinclear (Life Technologies, NY, USA), TruSeq RNA sample preparation kit V1 (Illumina, CA, USA); software described is publically available except for those cited for pathway analysis: Ingenuity Pathway Analysis (Ingenuity® Systems, Redwood City, CA, USA)) and GeneGo MetaCore (Thomson Reuters, New York, NY, USA).

3 Methods

3.1 Methods for Interrogation of Gene Expression

Most global gene expression studies to date has been performed using microarrays, but the recently available RNAseq technique provides a wider dynamic range, increased specificity, ability to examine isoform usage [17], and is adaptable to all forms of RNA [18].

3.2 Preparation of RNA-seq Libraries

Total RNA isolated from PAXgene RNA blood tubes using the PAXgene Blood RNA Kit, can be treated with Globinclear, to deplete the whole blood sample of the dominant mRNAs, alpha and beta globin mRNA, to allow more sensitive measurement of other RNAs. Library kits such as the Illumina TruSeq RNA sample preparation kit are needed to tag RNAs with sequencing primers. Samples can be multiplexed according to depth of read coverage required, which can be calculated with the Lander/Waterman equation [19].

3.3 RNAseq Analysis

Raw sequence data must be aligned to the UCSC human reference genome using software packages such as Tophat [20]. Aligned sequencing reads must then be summarized to counts per gene or per transcript. One way of achieving this is to use the Read Assignment via Expectation Maximization (RAEM) procedure [21] and subsequently calculate reads per kilobase per million mapped reads (RPKM) values, which can be performed using the sAMMate software package [22]. RPKM values may then be transformed by normalization procedures such as quantile normalization [23]. Dysregulation of expression in RNA-seq data can be analyzed using free statistical packages such as EdgeR [24], DESeq [25], and Cufflinks [26] (*see* **Note 5**). Data processing and analysis pipelines for RNAseq continue to be developed and are further discussed by others elsewhere [27, 28].

3.4 Pathway Analysis

Genes do not work alone, but in an intricate network of interactions. Pathway analysis is the search for sets of genes differentially expressed between distinct phenotypes. This approach enables gene expression data to be interpreted in the context of biological processes and networks [29]. Differentially expressed genes may tag particular signalling pathways that underpin the biologically relevant differences between phenotypes. The list of pathway genes used in to test enrichment may be derived from various sources including publically available databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) [30] and the Gene Ontology Project [31], or from subscription based services which utilize both publically available interaction data and pathways curated in-house from published literature. Examples of subscription based pathway analysis programs are listed in Subheading 2, **item 5**.

3.5 Gene Set Enrichment Analysis

Similar to pathway analysis, Gene Set Enrichment Analysis (GSEA) is an analytical method for interpretation of gene expression data by focusing on sets of genes that share common biological function, chromosome location, or regulation [32]. The goal of GSEA is to determine whether members of a gene set tend to occur towards the top, or bottom of a list of genes ranked by correlation between two phenotypic groups (e.g., multiple sclerosis vs. healthy control). An advantage of GSEA is that it does not rely on a specific significance threshold for an initial statistical test between groups of interest. Instead, GSEA ranks all genes measured from most correlated to least, and then assesses where the genes contained in the gene set of interest lie within that list. GSEA can be performed on any set of genes, and is a useful tool for assessing the enrichment of a previously published gene list in your data.

3.6 Clustering Analysis

Cluster analysis, also known as unsupervised learning, refers to statistical and computational approaches where the analytical goal is to find clusters of samples, or clusters of genes, such that observations within a cluster are more similar to each other than they are to observations in different clusters [33]. The goal in cluster analysis is to utilize the data itself to identify informative or meaningful subgroups. There are multiple clustering approaches described in the literature including hierarchical clustering [34], *k*-means clustering [35], and self-sorting maps [36]. Hierarchical clustering is often utilized in the context of microarray analysis. Hierarchical clustering algorithms produce a clustering tree, or dendrogram from a pair-wise distance matrix. The algorithms begin with each gene as a separate cluster, and then search for genes that have the smallest distance between them and merge these genes into a cluster. The distance matrix is then recalculated to now include the distance between genes not clustered and the new cluster formed in the previous step. This process continues until the desired level of clusters is achieved. A difficulty with cluster analysis is that clusters are guaranteed to be produced, even if there are little or no differences in the distribution of the data, and there is no generally accepted statistical method to test a null hypothesis of no clusters.

3.7 Heat Maps

In microarray gene expression analysis, hierarchical clustering is sometimes used to generate graphics termed "heat maps," which are a plotted grid of colored points where the color represents a gene expression value in the sample [33]. In the heat maps, colors at a certain point are assigned to represent the level of expression for that gene (row) in a particular sample (column). The intensity of the color red is often used to designate high level of expression, and blue used to indicate low levels of expression. The hierarchical clustering can determine the order of the rows and the columns in the heat map, and the associated dendrogram is sometimes included in the figure (Fig. 2).

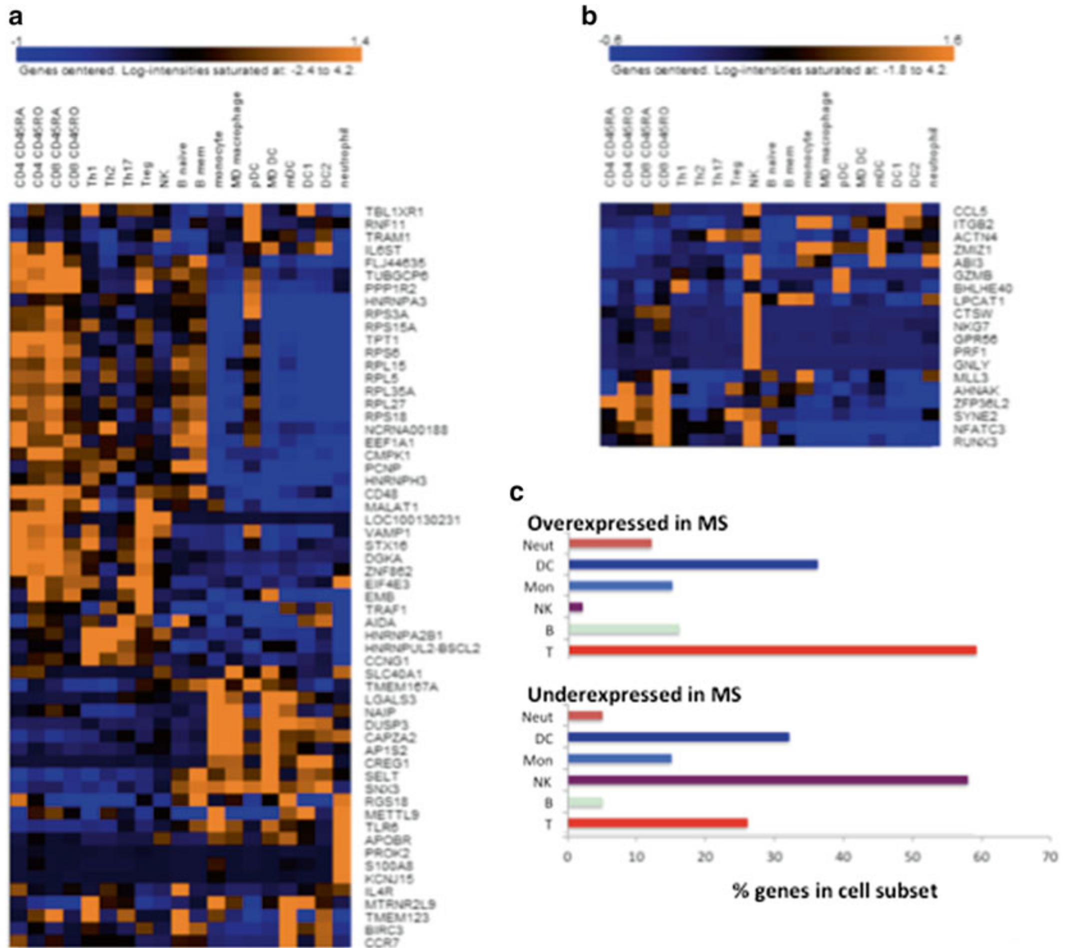

Fig. 2 Cell taggers are genes expressed predominantly in particular immune cell subsets. (**a**) This heat map depicts the cell subset expression of genes overexpressed in the whole blood of Multiple Sclerosis patients (MS); (**b**) Heat map of genes underexpressed in whole blood of MS patients; and (**c**). the percentage of dysregulated genes from each cell subset from the overexpressed and undexpressed gene sets

3.8 Deconvolution

A complex mixture of immune cell subsets all contribute to the overall gene expression profile observed when whole blood is assayed by microarray. It is often of interest to determine which cell subsets may be contributing to a particular gene signature. Several immune cell deconvolution methods have been developed to enable this [37, 38]. In addition, resources such as IRIS [39] and the ImmGen database [40] contain gene expression data for immune cell subsets that have been isolated. The genes of interest may be predominantly expressed in particular cell subsets, whose regulation is then implicated as driving the tested phenotypes. For example, this technique has been utilized to reveal that genes upregulated in

multiple sclerosis were predominately expressed in T lymphocytes [41]. Identification of particular immune cell subsets that are contributing to gene signatures for a particular condition may provide insights that can be followed up in subsequent experiments (Fig. 2, *see* **Note 6**).

4 Notes

1. Storage of cryopreserved PBMCs allows for their later culture for cell sorting by surface protein markers using flow cytometry. Particular marker/antibody combinations which work on fresh cells might not work on thawed cells. The transcriptome or target gene expression can be investigated in the sorted cell subsets to elucidate their role in generating investigated phenotypes. A particular advantage of this approach is that culture conditions can be manipulated to model disease/pharmacological effects in vitro.
2. Although the manufacturer has demonstrated expression of some genes is robust to storage of blood over several days at room temperature, we have found the quality of the mRNA, and the relative expression of labile genes such as MxA, are affected by such storage (Fig. 1). We routinely store PAXgene tubes at -80 °C within several hours of collection.
3. PAXgene tubes placed directly from room temperature to −80 °C tend to crack on thawing, and can be placed in to 50 ml falcon tubes to prevent loss of sample and contamination of lab surfaces. This cracking is reduced if samples are stored overnight at −30 °C before being placed in a −80 °C freezer.
4. Transcription of many RNA types is important in gene regulation. The cartridges used for standard RNA purification from PAXgene tubes do not capture the smaller RNA species, such as the miRNAs. These can be captured using alternative RNA purifications strategies, such as those using Trizol [42].
5. DNA sequencing platforms do not necessarily read all sequences with equal efficiency. For this reason, bias corrections such as those utilized for the Illumina HiSeq system may be beneficially employed [43].
6. Although the dysregulated genes may be predominantly produced in a particular cell subset or pathway, their role in other cell subsets or gene pathways may be critical to their pathogenic effect.

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Chapter 4

Diagnostic Procedures for Paraffin-Embedded Tissues Analysis in Pharmacogenomic Studies

Raffaele Palmirotta, Maria Laura De Marchis, Giorgia Ludovici, Patrizia Ferroni, Pasquale Abete, Fiorella Guadagni, and David Della-Morte

Abstract

In this book chapter we report our own experience of mutational analysis in selecting tailored anticancer treatments for solid tumors. Our Department of Advanced Biotechnologies and Bioimaging, IRCCS San Raffaele Pisana, Rome, Italy, routinely performs pharmacogenetic screenings for different genes such as K-ras, BRAF, KIT, PDGFRα, and EGFR on paraffin-embedded cancer sections. Therefore, the chapter describes the mutational analysis procedures on paraffin-embedded tumors aimed to predict individual response to anticancer therapy. These molecular diagnostic methodologies may help us in improving the translational impact of genetic information on clinical practice.

Key words Pharmacogenomic, Paraffin tumoral tissue, Diagnostic technique, Personalized therapy

1 Introduction

The biotechnological knowledge and expertise gained over the last decades have led us to the characterization of operational procedures in order to optimize the diagnostic approach for the identification of nucleic acid mutations relevant to personalized therapy [1]. The study of somatic molecular mutations, specific to certain tissues such as the neoplastic diseases, allowed not only the improvement of the molecular pathways interpretation but also the development of drugs whose mechanisms of action are closely related to genetic variants [2, 3]. Furthermore, the knowledge of sequence variants in the genes responsible for the effectiveness and toxicity of drugs permit to identify the appropriate and personalized medical therapy. This strategy is also important to define the most suitable dosage of used drugs, reducing or avoiding the risk of side effects or lack of effect which ultimately leads to a better outcome in patients with neoplastic diseases. In addition, this may

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also be relevant in cost-benefit analysis in applying genetic diagnostic tests. In fact, prediction of clinical response and potential drug-related toxicities is indispensable in managing the relevant costs of treating cancer and accounts for the great interest in studying somatic variants of specific genes.

In order to offer therapeutic indications for the clinical oncologist, in our laboratory we routinely perform pharmacogenetic screenings for different genes in different tissues: Kirsten Murine Sarcoma Virus 2 (K-ras) and V-Raf Murine Sarcoma Viral Oncogene Homolog B1 (BRAF) in metastatic colorectal cancer (mCRC), BRAF in melanoma, V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (C-KIT) and Platelet-Derived Growth Factor Receptor, Alpha (PDGFRα) in gastrointestinal stromal tumors (GISTs), and Epidermal Growth Factor Receptor (EGFR) in non-small-cell lung cancer (NSCLC) [1, 4–7].

This chapter illustrates the characterizations of technical procedures necessary in the course of these molecular evaluations, describing our laboratory experience and suggesting different diagnostic approaches: microdissection techniques, extraction of nucleic acids from paraffin-embedded tissues, polymerase chain reaction (PCR), direct sequencing, and allelic separation by cloning [4–7].

A better characterization of laboratory operational procedures in order to optimize the diagnostic approaches may allow a most accurate and sensitive identification of genetic markers likely associated with diseases. Moreover, it may consent a better definition of personalized therapy with obvious repercussions in patient outcomes with neoplastic diseases.

2 Materials

Prepare all solutions using molecular biology grade water. Prepare and store all reagents at room temperature (25 °C) (unless indicated otherwise).

2.1 Solutions

1. 0.5 M EDTA: 186 g of EDTA, 10 N NaOH, 1,000 ml water, pH 8. Add about 100 ml water to 1 l graduated cylinder or a glass beaker. Weigh 186 g of EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate, 99+%) and transfer to the cylinder. Add water to a volume of 900 ml. While stirring vigorously on a magnetic stirrer, add NaOH pellet or 10 N NaOH to adjust the solution pH 8.0. Mix and adjust pH with sodium hydroxide (*see* **Note 1**). Make up to 1 l with water.
2. 0.5 M Trizma Base: 60.57 g of Trizma Base, 1,000 ml water, 10 N NaOH, pH 8.5. Add about 100 ml water to 1 l graduated cylinder or a glass beaker (*see* **Note 2**). Weigh 60.57 g of

Trizma base and transfer to the cylinder. Add water to a volume of 900 ml. Mix and adjust pH with sodium hydroxide. Make up to 1 l with water.

3. Proteinase K: 20 mg/ml. Store at 4 °C. Add to digestion buffer only before use.
4. Digestion buffer: 0.5 M EDTA (pH 8), 0.5 M Tris base (pH 8.5), Tween 20, 1,000 ml water. Add 2 ml of a solution of EDTA 0.5 M (pH 8), 10 ml of a solution of Tris base 0.5 M (pH 8.5), and 5 ml of Tween 20 (Sigma Aldrich, St Louis, MO, USA) to a 1 l graduated cylinder or a glass beaker. Make up to 1 l with water.
5. Xylene.
6. Ethanol.
7. Saturated Salt Solution. Weigh out 357 g of NaCl. Add the salt to a 1 l graduated cylinder. Add water to the graduation line and stir until dissolved.
8. QIAamp DNA Blood Mini Kit (Qiagen). The features of the kit are available on the Web site: http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/Genomic-DNA/QIAamp-DNA-Blood-Mini-Kit. At the time of first use add the appropriate amount of ethanol (96–100 %) as indicated on the bottles of buffer AW1 and buffer AW2 (see the kit datasheet for more detailed information). Reconstitute Protease K in the storage buffer (Protease Solvent) provided by the kit. Store Protease stock solution at 2–8 °C for 2 months or at –20 °C for longer storage time (*see* **Note 3**).
9. Primers. Particular attention is paid to the design of the primers, a feature which is essential both for the PCR reaction and direct sequencing analysis. All oligonucleotides are designed in such a manner as to include coding regions and the flanking intron–exon junctions, in order to avoid PCR products more than 300 bp large and to minimize primer–primer interactions (Table 1).

   Each primer must be reconstituted in order to have a concentration of 100 pmol/μl. Calculate the value in picomoles multiplying the value in nanomoles (reported on the datasheet) by 1,000:

   $$\text{pmoles} = \text{nmoles} \times 1{,}000.$$

   Apply the following proportion to calculate the volume of RNase–DNase-free H_2O to add to the primer:

   $$100 \text{ pmol}: 1\ \mu\text{l} = \text{pmol}: x.$$

   Spin down the tube containing the lyophilized primers in order to avoid the dispersion at the time of opening. Add RNase–DNase-free water. Equilibrate at room temperature for 5–10 min before use (*see* **Note 4**).

Table 1
PCR primers, product size, and reaction conditions for amplification and direct sequencing of KRAS, BRAF, EGFR, PDGFRα, and CKIT genes

Gene	Exon	Primer	Sequence (5′–3′)	TA (°C)	μM primers	PCR cycles	bp DNA
KRAS	1	KRAS-1-exF	GTACTGGTGGAGTATTTGATAGTG	55	0.5	40	278
		KRAS-1-exR	GGTCAGAGAAACCTTTATCTGTATC				
		KRAS-1-exF[a]	GTACTGGTGGAGTATTTGATAGTG	54	1	25	174
		KRAS-1-intR[a,b]	GTCCTGCACCAGTAATATGC				
		KRAS-1-seqF[b]	TTTTTATTATAAGGCCTGCT				
	2	KRAS-2 exF	AGGTGCACTGTAATAATCCAG	52	0.5	40	309
		KRAS-2 exR	ATTATATGCATGGCATTAGC				
		KRAS-2 exF[a,b]	AGGTGCACTGTAATAATCCAG	55	1	25	256
		KRAS-2 intR[a,b]	AACTATAATTACTCCTTAATGTCAGC				
BRAF	11	BRAF EX 11 F	TCCTGTTTGGCTTGACTTGACTT	55	1	32	193
		BRAF EX 11 R	ACTTGTCACAATGTCACCACATT				
	15	BRAF EX 15 F	TCATAATGCTTGTTGCTCTGATAGGA	55	1	32	224
		BRAF EX 15 R	GGCCAAAAATTTAATCAGTGGA				
EGFR	18	EGFR EX18 F1	AGCATGGTGAGGGCTGAGG	60	0.5	40	233
		EGFR EX18 R1	CCACCAGACCATGAGAGG				
	19	EGFR EX19 F	AATTGCCAGTTAACGTCTTCC	57	0.5	40	242
		EGFR EX19 R	ATGTGGAGATGAGCAGGG				
	20	EGFR EX20 F1	CATTCATGCGTCTTCACC	55	0.5	40	332
		EGFR EX20 R1	ATGTGAGGATCCTGGCTCC				
	21	EGFR EX21 FF	TCGGATGCAGAGCTTCTTCC	59	0.5	40	247
		EGFR EX21 RR	TGCTGGCTGACCTAAAGCC				

PDGFRα	12	PDGFRA EX12 F	TCCAGTCACTGTGCTGCTTC	60	0.5	40	274
		PDGFRA EX12 R	TTGTAAAGTTGTGTGCAAGGG				
	18	PDGFRA EX18 F	TACAGATGGCTTGATCCTGAGTC	60	1	40	223
		PDGFRA EX18 R	TTAGAGAGTAAAGTGTGGGAGGATG				
CKIT	9	c-KIT EX 9 F	TTCCTAGAGTAAGCCAGGG	53	0.5	40	297
		c-KIT EX 9 R	TCATGACTGATATGGTAGACAG				
	11	c-KIT EX 11 F	TCCAGAGTGCTCTAATGACTG	58	0.2	40	276
		c-KIT EX 11 R	AGGAAGCCACTGGAGTTCC				
	13	c-KIT EX 13 F	ATCAGTTTGCCAGTTGTGCT	58	1	40	168
		c-KIT EX 13 R	GCTTTACCTCCAATGGTGC				
	17	c-KIT EX 17 F	TGTGAACATCATTCAAGGCGTAC	60	0.2	40	331
		c-KIT EX 17 R	CAGGACTGTCAAGCAGAGAATGG				

Exons 1 and 2 of KRAS are individually amplified using a semi-nested amplification protocol
TA PCR annealing temperature, *bp DNA* molecular length of PCR amplified products expressed in base pair
[a]Primers used for semi-nested PCR
[b]Primers used for the sequencing reactions

10. PCR amplification. HotStarTaq Master Mix Kit (Qiagen). Store the kit at −20 °C. The features of the kit are available on the Web site: (http://www.qiagen.com/Products/Catalog/Assay-Technologies/End-Point-PCR-and-RT-PCR-Reagents/HotStarTaq-Master-Mix-Kit).

2.2 Agarose Gel

1. Agarose: SeaKem® LE Agarose (Lonza), molecular biology grade agarose without DNase or RNase activity. The features of the product are available on the Web site: http://www.lonza.com/.
2. 0.5 M EDTA pH 8: *see* Subheading 2.1 and **Note 1**.
3. TBE 10×: 890 mM Trizma base, 890 mM Boric Acid, 0.5 M EDTA (pH 8). Add about 300 ml water to a 1-l graduated cylinder. Weigh 108 g of Trizma base and 55 g of Boric Acid and transfer to the cylinder. Add 40 ml of a solution of 0.5 M EDTA (pH 8). Make up to 1 l with distilled water (*see* **Note 5**). Alternatively, and depending on availability of the laboratory, you can use the buffer ready for use UltraPure™ 10× TBE Buffer (Life Technologies).
4. Ethidium bromide. All operations involving ethidium bromide should be carried out in a chemical fume hood. Ethidium bromide is a powerful mutagen.
5. Loading dye: 10× BlueJuice™ Gel Loading Buffer (Life Technologies).
6. Molecular weight marker: TrackIt™ 100 bp DNA Ladder (Life Technologies).

The percentage of agarose gel to use according to the size of the PCR products reported in this chapter is 2 %. An example table, which contains different amounts of gel according to the size of the electrophoretic platform, is shown below:

Gel volume (ml)	50	70	100	150	200	250
Agarose (2 %) (g)	1	1.4	2	3	4	5
TBE buffer 1× (ml)	50	70	100	150	200	250
Ethidium bromide (μl)	10	14	20	30	40	50

For the preparation of agarose gel proceed according to the following steps:

- Weigh out the required quantity of agarose (e.g., 1 g per 100 ml = 1 % gel). Place it into a glass flask or a glass beaker.
- Add the appropriate quantity of buffer TBE 1×.
- Microwave just until you start to see the appearance of boiling and remove the flask. Carefully, swirl the agarose mixture.

- Return the flask to the microwave and repeat until no residue of agarose is left.
- Add ethidium bromide to the liquid gel (1–5 μl of a 10 mg/ml stock solution per 100 ml of agarose gel) (*see* **Notes 6** and 7).
- Assemble the gel box and combs. Expect the gel to reach the temperature of 50–60 °C before pouring it in the box.

2.3 PCR Purification

1. ExoStar™ 1-Step (GE Healthcare). Store the kit at −20 °C. The features of the kit are available on the Web site: http://193.218.17.133/ex/downloads/brochures/life_science/ge_illustra_exostar.pdf.
2. QIAquick PCR Purification Kit (Qiagen) The features of the kit are available on the Web site: http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/DNA-Cleanup/QIAquick-PCR-Purification-Kit.

2.4 Direct Sequencing

1. BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Store the kit at −20 °C. The features of the kit are available on the Web site: http://tools.invitrogen.com/content/sfs/brochures/cms_081527.pdf.
2. BigDye® Terminator v1.1 and v3.1 5× Sequencing Buffer (Life Technologies) Store at 4 °C.
3. Hi-Di™ Formamide (Life Technologies). Store at −20 °C (*see* **Note 8**).

2.5 Sequencing Reaction Purification

1. DyeEx 2.0 Spin Kit. The features of the kit are available on the Web site: http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/DNA-Cleanup/DyeEx-20-Spin-Kit.

2.6 Cloning

1. Cloning kit: TOPO® TA Cloning® Kit for Sequencing, with One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies). Store at −20 °C and −80 °C, respectively. The features of the kit are available on the Web site: http://products.invitrogen.com/ivgn/product/K4575J10.

2.7 LB Plates

Add 20 ml of distilled water to the 200 mg ampicillin vial to reach a final concentration of 10 mg/ml. After reconstitution store for 2 weeks at 4 °C and for longer periods at −20 °C. Add 32 g di LB agar in 1 l of distilled and sterilize by autoclaving for 15 min at 121 °C.

Wait until the agar reaches a temperature of about 50 °C, then add 5 ml of ampicillin previously reconstituted (final concentration 50 μg/ml). Distribute the agar in petri dishes (20 ml of liquid medium for each petri dish). Culture plates with ampicillin can be stored at 2–8 °C for up to 2 weeks.

2.8 Instrumentation

1. Spectrophotometer (NanoDrop 2000c UV-Vis Spectrophotometer, Thermo Scientific).
2. Microcentrifuge (Heraeus Biofuge Pico, DJB Labcare).
3. Agitator with turntable (Inter Continental).
4. Chemical hood (SUPER-CHEMO, International PBI).
5. Magnetic stirrer (ARBO, International PBI).
6. Autoclave (Steristeam, CDL).
7. Thermoblock (THERMOBLOC, International PBI).
8. Incubator (Galaxy S, RS Biotech).
9. Centrifugal vacuum concentrator (SAVANT SPD 111 V SpeedVac Concentrator, Thermo Electron Corporation).
10. Thermocycler (Ab Veriti Thermal Cycler, Life Technologies).
11. Electrophoretic apparatus (Sub-Cell GT Agarose Gel Electrophoresis Systems, Bio-Rad) and power supply (PowerPac Basic Power Supply, Bio-Rad).
12. Gel imager (Gel Doc XR, Bio-Rad) and image acquisition and analysis software (Quantity One 1-D Analysis Software, Bio-Rad).
13. Automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Life Technologies) and DNA sequencing analysis software (AB DNA Sequencing Analysis Software v5.2, Life Technologies).
14. PC with sequence alignment software (*see* Subheading 3).

3 Methods

3.1 Paraffin Slide Microdissection

Each paraffin-embedded section is collected on microscope slides and first examined under a microscope (10×) to ensure that it contained sufficient tumor material and to eliminate possible contaminating normal tissues. Tumor and tumor-free areas are identified within 15 μm-thick deparaffinized sections lightly counterstained with hematoxylin and microdissected by gentle scraping with sterile scalpels into 1.5 ml polypropylene vials, using a hematoxylin and eosin-stained step section from the same block [4–7].

3.2 DNA Extraction and Purification

The DNA extraction method is an in-house developed protocol. Microdissected tumor area are dipped into xylene to remove paraffin, rehydrated in a series of ethanol, and incubated in a proteinase K digestion buffer. Two different DNA purification methods are used. The first method is a NaCl saturated solution precipitation, as previously described. In the second method we use reagents and materials provided by the QIAamp DNA Blood

Mini Kit. For more detailed information, refer to the Web site (www.qiagen.com).

1. Place a paraffin-embedded microdissected area in a 2 ml eppendorf (*see* **Note 9**).
2. Add 1 ml of xylene, mix at room temperature (15–25 °C, R.T.) overnight on an agitator with turntable (*see* **Note 10**).
3. Centrifuge for 30 min at R.T. to form the pellets (about 10,000 × *g* in microcentrifuge).
4. Remove supernatant (careful don't drag away the pellets).
5. Add to pellet 500 μl of 100 % ETOH, mix at R.T. for 30 min (*see* **Note 10**).
6. Centrifuge at 4 °C for 30 min at 10,000 × *g*.
7. Remove carefully the supernatant avoiding to drag away the pellet.
8. Add to pellet 500 μl of 70 % ETOH, mix at R.T. for 30 min.
9. Centrifuge at 4 °C for 30 min at 10,000 × *g*.
10. Remove the supernatant.
11. Air-dry the pellet in Savant or under the hood (the pellet should be completely dry and deprived of paraffin to permit enzymatic digestion).
12. Add 100–200 μl (containing 200 μg/ml of proteinase K) of digestion buffer, based on the amount of material present in the pellet.
13. Incubate at 45 °C overnight.
14. Centrifuge for 10 s (to remove the condensation under the cap).
15. Incubate at 95 °C for 10 min (*see* **Note 11**).
16. Spin down the samples.
17. Recover the supernatant and store it at −20 °C possibly aliquoting according to future needs.

3.2.1 Purification by High Salt Precipitation

1. Take an aliquot of DNA and add an amount of saturated NaCl solution equal to one third of the volume of the sample.
2. Place the sample on ice for 20 min.
3. Centrifuge at 16,000 × *g* for 30 min at 4 °C.
4. Recover the supernatant.
5. Precipitate the supernatant with an amount of 100 % EtOH equal to two volumes at R.T.
6. Centrifuge at 16,000 × *g* for 15 min at R.T.
7. Discard the supernatant.
8. Dissolve the pellet in 50 μl of DNase-free water.

3.2.2 Purification by QIAamp DNA Blood Mini Kit

1. Take an aliquot (100–200 μl) of each sample in 1.5–2 ml tubes.
2. Add 200 μl of ethanol (96–100 %) (*see* **Note 10**), vortex for 15 s and spin down.
3. Assembling the number of QIAamp columns necessary with related collecting tubes and identify with the laboratory code.
4. Load the mixture (about 650 μl) in a QIAamp columns assembled into the collection tubes.
5. Centrifuge at 6,000 × *g* for 1 min.
6. Put the columns into new collection tubes.
7. Load 500 μl of Buffer AW1 in the columns.
8. Centrifuge at 6,000 × *g* for 1 min.
9. Empty tubes (in a flask containing bleach) and reuse them for the next step.
10. Load 500 μl of Buffer AW2 in the columns.
11. Centrifuge at 20,000 × *g* for 3 min.
12. Place the columns in new tubes and centrifuge at 20,000 × *g* for 1 min.
13. Place the columns into new collection tubes.
14. Load 100 μl of Buffer AE in columns.
15. Incubate at room temperature for 1 min.
16. Centrifuge at 6,000 × *g* for 1 min.
17. Transfer the eluted DNA (100 μl) from collection tubes to 1.5–2 ml tubes and store at −20 °C.

3.3 Spectrophotometric DNA Assay

Concentration and quality of DNA is assessed spectrophotometrically [8, 9]. The features of the instrument NanoDrop 2000c are available on the Web site: http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf.

1. The amount of total DNA is quantified by measuring spectrophotometric optical density (OD) at 260 and reported as ng/ml, considering that one absorbance unit corresponds to 50 ng/ml (*see* **Note 12**).
2. Based on the concentration measurement, dilute the sample in such a way as to obtain a final concentration equal to 30 ng/μl.

3.4 PCR DNA Amplification

Be careful. DNA extraction and setup of PCR reactions are performed in a dedicated a laboratory distinct from that in which PCR reactions are performed and amplified DNAs are manipulate [10].

1. Prepare in a 1.5–2 ml tube a reaction mixture containing the necessary reagents for the amplification reaction (Primer forward and reverse and DNase-free water) except the DNA. To calculate the amount of the reactions programmed, multiply

the quantity by the number of samples to be analyzed and include a positive and a negative control (*see* **Note 13**). Moreover, to avoid pipetting error add in the calculation one reaction volume extra (*see* table below).

2. Spin down in mini-centrifuge to collect contents in bottom of tube.
3. Aliquot the Master Mix in 200 μl PCR reaction tubes.
4. Add 1.5 μl of DNA in all tubes as follows:

	1 sample	12 sample
HSTaq Master Mix	15 μl	225 μl
Primer F 100 μM (1 μM)	0.3 μl	4.5 μl
Primer R 100 μM (1 μM)	0.3 μl	4.5 μl
DNase-free H_2O	12.9 μl	193.5 μl
Total volume of reaction mixture	28.5 μl	342 μl
DNA template	1.5 μl	

5. Introduce an equal amount of distilled water in the negative amplification control tube.
6. Add 1.5 μl of control DNA in the positive amplification control tube.
7. Close the reaction tubes and place in the thermocycler using the previously created PCR program:
   - An initial denaturing step of 95 °C for 15 min
   - 35 cycles of:
     – 95 °C for 30 s
     – 54–60 °C for 30 s of annealing temperature (for specific annealing temperature *see* Table 1)
     – 72 °C for 30 s
   - A final extension at 72 °C for 10 min.
   - A storage step at 4 °C.

3.5 Agarose Gel Electrophoresis

1. Place the tray with agarose gel in the electrophoresis chamber, remove the comb being careful not to damage the wells, and cover with TBE buffer 1× until the wells are submerged (*see* **Note 7**).
2. Samples preparation:
   (a) Prepare a number of tubes equal to the number of samples to be examined.
   (b) Pipette about 2 μl of loading dye in 0.5 ml tubes.

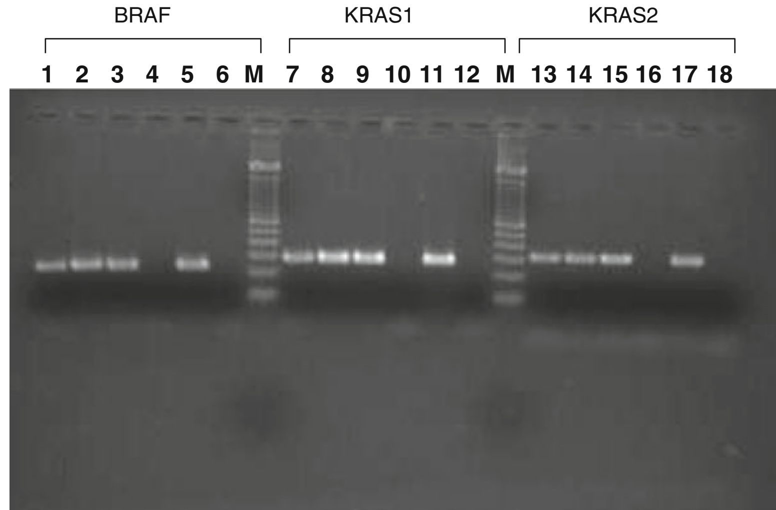

Fig. 1 PCR amplification of BRAF, Kras exon 1 and Kras exon2. *Lanes 1–3*, *7–9*, and *13–15*: amplifications product of BRAF exon 15 (224 bp), Kras exon 1 (278 bp) and Kras exon 2 (309 bp) of metastatic colorectal cancer samples. *Lanes 4*, *10*, and *16*: negative controls of amplification (PCR reagents and primers included without DNA). *Lanes 5*, *11*, and *17*: positive controls (with a Control DNA). *Lanes 6*, *12*, and *18* negative control of extraction (PCR reagents and primers in a negative control of extraction): *Lane M*: Molecular weight marker: TrackIt™ 100 bp DNA Ladder (Life Technologies)

(c) Add 7 μl of amplification product and mix.

(d) Carefully load the DNA samples onto the gel.

(e) Load 5 μl of the molecular size marker in the first well of the gel.

(f) Load the negative and positive controls.

3. Run at 120 V until dye markers have reached an appropriate distance, depending on the size of DNA to be visualized.
4. Place the gel into the imager Gel Doc XR Systems (Bio-Rad), evaluate the relative intensity of the DNA amplification product, and acquire the image using Quantity One 4.6.0 software (Fig. 1).

3.6 PCR Products Clean-Up for Sequencing

Two different commercial PCR purification methods are used. The ExoStar (GE Healthcare) method is a one-step enzymatic cleanup of PCR products that eliminates unincorporated primers and dNTPs. The QIAquick PCR Purification Kit (Qiagen) silica-membrane-based purification of PCR products (*see* **Note 14**).

3.6.1 PCR Products Purification by ExoStar (GE Healthcare)

1. Prepare and identify with a progressive number a number 1.5 ml vials equal to the number of samples to be purified.
2. Add to each tube 2 μl of ExoStar and 5 μl of amplified PCR product sample.
3. Place tubes in the thermal cycler using the previously created PCR program:
   - Activation of the enzyme at 37 °C for 15 min.
   - Inactivation of the enzyme at 80 °C for 15 min.
4. After the purification procedure the samples are subjected to the reaction sequence directly or stored at −20 °C.

3.6.2 PCR Products Purification by QIAquick PCR Purification Kit (Qiagen)

1. Prepare and identify with a progressive number so many 1.5 ml vials as there are samples to be purified.
2. Add 5 volumes of Buffer PB to 1 volume of the PCR amplification product and mix (i.e., 100 μl of Buffer PB to 20 μl of amplified DNA).
3. Place the QIAquick spin columns, identified by the code of each sample, in the provided 2 ml collection tubes.
4. Add the mix of Buffer PB and DNA to the QIAquick column and centrifuge for 60 s at 16,000 × *g*.
5. Discharge the contents of the collection tubes and reuse them for the next step.
6. Add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 60 s at 16,000 × *g*.
7. Discharge the contents of the collection tubes and reuse them for the next step.
8. Centrifuge for 60 s at 16,000 × *g*.
9. Place the QIAquick spin column in a new 1.5 ml tube.
10. Add to each spin column 50 μl of Buffer EB, after 1 min centrifuge for 60 s at 16,000 × *g*.
11. Store the eluate at 4 °C or at −20 °C.

3.7 Direct Sequencing Analysis

3.7.1 Sequencing Reaction by BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies)

1. Dilute the primer chosen for the sequence to obtain a concentration of 3.2 μM (*see* **Notes 15** and **16**).
2. Prepare and number the necessary 0.2 ml PCR tubes by the code of each sample.
3. For DNA template evaluate PCR gel and determine how much DNA is sufficient for the reaction sequence. Based on our experience the amount of DNA to be taken varies from 1 μl (band of medium intensity comparable to the ladder) to 3 μl (very weak band intensity).
4. Prepare the reaction mix sequence. To calculate the amount of the reactions programmed, multiply the quantity by the number

of samples to be analyzed and include the positive control pGEM. Moreover, to avoid pipetting error, add in the calculation one reaction volume extra.

5. Set up reaction as follows:

	1 sample	12 sample	pGEM
RR100 (Big-Dye Enzyme Mix)	4 μl	52 μl	4 μl
5× Buffer	2 μl	26 μl	2 μl
Primer 3.2 pmol	1 μl	13 μl	4 μl
DNA template	2 μl		2 μl
DNase-free H_2O	11 μl	143 μl	8 μl
Total volume of reaction mixture	20 μl	234 μl	20 μl

6. Run on PCR machine using the previously created PCR program:
   - An initial denaturing step of 95 °C for 1 min
   - 25 cycles of:
     - 95 °C for 10 s
     - 50 °C for 5 s
     - 60 °C for 4 min
   - A storage step at 4 °C.
7. If you do not perform immediately post-purification sequence, you can store samples at −20 °C.

3.7.2 Sequence Reaction Products Purification by DYE EX SPIN KIT (Qiagen)

1. Prepare and identify with a progressive number a number of spin column equal to the number of samples to be purified. Vortex the spin column to resuspend the resin.
2. Slightly open the cap to prevent the formation of vacuum inside the columns.
3. Open the bottom closure and place each column in the respective 2 ml collection tube (supplied by the kit).
4. Centrifuge for 3 min a 2,800 rpm.
5. Prepare a number of tubes of 1.5–2 ml (not supplied by the kit) with the date of execution and the sequence code number.
6. Gently transfer the column containing the gel (resin) in the 1.5/2 ml tubes prepared in the previous step.
7. Transfer the reaction sequence (20 μl) at the center of the resin surface avoiding to break with the tip; pipette slowly to the center of the gel so as to adsorb the mixture in the gel and avoiding to slide laterally in the column.

8. Centrifuge for 3 min at 2,800 rpm (*see* **Note 17**).
9. Remove the column. The tube now contains the purified DNA, which can also be stored at +4 or −20 °C.

3.7.3 Sequence Electrophoresis by ABI Prism 3130 (Life Technologies)

1. Dilute 5 μl of each purified DNA sample in 15 μl of formamide in the appropriate 3130 ABI sequencer 96-well plates. This mixture can be stored at +4 °C for 1 week or for 3 days at room temperature (Formamide).
2. Spin down.
3. Heat the samples for 2 min at 95 °C and put immediately on ice.
4. Run on the sequencer instrument (*see* **Note 18**).
5. After the electrophoretic run, recover the ABI files of the electropherograms corresponding to the results of sequencing reactions.

3.8 Interpretation of Sequence Electropherograms

1. Sequence analysis results will be delivered as .ABI files, containing the electropherogram, nucleotide sequence and other information that has been recorded by DNA analysis instrument during run conditions. The files with the .ABI extensions can be displayed as graphics files by using .ABI viewer programs running on Windows, Macintosh, and Linux operating systems (http://www.ehow.com/facts_6568843_file-extension-abi_.html#ixzz2Y5OGjOat). Table 2 lists the main software packages used to interpret the electropherograms in ABI format and the Web sites where it is possible to download the tool in demo, shareware, or freeware format.
2. Particular attention should be dedicated to the interpretation of electropherograms and the subsequent indication of mutations at the DNA and protein level by a description following the guidelines for sequence variation of the Human Genome Variation Society (HGVS; http://www.hgvs.org/mutnomen) [11, 12] (*see* **Note 19**).
3. The reference sequences used for our pharmacogenetic studies are as follows: KRAS (NM_004985 and M54968), EGFR (NM_005228.3.), CKIT (NM_000222.2), PDGFRA (NM_006206), and BRAF (NM_004333) mRNA sequences from GeneBank (http://www.ncbi.nlm.nih.gov). To compare all detected sequence variants we previously identified the most widely used mutations Internet databases listed in Table 3.

3.9 DNA Cloning by Topo TA Cloning Kit for Sequencing (Invitrogen)

In some cases it may happen that, for the identified mutation peculiarities, the electropherogram results will be ambiguous or difficult to follow for the polymorphic position as it might be a mixture of two variants processed simultaneously.

Table 2
Software packages used to interpret the electropherograms in .ABI format and the Web sites where it is possible to download the tool in demo, shareware, or freeware format

Software	Company	Web site
4Peaks	Nucleobytes.com	http://nucleobytes.com/index.php/4peaks
FinchTV	Geospiza	http://www.geospiza.com/Products/finchtv.shtml
CodonCode Aligner	CodonCode Corporation	http://www.codoncode.com/aligner/
Chromas	Technelysium	http://technelysium.com.au/?page_id=13
BioEdit	*Ibis Biosciences*	http://www.mbio.ncsu.edu/BioEdit/bioedit.html
TraceViewer	CodonCode Corporation	http://www.codoncode.com/TraceViewer/
Sequence Scanner	Applied Biosystems	http://www.appliedbiosystems.com/absite/us/en/home/support/software-community/free-ab-software.html
Lasergene	DNASTAR	http://www.dnastar.com/
Sequencer	Gene Codes Corporation	http://genecodes.com/
MacVector	MacVector	http://macvector.com/downloads.html

Table 3
Web databases used for comparing sequence variants obtained by mutational analysis CKit, PDGFRA, KRAS, BRAF, and EGFR genes

Gene name (OMIM code)	Web databases
KIT (*164920)	http://www.genomed.org/LOVD/CM/home.php?select_db=KIT http://www.hgmd.cf.ac.uk/ac/gene.php?gene=KIT http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=gene&ln=KIT
PDGFRA (*173490)	http://www.genomed.org/LOVD/CM/home.php?select_db=PDGFRA http://www.hgmd.cf.ac.uk/ac/gene.php?gene=PDGFRA http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=bygene&ln=PDGFRA
KRAS (*190070)	http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=bygene&ln=KRAS http://www.hgmd.cf.ac.uk/ac/gene.php?gene=KRAS
EGFR (*131550)	http://somaticmutations-egfr.org/index.html https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php?select_db=EGFR http://cancer.sanger.ac.uk/cosmic/search?q=egfr&domain=cosmic
BRAF (*164757)	http://www.hgmd.cf.ac.uk/ac/gene.php?gene=BRAF

OMIM Online Mendelian Inheritance in Man (http://www.omim.org/)

This issue may be related to the intrinsic nature of particular nucleotide variants such as mutations on different alleles [5, 6], presence of different cell clones and/or complex mutations [7].

In order to optimize the detection and the mutation reports, allelic separation by cloning PCR product allows separation of the two molecular variants of first sequencing and may represent an important step able to increase power of pharmacogenetic studies.

3.9.1 Topo Cloning Reaction by TOPO TA Cloning® Kit (Invitrogen)

1. Prepare and number by the code of each sample the necessary 0.2 ml vials.
2. Add to each tube
   - 1 µl of PCR amplification product
   - 1 µl of salt solution (provided by Kit)
   - 1 µl of Topo vector (provided by Kit)
   - 3 µl of H_2O (provided by Kit).
3. Mix gently and incubate for 5 min at room temperature (22–23 °C).
4. Place the vials in ice and proceed to the transformation (this cloning reaction can be left overnight at −20 °C).

3.9.2 Rapid One Shot Chemical Transformation Protocol

1. Prepare a plate and a vial of One Shot competent cells (stored at −80 °C) for each sample.
2. Preheat the plates using the stove at 37 °C for about 30 min. Preheat the thermostat at 42 °C.
3. Just before performing the transformation lay the vials of competent cells first at 20 °C and then at room temperature.
4. Add 4 µl of Topo Cloning in a reaction vials of One Shot (included in the kit) and mix gently without pipette.
5. Incubate on ice for 5 min.
6. Place the cells in the thermostat at 42 °C for 30 s.
7. Incubate on ice for 2 min.
8. Spread 50 µl of cells on the 37 °C preheated plate and incubate in oven overnight at 37 °C.

3.9.3 Analyzing Positive Clones

1. Withdraw all colonies eventually grew on the ground (Fig. 2) using a sterile loop and resuspend each colony in 200 µl of sterile saline or PBS (*see* **Note 20**).
2. Proceed with the extraction of the nucleic acid using QIAamp DNA Blood Mini (Subheading 3.2.2).
3. Proceed with the next steps until sequence analysis.

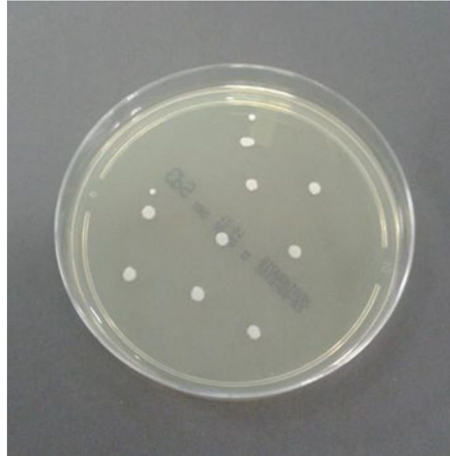

Fig. 2 An LB agar plate showing the result of a Topo Ta Cloning assay for allelic cloning separation

4 Notes

1. It is not easy to dissolve the EDTA. To dissolve the EDTA completely, solution pH 8.0 is required. All operations involving sodium hydroxide should be carried out in a chemical fume hood.
2. When using a glass beaker, the water can be heated to 37 °C to facilitate the dissolution of Tris. In this case, since pH is dependent on temperature, you should wait until the solution is cooled to room temperature before adjusting the pH.
3. Storage of aliquots of QIAGEN Protease is recommended.
4. Primers may be stored at 2–8 °C. Since too many cycles of freezing–thawing could damage the primers, for longer storage time aliquoting at −20 °C is recommended.
5. Precipitation of TBE 10× is likely to occur especially with colder temperatures. We noticed that autoclaving it for 20 min at 121 °C could solve the problem.
6. Ethidium bromide can be added to liquid gel cooled to 50–65 °C under a chemical hood.
7. Use extreme caution and work in a fume cupboard when handling ethidium bromide and agarose gel electrophoresis after adding ethidium bromide.
8. All operations involving formamide should be carried out under a chemical fume hood.

9. It may be necessary to increase the number of slices, while in case of large inclusions from surgical specimens may be necessary to use only part of the slice. In any case, the excess material can affect the amplification reaction.
10. Xylene and ethanol are flammable. Conduct experiments in a chemical fume hood avoiding the presence of potential sources of ignition (i.e., electrical equipment or flames) and other flammable solvents. Use the mandatory personal protective equipment.
11. This step is extremely important because the high temperature inactivates the proteinase K, which otherwise would inhibit the amplification reaction.
12. The ratio of absorbance at 260 and 280 nm are used to assess protein contamination. A DNA sample is pure if measurement provides a ratio of about 1.8. The ratio of absorbance at 260 and 230 nm are calculated to assess possible reagents contamination, with an optimal range from 2.0 to 2.2. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol, or other contaminants [8, 9, 13].
13. Exons 1 and 2 of *KRAS* are individually amplified using a semi-nested amplification protocol (*see* Table 1). The secondary internal PCR reactions are performed using the first PCR product as a template (dilution 1:1,000) [4, 5].
14. The purification of the PCR products is necessary to remove the residue of the reaction by the PCR amplified DNA, such as dNTPs, primers, Taq, and Mg, which interfere with direct DNA sequencing.
15. DNA sequencing is performed in both the forward and reverse directions so that the complementary strands can be compared to one another for quality control purposes.
16. Use the same primers in the PCR and sequencing reactions unless otherwise specified.
17. In this step, you must be careful to relocate in the centrifuge the column in the same direction of the first centrifuged, in order to avoid unpack the resin that in the first centrifugation was prepared according to the angle of the rotor, and thereby undermine the result of purification.
18. All sequencing instrument represent complex systems that requires considerable manual handling by the operator [14, 15]. Its use requires considerable experience both of the principles of operation that the various operational phases. ABI 3130 Genetic Analyzer (Applied Biosystems) is a four capillary electrophoresis system that uses fluorescently labeled dyes for detection of DNA (http://products.invitrogen.com/ivgn/product/313001).

The main operational steps are available consultation of the manual of the instrument (Applied Biosystems 3130 Genetic Analyzer Getting Started Guide—http://www.baylor.edu/content/services/document.php/186486.pdf). In addition to the operator in the vicinity of the instrument is necessary that there is a "Quick Reference Card" provided by the manufacturer and a summary diagram of the Standard Operative Procedures SOP provided by the laboratory.

19. Several mutations, especially those in homozygosis, may go undetected in a preliminary analysis of the electropherograms. It is therefore recommended to use an alignment program such as BLAST (basic local alignment search tool; http://blast.ncbi.nlm. nih.gov/Blast.cgi) to detect these kind of variants.
20. For the long-term storage of bacteria inoculate the colony in 1–2 ml of LB broth and ampicillin (100 mg/ml), place in culture overnight at 37 °C until saturation. Mix gently 85 μl culture medium with 150 μl of glycerol and store at −80 °C. The bacteria in this way can be preserved for long periods of time and can be possibly reseeded in liquid medium to perform the purification of the plasmids with commercial kits.

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Chapter 5

Approach to Clinical and Genetic Characterization of Statin-Induced Myopathy

QiPing Feng

Abstract

HMG CoA reductase inhibitors (statins) are among the most commonly prescribed medications in the industrialized world. They are generally regarded as safe. Mild myalgias can occur in up to 10 % of patients exposed to statins, but skeletal muscle damage (accompanied by an increase in circulating creatine kinase levels) occurs much less frequently. Clinical predictors of statin-induced rhabdomyolysis (severe muscle damage with end organ failure) include female gender, advanced age, and concomitant medications known to interact with critical pharmacokinetic and pharmacodynamic processes. The influence of genetic variations has been investigated by candidate gene association studies, genome-wide association studies, and whole-genome sequencing. This chapter summarizes current available approaches to clinical and genetic characterization of statin-related adverse effect.

Key words Genetic, Genomic, Myopathy, Rhabdomyolysis, Statin

1 Introduction

The rate-limiting enzyme in cholesterol biosynthesis is HMG Coenzyme A reductase (HMGCR) (Fig. 1) [1, 2]. Selective HMGCR inhibitors (statins) attenuate the onset and progression of cardiovascular disease in patients at risk [3–6]. In primary prevention trials, statin use can reduce the risk of first major coronary event by ~30 % [5, 7]. Secondary prevention trials reveal a risk reduction of similar magnitude [4, 8, 9], and aggressive interventions (higher doses and/or greater lipid lowering) have been associated with even further reduction in risk [8, 10]. Statins are therefore among the most commonly prescribed drugs in the industrialized world.

There are seven statins currently available for clinical use in the USA (Table 1). The first statin to be approved by the US Food and Drug Administration (FDA) was lovastatin in 1987, followed by simvastatin, 1988; pravastatin, 1991; fluvastatin, 1994; atorvastatin, 1997; rosuvastatin, 2003; pitavastatin, 2009 [11, 12]. Cerivastatin, initially approved by the FDA in 1998, was withdrawn

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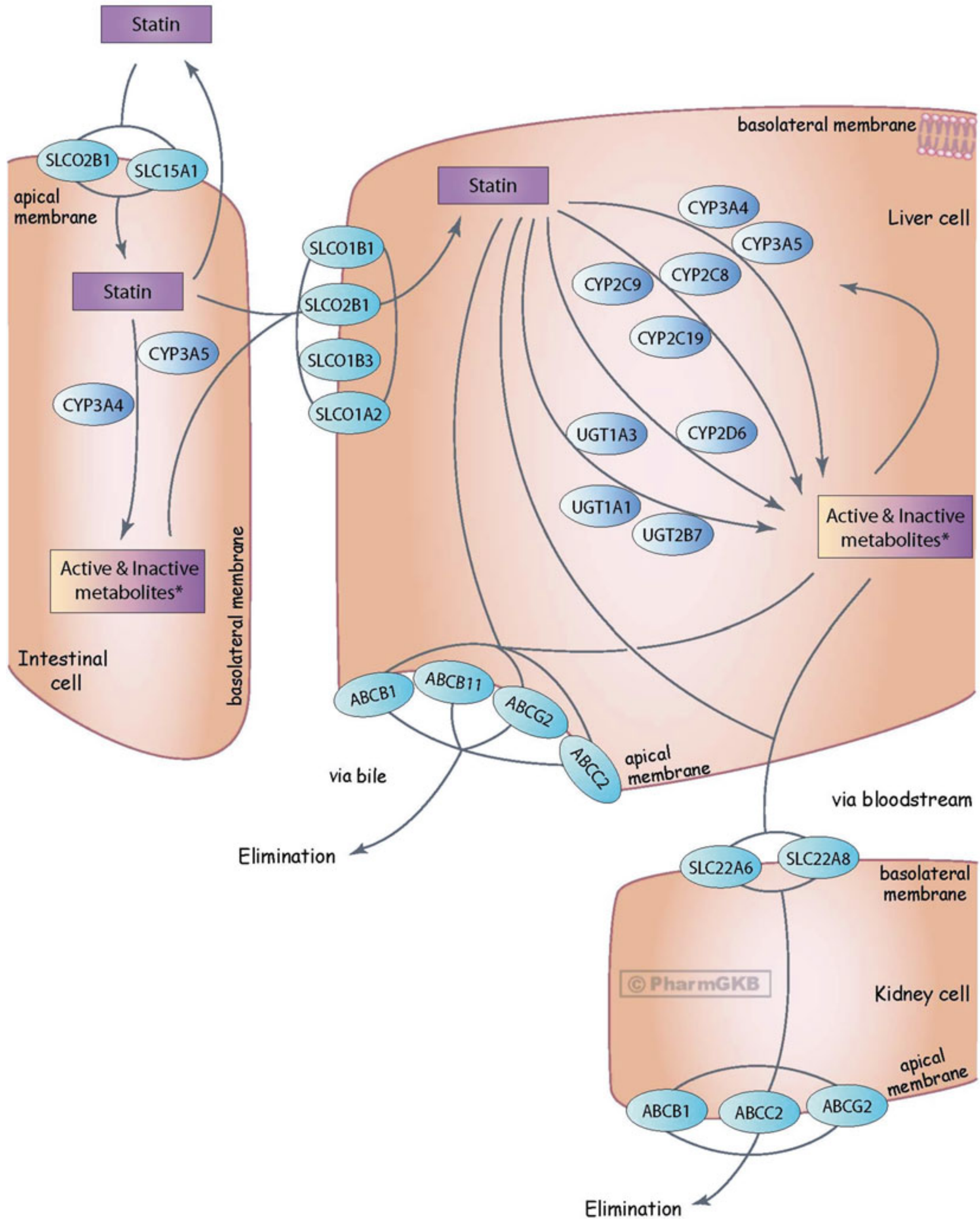

Fig. 1 Statin pathway—pharmacokinetics. Adapted from [119] with permission from PharmaGKB

from the US market in 2001 due to an increased frequency of musculoskeletal complications [13].

Although statin-related adverse drug reactions (ADRs) can involve the kidneys, liver, or brain [14], skeletal muscle toxicity is

Table 1
Currently available statins

Lovastatin (Mevacor)	1987
Simvastatin (Zocor)	1988
Pravastatin (Pravachol)	1991
Fluvastatin (Lescol)	1994
Atorvastatin (Lipitor)	1997
Cerivastatin (Baycol)[a]	1998
Rosuvastatin (Crestor)	2003
Pitavastatin (Livalo)	2009

[a]Withdrawn in 2001, due to increased frequency of adverse drug reactions

the most common ADR associated with this class of drugs [14, 15]. The clinical presentation of this ADR varies from mild myalgias to rhabdomyolysis (severe skeletal muscle damage accompanied by other end organ failure). Because this ADR varies along a fairly broad clinical continuum, the overall frequency of statin-related muscle damage has been difficult to quantify in clinical practice. Standardized phenotyping is needed within the context of large observational databases maintained across multiple institutions [16].

2 Phenotyping

The literature currently supports at least four diagnostic strata, based solely upon creatine kinase (CK) level: (1) Intermediate myotoxicity, defined as CK above upper limit of normal (ULN) but less than threefold ULN, (2) Incipient myopathy, defined as CK above threefold ULN but less than tenfold ULN, (3) Myopathy, defined as CK above tenfold ULN but less than 50-fold ULN, and (4) Rhabdomyolysis, defined as CK above 50-fold ULN. It is important to recognize that these strata are only weakly associated with myopathic symptoms [17]. Although some debate continues regarding the criteria necessary for establishing a diagnosis of statin-induced rhabdomyolysis [14, 15], many clinicians consider an elevation in serum CK level greater than 50-fold ULN to be necessary and sufficient to make the diagnosis. Graham and colleagues have published a now widely accepted stepwise approach for the identification of rhabdomyolysis cases in hospitalized patients [18]. Their algorithm is based on procedural codes for hospital admission, discharge diagnoses (e.g., International Classification of Diseases, 9th Edition [ICD-9] code 791.3, for myoglobinuria), and laboratory tests (reflecting indices of kidney function, liver function, and skeletal muscle integrity).

Myalgias related to statin use are quite common, occurring in up to 10 % of patients exposed [19]. Clinicians often measure circulating levels of nonspecific markers of myocyte damage (e.g., CK) to estimate severity. Myalgias accompanied by a mild elevation in serum CK level occur in approximately 1 % of patients exposed [20, 21]. Myopathy (CK >10-fold upper limit of normal) is less common, ~0.1 %, and rhabdomyolysis (CK >50-fold upper limit of normal) is extremely rare [14, 15]. Graham and colleagues surveyed more than 250,000 statin-exposed patients, and reported rhabdomyolysis rates of 0.000044 events per person-year [18]. Similar rates have been observed for more than 100,000 first-time statin users followed in the UK over a course of 20 months [22]. Event rates increase when statins are used in the presence of other medications known to alter their absorption, distribution, metabolism, and elimination (ADME) [23, 24]. Event rates also increase with comorbidity (e.g., thyroid disease) [21, 25].

2.1 Factors Influencing Severity

Statin-induced muscle toxicity is dose-dependent. McClure and colleagues quantified the frequency of this ADR in one of the largest managed care populations in the USA [21]. Using a relatively stringent definition of "myopathy" (CK level ≥10,000 Units/L, plus a relevant ICD-9 diagnostic code), they observed that the incidence rate for myotoxicity was roughly tenfold higher in patients on high-dose statin therapy (i.e., defined as a dose equivalent to 40 mg of lovastatin daily or greater). A meta-analysis of four large randomized trials [10, 26–28] also revealed a tenfold increase risk of myopathy associated with intensive statin treatment [29]. Since simvastatin is often prescribed at higher doses than other statins, it has been associated with a slightly higher incidence of myotoxicity [30]. This observation has been replicated in an independent clinical practice-based cohort [17]. From the records of nearly 2,000,000 unique individuals served by a single comprehensive system of care, 213 validated cases of statin-induced muscle toxicity were enrolled in a population-based study of genetic risk determinants (http://www.pharmgkb.org/contributors/pgrn/parc_profile.jsp). Within this observational cohort, the relationship between simvastatin dose and severity of myotoxicity was dose-dependent [17].

The severity of skeletal muscle toxicity is also increased by comorbid liver or kidney disease [21, 25]. In their initial assessment of dose–response, McClure and colleagues also observed that the relative risk for statin-related muscle toxicity was 4.3 [95 % CI = 1.5–13] in the context of liver disease and 2.5 [95 % CI = 1.3–5.0] in the context of kidney disease [21]. This increased risk may be due to perturbations in the clearance of either the parent drug or a statin metabolite [24, 25, 31, 32].

Additional clinical risk determinants include advanced age, small body mass index, Asian ancestry, female gender, metabolic

comorbidities (e.g., hypothyroidism), and vigorous physical exercise (Table 2) [16, 17]. Even in the absence of statin exposure (i.e., in healthy volunteers on no prescription medication), strenuous exercise can increase circulating CK level to 4,000 Units/L and above, an effect that remains evident 96 h after exercise [33]. A large fraction of statin ADRs are preceded by vigorous physical exercise or skeletal muscle trauma.

2.2 Case Finding

In general, statins are well tolerated, and ADR rates are low. Huge observational databases linked to the world's emerging electronic medical records (EMRs) may represent the most efficient way of finding statin-induced muscle toxicity cases within the community [16]. Frequency estimates derived from databases maintained by regulatory agencies tend to underestimate the problem because such event-reporting is voluntary [35]. Randomized clinical trials also underestimate the frequency of this ADR because patients with symptoms of intolerance are typically excluded during the run-in period [36–38].

The rates of myopathy and rhabdomyolysis are low [16], therefore, in order to find sufficient number of cases, multi-institutional consortia will be required. Genetics and Myopathy on Statins (GEMS) is an international consortium which represents a collection of statin-related muscle toxicity cases around the world. GEMS has exome-scanned 30 myopathy cases for simvastatin and atorvastatin, defining as CK greater than tenfold ULN (2,000 Units/L), by applying EMR algorithm and manual review from two large biobanks—Personalized medicine Research Project (PMRP) at Marshfield Clinic and Biobank at Vanderbilt (BioVU). GEMS also has access to exomes from >200 myopathy cases in the context of cerivastatin exposure [39].

Selection of control subjects is as important as case identifications, all known risk factors should be matched (drug exposure, dose, comorbid disease, age, race, gender, etc.) to ensure validity of statistical analysis (Table 2). In addition, accrual of sufficient numbers of cases and matched controls will be necessary for the efficient study of genetic factors underlying statin-induced adverse reactions. While association studies are used to carry out setting of clinical trials, the world's growing biobanks, especially those linked with EMRs, represent potential resources for studies identifying genetic and other determinants of statin-induced adverse reactions.

3 Genotyping

The degree to which genetic factors contribute to interindividual variability in myopathy risk has been an active area of investigation for more than a decade. There are two types of adverse reactions:

Table 2
Clinical risk factors of statin-induced myopathy

Clinical determinants	Form of toxicity	Magnitude of effect (odds ratio [95 % confidence interval])	Significant (p-value)	References
Age	Rhabdomyolysis	4.36 [1.5, 14.1]		Pharmacoepidemiol Drug Saf 16, 352–358 (2007) Am J Cardiol 97, 44C–51C (2006) J Am Coll Cardiol 40, 567–572 (2002)
Race	Rhabdomyolysis			Circulation 111, 3016–3019 (2005)
Asian		Twofold greater plasma exposure in Asian than in European		Clin Pharmacol Ther 78, 330–341 (2005) US Food and Drug Administration. FDA Public Health Advisory on Crestor (rosuvastatin). Media release, 2005
African American	Incidence of CK elevation	Higher in African American than in white	<0.001	Am J Cardiol 78, 420–424 (1996) Arch Intern Med 155, 1900–1906 (1995)

Female gender		3.1 [1.9–4.9]	<0.001	Atherosclerosis 211, 28–29 (2010)
	Discontinuation, myalgia, or CK >3× ULN		<0.01	J Am Coll Cardiol 54, 1609–1616 (2009)
History of muscle pain with another lipid-lowering therapy		10.12	<0.0001	Cardiovasc Drugs Ther 19, 403–414 (2005)
Unexplained cramps		4.14	<0.0001	
History of elevated CK		2.04	<0.0001	
Family history of muscular symptoms with lipid-lowering therapy		1.89	0.017	
Hypothyroidism		1.71	0.017	
McArdle disease	Myopathy			Muscle Nerve 34, 153–162 (2006)
Malignant hyperthermia	Myopathy			
Diabetes mellitus			0.01	Pharmacoepidemiol Drug Saf 19, 223–231 (2010)
Ongoing infection			0.005	
Renal disease			0.002	
Hyperuricemia			0.02	
Alcohol overconsumption			0.004	
Trauma			0.002	
Intense physical activity				Br J Clin Pharmacol 57, 525–528 (2004) Prev Cardiol 12, 88–94 (2009)
CYP3A4 inhibitor (with simvastatin)				Pharmacoepidemiol Drug Saf 18, 301–309 (2009)

(continued)

Table 2
(continued)

Clinical determinants	Form of toxicity	Magnitude of effect (odds ratio [95 % confidence interval])	Significant (p-value)	References
Inhibitors of CYP3A4 and SLCO1B1, including traconazole, ritonavir, verapamil, and diltiazem				Clin Pharmacol Ther 80, 565–581 (2006)
Antiretroviral drug and statin (inhibitor of CYP)				Cardiol Rev 17, 44–47 (2009)
Gemfibrozil and pravastatin				Clin Pharmacol Ther 75, 455–463 (2004)
Gemfibrozil and simvastatin				J Pharmacol Exp Ther 301, 1042–1051 (2002)
Fibrates				J Am Coll Cardiol 40, 567–572 (2002)

Adapted from [34] with permission

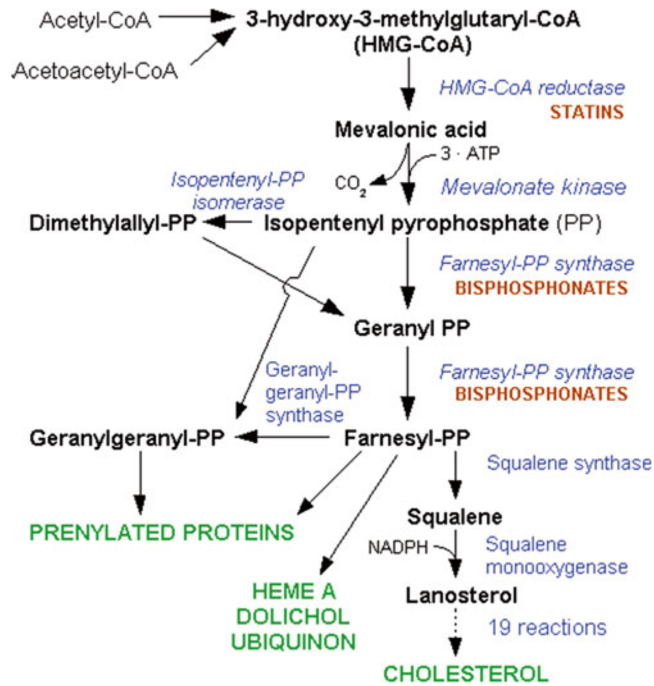

Fig. 2 Statin pathway—pharmacodynamics/cholesterol synthesis pathway. GNU Free Documentation License. Adapted from http://en.wikipedia.org/wiki/Statin

one represents an extension of statins' therapeutics effect; the other is unpredictable, occurring only in susceptible individuals, reflecting our lack of understanding of their underlying mechanisms. Much of our initial understating came from candidate gene association studies (CGAS), particularly within the context of pharmacokinetics (PK) candidates. Given the factor that retention time is strongly associated with both statin efficacy and toxicity, any factor that influences the disposition of these drugs—uptake, oxidation, conjugation, and efflux—would conceivably alter the severity of statin-induced skeletal muscle toxicity. Yet this only represents half of the story (i.e., what the body does to the drug). To fully understand the risk mechanism and risk determinants underlying this clinically important ADR, one must also consider pharmacodynamic (PD) factors (i.e., what the drug does to the body). The known PD and PK candidates are illustrated in the Figs. 1 and 2.

3.1 CGAS (Candidate Gene Association Studies)

3.1.1 Pharmacokinetic Candidate Genes

The clinical severity of statin-induced muscle toxicity is clearly influenced by variability in enzymes modulating statin disposition (absorption, distribution, metabolism, and elimination, ADME) (Fig. 1) [40]. While many statins undergo phase I oxidation (atorvastatin, fluvastatin, lovastatin, simvastatin), the impact of phase I oxidation on others (pitavastatin, pravastatin, rosuvastatin) is very limited [41].

Atorvastatin and lovastatin are oxidized primarily by the cytochrome P450 (CYP) 3A4 and 3A5 enzymes. Although the same enzymes are known to be responsible for the metabolism of simvastatin, fluvastatin, and cerivastatin, the oxidation of fluvastatin (and possibly pitavastatin) is influenced by CYP2C9 [42], whereas both simvastatin and cerivastatin metabolites are further oxidized by CYP2C8 [42–45]. Although controversial, the oxidation of simvastatin metabolites may also be influenced by CYP2D6 [44, 46–49]. Each of these genes—CY3A4/5, CYP2C8/9, and CYP2D6—is polymorphic, and variability in phase I drug metabolizing enzyme genes might therefore account for patient-to-patient differences in muscle-related ADRs.

The potential effect of CYP2D6 polymorphisms on statin intolerance was first explored in a cohort of 88 participants [48]. Frudakis et al. demonstrated that CYP2D6*4 was associated with the frequency of statin induced muscle events ($p = 0.001$), independent of demographic variables [50]. Kaspera et al. sequenced CYP2C8 in 126 rhabdomyolysis cases, and identified 12 novel single nucleotide polymorphisms (SNPs) with a potential to alter CYP2C8 enzyme function [43]. By measuring mRNA levels in 76 human liver samples, Wang et al. has identified an intron 6 SNP (rs35599367) affected mRNA expression and could predict lipid-lowering effect in statin users [51]. A common splice variant in CYP3A5 has been associated with the magnitude of CK elevation by our group, specifically within the context of atorvastatin [52]. The strength of the latter association was dependent upon the presence of concomitant medications known to interact with statins through processes other than phase I oxidation (e.g., phase II conjugation) [52].

Many statins and hydroxy-statin derivatives undergo further modification, through UDP-glucuronosyl transferase 1 (UGT1)-dependent processes [53]. It is therefore likely that genetic variability in the UGT1 enzyme family would contribute to myopathy risk as well. The entire family of UGT1 gene products (UGT1A1-12) is derived from the same locus. Because atorvastatin δ-lactone is associated with toxicity, Riedmaier et al. studied the role of UGTs in atorvastatin lactonization [54]. After analyzing 150 human liver samples, they showed that atorvastatin lactonization is associated with both UGT1A3 immunoreactive protein levels and mRNA levels. Genetic analysis UGT1A3 mRNA and protein levels are altered by the UGT1A3*2 allele, a variant also shown to influence the rate of atorvastatin lactonization. Interestingly, expression level of UGT1A3 mRNA was also positively influenced by the well-defined UGT1A1 variant allele—UGT1A1*28 ($p < 0.001$). This variant has previously been associated with clinical outcome within the context of a number of drug classes [55].

Beyond phase I (oxidative) and phase II (conjugative) statin metabolism, variability in membrane transport also contributes strongly to myopathy risk. The organic anion transporting

polypeptide OATP-1B1 (gene name SLCO1B1) is expressed on the sinusoidal membrane of human hepatocytes and facilitates the hepatic uptake of most statins. Other relevant hepatic uptake transporters include OATP1B3, OATP2B1, OATP1A2, and the sodium-dependent taurocholate co-transporting polypeptide, NTCP [56, 57]. Genetic variability in membrane transport clearly influences statin-related clinical outcome. Polymorphisms in candidate solute transporter genes are associated with the altered hepatic uptake of simvastatin [58] and pravastatin [59]. Much of this variability can be attributed to two coding variants in SLCO1B1 (Asn130Asp and Val174Ala) [60]. As outlined later, the latter variant has since been shown to be highly informative in determining risk for the development of toxicity to simvastatin.

Other transporters can influence the development and severity of statin-induced muscle toxicity as well. Many statins are substrates for efflux transporters such as multidrug resistance protein MDR1 (gene name ABCB1) or multidrug resistance-associated protein MRP2 (gene name ABCC2) [61]. Located on the canalicular membrane of hepatocytes, these ATP-binding cassette proteins mediate the final step in the hepatobiliary clearance of statins. It therefore seems likely that variability in the activity of these transporters would alter the course of statin-related clinical events. Genotype–phenotype association study performed in a cohort of 116 hypercholesterolemic patients has revealed that ABCB1 variants influence the efficacy of simvastatin. The same analysis also revealed that ABCB1 variants (1236T, 2677 non-G, and 3435T) were less frequent in patients with adverse muscle effects [62].

Change in the activity of another efflux protein from the same family (gene name ABCG2) further alters the pharmacokinetics of most statins [63, 64]. This is particularly true for atorvastatin and rosuvastatin, two of the most potent drugs in the class [63, 64]. In a recent study of 305 Chinese patients treated with 10 mg rosuvastatin daily, one SNP in ABCG2 (rs2231142) was strongly associated with statin efficacy [65]. Participants carrying a CC genotype at rs2231142 had a 6.9 % greater reduction in LDL cholesterol levels compared to those with AA genotypes. Because the frequency of this genotype differs widely by race, it may explain a significant portion of the increased myopathy risk observed in Asians [65, 66]. Other ABCG2 variants may be involved as well [67, 68].

3.1.2 Pharmacodynamic Candidate Genes

The rate-limiting enzyme in cholesterol biosynthesis is HMGCR (Fig. 2). Statins inhibit the activity of this enzyme. It has been demonstrated in multiple studies that genetic variants in the HMGCR gene are important determinants for statin efficacy [69–75]. Although it seems reasonable to assume that those polymorphisms would also alter risk of statin toxicity, Frudakis et al. failed to observe any association between HMGCR variants and statin myopathy in a

well-designed case–control study (263 samples) [50]. Thus, alteration in cholesterol biosynthesis alone might not be sufficient to induce myopathy [76]. Inhibition of HMGCR also attenuates the levels of many distal intermediates [2]. After the generation of mevalonic acid, the pathway subsequently produces geranyl pyrophosphate (10 carbons), farnesyl pyrophosphate (15 carbons) and geranylgeranyl pyrophosphate (20 carbons) (Fig. 2). The isoprenoid side chains of these biosynthetic intermediates can transfer farnesyl or geranyl moieties to C-terminal cysteine(s) of target proteins, through a process call "protein prenylation."

Because prenylation is necessary for synthesizing the side chain within ubiquinone (coenzyme Q10, CoQ10), statins may disturb the integrity of electron transport within the mitochondria. As such, mitochondrial dysfunction due to altered levels of CoQ10 has been suggested as a potential mechanism for statin myopathy. The role of mitochondrial dysfunction in the pathogenesis of statin-induced myopathy is supported by extensive pathological evidence [77–79]. Vladutiu et al. have demonstrated that 52 % of muscle biopsies from patients with statin-related myalgias revealed mitochondrial abnormalities, and 31 % of these biopsies revealed multiple defects [79]. Further work by the same group identified variants in adenosine monophosphate deaminase (AMPD1), myophosphorylase (PYGM), and carnitine palmitoyltransferase II (CPT2) as contributors to risk [79]. Additional pharmacodynamic variants contributing to myopathy risk have been reviewed by Peters et al. (including subclinical McArdle disease) [80]. Oh et al. genotyped two SNPs in COQ2 (encoding an important enzyme in CoQ10 biosynthesis) in 133 statin-induced myopathy cases and 158 matched controls [81]. Both SNPs were associated with increased risk of statin intolerance, and a haplotype based on these variants yielded an even stronger association (2.5-fold increase in risk) [81]. These observations have led a number of investigators to explore the possibility that statin myopathy could be attenuated by co-administration of oral CoQ10 [82]. The trials, however, have been small, and the results have been disappointing; for example, Young et al. randomized 44 patients, who had previously failed statins due to muscle pain, to receive simvastatin with either placebo or CoQ10 supplementation (200 mg/day). No difference in myalgia score was observed between the treatment groups. Thus, oral CoQ10 did not improve statin tolerance [83].

Prenylation also influences the balance between myocyte viability and apoptosis. Statin-induced apoptosis has been demonstrated in vitro, using myotubes [84], myoblasts [85], and differentiated primary human skeletal muscle cells [86]. This effect can be reproduced by geranyl-geranyl-transferase inhibitors, and rescued by replacement of mevalonic acid [84]. Compelling evidence suggests that statins cause apoptosis in skeletal muscle by disrupting the prenylation of small G proteins like Rho [85],

Rab [87], and Rap [84]. For example, statins induce apoptosis at concentrations that suppress the prenylation of Rap1a (a 21 kDa GTPase) [84], and Itagaki et al. have shown that this process is accompanied by the redistribution of small G proteins in myoblasts [88]. It remains unclear, however, whether the altered prenylation of small G-proteins is necessary and sufficient to produce myopathy in vivo, or whether myocyte apoptosis is first activated by disrupted Ca^{2+} homeostasis following mitochondrial injury.

3.2 GWAS (Genome-Wide Association Studies)

Although the candidate gene approach was widely applied in the identification of genes responsible for complex diseases, evolutionarily important quantitative traits, and drug induced adverse effect, the utility of this approach is largely limited by its reliance upon a priori knowledge [89]. On the other hand, genome-wide approaches usually proceed without any presuppositions regarding the importance of specific functional features of the traits being investigated. Genome-wide approaches include linkage studies (in families) and genome wide association studies (in unrelated individuals). Both approaches represent unbiased hypothesis-free experiments that hold the potential to identify new biology [90].

Linkage studies represent the earliest type of whole-genome scanning. By constructing pedigrees, early linkage analyses tested for the joint transmission of chromosomal segments and complex phenotypic traits within families. Linkage is the method of choice to identify rare variants with a large impact on disease risk if the trait aggregates in families. The diseases caused by such variants show obvious inheritance patterns and are typically called Mendelian diseases [91]. Although powerful, linkage analyses typically only localize ~10 to 100 cM (centiMorgans) intervals because of the limited number of recombination events within pedigrees [92, 93]. Furthermore, this approach has limited capacity for identifying genes with low penetrance and modest effect size. Thus, the main advantage of family-based studies is that they are not susceptible to false positives from racial admixture and population stratification. Linkage studies have typically not been applied routinely within a pharmacogenomic context, due to the difficulty in identifying families with multiple members exposed to the same drug at the same dose.

Conversely, genome-wide association studies (GWAS) are applied often within the context of pharmacogenomics, for large cohorts of unrelated individuals [94]. GWAS conducted in randomized controlled trials (RCTs) can provide an unbiased survey of the genomic architecture underlying treatment outcome. It is now possible to examine large numbers of polymorphisms, on the order of 100,000–1,000,000, across the entire genome using highly parallel genotyping arrays [95]. In 2008, the SEARCH Collaborative Group applied a 317K SNP scan to 85 cases of incipient myopathy and 90 frequency-matched drug exposed controls,

to identify markers of muscle toxicity specifically within the context of high dose simvastatin (80 mg daily) [96]. This was the first published genome-wide association study (GWAS) of statin-induced muscle toxicity. A single variant survived statistical correction for multiple testing: a base substitution in the SLCO1B1 gene [96].

After genomic re-sequencing of SLCO1B1, the putative causative allele (Val174Ala) was retested for association in a subset of definite myopathy cases from the original SEARCH study cohort, revealing an odds ratio for myopathy of 4.5 per copy of the variant allele [96]. This association has since been replicated in several independent study populations [96–99]. In the Heart Protection Study (HPS), 24 cases of myopathy were identified in 10,269 participants receiving primary prevention with a lower dose of simvastatin (40 mg daily); 21 were genotyped retrospectively for the variant identified in SEARCH [96], and the relative risk was 2.6 per copy of the variant allele. In a practice-based setting, where the definition of intolerance includes discontinuation of the drug for any reason, the relative risk appears to be closer to 1.5 [97–99]. Efforts are now being made to move this pharmacogenetic association into clinical practice through the application of novel decision-support mechanisms [100, 101].

GWAS using statin-induced myopathy cases may also provide deeper insight into the underlying mechanism of toxicity (i.e., leveraging the genetics to inform the biology) [39, 102]. In 2011, Marciante et al. published a combined CGAS–GWAS using a cohort of 185 confirmed cerivastatin-induced myopathy cases (CK >10×ULN with pain) and 732 matched controls [39]. In addition to replicating the well-established SLCO1B1 association for another statin (odds ratio 1.9, $p = 0.002$), Marciante et al. also leveraged GWAS to identify an association between cerivastatin-induced myopathy and an intronic SNP (rs2819742) in the ryanodine receptor 2 gene (RYR2) (odds ratio 0.48, $p = 1.74E{-}07$) [39]. Other GWAS cohorts are providing new candidates for statin-related myopathy. Muscle-specific genes (e.g., gene products modulating Ca^{2+} flux and excitation–contraction coupling) represent attractive targets for mechanism-based study in vitro.

3.3 WGS (Whole-Genome Sequencing)

Since the first publication in 2005, GWAS studies have successfully identified hundred of genetic variants associated with complex diseases and important phenotypes. Despite the early success, most identified variants individually or in combination confer relatively small increments in risk and explain only a small proportion of heritability. For example, in the genetics analyses of height, a classic complex trait with an estimated heritability of 80 %, at least 40 loci showed significant association; however, in total they only explain about 5 % of phenotypic variance in analyses of tens of thousands of people [103]. One possible contributor to the small genetic effect sizes observed so far is that researchers have incompletely

surveyed the potential causal variants within each gene. Currently, popular genotyping platform capture mostly common "marker" SNPs, the observed relative risks of which may underestimate the actual risk associated with the true causal SNPs. Although the underlying rational for GWAS is "common disease, common variants" hypothesis, it is now increasing clear that rare or low frequency variants might contribute to genetic susceptibility to common disease as well. One solution to detection of rare or low frequency variants is sequencing. The emerging sequencing strategies include targeting the region with significant association from GWA study in large population, or targeting coding region (or all exons) within subjects at the extreme of trait distribution. Effort has been made by our group and others to sequencing all exons in 30 extreme rhabdomyolysis cases, which were identified from two large EMR-linked biobanks in the USA—biobank at Vanderbilt (BioVU) and biobank at Marshfield Clinic (PMRP). The first path of analyses identified rare variants in ryanodine receptor 1 (RYR1) gene might involved in development of myopathy after statin exposure [104].

4 Statistical Analyses Strategies

Since the emerging of WGS, different strategies have been applied to characterize the contribution of rare variants. For example, one could sequence candidate gene/loci; or the genomic region identified from linkage or GWAS; or only potential functional region, such as exons; or, with sufficient funding, one could sequence the whole genome for individuals carry phenotype of interest. Then, along with the growing demand of sequencing technology, there is an increasing need to establish appropriate analytical technologies.

4.1 Adjusting for Admixture

Admixture forms from the process whereby two or more genetically and phenotypically diverse populations begin to mate and form a new mixed population [105]. Therefore, each chromosomal segment derived from a particular ancestral population (Fig. 3). Without properly accounting for admixture, the association between gene and adverse drug effect may be incorrectly estimated. Admixed mapping is desirable when variability in a given drug outcome is significantly different between the ancestral population. For association studies, race/ethnicity is usually estimated by a study coordinator's visual inspection (observer-reported) at the time of enrollment; or study participants are asked to self-identify a single race/ethnicity. However, both observer- and self-reported race do not reveal the extent of admixture. In order to estimate admixture proportion, a panel of ancestry informative markers (AIMs) that differentiate well between ancestral populations should be designed and each individual should be genotyped to

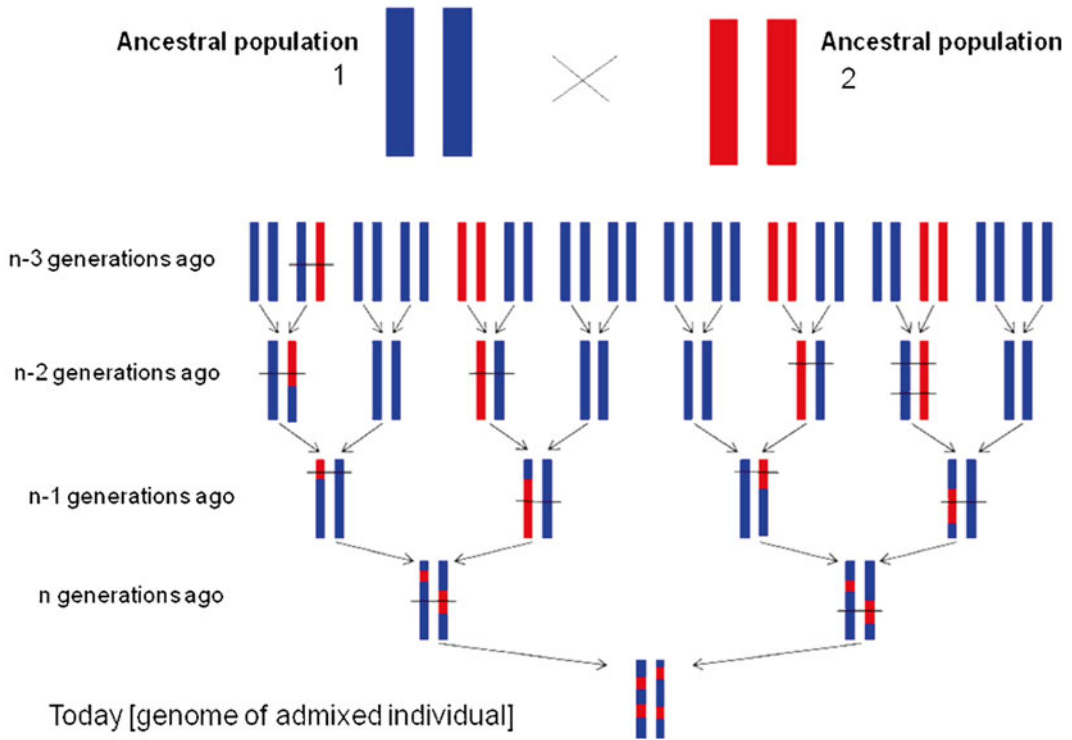

Fig. 3 Schematic presentation of an admixed individual. Adapted from [120] with permission

infer the mosaic of ancestries. The ideal AIM has one allele that is monomorphic in one population and that is not present in another. Several SNP panels have been used to adjust the results of genetic association studies according to population admixture [106–112], and it is likely that different populations need different sets of markers. Bayesian methods and assignment of maximum likelihood are two commonly used approaches to estimate admixture. Both of them estimate the transmission of allele at specific loci comparing to parental population. Several software are available based on these methods, e.g., Structure, AdmixMap, AncestryMap, LAMP, LEA, SABER, PSMIX, FRAPPE, LEADMIX, MEADMIX, EIGENSTRAT.

4.2 Collapsing Rare Variants

As we state above, the identified variants from GWAS together could only explain a small fraction of the overall genetic contribution to common disease risk. The "missing inheritability" suggests that other factors could influence common disease risk as well. The "common disease common variant" hypothesis is now replaced by the "common disease multiple rare and common variants." Rare variants, or private variants, which are poorly captured by GWA studies, are believed to influence prevalence of disease in human population.

In GWAS studies, common variants are generally analyzed individually, however, with only one or two carriers for each rare variant, the power to detect an association between a single rare variant and phenotype of interest is low even with large sample size. One possible solution is to assess individuals with extreme phenotype, hypothesizing this subset of individuals possess a larger number of rare variants which contribute to the phenotype in a similar way. For example, as discussed above our group and other has sequencing all exons in 30 extreme rhabdomyolysis cases and identified RYR1 as a candidate gene. Rare variants could influence phenotype independently from one another; they could also act synergistically with common variants to influence phenotype. Therefore, statistical analysis model should test synergistic effect of both common and rare variants.

Since the power is low for a single rare variant event in a large sample, association analysis considering both common and rare variants is challenging. Thus, to overcome the power issue associated with testing rare variants individually is to consider a set of rare variants collectively, a method called "collapsing." Rare variants can be collapsed based on proximity or functionality. While proximity-based collapsing methods are straightforward, functionality-based collapsing methods rely on currently available assessment for genetic polymorphisms. Proposed functional assessment including: functional element annotation, pathway and process assessment, functional impact prediction modeling and generality of annotators [113]. Functional element annotation focuses on the expression regulation of single gene and collectively considers multiple prediction methods for transcription factor binding (TF search, ConSite, and TRANSFAC), enhancer (VISTA enhancer browser), miRNA (miRBase), miRNA-binding site (TargetScan), etc. Pathway and process assessment focuses on gene–gene interaction and includes several widely used pathway analysis tools. Functional impact prediction modeling analyzes variants based on sequence conservation. Generality of annotator tries to prioritized variants based on function prediction.

5 Beyond Genomics—Refining the Endophenotype with Other Approaches

There are emerging roles for other rigorous phenotyping approaches, such as transcriptomics, proteomics, and lipidomics. Transcriptional profiling arrays now consider alternative splicing [114], tissue specific gene expression [115], and evolutionary aspects of gene expression [116]. Laaksonen et al. analyzed the expression of over 46,000 genes in muscle biopsy samples obtained from six subjects receiving atorvastatin, five receiving simvastatin (one of six cases yielded insufficient RNA), and six receiving placebo

(limited to males, and frequency-matched according to age) [117]. Simvastatin treatment resulted in expression change in 111 genes—26 downregulated and 85 upregulated. More than 20 biological pathways were affected according to their bioinformatics analysis. The most significant upregulated genes included ALOX5AP, CCL5, COL3A1 MYL5, and MYBPH. The same muscle biopsy specimens were then also characterized by lipidomics (LC tandem MS), quantifying 132 unique molecular lipid species [117, 118]. Regression of lipidomic data on gene expression data for pathway-based signaling networks confirmed the involvement of lipid-derived signaling pathways (e.g., prostanoid biosynthesis) and suggested a role for Ca^{2+}-dependent pathways capable of modulating excitation contraction coupling and apoptosis (e.g., Phospholipase C, PLC) [117, 118]. Thus, comprehensive approaches linking genomics and transcriptional profiling, with novel phenotyping strategies in the context of larger populations hold the potential to define the genetic architecture of statin-induced myopathy with unprecedented power.

Web Sites

Structure: http://pritch.bsd.uchicago.edu/
AdmixMap: http://www.lshtm.ac.uk/eu/genetics/admix.html
AncestryMap: http://genepath.med.harvard.edu/~reich/contents.htm
LAMP: http://lamp.icsi.berkeley.edu/lamp
LEA: http://www.rubic.rdg.ac.uk/~mab/software.html
SABER: http://med.stanford.edu/tanglab/software/saber.html
PSMIX: http://bioinformatics.med.yale.edu/PSMIX
FRAPPE: http://med.stanford.edu/tanglab/software/frappe.html
LEADMIX, MEADMIX: http://www.zoo.cam.ac.uk/ioz/software.htm
EIGENSTRAT: http://genepath.med.harvard.edu/~reich/Software.htm
TFSEARCH: Searching Transcription Factor Binding Sites. www.cbrc.jp/research/db/TFSEARCH.html
ConSite: http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite
TRANSFAC®: www.gene-regulation.com/pub/databases.html
VISTA Enhancer Browser: http://enhancer.lbl.gov/
miRBase: www.mirbase.org
TargetScan: http://www.targetscan.org

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Chapter 6

Pharmacogenetics of Membrane Transporters: A Review of Current Approaches

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Abstract

This chapter provides a review of the pharmacogenetics of membrane transporters, including ABC transporters and OATPs. Membrane transporters are heavily involved in drug disposition, by actively transporting substrate drugs between organs and tissues. As such, polymorphisms in the genes encoding these proteins may have a significant effect on the absorption, distribution, metabolism, excretion, and activity of compounds. Although few drug transporter polymorphisms have transitioned from the bench to the bedside, this chapter discusses clinical development of transporter pharmacogenetic markers. Finally, development of SLCO1B1 genotyping to avoid statin induced adverse drug reactions is discussed as a model case for transporter pharmacogenetics clinical development.

Key words ABCB1, ABCG2, ABCC1, ABCC2, OATP1B1, OATP1B3, Transport, Polymorphisms

1 Background

The fate of a drug in vivo is dictated by a variety of physiochemical properties including: size, lipophilicity, and charge. These properties determine how a drug is absorbed, distributed throughout the body, metabolized, and eventually eliminated. While movement of a drug molecule can occur through simple diffusion, there are many transporter proteins expressed on cell membranes to assist with efflux or influx via active transport. Transporters generally move substrates in an intracellular to extracellular direction, efflux transporters; however, some transporters actively move substrates in an extracellular to intracellular direction. Both efflux and influx transporters significantly affect drug disposition. For example, influx of a drug from the blood to the liver, where it is subsequently metabolized and excreted, may increase the rate of elimination. Transport proteins and the genes that encode them are essential to drug uptake, bioavailability, targeting, efficacy, toxicity, and clearance. The genes encoding these transporters are polymorphic,

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phenotypically resulting in transporters with different expression patterns and transport efficiency. Consequently, common variants in genes coding for transport proteins contribute to variability in drug pharmacokinetics and ultimately the patient's response to treatment.

Many drugs undergo transport mediated by the ATP-binding cassette (ABC) family of transporters. There are a total of 49 known ABC genes including, but not limited to: *ABCB1* (P-glycoprotein, MDR-1), *ABCC1* (MRP1), and *ABCG2* (BCRP, MXR, ABCP). ABC transporters utilize ATP to move substrates across membranes [1–5]. These transporters generally counteract uptake through the intestinal wall, efflux substrates out of tissues into the systemic circulation, and eventually promote the clearance of drugs through the kidneys and liver. Proteins in the ABC family are primarily known to be efflux transporters, moving substrates across the cell membrane and out of the cell.

ABCB1 and ABCG2 are the best characterized polymorphic transporters to date [6, 7]. Many current FDA approved drugs are substrates of these transporters, although both transporters efflux a plethora of other compounds including naturally occurring toxins. ABCB1 and ABCG2 are expressed in enterocytes, the canalicular plasma membrane of hepatocytes, and the proximal renal tubule [8–12]. As such, these transporters often mediate bioavailability and exposure to their substrate drugs mentioned in Table 1 [13, 14]. Additionally, they have been shown to be expressed in hematologic tissues including hematopoietic stem cells and endothelial cells composing blood–tissue barriers of the brain, heart, nerves, testes, and placenta, where they efflux substrates out of these tissues into the systemic circulation [9, 15–17]. An exception includes the expression of ABCB1 in the choroid plexus where it transports molecules from the circulation into the cerebrospinal fluid [18–20]. It is believed that the evolutionary role of these transporters is to limit the penetration of toxic molecules into critical organs, thereby serving a protective role in blood–tissue barriers.

Two other efflux transporters, ABCC1 (MRP-1) and ABCC2 (MRP-2) are also involved in drug disposition. ABCC1 is expressed ubiquitously and is localized to the basolateral, rather than apical, membranes of epithelial cells. Due to its basolateral localization, ABCC1 pumps drugs into the body rather than into the bile, urine, or intestine. For this reason, it is thought to serve mainly as a protective barrier in epithelial cells of tissues rather than a classic drug efflux pump [21, 22]. ABCC2 is similar in function to ABCB1. ABCC2 is expressed on the apical domain of epithelial cells. It is involved in luminal excretion in organs such as the liver, the intestine, and the kidney, but also plays a role in blood–tissue barriers. Additionally, ABCC2 actively exports anionic drug conjugates and many unconjugated substances, making it an important part of

Table 1
Substrates and inhibitors of ABCB1, ABCG2, ABCC2, OATP1B1, and OATP1B3

	Substrates	Inhibitors[a]
ABCB1 (P-gp) Antibiotics	Ciprofloxacin (Systemic, UTD) Erythromycin (UTD) Rifampicin (UTD)	Azithromycin [116, 138] Clarithromycin [138, 139] Erythromycin [138, 139] Telithromycin [1]
Antifungals	Posaconazole [138, 139]	Itraconazole [138, 139] Ketoconazole [138, 139]
Antihistamines	Fexofenadine [138, 139] Cetirizine (UTD) Desloratadine (UTD) Loratadine (UTD)	
Antihypertensive drugs	Aliskiren [138, 139] Ambrisentan [138, 139] Talinolol [138, 139] Amiodarone (UTD) Carvedilol (UTD) Diltiazem (UTD) Nadolol (UTD) Nicardipine (UTD) Verapamil (UTD)	Captopril [138, 139] Carvedilol [138, 139] Conivaptan [138, 139] Diltiazem [138, 139] Felodipine [138, 139] Verapamil [138, 139] Reserpine [138] Nicardipine (UTD) Propranolol (UTD)
Heart medications	Digoxin [138, 139] Ranolazine [139] Tolvaptan [138, 139] Quinidine [138]	Amiodarone [138, 139] Dronedarone [138, 139] Quinidine [138, 139] Ranolazine [138, 139]
	Digitoxin (UTD)	Quinine (UTD)
	Quinine (UTD)	
Antiviral drugs	Maraviroc [138, 139] Ritonavir (UTD) Indinavir [138] Fosamprenavir (UTD) Nelfinavir (UTD)	Lopinavir [138, 139] Indinavir [138] Ritonavir [138, 139] Nelfinavir [138] Saquinavir [138]
	Saquinavir (UTD)	Telaprevir [139]
	Telaprevir (UTD)	Tipranavir [138]
		Cobicistat (UTD)
		Darunavir (UTD)
Immunosuppressants	Sirolimus [138] Ciclosporin (UTD)	Ciclosporin [138, 139] Tacrolimus [138]
	Hydrocortisone (UTD	
	Dexamethasone (UTD)	
	Tacrolimus (UTD)	

(continued)

Table 1 (continued)

	Substrates	Inhibitors[a]
Platelet aggregation inhibitors	Dabigatran etexilate [138, 139]	Ticagrelor [139]
	Rivaroxaban (UTD)	
		Dipyridamole (UTD)
Flavonoids		Quercetin [138, 139]
Anticancer drugs	Everolimus [138, 139] Imatinib [138, 139] Lapatinib [138, 139] Nilotinib [138, 139] Topotecan [138, 139] Paclitaxel [138] Vincristine [138] Vinblastine [138] Crizotinib [143] Erlotinib [144] Barasertib [145] Vismodegib (UTD)	Valspodar (PSC833) [138] Lapatinib [139] Everolimus [140] Bosutinib [79] Nilotinib [79] Dasatinib [79] Crizotinib [141] Erlotinib [142] Gefinitib Abiraterone acetate (UTD + product label) Sunitinib (UTD)
	Afatinib (UTD)	Tamoxifen (UTD)
	Bosutinib (UTD)	Vandetanib (UTD)
	Carfilzomib (UTD)	Vemurafenib (UTD)
	Gefinitib	
	Daunorubicin (UTD)	
	Docetaxel (UTD)	
	Doxorubicin (UTD)	
	Etoposide (UTD)	
	Idarubicin (UTD)	
	Irinotecan (UTD)	
	Methotrexate (UTD)	
	Mitomycin (UTD)	
	Pazopanib (UTD)	
	Pomalidomide (UTD)	
	Romidepsin (UTD)	
	Temsirolimus (UTD)	
	Teniposide (UTD)	
	Trabectedin (UTD)	
	Vemurafenib (UTD)	

(continued)

Table 1 (continued)

	Substrates	Inhibitors[a]
Statins and other cholesterol-lowering drugs	Atorvastatin (UTD)	Atorvastatin (UTD)
	Lovastatin (UTD)	Lomitapide (UTD)
	Pravastatin (UTD)	
Miscellaneous	Colchicine [138]	Elacridar (GF120918) [138]
	Saxagliptin [138, 139]	Tariquidar (XR9576) [146]
	Sitagliptin [138, 139]	Zosuquidar(LY335979) [138]
	Loperamide [138]	Laniquidar (R101933) [147, 148]
	Cimetidine (UTD)	Grapefruit juice (UTD)
	Estradiol (UTD)	Ivacaftor (UTD + product label)
	Ivermectin (UTD)	Mefloquine (UTD)
	Linagliptin (UTD)	Progesterone (UTD)
	Ondansetron (UTD)	Ulipristal (UTD)
	Paliperidone (UTD)	
	Risperidone (UTD)	
	Ranitidine (UTD)	
	Silodosin (UTD)	
ABCG2 (BCRP)		
Anticancer drugs	Daunorubicin [138]	Gefitinib [138, 139]
	Doxorubicin [138]	Lapatinib [140]
	Methotrexate [138, 139]	Everolimus [140]
	Mitoxantrone [138, 139]	Nilotinib [79]
	Imatinib [138, 139]	Dasatinib [79]
	Irinotecan [138, 139]	Bosutinib [79]
	Lapatinib [138, 139]	Erlotinib [142]
	Topotecan [138, 139]	
	Barasertib [145]	
	Nilotinib [79]	
	Dasatinib [79]	
	Erlotinib [144]	
	Gefinitib	

(continued)

Table 1
(continued)

	Substrates	Inhibitors[a]
Immunosuppressants		Ciclosporin [138]
Statins	Rosuvastatin [139]	
Miscellaneous	Sulfasalazine [138, 139]	Eltrombopag [138] Elacridar (GF120918) [138, 139]
ABCC2		
Anticancer drugs	Cisplatin [138]	
Antiviral drugs	Indinavir [138]	
Immunosuppressants		Ciclosporin [138]
OATP1B1		
Antibiotics	Rifampicin [138, 139]	Rifampicin [139] Clarithromycin [1]
Anticancer drugs	Atrasentan [139] Methotrexate [138] SN-38 (active metabolite of irinotecan) [139]	
Antihypertensive drugs	Bosentan [139] Valsartan [139] Olmesartan [139]	
Antiviral drugs		Atazanavir [139] Lopinavir [139] Ritonavir [139] Saquinavir [139] Tipranavir [139]
Blood-glucose lowering drugs	Glibenclamide (Glyburide) [139] Repaglinide [139]	
Immunosuppressants		Ciclosporin [139]
Statins and lipid lowering drugs	Atorvastatin [139] Ezetimibe [139] Cerivastatin Fluvastatin [139] Rosuvastatin [138, 139] Simvastatin acid [139] Pitavastatin [139] Pravastatin [138, 139]	Gemfibrozil [1, 138, 139]
Miscellaneous	Thyroxine [139]	Eltrombopag [139]
OATP1B3		
Antibiotics	Rifampicin [138]	Rifampicin [139] Erythromycin [1]

(continued)

Table 1 (continued)

	Substrates	Inhibitors[a]
Anticancer drugs	Methotrexate [138]	
Antihypertensive drugs	Telmisartan [139] Valsartan [139] Olmesartan [139]	
Antiviral drugs		Atazanavir [139] Lopinavir [139] Ritonavir [139] Saquinavir [139]
Heart medications	Digoxin [138]	
Immunosuppressants		Ciclosporin [139]
Statins	Atorvastatin [139] Rosuvastatin [139] Pitavastatin [139] Pravastatin [1]	

[a]Inhibitors listed for P-gp are those that showed >25 % increase in digoxin/fexofenadine/talinolol AUC.
UTD UpToDate (http://www.uptodate.com/contents/search)

drug detoxification [23, 24]. Both ABCC1 and ABCC2 primarily secrete drugs that have undergone phase II metabolism into glutathione, glucuronide, or sulfate conjugates, but both efflux a wide range of drugs [25].

There are also several classes of "influx" or "uptake" transporters that mediate the cellular uptake and the reabsorption of drugs by moving substrates against a concentration gradient. Uptake transporters include organic anion transporting proteins (OATPs), organic cation transporters (OCTs), concentrative nucleoside transporters (CNT), dipeptide transporters (PEPT), and monocarboxylate transporters (MCT) [24, 26]. In the interest of time, we will limit our discussion to two members of the OATP1B family of proteins, as these are well-characterized influx transporters. OATP1B1 and OATP1B3 are expressed in liver tissues and are responsible for hepatocellular uptake of drugs from blood across the basolateral membrane [27–29]. For instance, all statins are transported from the circulation into the liver by OATP1B1, which affects the systemic exposure to many statins and thereby statin-induced myopathy [30]. Most are also transported by OATP1B3. It has previously been thought that these transporters were primarily involved in uptake of substrates into the liver where metabolism occurs [28]. However, more recent evidence suggests that OATP1B3 is overexpressed in several tumor types such as prostate, colon, lung, pancreas, breast, and liver [31–36].

Thus, since OATP1B3 influences drug treatment with docetaxel, paclitaxel, and irinotecan (along with active metabolite SN-38), it is possible that those tumors will be more sensitive to OATP1B3 substrate drugs [31, 37, 38]. Therefore, the OATP1B family is important in regulating the pharmacokinetics, toxicity, and potentially the response to several substrate drugs.

There is significant variation in the genes encoding all of the aforementioned transporters. Several of these genetic variants result in alterations in mRNA expression levels (e.g., promoter variants), translational efficiency (e.g., alterations in mRNA folding), and protein function (e.g., coding polymorphisms). Such genetic variability in transporters often explains a component of the interindividual variability in drug disposition, ultimately resulting in differences in clinical endpoints including toxicity and response. The field of transporter pharmacogenetics is concerned with elucidating the mechanisms by which genetic variation in transporters determines individual differences in drug transport, with a goal of eventually personalizing treatment with substrate drugs based on genotype. This chapter provides an overview of the methods by which investigators have discovered and characterized such associations in the ABCB1, ABCG2, ABCC1, ABCC2, OATP1B1, and OATP1B13 transporters. This methodology could be readily applied to the study of many additional transporters.

2 Genetic Variation and Genotyping Methods

More than 66 coding Single Nucleotide Polymorphisms (SNPs), three insertions/deletions, and several promoter alterations that modify gene transcription have been described in the ABCB1 gene. Twenty-four of the SNPs are synonymous and forty-two are non-synonymous [39, 40]. Three of the SNPs are common in most ethnic groups and demonstrate strong linkage disequilibrium: the synonymous transition at nucleotide 1236C>T (Gly411Gly) in exon 12, the non-synonymous tri-allelic transition 2677G>T/A (Ala893Ser/Thr) in exon 21, and the synonymous transition 3435C>T (Ile1145Ile) in exon 26. Studies have found evidence to suggest that these three SNPs may be implicated in altered transcription of mRNA [23], folding of ABCB1 protein [41], and the pharmacokinetics of drugs [42]. Other studies, however, have failed to confirm these findings [43]. Of the three SNPs mentioned above, only the 2677G>T/A (Ala893Ser/Thr) polymorphism causes an amino acid change. This change is located in a structurally important transmembrane domain of the translated protein. The effects of this transition are controversial and drug specific [26, 44–47]. The 3435C>T SNP is associated with decreased mRNA stability and expression levels [48]. Synonymous polymorphisms in ABCB1 may be responsible for altered protein confirmations due to

ribosomal stalling [41]. Because the genetic code is degenerate and relative frequencies of codons vary, there is occasion for frequent-to-rare synonymous codon substitutions to appear. The substitution of a rarer codon can lead to pauses in ribosomal translation, during which the protein can adopt different secondary structures that may result in functional changes. This mechanism may apply broadly to several transporters [49].

Additive effects cause haplotype combinations to potentially result in greater protein functional differences when compared to single polymorphisms alone. The combination of the 3435C>T, 1236C>T, and the 2677G>T/A polymorphisms, also known as ABCB1*13, has been found to result in a change of ABCB1 transporter characteristics when compared to the polymorphisms alone [41]. Overall, nearly 64 haplotypes have been identified. Linkage between SNPs should be studied for confounding factors. For example, the 1236C>T polymorphism is in ~90 % D′ linkage with the 2677G>T/A polymorphism in several populations, and by virtue of that linkage may only be artificially associated with interindividual ABCB1 transport alterations.

While there are many polymorphisms in ABCG2, the most characterized and common is the ABCG2 421C>A allele in exon 5. This SNP results in a in an amino acid change of Gln to Lys at codon 141 and has been shown in Flp-In-293 cells to have half the protein expression of the wild-type [50]. The variant alleles (i.e., 421A and 141K) have also been associated with lower ATPase activity compared with the wild-type ABCG2 [51]. Thus the ABCG2 421C>A SNP, much like the ABCB1 2677G>T/A allele, may alter both expression and activity of the encoded protein. The variant alleles have also been associated with reduced transport of tyrosine kinase inhibitors because of lower protein concentrations [52]. The frequency of this mutation varies significantly by race; it occurs at 35 % frequency in Chinese populations, whereas the mutation is very rare in African Americans (1 %) [53]. Another potentially important SNP exists at nucleotide 34 and results in an amino acid of V12M. This mutation is most notably associated with poor ABCG2 protein localization [51, 54]. Conclusions vary on whether or not the change results in a difference in expression levels [52, 55]. Surprisingly, this mutation does not appear to modify substrate transport [56]. Furthermore, mutations at R482 which result in non-synonymous protein changes have been identified in numerous cancer cell lines (presumably a mechanism of multidrug resistance) but have never been found in humans. This mutation affects both transport and substrate specificity [57–60].

There are several polymorphisms in *ABCC1*, many of which are non-synonymous. Those studied include C43S, T73I, S92F, T117M, R230Q, V353M, R433S, R633Q, G671V, R723Q, A989T, C1047S, R1058Q, A1337T, and S1512L. A majority of these SNPs do not alter the functionality of the expressed protein

and are unlikely to significantly influence the expression [61, 62]. However, it has been noted that C43S, R433S, and A989T result in decreased ABCB1 function [61]. The G1299T mutation of exon 10 resulted in decreased transport of multiple organic anions but increased the resistance of doxorubicin [62]. It has been noted that C43S, R433S, and A989T all result in decreased ABCC1 function [61]. In vitro analysis has shown that the G128C mutation causes changes in membrane localization [62]. Others have evaluated non-synonymous polymorphisms to assess their impact on mRNA expression, but have found no significant results [63].

The *ABCC2* gene also contains several polymorphisms. In particular, patients with Dubin Johnson Syndrome (DJS) commonly have the 2302C>T A768W polymorphism [23]. Four other SNPs have recently been studied more extensively. They are −24C>T, 1249G>A, 3972C>T, and 4544G>A. In vitro studies with the −24C>T polymorphism showed a 20 % reduction in transcription in HepG2 cell lines. In kidney tissues carrying the −24C>T polymorphism, lower ABCC2 mRNA levels were detected [64].

The gene SLCO1B1, which codes for transporter OATP1B1, contains many polymorphisms that have been associated with a decreased transport phenotype towards several drugs (*see* Table 1) and endogenous substrates [28, 65]. It was also noted that nearly five variants have been shown to effect expression of OAT1B1 on the membrane surface (SLCO1B1*2, *3, *5, *6, *9) [65]. A few of the SNPs have been well studied. These are the −11187G>A, the 388A>G (SLCO1B1*1b), and the 521T>C (SLCO1B1*5). These three variants have been shown to influence clinical outcomes. Studies found that SLCO1B1*5 affected the maximum transport velocity, not the substrate affinity of transport kinetics [65]. The affects of SLCO1B1*1b however remain controversial. Studies have been published showing increased activity, decreased activity, or no activity change at all. These results were very experiment and substrate specific [65]. The polymorphism −11187G>A found in a promoter region has not been shown to reduce or increase expression levels [65]. Allele frequencies differ between populations. The SLCO1B1*5 polymorphism is present in approximately 14 % of the Caucasian population [66] but only 1 % in Japanese populations [67]. For this reason, studies evaluating associations between SLCO1B1*5 and clinical outcome in Caucasians have been more statistically powered and have resulted in clearer clinical outcomes [66, 68, 69]. SLCO1B1*b1 and SLCO1B1*5 may be in linkage disequilibrium. This leads to four functionally distinct haplotypes. The 388G/521C variant is classified as SLCO1B1*15 and showed nearly a decrease in activity of 70 % when transporting estradiol-17β-D-glucuronide in vitro compared to the wild-type allele [65].

Genetic variations for SLCO1B1 are limited and have been much less characterized [65]. Three variations found in the Caucasian population are 334T>G, 699G>A, and 1563G>T and have been studied in HEK293 and MDCKII cells. 334T>G and 699G>A variants did not result in altered expression of six substrates. The frequency of the variants in the Caucasian population are 334T>G 74 %, 699G>A 71 %, and 1563G>T 1.9 % [65]. Variant 699G>A was associated with decreased uptake of cholecystokinin-8 and rosuvastatin in HeLa cells [70]. Recently, variants 332T>G and 699G>A have shown to decrease uptake of testosterone and mycophenolic acid [65]. Two other variants, 1679T>C and 1559A>C lead to decreased cell surface expression and for this reason lower transport functions when compared to the wild-type. In vivo, 334T>G and 699G>A have not been associated with differences in clearance or exposure of paclitaxel or docetaxel [65]. A common haplotype consisting of the 334T>G (S112A) and 699G>A (M233I) SNPs was related to altered OATP1B3 transport characteristics in COS-7 cells, while no differences in the transport of cells transfected were observed with either variant alone [71]. However, this observation may be substrate- or assay-specific given that paclitaxel transport was not altered based on any of the SNPs (334T>G, 699G>A, 1564G>T) or haplotype combinations thereof in *Xenopus oocytes* [72].

Many of the recent publications regarding transporter genotyping have utilized restriction fragment length polymorphism (RFLP) analysis or direct sequencing, although several other methods of genotyping are available such as resequencing, allele-specific PCR, TaqMan PCR, and Fluorescence Resonance Energy Transfer (FRET). Next generation sequencing has brought many new methods including; DNA nanoball sequencing, pyrosequencing, Illumina sequencing, Single Molecule Real-Time (SMRT) sequencing, ion torrent semiconductor sequencing, SOLiD sequencing, and HeliScope single molecule sequencing.

Genotyping for SNPs in genes that may have an effect on drug transport is recommended by the FDA and can be achieved by CLIA-certified genotyping services, many of which use the AmpliChip P450 or the CodeLink P450 genotyping platforms. However, to our knowledge, no genetic variation in a drug transporter has yet been evaluated by the FDA. Recently, some hospitals have begun to offer SLCO1B1 genotyping assays for statin use, these assays must be CLIA-certified if they will be used to inform a patients clinical decisions. Although FDA approval and CLIA certification remain to be worked out, the drug metabolizing enzyme transporter (DMET) platform may provide a basis to evaluate hundreds of polymorphisms in drug transporters and factors that regulate transporter expression (i.e., PXR) in future clinical trials. A brief overview of these genotyping platforms is reviewed in [73].

3 Substrate Identification

ABCB1 and ABCG2 substrates (*see* Table 1) are typically hydrophobic molecules including lipids, peptides, steroids, and xenobiotics—such as anticancer, HIV, atypical antipsychotics, and immunosuppressant drugs. There is often broad overlap between ABCB1 and ABCG2 substrates. The ABCC proteins are multispecific anion transporters. ABCC1 is known to be involved in anthracycline transport [74], but ABCC2 effluxes a wider range of drugs such as cyclosporine, cisplatin, vinblastine, and camptothecin derivatives [21, 75]. OATP1B1 and OATP1B3 interact with a wide range of substrates (not only organic anions as the nomenclature implies) including bilirubin, bile acids [76], peptides, eicosanoids, hormones, and prescribed drugs, including fexofenadine [77]. However, each transporter has distinct substrate specificity, so some compounds are transported by one transporter but not another in the same family.

For investigational drugs, the FDA recommends that all investigational drugs should be evaluated whether they are substrates for drug transporters [78]. In short, all investigational drugs should be tested whether they are ABCB1 and/or ABCG2 substrates in vitro. If results are positive, these drugs should undergo further testing in humans. This does not apply to highly permeable and highly soluble drugs since intestinal absorption is not a rate-limiting step. In addition, drugs that undergo extensive (e.g., ≥25 % of total clearance) hepatic or biliary secretion should be investigated whether they are substrates of OATP1B1/OATP1B3. Several test systems are used to identify ABC and OATP substrates.

3.1 ABCB1 and ABCG2 Substrates

Substrates for ABC drug transporters can be identified using several assays, which can be classified into membrane-based assay systems (including ATPase assay, vesicular transport assay and photoaffinity labeling) and cell-based assay systems (including monolayer assay, cytoxicity assay, and sandwich-cultured hepatocytes) [79]. The FDA recommends to perform a bidirectional transporter assay using cell lines overexpressing the transporter of interest (e.g., transfected polarized cells: MDCK, Caco-2, LLC-PK1, endothelial cell lines; or unpolarized cells: HEK293, CHO) [78]. These cell types are grown in a monolayer on a membrane separating two chambers of culture medium (i.e., the Transwell Cell Culture Assay, Corning Costar Corp., Cambridge, MA). Drug is administered into one chamber in the presence or absence of a specific inhibitor of the transporter of interest, and drug transport across the monolayer is evaluated by sampling from the other chamber. The experiment is then repeated applying drug to the opposite chamber. Due to the directionality of the transporters, these experimental systems allow investigators to assess the basolateral to apical (B-A), and apical to basolateral (A-B), transport of drug. A drug is considered to

be a substrate for ABCB1 or ABCG2, if the efflux ratio B-A to A-B is ≥2. In addition, ABCB1- or ABCG2-mediated inhibition is further confirmed when specific inhibitors (e.g., itraconazole and verapamil for ABCB1; and fumitremorgin C for ABCG2) reduce the efflux ratio by more than 50 % [78, 80]. In that case, clinical drug–drug interaction studies may be warranted.

Another widely applied cell-based system is the cytotoxicity assay. In this system, the cytotoxic effect of the investigational compound is determined after incubation of ABC transporter expressing cells. This assay can be performed with the test compound alone (*direct*) or in the presence of a cytotoxic ABC transporter substrate (*indirect*). The test compound is considered a ABC transporter substrate, when the inhibitory drug concentration causing 50 % cell death (IC_{50} value) is increased in ABC-transporter-expressing cells compared to wild-type parental cells. When using the indirect method, the IC_{50} value of the ABC transporter substrate is decreased when the test compound is a competing substrate.

The third cell-based approach concerns sandwich-cultured rat or human hepatocytes (SCH) [80], which closely mimic the hepatic environment in terms of expression of transporters and metabolizing enzymes. In the SCH model, hepatocytes are cultured in a sandwich configuration between two layers of gelled matrix to form intact bile canaliculi [81]. The advantage of this model is that both hepatic uptake and biliary excretion can be studied.

Among the membrane-based assay systems, the ATPase assay can be used to identify ABC substrates, since ABC transporters require ATP to transport substrates across the cell membrane. Using isolated membranes containing the ABC transporter of interest or reconstituted ABC protein preparations, ABC substrates would be revealed by an observed increase in ATPase activity (colorimetric detection of inorganic phosphate). In an alternative, inhibition-type (indirect) setup, the test compound is added to a well-established ABC substrate, which creates high ATPase activity. If the test compound is also an ABC substrate, the increased ATPase activity will decrease.

By use of the vesicular transport assay, the direct transport of ABC substrates into inside-out plasma membrane vesicles can be detected. These vesicles can be derived from several different cell lines, such as drug-selected cells, transfected cells, and baculovirus-infected insect cells [80]. Similar to the cytotoxicity assay, this assay can be executed using a direct or indirect setup.

3.2 OATP1B1 and OATP1B3 Models

Identification of OATP1B/3 substrates is usually performed in stable OATP1B1- or OATP1B3-overexpressing systems, such as Chinese Hamster Ovary (CHO) and Human Embryonic Kidney 293 (HEK293) cells, *X. Laevis* oocytes, and recombinant virus [80, 82]. The criterion for test compounds to be considered as

OATP substrates is a ratio of uptake in OATP-expressing cells versus control (or empty vector cells) statistically greater than 1 [80]. Furthermore, uptake should be inhibited by a known inhibitor of the transporter.

In addition, similar to the identification of ABC substrates the uptake of OATP substrates can also be studied in primary isolated hepatocytes and the SCH model [82].

The FDA utilizes the following criteria to determine whether an investigational drug is a substrate of OATP1B1 or OATP1B3: "uptake in OATP1B1- or OATP1B3-transfected cells greater than 2-fold of that in empty vector transfected cells and is inhibitable (e.g., >50 % reduction to unity) by a known inhibitor (e.g., rifampin) at a concentration at least 10 times of its Ki. Michaelis–Menten studies may be conducted in the transfected cells to determine the kinetic parameters of the investigational drug. A positive control should be included. In an acceptable cell system, the positive control should show a ≥2 fold increase in uptake compared to vector-transfected cells. An uptake ratio (transporter transfected vs. empty vector transfected cells) other than 2 may be used if a ratio of 2 is deemed non-discriminative as supported by prior experience with the cell system used."[78].

4 Assessing Functional Significance of Polymorphisms In Vitro

4.1 Cell-Based Assays

Polymorphic efflux of ABCB1 substrates was initially evaluated using flow cytometry, although such assays are limited in that only fluorescent compounds can be assayed and differences in polymorphic transporter expression and function are not made clear. To date, the influx of Rhodamine 123, calcein, doxorubicin, and daunorubicin have been evaluated using such methods, and are still used in drug–drug interaction studies (covered later). The same technique has been used with mitoxantrone to assess transport by, and inhibition of, ABCG2. Such assays were initially used in the field of transporter pharmacogenetics to show that Rhodamine 123 transport is lower in 3435TT human $CD56^+$ cells [83]. As the pharmacokinetics of many other drugs could potentially also be differentially altered based on polymorphic ABCB1 expression and function, with ensuing clinical implications, many have evaluated ABCB1 efflux using other in vitro assays. Some have used transfected cell lines to evaluate the functional significance of non-synonymous polymorphisms in ABCB1 and have demonstrated that differences in activity exist between proteins carrying a single amino acid difference brought on by these SNPs. For example, using this technique, it was found that the 2677G>T/A (893S>T/A) polymorphism results in activity differences toward vincristine such that V_{max} 893T>893S>893A, while Km 893S>893T/A [84]. Other investigators have employed ATPase

assays to evaluate the ATP-dependent active transport of substrates. In this assay, vesicles obtained from Sf9 cells transfected with ABCB1 variants have been studied and have validated the previously mentioned finding with ABCB1 [85]. The effect of different polymorphisms on substrate transport by ABCG2 has been assessed using stably transfected HEK293 cells [86]. Following incubation of the cells with the drug, concentrations can be measured via flow cytometry [59], liquid scintillation counting if radiolabelled drug is available [87], or LC-MS [88]. In vitro analyses of OATP1B1 functional polymorphisms were evaluated similarly [66, 67, 89–92]. Interestingly, the above assays have been employed to address the functional consequences of polymorphisms in the ABCC family of transporters, but to no notable alterations in transport capacity have been found [61]. It seems that while ABCC transporters contain several potentially important polymorphisms and are very important in drug transport overall, functional variability is actually quite low. This is perhaps the reason for the multiple negative studies that have assessed ABCC polymorphisms as they relate to drug bioavailability [23].

4.2 Assessing the Cause of Phenotypic Differences

Polymorphic differences that result in altered transporter kinetics, and possibly subsequent changes in drug disposition, can affect this change via multiple mechanisms, including modulated tissue expression. For example, the *ABCB1* 2677TT genotype was associated with decreased mRNA expression in several human tissues as compared to the wild-type allele [83, 93, 94], and thus the functional consequences of the 2677G>T/A polymorphism may be explained by expression alterations alone and not necessarily by altered substrate binding or transport efficiency of the protein. Some postulate that polymorphisms encoding rarer codons for the same amino acid (a synonymous or silent mutation) result in decreased translation efficiency of the mRNA, resulting in lower protein levels, and that it is possible that alterations in polymorphic mRNA secondary structure could also result in inefficient translation. This mechanism has been suggested as one possible explanation for the effects seen with a synonymous mutation in ABCB1 because the 3435C>T transition does not result in an amino acid change, but is still associated with differential drug efflux capability. An alternate, though not mutually exclusive explanation has also been proposed; the 3435C>T SNP is in linkage with the nonsynonymous 2677G>T (893T>S) transition and therefore, it may be associated with a protein product with attenuated efflux capacity through lowered efflux efficiency.

The former hypothesis has been evaluated using mRNA expression measurements in human tissues and it was found that ABCB1 is generally expressed at higher levels with the 3435C [83, 93–95]. These observations were replicated with cotransfection of equal amounts of plasmid and it was concluded that the 3435T allele

lowers mRNA stability and is therefore responsible for decrease efflux capacity [96]. In the case of ABCG2, the effect of the 421C>A polymorphism has been debated. Originally, the resulting amino acid change was believed to reduce protein expression, due to instability [55], but this finding was not confirmed by human intestinal samples that did not reveal a difference [97]. Subsequently, it has been shown that the transport efficiency of the protein is decreased. This was demonstrated by measuring ATPase activity in wild-type and mutant cells, normalizing for expression [51].

When OATP1B1 variants were expressed in HeLa cells, it was noted that *OATP1B1**2, *3, *5, *6, *9, *12, and *13 alleles were associated with reduced transport toward OATP1B1 substrates [66]. Others noted that when the OATP1B1*15 variant was expressed in HEK293 cells, and *Xenopus laevis* oocytes, these cells also had reduced transport capability [89, 91]. The reasons for the reduced transport capacity of these alleles was made clear after it was demonstrated that the plasma membrane localization of many of these polymorphic transporters was impaired due to a cell surface trafficking defect [66]. It was also shown that some polymorphisms encode for impaired protein maturation that results in the encoded OATP1B1 protein to be retained intracellularly [90]. Studies evaluating OATP1B3 polymorphisms are currently undergoing similar validation, but no significant results have yet been reported.

Despite the encouraging results of the above investigations, not all studies using the above experimental systems have consistently validated these observations in other tissues and cell types. For example, associations between genotype and expression seem to be tissue-specific, as lymphocytes and the small intestine both express ABCB1, but expression levels were not associated with polymorphic variants, and it is often the case that reports evaluating the same tissues conflict [7]. Furthermore, some tissues such as cardiac endothelium actually express ABCB1 at greater levels in patients carrying variant alleles which is the direct opposite of data generated in other tissues [95]. Others have used nonhuman in vitro expression systems in an attempt to validate the effect of ABCB1 polymorphisms although transfected variant alleles do not seem to influence ABCB1 transport in some of these experimental systems—perhaps due to differences in mRNA processing membranes in different cell lines and between species [98].

5 Assessing Functional Significance of Polymorphisms In Vivo

Mice carry two homologues of *ABCB1* (*Abcb1a*, *Abcb1b*), and viable single (*Abcb1a*), and double knockout mice are commercially available (Taconic Laboratories Additionally, triple knockout (TKO) mice have recently become available in which homologous

genes encoding ABCB1, and ABCC family members (covered later) have been removed from the mouse genome. An Abcg2 (the mouse homologue of ABCG2) knockout mouse is also commercially available from, in addition to a triple knockout, null for Abcb1a, Abcb1b and Abcg2. Many have utilized such mice to evaluate the influence of ABC transporters on the pharmacokinetics and toxicity of drugs. Based on data obtained from these mice, ABCB1 has been shown to play a major role in detoxification and serves as a protective barrier against the toxic effects of xenobiotics [99]. Mice lacking Oatp1a and mice lacking Oatp1a/1b are also available. However, while these uptake transporters are expressed in human liver and few other tissues and tumors, mice express these transporters more ubiquitously thereby limiting the usefulness of this model in drug–drug interaction studies and other "translational" endpoints [100, 101]. Several humanized models are also readily available. Genetically engineered mice have been used as animal models of compromised blood–brain barrier function [11, 102], intestinal drug absorption [103], fetal drug exposure [104], and drug-induced damage to testicular tubules, choroid plexus epithelium [19], oropharyngeal mucosa [18], and peripheral nervous tissues [105].

Mice lacking the expression of a transporter generally have less ability to eliminate substrate drugs, except in cases where compensatory pathways are upregulated that circumvent transporter-mediated clearance [106, 107]. Alterations in plasma pharmacokinetics result from the lack of transporter expression in gut, liver, and renal tissues where several transporters are involved in the elimination of substrate drugs through hepatobiliary pathways, and glomerular filtration. Such mice generally also demonstrate increased uptake of oral substrate drugs as efflux transporters are involved in the excretion of toxic substances back into the gut lumen in normal mice. As such, bioavailability and exposure are usually increased in knockout mice, while clearance is decreased. This can have both positive and negative effects and can allow translational researchers to make clinical decisions based on the outcome of these drug-treated mouse models. However, this is not necessarily always the case. Compounds that are highly bioavailable in wild-type mice are unlikely to show great increases in absorption when the transporter protein is impaired. Also, as mentioned previously, many drugs have alternate routes of elimination, which may become more important when the primary transport mechanism is not functioning. As such, it is critical that in vivo testing is carried out for each compound, rather than assuming that because a drug is a substrate, it will be greatly affected by these polymorphisms.

Mice that do not express a specific transporter are generally more likely to experience benefit from treatment with a substrate drug because bioavailability and exposure to the drug are usually increased along with the beneficial aspects of treatment. Lack of

transporter function may also allow penetration into tissues that were previously impermeable to the agent. For example, Abcb1 knockout mice with brain metastases can be successfully treated with drugs that otherwise would not penetrate the blood–brain barrier such as paclitaxel [108]. ABCB1a$^{-/-}$ mice also showed ten times more brain–serum ratios of both risperidone and its active metabolite, 9-hydrorisperidone than control mice [109], and most central nervous drugs showed 1.1- to 2.6-fold greater brain-to-plasma ratios in double knockout mice compared to wild-type mice [110].

Although the efficacy of drug treatment may increase, this is counterbalanced by increases in toxicity through routes other than increased plasma concentrations as blood–tissue barriers are disrupted allowing increased penetration of drugs into organs—especially the brain where ABCB1 is an important mediator of drug exposure. In drugs with a narrow therapeutic window (e.g., many anticancer agents), the toxicity can outweigh the beneficial aspects of drug treatment. Following the above example, ABCB1 knockout mice treated with paclitaxel are more susceptible to treatment-related peripheral neuropathy due to increases in drug concentrations in nerve cells [105].

6 Transporter Genetics in Clinical Pharmacokinetics

Numerous clinical trials have investigated the effects of ABCB1 polymorphisms on the pharmacokinetics of ABCB1 substrates [111]. Initially, investigators determined that the *ABCB1* 3435C>T SNP was associated with lowered ABCB1 expression and higher digoxin levels in human volunteers [93]. The association was stronger when the ABCB1 2677G>T/A and 3435C>T polymorphisms were evaluated together as a haplotype—those patients variant at both alleles having both the lowest ABCB1 expression and the highest digoxin AUC [112, 113]. Since then, many investigators have found similar associations between these polymorphisms and plasma concentrations of several other drugs, although these observations have not been consistently confirmed [23, 114, 115]. Overall, the relationship between ABCB1 polymorphisms (e.g., common coding SNPs 1236T>C, 2677T>G/A, and 3435T>C) and the pharmacokinetics of ABCB1 substrates is yet unclear, since clinical studies often report discordant results [80, 116]. It should be noted that polymorphic ABCB1 expression not only influences plasma pharmacokinetics, but also the degree to which drugs are able to penetrate into tissues that express ABCB1 (e.g., tumors, brain, HIV-infected cells, etc.) [117, 118]. As previously mentioned, drug penetration into tissues can be both efficacious (i.e., by increasing therapeutic efficacy) and deleterious (i.e., by increasing toxicity).

For ABCG2, the most common and best-studied polymorphism concerns the 421C>A SNP, which is associated with reduced protein expression levels and impaired ABCG2 activity [119]. Thus far, associations between the 421C>A mutation and plasma pharmacokinetics have been evaluated for several drugs. As reviewed by Schnepf and Zolk [119], individuals with the ABCG2 421AA genotype displayed significantly higher systemic levels of statins (rosuvastatin, atorvastatin, fluvastatin), compared with the ABCG2 421CC genotype. Concordant with these results, impaired transporter activity by the 421C>A SNP also led to increased bioavailability of the anticancer drugs topotecan, diflomotecan, gefitinib, and sunitinib [120–123]. In contrast, for certain other drugs (e.g., pitavastatin, irinotecan, sulfasalazine) no significant association was found between the ABCG2 421AA variant and their pharmacokinetics [53, 124, 125].

The clinical consequences of OATP1B1 polymorphisms on drug exposure have been investigated in several studies [126, 127]. In particular, the relatively common 521T>C SNP is associated with decreased transporter activity and consequently higher systemic exposure to OATP1B1 substrates, including statins, repaglinide, lopinavir, and eythromycin [127]. For example, individuals expressing the 521CC genotype displayed twofold and threefold higher plasma levels of simvastatin acid than those with the TC and TT genotype, respectively [128]. In line with this finding, the 521T>C SNP is associated with simvastatin-induced myopathy [129]. In addition, the effects of genetic ABCC1 variants on drug transport are largely unknown, while ABCC2 polymorphisms seem less likely to alter transporter expression or function [130].

Table 2 shows the effects of common transporter polymorphisms on certain substrates in vitro and in the clinical setting. This table demonstrates that for certain drugs, data obtained in vitro are not always extrapolatable to humans. For example, the ABCG2 421C>A variant significantly increased imatinib accumulation in cells, while this polymorphism did not significantly affect imatinib pharmacokinetics in patients carrying this variant [131]. In addition, conflicting clinical results with the same drug substrate are shown (e.g., OATP1B3 effects on docetaxel pharmacokinetics) [38, 132].

7 Transporter Genetics in Clinical Endpoint Analysis

The ultimate research goal of transporter pharmacogenetics is to further our understanding of the ways in which transporter genetics influences clinical endpoints so that current drug treatment can be made safer and more efficacious, and investigational therapies can be better developed. The literature consists of a multitude of studies that have evaluated drug efficacy and toxicity and have

Table 2
Examples of in vitro and clinical studies that have investigated the effects of common drug transporter polymorphisms on drug transport

Drug transporter and polymorphism	Drug	In vitro effect on drug transport	Reference	Clinical effect on PK	Reference
ABCB1					
3435C>T	Digoxin	No sign. change in digoxin transport in LLC-PK1 cells expressing SNP	[150]	Increased digoxin C_{max} in T/T genotype Decreased digoxin AUC in T/T genotype	[93] [151]
ABCG2					
421C>A	Topotecan	Increased topotecan accumulation in cells transfected with 421A	[121]	Increased AUC_{oral} of topotecan in heterozygous C421A patients	[131]
	Imatinib	Increased imatinib accumulation in cells transfected with 421A	[131]	No significant differences in PK parameters of imatinib in heterozygous C421A patients (vs. wild-type)	[131]
OATP1B1					
521T>C 388A>G	Simvastatin, Pravastatin, Atorvastatin, Cerivastatin	Decreased statin uptake in HEK293 cells transfected with OATP1B1*5 (521T>C), *15 (388A>G, 521T>C) and *15+C1007G	[152]	Increased pravastatin AUC in heterozygous OATP1B1*5 and *15 healthy volunteers compared to noncarriers of these variants	[153]
	Docetaxel	Decreased docetaxel uptake in Flp-In T-Rex293 cells transfected with OATP1B1*5 or *15	[38]	No significant association between docetaxel CL and 521T>C or 388G>A variants in cancer patients	[38]
OATP1B3					
334T>G 699G>A IVS 12-5676A>G	Docetaxel	No in vitro studies regarding OATP1B3 genetic variants and docetaxel transport		In 334T>G, 699G>A, IVS 12-5676A>G variants no association with docetaxel CL was observed Homozygous IVS 12-5676A>G variant showed sign. higher AUC and lower CL of docetaxel compared to AA+AG genotypes	[38] [132]

PK pharmacokinetics, C_{max} maximum plasma concentration, *CL* systemic clearance, *AUC* area under the concentration vs. time curve, *SNP* single-nucleotide polymorphism

made associations between these parameters and polymorphisms in drug transporters [7, 23, 133]. The FDA recommends several endpoints to evaluate specific diseases and those endpoints should be evaluated when making associations between a genetic variation and the treatment of diseases with drugs (see: www.fda.gov/cder/guidance; last accessed November 25, 2013). In pharmacogenetic studies, these endpoints should be evaluated in a standard fashion in similar populations in order to establish the predictive value of a polymorphism. Unfortunately, the literature has not typically been consistent mainly due to the availability of samples for analysis, and perhaps this is the reason that transporter polymorphisms have not been consistently validated. Thus far, all studies linking pharmacogenomics of membrane transporters with clinical outcome have been retrospective, taking place in eclectic populations with relatively low statistical power. It is essential that well-powered and prospective studies are undertaken, prior to any treatment modification, to assess the true effects of these polymorphisms and determine whether the effect is drug-specific or disease related.

8 Case Study: SLCO1B1 Genotyping for Statin-Induced Adverse Drug Reactions

The FDA has listed numerous markers of variability in: drug exposure, clinical response, adverse event risk, dosing, drug action, targeting, and disposition (http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm; last accessed 10 January 2013). Notably, while this list consists of over 100 gene/drug pairings, not a single drug transporter is listed. This is in stark contrast to the number of CYP enzymes and other regulators of absorption, distribution, metabolism, and elimination that are frequently listed by the FDA as important regulators of drug disposition and outcome. It is also important to consider that over 35,000 articles have been published on ATP-binding cassette (ABC) drug transporters alone while a similar search of "cytochrome P450" only reveals approximately 75,000 articles. Clearly, the disparity between the clinical utility of information about a patient's genetic status at a transporter versus a CYP is not for a lack of research; rather, current methodologies are not breaking the barrier between discovery, development, and ultimate translation of transporter pharmacogenetics.

Nonetheless, there is a single drug transporter where genetic testing has led to clinically actionable information. The Pharmacogenetics Research Network (PGRN) has also listed annotations for polymorphisms that have either been endorsed by a medical society, have been implemented in a major hospital site, or where the preponderance of the evidence suggests that there is an association between polymorphic variation and clinical drug use (http://www.pharmgkb.org/search/clinicalAnnotationList.action?levelOfEvidence=top; last accessed 10 January 2013).

While there are 45 polymorphism/drug pairings listed on this Web site, the PGRN lists a single transporter polymorphism *SLCO1B1* (rs4149056) which encodes an amino acid transition in the OATP1B1 protein. This, rather common, allelic variant alters the disposition of various statins and is a strong marker of the risk to develop statin-induced myopathy. It is widely expected that the FDA will embrace this genetic approach to adverse drug reaction (ADR) avoidance in certain clinical situations. Therefore, OATP1B1-related statin pharmacogenetics may serve as a rubric for successful translation of transporter pharmacogenetics.

In 2001, Tirona et al. identified and functionally characterized polymorphisms in SLCO1B1. Although several SNPs altered OATP1B1 transport activity, the 521T>C SNP (V174A) caused a significant decrease in transporter activity toward multiple substrates (Tirona et al. [66]). Haplotype analysis was also conducted in several world populations by various investigators [27]. Statins were later discovered to be OATP1B1 substrates using several transwell and overexpression systems [27]. In vivo studies were conducted concurrently with retrospective clinical pharmacogenetics studies in humans. The *Slco1b2* knockout mouse model showed that there was a fourfold decrease in the liver–plasma ratio of pitivastatin although humanized mice were never studied [134]. Several candidate SNP studies also largely demonstrated that plasma exposure of statins was significantly increased in individuals carrying 521CC, and modestly in those carrying 521CT [27] suggesting, by and large, that the *5 and *15 haplotypes were both associated with increased plasma AUC of several statins. Several other studies suggested that the 521C allele was also related to attenuated cholesterol response [151]. A very large retrospective GWAS study was published in 2008 [136] that suggested the 521C allele was associated with both an impaired cholesterol response and an increased incidence of statin-induced myopathy. This led to ultimate clinical guidelines for implementation published by the Clinical Pharmacogenetics Implementation Consortium, consisting of individuals from academia, the pharmaceutical industry, and clinicians from numerous institutions [137].

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Chapter 7

G Protein-Coupled Receptor Accessory Proteins and Signaling: Pharmacogenomic Insights

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Abstract

The identification and characterization of the genes encoding G protein-coupled receptors (GPCRs) and the proteins necessary for the processes of ligand binding, GPCR activation, inactivation, and receptor trafficking to the membrane are discussed in the context of human genetic disease. In addition to functional GPCR variants, the identification of genetic disruptions affecting proteins necessary to GPCR functions have provided insights into the function of these pathways. $G_s\alpha$ and $G\beta$ subunit polymorphisms have been found to result in complex phenotypes. Disruptions in accessory proteins that normally modify or organize heterotrimeric G-protein coupling may also result in disease states. These include the contribution of variants of the regulator of G protein signaling (RGS) protein to hypertension; the role variants of the activator of G protein signaling (AGS) proteins to phenotypes (such as the type III AGS8 variant to hypoxia); the contribution of G protein-coupled receptor kinase (GRK) proteins, such as GRK4, in disorders such as hypertension. The role of accessory proteins in GPCR structure and function is discussed in the context of genetic disorders associated with disruption of the genes that encode them. An understanding of the pharmacogenomics of GPCR and accessory protein signaling provides the basis for examining both GPCR pharmacogenetics and the genetics of monogenic disorders that result from disruption of given receptor systems.

Key words G protein-coupled receptor, Accessory proteins, G protein-coupled receptor kinases (GRK), Regulator of G protein signaling (RGS), Activator of G protein signaling (AGS), Hypertension, Pharmacogenomics, Signaling

1 Introduction

Pharmacogenomics—the genomics of pharmaceutical targets, such as the G protein-coupled receptors (GPCRs)—involves classification of the genes encoding the proteins that are necessary for a pharmaceutical target to function. With respect to the GPCRs themselves, there are three subclasses of receptors that are of particular importance in to pharmacogenomics: class A receptors share sequence similarity to rhodopsin and the calcitonin receptor; class B receptors consist of secretin/glucagon-like receptors that share little structural similarity to the other classes of GPCRs;

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class C receptors, such as the calcium sending receptor (CASR), which signal as a result of conformational changes in response to allosteric ligands [1–3]. The genomic classification of GPCRs allows for more accurate prediction of the changes in receptor function that may result from sequence variants that occur in nature or in vitro.

The manner in which GPCRs are able to regulate subtle physiological processes, however, suggests that the specificity of GPCR signaling is also determined by which heterotrimeric G protein, effector, and accessory proteins are recruited. The accessory proteins involved in receptor inactivation may be as important as the structure and function of a given GPCR.

Genetic variations in accessory proteins that disrupt receptor function have been identified in nature. Examples include (1) variants of a regulator of G protein signaling (RGS) protein that confer risk for essential hypertension through dopamine D_1 receptor-mediated kidney function; (2) variants of the *GNAS* gene, which encodes Gαs, the ubiquitously expressed Gαs-subunit; (3) variants of the Gβ subunits in essential hypertension, obesity, stroke, and myocardial infarction; and (4) variants of G protein-coupled receptor kinase 4 (GRK4) that alter dopamine D_1 receptor-mediated kidney function in essential hypertension. Given their importance, the role of accessory proteins in GPCR activation and inactivation is perhaps best discussed in the context of representative receptor systems.

1.1 The G Protein-Coupled Receptors

The largest GPCR subfamily is known as class A. It comprises approximately 90 % of all GPCRs [1]. Members of this class of GPCRs have been studied at both the molecular and the structural levels [1]. Identification of the properties of class A receptors has resulted in the identification of orphan receptors [4, 5] that have become reagents for drug discovery in drug screens [5].

These receptors share many common features: some of which are illustrated by the cysteinyl leukotriene 2 ($CysLT_2$) receptor (*see* Fig. 1) [6]. These features include (1) insertion into the membrane and targeting to the plasma membrane, (2) the presence of seven conserved transmembrane domains, (3) three extracellular and three intracellular loops, (4) an extracellular amino terminus, and (5) an intracellular carboxyl terminus [1, 2].

All of the known class A receptors are subject to posttranslational modification at one or more N-linked glycosylation sequences, located in either the extracellular amino terminus or in the second extracellular loop. Glycosylation is essential for the expression of some GPCRs at the plasma membrane [7, 8]. Furthermore, many receptors are also subject to other posttranslational modifications, such as palmitoylation at the intracellular domains [9]. These palmitoylation sites probably serve to anchor the intracellular carboxy tail to the plasma membrane [10].

Fig. 1 Schematic representation of the cysteinyl leukotriene 2 ($CysLT_2$) receptor. Ribbon model of this family A G protein-coupled receptor (GPCR) is pictured in its heptahelical configuration. The extracellular amino terminus of the receptor, the transmembrane domains, and the intracellular carboxyl tail extend behind the intracellular palmitoylation site. The putative "binding pocket" for cysteinyl leukotriene ligands is derived from a rhodopsin model

Indeed, X-ray crystallography studies have suggested that the prototypic class A receptor, rhodopsin, may effectively form an additional helical structure as a result of membrane anchoring [11, 12].

Activation has most often been studied by analyzing the in vitro consequences of mutated GPCRs or G protein subunits. Receptors targeted by bulky ligands, such as large peptides and protein hormones, tend to bind at the N-terminal extracellular loops and in the transmembrane domains. Ligands as diverse in structure as dopamine and the cysteinyl leukotrienes (CysLT), however, bind to their cognate recognition sites within the hydrophobic core formed by the membrane-spanning α-helices [13, 14]. In the case of the $CysLT_2$ receptor (*see* Fig. 1), naturally observed variants have been discovered that alter the region defining the putative binding pocket (discussed in Chapter 9). Thus, multiple motifs define the ligand–receptor interaction [15].

Still other receptors have poorly defined binding pockets: they accommodate ligands in many orientations and at alternative binding domains. In addition, many receptors assume different conformations with distinct signaling functions, potentially as a result of receptor homo- or hetero-oligomerization. As a result of these and

other factors, single receptor types may trigger multiple signaling pathways, while groups of receptors may all act on a single intracellular signaling cascade [3, 16–18].

A special problem arises in assessing the therapeutic relevance of receptor families across the genome, as there may be complex interactions via multiple closely related receptors that bind a single drug in a variety of different ways [3]. For example, although the $CysLT_1$ and $CysLT_2$ receptors have a unique rank order of ligand potency [19, 20], the fact that their distribution in mast cells overlaps suggests that they need not always act as autonomous leukotriene-binding sites [21–23].

Like many GPCRs, the $CysLT_1$ and $CysLT_2$ receptors contain a number of structures capable of facilitating functional interactions. As reported for other receptors, dimerization or higher order oligomerization may occur as the result of posttranslational modification or the interaction between transmembrane domains [24, 25], although the functional relevance in vivo is often unclear. Oligomers of receptors such as angiotensin II type I [26, 27], M_3 muscarinic [28], dopamine [29, 30], and the metabotropic glutamate (mGluR) [31] may form through a variety of protein–protein interactions. These interactions may play a role in modifying the orientation of high-affinity ligand-binding sites [31–34]. The effects of naturally occurring GPCR variants on functions relating to receptor dimerization and G protein coupling, however, remain largely unknown [35–37].

2 GPCR Signaling

Significant advances in the understanding of GPCR structure and function have resulted from the identification of particular residues critical to the cell signaling that results from ligand binding, receptor activation, and receptor inactivation [38]. When exposed to continuous stimulation by an agonist, GPCRs can trigger a variety of negative feedback mechanisms that limit further signaling. The process of activation will be reviewed in the context of what is known about the genomics of G protein subunits and accessory proteins and the human disorders that result from disruption of these processes [39].

Several human disorders result from genetic abnormalities in G protein structure. Several involve the imprinted *GNAS* gene, which encodes Gαs: a ubiquitously expressed Gα-subunit that couples receptors to adenylyl cyclase (AC) to increase cellular levels of the second messenger cyclic adenosine monophosphate (cAMP) [40]. Loss-of-function, gain-of-function mutations and imprinting effects lead to many clinical phenotypes. Mutations of *GNAT1* [41, 42] and *GNAT2* [43, 44], which encode the retinal G proteins (transducins), cause specific congenital visual defects.

Common polymorphisms of the *GNAS* and *GNB3* (which encodes Gβ_3) genes have been associated with multigenic disorders such as hypertension [45], metabolic syndrome [40, 46], cancer [47], and pseudohypoparathyroidism (PHP) [48, 49].

PHP, a rare heterogeneous genetic disorder characterized by end-organ resistance to parathyroid hormone, is discussed further in Chapter 8. Heterozygous inactivating GNAS mutations lead to PHP type Ia (PHP-Ia) when maternally inherited, or pseudopseudohypoparathyroidism (PPHP), if paternally derived [48]. To date, only variants of the Gα- and Gβ-subunits of the G protein have been implicated in human disease—no Gγ-subunit disruptions have been identified.

A general overview of G protein coupling is necessary before a description of the G protein, accessory protein, and GPCR variants associated with disease is undertaken.

2.1 G Protein Coupling: Molecular Mechanism of GPCR Activation

The G protein-mediated signal transduction that results from GPCR activation by an extracellular agonist takes the form of a cascade of intracellular chemical signals. The release of second messengers in response to agonist allows an individual ligand binding event to be amplified within the cell, a process that accounts for the great sensitivity of GPCR signal transduction [1, 2]. These pathways, however, can be disrupted when a receptor is subjected to natural or in vitro mutation [1, 50–54].

Amplification of the signal is an elaborate process that depends on specific properties of the receptor, which G protein system is involved, and on the presence of auxiliary proteins that amplify or quench the signal [18]. A single amino acid variation in GPCR sequence can cause a dramatic gain or loss of function: depending partly on the G protein species it is able to interact with [51]. When the signal from a receptor with a gain-of-function mutation is amplified, pathophysiological dysregulation can result. Conversely, when the signal from a receptor with a loss-of-function mutation is amplified, signaling activity may be reduced to below what would otherwise be considered basal levels [16, 52].

2.2 G Protein Subunits

In classic models of G protein coupling, the process is often described as involving several steps. First, as ligand is bound to the GPCR, the GPCR assumes an “activated” conformation. An activated GPCR then interacts with an inactive G protein complex, consisting of three subunits: the Gα-, Gβ-, and Gγ-subunits. The inactive G proteins exist as heterotrimers with one guanosine 5′-diphosphate (GDP) bound to each Gα-subunit, while the other two subunits together form a stable Gβγ dimer. It is the interaction of an activated GPCR with a heterotrimeric G protein that results in an activated, or high-affinity, receptor–G protein complex [2, 18].

The complex subsequently releases GDP, and guanosine 5′-triphosphate (GTP) binds to the Gα-subunit in its place [42, 53, 54].

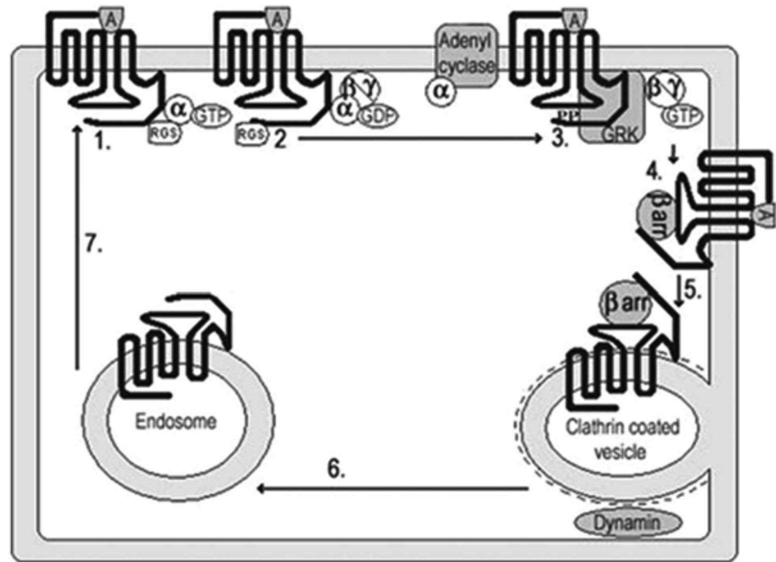

Fig. 2 Schematic of G protein-coupled receptor (GPCR) activation and inactivation. Following short-term exposure to agonist, common pathways of GPCR desensitization, internalization, and downregulation are initiated. The rapid effects, often described as resulting in homologous desensitization, are mostly associated with the G protein-coupled receptor kinase (GRK)-mediated phosphorylation of agonist-occupied receptor. They are summarized in this schematic as follows: (*1*) agonist (A) binds to GPCR, initiating conformational changes in the receptor, resulting in the recruitment of the regulator of G protein signaling (RGS); (*2*) G protein (α, β, and γ) couples, RGS facilitates guanosine triphosphatase (GTPase) activity, and the second-messenger cascade results after Gα binds to adenylcylase; (*3*) GRK is recruited, displacing enzyme and phosphorylating (PP) agonist-occupied receptor; (*4*) β-arrestin (βarr) forms a complex with the receptor; (*5*) the receptor is internalized at clathrin-coated pits; (*6*) internalization results in degradation of the endosome-internalized receptor; but (*7*) dephosphorylated receptor may be recycled to the plasma membrane [2, 53, 114]. *GDP* guanosine 5′-diphosphate, *GTP* guanosine 5′-triphosphate

There is evidence supporting a model that allows for the dissociation of both the active Gα–GTP and the non-covalently bound βγ-heteromeric complex from the receptor–effector complex; however, other models can also account for these data [55, 56]. Auxiliary proteins may regulate the potentiation of the GPCR–G protein effector complexes that generate second messengers or specific transmembrane proteins such as ion channels [39]. These processes are outlined schematically in Fig. 2.

2.3 The G Protein Gβ- and Gγ-Subunits

The Gβ- and Gγ-subunits (apart from the special case of Gβ5) are generally less diverse than the Gα-subunits, however, they have a role in both activation and inactivation of GPCRs [57–59]. In addition to their essential role in G protein activation, the Gβγ-subunits

bind three classes of GRKs (GRK1, GRK2, and GRK3)—allowing translocation of these kinases to the membrane. It is the membrane co-localization of GRKs and GPCRs that makes possible the GRK phosphorylation of GPCRs that is integral to the process of receptor desensitization [60].

The diversity in tissue expression of Gβ- and Gγ-subunits also plays a role in regulating such processes. Ignoring splice variants, at least 4 β-subunits (Gβ1 to Gβ4) and 11 γ-subunits (Gγ1 to Gγ11) have been isolated [61]. The considerable overlap in the distribution [61] of these subunits gives rise to subtle phenotype penetrance.

2.4 Gβ-Subunits Associated with Complex Phenotypes

While no variants of the Gβ- and Gγ-subunits have been associated with monogenic disorders, polymorphisms have been associated with a variety of subtle phenotypes. For example, a single-base substitution (c.825C>T) of the Gβ_3 gene (*GNB3*) is associated with hypertension. The variant leads to alternative splicing, leading to a shortened Gβ_3 protein [63], which may result in enhanced G protein signaling [63–65].

While an association between the C825T allele of *GNB3* and other features of the metabolic syndrome, including obesity, insulin resistance, autonomic nervous changes, and dyslipidemia have often been reported [65–68], some studies have failed to identify such phenotypes [69–72]. Beyond this, the polymorphism has also been implicated in Alzheimer's disease [73], sudden death [74], and tumor progression [75, 76]—as well as being a pharmacogenetic marker for drug response [64, 77, 80]. The mechanisms linking the C825T polymorphism to these various clinical outcomes have not been identified. The *GNB3* polymorphisms, however, may become a useful markers for disease risk and drug response.

2.5 The G Protein α-Subunits

All three heterotrimeric G proteins are required for GPCR coupling [51, 52]. Moreover once GTP binds both the Gα- and Gβγ-subunits can activate effector proteins and ion channels, such as AC, phospholipases C, Ca^{2+} and K^{+} channels [81]. For example, while the activated Gαs tends to activate AC [82, 83], Gαi tends to inhibit AC, and activated Gαq tends to activate phospholipase C-β [39, 84]. Variations in receptor structure can change the rate at which G protein subunits are liberated. Enhanced or diminished GPCR signaling can result from changes in these processes at any step.

2.6 Tissue Variability of G Protein Subunits and GPCR Signaling

Since there are more than 20 distinct Gα subunit proteins, their activities can be a major determinant of the specificity of GPCR signaling and its variability in both health and disease. By definition, the characteristics of variant GPCR signaling will depend on the G protein subunits co-expressed in tissues or cells. In particular, the rate of GTP hydrolysis varies, depending on the type of Gα subunit

[85, 86] and which if any RGS proteins are present that might be targeted to them [87]. The persistence of the signal depends on the rate of guanosine triphosphatase (GTPase) activity, which inactivates G protein signaling to restore the low-energy Gα–GDP conformation [85, 86].

Four Gα subfamilies, identified by sequence homology, exert a physiological influence through their expression in different tissues. The ~20 different types are categorized into the Gαi, Gαs, Gαq, and $G\alpha_{12}$ subfamilies. The widely expressed Gαi subfamily, including (1) $G\alpha\tau_{1,2}$; (2) the transducins (expressed in rods and cones); (3) $G\alpha_{gust}$, the gustatory G protein that transduces signals from the taste receptors on the tongue; and (4) Gαz, which stimulates cyclic guanosine monophosphate (cGMP) phosphodiesterase, inhibits AC, and regulates the Ca^{2+} and K^+ channels. Next is the Gαs family, including Gαs and $G\alpha_{olf}$ (the olfactory G proteins), which stimulate AC and regulate both Ca^{2+} and K^+ channels. Third, is the Gαq family, Gαq and $G\alpha_{11,14,15,16}$, which activate phospholipase C (PLC), p63 RhoGEF, and potentially other effectors [88]. Finally, there is the $G\alpha_{12}$ family, $G\alpha_{12}$ and $G\alpha_{13}$, which stimulate Rho via certain Rho-GEF proteins, adenylyl cyclase (isoform VII), and Na^+–H^+ exchangers [51, 89–91].

2.7 Gαs Subunit Disrupted in Disease

The Gαs subunit, encoded by the *GNAS* gene on chromosome 20q13, is one multiple-gene product that results from alternative promoters and exon splicing. This section serves to introduce the functions of the *GNAS* gene in the context of a *GNAS* mutation that results in testotoxicosis combined with pseudohypoparathyroidism type Ia. The phenotype, discussed in Chapter 8 (Subheading 2.7.1), is associated with increased GDP dissociation resulting in protein denaturation at normal body temperature, while sparing Gs function in the testes [49].

Gαs is the ubiquitously expressed Gα subunit required for receptor-mediated cAMP production. A number of widely distributed activating variants, such as Arg201Leu, lead to McCune–Albright's syndrome (MAS) [92], in which patients can develop fibrous dysplasia (FD) of bone, café-au-lait skin lesions, gonadotropin-independent sexual precocity, or tumors (or nodular hyperplasia) of pituitary somatotrophs, thyroid, or adrenal cortex with associated hormonal oversecretion [93]. Similar genetic variants have been identified in cases of adrenocorticotropin-independent macronodular adrenal hyperplasia [94] and premature breast development [95]. The activating Gαs variants result in various phenotypes due to constitutive cAMP production [92]. Inactivating Gαs variants lead to Albright's hereditary osteodystrophy (AHO) in the heterozygote, suggesting that Gαs haploinsufficiency causes the disorder. AHO is characterized by short stature, obesity, brachydactyly (shortening of metacarpal and metatarsal bones), subcutaneous ossifications, and developmental deficits [96, 97].

The severity of the phenotype, however, is variable, as some patients with Gαs mutations have few or no symptoms.

The mechanism of Gαs disease in chondrocytes may result from insufficient parathyroid hormone-related peptide signaling by the parathyroid hormone receptor 1 (PTHR1) due to the inability of the receptor to activate mutant forms of the G protein. This deficiency may inhibit chondrocyte differentiation within the endochondral growth plate [98, 99]. A variety of parathyroid hormone abnormalities can result.

The *GNAS1* gene imprinting causes those patients who inherit Gαs mutations from their fathers to develop only AHO or pseudopseudohypoparathyroidism (PPHP). On the other hand, those who inherit mutations from their mothers develop both AHO and resistance to a variety of hormones, including parathyroid hormone (PTH), thyrotropin (TSH; formerly called thyroid-stimulating hormone), growth hormone-releasing hormone, and gonadotropins. This array of hormone resistance resulting from Gαs insufficiency is known as pseudohypoparathyroidism (PHP) type 1A [97, 100, 101]. Maternal-specific inheritance of hormone resistance results from expression of Gαs from the maternal allele in tissues such as the renal proximal tubule, thyroid, pituitary, and gonads [102–106]. In other tissues, where Gαs is not imprinted, however, expression of both mutated alleles produces Gαs haploinsufficiency, leading to the AHO phenotype.

Gαs loss-of-function mutations do not always result in pluripotent phenotypes. Those with pseudopseudohypoparathyroidism type 1B (PHP1B), for example, have renal PTH resistance without AHO or resistance to any other hormone. In fact, Gs function is normal in some tissues from PHP1B patients. In such cases, imprinting of *GNAS1* exon 1A region determines the transcriptional status of the Gαs promoter in proximal tubules. Loss of this imprinting pattern due to the deletion of nearby genes, such as STX16 or NESP55, results in the loss of maternal imprinting pattern throughout *GNAS* [107–109]. Since Gαs is usually expressed primarily from the maternal allele in renal proximal tubules [102], an abnormal paternal imprinting pattern would lead to Gαs deficiency and renal PTH resistance. It has been proposed that this may result from the activation of a repressor(s) due to the effect of demethylation, thereby causing the Gαs promoter to cease activity. The Gαs deficiency in affected tissues causes PTH resistance [102].

The study of activating and inactivating *GNAS1* mutations, therefore, has identified tissue-specific regulation of GPCR signaling. On one extreme, disruptions to the Gαs subunit, can resemble phenotypes caused by numerous constitutively active receptor variants, while on the other extreme they can resemble complex phenotypic patterns of tissue-specific receptor inactivation. In addition to G protein subunits, accessory proteins also have a significant influence on the activity of a multitude of receptors.

3 Accessory Proteins

The complexity of the disruptions possible in GPCR signaling becomes increasingly evident as accessory proteins are studied in disease. In addition to the accessory proteins involved in regulating the duration of the GPCR signal, such as β-arrestin (reviewed in Subheading 4.2), other classes of accessory protein facilitate and focus GPCR signaling. These proteins include the regulators of G protein signaling (RGS) proteins and the activators of G protein-signaling (AGS) proteins [110, 111]. While RGS proteins act to enhance the GTPase activity of Gα that follows G protein coupling [87, 112–114], the actions of AGS proteins are receptor independent [110, 111]. In selected cases, examples of accessory proteins implicated in human disease (*see* Table 1) provide an insight into signaling pathways.

3.1 Activators of G Protein Signaling

The AGS proteins comprise a group of about ten structurally diverse proteins that have in common the ability to activate Gβγ-dependent signaling, as originally discovered through a yeast-based screening system developed by Lanier and coworkers [118]. The largest subgroup of these, the Group II AGS proteins, includes most of the known proteins that contain one or more G protein signaling modifier (GPSM) domains (also referred to as GPR or GoLoco domains) [119]. Such domains bind to a subset of Gαi proteins and impede GDP dissociation, and the GPSM proteins have been implicated in regulating functions as diverse as asymmetric cell division, differentiation, autophagy, receptor trafficking, and addictive behavior [118]. The remaining Group I and III AGS proteins activate signaling by a variety of incompletely understood mechanisms and essentially lack any homology with one another [118].

It is thought that AGS proteins may contribute to the pathological GPCR-mediated responses to environmental stressors characteristic to some disease states. Although not a typical example, AGS8, a member of group III, has been implicated in remodeling the G protein signaling networks of cardiomyocytes that are subjected to hypoxia [111]. AGS8 is hypoxia inducible and enhances GPCR signals by directly interacting with Gβγ. The upregulation of AGS8 in hypoxic cardiomyocyte cells is probably major a component of the signal remodeling that occurs during ischemic heart disease. Thus, the kinase-dependent pathways involved in the collateral growth characteristic of remodeling can be engaged independent of GPCR activation. AGS proteins, therefore, represent a class of accessory proteins that may be critical to refining GPCR signaling pathways.

Table 1
Genes encoding accessory proteins for G protein-coupled receptors that are disrupted in human genetic disease

Gene	Variant/allele	Disease/phenotype	Pharmacology	References
$G\beta_3$, guanine beta-3 (*GNB3*) 12p13	825C>T SNP alternative splice	Hypertension	Shortened $G\beta_3$	[63–65]
		Metabolic syndrome, obesity, insulin resistance, dyslipidemia	↑G protein signal Abnormal stability of the functional interactions of the shortened $G\beta_3$ proteins	[66–68]
		Alzheimer's disease, autonomic nervous system changes, sudden death		[73, 74]
		Tumor progression		[75, 76]
		Polymorphic drug response marker		[64, 77, 80]
	Arg201Leu	McCune–Albright's syndrome; fibrous dysplasia of bone; café-au-lait skin lesions; sexual precocity; pituitary, thyroid, or adrenal tumors	Activating $G\alpha s$ variants with constitutive cAMP production	[92–95]
Gs, alpha (*GNAS*) 20q13.2	Insertions/deletions and SNPs, 20 % in exon 7 Haploinsufficiency	Albright's hereditary osteodystrophy (AHO), short stature, obesity, brachydactyly, subcutaneous ossifications, developmental deficits	Inactivating $G\alpha s$ variants lead to variable phenotype related to insufficient parathyroid hormone receptor (PTHR1) in chondrocytes	[96–99]
	Inheritance of paternally imprinted gene in exon 1A	Pseudopseudohypoparathyroidism type 1B (PHP1B)	Renal PTH (parathyroid hormone) resistance without AHO	[98, 99]

(continued)

Table 1 (continued)

Gene	Variant/allele	Disease/phenotype	Pharmacology	References
	Inheritance of maternally imprinted gene	Pseudohypoparathyroidism type 1A (PHP)	AHO and resistance to multiple hormones	[97, 100, 106]
Regulator of G protein signaling 2 (*RGS2*) 1q31	1166A > C variant located in the 3′UTR	Bartter's/Gitelman's (BS/GS) angiotensin II-related vasomotor tones are blunted	RGS2 maximally stimulated: failure to regulate nitric oxide and cGMP	[130–133]
	Many SNPs, insertions/ deletions: 1891–1892 TC 2138–2139 AA	Haplotypes associated with hypertension	RGS2 mRNA ↓ in fibroblasts and peripheral blood mononuclear cells	[125, 134]
G protein-coupled receptor kinase 1, rhodopsin kinase (*RHOK/GRK1*) 13q34	Exon 5 deletion	Oguchi disease, recessively inherited stationary night blindness	Impairment of GRK1-mediated desensitization of rhodopsin	[204–206]
G protein-coupled receptor kinase 4 (*GRK4*)	Arg65Leu, Ala142Val, and Ala486Vval	Hypertension, sodium sensitivity	GRK4 activity increased: ↑ Dopamine D_1 receptor desensitization ↑ Angiotensin II type 1 receptor expression	[201–203]

cAMP cyclic adenosine monophosphate, *cGMP* cyclic guanosine monophosphate, *mRNA* messenger RNA, *SNP* single-nucleotide polymorphism

3.2 Regulators of G Protein Signaling

The RGS proteins are GTPase accelerating proteins (GAPs) and are involved in the inactivation of the signal resulting from the coupling of GPCRs to heterotrimeric G proteins. G protein deactivation occurs upon the hydrolysis of Gα-bound GTP to GDP. As shown in Fig. 2, the RGS proteins bind directly to activated Gα–GTP to serve as GAPs. These proteins limit the half-life of Gα–GTP by accelerating the GTPase activity of the Gα subunit, thereby facilitating the termination of signaling [87, 112, 116]. RGS polymorphisms have been associated with disease states [115].

The RGS proteins exemplify the importance of accessory proteins to receptor function [115–135]. In mammals there are 21 different genes that encode RGS proteins, with several having multiple splice variants [87]. These are categorized into four subfamilies based on structural and sequence similarities, and as well there are a number of related "RGS-like" proteins, some of which can also act as GAPs on heterotrimeric G proteins [87]. All RGS proteins have the ability to promote GTP hydrolysis by members of the Gαi subfamily, although RGS2 has uniquely low affinity for these [116]. About half of the RGS proteins additionally are GAPs for Gαq proteins. Gαq GTPase activity is also accelerated by its effector phospholipase Cβ [121]. Similarly the $G\alpha_{12/13}$ effectors p115-RhoGEF, PDZ-RhoGEF, and leukemia-associated RhoGEF (LARG), each of which contains an RGS-like domain, can act as GAPs for Gα12/13 [113, 116].

The GTPase activity of Gαs is unaffected by RGS proteins; however, some RGS proteins such as RGS2, RGS3, and RGS13 appear to be able to block Gs-stimulated cAMP production by AC [116]. The inhibition of G protein–effector coupling, absent any measurable effects on GTPase activity (sometimes referred to as "effector antagonism"), has been observed with Gq signaling as well. This presumably reflects the physical disruption of G protein–effector complexes by RGS proteins [87].

In solution, the affinity of RGS proteins for their Gα binding partners tends to be increased when the latter are activated, and several studies have shown RGS protein localization to the plasma membrane to be increased by the presence there of activated G proteins [116]. Other evidence suggests, however, that RGS recruitment to the membrane can occur in a manner independent of the state of activation of the G protein, and that RGS protein binding to phospholipids is also an important consideration in this context [116]. Recruitment may facilitate signal quenching. A combination of 30 RGS proteins and 20 Gα subunits allows for a diverse pattern of inactivation. RGS proteins, therefore, are recruited to the plasma membrane in cells expressing either Gα subunits (Gαs) or linked GPCRs in preparation for the GAP activity that quenches G protein signaling [87, 114].

Regardless of whether or not RGS recruitment depends on the activation state of either receptor or G protein, there is evidence

that RGS proteins can bind directly to GPCRs [116]. It thus is possible that the receptors recruit RGS proteins nearer to their G protein targets [117]. In other cases, targeting of RGS proteins to G proteins may be enhanced via scaffolding proteins, such as spinophilin and GIPC, or alternatively by G protein effectors [87]. Thus, the selective sorting of RGS proteins at the plasma membrane through various scaffolding mechanisms may serve to orient and optimize their GAP activity toward the linked Gα, shadowing their function in regulating G protein function.

Insights into GPCR signal termination may suggest strategies for designing drugs that selectively optimize RGS activity [87, 114] in a specific disease, such as essential hypertension. As with the other systems described, naturally occurring RGS variants may alter receptor function by altering the interaction of RGS proteins with the receptor.

3.3 Polymorphisms of the RGS2 Gene in Hypertensives

RGS2 preferentially alters Gαq-mediated signaling [50, 87, 116, 121, 128, 129]. In hypertension, this may be particularly relevant with respect to the signaling of the angiotensin II type I receptor. While the receptor itself has been independently implicated in hypertension because of the 1166A>C variant located in the 3′ untranslated region (3′UTR) [87, 130, 131], in Bartter's/Gitelman's syndrome (BS/GS) patients, angiotensin II-related signaling and vasomotor tone can be blunted independent of the 3′UTR variant. In BS/GS, *RGS2* gene expression is maximally stimulated in BS/GS: suggesting a link between BS/GS genetic abnormalities and abnormal vascular tone regulation [132]. Pathogenic effects may result from the failure of RGS2 to regulate nitric oxide and cGMP through adequate phosphorylation of RGS2 by cGMP-dependent protein kinase 1α (PKG) [127–129, 132]. *RGS2* knockout mice exhibit an alteration in smooth muscle relaxation that is associated with hypertension [128, 129]. Although BS/GS pathogenesis may not be directly attributed to *RGS2* variants, these data do provide a better insight into the regulation of RGS proteins by Rho inhibition of PKG [133].

The *RGS2* gene variants are found at various frequencies in different populations. Genetic variation in the human *RGS2* gene consists of at least 14 single-nucleotide polymorphisms (SNPs) and 2 two-base insertion/deletions (in/del; 1891 to 1892 TC and 2138 to 2139 AA) [115, 134]. Most coding variants are reported at low allelic frequency; however, the C1114G polymorphism was associated with lower *RGS2* gene expression in some populations [135].

The intronic 1891 to 1892 TC and 2138 to 2139 AA in/del variants, however, are more common. These variants have been reported to be in linkage disequilibrium and are associated with hypertension in African Americans. Two haplotypes are reported to have significantly different frequencies between hypertensives

and normotensives—but only among African American groups—reflecting the unique epidemiology of essential hypertension in the African American population. The intronic in/del haplotypes may serve as ethnicity-specific genetic variants for essential hypertension [115, 134].

Various measures suggest that RGS2 expression is reduced in these patients. RGS2 messenger RNA (mRNA) expression was significantly lower in peripheral blood mononuclear cells (PBMC) and in fibroblasts from hypertensives in comparison to normotensives. C1114G polymorphism was associated with RGS2 expression, with the lowest values in GG hypertensives. The 1114G allele frequency was increased in hypertensives compared with normotensives. These finding suggest that insufficient RGS2 expression results in a failure to limit the half-life of Gα–GTP that would normally result from RGS activation of the Gα subunit's GTPase activity: preventing the termination of signaling [87, 112–114].

4 Inactivation of GPCRs

Whereas continuous exposure of a GPCR to an agonist normally produces a self-limited signal [39–44], disease states are often characterized by unlimited signaling. Two examples worthy of discussion are Oguchi disease, caused by disruption of GRK1 inactivation, and essential hypertension associated with GRK4 variants. Disruption of GRK activity is discussed with respect to Oguchi disease and to essential hypertension in separate discussions in Subheading 4.3.

Inactivation, a process that reduces the cellular response to the agonist, is illustrated schematically in Fig. 2. It is often measured by quantitating the change in second-messenger production, such as cAMP production by AC, following prolonged exposure of one type of receptor to an agonist [136]. The study of natural and artificial mutations of GPCRs and the genes encoding proteins involved in inactivation, such as GRK1 and GRK4, has identified many protein motifs that are essential to the inactivation process. Residues that may be involved in the inactivation in the dopamine D_1 receptor are shown in Fig. 3. The contribution of specific residues to these processes is determined by the extent to which the signal is limited by the ability of wild-type and mutated GPCRs to inactivate in response to agonist [137].

4.1 Desensitization

The process known as desensitization, taking place within a time frame of seconds to minutes following agonist exposure, occurs when the receptor uncouples from its G protein. This results from conformational changes that result from agonist-dependent phosphorylation, often as a result of GRK activity. The desensitized receptors undergo plasma membrane clustering and

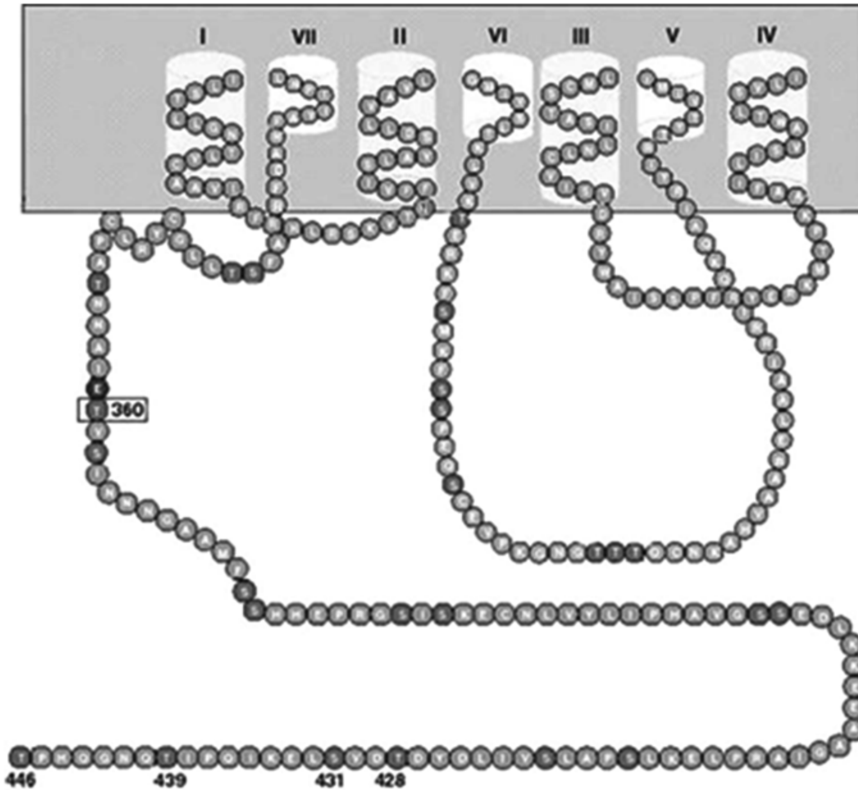

Fig. 3 Amino acid residues required for receptor desensitization and internalization: the dopamine D receptor example. The substitution of 359Glu or 360Thr by Ala results in desensitization-deficient mutants of the dopamine D1 receptor, but they are still able to internalize to some extent. Phosphorylation sites in a 12-amino acid stretch of the distal carboxyl tail (428Thr to 439Thr and 446Thr) may be involved in internalization of the receptor. The variant constructs (substitutions by Ala) were generated by site-directed mutagenesis and expressed in cultured Chinese hamster ovary (CHO) cells [137]

endosome-mediated internalization and are finally targeted for degradation unless they are recycled back to the cell surface. If receptors are lost from the cell surface, down-regulation is said to have taken place. This may be transient, in the case of intracellular sequestration, or longer term if protein synthesis is unable to keep pace with receptor loss [39]. Two patterns of desensitization, homologous and heterologous, have been characterized [138]. While phosphorylation of GPCRs is associated with both forms [139, 140], it is the GRK enzymes that tend to be implicated in the homologous form that will be of interest in discussing the events relevant to Oguchi disease and various hypertension phenotypes.

Agonist-specific desensitization, generally termed homologous desensitization, is associated with agonist-dependent GRK phosphorylation. Originally characterized in the case of rhodopsin, it was later found to be common among GPCRs.

Homologous desensitization occurs rapidly when GPCRs are exposed to high (micromolar) agonist concentrations [141–143]. Non-activated receptor systems are spared, however, and continue to function normally.

Historically, heterologous desensitization was described as a slower response to agonist (minutes rather than seconds) that occurs even when GPCRs are exposed to lower agonist concentrations. It may involve the diminished response of many kinds of GPCRs, including receptors that have not been exposed to ligand. This appears to occur even if GPCRs share few, if any, common signaling pathways or effectors [141–143].

Second-messenger-dependent kinases, such as cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC), are most often implicated in heterologous desensitization [138, 144]; however, the systems involved may vary between cell types [145]. These protein kinases are associated with GPCR desensitization that occurs at slower rates than that reported for the GRKs ($t_{1/2}$ of 3 min compared to 15 s). This probably accounts for the slower time course of heterologous desensitization [146]. For the most part, the following discussion centers on homologous desensitization.

4.1.1 Mechanisms of Homologous Desensitization

The desensitization of most GPCRs appears to be dependent on the carboxyl tail or third intracellular loop regions. For example, the α_{2A}-adrenergic [147], the α_{1B}-adrenergic [148], the *N*-formyl peptide [149], and the M_2 muscarinic acetylcholine [150, 151] receptors all contain clusters of residues in the third intracellular loop that are required for desensitization.

While GRK2, 3, and 5, phosphorylation has been associated with agonist activation of many receptors [39, 152], only discrete regions of phosphorylation that are attributable to one specific enzyme appear to be essential for desensitization [137]. With respect to the β_2-adrenergic [153–156], the dopamine D_1 [137], the μ-opioid [157], the δ-opioid [158], the α_{1B}-adrenergic [148], the A_3 and A_{2a} adenosine [159–161], and the *N*-formyl peptide [149] receptors, the motifs may be located in the carboxyl tail.

The desensitization motifs in the dopamine D_1 receptor, as an example, may be at least partly located in the proximal carboxyl tail of the receptor [137]. It is likely that this region interacts with portions of the third intracellular loop in order to promote desensitization. These structures may also be involved in recycling and trafficking of inactivated receptors [162, 163]. A portion of the proximal carboxyl tail of the dopamine D_1 receptor may contain some of the residues necessary, but not sufficient on their own, for GRK2 mediated desensitization. A motif consisting of a serine or threonine preceded by an acidic amino acid may define the GRK2 recognition sequence [163].

For the dopamine D_1 receptor, the 360Thr and preceding 359Glu may play a role (Fig. 3). Normal desensitization of the

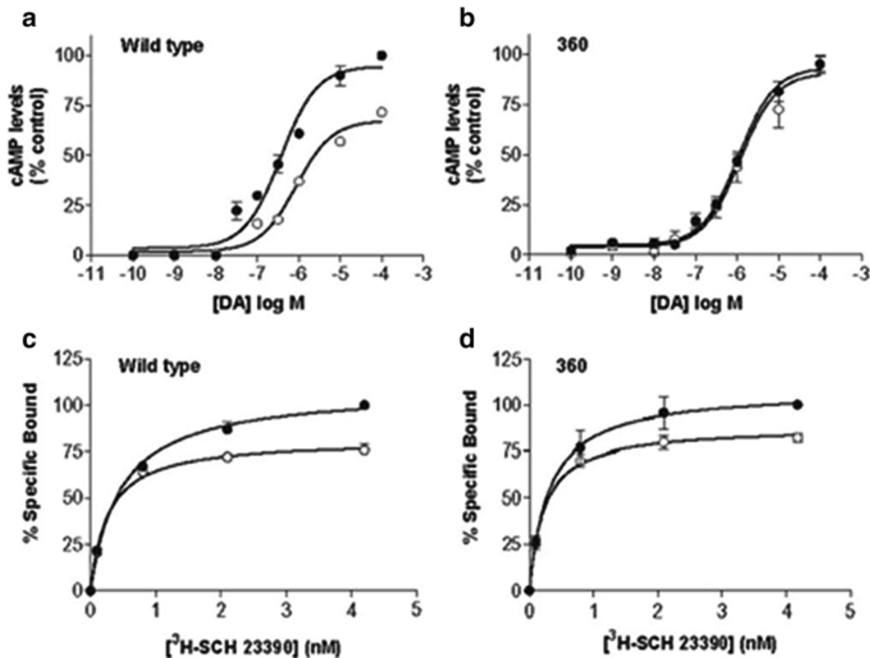

Fig. 4 In vitro effects of mutation on desensitization and internalization of the dopamine D1 receptor. Shown here are effects of mutation on dose-dependent intracellular cyclic adenosine monophosphate (cAMP) accumulation (**a** and **b**) and binding curves (**c** and **d**) for artificial ligand (SCH 23390) using three constructs: controls (wild-type, **a** and **c**) and the Thr360Ala mutant (360, **b** and **d**). In the desensitization experiments, cells were preincubated with 10 μM dopamine (*open circle*) or vehicle (*closed circle*) for 20 min, and increasing concentrations of dopamine (10^{-10} to 10^{-4} μM) were added to assess cAMP accumulation. Desensitization of the wild-type receptor (**a**), defined by an increase in K_m and decrease in V_{max} for agonist-pretreated compared with naïve cells was abolished (with respect to efficacy and potency) disappeared with the Thr360Ala mutation (**b**). Conversely, internalization, defined as a loss of cell surface receptors (measured by decreased maximal binding or B_{max} assessed by SCH23390 binding) is unchanged from wild-type (**c**) after pretreatment with 10 μM dopamine (*open circle*, compared to vehicle *closed circle*), for the Thr360Ala mutation (**d**)

wild-type dopamine D_1 receptor (Fig. 4a), was abolished when the Thr360 residue was substituted for Ala (*see* Fig. 4b). Although desensitization appeared intact when other carboxyl terminal serine and threonine residues were eliminated (Fig. 3, distal carboxyl tail), it was eliminated when the acidic residue present at 359Glu was mutated to alanine (data not shown). In this model, the acidic 359Glu may be necessary to potentiate basal levels of phosphorylation of the critical 360Thr residue [137]. In principle, these findings are analogous to evidence suggesting that the rhodopsin receptor requires critical acidic residues, such as 341Glu, to maintain both basal phosphorylation and agonist-induced phosphorylation of 338Ser [163].

GRK-related mechanisms of agonist-induced desensitization, however, are likely to depend on patterns of GRK phosphoacceptors at many serines and threonines [38, 137]—in a barcode-like

fashion that may depend on receptor conformation [164]. There is evidence that phosphorylation of the serines and threonines located in the third intracellular loop may, in at least some cases, be a co-requisite for desensitization [165] in many receptors. Third-loop mutations exhibit attenuated agonist-induced receptor phosphorylation that correlates with an impaired desensitization response [165]. It seems likely that, for some receptors, the role of the third loop and the distal proximal tail in desensitization is dependent on the complementary structure. This may reflect a requirement for an interaction between the third intracellular loop and portions of the carboxyl tail in sustaining agonist-dependent desensitization that is dependent on GRK phosphorylation of the carboxyl tail. Thus, the role of receptor phosphorylation may be to create a receptor conformation that will allow its interaction with proteins integral to the desensitization process [3, 164]. One such group of proteins, indicated in Fig. 2, are the arrestins.

4.2 Internalization

GRK-mediated phosphorylation of the receptor is often required to promote the formation of the β-arrestin complex that can be internalized [166, 167]. The pathway of arrestin-mediated GPCR internalization that involves the transfer of ligand-activated receptors from the plasma membrane to an intracellular compartment [168] is shown in Fig. 2.

Although internalization is also often described to be a phosphorylation-dependent process, Fig. 4c, d shows that receptors do not always require phosphorylation of the same residues to desensitize [137]—and for the recycling of inactivated receptors to the cell membrane [162]—as they do for receptor endocytosis [137, 162]. The process of internalization, however, is integral to the membrane trafficking of GPCRs. The mechanisms that are critical to the maintaining the appropriate quantity of receptors at the cell surface [169] can be teased apart using examples from a number of different receptors [170].

In the case of the β_2-adrenergic receptor, phosphorylation of serine and threonine residues in the carboxyl tail can be shown to be involved in desensitization and internalization [156, 171]. Other GPCRs—such as the μ- and δ-opioid receptors [172, 173] and the A_{2b} adenosine receptor [174]—require analogous serine and threonine residues in the carboxyl tail for both desensitization and internalization [172, 173].

While reproducible for many receptors, this phenomenon is not universal for GPCRs. For example, in the case of the M_2 muscarinic receptor, while two-thirds of intracellular loop clusters of Ser/Thr residues (286Ser-290Ser and 307Thr-311Ser) mediate internalization, only the carboxyl terminal (307Thr-311Ser) cluster mediates desensitization [151]. In conclusion, internalization

may follow desensitization, or it may occur independently [175] with or without the influence of other regulatory processes [176].

For the dopamine D_1 receptor, normal internalization may be dependent on distal carboxyl terminal residues (*see* Fig. 3) that are independent of the 360Thr that may be required for desensitization (*see* Fig. 4c, d). Therefore some, although not all, GPCRs show radical dissociation between desensitization and internalization. This is found not only in the dopamine D_1 receptor [137] but also in the *N*-formyl peptide [149] and the M_2 muscarinic [170] receptors.

Regardless of the GPCR residues involved, the involvement of β-arrestin in GPCR internalization has been particularly well elucidated. First, the binding of β-arrestin to the GPCR sterically inhibits interaction of the receptor with G proteins [177]. The displaced receptor–β-arrestin complex is then free to bind with high affinity to clathrin chains [178]. This recruitment of the complex to clathrin-coated pits allows the incorporation of the GPCRs into lipid vesicles. Internalization follows when the vesicles are pinched off the cell membrane by the GTPase dynamin [179–181]. Subsequently, the internalized receptors are either recycled back to the plasma membrane or are targeted, within days or hours, for degradation in lysosomes [182].

In some cases, for example, in the case of the β_2-adrenergic receptor, internalization has been found to be a precursor to resensitization of the receptor [183, 184]. This phenomenon may be common to many GPCRs. Internalization may afford the opportunity of receptor dephosphorylation through the action of an endosomic acid phosphatase [185], resulting in resensitization of the receptor [186].

While it is often convenient to model internalization as a process that follows desensitization, the evidence now suggests that, although often linked, these processes can be distinct [187]. For some receptors, such as the β_2-adrenergic receptor [153], the forms of internalization that are distinct from desensitization may include those that are arrestin independent. Less is known, however, about the pathways of internalization that may not involve arrestin.

The residues required for internalization, like those implicated in desensitization motifs, do not always meet the requirements for putative sites of kinase-mediated phosphorylation. Among the numerous motifs that have been implicated, an NPXXY motif [169, 188] may be required for agonist-induced activation and internalization of the β_2-adrenergic receptor, and a dileucine motif in the carboxyl tail of many GPCRs [169] may be involved in internalization of receptors such as the β_2-adrenergic [189] and the vasopressin V_{1a} receptors [190].

While GPCR phosphorylation at serine and threonine residues is involved in the internalization pathways of many receptors

[149, 191–193], it is likely that for some GPCRs internalization pathways may be distinct [149, 191–193]. These apparently non-arrestin mechanisms of internalization, however, may vary more between receptors than those identified for GRK-dependent processes [194–195].

4.3 The Family of GRK Enzymes

The GRK family consists of seven well-characterized enzymes. These enzymes are distinguished by (1) the structural homology within the family, (2) the specific amino acid sequences that a given GRK can phosphorylate, (3) enzyme kinetics [184, 196], and (4) GPCR disease phenotypes that are often manifested by dysregulation of GRK activity. Gain-of-function GPCR mutations are frequently found to be constitutively phosphorylated. Conversely, inadequate receptor desensitization and sequestration often result.

Much has been learned about GPCR biochemistry from contrasting the GRK1-like, GRK2-like, and GRK3-like subfamilies in health and disease [184]. The role of the GRKs is indicated schematically in Fig. 2. Substrate specificity of the GRKs may be a factor in the degree to which specific tissues are affected by deleterious GPCR mutations [197]. Of all the GRK family, the GRK2 amino acid sequence is most widely divergent from GRK1, which may also be a factor in defining which tissues are affected by ectopic GPCR phosphorylation [163]. However, substrate specificity is also defined by the amino acid sequence of GPCRs adjacent to serine/threonine residues. While GRKs 1 and 2 require adjacent acidic residues, respectively, on the carboxyl and amino terminal flanks of the phosphorylation site, GRK4 specifically phosphorylates at sites adjacent to basic amino acid residues. This evidence for GRK substrate specificity affords us a significant insight into the molecular pathology of phenotypes that may involve GRK activity [184].

The GRK1 subfamily, consisting of GRK1 and GRK7, is known to be involved in the pathophysiology of deleterious rhodopsin mutations that underlie several inherited retinal disorders, including Oguchi disease. While GRK1 is the prototypic GRK enzyme rhodopsin kinase [184], both the GRK1 and GRK7 enzymes are expressed in the retina and act to quench the rhodopsin signal transduction after light activation [198]. The involvement of GRK7 in retinal disease has not been confirmed.

The GRK2 subfamily, consisting of GRK2 and GRK3, acts on a wide range of GPCRs that are expressed in many tissues. The GRK2 enzymes were first characterized in studies of the phosphorylation of agonist-occupied β_2-adrenergic receptors [184]. GRK2 enzymes contribute to disease. For example, GRK2 gain-of-function mutations affect the leuteinizing hormone (LH) receptors that are associated with Leydig cell hyperplasia [199].

The GRK4 subfamily is best understood in the context of the prototypical GRK1 and GRK2 subfamilies [184]. The GRK4 subfamily

consists of the GRK4, GRK5, and GRK6 enzymes [200]. In contrast to GRK1 and GRK2 enzymes, GRK4 enzymes selectively phosphorylate residues with an amino terminal basic amino acid. GRK4 has been found to have potential significance in systems as well characterized as dopamine D_1 receptor desensitization [201]. In the context of the role of the dopamine D_1 receptor in the kidney, GRK4 enzyme variants are in the subheading that deals with phenotypes associated with essential hypertension [202, 203].

4.3.1 Oguchi Disease: Defective GRK1 Phosphorylation of Rhodopsin

Receptors that remain in the activated state even in the absence of ligand are often known as constitutively active mutants (CAMs). The resulting disruptions in rhodopsin signaling also often result in alterations in the phosphorylation of rhodopsin by rhodopsin kinase (GRK1), the specialized GRK enzyme expressed in the retina that is largely responsible for rapidly desensitizing the receptor when it is exposed to light.

In fact, a group of rhodopsin-related disorders results from mutations in the *GRK1* gene itself. The result is Oguchi disease, a rare, recessively inherited retinopathy [204]. The Oguchi mutations result in the impairment of GRK1-mediated desensitization of rhodopsin that is not compensated by normal expression of another GRK enzyme, such as GRK7 [198]. The *GRK1* mutations disrupt the pathway of light-dependent rhodopsin phosphorylation that is normally required for quenching light-induced signal transduction in photoreceptor cells.

In vitro experiments have demonstrated that a deletion of exon 5 of the *GRK1* gene is a null mutation that abolishes the enzymatic activity of GRK1 [204]. Because both homozygous and heterozygous states for this mutation lead to disease [205], it is likely that GRK1 integrity is critical to retinal health. As a result of these observations, it is possible that a dominant negative effect or a *GRK* gene dose effect may be involved in retinal disease.

In vivo functional characterization of *GRK1* gene mutations has demonstrated that they prevent rhodopsin phosphorylation and subsequent arrestin binding. Interestingly, when studied ex vivo, rod cells expressing *GRK1* gene mutations also exhibited a greatly diminished attenuation of light sensitivity [206]. Thus, the function of GPCRs in healthy tissues may depend on the integrity of GRK-dependent processes.

4.3.2 Essential Hypertension: GRK4 Polymorphisms and Excessive Phosphorylation of the Dopamine D_1 Receptor

The GRKs have been implicated in genetic and acquired hypertension because they participate in the desensitization of GPCRs, including D_1 receptor and the angiotensin II type I receptor [201, 203]. For example, basal GRK-dependent phosphorylation of serine residues of the D_1 receptor is increased in the renal proximal tubules in animal models as well as in humans with essential hypertension. Of the α/β- and γ/δ-isoforms of GRK4 expressed in the kidneys, the γ-isoform was found to be

polymorphic, confirming the *GRK4* locus linkage with essential hypertension [201, 202].

The *GRK4* SNPs include Arg65Leu, Ala142Val, and Ala486Val. Dopamine D_1 receptor-mediated cAMP production is reported to be markedly impaired by these variants. Expression of these SNPs is also associated with increased basal phosphorylation of the dopamine D_1 receptor. This suggests that increased basal phosphorylation of the dopamine D_1 receptor by GRK4 may be associated with the decreased responsiveness of the dopamine D_1 receptor in hypertension [202, 203].

In vitro studies suggest that the *GRK4* SNPs impair the function of D_1 receptors, increase blood pressure, and impair the diuretic and natriuretic effects of dopamine D_1-like agonist stimulation. Inappropriate desensitization of the dopamine D_1 receptor in renal proximal tubules in hypertension may result in the decreased ability of the kidney to eliminate a sodium chloride load—a key risk factor in the development of hypertension.

The effect of GRK4 disruption is widespread in affected tissues. In addition to abnormal desensitization of the dopamine D_1 receptor, GRK4 polymorphisms are associated with increased expression of another regulator of sodium load, the angiotensin II type 1 receptor. The findings suggest that dysregulation of GPCR systems might be corrected by blocking the effects of GRK4 in patients who harbor GRK4 polymorphisms. The principle of targeting accessory proteins might be applied to other disorders that involve disruptions to normal GPCR signaling [201, 203].

5 Conclusion

Insights into the processes of GPCR activation and inactivation have developed hand in hand with an appreciation of the accessory proteins necessary to these processes. This has accelerated progress in understanding the fundamental mechanisms involved in GPCR synthesis, transport to the membrane, ligand binding, and activation and inactivation by GRK-mediated (and other) phosphorylation [207]. The catalog of Gαs and Gβ subunit polymorphisms that result in complex phenotypes has complemented this effort.

Significantly, the study of GPCR accessory proteins has provided an insight into pathways of disease, such as the contributions of RGS proteins to hypertension and AGS proteins to myocardial hypoxia. In the case of the GRKs, identified originally in the retina as integral to the pathways that involve rhodopsin, proteins such as GRK4 have been identified that have been subsequently associated with hypertension. These studies show how classical human genetics can become an entrez into the genomics and pharmacogenomics of an entire class of receptors and associated systems.

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Chapter 8

G Protein-Coupled Receptor Mutations and Human Genetic Disease

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Abstract

Genetic variations in G protein-coupled receptor genes (*GPCRs*) disrupt *GPCR* function in a wide variety of human genetic diseases. In vitro strategies and animal models have been used to identify the molecular pathologies underlying naturally occurring *GPCR* mutations. Inactive, overactive, or constitutively active receptors have been identified that result in pathology. These receptor variants may alter ligand binding, G protein coupling, receptor desensitization and receptor recycling. Receptor systems discussed include rhodopsin, thyrotropin, parathyroid hormone, melanocortin, follicle-stimulating hormone (FSH), luteinizing hormone, gonadotropin-releasing hormone (GNRHR), adrenocorticotropic hormone, vasopressin, endothelin-β, purinergic, and the G protein associated with asthma (GPRA or neuropeptide S receptor 1 (NPSR1)). The role of activating and inactivating calcium-sensing receptor (*CaSR*) mutations is discussed in detail with respect to familial hypocalciuric hypercalcemia (FHH) and autosomal dominant hypocalemia (ADH). The *CASR* mutations have been associated with epilepsy. Diseases caused by the genetic disruption of *GPCR* functions are discussed in the context of their potential to be selectively targeted by drugs that rescue altered receptors. Examples of drugs developed as a result of targeting *GPCRs* mutated in disease include: calcimimetics and calcilytics, therapeutics targeting melanocortin receptors in obesity, interventions that alter GNRHR loss from the cell surface in idiopathic hypogonadotropic hypogonadism and novel drugs that might rescue the P2RY12 receptor congenital bleeding phenotype. De-orphanization projects have identified novel disease-associated receptors, such as NPSR1 and GPR35. The identification of variants in these receptors provides genetic reagents useful in drug screens. Discussion of the variety of *GPCRs* that are disrupted in monogenic Mendelian disorders provides the basis for examining the significance of common pharmacogenetic variants.

Key words G protein-coupled receptor, Gain of function, Loss of function, Monogenic disease, Rhodopsin, Follicle-stimulating hormone receptor (FSHR), Gonadotropin-releasing hormone receptor (GNRHR), Luteinizing hormone receptor (LHCGR), Melanocortin receptor (MC1R, MC4R), Parathyroid hormone receptor, Thyrotropin receptor (TSHR), Calcium-sensing receptor (CaSR), Vasopressin receptor (AVPR2), De-orphanized receptors (GPRA, NPSR1, GPR35, GPR55)

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1 Introduction

The properties that define G protein-coupled receptors (*GPCRs*), their utility in drug discovery, and their signaling characteristics have been reviewed in Chapter 7 [1–5]. *GPCR* sequence variability is significant and each variant provides an opportunity to study receptor function in vivo that complements a plethora of available in vitro data on the pharmacology of the *GPCRs*. Variation in the genes encoding the *GPCRs* are associated with a spectrum of predispositions and drug responses. In Chapter 9, the discussion extends to *GPCR* variants that are associated with a phenotype of altered drug efficacy or altered susceptibility to disease.

Here, we discuss the wide variety of *GPCR* gene variants and mutations as they contribute to human Mendelian disorders. The receptors mutated in monogenic disease phenotypes are discussed in the context of signaling disruptions [1–3]. Refining our knowledge of the genes that encode *GPCRs* helps to define (1) the properties of the largest class of transmembrane (TM) receptors with respect to their genomic, protein, and signaling properties and the many putative drug targets available for drug discovery using "reverse pharmacology"; (2) the genetic predisposition to disease states that can result from sequence variation in the genes encoding these receptors; and (3) the basis of variability in drug response and toxicity (pharmacogenetics) and subsequent alterations in drug efficacy.

Estimates of receptor efficacy and potency are two of the common ways to determine whether a *GPCR* variant results in the radically disrupted signaling characteristic of disease or the more subtle alterations in signaling relevant to pharmacogenetics. Drug efficacy describes the extent to which ligand activation of a receptor results in maximal stimulation (Vmax) of a relevant signaling pathway (e.g., adenylyl cyclase generation of cyclic adenosine monophosphate [cAMP]). By contrast, drug potency denotes the concentration of ligand that results in half-maximal response EC_{50} of a signal such as cAMP stimulation. Variants or polymorphisms in *GPCR* genes include coding and noncoding protein variants that sometimes alter efficacy and potency.

Here we discuss mutant *GPCR* genes that are known to cause disease through the expression of defective receptor proteins that have been shown in vitro to result in receptors that are inactive or constitutively active. Mutations that cause inactive receptor proteins are often referred to as loss-of-function (LOF) mutations. Among the LOF mutations, some result in a dominant negative phenotype, indicating that, among heterozygotes, expression of the LOF variant impairs the function of the wild type. By contrast, constitutively active mutants (CAMs) result in autonomous signaling in the absence of agonist.

Although originally described in vitro, CAMs have now been described for many members of class A, B, and C *GPCR* families [3, 4]. The two extreme receptor states, LOF and CAM, result from changes in ligand binding, G protein coupling, receptor desensitization, and receptor recycling [3, 4]. The investigation of these mutations is critical to understanding the causes of human genetic disease and provides perspective on strategies for drug discovery that take into account the potential for the development of drugs targeted at mutated and wild-type *GPCRs* [3–7]. Advances in our knowledge of both receptor structure and function also facilitate the discussion of *GPCR* pharmacogenetics outlined in Chapter 9.

Selected examples include those disorders resulting from mutations in rhodopsin, thyrotropin (formerly called thyroid-stimulating hormone, TSH), luteinizing hormone (LH), vasopressin, angiotensin receptors, and the de-orphanized *GPCR* associated with asthma (GPRA), now known to be the neuropeptide S receptor 1 (NPSR1). A de-orphanized receptor is one whose endogenous ligand has been identified subsequent to the gene's identification.

Recurrent pharmacogenetic variants, however, may not result in monogenic disorders but are likely to result in an altered predisposition to developing a complex disease or drug response phenotype. In some cases, such as the calcium-sensing receptor (CaSR), different classes of receptor variant may result in either monogenic disease or variable pharmacology. The pharmacological phenotypes are often reported to result from either a partial gain or a partial loss of receptor signaling. These phenomena, reviewed in Chapter 9, are often defined in terms of alterations of efficacy or potency of the variant receptor with respect to the wild-type receptor. As a result, some of the *GPCRs* mutated in disease are also discussed in Chapter 9 in relation to a different group of variants that are primarily pharmacogenetic variants.

2 Receptor Genes and Disease

The properties of *GPCR* variants are reviewed with respect to what can be learned from prototypical receptors, beginning with rhodopsin. The selected examples are summarized in Table 1 with respect to the common single-nucleotide polymorphisms (SNPs) that cause the disorders.

Disease phenotypes have been associated with both LOF mutations leading to ligand resistance (or reduced binding) and gain-of-function mutations leading to constitutive activation of signaling pathways (or enhanced binding). Examples include the inactivating Met201Val variant of the cysteinyl leukotriene 2 receptor [8] and the activating Gly300Ser variant of the cysteinyl leukotriene 1 receptor [9]: variants that are commonly inherited

Table 1
G protein-coupled receptor (GPCR) sequence variants associated with human genetic disease

Receptor	Variant/allele	Disease/phenotype	Pharmacology	References
Rhodopsin(RHO) 3q21–q24	G90D, A292E, T4K, N15S, T17M, P23H	Retinitis pigmentosa, congenital night blindness	Constitutively active mutant (CAM) receptor	
	L125R			
	K296E, E113, and substitution of adjacent residues		Potentially ruptures the salt bridge by a competitive mechanism	
	E134Q, E134D		↑ Activity; ↓ activity: Substitution of E134 may disrupt structure	[7, 11, 13–22]
Luteinizing hormone/ chorionic gonadotropin receptor (LHCGR) 2p21	Truncated TM5	Leydig's cell hyperplasia	Constitutively activated luteinizing hormone (LH) receptor	[99–105]
	T398M, A568V, M571I, T577I, D578G	Association with familial male precocious puberty		
Follicle-stimulating hormone receptor (FSHR) 2p21–p16	A189V	Ovarian dysgenesis	↓ Affinity for ligand	[92–94]
	D567G	Semen production normal despite ↓ gonadotropins	Constitutively active	[82–88]
	N680S	Pharmacogenetic variant	Population study	[92–98]
	D294H	Redhair/fairskin	↓ Affinity for ligand	
Melanocortin1 receptor (MC1R) 16q24.3	D84E	Development of melanoma		
	V92M	Redhair/fairskin	Activating/inactivating	[70–73]
Melanocortin 4 receptor (MC4R) 18q22	V103I, many SNPs	Morbid obesity, monogenic form of binge eating		[71, 74–80]

Endothelin receptor, type B (EDNRB) 13q22	Many SNPs, W276C	Hirschsprung's disease (one of nine genes at four loci)	↓ Gq coupling in vitro	[159–167]
Adrenocorticotropin receptor (ACTHR/MC2R) 18p11.2	S120R, R201Stop, S74I, V254C, C360G	Isolated glucocorticoid deficiency	Altered/loss of function	
	Promoter polymorphism	Adrenocortical tumors	↓ Expression; loss of heterozygosity in tumors	[119, 120]
Gonadotrophin releasing hormone receptor (GNRHR) 4q21.2	N10K, N10R, E90K, R139H, S217R, T321I	Idiopathic hypogonadotropic hypogonadism (IHH)	Reduced or loss of function	[106–118]
Parathyroid hormone receptor (PTHR1) 3p22–p21.1	H223R, T410P, I458R	Jansen's metaphyseal chondrodysplasia	Constitutively active receptor	[62]
	P132L, Delete. bp 1122 (frame shift), 1176 G/A	Blomstrand's chondrodysplasia	No accumulation of cAMP	
	R150C	Enchondromatosis	Inactivating mutations	[61–69]
Thyroid-stimulating hormone receptor (TSHR) 14q31	P52T, G431S, V509A, C672Y	Autoimmune thyroid disease	Altered receptor function/conformation	[24–38]
	D727E	Grave's disease	Population studies	[26]
	D619G, A623I (somatic)	Toxic multinodular goiter	Constitutive activation of adenylyl cyclase	[31]
		Hyperfunctioning thyroid adenomas		[25, 34]
Arginine vasopressin receptor 2 (AVPR2) Xq28	W71 truncation and many SNPs	Nephrogenic diabetes insipidus	↓ Ligand binding/reduced expression of receptor	[123–158]
G protein-coupled receptor 154, associated with asthma(GPR154/GPRA) 7p15–p14	GPRA-B isoform overexpressed in bronchial epithelia of asthmatics	Asthma	Unidentified ligand suggests that GPRA is a potential drug target	[171–174]

(continued)

Table 1 (continued)

Receptor	Variant/allele	Disease/phenotype	Pharmacology	References
Chemokine, C-C motif, receptor 5(CCR5) 3p21	Δccr5(32-bp deletion)59029 A/G	Partial resistance to HIV infection		
	Homozygous	↓ AIDS progression	Altered binding affinity	[2]
	Heterozygous	↓ Non-Hodgkin's lymphoma		Chapter 9
Purinergic receptor, P2Y, G-protein coupled, 12(P2RY12) 7p13	2-nt deletion	Bleeding disorder	Disrupted Gi/Go inhibition of cAMP accumulation	[168–170]
Calcium-sensing receptor(CASR) 3q13.3–q21	R185Q, E297K, R795W, Arg185Q, R220W	Familial hypocalciuric hypercalcemia (FHH)/ neonatal severe hyper parathyroidism	Loss of function	[42, 48]
	0.9-kb*alu* insertion in exon7	Adenylyl cyclase		
	A116T, N118K, etc.	Familial hypocalcemia	↑ IP3 response	[55–59]
	A986S, R990G.	Common polymorphisms	Predictive of serum Ca^{2+}	[39–59]

together among asthmatics of the founder population living on Tristan da Cunha [10]. Pharmacological phenotypes, including either gain or loss of receptor efficacy or potency attributed to variants in these and other receptors, are reviewed in Chapter 9.

2.1 Rhodopsin Variants in Retinal Disease

Constitutively active *GPCR* mutants (CAMs) encode receptors capable of enhanced signaling when they are activated without exposure to ligand. The majority of rhodopsin variants are CAMs. As a result, they have become useful tools in the study of conformational changes leading to receptor activation. Study of CAMs has also identified a class of ligands that act as inverse agonists: agents causing conformational changes in a receptor that restore basal levels of receptor signaling by uncoupling a constitutively activated receptor from the G protein. In the example of rhodopsin, it is the retinoic acid derivative, 11-*cis*-retinal, that acts as an inverse agonist [6, 7, 11].

These CAM mutations not only activate the G-protein complex, transducin, constitutively but also often result in constitutive phosphorylation of rhodopsin by rhodopsin kinase or GRK1 (G protein-coupled receptor kinase 4). As discussed in Chapter 7, GRK1 is a specialized enzyme expressed in the retina that is responsible for rapidly desensitizing the receptor when it is exposed to light. In turn, phosphorylated rhodopsin binds tightly to the inhibitory protein, beta-arrestin. This reaction quenches the activated receptor's interaction with transducin, and inhibits further G protein signaling. A reciprocal relationship exists between *GPCR* activation during G protein coupling and rapid quenching, or desensitization, by one of the GRKs [11, 12]. The identification of the aberrant rhodopsin phosphorylation and desensitization [13] that results from a wide variety of rhodopsin mutations is suggestive that the retinitis pigmentosa phenotype may result partly from a pathology of GRK phosphorylation.

2.1.1 Night Blindness, Retinitis Pigmentosa, and Rhodopsin Phosphorylation

Rhodopsin CAMs are responsible for various ocular abnormalities, including night blindness and various retinal dystrophies, generically termed retinitis pigmentosa (RP). The rhodopsin variants include Thr4Lys [14, 15], Asn15Ser [16], Thr17Met, Pro23His [17, 18], Pro23Leu, Gln28His, Gly90Asp, Glu113Gln, Ala292Glu, and Lys296Glu [11, 13]. In the case of each variant, both the disease phenotype and the effect of the mutation on receptor structure and function may vary. The mutations at positions Gly90Asp and Ala292Glu result in complete night blindness, while other mutations cause RP [13]. In many cases, such as the Gly90 variants, different amino acid substitutions at the same position have been found to result in distinct phenotypes [19].

Study of another constitutively phosphorylated rhodopsin mutant, the Leu125Arg variant in TM domain 3, has resulted in an understanding of the specificity with which an amino acid

substitution can determine whether a receptor is able to desensitize. When the amino acid at position 125 of rhodopsin was individually modified in vitro to each of the remaining amino acids, it was found that receptors with smaller residues at position 125 were better able to activate transducin. In the case of the bulkier Leu125Tyr and Leu125Trp substitutions, very little G protein signaling was detected. This suggests that amino acid side chains exert a steric effect, leading to inhibition of G protein activation [20]. In view of this, it seems likely that the Leu125 in TM helix III of rhodopsin, which is located near the ligand-binding pocket for 11-*cis*-retinal, may be important for the structure of the chromophore-binding pocket [20]. This structural information provides new information about the structure of the ligand-binding site of the prototypical *GPCR*, rhodopsin [21].

2.1.2 Oguchi Disease and Defective GRK1 Phosphorylation of Rhodopsin

The group of rhodopsin-related disorders has been demonstrated to result from mutations in the S-antigen (*SAG*) gene and the *GRK1* gene, (reviewed in Chapter 7 in relation to its significance to *GPCR* signaling). One example is autosomal recessive Oguchi disease. Nonsense mutations in the *SAG* gene (eg. c.916G>T; p.Glu306*) can result in impairment of GRK1-mediated desensitization of rhodopsin Oguchi [11, 22]. This disrupts the normal pathway of light-dependent rhodopsin phosphorylation and subsequent quenching of light-induced signal transduction in photoreceptor cells [23]. Thus, regardless of the integrity of the receptor itself, disruption of *GPCR* accessory proteins can result in a disease phenotype attributable biochemically to receptor dysregulation.

2.2 Thyroid Disease and Thyroid-Stimulating Hormone Receptor Mutations

Activating and inactivating mutations of the thyroid-stimulating hormone (*TSH*) and TSH receptor (*TSHR*) genes, analogous in many ways to the rhodopsin receptor disorders, underlie many cases of thyroid disease. The *TSHR* mutations disrupt TSH signaling by blunting the G-protein mediated stimulation of adenylyl cyclase. Disruption of the TSHR, resulting in dysregulation of the TSH function, results in the abnormal growth of thyroid hormone-secreting cells.

In humans, hyperthyroidism, for example, can result from mutations located in the TSHR TM domains that activate the receptor. By contrast, thyroid adenomas and multinodular goiter [24–30] result from many somatic mutations affecting other regions of the TSHR. Such an example is a constitutively active *TSHR* mutation in the first TM domain that results from a Gly substitution at the conserved 431Ser position [27].

Mutations with similar outcomes have been identified in non-autoimmune autosomal dominant hyperthyroidism (toxic thyroid hyperplasia) [24, 25, 27, 31, 32]. Variants have been located in the third TM (Val509Ala), the seventh TM (Cys672Tyr), and the carboxyl tail (Asp727Glu) regions [33] of the TSHR.

These germ-line variants, resulting in a form of congenital hyperthyroidism, share similar functional characteristics somatic mutations present in hyperfunctioning thyroid adenoma [24, 32].

2.2.1 Toxic Multinodal Goiter and Activating TSHR Mutations

Although toxic multinodular goiter is pathogenetically heterogeneous, it can nonetheless result in hyperthyroidism. The molecular pathology of this disorder is complicated by the fact that activating mutations of both the Gαs subunit (reviewed in Chapter 7) and the TSHR have been identified in goiter. These variants result in autonomously hyperfunctioning thyroid adenomas [25] as well as the majority of nonadenomatous hyperfunctioning nodules that are found throughout the gland in patients with toxic or functionally autonomous multinodular goiter [34].

2.2.2 Variable Thyroid Phenotypes

There is wide variability in phenotypic presentation of *TSHR* gene mutations even though they are tightly clustered within TM domains. Single amino acid changes, such as Ser505Arg, have been associated with a broad clinical phenotype that is not consistent with constitutive activity of these TSHR mutants when evaluated for second messenger production. These variants are pharmacogenetically relevant, however, and should be taken into account due to their number and variety. On the continuum of receptor activity, they demonstrate enhanced sensitivity to agonists [36], suggesting their relevance to congenital nonautoimmune hyperthyroidism of varying severity [35]. To complicate matters, these variants may be of variable clinical significance depending on the genetic background [26, 37]: many TSHRs also have defects in co-repressor interaction that influence variable thyroid phenotype within kindreds [38]. A more detailed discussion of *GPCR* variants that are associated with intermediate phenotypes is found in Chapter 9.

2.3 Calcium-Sensing Receptor Mutations and Hypercalcemia/ Hypocalcemia

The calcium sensing receptor (CASR) functions as an extracellular calcium sensor for the parathyroid gland and the kidney. The CA SR is a plasma membrane *GPCR* that is abundantly expressed in the parathyroid hormone (PTH) secreting cells of the parathyroid gland and the cells lining the renal tubule lumen [39–41]. By its ability to sense small changes in circulating calcium concentration ([Ca^{2+}]o) and to couple this information to intracellular signaling pathways that modify PTH secretion and renal cation handling, the CASR plays an essential role in maintaining the [Ca^{2+}]o within the normal range [42]. The activity and/or expression levels of the CaSR dictate the so-called calcium set-point, defined as the [Ca^{2+}] o at which PTH secretion from the parathyroid gland (or calcium reabsorption across the kidney tubule) is half-maximal [41]. CASR variants appear to influence calcium set-points integral to many physiological processes, including alterations in seizure threshold in the brain, as seen in autosomal dominant hypocalcemia (ADH). Because the *CASR* gene is highly polymorphic [39–41], the

contribution of common polymorphisms to individual differences in calcium metabolism is under increasing scrutiny. These studies are reviewed in Chapter 9. A schematic representation of the *CASR* gene and selected variants is shown in Fig. 1.

Previous studies showed that a cluster of missense polymorphisms located in the cytoplasmic tail of the CASR is associated with interindividual population differences in $[Ca^{2+}]o$ [43]. Different haplotypes are associated with primary hyperparathyroidism and the frequency of kidney stones [44]. More recent genome-wide association studies in ~33,000 individuals of European and Indian Asian ancestry confirmed that the blood calcium concentration associated most significantly with SNPs in the *CASR* gene [45, 46].

The key structural elements of the CASR protein, including the bilobed Venus Flytrap (VFT) domain and cysteine-rich region in the extracellular domain (ECD), and the fact that it is a constitutive and functional dimer, are largely conserved with other members of the Class C of the *GPCR* superfamily such as metabotropic glutamate receptors (mGLuRs), gamma aminobutyric acid receptor type B ($GABA_B$), amino acid, taste and odorant receptors [47]. The identification of multiple Ca^{2+} binding sites in the VFT domain of the CASR is fully consistent with the cooperative nature of binding of this divalent cation [48].

Inherited abnormalities of the *CASR* gene give rise to a variety of disorders of mineral ion homeostasis [2]. Heterozygous loss-of-function mutations cause familial (benign) hypocalciuric hypercalcemia (FHH) is which the lifelong mild hypercalcemia is generally asymptomatic. Homozygous inactivating mutations give rise to neonatal severe hyperparathyroidism (NSHPT) with extreme hypercalcemia and marked skeletal changes. Heterozygous activating mutations of the *CASR* cause autosomal dominant hypocalcemia (ADH) that may be asymptomatic or present with seizures in the neonatal period or childhood or later in life. Phenocopies of FHH or ADH are due to circulating CASR inactivating or activating autoantibodies, respectively. The CASR is the target of orally active small molecule allosteric modifiers, either activators, calcimimetics, or inhibitors, calcilytics.

2.3.1 Familial Hypocalciuric Hypercalcemia and Neonatal Severe Hyperparathyroidism

The syndrome known as familial hypocalciuric hypercalcemia (FHH) was first called familial benign hypercalcemia in order to emphasize the asymptomatic nature of the lifelong hypercalcemia that results from inactivating *CASR* mutations. The degree of hypercalcemia in the majority of FHH patients is similar to that of mild primary hyperparathyroidism and their serum concentrations of PTH are inappropriately normal, given the degree of hypercalcemia [49]. An important characteristic of FHH is the unusually high renal tubular reabsorption of calcium and magnesium in the face of hypercalcemia [50]. However, some FHH families have

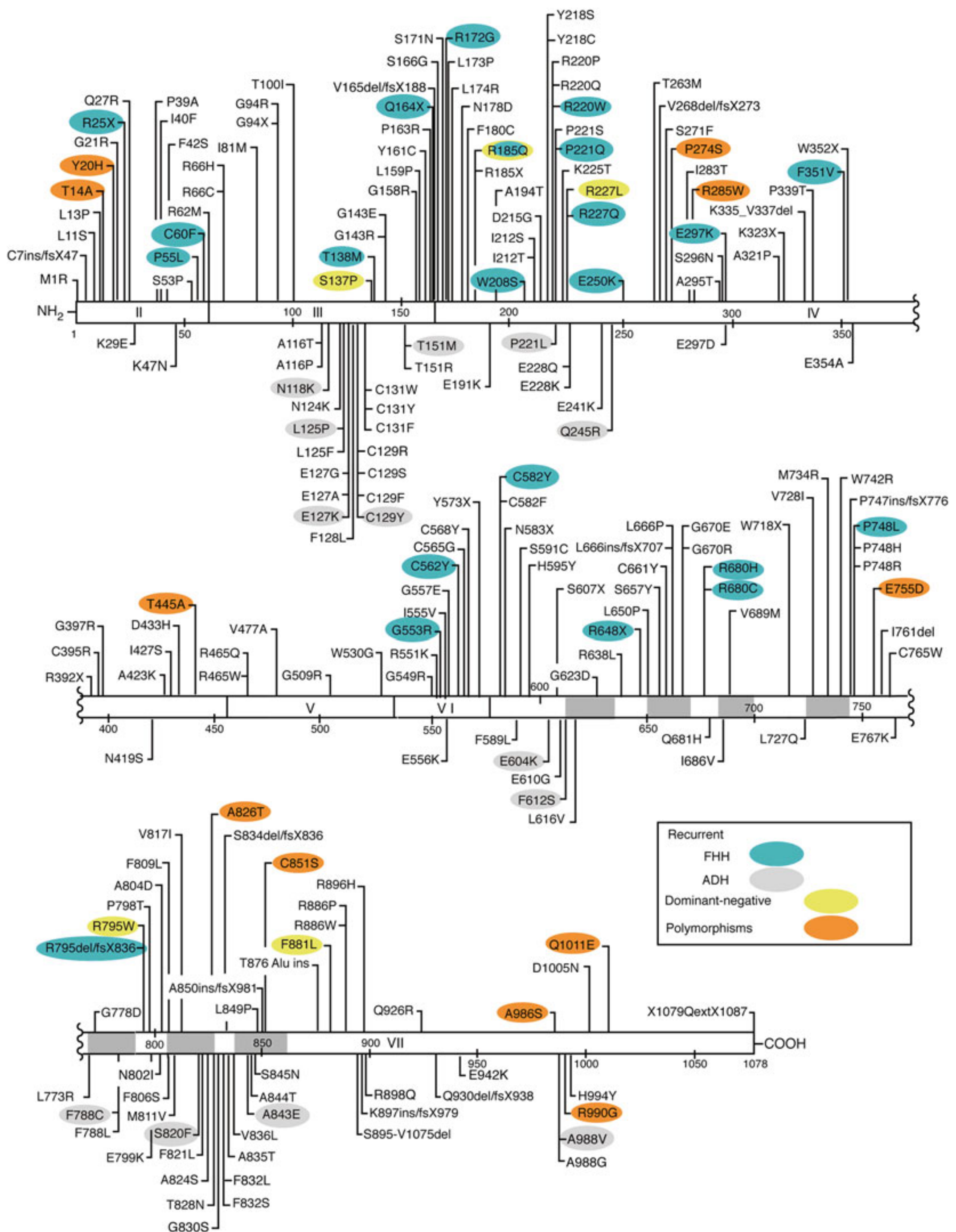

Fig. 1 Mutations and polymorphisms in the CASR. Schema of the CASR (which has 1,078 amino acids) showing the relationship between gene exons (II to VII) and the portions of the protein they encode. Exons II to VI and the beginning of exon VII encodes the ECD of ~610 amino acids, exon VII encodes the TMD of ~250 amino acids including membrane-spanning helices TM1–TM7 (indicated by the *hatched boxes*), ECL1 to ECL3, ICL1 to ICL3, and the ICD of ~200 amino acids (*ECD* extracellular domain, *TMD* transmembrane domain, *TM* membrane-spanning helix, *ECL* extracellular loop, *ICL* intracellular loop, *ICD* intracellular domain). The locations of the inactivating mutations found in patients with FHH and/or NSHPT (as well as some polymorphisms) are shown *above*, and of activating mutations found in patients with ADH (as well as polymorphism R990G) are shown *below the bars* representing the protein-coding exons. http://www.casrdb.mcgill.ca/

affected members for which calcium excretion is increased. This may reflect the particular *CASR* mutation involved.

NSHPT involves multiglandular parathyroid hyperplasia and affected children under the age of 6 months develop severe, symptomatic hypercalcemia with bony changes of hyperparathyroidism that increase in severity with time after birth. Delay in effective treatment can lead to a devastating neurodevelopmental disorder, if it is not fatal [51]. Some forms of neonatal hyperparathyroidism, involving either a de novo or paternal inheritance of a mutated *CASR* allele, present with milder, less symptomatic disease that can be transient.

Upwards of 200 unique inactivating, FHH/NSHPT type mutations in the *CASR* have been identified [42, 48] (*see* http://www.casrdb.mcgill.ca/). The majority of the inactivating mutations are missense (single amino acid substitution), but insertion/deletion, truncation (nonsense or frameshift), and splice-site mutations have been described. The mutations are scattered throughout the protein sequence with some clustering in the first half of the ECD (within the VFT and closely associated with the Ca^{2+} binding sites) and the latter part of the ECD (within the cysteine-rich region—and parts of the transmembrane-spanning region) (Fig. 1). The scattering of mutations is consistent with the notion of the CaSR having multiple functional components that collectively contribute to activity and that a critical mutation in any one of them can cause major impairment in function.

FHH is inherited in an autosomal dominant manner with almost 100 % penetrance, but variable expressivity; the population prevalence is not well defined. The FHH trait was initially mapped to chromosome 3q21, the locus of the *CASR* gene. Two-thirds of FHH cases are due to mutations in the *CASR* gene and the disorder is FHH type 1. However, the FHH trait is heterogeneous and in some kindred maps to either chromosome 19p13.3 (FHH type 2) or 19q13.3 (FHH type 3). FHH2 result from heterozygous loss-of-function mutations in *GNA11*, the gene encoding the alpha subunit of $G\alpha_{11}$ that couples the activated CASR to intracellular signaling pathways [52]. By contrast, in an osteogenic cell line, UMR 106-01, increased expression of $G\alpha_{11}$ resulted in enhanced PLC signaling in response to PTH stimulation [53]. FHH3 is due to inactivating mutations in the *AP2S1* gene that encodes the sigma subunit of adaptor protein complex 2 critical for clathrin-mediated endocytosis of a variety of cell surface proteins including *GPCRs* such as the CASR [54].

2.3.2 Hypocalcemia, Hypoparathyroidism, and Hypocalcemic Hypercalciuria

Gain-of-function mutations in the *CASR* gene have been identified in several families previously diagnosed with autosomal dominant hypocalcemia (ADH), autosomal dominant hypoparathyroidism, and hypocalcemic hypercalciuria [55]. In the parathyroid gland, the activated CASR suppresses PTH secretion and in the kidney, it

induces hypercalciuria. De novo mutations are also common [55]. Mosaicism for de novo mutation in an otherwise healthy parent has been described and this has important implications for counseling parents about the risk of recurrence [56]. *CASR* activating mutations present as Bartter Type V in a few severe cases. Another consequence of *CASR* disruption takes place in the cortical thick ascending limb, where there is sodium chloride wasting linked to increasing losses of calcium and magnesium in the urine.

In a subset of ADH families, *CASR* gain-of-function mutations have been associated with the onset of tonic–clonic seizures. The neurological implications of *CASR* mutations have not been widely explored [40]. In ADH, brain calcifications—sometimes accompanied by seizures—suggest that activating mutations may alter calcium homeostasis in the brain. Further, expression of the CASR in the hippocampus suggests that many neurological functions relating to seizure threshold may be regulated by the CASR. Up to a third of all cases of idiopathic hypoparathyroidism may be found to have activating *CASR* mutations. This suggests that the frequency of neurological symptoms caused by activating *CASR* mutations may be higher than expected. The suppression of PTH secretion from the parathyroid gland that accompanies the constitutive activation of the CaSR makes the disorder difficult to recognize and treat. In some cases, it has been reported that seizures can be intractable. The abnormal set point of calcium regulation complicates treatment with calcitriol and dietary calcium supplementation because the CASR expressed in the kidney controls calcium excretion. The constitutively activated *CASR* mutant induces hypercalciuria, which may exacerbate the hypocalcemia [57]. Further work on the biochemistry of activating *CASR* mutations may refine therapy for ADH patients (and other hypoparathyroid patients) who harbor *CASR* mutations.

The CASR has been further implicated in neurological functions since an idiopathic epilepsy locus was mapped to the *CASR* locus and novel, rare missense *CASR* variants were identified in idiopathic generalized epilepsy [58, 59]. Mutations involved in the idiopathic epilepsy syndrome disrupt an arginine-rich retention motif in the proximal part of the CASR cytoplasmic tail promoting greater cell-surface expression and activity of the CASR than normal [59].

Almost 100 unique activating mutations (virtually all missense) have been identified and appear almost equally divided between the amino-terminal third of the extracellular domain (ECD) and the transmembrane domain [42, 48] (*see* http://www.casrdb.mcgill.ca/). Of note is the cluster of ECD mutations (from A116T to C131W), which cause a leftward (activating) shift in receptor sensitivity, suggesting that this region is critical for receptor activation (Fig. 1). This cluster overlaps the two cysteine residues—C129 and C131—involved in the interface of the mature protein dimer.

The ADH trait is heterogenous. Those cases resulting from activating mutations in the *CASR* are referred to as ADH type 1. Recently, some individuals and kindred having ADH but without *CASR* mutations have been shown to be due to heterozygous gain-of-function mutations in *GNA11*, the gene encoding the alpha subunit of G_{11} [52, 60]. This disorder is ADH type 2.

2.3.3 Parathyroid Hormone Receptor, Skeletal Dysplasias, and Primary Failure of Tooth Eruption

The parathyroid hormone receptor type 1 (PTHR1) binds the two ligands, PTH and PTH-related protein (PTHrP). The PTHR1 belongs to subgroup B of the *GPCR* superfamily that also includes the receptors for secretin, calcitonin, vasoactive intestinal peptide, glucagon, glucagon-like peptide-1, and growth hormone-releasing hormone. The PTHR1 is found widely in fetal and adult tissues, but is most abundant in kidney, bone, and metaphyseal growth plates. Polymorphisms in the PTHR1 are associated with differences in adult height and bone mineral density, consistent with the role that the receptor and its ligands play in endochondral bone formation and bone metabolism [61].

The rare autosomal dominant Jansen-type metaphyseal chondrodysplasia (JMC), characterized by short-limbed dwarfism secondary to severe growth plate abnormalities—along with symptomatic hypercalcemia and hypophosphatemia in the presence of low or undetectable PTH—is due to heterozygous gain-of-function missense *PTHR1* mutations giving rise to constitutively active receptors [62]. Most reported cases are caused by de novo mutations. Homozygous or compound heterozygous loss-of-function mutations in the *PTHR1* have been implicated in the molecular pathogenesis of Blomstrand lethal chondrodysplasia (BLC) and associated skeletal dysplasias and dental abnormalities. The rare autosomal recessive BLC disease is characterized by advanced endochondral bone maturation, short-limbed dwarfism, abnormal breast and tooth morphogenesis, and fetal death, mimicking the phenotype of *PTHR1* knockout mice [63]. Although both forms can be lethal, Blomstrand disease has been subdivided into type I, the severe (classical) form and type II, a relatively milder variant. The difference in severity can be attributed to complete or incomplete inactivation of the PTHR1, in Type I versus Type II, respectively [64]. Eiken syndrome, a milder form of recessively inherited skeletal dysplasia, has also been linked to mutations of *PTHR1* [65].

Dominantly acting heterozygous *PTHR1* mutations have been identified in the enchondromas of ~10 % of patients with Ollier's disease, characterized by multiple benign cartilage tumors, and a predisposition to malignant enchondromatosis [66]. The nonhereditary asymmetrical polyostotic distribution of the lesions suggests that they are activating somatic mosaic mutations. This would be similar to the McCune–Albright syndrome/polyostotic dysplasia in which an activating mutation in *GNAS* occurs during early embryogenesis

with the somatic mosaic state resulting in fibrous dysplasia affecting several bones or, in rare instances, much of the skeleton.

Dominantly inherited symmetrical enchondromatosis is associated with duplication of 12p11.23 to 12p11.22 that includes the *PTHLH* gene encoding PTHrP, suggesting abnormal PTHR1 signaling may underlie this unusual form of enchondromatosis [67]. In addition, cases of autosomal dominant primary failure of tooth eruption (PFE) are due to loss-of function mutations in the *PTHR1*, including those identified in BLC [68]. The phenotype associated with loss of function mutations results from haploinsufficiency of the receptor [69].

2.4 GPCR Mutations and Obesity

Specific brain regions, including parts of the hypothalamus, are known to be involved in the regulation of feeding, body adiposity, and sensory integration of satiety and body fat inputs—functions that are also regulated by other systems such as the orexin/hypocretin system. Candidates for genetic obesity include melanin-concentrating hormone (MCH), a 19-amino acid hypothalamic neuropeptide that is important in the regulation of energy homeostasis [70–72] and the hormone, melanocortin. Two MCH receptors have been identified: MCHR1: isolated from rodents and humans, and MCHR2, present only in humans. MCH signals via *GPCRs* coupled to Gi/o downstream of the leptin pathway and is expressed in neurons known to regulate body weight [73]. The variants of MCHR1 and MCHR2 that are known, however, have little clinical correlation compared with melanocortin.

2.4.1 Melanocortin Receptor Mutations and Obesity

The melanocortin 4 (MCH) receptor (*MC4R*) gene may contribute substantially to the genetics of obesity that involve the hypothalamus [71, 74–80]. The natural ligand for this receptor, melanocyte-stimulating hormone (αMSH), is a neuropeptide derived from pro-opiomelanocortin (POMC). MC4R is also negatively regulated by endogenous inverse agonists, such as the agouti (Ag) and agouti-related proteins (AgRPs). Since the MC4R is constitutively active, it is the balance between the activity of AgRP-containing neurons and αMSH-containing neurons that determines the extent of melanocortin pathway activation [81].

The contribution of the MCH4–αMSH pathway to obesity has been primarily identified from the study of MC4R knockout mice that are hyperphagic and severely overweight [82, 83]. More recently, however, large association studies in humans have identified polymorphisms such as Val103Ile, as well as private mutations, that account for a monogenic form of binge eating and obesity [84–87]. The discovery of a rare form of autosomal dominant obesity that results from an inactivating (frame-shift) *MC4R* mutation confirmed the role of the MCH4 receptor in energy homeostasis. Loss-of-function *MC4R* mutations were identified as a result of the linkage studies in families with severe autosomal dominant obesity [76–78].

The loss of constitutive activity in these receptors resulted in the identification of an important pathway that contributes to regulation of energy homeostasis.

The correct balance of agonists and inverse agonists may be achieved by pharmaceutical interventions which target the MC4R functions that maintain weight homeostasis. Since several regions of intracellular loop 2 (ICL2) and adjacent regions of transmembrane helix 3 (TMH3) and TMH4 may be required for dimer formation, the effect of dimerization on the capacity for signaling activity must be considered for MC4R variants. For example, dimer dissociation has been reported in the naturally occurring His158Arg activating *MC4R* mutation in ICL2. These considerations facilitate MC4R drug design [88–91].

2.5 Follicle-Stimulating Hormone Receptor Mutations and Gametogenesis

The follicle-stimulating hormone (FSH) receptor (FSHR) is a key component of the endocrine axis governing gonadal function. FSH is essential for normal gametogenesis in both males and females. Inactivating *FSHR* mutations identified in female ovarian dysgenesis, however, appear to be benign in males, who instead occasionally harbor an asymptomatic, constitutively active *FSHR* mutation. This difference reflects gender-based developmental differences: In females, FSH is required for ovarian development and follicle maturation, whereas in males FSH determines Sertoli cell number and normal spermatogenesis. The prototypic inactivating (Ala189Val) and activating (Asp567Gly) *FSHR* mutations are reviewed with respect to the genetic causes of ovarian dysgenesis and disease in hypophysectomized males [92, 93].

2.5.1 FSHR Mutants, Ovarian Dysgenesis, and Infertility

The Ala189Val mutation in the FSHR was first identified in a female patient with severely affected gametogenesis [92]. The resultant female infertility phenotype is a dominantly inherited pattern of ovarian dysgenesis. Identified in a homozygous form in affecteds, the FSHR mutation disrupts the large ECD implicated in ligand binding, while leaving the remaining TM-spanning domains and the carboxyl tail intact [92, 93]. In vitro studies suggested that the mutation probably affects FSH binding by disrupting the proper protein folding, thereby inactivating the receptor [93, 94].

2.5.2 FSHR Mutations Unmasked in Hypophysectomized Males

Male patients hypophysectomized because of a pituitary tumor have been discovered to harbor constitutively active forms of the *FSHR* gene. These patients had normal semen counts despite undetectable serum gonadotropins after surgery. Because the benign phenotype is only unmasked by the development of an unrelated tumor, however, the frequency of the mutations themselves in the general population is difficult to evaluate [92, 95].

The constitutive Asp567Gly FSHR mutation is encoded by a SNP located in exon 10 of the gene. The substitution probably affects the structure of the third cytoplasmic loop. The constitutive

mutation was found to result in an increase in basal cAMP production compared in vitro to the wild-type FSHR. The ligand-independent activation of the FSHR in the constitutive mutant explains why this heterozygote is capable of maintaining spermatogenesis in hypophysectomized patients [92, 93, 96, 97].

Interestingly, although Ala189Val variants have been identified in both sexes, the Asp567Gly variant has been found only in males, suggesting that this activating FSHR mutation may have a lethal phenotype in females [92, 93]. In this context, it is intriguing that there is evidence for an association between homozygosity for the common Asn680Ser variant with increased FSH serum levels in normogonadotropic anovulatory infertile women [94].

Although inactivating FSH mutations are the only FSHR mutations known to cause monogenic disease [92], FSH variants, such as Asn680Ser, create a spectrum of pathophysiologic phenotypes, differentially affecting the fertility of women from different genetic backgrounds [94]. A contrasting example is provided by ovarian hyperstimulation syndrome (OHSS). In some cases, this potentially life-threatening complication of ovarian stimulation treatments has been associated with an activating FSHR mutation [98]. This is one example of how pharmacogenetics can focus attention on genetic predispositions that would not have otherwise attracted full scrutiny.

2.6 Luteinizing Hormone Receptor Mutations

Luteinizing hormone (LH) is critical to male fertility because it stimulates testicular Leydig cells to produce the testosterone that maintains male secondary sex characteristics. The LH receptor mediates these functions by stimulation of cellular adenylyl cyclase via G-protein transduction [99].

There are a variety of constitutively active mutations in the gene encoding the LH receptor. These variants result in gonadotropin-independent disorders such as testotoxicosis and familial male precocious puberty (FMPP) [100]. These disorders are inherited in an autosomal dominant, male-limited pattern [101].

Inactivating mutations in the *FSHR* gene cause infertility in women featuring amenorrhea, hypergonadotrophic hypogonadism, ovarian failure, and/or dysgenesis. Loss-of-function mutations interfere with sexual development and ovarian function. This diverse group of mutations can cause either the suppression of hormone binding or abnormalities in signal transduction, including diversion from the normal cascade [102].

2.6.1 Testotoxicosis

Testotoxicosis is a form of male precocious puberty. The disorder results from a constitutive activation of the Gαs protein (reviewed in Chapter 7). This results in LH receptor activation that is analogous to the LH receptor mutant phenotypes. The disorder often presents alongside paradoxical pseudohypoparathyroidism type Ia (PHP-Ia), a condition that is marked by resistance to hormones acting through cAMP (PTH and TSH) [100].

Molecular studies showing a temperature-sensitive Gαs mutation (Ala366Ser) may help to explain this apparent paradox. At 32 °C (testicular temperature), the Gαs 366Ser mutation results in the constitutive cAMP accumulation that causes the testosterone secretion that is the hallmark of the testotoxicosis phenotype. At 37 °C (core body temperature), however, the Gαs 366Ser mutation results in loss of adenylyl cyclase signaling, causing PHP-Ia. As a result, a single mutation that acts differently in different tissues—based solely on differences in temperature optima—causes precocious puberty and abnormalities of PTH and TSH receptor transduction [100].

2.6.2 Familial Male Precocious Puberty and Constitutive LH Receptor Mutants

Activating LH mutations are rare; although they have been identified in men with normal spermatogenesis [103]. Familial male precocious puberty, however, is associated with Leydig cell hyperplasia, which may contribute to low sperm cell counts. Molecular studies have identified substitutions in the TM 6 domain of the LH receptor in affected males [103, 104]. The Asp567Gly mutation of the LH receptor, for example, was found to result in a constitutively active phenotype. The disorder was also found to result from a nearby Ala568Val mutation [104] and from Met571Ile and Thr577Ile mutations in the more cytoplasmic portion of helix 6. These mutations were found to result, in vitro, in receptors with constitutively active phenotypes characterized by significantly increased basal cAMP production. Although these variants have been reported in kindred from various ethnic origins, including European [105] and Brazilian [103, 104] populations, it is unclear whether these variants constitute founder mutations.

2.6.3 Models of LH Receptor Structure and Function

Constitutively active mutations, such as those reported in the LH receptor, provide an insight into the dysregulated G protein coupling observed in a variety of disease states. Various important structural components of *GPCRs* have been highlighted. For example, in vitro studies have shown that a constitutively active α1-adrenergic receptor can be generated by mutating the alanine residue homologous with 568alanine of the FSHR. Similar to the LH receptor variant, the resulting α1-adrenergic receptor variant is characterized by high basal adenylyl cyclase activation. These studies suggest that the alanine residue conserved in the TM 6 domain may be critical for downregulation of signal transduction [103–105].

2.7 Gonadotropin-Releasing Hormone Receptor Mutations and Idiopathic Hypogonadotropic Hypogonadism

Idiopathic hypogonadotropic hypogonadism (IHH) consists of those patients without a deficient sense of small, anosmia, or adrenal insufficiency. This subset of IHH results in reproductive failure that may be caused by at least 20 different mutations of the GNRH (gonadotropin-releasing hormone) receptor (*GNRHR*) gene.

Subsequently, mutations in three other *GPCRs* were found to cause related, but somewhat broader range of phenotypes. For example, at least 12 mutations of GPR54, the kisspeptin (metastin) receptor (*KISS1R*), have been identified in consanguineous families [106, 107]. Disruption of the Kisspeptin pathway, the ligand for KISS1R, may result in IHH due to its role as a potent stimulator of GNRH and gonadotropin. Like all IHH patients, affecteds have delayed sexual development and low or apulsatile gonadotropin levels. The impairment in sexual development, however, occurs in the absence of the anatomical abnormalities common to fertility disorders that affect the hypothalamic–pituitary axis [3, 8].

The genetic defects for two of the more common X-linked subtypes of IHH, congenital IHH with anosmia (or Kallmann syndrome, KS), and IHH with adrenal insufficiency (adrenal hypoplasia congenita) are distinct from the forms of the disease caused by GnRH receptor (*GNRHR*) mutations. The KS mutations were identified in the *KAL* gene and result in abnormal olfactory bulb development [108, 109]. The mutations responsible for the X-linked IHH with adrenal hypoplasia congenita were identified in the *DAX1* gene. *DAX1* encodes an orphan nuclear hormone receptor that regulates various aspects of reproductive development [110, 111].

Mutations in the *PROKR2* and *PROK2* genes, encoding a receptor–ligand pair that regulates intestinal contraction, circadian rhythms, vascular function, have also been identified in patients with kallamann syndrome (KS) [112]. At least 26 missense mutations in *PROKR2* have been reported in patients with IHH or KS. While the majority of mutations in *GNRHR* and *KISS1R* in patients with GnRH deficiency are found in either compound heterozygous or homozygous states, many *PROKR2* mutations reported in patients with KS or IHH are heterozygous.

Finally, the NK3R receptor for neurokinin B (NKB), a member of the tachykinin family which includes substance P, has been shown to have excitatory roles in synaptic transmission in pyramidal neurons of the medial prefrontal cortex [113], has been shown to be disrupted in IHH phenotypes. NKB binds to its *GPCR*, NK3R. The emphasis of this discussion of IHH, however, is on the role of *GNRHR* mutations in IHH etiology.

2.7.1 GnRHR Mutations That Result in Idiopathic IHH

Comparatively little is known about the molecular biology of the *GNRHR* mutations that result in idiopathic IHH. At least 15 *GNRHR* mutations have been described in IHH [97, 104–106]. Some of these mutations, such as Glu90Lys and Ser217Arg, have been shown in vitro to be loss-of-function mutations. Other *GNRHR* mutations, such as Asn10Lys, Thr32Ile, and Gln10Arg, have a somewhat reduced ability to elicit an intracellular inositol phosphate response in vitro [107].

Site-directed mutagenesis has been used to identify the significance of GNRHR variants in relation to receptor function. The Glu90Ala and Arg139His [117, 118] mutations are inactive in vitro, suggesting that these residues are probably critical to receptor activation. The 217Ser variant of TM 5, however, illustrates how the effect of an amino acid substitution can be context sensitive. Although the GNRHR variant identified in patients, Ser217Arg, is completely inactive; a substitution of Ser217Gln and Ser217Tyr using site-directed mutagenesis results in a GNRHR with partial function. Therefore, some residues may not always be critical to receptor function if the substitution does not elicit steric hindrance that is disruptive to receptor structure [107]. This approach has resulted in the isolation of portions of the GNRHR that are involved in specific molecular functions [118].

2.7.2 GnRHR Pharmacogenomics

The advances made possible by isolating *GNRHR* gene variants illustrate the potential applications of pharmacogenomics. The application of structural biology to clinical problems has resulted in the identification of an antagonist, permeant, that can selectively rescue most of the naturally occurring *GNRHR* mutants by increasing their cell surface expression [118]. This is an example of a therapeutic strategy that would have been unimaginable before the pharmacogenomic paradigm of drug discovery.

This antagonist may act on *GPCRs* to stabilize misfolded proteins and prevent them from being targeted for degradation [106, 107, 118]. The permeant antagonist is named after its ability to recover the function of receptors before they are degraded or expressed incorrectly at the membrane. While still experimental, this example illustrates how an understanding of *GPCR* genomics and *GPCR* protein structure facilitates the identification of drugs with novel mechanisms of action that may provide clinical intervention for complex developmental disorders.

2.8 Adrenocorticotropic Hormone Receptor Mutations and Familial Glucorticoid Deficiency

Familial glucocorticoid deficiency (FGD) is an autosomal recessive disorder characterized by progressive primary adrenal insufficiency but normal mineralocorticoid metabolism. As a result of screening affected families, the human corticotropin (ACTH, adrenocorticotropic hormone) receptor gene, *MC2R*, was found to be involved in the etiology of type 1 FGD [119–122]. Subsequently, mutations in the melanocortin 2 receptor accessory protein accessory protein (MRAP) were found to cause early onset, or type 2 disease, which manifests at a median age of 0.1 years [121]. Type 3 FGD was reported to result from mutations of the steroidogenic acute regulatory protein [122].

With respect to type 1 FGD, several compound heterozygous mutations of the ACTH receptor (*ACTHR*) gene appear to be associated with disease. For example, a germ-line nt.201C>T substitution results in the truncation of the entire carboxyl portion of

the receptor because of the introduction of a premature stop codon (TGA). A germ-line substitution at nt.360C>G, resulting in a Ser120Arg ACTHR mutation in TM 2, was also identified. In another family, the Ser120Arg mutation was found concurrently with a Tyr254Cys variant in the third extracellular loop of the receptor protein [119, 120].

The identification of ACTHR variants has therefore allowed a better assessment of the significance of a given compound heterozygote to FGD. Some variants of the ACTHR may be expressed on a background entirely lacking in functional ACTHR [119]. For example, a truncation of the protein at Gly217 was found on the paternal chromosome concurrently with a substitution in the maternal chromosome located two bases upstream from the transcription start site. Although this substitution may be present in 6.5 % of healthy individuals, it is only penetrant when the maternal variant is inherited concurrently with the truncation mutant [119–122]. These studies exemplify how an understanding of the inherited defects in a receptor gene may help to define not only the regulation of cell signaling but also the role that tissue expression can play in regulating the function of some *GPCRs* [119].

In general, type 2 FGD results from *MRAP* gene mutations. *MRAP* mutations result in early onset disease; however, two missense mutations, Tyr59D and Val26Ala have been shown to result in a phenotype more reminiscent of later-onset type 1 disease. Both mutations impair cAMP generation: resulting in a shift in the dose-response curve to the right when compared to wild type. While trafficking of MC2R appeared to be normal, mutant MRAPs appear to be responsible for an ACTH resistance phenotype that most resembles the type 1 FGD otherwise most often attributable to MC2R mutations [121].

2.9 Vasopressin V2 Receptor Mutations are responsible for X-linked Nephrogenic Diabetes Insipidus

Nephrogenic diabetes insipidus (NDI) is characterized by renal tubular resistance to the antidiuretic effect of arginine vasopressin (AVP). NDI may be inherited as an autosomal dominant or X-linked disorder. More than 90 % of cases are X-linked and rapidly diagnosed by sequencing analysis of the gene encoding the vasopressin V2 receptor (*AVPR2*) [123]. The autosomal dominant form of NDI results from mutations of the aquaporin 2 gene (*AQP2*). *AQP2* encodes a water channel of the renal collecting duct. Its disruption causes autosomal dominant NDI and recessive forms of the disease [124].

2.9.1 Vasopressin Receptor

The gene encoding the V_2 vasopressin receptor (*AVPR2*), located in the Xq28 region, is responsible for the X-linked nephrogenic diabetes insipidus [125]. AVPR2 belongs to the cyclic nonapeptide-binding *GPCR* subfamily that also includes the V1a and V1b vasopressin receptors and the oxytocin receptor. AVPR2 is expressed predominantly in the distal convoluted tubule and collecting ducts

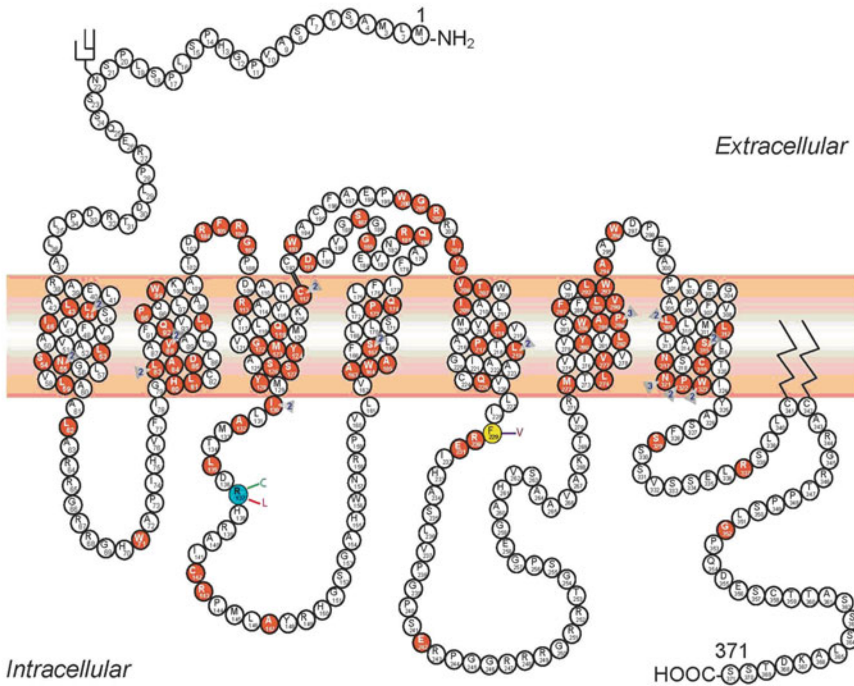

Fig. 2 Schematic representation of the V_2 receptor and identification of 193 putative disease-causing AVPR2 mutations. Predicted amino acids are shown as the *one-letter amino acid code*. A *solid symbol* indicates a codon with a missense or nonsense mutation; a *number* indicates more than one mutation in the same codon; other types of mutations are not indicated on the figure. There are 95 missense, 18 nonsense, 46 frameshift deletion or insertion, 7 inframe deletion or insertion, 4 splice-site, and 22 large deletion mutations, and 1 complex mutation. The gain-of-function mutations affecting codons R137 and F229 are indicated

of the nephron. Its primary role is to respond to the pituitary hormone AVP by stimulating mechanisms that concentrate the urine and maintain water homeostasis. A "noncanonical" control of the vasopressin V2 receptor signaling by retromer and arrestin has recently been suggested, since beta-arrestins promote rather than attenuate cAMP signaling mediated by vasopressin [126]. This noncanonical signaling could possibly explain why a mutant V2R causing NDI remained in the endoplasmic reticulum, still generated cAMP when challenged with a membrane permeant agonist [127] and raises the possibilty that internalized vasopressin-V2R complexes can access the machinery for signaling from membranes inside the cell and continue to stimulate the cAMP/PKA pathway. More than 211 different mutations, without any significant major differences in phenotypic expression, have been reported in 326 different families [123].

The *AVPR2* mutations that are known to cause X-linked NDI include missense and nonsense mutations, small or large insertions, and deletions [123]—some of which are shown in Fig. 2. For example, familial NDI may result from substitutions of

Ser167Thr—a residue conserved across many *GPCRs*—and Leu44Pro. The Hopewell mutation, a Trp71 truncation (*W71X*), results in NDI in the largest (known) North American NDI pedigree. Most affecteds originate from Colchester County in Nova Scotia. Most other mutations are ancestrally independent.

2.9.2 Loss of Function of the Vasopressin V2 Receptor

X-linked NDI (OMIM 304800) [128] is secondary to *AVPR2* mutations, which result in a loss of function or dysregulation of the V2 receptor [129]. Males who have an *AVPR2* mutation have a phenotype characterized by early dehydration episodes, hypernatremia, and hyperthermia as early as the first week of life. Dehydration episodes can be so severe that they lower arterial blood pressure to a such degree that it is not sufficient to sustain adequate oxygenation to the brain, kidneys, and other organs. Mental and physical retardation and renal failure are the classical "historic" consequences of a late diagnosis and lack of treatment. Heterozygous females exhibit variable degrees of polyuria and polydipsia because of skewed X chromosome inactivation [130, 131].

Population Genetics of *AVPR2* Mutations

In Quebec, the incidence of NDI among male individuals was estimated to be approximately 8.8 in 1,000,000 male live births [130]. A founder effect of two particular *AVPR2* mutations [132], one in Ulster Scot immigrants (the Hopewell mutation, W71X) and one in a large Utah kindred (the Cannon pedigree), result in an elevated prevalence of X-linked NDI in their descendants in certain communities [132]. However, these founder mutations have now spread all over the North American continent. For example, the W71X mutation was identified in 42 affected male individuals who reside predominantly in the Maritime Provinces of Nova Scotia and New Brunswick and the L312X mutation was identified in eight affected males who reside in the central United States. There are approximately 98 living affected male individuals of the Hopewell kindred and 18 living affected male individuals of the Cannon pedigree.

As a result, it can be argued that all families with hereditary diabetes insipidus should have their molecular defect identified. The molecular identification underlying X-linked NDI is has immediate clinical consequences, since early diagnosis and treatment can avert the physical and mental retardation that results from repeated episodes of dehydration.

Expression Studies

Classification of the defects of naturally occurring mutant human V2 receptors can be based on a scheme similar to that used for the LDL receptor. Mutations have been grouped according to the function and subcellular localization of the mutant protein whose cDNA has been transiently transfected in a heterologous expression system [133]. Using this classification, type 1 mutant V2 receptors reach the cell surface but display impaired ligand binding

and are consequently unable to induce normal cAMP production. The presence of mutant V2 receptors on the surface of transfected cells can be determined pharmacologically. By carrying out saturation binding experiments using radiolabelled AVP, the number of cell surface mutant V2 receptors and their apparent binding affinity can be compared with that of the wild-type receptor. In addition, the presence of cell surface receptors can be assessed directly by using immunodetection strategies to visualize epitope-tagged receptors in whole-cell immunofluorescence assays.

Type 2 mutant receptors have defective intracellular transport. This phenotype is confirmed by carrying out, in parallel, immunofluorescence experiments on cells that are intact (to demonstrate the absence of cell surface receptors) or permeabilized (to confirm the presence of intracellular receptor pools). In addition, protein expression is confirmed by Western blot analysis of membrane preparations from transfected cells. It is likely that these mutant type 2 receptors accumulate in a pre-Golgi compartment, because they are initially glycosylated but fail to undergo glycosyl-trimming maturation.

Type 3 mutant receptors are ineffectively transcribed and lead to unstable mRNA, which are rapidly degraded. This subgroup seems to be rare, since northern blot analysis of cells expressing mutant V2 receptors showed mRNA of normal quantity and molecular size.

Most of the *AVPR2* mutants are type 2 mutant receptors. They do not reach the cell membrane and are trapped in the interior of the cell [134–137]. Other mutant *GPCRs* [138] and gene products that cause genetic disorders are also characterized by protein misfolding. Mutations that affect the folding of secretory proteins; integral plasma membrane proteins; or enzymes destined to the endoplasmic reticulum, Golgi complex, and lysosomes results in loss-of-function phenotypes irrespective of their direct impact on protein function because these mutant proteins are prevented from reaching their final destination on the cell surface [139]. Folding in the endoplasmic reticulum is the limiting step: Mutant proteins which fail to correctly fold are retained initially in the endoplasmic reticulum and subsequently often degraded. Key proteins involved in the urine countercurrent mechanisms are good examples of this basic mechanism of misfolding. *AQP2* mutations responsible for autosomal recessive NDI are characterized by misrouting of the misfolded mutant proteins and are trapped in the endoplasmic reticulum [140]. Mutants that encode other renal membrane proteins that are responsible for Gitelman syndrome [141], Bartter syndrome [142, 143], and cystinuria [144] may also be retained in the endoplasmic reticulum.

The *AVPR2* missense mutations are likely to impair folding and to lead to rapid degradation of the misfolded polypeptide and not to the accumulation of toxic aggregates (as is the case for *AVP*

mutants), because the other important functions of the principal cells of the collecting duct (where *AVPR2* is expressed) are entirely normal. These cells express the epithelial sodium channel (ENac). Decreased function of this channel results in a sodium-losing state [145]. This has not been observed in patients with *AVPR2* mutations. By contrast, another type of conformational disease is characterized by the toxic retention of the misfolded protein. The relatively common Z mutation in a_1-antitrypsin deficiency not only causes retention of the mutant protein in the endoplasmic reticulum but also affects the secondary structure by insertion of the reactive center loop of one molecule into a destabilized beta sheet of a second molecule [146]. These polymers "clog up" the endoplasmic reticulum of hepatocytes and lead to cell death and juvenile hepatitis, cirrhosis, and hepatocarcinomas in these patients [147].

If the misfolded protein/traffic problem that is responsible for so many human genetic diseases can be overcome and the mutant protein transported out of the endoplasmic reticulum to its final destination, these mutant proteins could be sufficiently functional to mitigate the severity of the pathological phenotype [148]. Therefore, using pharmacological chaperones or pharmacoperones to promote escape from the endoplasmic reticulum is a possible therapeutic approach [137, 148, 149]. We used selective non-peptide V2 and V1 receptor antagonists to rescue the cell-surface expression and function of naturally occurring misfolded human V2 receptors [136]. Because the beneficial effect of non-peptide V2 antagonists could be secondary to prevention and interference with endocytosis, the R137H mutant has previously been reported to lead to constitutive endocytosis [151]. It was found that the antagonist did not prevent the constitutive beta-arrestin-promoted endocytosis [150]. These results indicate that as for other *AVPR2* mutants, the beneficial effects of the treatment result from the action of the pharmacological chaperones. These studies were confirmed in vitro with the use of non-peptide V2 agonists [127]. In a clinical study, a non-peptide vasopressin antagonist SR49059 was administered to five adult patients with NDI and who bear the del62_64, R137H and W164S mutations. SR49059 significantly decreased urine volume and water intake and increased urine osmolality whereas sodium, potassium, and creatinine excretion, and plasma sodium levels were constant throughout the study [136]. This new therapeutic approach could be applied to the treatment of several hereditary diseases resulting from errors in protein folding and kinesis [148, 149].

2.9.3 Gain of Function of the Vasopressin V2 Receptor: Nephrogenic Syndrome of Inappropriate Antidiuresis

The clinical phenotype in this disorder is opposite to NDI. Rare cases of infants or adults with hyponatremia, concentrated urine, and suppressed AVP plasma concentrations have been described bearing the mutations R137C , R137L [152–155] or F229V [156] in their *AVPR2* gene. It is interesting to note that another

mutation in the same codon (R137H) is a relatively frequently recurring mutation causing classical NDI, albeit the phenotype may be milder in some [157]. With cell-based assays, R137C and R137L were both found to have elevated basal signaling through the cAMP pathway and to interact with beta-arrestins in an agonist independent manner [156]. In general, *AVPR2* gain-of-function mutations are extremely rare. The *AVPR2* gene has been sequenced in many patients with hyponatremia without ever finding a mutation. By contrast, new and recurrent loss-of-function *AVPR2* mutations are frequently identified in patients with classical NDI.

2.10 Endothelin-β Mutations Associated with Hirschsprung's Disease

Hirschsprung disease is a disorder that involves an enlargement of the colon that is defined by the absence of ganglion cells in the myenteric and submucosal plexuses of the gastrointestinal tract. At least, nine genes and four loci for susceptibility to Hirschsprung disease are known [159]. The disorder is characterized by incomplete penetrance and variable expressivity [160]. Although the RET proto-oncogene accounts for the highest proportion of familial and sporadic cases [160], mutations in the endothelin 3 (EDN3) ligand and the endothelin-β (ETB) receptor gene (*EDNRB*) are important because of the extent to which they disrupt normal human development [161]. In particular, *EDNRB* may be essential to the formation of the enteric nervous system [161].

Although the endothelin system consists of two *GPCRs*, the ETB and endothelin-α (ETA) receptors, and three peptide ligands [161], Hirschsprung disease is most frequently associated with ETB receptor variants such as the Trp276Cys mutation. Rare mutations in the *EDN3* ligand gene [161–164] and the gene encoding the endothelin-converting enzyme 1 (ECE-1) [165], however, are also associated with Hirschsprung [159].

Other ETB receptor mutations have been reported in sporadic cases of Hirschsprung. These include the Gly57Ser, Arg319Trp, and Pro383Leu ETB receptor variants. In each case, the variants appear to inactivate the receptor [166].

The study of the ETB Trp276Cys receptor, however, has resulted in a useful insight into the molecular pathology of Hirschsprung. The extensive evolutionary conservation between the endothelin receptor subtypes A and B has facilitated detailed molecular characterization [167]. The homologous 257Trp and 258Trp mutations of the ETA and ETB receptors have been characterized with respect to their coupling properties with G_i, G_o, and G_q in vitro. The mutants have a similar affinity for endothelin 1, but the naturally occurring Trp276Cys ETB receptor mutation shows reduced G coupling in comparison to the engineered Trp276Ala ETB and Trp258Ala ETA receptor mutations.

2.11 Purinergic Receptor Mutations

The purinergic receptors are a large family of *GPCRs*. Some subtypes have overlapping pharmacological selectivity for purinergic nucleotides. The purinergic (P2RY12) receptor is involved in

platelet aggregation and is a potential pharmacogenetic target for treatment of thromboembolism and other clotting disorders.

The P2RY12 receptor was identified as the result of linkage mapping of a pedigree exhibiting a severe bleeding disorder that was refractory to many treatments. This became evident because the wild-type P2RY12 receptor is the pharmacological target for the anticlotting agents triclopine and clopidogrel. One P2RY12 receptor mutation, located in the TM 6 domain, is a two-nucleotide deletion that was found to have reduced efficacy and potency for these anticlotting agents. By expressing the P2RY12 mutations in vitro, novel pharmacological agents with efficacy in bleeding disorders may be developed [168].

Molecular cloning of purinergic receptors has refined the pharmacology of anticlotting agents for patients not affected by the prototypical bleeding disorder. For example, dual antiplatelet therapy with aspirin and clopidogrel, an antagonist of P2RY12, has been examined in patients with acute coronary syndromes and associated percutaneous coronary intervention (PCI) [169, 170]. The slight difference may be partly explained by a C893T P2Y purinoceptor 1 (*P2RY1*) polymorphism that results in a threefold increase in aspirin resistance in Caucasian male patients with a history of myocardial infarct [169]. More importantly, P2RY12 receptors have been shown to be critical in the thromboxane (TXA_2)-dependent pathways of platelet aggregation [168–170]. Studies suggesting a link between adenosine receptors and aspirin mechanism, therefore, have been furthered by work done on rare monogenic bleeding disorders.

2.12 Neuropeptide S Receptor 1 (NPSR1): The GPCR Associated with Asthma

De-orphanization studies have identified GPR154 (GPRA) as the Neuropeptide S Receptor 1 (NPSR1). The gene was positionally cloned to asthma on chromosome 7p13 from linkage studies of asthma in a Finnish population and five other Western European populations [171–173].

NSPR1 was identified as a candidate gene in the pathogenesis of asthma and other diseases mediated by immunoglobulin E (IgE). Like other de-orphanized *GPCRs*, NSPR1 may act as a receptor for other ligands and is a potential drug target. Variability in receptor structure has been reported that may be relevant to its pharmacology. The NPSR1 has two main isoforms: NPPR1-A and NSPR1-B. NPSR1-A encodes the shorter isoform with a 29-residue long distinct C-terminus, whereas the NPSR1-B uses an alternate 3′ exon (E9b), encoding a larger protein with a distinct 35-residue C-terminus [174].

The NSPR1 ligands define a distinct signaling pathway that is dysregulated in asthma [173]. While the B isoform is more highly expressed in the bronchial epithelia and smooth muscle of asthmatics compared with healthy individuals [139], NPSR1-A has stronger signaling effects [174]. The unique phosphorylation sites in the C-terminus are a possible explanation for the discrepancy, but

the studies are inconclusive. The NSPR1-A receptor, therefore, may be a promising target against which to screen asthma drugs [174] that may address an isoform-specific pathogenetic process in allergic airways [174].

3 Conclusion

Mutated forms of *GPCRs* are associated with a wide spectrum of disease phenotypes and predispositions. Monogenic disorders that result from disrupted *GPCR* signaling provide a unique window on receptor function that complements the plethora of data available from in vitro studies of variant receptors generated purely by site directed mutagenesis. In particular, an understanding of how mutant *GPCR* genes cause disease—especially through loss of function (LOF) or constitutively active mutations (CAMs)—may suggest novel pharmacological interventions.

Since disrupted receptors are also pharmacological targets, the identification of *GPCRs* mutated in disease provides the opportunity to identify dugs that specifically compensate for the disruption. These endeavors are intimately related to the field of *GPCR* pharmacogenomics that is discussed further in Chapter 9. Many receptors are known to have variants that, although not always directly resulting in a monogenic disease phenotype, may confer a phenotype that alters risk for a disease or altered reaction to a pharmaceutical [3, 5–7].

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Chapter 9

Pharmacogenetics of the G Protein-Coupled Receptors

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Abstract

Pharmacogenetics investigates the influence of genetic variants on physiological phenotypes related to drug response and disease, while pharmacogenomics takes a genome-wide approach to advancing this knowledge. Both play an important role in identifying responders and nonresponders to medication, avoiding adverse drug reactions, and optimizing drug dose for the individual. G protein-coupled receptors (GPCRs) are the primary target of therapeutic drugs and have been the focus of these studies. With the advance of genomic technologies, there has been a substantial increase in the inventory of naturally occurring rare and common GPCR variants. These variants include single-nucleotide polymorphisms and insertion or deletions that have potential to alter GPCR expression of function. In vivo and in vitro studies have determined functional roles for many GPCR variants, but genetic association studies that define the physiological impact of the majority of these common variants are still limited. Despite the breadth of pharmacogenetic data available, GPCR variants have not been included in drug labeling and are only occasionally considered in optimizing clinical use of GPCR-targeted agents. In this chapter, pharmacogenetic and genomic studies on GPCR variants are reviewed with respect to a subset of GPCR systems, including the adrenergic, calcium sensing, cysteinyl leukotriene, cannabinoid CB1 and CB2 receptors, and the de-orphanized receptors such as GPR55. The nature of the disruption to receptor function is discussed with respect to regulation of gene expression, expression on the cell surface (affected by receptor trafficking, dimerization, desensitization/downregulation), or perturbation of receptor function (altered ligand binding, G protein coupling, constitutive activity). The large body of experimental data generated on structure and function relationships and receptor-ligand interactions are being harnessed for the in silico functional prediction of naturally occurring GPCR variants. We provide information on online resources dedicated to GPCRs and present applications of publically available computational tools for pharmacogenetic studies of GPCRs. As the breadth of GPCR pharmacogenomic data becomes clearer, the opportunity for routine assessment of GPCR variants to predict disease risk, drug response, and potential adverse drug effects will become possible.

Key words Agonist, Antagonist, Efficacy, Potency, G protein-coupled receptor, Pharmacogenetics, GPR55, Cannabinoid, Database, Variant

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1 Introduction

Response to therapeutic treatment can be considered a complex trait influenced by inherited and environmental factors and their interaction. Interindividual variation in drug response spans phenotypic variation in drug efficacy and severity of adverse reactions. A goal of personalized medicine is to incorporate information about a patient's genetic background into his or her therapeutic drug regimen as a supplement to the patient's basic demographic information (e.g., gender, age, and ethnicity) that is traditionally considered when recommending treatment. Since G protein-coupled receptors (GPCRs) are the most abundant cell surface receptors that can be targeted clinically (*see* Chapter 7) their study has become a priority in pharmacogenetic investigations aimed at advancing personalized medicine. This review outlines our current knowledge of genetic variation at *GPCR* genetic loci—variation that perturbs receptor function by altering ligand binding, G protein coupling, receptor activity, or cell surface expression through genomic mechanisms or cellular processes (e.g., receptor trafficking, dimerization, and desensitization) [1–3]. Examples discussed include the variants of the cannabinoid CB1 and CB2 receptors; the GPR55 receptor that binds cannabidiol; and the adrenergic receptors and neurotransmitter receptors [1, 4, 5]—to name a few.

We conclude this chapter with a survey of publically available online resources holding a large body of experimental data on GPCR variants that can be harnessed for *in silico* functional prediction of naturally occurring GPCR variants identified in pharmacogenomic studies.

1.1 Pharmacogenetics and Pharmacogenomics

Pharmacogenetics investigates how RNA and DNA variations influence physiological phenotypes related to drug response [1, 3]. Pharmacogenomics employs genome-wide approaches (genomic wide association studies, GWAS) and other high-throughput tools (microarrays and next-generation sequencing) to identify inherited variants responsible for interindividual differences in drug response, adverse drug effects, and phenotypes related to altered pharmacological properties of the receptor. These studies have been employed to classify the structure and function of putative drug targets across the entire genome [1]. Pharmacogenomics is not limited to identifying DNA sequence variants such as single-nucleotide polymorphisms (SNPs). The field includes the study of changes in gene expression and epigenetic regulators of gene expression. Pharmacogenomic approaches have also enabled the identification of novel therapies by means of reverse pharmacology which uses the receptor class as "substrate" for novel compounds [6]. Pharmacogenetics, by contrast, makes use of the genetic basis of drug response to optimize treatment [4, 5].

1.2 Application of Pharmacogenomic to Personalized Medicine

Personalized medicine strives to predict disease risk, outcome, and treatment responses based on the individual's genetic background. Pharmacogenomics plays an important role in identifying responders and nonresponders to medication, particularly for the purpose of avoiding adverse drug reactions (ADRs), and optimizing individual drug responsiveness. In response to FDA rulings, some drug labels now contain information on genetic biomarkers that describe variability in clinical response, risk for ADRs, and genotype-specific dosing.

Despite the fact that GPCRs are a primary target of therapeutic drugs, however, no drug label currently includes information on screening for GPCR variants. Yet there is a growing body of evidence for key influence of GPCR variants on drug efficacy or ADRs that should ultimately lead to the recognition of GPCR variants as informative biomarkers for drug treatment. For example, a dopamine D_2 receptor variant (rs1799978) has been implicated in variable risperidone efficacy in schizophrenia treatment [5, 6]. This example suggests the potential for GPCR-specific "personalized medicine" in which pharmacology is selected for a given variant. Such phenomena are reviewed below with respect to a number of GPCR systems.

The limited pharmacogenomic data validating the role of GPCR variants in differential drug response or ADRs is not surprising because the identification of variants contributing to complex phenotypes is confounded by genetic complexity and heterogeneity and the influence of environmental factors. Unlike the rare GPCR variants responsible for monogenic diseases, common genetic variants (e.g., SNPs) contribute to small effects (as little as 1 %) to the observed population variation of a complex trait or disease. Such variants may only display subtle to moderate molecular effects in controlled in vitro systems and as such their in vivo effects may be very difficult to identify.

2 GPCR Pharmacogenetics

GPCR pharmacogenetic studies provide information on the effect of genetic variants on the pharmacological properties of GPCRs including drug absorption and disposition (pharmacokinetics) and drug action (pharmacodynamics). The identification of rare variants in the prototypical GPCR rhodopsin associated with disease, followed by discovery of more common naturally occurring β2-adrenergic and melanocortin receptor variants that defined disease, traits, and pharmacological differences [1], laid the foundation for studying the pharmacology of naturally occurring GPCR variants. These findings provided the first insight into the locations and types of mutation that could alter GPCR function [2].

The past two decades of novel variant discovery studies have generated data on a plethora of common and rare variants in the GPCR pathway. Molecular pharmacological experiments provide data demonstrating both subtle and major effects of these mutations on GPCR function in vitro, and advances in bioinformatics now provide tools for predicting the function of these variants. While the role of the majority of these variants in complex traits and diseases is still unknown, genome-wide approaches suggest that combinations of GPCR pathway variants may be associated with disease, and that individual mutations within a GPCR pathway can result in similar disorders, such as in the case of *CASR* and *GNA11* variants identified in ADH [7]. Next-generation sequencing and genome-wide association studies will ultimately provide data on the role of these variants and their combination in complex disease and traits, including drug response.

Although early discoveries were contingent on the proof of principle that GPCR mutations may be associated with disease or with an altered phenotype, GPCR pharmacogenetics has increasingly become an independent field that studies the genetic basis of drug response phenotypes irrespective of disease state. As a result, the focus is now on defining GPCR variants that are important clinical indicators regardless of whether they are associated with disease predisposition [8, 9]. For example, although the rare variants of the cysteinyl leukotriene $CysLT_1$ and $CysLT_2$ receptors shown in Fig. 1 have been associated with atopic asthma [10, 11], these variant receptors, which have higher and lower efficacy for CysLT ligands, respectively, provide useful insight into the mechanism of drug action.

The significance of a GPCR variant may be best judged by its contribution to an in vitro pharmacological phenotype. The phenotype is often described with respect to altered efficacy (measured by EC_{50} and V_{max}) or altered binding (measured by B_{max} and K_d). In the following discussion, GPCR pharmacogenetics and pharmacogenomics are presented through specific examples of variant GPCR receptor systems (summarized in Table 1). This experimental data on structure and function relationships of variant receptors is then discussed in the context of the in silico functional prediction of naturally occurring GPCR variants [12–14]. Information on online resources dedicated to the evaluation of variant GPCRs concludes the discussion of GPCR variants that influence disease risk, drug response, and adverse drug effects.

2.1 The Calcium-Sensing Receptor: GPCR Variability in the Population

Although the majority of GPCRs are family A receptors, the calcium-sensing receptor (CASR), a member of family C, shares fundamental characteristics (e.g., ligand binding, signaling, and downregulation) with other GPCR families. This allows valid and informative comparisons between CASR and GPCRs.

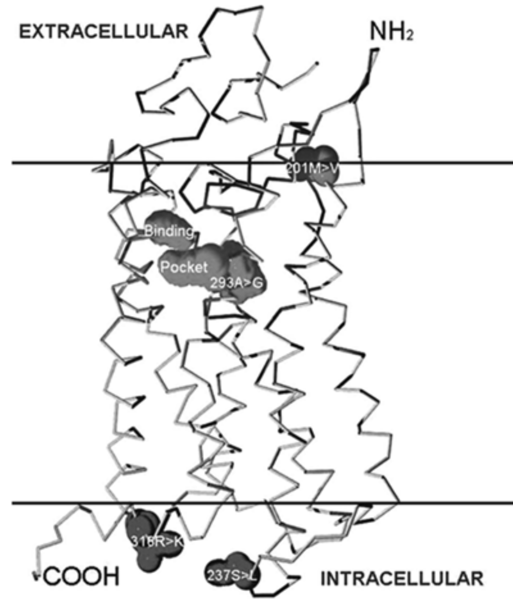

Fig. 1 Structure of the cysteinyl leukotriene 2 (CysLT) receptor and variants. The positions of the transmembrane (TM)-spanning domains of the CysLT receptor, the putative binding pocket, and four naturally occurring amino acid substitutions are shown in relation to the cutaway plasma membrane. Of the four single-amino acid variants discovered (Met201Val, Ser237Leu, Ala293Gly/Arg316Lys), only the partially inactivating Met201Val variant may be associated with the asthma or the atopy phenotypes. The Ala293 variant, found in the context of a compound heterozygote, Ala293Gly/Arg316Lys, results in an activating variant that is predicted to disrupt the putative binding pocket that was predicted from rhodopsin

CASR variants that are known to alter the sensitivity of the CASR may result in altered [Ca^{2+}] set points in tissues. Searching http://www.casrdb.mcgill.ca/ or dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) reveals a number of SNPs scattered across the more than 100 kb region of genomic DNA that encompasses the *CASR* gene. A number of common missense SNPs (Ala986Ser, Arg990Gly, and Gln1011Glu) are clustered in the DNA region encoding the cytoplasmic tail of CASR [15–22]. The most common of these, the Ala986Ser variant, has proven to be predictive of the unbound, extracellular calcium fraction [15, 16]. The Ala986Ser variant is, however, a relatively mild inactivating variant that may predispose to hypercalcemia without being fully predictive of hypocalciuria. By contrast, the Arg990Gly variant results in the increased calcium excretion that characterizes idiopathic hypercalciuria and is predictive of nephrolithiasis [21].

While *CASR* variants contribute to monogenic disorders such as FHH and ADH, common *CASR* polymorphisms may also account for the observed population variation in calcium response that is a risk factor for a variety of disease susceptibilities. In fact,

Table 1
Human G protein-coupled receptor (GPCR) sequence variants associated with altered risk for disease or altered pharmacology

Receptor	Variant/allele	Disease/phenotype	Pharmacology	Reference
α_{2A}-Adrenergic receptor (ADRA2A) 10q24–q26	N251K, third intracellular (IC3) loop		↑ Agonist-dependent G protein coupling—gain of function	[153]
β_1-Adrenergic receptor (ADRB1) 10q24–q26	G389R (C-terminus)		↑ Basal and agonist-dependent G coupling—gain of function	[152, 356]
β_2-Adrenergic receptor (ADRB2)	R16G	Nocturnal asthma/ severity	Agonist-dependent downregulation enhanced	[12, 357]
		Hypertension risk ↑		[358]
		↓ Albuterol response		[359]
		Myasthenia gravis		[159, 360]
	Q27E	Hypertension risk ↑	Resistance to downregulation	
5q32–q34		Heart failure/ performance		[12, 154, 361]
		Drug hypersensitivity	Albuterol response ↑	[163, 165]
	R16G/Q27E “2/2” haplotype			
	T164I	Asthma, heart disease, and immune disorders	↓ β_2-agonist affinity	[156–158, 161, 163–165]
	C341G	Obesity; heart failure	Altered Gs coupling adenylyl cyclase	[12, 148]
μ_1-Opioid receptor (OPRM1)	A6V, N40D, N152D		↑ Potency of β-endorphin; ↓ membrane trafficking ↓ Basal G protein coupling	[141–144]

	A6V, N40D	Haplotype associated with substance abuse		[145, 146]
6q24–q25				
	H260R	Idiopathic absence epilepsy		[362]
	H265R	Substance abuse/addiction		[145]
	S268P, N273D		↓ CaM, ↓ desensitization	[141, 142]
Dopamine receptor D_1 (DRD1)	*Dde* I (−48G, 5′ UTR) SNP and haplotype association	Alcoholism, bipolar disorder, attention-deficit/hyperactivity disorder (ADHD)	Polymorphisms may be in linkage disequilibrium with regulators of D_1 expression	[41, 60, 62, 64, 65]
5q35.1				
Dopamine receptor D_2 (DRD2)	Short/long/longer nt.414/443/TG splice	↑ Expression of all dopamine D variants in schizophrenia	Short is three times more sensitive to dopamine	[363]
	A1 (*Taq*I RFLP)	Reward deficiency/addiction	↓ Receptor expression	[91, 364–366]
		Obesity		[87, 89]
11q23		Alcoholism		[91–93]
	A2, nt. C957T	Pathological gambling		[60, 367]
	S311C, P310S, V96A, nt.			
	A241G	Tardive dyskinesia	Altered drug affinity or clinical efficacy	[88, 94, 95] [368, 369]

(continued)

Table 1 (continued)

Receptor	Variant/allele	Disease/phenotype	Pharmacology	Reference
Dopamine receptor D_3 (DRD3)	*Bal*I	Unipolar depression		[370, 371]
3q13.3	S9G (*Msc*I RFLP)	Tardive dyskinesia		[85, 86]
	S9G + SNPs	SNPs and haplotypes associated with schizophrenia		[81, 82]
Dopamine receptor D_4 (DRD4)	48 bp Repeat in IC3	Effect on clozapine binding	Effect on G protein coupling	[56, 67, 69]
11p15.5		Novelty seeking; ADHD		[74–80, 372]
		Schizophrenia		[68, 69, 73]
	V194G	Protein expression ↑		
	Promoter SNPs		↓ Sensitivity to dopamine and clozapine	[67, 68, 73]
Dopamine receptor D_5 (DRD5)	L88F		↑ Affinity of agonist	
4p16.1–p15.3	N351D		↓ Affinity of agonist	[373]
5-Alphahydroxytryptamine receptor 1B (HTR1B)	F124C		↑ Affinity for ligand	[132, 133]
6q13	SNPs	ADHD uncertain		[130, 135–138]
5-Alphahydroxytryptamine receptor 1D (HTR1D)				

6q13	G861, SNPs	Obsessive-compulsive disorder		[135–138]
5-Alphahydroxytryptamine receptor 2A (HRT2A)	102T/C (silent)	ADHD; Alzheimer's disease psychotic symptoms	↓ Response to clozapine	[94, 100, 117, 118]
		Schizophrenia marginal; Suicide association marginal; Alcohol/behavioral possible		[98, 99, 109–116]
13q14–q21		↓ Response to clozapine; ↓ G(q) and G(13) signaling		[97, 102–106]
	T25N, I197V, A447V, H452Y	Citalopram response; depression, negative symptoms of schizophrenia	↓ Calcium mobilization	[107, 108]
	−1438A/G promoter	Possibly eating disorders		[374–376]
5-Alphahydroxytryptamine receptor 2C (HRT2C)	C23S	Alzheimer's disease psychosis: suicide ideation	May be associated with ↑ clozapine response	[97, 117, 125, 126]
Xq24	Promoter SNPs	May be associated with obesity, type 2 diabetes	May be associated with clozapine-induced obesity	[119–124]
5-Alphahydroxytryptamine receptor 6 (HRT6)	C267T (267C allele)	May be associated with an ↑ risk for Alzheimer's		[127–129]
Histamine receptor H1 (HRH1)	E349D, L449S, many SNPs	No association with atopic asthma	L449S: Probably no effect on clozapine response	[97, 377–379]
3p21-p14				

(continued)

Table 1 (continued)

Receptor	Variant/allele	Disease/phenotype	Pharmacology	Reference
Histamine receptor H2 (HRH2)	R649G, SNPs	Some evidence for ↑ frequency in schizophrenia	No effect on clozapine response	[378, 380, 381]
	Promoter SNPs (1018 G/A)	No association with atopic asthma or schizophrenia		[378]
Histamine receptor H3 (HRH3)			↑ Constitutive activity of this receptor subtype	[382–384]
Cysteinyl leukotriene receptor 1 (CYSLTR1)	G300S	Atopy/asthma association: Tristan da Cunha population	Gain of function	[10, 385–390]
Xq13–q21				
Cysteinyl leukotriene receptor 2 (CYSLTR2)	M201V, SNPs	Tristan da Cunha, Caucasian, and Japanese populations	Loss of function	[11, 385–390]
13q14				
Angiotensin receptor 1 (AGTR1) 3q21–q25	1166 A/C (3′ UTR)	↑ Risk of hypertension	↑ Angiotensin response	[27, 391–393]
		Hypertension and vasoconstriction		[24, 32]
		↑ Risk of ischemic events		[23, 29, 394]
		Influences aortic stiffness		[32]
	Many SNPs	Pharmacogenetic variants	Interact with ACE deletion	[29, 31, 34–36]

Endothelin receptor, type A (EDNRA)	Lys198Asn and −134delA, SNPs	Affect pulse pressure/ baroreflex/heart failure association possible	↓ G_q coupling, in vitro	[395–405]
4q31.2				
	Afi II	Rare reports of ↑ immunoglobulin E levels/atopy	Altered receptor expression	[181, 182]
Prostaglandin D receptor (PTGDR)	Promoter SNPs undermine normal helper T anti-inflammatory response	Asthma	Altered receptor expression ↓ Anti-immune response to PGD_2	[184, 185, 406, 407]
14q22.1				
G protein-coupled receptor 44 (GPR44/CRTH2)	3′ UTR SNPs: n.G1544C	Asthma	Altered receptor expression ↑ Inflammatory response to PGD expressed on helper T, type 2	[186–188, 408]
11q12–q13.3	n.G1651A			[193, 408]
	n.G1544C/G1651A haplotype			
Chemokine, CC motif, receptor 2 (CCR2)	V64I	↓ AIDS progression	CCR5/CXCR4 heterodimer	[326]
3p21		↓ Sarcoidosis progression		[12, 326]
	R275Q, L351P, L302P	Susceptibility to insulin-dependent diabetes mellitus		[12, 323, 409]
Chemokine, cc motif, receptor 2 (CCR3)	R275E, L302P	Population studies		[327, 328]

(continued)

Table 1 (continued)

Receptor	Variant/allele	Disease/phenotype	Pharmacology	Reference
Chemokine, cc motif, receptor 5 (CCR5)				
3p21	Δccr5 (32-bp deletion) 59029 A/G	Partial resistance to HIV infection; protection against hepatitis B infection possible	Altered binding affinity	[319, 320, 410–412]
	Homozygous	↓ AIDS progression		[410–412]
	Heterozygous	↓ Non-Hodgkin's lymphoma		[410–412]
Chemokine CX3C motif, receptor 1 (CX3CR1)				
3pter-p21	V249I, T280M	↑ AIDS progression		[413]
Calcium-sensing receptor (CASR)	0.9 kb *alu* insertion in exon 7	↓ Adenylyl cyclase	Loss of function	[414, 415]
3q13.2–q21	A116T, N118K, etc.	Familial hypocalcemia	↑ IP3 response	[416–419]
	A986S, R990G	Common polymorphisms	Predictive of serum Ca^{2+}	[14–22]

SNP single-nucleotide polymorphism, *Ip3* inositol triphosphate

CASR SNPs have been associated with a number of complex phenotypes. The Ala986Ser variant has been associated with bone mineral density [18], primary hyperparathyroidism [19], and Paget disease [20].

2.2 Polymorphisms of the Angiotensin II Receptor in Hypertension

The complexity of GPCR pharmacogenetics is illustrated by the enormous heterogeneity in efficacy of antihypertensive medications. This is particularly true for antihypertensives that target the renin–angiotensin system. The renin–angiotensin system consists of a two-enzyme cascade involved in the regulation of blood pressure and electrolyte homeostasis. The renin enzyme cleaves the substrate, angiotensinogen, to angiotensin I, which is in turn cleaved by angiotensin-converting enzyme (ACE) [23] to generate angiotensin II (an octapeptide). Angiotensin II acts at the angiotensin II type 1 GPCR (AT1R) as a potent vasoconstrictor.

The identification of the AT1R [24] that binds angiotensinogen ligand [25] produced by ACE [26] led to the discovery of *AT1R* polymorphisms implicated as risk factors for cardiovascular disease. Because antagonism of the AT1R is used to decrease blood pressure in hypertensive patients [27], *AT1R* polymorphisms, such as 1166 A>C, may be clinically significant. Located in the 3′ untranslated region of the *AT1R* gene, the A1166C polymorphism is associated with hypertension [24], left ventricular hypertrophy [28], coronary heart disease, myocardial infarction [29], and progression of diabetic nephropathy [23, 30]. Pharmacological evidence suggests that the 1166 A>C substitution is associated with altered receptor sensitivity. Further evidence suggests that the 1166C allele may be predictive of the success of antihypertensive drug treatment [31].

Homozygosity for the *AT1R* A1166AC polymorphism is associated with greater vasoconstriction in in vitro studies, suggesting that the variant may alter gene expression in vivo and may render carriers at risk for increased vasoreactivity and/or cardiovascular disease [32, 33] and consequent higher blood pressure [24].

The frequency of the C allele is approximately 25 % in the Caucasian population and as such may account for a variety of symptoms of heart disease in the population [23]. It is also possible that epistatic interactions between the *AT1R* gene polymorphism, an ACE deletion/insertion variant, and the Met235Thr variant of the angiotensinogen gene [29] lead to poor treatment outcome [23]. The heterogeneity of vascular disease [34, 35] may underlie some paradoxical *AT1R* findings [36, 37] .

2.3 Neurotransmitter Pharmacogenetics

Psychoactive drugs bind to many GPCRs involved in neurotransmitter pathways. These GPCRs include the dopaminergic, serotonergic, and muscarinic receptors. Thus, the genomic structure and expression of the genes encoding these receptors may be relevant to understanding disease progression and therapeutic outcome of neuropsychiatric disorders. Data from association studies examining

GPCR variant relevance to disease and drug response phenotypes may be useful in modeling systems suitable for drug development—such as targeting such systems as the orexin/hypocretin neuropeptides [37]. Such analyses are, however, complicated by the variability across different populations.

Interestingly, candidate gene studies of both the dopamine and serotonin receptors have revealed some GPCR variants to be associated with both the risk for developing a disorder and the treatment outcome [38–45]. In view of this complexity, studies of GPCR gene variant contributions to phenotypes such as drug response, nonresponse, and adverse events have increasingly made use of a genome-wide association survey (GWAS) strategy. This approach has implicated many genes in disease that are not directly implicated in GPCR function. For the purposes of this review, however, the focus will be on understanding GPCR variant phenotypes [46] in the context of the studies of altered receptor function, such as receptor activation, signaling, and inactivation [47, 48]. The role of *GPCR* mutations in monogenic human disease [49] is reviewed in Chapter 7.

2.3.1 Dopamine Receptor Pharmacology

Dopamine is a major catecholamine neurotransmitter in the central nervous system that has been implicated in the regulation of locomotor activity, emotion, cognition, and behavior reward and in neuroendocrine regulation [39]. Clinically, dopaminergic drugs (e.g., risperidone) that block or activate dopamine receptors are used to treat neurodegenerative, neuropsychiatric, and neurodevelopmental conditions such as Parkinson's, schizophrenia, bipolar disorder, and autism [40].

Dopamine Receptor Variants

The pharmacological properties used to distinguish dopamine receptors as dopamine D_1-like or dopamine D_2-like have been exploited in a number of dopamine receptor gene-related association studies. For the most part, the D_1-like dopamine receptors D_1 [50–52] and D_5 [53] have not shown as much association with disease as the D_2 dopamine receptors.

The high sequence conservation of the dopamine D_1 receptor may reflect its importance to central nervous system function as is also suggested by its wide expression in the brain and relatively high affinity for dopamine. The dopamine D_1 receptor gene is essentially nonpolymorphic, in its exon–intronic regions [41]. A 5′ untranslated region (UTR) promoter SNP, however, has been associated with a number of neuropsychiatric disorders and drug response phenotypes.

The D_2-like receptors—D_2 [54], D_3 [55], and D_4 [56]—have similar dopamine sensitivities and are much more polymorphic than the D_1-like receptors [46]. The dopamine D_4-like receptor polymorphisms include SNPs, variable-number tandem repeats (VNTRs), and splice variants [56]. The polymorphic forms of the

dopamine D_4 receptor also include variable numbers of 48 bp repeat sequences (denoted $D_{4.1}$ to $D_{4.7}$) [56].

The efficacy of antipsychotics that target dopamine receptors appears to result mostly from blockade of D_2-like receptors [57–59], a possibility consistent with studies suggesting that D_2 receptor dysregulation may be the downstream result of pathophysiology present in disorders such as schizophrenia [57]. Binding of the classical antipsychotic drugs (e.g., bromocriptine and raclopride) is about two orders of magnitude stronger for D_2 compared to D_4 receptors. The atypical antipsychotics, such as clozapine, however, have less potent effects on the dopamine D_2 and D_3 receptors compared with the D_4 receptor [42, 56]. Clinically, the effects of clozapine on dopamine D_4 receptor variants, such as $D_{4.2}$, $D_{4.4}$, and $D_{4.7}$, are probably similar under therapeutic conditions [42, 56, 57].

Dopamine Receptor Association Studies

Dopamine receptor variants have been widely studied for associations with psychiatric and drug response phenotypes [42]. Many of these studies have been based on a candidate gene hypothesis, i.e., that the efficacy of pharmaceutical agents targeting dopamine receptors (especially the D_2 receptor) in ameliorating psychotic illness implies that alterations in dopamine receptor pathways may be associated with the risk for these diseases [3, 57].

With respect to the dopamine D_1-like receptors, including the dopamine D_5 receptor (which is ten times more sensitive to dopamine and has a much more narrow tissue expression than the dopamine D_1 receptor), very few associations of coding variants with disease have been identified [41, 42]. Associations of noncoding promoter region SNPs, however, have been associated with various disease phenotypes. These findings, while far from unanimous, suggest associations with bipolar disorder, alcoholism, and attention-deficit disorder, to name a few [60–65].

Reports of association of the dopamine D_2-like receptors with psychiatric phenotypes are more common [57, 66]. One such finding is an association between schizophrenia and dopamine D_4 receptor variants that appear to alter dopamine D_4 receptor expression. Dopamine D_4 polymorphisms also appear relevant to the efficacy and the neuromuscular toxicity (tardive dyskinesia) of antipsychotics such as clozapine [67–73].

In contrast to these data on the dopamine D_2, the contribution of dopamine D_4 polymorphisms to disease is less well understood—although increased levels of dopamine D_4 receptor expression in schizophrenia appear to be reproducible in independent studies [71, 72] and may be associated with a Val194Gly polymorphism [68, 69]. Disorders such as attention-deficit/hyperactivity disorder (ADHD) [74–77] and novelty-seeking behavior [78–80] have also been associated with the D_4 dopamine receptor, albeit not consistently [74–80].

Studies of the dopamine D_3 receptor variants in schizophrenia [81–83] have yielded variable results. Tardive dyskinesia in schizophrenic patients treated with clozapine, however, has been associated with D_3 receptor variants [84–86]. These findings suggest that although *GPCR* gene variants may not always contribute to a disease phenotype, they may be associated with genetic variability in pharmacology or pharmacogenetics.

Associations have also been reported between dopamine D_2 receptor variants with obesity [87], tardive dyskinesia [88], smoking and obesity [89] [87–89], and alcohol dependence [90–93]. A polymorphism of the dopamine D_2 receptor has also been associated with development of tardive dyskinesia in schizophrenics undergoing antipsychotic treatment [88, 94, 95]. While the results of association studies may vary [3, 12], these data will be increasingly clarified as GWAS approaches are used to assess dopamine receptor pharmacogenetics—including the possible role of the dopamine D_2 receptor in many of the psychoses [57, 96]. Together, these data suggest that *GPCR* gene variants may contribute to both disease phenotypes and genetic variability in pharmacology or pharmacogenetics.

2.3.2 Serotonin Receptor Polymorphisms

The serotonergic system is targeted by a variety of pharmaceutical agents including antidepressant medications. It has however been challenging to identify reproducible associations between the psychiatric symptoms and specific receptor variants, although, as described below, polymorphisms in receptors for serotonergic drugs have been associated with both clinical nonresponsiveness [97–100] and adverse events [40].

Associations of SNPs in such genes as the serotonin 5-HT_{2A} and 5-HT_{2C} receptors and the histamine H_2 receptor with drug response/nonresponse phenotypes have been widely reported [97]. The 5-HT_{2A} receptor gene and the 5-HT_{2C} Cys22Ser gene variants have, for example, been associated with altered responses to clozapine [97]. The 5-HT_{2A} variants, such as the His452Tyr variant, have specifically been associated with decreased calcium flux in response to clozapine that likely results from decreased Gq signaling [97, 102–106]. Another *5-HT_{2A}* polymorphism −1348A/G in the promoter region appears to be associated with abnormal or reduced response to the antidepressant citalopram, possibly due to an associated altered receptor density [107–109].

Some studies have reported associations of serotonin receptor variants with clinical phenotypes. Cys23Ser and Gly22Ser 5-HT_{1A} receptor variants, for example, have been associated with phenotypes such as intractable suicidal ideation [98], ADHD [100], bipolar disorder, and schizophrenia [98, 99, 109–116] and the −1348 A/G polymorphism of the 5-HT receptor has been associated with schizophrenia, eating disorders, and psychotic symptoms in Alzheimer's patients [94, 100, 117, 118], although these associations are not consistent across different studies.

Data on 5-HT_{2C} receptor polymorphisms are also inconsistent. However, promoter polymorphisms for this gene have been associated with clozapine-induced weight gain [119–124] and the Cys23Ser variant has been associated with increased clozapine response, psychotic symptoms in Alzheimer's disease, and suicide ideation [97, 117, 125, 126]. A *5-HT_6* gene variant (C267T) has also been associated with increased risk for Alzheimer's [127–129].

Polymorphisms in the receptors for the triptan drugs used to treat migraine, such as the 5-HT_{1B} and 5-HT_{1D} receptors, have also been studied with respect to their relevance to pharmacogenetics and disease [130, 131]. For example, polymorphisms at the *5-HT_{1D}* receptor locus on chromosome 1p36 have been found to be associated with migraine although this association may derive from an adjacent gene, *PRDM16*, which was associated with migraine without aura [131]. This may account for what may be false-positive associations of the *5-HT_{1D}* receptor locus with undifferentiated migraine [130].

Coding SNPs for the *5-HT_{1D}* seem rare [130], although a variant of the 5-HT_{1B} receptor, Phe124Cys, has been described and shown to have a higher affinity for ligand [132]. Other studies have also reported associations of *5-HT_{1B}* and *5-HT_{1D}* receptor variants with ADHD [133, 134] and obsessive–compulsive disorder [135–139].

In view of this, understanding the surprisingly large number of poor responders, both to antidepressants and antipsychotics, may result not from *GPCR* candidate gene studies as much as from GWAS [44, 140]. The pharmacogenomic approach to identifying candidate loci in psychiatric disorders depends on genome-wide mapping of the contribution of SNPs to altered drug response [12]. The pharmacogenomic strategy may also identify novel GPCR targets and other genes that interact to create a polygenically determined responder/nonresponder phenotype [5].

2.3.3 Opioid Receptor Polymorphisms

Opioid receptor variants have been associated with altered pharmacology, an issue of particular clinical relevance in view of the roles for opioid neurons in addictions [12]. The pharmacogenetics of μ-opioid receptor coding variants (e.g., Asn40Asp, Asn152Asp [141, 142], Val158Met, His260Arg, His265Arg, and Ser268Pro) have been extensively studied [142, 143]. Among these polymorphisms, the Asn40Asp and Val158Met variants have been shown to bind the natural β-endorphin ligand with significantly higher affinity and to be trafficked to the cell membrane with reduced efficiency [141]. The Asn40Asp variant specifically appears to confer resistance to surgical analgesia, patients with this variant requiring 63–93 % higher morphine doses [142]. A study of the μ-opioid receptor found that SNP haplotypes including the synonymous 188A>G variant were not only more frequent in opiate addicts [145] but also associated with opioid resistance [142].

Interestingly, not all opioid receptor gene variants lead to clinically meaningful alterations in opioid signaling [12]. For example, while the Ser268Pro variant disrupts a calmodulin kinase II-binding site required to maintain a basal level of receptor signaling [141], resulting in diminished receptor desensitization, the low frequency of the variant, even among addicted individuals, limits its significance [12]. Mechanistically, however, the variant is interesting. The effect may be attributable to the elimination of the normal competition for the Ser268 residue that normally exists between calmodulin kinase and the G_i/G_o protein [144]. As a result, the 268Pro receptor variants are more frequently found in the active conformation necessary for ligand binding. The variant may also be relevant to addiction because people expressing the receptor variant are predicted to have an altered tolerance for opioid ligands.

The limitations associated with studying vary rare *GPCR* variants illustrate why studies of pain genetics have tended to adopt a genome-wide approach [146]. However, more common opioid variants, such as the 188A>G variant, are likely to be associated with drug response in GWAS studies, especially given the significant difference in variant receptor pharmacology. The Opioid Receptor Database (http://www.opioid.umn.edu) represents a useful resource on this subject.

2.4 Pharmacogenetics of Adrenergic Receptors

One of the better examples of the potential of personalized medicine comes from data on adrenergic receptors. The adrenergic receptors are targeted by endogenous catecholamine ligands (epinephrine and norepinephrine) and by therapeutic antagonists and agonists such as albuterol for asthma and isoproterenol for heart failure treatment, respectively [147, 148]. Although the adrenergic receptor variants were among the first *GPCR* polymorphisms to undergo extensive in vitro study [149], and allelic variants of these receptors were found to be common, their clinical relevance has only emerged relatively recently [147]. Variant forms of adrenergic receptors can elicit a wide spectrum of disease phenotypes or altered drug efficacies. Polymorphic adrenergic receptors have been reported to result in both gain and loss of receptor efficacy or potency phenotypes. Since adrenergic receptors are widely expressed, variants in these proteins are highly physiologically significant.

For example, β_1-adrenergic receptor variants can elicit a wide spectrum of altered drug efficacies that result from both gain and loss of receptor efficacy or potency. Pharmacogenetic variants do not always result in molecular defects so dramatic that they constitute a measurable risk for disease phenotype—although the β_1-adrenergic receptor [147, 151] may be one. Clinically, however, adrenergic GPCR variants can be important in determining drug response.

For example, while not constitutively activated, the Arg389Gly β_1-adrenergic receptor variant results in a gain in second-messenger signaling (efficacy and potency). This results in a shift to the left of the dose-response curve in agonist-elicited second message. This variant is common in the population and may be significant with respect to drug efficacy and disease risk [152–154]. Similarly, the Asn251Lys variant of the α_{2A}-adrenergic receptor results in increased efficacy and potency that may be of clinical relevance [152, 153].

Coding and promoter polymorphisms of other adrenergic receptors may also cause altered expression, ligand binding, coupling, or regulation phenotypes. For example, the Pro64Gly variant of the β_3-adrenergic receptor, expressed in adipose tissues, has been associated with obesity [150].

2.4.1 Downregulation Polymorphisms in the β_2-Adrenergic Receptor

The β_2-adrenergic receptor gene displays a fair degree of polymorphism in human populations. Constitutively active mutant (CAM) and loss-of-function (LOF) variants, however, are in evidence. Like the dopamine receptors, the β_2-adrenergic receptor variants are often relevant to pharmacogenetics—although genome-wide studies suggest that the effect is nominal [147]. β_2-adrenergic receptor pharmacogenetics is complex. For example, the allele distributions of SNPs at amino acid positions 16, 27, and 164 are skewed in asthma, hypertension, obesity, and some immune disorders. Among these, the Arg16Gly receptor displays enhanced agonist-promoted downregulation, suggesting that this receptor may be rapidly lost from the cell surface and degraded in lysosomes. By contrast, the Gln27Glu polymorphism is actually resistant to downregulation [148, 155].

2.4.2 Heart Disease Associated with β_2-Adrenergic Receptor Polymorphism

Variants of the β_2-adrenergic receptor, especially the Thr164Ile polymorphism, have been associated with increased severity of congestive heart failure (CHF). CHF subjects with the Thr164Ile mutation have a 1-year survival rate of 42 %, as compared to 76 % for a control group with the wild-type β_2-adrenergic receptor [154, 156]. Carriers of the 164Ile polymorphism may therefore be candidates for more aggressive treatment [156]. By contrast, the Arg16Gly and Gln27Glu polymorphisms may have less influence on disease course.

2.4.3 Myasthenia Gravis and β_2-Adrenergic Receptor Polymorphisms

Myasthenia gravis (MG), an autoimmune-based failure of cholinergic transmission at the neuromuscular junction, has been associated with variant forms of the β_2-adrenergic receptor. The disorder is associated with decreased density of β_2-adrenergic receptors on peripheral blood mononuclear cells: particularly in patients with the Arg16Gly variant. 16Gly is also associated with antibodies to the variant β_2-adrenergic receptor and the secretion of cytokines in response to β_2-adrenergic receptor peptide fragments.

In addition, acetylcholine receptor antibodies have been measured at higher levels in patients homozygous for β_2-adrenergic receptor variants [158].

The role of the β_2-adrenergic receptor in development of MG is supported by the evidence showing that increased 16Gly homozygosity and lower prevalence of 16Arg homozygosity are characteristics of MG patients [158]. These data suggest that pharmacogenetic variants can sometimes also be associated with disease susceptibility.

2.5 Asthma GPCR Pharmacogenomics

Studies of GPCRs in asthma can be differentiated on the basis of whether they measure the contribution of candidate genes to atopy, bronchial hyperreactivity (BHR), drug response/nonresponse, or another phenotype. For example, de-orphanization studies have identified GPR154 (GPRA) as the neuropeptide S receptor 1: a locus positionally cloned in asthma [159]. Additional genome-wide studies, however, have also reported an association between a coding SNP of *GPR35*, an orphan GPCR, and primary sclerosing cholangitis, as well as ulcerative colitis [160].

Other GPCRs implicated in asthma include one of the three α_1-adrenergic receptor subtypes, the *ADRA1B* gene. The gene product is expressed in the lung and may be functionally relevant to asthma. Although more commonly implicated in physiologic responses such as fight-or-flight signaling, the α_1-adrenergic receptor may also be involved with proinflammatory responses [161].

2.5.1 The β_2-Adrenergic Receptor

Variants of the β_2-adrenergic receptor (ADRB2) are likely to be involved in the development of asthma: a phenomenon that has bearing on the pharmacogenetics of adrenergic drugs [158, 161]. Although the β_2-adrenergic receptor Arg16Gly variant is associated with reduced lung function [158] and familial nocturnal asthma [161], it is also commonly resistant to some β_2-adrenergic receptor agonists [162]. This may result from receptor loss at the cell surface during defective downregulation. As a result of the drug response phenotypes and disease phenotypes stemming from the same β_2-adrenergic receptor genetic variants, clinical management can become very difficult.

Given the difficulty of analyzing the contribution of the many β_2-adrenergic receptor variants to various phenotypes, many studies have refined the analysis by constructing haplotypes consisting of two or more variants on the same chromosome (i.e., in *cis*). For example, the variants encoding the Arg16Gly and Gln27Glu variants form a haplotype that may predict treatment outcome to the β_2-adrenergic receptor agonist albuterol [12]. Carriers of these variants have a complicated phenotype because the downregulation-resistant 27Glu receptor results in β_2-adrenergic receptor hypersensitivity that potentially complicates the treatment [163]. These findings suggest the complexity of albuterol hypersensitivity [164].

The β_2-adrenergic receptor gene mutations have, therefore, been associated with a wide spectrum of respiratory phenotypes that include altered drug responses and bronchial hyperreactivity disease. The β_2-adrenergic receptor polymorphisms probably represent only a few of the genetic variables involved in asthma pathophysiology [157, 165]. There may be potential to use these variants more widely to personalize diagnosis and treatment options.

2.5.2 The Cysteinyl Leukotriene Receptor System

It is possible that genetic variability in the genes encoding proteins critical to the CysLT pathway (*see* Fig. 1) contribute additively or synergistically to altered drug responses. Studies of $CysLT_1$ and $CysLT_2$ receptors, with high affinity for the leukotriene, LTD_4, have focussed on how receptor variants might alter the response to agonists and on their possible contribution to the atopy phenotype [10, 11, 166]. The identification of the $CysLT_3$ receptor with higher affinity for LTE_4 underscores the known complexity of the system; however, many pharmacological reactions are attributable to the action of LTD_4 ligand with its cognate $CysLT_1$ and $CysLT_2$ receptors [167].

$CysLT_1$ Receptor Pharmacogenetics

The $CysLT_1$ receptor has been associated with atopic asthma in at least one geographically isolated population—the residents of Tristan da Cunha in the Southern mid-Atlantic. This is intriguing from the point of view of personalized medicine because drugs that act as high-affinity antagonist ligands of the $CysLT_1$ receptor (e.g., montelukast, pranlukast, zafirlukast) [168–176] or allergic rhinitis [177] have been reported to be ineffective in approx. 20 % of patients [178]. The discovery that there are at least four *CysLT*$_1$ transcripts generated by alternative splicing suggests the heterogeneity possible in the system [179].

Although the *CysLT*$_1$ receptor gene may harbor inactivating mutations in some populations, studies of the Tristan da Cunha population have only identified the unremarkable Ile206Ser variant and an activating Gly300Ser mutation. Unfortunately, additional clinical correlations between *CysLT*$_1$ receptor genotypes and drug response have not yet been reported for the study population.

$CysLT_2$ Receptor Pharmacogenetics

The $CysLT_2$ receptor may also be important to the pharmacology of $CysLT_1$ pharmaceuticals if, like many GPCRs [2], these receptors form functional heterodimers with unique pharmacological properties. While specific $CysLT_2$ receptor antagonists have not been marketed, drug development based on targeting the $CysLT_2$ receptor may be important, given that approx. 20 % of patients treated with CysLT agents fail to respond. This problem may become particularly important in patients for whom both the $CysLT_1$ and $CysLT_2$ receptors are polymorphic.

A Met201Val variant has been associated with atopy in populations including the Tristan da Cunha isolate. Unlike the p.G300S $CysLT_2$ variant, however, the Met201Val variant is partially

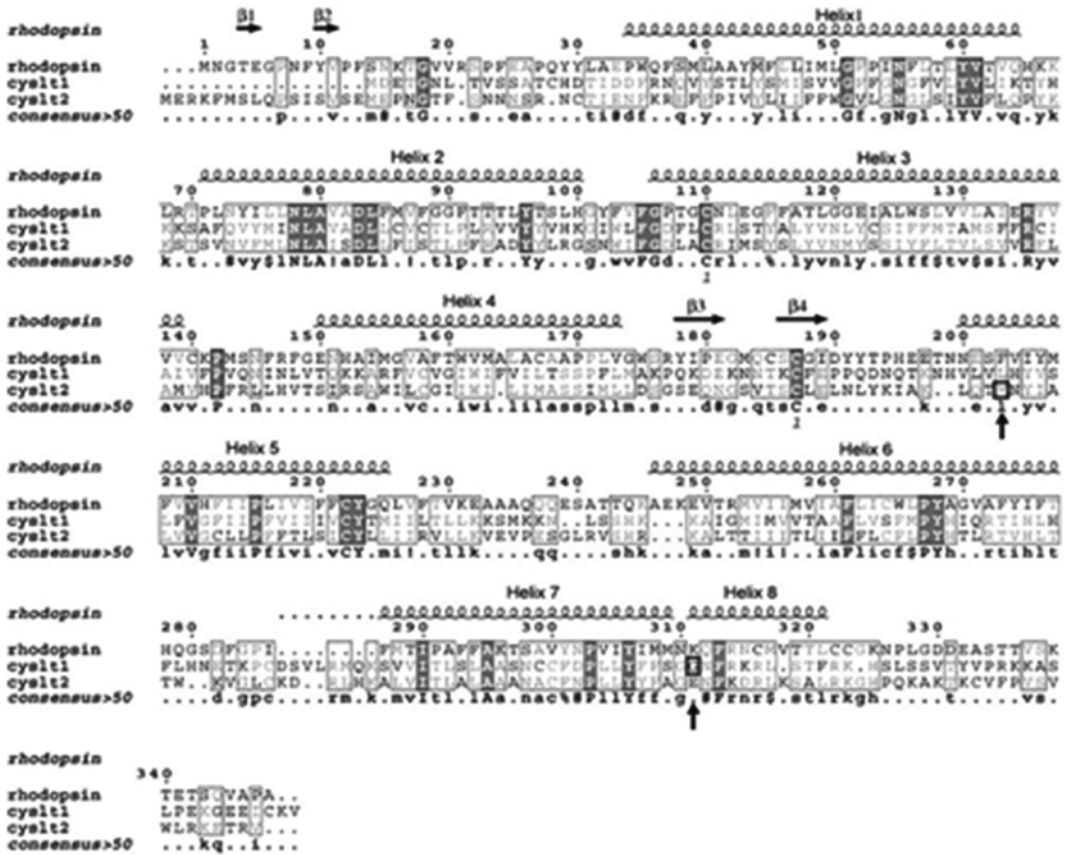

Fig. 2 Alignment of the protein structure of the cysteinyl leukotriene 1 (CysLT) and 2 (CysLT) receptors in relation to rhodopsin. The amino acids conserved between these family A receptors are shown. The consensus is greater than 50 %. These data formed the basis of the model predicting the CysLT and CysLT transmembrane domains (helices 1–7), the four β-sheets, and the putative cysteinyl leukotriene-binding domain. The amino acid variants that are associated with atopy or asthma, the G300S CysLT variant, and the M201V CysLT variant are each *boxed* and noted with *arrows*

inactivating (*see* Fig. 2). The fact that $CysLT_1$ and $CysLT_2$ are both polymorphic in some individuals suggests that the co-expression of variant receptors may alter CysLT signaling.

Interaction of $CysLT_1$/$CysLT_2$

In the study of the Tristan da Cunha isolate, the activating $CysLT_1$ Gly300Ser variant and the inactivating $CysLT_1$ Met201Val variant receptor were both associated with atopic asthma. It is possible, therefore, that these variants interact in other populations to confer the risk for atopy and/or altered leukotriene pharmacology. While evidence for a functional interaction between $CysLT_1$ and $CysLT_2$ receptors in mast cells seems likely [180], the pharmacological consequence of heterodimers formed from variants remains to be fully addressed. However, the fact that all Tristan da Cunha individuals reported to be heterozygous for both $CysLT_1$ and $CysLT_2$ receptor variants were atopic suggests that more work in this area might be rewarding.

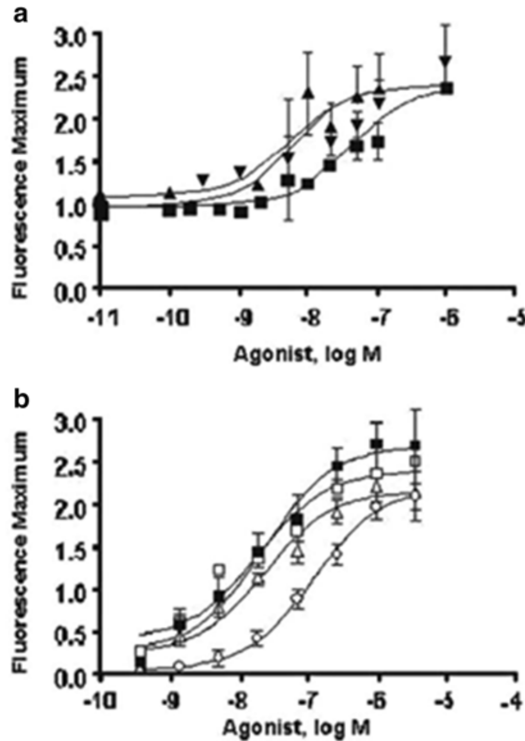

Fig. 3 In vitro effects of Gly300Ser cysteinyl leukotriene 1 (CysLT) receptor and Met201Val on CysLT receptor signaling compared with wild type. (**a**) Cysteinyl leukotriene D (LTD) concentration–response curve for CysLT receptors in transfected cells. Inositol triphosphate (InsP) generation assay of the variants and wild-type forms of the CysLT receptor. Both 300 S and 206 S variants' EC were significantly different from wild type. The concentrations of LTD required to produce the InsP effect were much higher than those used in the [Ca^{2+}] assay shown in (**b**), in which calcium flux was assayed for the variants and wild-type and variant forms of the CysLT receptor challenged with LTD. The resulting changes in intracellular calcium concentrations were measured as fluorescence maximum. For LTD4, the Met201Val variant (*open circle*) had a significantly greater EC compared to wild type (*filled square*), while the Ser237Leu (*open triangle*) and Ala293Gly/Arg316Lys (*open square*) variants were not different. However, the Ala293Gly/Arg316Lys variant showed decreased efficacy (V). Interestingly, when the Ala293Gly/Arg316Lys receptor was challenged with the agonist max Bay u9773 (data not shown), this rare variant was demonstrated to have a significantly smaller EC compared to wild type, indicating that, under some circumstances, the variant is activating [10, 11]

The relative location of each variant is shown in the alignment of each CysLT receptor with rhodopsin (*see* Fig. 3). This alignment was used to predict the transmembrane spanning and the putative binding pocket of the receptors. This suggests that variants of these receptors modify the putative CysLT-binding site that is partially determined by the integrity of their respective transmembrane domains. The abnormal but opposite pharmacology of the variants

of these receptors, causing increased potency of LTD_4 at the Gly300Ser receptor variant (located in the intracellular portion of TMD7) and decreased potency of LTD at the Met201Val receptor variant (located in the extracellular portion of TMD5), deserves further investigation.

2.5.3 Endothelin Receptors

Endothelin 1 (ET1) is a 21-amino acid peptide released from bronchial cells that is derived from its biologically stable surrogate, C-terminal-pro-endothelin-1 (CT-pro-ET-1). It has potent vaso-constrictive agonist properties mediated by two receptor types—A and B. The endothelin 1 type A (EDNRA) gene *AflI* SNP was associated with atopy concurrent with elevated antigen-specific immunoglobulin E (IgE) levels in a British population [181]. While the involvement of the EDNRA *AflI* SNP in atopy may be uncertain; however, there is evidence from GWAS studies of an association of the endothelin system with asthma [182].

2.5.4 Prostaglandin D2 Receptors

The traditional prostaglandin D_2 receptor (PGD2), a target for prostaglandin D2 (PDG2), is encoded by the *PTGDR* gene located on chromosome 14q22.1. The PGD2 receptor SNPs associated with asthma are located in the gene's promoter. Determining the functional relevance of these variants is complicated by the fact that PDG2 also acts on the PGD2 receptor, formerly known as CRTH2 (or chemoattractant receptor homologous molecule) expressed on T-helper type 2 (T 2) cells. Although these receptors both bind the proinflammatory eicosanoid PGD2, they appear to have opposite signaling properties [183].

While PGD1 receptor activation is associated with ameliora-tion of asthma pathology, the activation of PGD2 increases eosino-phil recruitment at inflammatory sites—pathological changes characteristic of atopic dermatitis and allergic asthma [70]. It is possible that maintaining a greater expression of prostanoid PGD1 relative to *PGD2* may protect against the deleterious effects of *PGD2*. The *PGD1* receptor gene promoter polymorphism there-fore appears to alter receptor expression to protect against bron-chiole hyperreactivity (BHR) [184, 185].

The *CRTH2* gene, which encodes the receptor for PGD2, is located within a linkage region for asthma on chromosome 11q [186–188]. Since CRTH2 is expressed on basophils and eosino-phils [189–191] it is involved in the regulation by PDG2 of aller-gic inflammation [192–194]. Although possibly confounded by population effects, two common SNPs, 1544G>C and 1651G>A, in the 3′-untranslated region, show evidence of linkage with asthma. This was refined, by haplotype analysis, to a linkage dis-equilibrium haplotype of the 1544G+1651G SNPs [193].

2.5.5 Thromboxane A2 Prostanoid Receptors

The prostanoid thromboxane (TP) receptor (*TBXA2R*) gene has been studied in asthma as a putative candidate gene due to its phar-macology. Thromboxane A2 (TXA_2) binds to a specific receptor,

the TP receptor, which in turn signals, through activation of the Gq/11 family of G proteins, the mitogen-activated protein kinase (MAPK) pathway and the protein kinase A pathway. TXA_2 is the most potent of the prostanoids. TXA_2 plays a role in inflammation, platelet aggregation, and the degree of vasoconstriction. The *TBXA2R* gene, located on chromosome 19p13.3, encodes two receptor isoforms, expressed as a result of alternative splicing of the carboxyl terminus. These GPCR isoforms, TPα and TPβ, share the first 328 amino acids [195]. TP receptor gene alternative splicing may represent a source of variability in BHR. The TPβ isoform, for example, undergoes agonist-induced internalization [195] that results in the loss of this isoform from the population of cell surface receptors.

Many aspects of BHR are potentially mediated by the TP receptor isoforms, making these variants candidates in the pathophysiology of asthma [196, 197]. The relevance of the TXA_2 system to BHR phenotypes and asthma pharmacology [199] derives from the fact that alveolar macrophages, eosinophils, and platelets increase the production of TXA_2 during lung inflammation. Blocking TXA_2 action may prevent constriction of pulmonary vasculature and airway smooth muscle (ASM). Thus, TXA_2 appears to be involved in microvascular leakage, mucus secretion, and ASM proliferation [198]. The association of *TBXA2R* has only been nominally replicated in the context of GWAS studies [199], however.

Physiologically, the TP receptor seems relevant to asthma. Its signaling has been extensively documented in vascular smooth muscle and platelets, but its characterization in human ASM cells has been less extensive. ASM cells express messenger RNA (mRNA) for both TP receptor isoforms, and functional receptors respond to agonist with an increase in intracellular Ca^{2+} concentration [200]. As a consequence, besides potentiating the epidermal growth factor (EGF) mitogenic response independently from transactivation of the EGF receptor (EGFR) [200], TP receptor stimulation induces a concentration-dependent increase in DNA synthesis.

The TP receptor requires the G_i/G_o protein to activate the Src-Ras-ERK1/2 (extracellular signal-regulated kinase 1 and 2) cascade to induce the proliferative response, which in turn promotes the rapid nuclear translocation of activated ERK1/2 [201]. Because TP receptor may be activated by many inflammatory mediators [202–204], these findings suggest new therapeutic strategies that alter the ASM hypertrophy or hyperplasia observed in the chronic airflow obstruction and airway inflammation that characterizes asthma, chronic bronchitis, bronchiolitis obliterans, and chronic obstructive pulmonary disease.

TBXA2R gene variability may also contribute to interindividual differences in the efficacy of pharmaceutical agents that act on this system. A positive association between a polymorphism in the *TBXA2R* gene and risk of asthma, atopy, and the aspirin-intolerant

asthma (AIA) phenotype has been identified [205–208]. These drugs include the synthase inhibitor ozagrel hydrochloride (OKY-046); the TP receptor antagonist seratrodast (AA-2414); and ramatroban (Bay u3405), a TP receptor antagonist [198].

In addition to asthma, a rare bleeding disorder that results from failure of platelet aggregation has been attributed to distinct variants of the TP receptor. The Arg60Leu variant of the TPα isoform has been associated with the failure of platelet aggregation [193] that probably results from a mechanism distinct from that resulting in BHR. Located in the first cytoplasmic loop of the receptor, the Arg60Leu variant impairs cyclic adenosine monophosphate (cAMP) accumulation and phospholipase C (PLC) activity while leaving ligand binding intact. Interestingly, the homologous mutation of the TPβ isoform was not deleterious, possibly because it acts through Gi/Go systems [193], while the TPα isoform may act through Gαs.

The *TBXA2R* gene splice variants result, therefore, in protein structures with distinct functions. An amino acid substitution that is deleterious in one splice isoform, however, may only be a polymorphic marker in another. This phenomenon may have pharmacogenetic consequences because one copy of the mutation is adequate to prevent TPα signaling and possibly disrupt receptor dimerization [193].

2.5.6 Cannabinoid GPCR Pharmacogenetics

Synonymous and non-synonymous SNPs have been reported in the genes encoding the cannabinoid GPCRs [209–217]. In addition to the sequence variability in prototptypical cannabinoid receptors, we will review the pharmacogenetics of the deorphanized GPR55 receptor [218–229]. The analysis of allelic variants in humans and animal models may be relevant to the study of a wide variety of human disease predispositions including neuropsychiatric disorders, the genetic basis of marijuana use, and addiction. Since there are probably more than 400 compounds present in cannabis, at least 70 of them known to be cannabinoids [230, 231], cannabinoid receptor variants may be important pharmacogenetic determinants of drug response.

Cannabinoid CB1 Receptor

The *CB1* receptor gene (*CNR1*), located on chromosome 6q14-15, has a number of variants [232] including the intron 2 C/T polymorphism (rs9444584). This ancient polymorphic sequence variant acts as an enhancer in hypothalamic and dorsal root ganglia cells. While the wild-type allele is more active in hypothalamic and dorsal root ganglia cells, however, the C allele is highly active in hippocampal cells where it also responds strongly to MAPK activation. The C allele is in strong linkage disequilibrium with two SNPs (rs9450898 and rs2023239) associated with addiction, obesity (rs2023239), and

reduced fronto-temporal white matter volumes in schizophrenia patients as a result of cannabis misuse (rs9450898). Since the T allele elicits increased MAPK signaling when compared with the C allele, it is possible that the functional effects of the different alleles may play a role in these conditions [233].

In addition to these polymorphisms, a 3813G>A polymorphism in the exon 4 sequences that encode 3′ UTR [234] and an $(AAT)_n$ polymorphism more than 12 kb from the *CB1* amino acid-coding exon 4 [235] have been identified. With respect to the 5′ UTR variant, the excision of an intron at the 5′-extremity of the coding region of the human receptor mRNA results in a receptor that is functional but demonstrates slightly attenuated signaling in vitro [236]. These and other *CB1* variants have been studied with respect to a wide variety of disease phenotypes as well as phenotypes that are primarily pharmacogenetic or cytotoxic [237]: creating a complex pattern of associations that may benefit from being tested in a GWAS paradigm.

For example, the *CB1* gene has been associated with Huntington's age of onset [238], depression in Parkinson's [239], and at least some aspects of multiple sclerosis progression [240, 241]. With respect to schizophrenia, a psychosis that may be elicited by components of cannabis [242, 243], in some [244–248] but not all studies [249–252], implicates the *CB1* in certain endophenotypes. Similarly, a complex pattern of associations of the *CB1* gene with unipolar and bipolar depression, and related disorders, is emerging [253–256] that may involve other genes.

Associations of *CB1* gene with various forms of substance use have been reported. With respect to cannabis use itself, the associations with withdrawal seem compelling [247–262]. Interestingly, the *CB1* gene has also been associated with nicotine cessation [263] and addiction [264]. In addition, alcohol [265–269], cocaine [270–273], and various other drugs of abuse [274, 275] have also been associated with *CB1* variation. Although not unanimous [276], taken together these data suggest that traits that raise the risk for polysubstance abuse, such as impulsivity and attention-deficit hyperactivity disorder [277–279], may be influenced by *CB1* variability [270, 280]. In many instances, however, *CB1* variants may be associated with drug responses of compounds that target other receptor classes in the treatment of the psychiatric disorders that may underlie addiction [281–284].

Other endophenotypes that may also be influenced by *CB1* variants include the risk for anorexia [285, 286] or the set point of body mass index [287]. Paradoxically, the *CB1* variants have also been consistently associated with various clinical measures of obesity [288–294], the consequences of hyper- or hyponutrition [292, 294], and type II diabetes [295, 296]. While the influence of *CB1*

variants on metabolism seems robust, for example, it has not been confirmed in all populations [297].

Large GWAS studies will have the benefit of ultimately confirming these associations. While the *CB1* association with addiction has not been fully replicated, the *CB1* gene does seem to be associated with an endophenotype found in drug users [270]. In this context, the likely role of the *CB1* variants as pharmacogenetic variants should not be ignored [281–284].

Cannabinoid CB2 Receptor

The *CB2* gene (*CNR2*), located at chromosome 1p36, has not been as widely associated with disease phenotypes. Some of those reported, however, seem to share an immunological basis [298]. CB2 variants, including the Glu63Arg and His316Tyr functional variants, respond to endocannabinoid agonists such as 2-arachidonoylglycerol with reduced efficacy; however in constitutive activity assays, the His316Tyr and Glu63Arg/His316Tyr polymorphic receptors exhibited higher constitutive activity than the CB2 wild-type receptor [211].

The *CB2* gene has been associated with the risk for celiac disease [299], hepatic anomalies [300, 301], osteoporosis [302–304], and drug addiction [305]. An endophenotype consisting of reduced immune modulation may underlie some of these conditions. Risk for immunological compromise may be associated with the *CB2* variants [298]: suggesting that novel cannabinoid ligands could be developed to reverse this effect [304].

In some cases, the pharmacology of the variants has not been fully characterized. For example, while the 524C>A, Leu133Ile CB2 variant has been associated with bipolar disorder, the pharmacological consequences of the variant are not fully unknown [306]. By contrast, the *CB2* variant, 315A>G, has been associated with major depression (MD) in Japanese population by [307].

GPR55 Pharmacogenetics

This overview of cannabidiol pharmacogenetics serves as an introduction to a field that is developing rapidly. It is has become evident that cannabinoid compounds other than THC are likely to be developed as pharmaceuticals. Among these, the cannabidiol-derived compounds are of interest since most are putative antagonists at cannabinoid receptors including GPR55 [209, 218–229]. For example, while rimonabant is an antagonist/inverse agonist CB_1 receptor at the CB1 receptor that was developed as an anti-obesity agent, it has been shown to activate GPR55 [222]. This pharmacology is interesting given the association of CB receptors with obesity [288–294].

The potential role of the GPR55 receptor in weight regulation is also of interest due to pharmacogenetic data. A Gly195Val variant of GPR55 has been reported. The Val195 allele itself and homozygosity for the Val195 allele have both been reported to be more abundant in patients diagnosed with anorexia nervosa [307]:

suggesting the potential metabolic significance of GPR55 pharmacology. In vitro functional analysis of the 195Val *GPR55* variant resulted in less phosphorylated ERK than Gly195-type *GPR55* when challenged with LPI. The possible utility of drugs derived from cannabidiol that target the GPR55 receptor in treating disorders including anorexia and addiction may be predicated on its tendency to have actions that are distinctly opposite to those drugs that act primarily at the CB1 and CB2 receptors [209]. Furthermore, the non-psychogenic properties of cannabidiol-derived compounds [209] suggest their potential utility in conditions such as obesity and osteoporosis: conditions that have been associated with CB1 and CB2 variants, respectively [291, 302]. Given the variety of disorders associated with CB1 and CB2 receptor variants, including mood disorders [253, 305], cancer [299], and neurological conditions [239–241], there is clear justification to study the relevance of GPR55 and its ligands to these and other disorders.

2.6 Polymorphisms of the Chemokine System in Infection and Immunity

Chemokines are the largest family of cytokines. Four invariant cysteines define the chemokine proteins. They are grouped on the basis of the conservation of the domain containing the first two cysteines.

The involvement of the chemokine system in disease predisposition has been widely discussed ever since the chemokine receptor gene family was identified as part of the chemokine receptor gene cluster region on 3p21. Among these receptors, the CCR5 receptor binds natural ligands such as the CC chemokines, including RANTES (regulated on activation, normal T cell expressed and secreted), the macrophage inhibitory proteins MIP1α and MIP1β, and the monocyte chemoattractant protein 2 (MCP2).

The *CCR5Δ32* polymorphism, a 32-bp deletion in the receptor promoter, has been associated with protection against human immunodeficiency virus (HIV) infection, asthma, and other disease states [308–310]. While the potential contribution of the *CCR5* variants to immune diseases is stronger in the case of HIV infection [311], it is not necessarily significant in genome-wide studies of HIV resistance (*see* Subheading 3.6.2).

2.6.1 Chemokine Receptors and Asthma

The role of chemokines in asthma often depends on T cell activation [312–316]. The *CCR5Δ32* polymorphism diminishes CCR5 receptor expression in type 1 T-helper (Th1) cells, which may result, indirectly, in the greater Th1 cell activity that is associated with asthma. The variant causes a decrease in CCR5 binding to endogenous CC chemokine agonists such as RANTES MIP-1α and MIP-1β [312]. This suggests that asthma is associated with a systemic increase in the production of the allergic Th2 cytokines [313], as noted in the discussion of the prostenoid DP receptor. Interestingly, the mechanism that preferentially maintains Th2

cells, TIM-1, has been implicated in pathways maintaining allergic responses [314]. Individual loci encoding enzymes required by this pathway, such as the region of chromosome 2q14–32 that encodes a dipeptidyl peptidase (*DPP10*), have been positionally cloned [315].

The biochemistry of cytokine and chemokine involvement in asthma is complex, however. While the cytokine pathway is likely to be involved in asthma, the role of specific chemokine receptor variants in disease is still a subject of active investigation. In fact, GWAS studies suggest a modest role for two chemokine genes other than *CCR5–CCL18* and *CXCL12* [316].

2.6.2 Chemokine Receptors and HIV

CCR5 is known to be an important co-receptor for macrophage-tropic viruses, including HIV. Expression of CCR5 is detected in promyeloblastic cell lines, suggesting that this protein plays a role in granulocyte lineage proliferation and differentiation. The polymorphic 32-bp *CCR5* promoter deletion, resulting in promoter inactivation, may confer some degree of resistance to HIV-1 infection.

Studies of co-receptors have suggested novel avenues for developing therapeutic and preventive strategies against HIV and acquired immunodeficiency syndrome (AIDS). These strategies build on an understanding of the role of chemokine receptors in HIV-1 transmission and pathogenesis [317–320].

The other GPCRs that act as co-receptors for the HIV virus include the CCR2 and CCR4 receptors, which have been identified as receptors for T-cell line-tropic and macrophage-tropic HIV-1 isolates. The roles of CCR2 and CCR4 were identified partly because another CCR5 variant, the Val64Ile SNP, was found to be genetically associated with resistance to HIV infection and to result in abnormal heterodimerization with CCR2 and CCR4 in vitro [221–224]. Thus, it is possible that aberrant CCR heterodimerization may be another contributor to the modulation of HIV resistance.

In contrast, it has been suggested that the expression of G protein-coupled receptor 1 (GPR1) in the kidney mesangial tissues results in increased susceptibility to variant HIV-1 infection. The GPR1 protein may also be involved in nephritis associated with AIDS progression [319]. The transmission of macrophage-tropic variants and the subsequent appearance of T-cell line-tropic variants may be worth examining with respect to co-receptor polymorphisms [325].

2.6.3 CCR2 and CCR3 Polymorphisms

Of the HIV co-receptors that may be polymorphic, the CCR2B and CCR3 receptors appear to function as minor co-receptors. A common Val64Ile substitution of the CCR2 receptor is associated with the delayed progression of HIV infection to AIDS. Although the variant has been shown to delay disease progression, it does not reduce the risk of infection [326].

CCR3 missense polymorphisms, encoding a Arg275Glu substitution in the third extracellular loop and a Leu302Pro substitution in the intracellular cytoplasmic tail, have been identified. As yet, however, no phenotype has been associated with these polymorphisms [327]. Since polymorphisms in a variety of GPCR co-receptors have been identified, however, further phenotyping remains critical to understanding their consequence.

Genetic differences might be useful in identifying persons with a specific disease-modifying phenotype that might in turn be targeted by a specific drug response. While polymorphism-induced alterations in receptor–host interaction will be a valuable focus of drug development efforts [328], the GWAS data suggest that, at least from a genetic point of view, they may play a minor role in infection resistance and disease progression [316].

2.7 Polymorphisms of the Platelet-Activating Factor Receptor

The platelet-activating factor (PAF) receptor (PAFR) mediates the proinflammatory and vasoactive actions of PAF. Interindividual variation in PAF-related physiological response and anti-inflammatory drug responsiveness results from the substitution of Ala224Asp in the third intracellular loop of the PAFR [329]. In vitro studies suggested that the Ala224Asp results in a significant reduction of the PAF-induced intracellular signals that include calcium mobilization, inositol phosphate production, and inhibition of adenylyl cyclase. The reduction in these signals is associated with a phenotype in vitro of reduced chemotaxis. These data suggest that this PAF variant may be selectively targeted in some patients. The pharmacological potential of targeting such variants by reverse pharmacology is suggested by the fact that the variant was present at an allele frequency of 7.8 % in a sample from a Japanese population [329].

2.8 GPCR Mediation of Interactions Between Virus and Host

The study of the GPCRs involved in infection, inflammation, and disease progression has aided in the identification of novel receptors that may encode potential pharmaceutical targets. There is complex interaction between human GPCR genes and those from viral sources [330, 331]. While the expression of some nonhuman GPCRs may be beneficial in that they protect the host against the virus by providing constitutional resistance, other variants may compound the difficulty of treating viral infections with new antiviral agents.

The potential for pharmaceutical intervention in viral infection at GPCRs may exist not only for HIV but also for Kaposi sarcoma (KS), a common result of infection by the Kaposi herpes virus, KSHV. Experimental evidence supports a key role for a particular viral gene, encoding a constitutively active G protein-coupled receptor (vGPCR), in the development of KS. Although this receptor, like the cytomegalovirus (CMV)-encoded GPCR [331], originates in a nonhuman genome, it is able to function in human cells

and thereby co-opt many host functions. In particular, it is able to function as a receptor for human ligands affecting immunomodulating cytokines such as interleukin 6. This GPCR may facilitate viral control of the host pathways that regulate angiogenesis needed to sustain tumor growth [332–335].

In the case of CMV, viral strains may encode four potential chemokine receptors (US27, US28, UL33, and UL78). Of these virally encoded chemokine receptors, US28 binds many endogenous human CC chemokines [336, 337] in vitro. The US28, therefore, is a functional chemokine receptor. It may enhance cell growth in some cell types while inducing apoptosis in others. In contrast, the *US27* gene product may not signal either constitutively or in a ligand-induced manner. When expressed in transfected cells, however, US27 induces both cell proliferation and DNA synthesis. UL33 and UL78 may form heteromers with CCR5 and CXCR4 chemokine receptors in transfected cells: having predominantly negative effects on CCR5 and CXCR4 cell surface expression, ligand-induced internalization, signal transduction, and migration without modifying the chemokine binding properties of CCR5 and CXCR4 [335]. The expression of the foreign genomic material in human cells may therefore promote CMV infection [330–337].

Another example of the complex interaction between host and virus genomes has been demonstrated in the case of human Epstein–Barr virus infection. The Epstein–Barr virus transcription factors facilitate the spread of infection [336, 337] by interacting with human promoter elements—a common site for polymorphic mutation. The expression of the human GPCR genes encoding the Epstein–Barr virus-induced EBI receptors during the course of infection facilitates the spread of infection. The severity of infection therefore may hinge on whether a certain viral strain can co-opt the regulation of human GPCR genes that are critical for infection [330–337].

EBV ultimately induces expression of many endogenous chemokine GPCRs such as EBI1/CCR7, EBI2/GPR183/oxysterol, CCR6, and CCR10 receptors in host cells. In addition, the EBV genome open reading frame, *BILF1*, which encodes a functional, constitutively active GPCR, is expressed. BILF1 couples to Gαi and modulates CRE-mediated signaling, activates NF-κB, and inhibits the phosphorylation of RNA-dependent protein kinase—thereby activating a positive-feedback loop for cytokine production. In particular, *GPCR* genes that are transcribed during the lytic-replication cycle may contribute to the viral reactivation that leads to tumorigenesis [330–337].

2.9 GPCR Mutations in Developmental Disorders and Cancer

In addition to their effects on metabolism, GPCRs and G proteins also play a role in the regulation of cell growth, differentiation, dysplasia, and neoplasia. Autonomous cell growth, resulting in neoplastic transformation, is associated with naturally occurring mutations both in GPCRs and in G protein α-subunits.

These phenotypes suggest that the GPCR component of the genome is critical to normal differentiation and development.

Cell division can be induced by a number of mechanisms, including those transducing mitogenic signals from the cell membrane to the nucleus. Mitogenic signaling by GPCRs results from the convergence of signals emanating from many different classes of GPCRs expressed on the cell surface. The common pathway involves the ERK MAPK cascade, although receptor and non-receptor tyrosine kinases also play central role.

GPCR pharmacogenomics has facilitated the understanding of how receptor, G protein, and tyrosine kinases contribute to the mitogenic signaling of normal and transformed cells. Reverse pharmacology may ultimately allow the rational design of pharmaceuticals to treat diseases involving uncontrolled cell proliferation [338, 339].

2.9.1 CCKβ/Gastrin Receptor Mutations and Gastrointestinal Carcinoma

The receptors for cholecystokinin (CCK) and gastrin (CCKR and CCKβ/gastrin, respectively) have been implicated in the risk for a spectrum of human diseases that includes metabolic and neoplastic disorders [340–342]. The serious consequences of disrupting these receptors may reflect the role of the wild-type receptors in regulating food intake and pancreatic endocrine function. For example, the role of mature amidated gastrin, progastrin, and its intermediates has been identified in gastrointestinal neoplasia [340–342]. Other disorders of gene regulation and development, including type 2 diabetes, have also been associated with activating variants of CCKRs [342]. This insight into the disruption of gastrin signaling may allow development of pharmacological interventions at the gastrin receptor for affected patients.

Since the epidemiological evidence does not always confirm that elevated gastrin levels contribute to increased risk for colon cancer, it is worth reviewing the evidence of the molecular pathology of gastrin-related systems in colorectal cancer. This evidence is mostly derived from the study of colorectal cells cultured from biopsied tissue. It suggests that prolonged hypergastrinemia is associated with an increased risk for neoplastic changes.

Within this cohort, abnormal expression of CCKβ/gastrin receptor has been associated with colon cancer since the receptor protein was expressed in 44 % of colorectal cancers compared with 13 % of controls [341]. Mutation screening of tissues collected from colon cancer patients and controls discovered variants of the genes encoding the peptide G17 amide and the G protein-coupled CCKβ/gastrin receptor [341]. Several somatic mutations have been directly associated with disease. CCKβ/gastrin receptor variants were associated with abnormal gastrin binding in vitro [340, 341].

For example, the Val287Phe CCKβ/gastrin receptor somatic mutation was found in some colon cancer patients. In vitro, the Val287Phe variant results in a loss of gastrin-induced MAPK p44/p42 signaling compared to wild type. It is associated with a 51 %

increase in clonal expansion. This structural alteration may be informative in the study of other GPCRs that are candidates in oncogenesis [341–343], particularly those with disruptions in the third intracellular loop.

Clinically, growth of gastric tumours is inhibited by antagonists of the cholecystokinin and gastrin receptors. Therefore, these receptors may provide an opportunity for specific tumor targeting and therapy of tumors overexpressing gastrin receptors [341, 343]. Future studies might be based on targeting the variant GPCRs that are expressed in tumors because they are known to be both pharmacogenetically distinct and associated with tumorigenesis.

2.10 Thrombin, Inflammation, and Protease-Activated Inhibitor Receptors

Protease-activated receptors (PARs), a subclass of GPCRs that function in the coagulation cascade, have been identified. A comprehensive survey of the GPCR portion of the proteome may provide information about the structure and function of these receptors. The PAR factor II (thrombin) receptor-like 2 (PAR2), encoded by the *F2RL2* gene, is inactive in the cascade until proteolytic cleavage of its extracellular amino terminus. A Phe240Ser variant that is located in the second intracellular loop, found at a frequency of approximately 8 %, disrupts receptor activation by proteolysis.

This illustrates how GPCR function can be influenced by structural changes that are genetically determined. In the wild type, the terminus created by proteolytic cleavage that activates the receptor by creating a tethered ligand is absent in the variant. As a result, the variant causes the loss of the PAR2 receptor as a cofactor in PAR3 activation and subsequent thrombin-triggered phosphoinositide hydrolysis [344, 347]. In addition, any biological activity associated with the cleaved fragments is also absent. The relevance of PAR2 receptor variants in primary cultures of human cells and cancer [344–346] may be useful in the development of small-molecule PAR2 agonists and antagonists [347].

3 GPCR Databases: Applications to Pharmacogenomics

GPCRs represent the largest group of functional genes in mammalian genomes. This creates a challenge to organizing information on natural variation within these genes and the function of the encoded variants. In addition, considerable data has been generated on in vitro and in silico mutations that can aid in pharmacogenomic studies. In addition to databases that are widely applicable to predicting the effect of DNA variation on protein function [348], a number of databases in the public domain have integrated in silico and naturally occurring GPCR variant data with computational tools for specifically predicting the function of GCPR variants [349–351].

In this section, however, we provide information and Uniform Resource Locators (URLs) for online GPCR resources. These URLs are curated and kept current at https://sites.google.com/site/gproteincoupledreceptors/.

3.1 Databases of GPCR Sequences and Variants

Several databases integrate information on GPCR sequences and their variants—primarily in silico variants. For almost two decades, the growing GPCRDB (http://www.gpcr.org/7tm/) has provided the GPCR community with resources for GPCR sequence search and alignments, mutation information, and ligand-binding constants. The site is continually maintained and new resources are added to facilitate GPCR-related studies.

Additional databases provide tools for querying a new GPCR sequence to determine its relationship with previously characterized receptor subtype (GPCR pattern recognition, http://www.biochem.ucl.ac.uk/bsm/dbbrowser/GPCR/) or facilitate comparative genomic studies by cataloguing GPCR sequence information across species (http://sevens.cbrc.jp/).

Of particular relevance to pharmacogenetic and genomic studies are resources for naturally occurring variants. Several databases maintained by research groups may be limited to specific GPCR families and provide data on those GPCR mutations and polymorphisms. These include the Olfactory Database (http://senselab.med.yale.edu/ordb), the Opioid Receptor Database (http://www.opioid.umn.edu), and the Calcium-Sensing Receptor Database (http://www.casrdb.mcgill.ca).

The GPCR Natural Variants Database (NaVa, http://nava.liacs.nl/) contains information on both rare mutations and polymorphisms, including SNPs found in the superfamily of GPCRs. NaVa contains allele frequencies and reported disease associations when available and is designed for studies of pharmacogenetics, genotype-phenotype association, and structure-function relationships of GPCRs [13]. The International Union of Basic and Clinical Pharmacology hosts a database (http://www.iuphar-db.org) of GPCRs which, in addition to pharmacological information on the receptors, lists information on biologically significant human variants, mouse mutant phenotypes, and data on altered gene expression.

3.2 In Silico Analysis of GPCR Variant Function

Next-generation sequencing studies are likely to generate a large number of novel and uncharacterized GPCR variants. Efficiently assessing the pharmacodynamic effects of these GPCR variants will become increasingly important. A few computational tools are available for predicting the function of non-synonymous variants: PolyPhen, SIFT, SNAP, and Mutation Taster. SNAP (https://www.rostlab.org/services/snap/) [348] evaluates the effects of single-amino acid substitutions on protein function through a neural-network-based method for the prediction of the functional effects of non-synonymous SNPs.

A study by Bromberge et al. [349] outlined an approach for applying SNAP to predict the function of GPCR variants using the human melanocortin 4 receptor (MC4R) as a model. They found that the results obtained from SNAP were generally consistent with available in vitro experimental data and identified 12 additional potentially functionally important amino acid residues in human MC4R that had not previously been studied experimentally.

PolyPhen-2 (http://genetics.bwh.harvard.edu/pph) is a tool which predicts the possible impact of an amino acid substitution on the structure and function of human proteins and has been successfully used to predict GPCR variant function [350]. SIFT (http://sift.bii.a-star.edu.sg/) uses sequence homology and physical properties of amino acids to predict the effect of non-synonymous polymorphisms and missense mutations on protein function.

SIFT has been applied to GPCR SNPs from dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) to predict GPCR variants that are likely to be associated with disease for use as candidate SNPs for genotyping in genetic association studies [351]. Using a combination of these tools will be informative for pharmacogenetic studies on GPCR variants when experimental or genotype-phenotype data on GPCR variants are not available in GPCR databases.

4 Conclusion

Investigations of naturally occurring GPCR variants have provided insight into the role of GPCR variants as genetic risk factors for disease, altered drug response, or ADRs (*see* Table 1). At the same time, the discovery of these variants has provided pharmacogenomic reagents used to refine drug discovery [352, 353].

In addition, the relevance of in vivo mutations with respect to structural in vitro data will provide a detailed population model of the receptors of family A, which show structural similarity to rhodopsin. Comparison of these data with the family B GPCRs, the glucagon-like receptors [354, 355], and the family C receptors, such as the *CASR*, may provide the detail necessary to model how GPCR structure and function are altered by common genetic variants. Next-generation sequencing will add to the already large inventory of GPCR variants identified in the past two decades and surveyed in this chapter. Further, technologies emerging from next-generation sequencing will generate datasets for integrating genomic information into pharmacogenomic studies. For example, RNA-Sequencing, which utilizes the capabilities of next-generation sequencing to capture a snapshot of RNA presence and quantity at a given time, enables the simultaneous profiling of expressed genes, splice variants, RNA-edited isoforms, and haplotypes.

Using such data, the pharmacogenomic investigator can move beyond investigating the association of a phenotype with a single GPCR variant and, instead, determine whether specific haplotype is differentially expressed and how its differential expression is related to the phenotype of interest. The investigator can also identify variants that could not be efficiently screened for using high-throughput technologies of the last decade such as rare variants and isoforms resulting from RNA editing. Thus, next-generation sequencing technologies have great potential for the future of GPCR pharmacogenomics and will provide data helpful in overcoming many of the factors confounding genome-wide association studies in the past decade.

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Part II

Clinical Applications of Pharmacogenomics

Chapter 10

Pharmacogenomics of Heart Failure

Anastasios Lymperopoulos and Faren French

Abstract

The combination of angiotensin-converting enzyme (ACE) inhibitors and β-adrenergic receptor (βAR) blockers remains the essential component of heart failure (HF) pharmacotherapy. However, individual patient responses to these pharmacotherapies vary widely. The variability in response cannot be explained entirely by clinical characteristics, and genetic variation may play a role. The purpose of this chapter is to examine the current knowledge in the field of beta-blocker and ACE inhibitor pharmacogenetics in HF. β-blocker and ACE inhibitor pharmacogenetic studies performed in patients with HF were identified from the PubMed database from 1966 to July 2011. Thirty beta-blocker and 10 ACE inhibitor pharmacogenetic studies in patients with HF were identified.

The ACE deletion variant was associated with greater survival benefit from ACE inhibitors and beta-blockers compared with the ACE insertion. Ser49 in the β_1AR, the insertion in the α_{2C}AR, and Gln41 in G protein-coupled receptor (GPCR) kinase (GRK)-5 are associated with greater survival benefit from β-blockers, compared with Gly49, the deletion, and Leu41, respectively. However, many of these associations have not been validated. The HF pharmacogenetic literature is still in its very early stages, but there are promising candidate genetic variants that may identify which HF patients are most likely to benefit from beta-blockers and ACE inhibitors and patients that may require additional therapies.

Key words Heart failure, β-Blocker, ACE inhibitor, Pharmacogenetic, Pharmacogenomic, Polymorphism, Variant

1 Introduction

Advances in pharmacotherapy over the past 20 years have significantly improved heart failure (HF) morbidity and enhanced survival. The combination of angiotensin-converting enzyme (ACE) inhibitors and β-adrenergic receptor (AR) blockers remains the essential component of HF pharmacotherapy [1]. Both drug classes significantly improve survival, particularly β-blockers, which have been firmly entrenched in HF evidence-based guidelines since the late 1990s [2, 3]. However, physiologic actions, side effects, and efficacy vary substantially from patient to patient. For example, ACE inhibition may fail to suppress angiotensin II in HF patients, and aldosterone escape is common [4]. The prevalence and severity of

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ACE inhibitor-induced cough varies significantly, and angioedema, a rare but potentially serious side effect, remains unpredictable [5]. Treatment with β-blockers results in widely variable effects on left ventricular ejection fraction (LVEF). A distinct minority of patients experience a marked and sustained improvement in ventricular function, whereas others have no change or rarely may experience a decline [6]. HF patients may experience worsening of their symptoms during beta-blocker titration, requiring increased diuretic doses and rarely discontinuation of β-blocker therapy [7]. Unfortunately, variability in ACE inhibitor and β-blocker clinical response is typically not predictable based on clinical characteristics. Clearly, a better understanding of the basis of variable therapeutic response to ACE inhibitors and β-blockers would be clinically useful.

Genetic variation is suspected to account for a significant part of the individual patient response to cardiovascular medication. Differences in genetic coding have been shown to influence pharmacokinetics and pharmacodynamics, which may translate into clinical outcomes such as therapeutic efficacy and adverse events. Whether genetic tailoring of ACE inhibitors and β-blockers will improve the response in HF to these agents is unknown. Therefore, it is important to understand the current state of the pharmacogenetic literature for HF. The purpose of the present chapter is to specifically examine genetic variants influencing the response to the mainstay of HF pharmacotherapy: ACE inhibitors and β-blockers.

2 Methods

β-Blocker and ACE inhibitor pharmacogenetic studies were identified in the PubMed database from 1966 to July 2013 by combining the following search terms: heart failure, variant, polymorphism, pharmacogenetics, pharmacogenomics, β-blocker, ACE inhibitor, and each individual drug name. Studies were also identified from the reference lists of articles. Studies were limited to those performed in patients with HF and those published in English.

3 Results

3.1 β-Blockers

Among the β-blocker pharmacogenetic studies in patients with HF published from 2000 to date, the smallest study investigated 33 HF patients [8] and the largest one 2,460 [9]. The end points ranged from intermediate phenotypes, such as heart rate and LVEF, to clinical outcomes, such as survival. The majority of studies tested genetic variants related to the sympathetic adrenergic system, but there were some that investigated genetic variants related to the renin–angiotensin–aldosterone system (RAAS) [10–12].

3.2 β_1AR

The largest amount of pharmacogenetic data for β-blockers is for the primary drug target, the β_1AR (ADRB1). ADRB1 is the principal βAR subtype expressed on the cardiac myocyte, and it mediates cardiac contractility. There are two variants in ADRB1 that have been studied: an amino acid substitution of glycine for serine at position 49 (Ser49Gly) and a glycine substituted for arginine at position 389 (Arg389Gly) in the receptor protein (Fig. 1). These variants are common in the general population, and there are racial differences in their frequencies. The frequency of these variants and other variants discussed in this chapter are presented in Table 1. Gly49 results in greater agonist-promoted downregulation of ADRB1 compared with Ser49 [13, 14]. Downregulation of βARs is thought to be a protective adaptation in HF, where chronic sympathetic activity is toxic to the cardiac myocyte [15]. This concept suggests that the Gly49 variant may be protective in patients with HF, but patients with this variant may be less responsive to βAR blockade. In contrast, in vitro experiments demonstrate that cells expressing Gly49 are more sensitive to the inhibitory effects of metoprolol [13]. A number of clinical studies have examined the association of the Ser49Gly genotype with ventricular remodeling parameters such as LVEF, left ventricular end diastolic diameter (LVEDD), and left ventricular fractional shortening (LVFS) and outcomes during βAR blockade, with complex and somewhat inconsistent results that are discussed in detail below. Terra and colleagues [16] studied 54 patients with systolic dysfunction receiving metoprolol. Patients carrying Gly49 had a significant

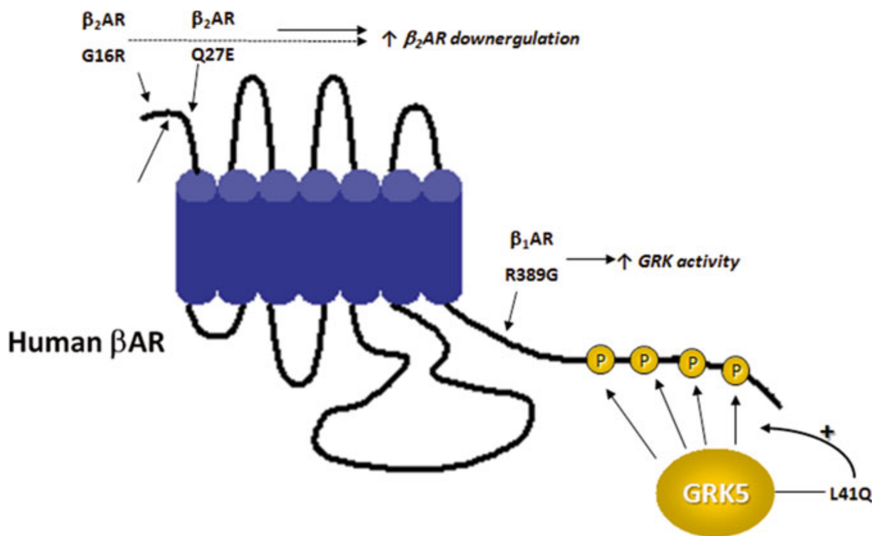

Fig. 1 The location within the encoded receptor protein of the main human ADRB1 and ADRB2 gene polymorphisms discussed in this chapter. See text for details and abbreviations

Table 1
Minor allele frequencies of the polymorphisms discussed in this chapter

Gene name	Gene symbol	Polymorphism	Minor allele	Frequency in Caucasians (%)	Frequency in African-Americans (%)
Angiotensin-converting enzyme	ACE	Ins/Del 17q23.3eq23.3	Ins	44	43
Alpha2C-adrenergic receptor	ADRA2C	Ins/Del 322–325	Del	4	43
Beta1-adrenergic receptor	ADRB1	Ser49Gly Arg389Gly	Gly Gly	17 27	25 38
Beta2-adrenergic receptor	ADRB2	Gly16Arg Gln27Glu Thr164Ile	Arg Glu Ile	40 42 2	50 20 <2
G protein-coupled receptor kinase-5	GRK5	Gln41Leu	Leu	2	24

decrease in LVEDD compared with Ser49-homozygous patients in response to metoprolol CR/XL. However, changes in LVEF were not significantly different between Ser49Gly subgroups. de Groote and colleagues [17] studied 199 patients with systolic dysfunction and 3 months of the maximum tolerated dose of bisoprolol or carvedilol. They found no difference in LVEF or RVEF among Ser49Gly subgroups, but they did not compare LVEDD responses. Nonen and colleagues [18] studied LVFS response in 80 patients with IDC on 6 months of a variety of β-blockers, in which they did not find an influence of Ser49Gly. There are limitations to these studies: duration of β-blocker therapy too short, different HF etiologies (ischemic etiology may damage the myocardium irreversibly), etc. These limitations were addressed in a study of 135 non-ischemic cardiomyopathy patients after 1.5 years of treatment with carvedilol, and there was still no significant impact of Ser49Gly on LVEF [19]. Although there is little data supporting an interaction between Ser49Gly and ventricular remodeling response after β-blocker treatment, Ser49Gly may have an impact on long-term response. Because Gly49 is protective against chronic sympathetic stimulation, it has been hypothesized that long-term exogenous β–blockade is more critical for HF patients with Ser49. Indeed, this is supported by a retrospective study of 184 patients with IDC, in which the survival curve for Ser49-homozygous patients treated with β-blockers was almost identical to Gly49-carrying patients not receiving β-blockers [20]. However, this study consisted entirely of Swedish patients; racial and ethnic stratification is

especially a concern for genetic association studies, owing to differences in allele frequencies, haplotype structure, and the genetic admixture of populations [21].

Sympathetic stimulation of ADRB1 results in activation of the stimulatory G protein G_s, which in turn activates adenylyl cyclase and the production of cyclic adenosine monophosphate [22]. Arg389 of ADRB1 displays increased coupling to G_s compared with Gly38934; thus, Arg389 has greater basal and agonist-stimulated activity [23]. Therefore, it has been hypothesized that HF patients possessing Arg389 would have a greater response to β-blockers. Regarding ventricular remodeling responses, this has been studied in a series of small HF cohorts. Liggett and colleagues [24] retrospectively studied 224 patients with systolic dysfunction receiving carvedilol. Those researchers were the first to report that patients who were homozygous for Arg389 had a significantly greater improvement in LVEF after treatment with β-blocker than patients who were homozygous for Gly389. Patients who were heterozygous at position 389 had a similar improvement in LVEF compared with Arg389 homozygotes This association was confirmed in 3 prospective studies totaling 345 patients among a variety of etiologies (ischemic and non-ischemic), β-blockers (metoprolol and bisoprolol), and ethnic groups (European, African-American, and Chinese) [16, 19, 25]. However, there are also three studies totaling 416 patients that failed to find a significant association [17, 26, 27]. Given that the series of studies investigating LVEF response were small, it is difficult to conclude if Arg389Gly is a good predictor of LVEF response to β-blocker. Liggett et al. also conducted a ground-breaking prospective pharmacogenetic substudy using patients from the Beta-Blocker Evaluation of Survival Trial (BEST) study [28]. BEST was a randomized placebo-controlled trial of the investigational novel β-blocker bucindolol which found that bucindolol did not significantly decrease mortality in HF patients. However, in the pharmacogenetic substudy of 1,040 patients [29], the investigators found that response to bucindolol varied by genotype. Patients homozygous for Arg389 had a statistically significant improvement in survival compared with placebo, whereas Gly389 carriers did not. In contrast, these results do not seem to apply to β-blockers currently used to treat HF. In another substudy of 600 patients from MERIT-HF [30], no association of Arg389Gly with the primary outcome of all-cause mortality or hospitalization in either the metoprolol CR/XL or the placebo group was found. These findings are consistent with the lack of association of Arg389Gly genotype with mortality described in a prospective registry study of 637 patients that were all treated with beta-blockers [31]. Only a small study of 201 HF patients with a limited number of events found results consistent with Liggett et al, where metoprolol and carvedilol appeared to be more effective at high doses in decreasing HF-related

mortality in patients carrying the Arg389 allele [32]. Although additional studies are needed, these discrepant results are most likely related to the unique pharmacologic properties of bucindolol, which include marked suppression of β_1AR activity in patients homozygous for Arg389.

3.3 β_2AR

In HF, chronic adrenergic stimulation causes downregulation of ADRB1, but not of the β_2AR (ADRB2). This causes a change in the ratio of ADRB1:ADRB2 from ~80:20 in healthy heart tissue to ~60:40 in the failing heart [33]. Therefore, the use of β_1AR-selective versus non-selective βAR blockers in HF remains a clinical issue. There are three variants in ADRB2 that have been studied: Gly16Arg, Gln27Glu, and Thr164Ile. Although the density of ADRB2 in HF is unchanged compared with ADRB1, ADRB2 is subject to desensitization via functional uncoupling from the intracellular G protein, G_s [22]. A glycine at amino acid position 16 results in increased agonist-promoted desensitization compared with arginine [34]. The pharmacogenetic interaction between this variant and β-blockers has not been studied in vitro. However, it has been hypothesized that because Gly16 allows for greater desensitization of ADRB2, HF patients possessing Gly16 have "genetic β-blockade." Genetic β-blockade, or the lack thereof, may interact with exogenously administered β-blocker. Six clinical studies tested the Gly16Arg variant in 738 HF patients [17–19, 27, 35, 36] and none found a significant association between Gly16Arg and β-blocker response in terms of β-blocker tolerability, LVEF, or LVFS. Thus, it seems unlikely that this variant could have a clinically meaningful pharmacogenetic interaction with β-blockers.

Glutamine (Glu) at amino acid position 27 of the ADRB2 gene results in a receptor resistant to agonist-promoted desensitization [34], and in contrast to Gly16Arg, there is clinical literature to support a pharmacogenetic interaction with β-blockers. Although the pharmacogenetic interaction has not been studied in vitro, it has been hypothesized that patients with Glu27 are responsive to β-blockers because they have more sensitive ADRB2. Indeed, this has been confirmed in 3 clinical studies evaluating LVEF changes [8, 27, 36]. Glu27 was also found to be associated with a favorable β-blocker response in other parameters, such as heart rate, malondialdehyde levels (a marker of oxidative stress), and pulmonary wedge pressure both at rest and peak exercise [8, 27]. There are also studies that did not find a significant association between Gln27Glu and ventricular remodeling response to β-blockers [16–19]. However, the majority of the data still support the pharmacogenetic interaction, because the negative studies are small ($n < 200$) and most included β_1AR-selective β-blockers.

An isoleucine substitution for threonine at amino acid position 164 in ADRB2 has profound effects on receptor function in vitro. Ile164 demonstrates a substantial decrease in basal and

agonist-stimulated activity owing to defective coupling of the receptor to the stimulatory G protein, G_s [37]. Ile164 also has a lower affinity for beta-blockers [37]. This is a rare allele (Table 1), so definitive studies are lacking, but Liggett's group [37] found suggestive evidence of a counterintuitive adverse association between the presence of the Ile164 genotype and poor outcome in HF. This finding, coupled with the observation that Ile164 also has a lower affinity for β-blockers, led to the hypothesis that HF patients with Ile164 are less responsive to β-blockers. However, an exploratory clinical pharmacogenetic study found that Thr164 homozygotes demonstrated the expected mortality benefit from an average 3.09 years of β-blockade (55.2 % mortality rate without β-blocker and 39.5 % mortality rate with β-blocker; $p = 0.004$) [38]. Only 14 patients were heterozygous for Ile164, and no homozygotes were found. Surprisingly, the β-blocker effect was reversed in patients carrying Ile164. There was a twofold higher mortality rate in the 7 Ile164-heterozygous patients treated with β-blocker (57.1 %) compared with the 7 Ile164 heterozygous patients not treated with β-blocker (28.6 %). This could be due to the excessive impairment of cardiac function via the combination of dysfunctional ADRB2 and pharmacologic blockade. The difference in mortality between β-blocker treated and untreated Ile164-heterozygous patients was not statistically significant, but the sample size was small. Three other studies failed to find a significant association [17–19].

3.4 α_{2C}AR

The function of the α_{2C}AR (ADRA2C) is presynaptic autoinhibition of norepinephrine release. An insertion/deletion variant in ADRA2C results in a 4 amino acid loss at positions 322–325. The deletion results in the loss of normal autoinhibitory receptor function and therefore increased presynaptic release of norepinephrine [39]. Although not studied in vitro, it is possible that the deletion is associated with β-blocker response, especially when it is inherited with other genetic variants affecting sympathetic activity. For example, HF patients with ADRB1 Arg389 (with increased agonist-promoted activity) and the ADRA2C deletion (with increased presynaptic release of norepinephrine) could have enhanced βAR activity and therefore greater response to β-blockade. In one study, the ADRA2C deletion carriers had an increased improvement in LVEF compared with insertion homozygotes [40]. Synergy between the ADRB1 and ADRA2C variants was supported by the magnitude of results, in that patients both homozygous for Arg389 and carrying the deletion exhibited the greatest LVEF response compared with all other genotypes. Another study also investigated ventricular changes (LVFS) in response to β-blocker and ADRA2C status in 80 IDC patients but did not find a significant association [18]. However, these investigators did not test for synergy between ADRB1 Arg389 and the ADRA2C deletion.

This discrepancy in results between these two studies could be due to population differences (European- and African-Americans with systolic dysfunction due to ischemic and non-ischemic etiologies vs. Japanese patients solely with IDC). Whether the influence of ADRA2C on LVEF is population specific or only important when inherited in combination with Arg389Gly variants remains unknown. The complexity of adrenergic regulation through ADRA2C was highlighted in a pharmacogenetic substudy consisting of 1,040 patients from BEST by Bristow and colleagues [41]. This study found that deletion carriers did not experience survival benefit from β-blockade. However, insertion-homozygous patients did experience survival benefit. Importantly, the β-blocker investigated (bucindolol) had previously shown to cause marked sympatholysis resulting in increased mortality and HF hospitalizations, compared with patients with little or no sympatholytic response [42]. Indeed, this was the case in deletion carriers. In bucindolol-treated patients, a comparison of homozygous ADRA2C insertion and deletion carriers revealed that deletion carriers had a 3.1-fold greater reduction in norepinephrine. Marked sympatholysis is unique to bucindolol; therefore, it is unclear whether these can be applied to other β-blockers.

3.5 GRK5

The function of the GRKs is to desensitize ligand-occupied GPCRs, such as βARs [43]. Liggett's group studied a variant in GRK5 that changes amino acid 41 from glutamine to leucine both in vitro and in association with outcomes in HF patients [43]. The Leu41 allele more effectively desensitizes agonist-stimulated responses. These investigators examined the potential pharmacogenetic interaction both retrospectively and prospectively in HF patients. In a case-control study, they found a significant pharmacogenetic interaction, but only in the African-American subgroup. They then confirmed these findings in a prospective observational study of a second cohort of 375 African-Americans with HF, where they found that only individuals who were homozygous for Gln41 had significantly improved transplant-free survival with β-blocker treatment. There was no difference in this outcome in patients carrying Leu41 with or without β-blocker. Similar results were found in a combined cohort of African-American HF patients [9]. In the overall cohort, there was a trend for a β-blocker treatment effect, but in a subgroup of ADRB1 Gly389-homozygous/GRK5 Gln41-homozygous African-Americans, β-blockers did provide mortality benefit. When the investigators matched African- and European-Americans by GRK5 genotype and β-blocker treatment, survival was similar in the 2 groups. These findings must be considered with some caution, owing to the limited number of events in the first prospective cohort, overlapping composition of the study populations, and the registry design used in

these studies. Whether there are differences in β-blocker treatment effect between European- and African-Americans has been a subject of controversy [44]. Additional prospective studies are definitely warranted.

3.6 ACE

The genetic variants discussed to this point are related to the sympathetic adrenergic system, but the RAAS also contributes to worsening of the HF syndrome. ACE plays a critical role in the RAAS, where it converts angiotensin I to angiotensin II, resulting in downstream effects including sodium and water retention and vasoconstriction. The ACE gene is localized to chromosome 17q23.3-q23.3, and it comprises 26 exons that are alternately spliced to give 2 isoforms. Since its discovery, a 287-basepair insertion/deletion in intron 16 of ACE has been the most studied cardiovascular-related variant. The ACE insertion/deletion accounts for one-half of the variance in serum ACE levels [45], with the deletion allele conferring significantly higher levels. β-blockers have been shown to decrease RAAS activity in HF [46], probably via inhibition of ADRB1 present in the kidney, where β_1AR activation leads to release of renin and ultimately aldosterone [47]. Because the ACE deletion results in higher RAAS activity, it has been hypothesized that HF patients with the ACE deletion have a greater response to β-blockers. In 2001, McNamara and colleagues [10] were the first to publish this pharmacogenetic interaction in a cohort of 328 HF patients followed for a median 21 months. In the overall cohort, there was a trend for increased transplant-free survival in patients receiving β-blockers. However, when the ACE insertion/deletion subgroups were analyzed individually, only patients homozygous for the deletion had a significant improvement in transplant-free survival from β-blockade. These results were validated in a later study [11] with 479 patients.

3.7 ACE Inhibitors

The majority of pharmacogenetic literature in HF patients has focused on beta-blockers. There have been 10 studies since 1998 evaluating pharmacogenetic interactions with ACE inhibitors. Because the ACE deletion allele results in significantly higher ACE levels [45], it has been hypothesized that HF patients possessing the deletion require a higher dose of ACE inhibitor to achieve the same response as a patient without a deletion allele. Most of the studies investigating intermediate phenotypes, such as mean arterial pressure, aldosterone escape, and serum ACE activity support this hypothesis. In a small double-blind crossover study of captopril and lisinopril, the insertion allele was associated with a greater decrease in mean arterial pressure in patients with HF [48]. Another study of 132 patients with HF addressed the clinical issue of "aldosterone escape," reporting that 13 patients had aldosterone escape, and there was a significantly higher frequency of the deletion allele in those patients compared with those who did not

experience aldosterone escape [49]. Of those that experienced aldosterone escape, none were homozygous for the insertion allele. The relationship between the ACE genotype and the intermediate phenotype of LVEF improvement after ACE inhibitor is not clear. In a study of 107 IDC patients, LVEF improvement was similar among ACE genotype after 2.5 years of ACE inhibitor therapy [50], but another study of 168 patients with systolic dysfunction found that deletion carriers responded better to ACE inhibitor than insertion homozygotes [51].

The relationship between the ACE variant and survival benefit from ACE inhibitors is more clear than the intermediate phenotypes. In the largest ACE inhibitor pharmacogenetic study in HF patients to date, this pharmacogenetic interaction with the clinical end point of death or cardiac transplantation was investigated and a dose-dependent relationship between the ACE insertion/deletion and transplant-free survival was found [11]. After a median follow-up of 33 months, patients on low-dose ACE inhibitors had poorer transplant-free survival associated with the deletion allele, with a relative risk for deletion homozygotes of 2.07. This was exaggerated in patients who were also not receiving a β-blocker, with a relative risk for deletion homozygotes of 2.75. However, high-dose ACE inhibitor, with or without concomitant β-blocker, eliminated the adverse effect of the ACE deletion. Although the deletion allele was associated with poorer transplant-free survival, it seemed that deletion homozygotes benefited the most from ACE inhibitor and β-blocker therapy. When this pharmacogenetic interaction was studied with the end point of death from any cause, the deletion allele was again found to be associated with all-cause mortality in patients not receiving an ACE inhibitor, but not for patients receiving an ACE inhibitor [52]. Both of these latter studies were performed in observational cohorts, in which the patients were not randomized to ACE inhibitor treatment. Nevertheless, they both seem to indicate that HF patients with the deletion allele need to be treated with ACE inhibitors to compensate for the increased ACE activity associated with this allele, as well as to demonstrate outcomes similar to patients with the insertion allele.

4 Discussion

The first report of a pharmacogenetic interaction in HF patients was published 13 years ago [48]. Since then, the work of Liggett and colleagues concerning the investigational drug bucindolol provides the best evidence yet to support that genetic variation can be associated with differential response to HF pharmacotherapy, which can in turn affect the risk of adverse outcomes. Unfortunately, the literature as a whole does not provide sufficient evidence to guide application of available HF drug therapy based on genetic testing.

There are numerous possible explanations for why initial pharmacogenetic associations have failed to be replicated in subsequent studies. Outcome studies are particularly problematic in this field, because they almost uniformly lack statistical power owing to insufficient event rates (due to small sample size and/or short follow-up time). The choice of end point is also important, because studies more often than not note differences in clinical outcome end points without detecting differences in surrogates such as LVEF or heart rate [29, 41].

There are still many gaps in investigation in the HF pharmacogenetic literature. Many of the genetic candidates described to date are common in the population; therefore, any given HF patient is likely to possess multiple genetic variants, and the consequences of that have not yet been studied. There is some evidence that inheriting two genetic variants within the sympathetic adrenergic system has synergistic effects. The literature for ACE inhibitors is not nearly as developed as that for β-blockers. To move the field of HF pharmacogenomics forward, adequately powered prospective HF patient cohorts with extensive genotyping and association analyses are needed. Some elegant candidate genetic variants have already been identified and begun to get validated in small clinical trials. The next steps would thus be studies in large independent HF patient cohorts and then prospective evaluation of interventions based on genotype. In a syndrome as fatal and prevalent as HF, any information that could improve and "personalize" pharmacotherapy tailoring will have profound patient and public health benefits.

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Chapter 11

Pharmacogenomics in the Development and Characterization of Atheroprotective Drugs

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Abstract

Atherosclerosis is the main cause of cardiovascular disease (CVD) and can lead to stroke, myocardial infarction, and death. The clinically available atheroprotective drugs aim mainly at reducing the levels of circulating low-density lipoprotein (LDL), increasing high-density lipoprotein (HDL), and attenuating inflammation. However, the cardiovascular risk remains high, along with morbidity, mortality, and incidence of adverse drug events. Pharmacogenomics is increasingly contributing towards the characterization of existing atheroprotective drugs, the evaluation of novel ones, and the identification of promising, unexplored therapeutic targets, at the global molecular pathway level. This chapter presents highlights of pharmacogenomics investigations and discoveries that have contributed towards the elucidation of pharmacological atheroprotection, while opening the way to new therapeutic approaches.

Key words Pharmacogenomics, Atherosclerosis, Therapeutic targeting, Therapeutic mechanisms, Adverse effects

1 Introduction

1.1 Epidemiology Risk Factors and Molecular Background of Atherosclerosis

Atherosclerotic coronary disease is the major cause of cardiovascular disease (CVD) [1, 2]. It is currently the underlying cause of approximately 50 % of deaths across the Western world, while having a major impact on the quality of life of many more individuals, as a result of chronic pain, activity restriction, unemployment, and disability [1, 2]. As a consequence atherosclerosis places a significant psychological and financial burden for patients and the health-care system [3]. According to the American Heart Association, the direct and indirect cost of CVD for 2009 in the USA, including health expenditures and loss of productivity, has been estimated to a total of $475.3 billion, and in the EU approximately €192 billion a year [3]. By 2030, the prevalence of CVD and the cost of treating CVD are predicted to increase by almost 18 % and 100 %, respectively [4].

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Atherosclerosis has been attributed to both genetic and environmental risk factors, as well as their interactions. Among them are age, gender, smoking, high blood pressure, hyperlipidemia, low HDL cholesterol, metabolic syndrome, diabetes mellitus, obesity, physical inactivity, and stress [1, 2, 4–6]. Atherosclerosis is a chronic inflammatory disease of the large arteries. Damage of arterial endothelial integrity induced by pro-inflammatory factors, such as oxidation of the accumulated LDL in the subendothelial matrix, is the primary initiating event in atherosclerosis [1, 2]. Injury of the arterial wall can disrupt the permeability of the endothelial barrier, reduce NO (nitric oxide) production, and increase expression of pro-inflammatory molecules, including cell surface adhesion molecules, cytokines, and growth factors. Endothelial dysfunction facilitates the recruitment of T-lymphocytes and circulating monocytes to the intima of the arterial wall. The subendothelial monocytes differentiate into macrophages and become foam cells by accumulating cell debris and oxidatively modified lipoproteins, which mediate further the chemotaxis of T cells by secreting cytokines and growth factors. These initial atherosclerotic lesions, called "fatty streaks," progress further with the smooth muscle cell (SMC) migration from media into the intima, SMC proliferation, extracellular matrix (ECM) production, and fibrous tissue formation, resulting in the formation of mature atherosclerotic plaque. The most advanced and unstable lesions within the intima are characterized by a fibrous cap containing SMCs and ECM enclosing a lipid-rich necrotic core that upon rupture, leads to thrombosis and major clinical complications, such as myocardial infarction and stroke [1, 2, 5, 7].

1.2 Atheroprotective Treatments and Their Limitations

Currently available treatments against atherosclerosis include cholesterol-lowering drugs such as statins, fibrates, nicotinic acid (NA) [8–13] and the cholesterol intestinal absorption inhibitor, ezetimibe (Fig. 1) [14].

Statins are widely used as the first-line treatment for dyslipidemias, and both primary and secondary prevention of atherosclerosis [9, 15, 16], reducing CVD events by 25–54 % [13, 17]. However, significant residual cardiovascular risk remains even after considerable LDL-C reduction through statins in many high-risk patients with established atherogenic dyslipidemia [17, 18]. Statins act by competitively inhibiting the cholesterol biosynthesis pathway, and thus reducing plasma LDL, without significantly affecting HDL [19, 20]. Additionally, they have direct antioxidant [21], vasodilatory [22], anti-inflammatory [23, 24], plaque-stabilizing [25], and antithrombotic [26] properties. Meanwhile, statins have a number of adverse effects, including myalgia (10 % of patients) [27], and an increased risk of type 2 diabetes [28]. Rare statin-treated patients experience severe muscle weakness (clinical myopathy characterized by marked elevations of creatine kinase),

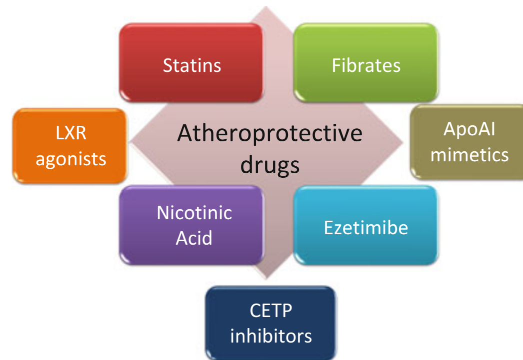

Fig. 1 Clinically available drugs against atherosclerosis and novel atheroprotective HDL-based therapeutic approaches that have been assessed by pharmacogenomics

rhabdomyolysis, hemoglobinuria, and acute renal failure. Of note, the fatal rhabdomyolysis associated with cerivastatin led to its withdrawal from the US market in 2001 [29, 30].

For patients with hypertriglyceridemia or statin intolerance, fibrates and NA serve as valuable alternatives [31]. Fibrates, including fenofibrate, gemfibrozil, and bezafibrate [32], have been used for more than three decades for the treatment of primary and secondary dyslipidemias [33]. They modestly raise HDL plasma levels, without affecting LDL, and effectively promote lipolysis of triglycerides (TG) by inducing LP (lipoprotein lipase) activity, leading to reduced atherosclerosis [34–36], and to an approximately 10 % reduction in the risk of nonfatal cardiovascular events. A recent meta-analysis of fibrate use for stroke prevention revealed that fibrate therapy might decrease the risk of fatal strokes in patients with previous CVD or stroke [37]. However, fibrates do not reduce cardiovascular total mortality [38, 39]. Fibrates exert their hypolipidemic effects through their action as peroxisome proliferator-activated receptor alpha (PPARα) synthetic agonists that regulate the transcription of energy metabolism-related genes, such as APOA-I (apolipoprotein A-I), APOA-2, ABCA1 (ATP-binding cassette A1 transporter), ABCG1 (ATP-binding cassette transported 8), APOC3 (apolipoprotein C3), and LP [40, 41]. Additionally, the atheroprotective properties of fibrates include their anti-inflammatory effects in vascular endothelial cells (ECs) and SMCs [42]. However, they have also been associated with a number of adverse effects including acute renal failure related with increased serum creatinine levels [43], nausea, headache, skin rash, and hepatic toxicity [44, 45]. Upon combination with statin treatment, the risk of myopathy and rhabdomyolysis increases considerably, as compared to statin or fibrate monotherapy [46–48]. Of note, the increased rate of adverse effects, including mortality, led to the withdrawal of clofibrate in 2002 [49].

Nicotinic acid effectively lowers TG, moderately reduces LDL-C and raises HDL-C, leading to reduced subclinical atherosclerosis [10], cardiovascular events, and total mortality when combined with a statin [50, 51]. However, two recent, large-scale randomized clinical trials, AIM-HIGH [31] and HPS2-THRIVE (HPS2-THRIVE Collaborative Group, 2013 [52]), showed that the extended addition of NA to intensive simvastatin therapy had no incremental clinical benefit in reducing atherosclerotic cardiovascular risk among patients with atherosclerotic coronary artery disease compared to statin therapy alone, despite significant improvements in HDL and TG levels [31, 53]. Additionally, the initial reduction in plasma FFA levels often rebounds after long-term NA treatment, whereas its lipid-lowering effect persists [54]. NA acts by binding and stimulating a G protein coupled receptor (GPR109A) in the plasma membrane of adipocytes and thus reducing the plasma levels of FFA, VLDL formation, LDL, and lipoprotein LP(a), while increasing HDL levels [55, 56]. Growing evidence is now suggesting that the FFA-lowering effect may account for only a fraction of NA effects on plasma lipids, while other yet unknown mechanisms may be implicated [57]. Meanwhile, the clinical use of NA for the prevention of atherosclerotic CVD has been limited mainly due to its adverse effects including cutaneous flushing of the skin [58], gastrointestinal symptoms, hepatoxicity [56, 58], blurred vision, diabetes, and myopathy [52].

Ezetimibe is used for secondary prevention against established atherosclerotic CVD to achieve an optimal atherogenic cholesterol level in patients with intolerance to high-doses of statins. It can further be used in combination with statins to achieve lower LDL-C levels in very-high-risk patients [59]. Ezetimibe inhibits the Niemann-Pick C1-Like 1 (NPC1L1)-dependent intestinal cholesterol absorption in the apical brush border membrane of jejuna enterocytes [14], and thus it only moderately lowers LDL-C (12–25 %) [60]. Meanwhile, common adverse effects associated with ezetimibe therapy include gastrointestinal disturbances, while infrequent adverse effects such as rash, angioedema, anaphylaxis, hepatitis, cholelithiasis, cholecystitis, thrombocytopenia, raised creatine kinase, myopathy, and rhabdomyolysis may occur [46].

Interestingly, clinical studies have demonstrated that pharmacological inhibition of RAAS (rennin–angiotensin–aldosterone system) pathway could mediate atheroprotection [61, 62]. Angiotensin I converting enzyme (ACE) inhibitors offer a mechanism of RAAS inhibition and several large-scale clinical trials have demonstrated that they reduced the cardiovascular events (myocardial infarction, stroke, and mortality) among patients with coronary artery disease [62, 63]. ACE inhibitors reduce angiotensin II production [64] leading also to the prevention of atherosclerosis development in several animal models of atherosclerosis [65]. ACE

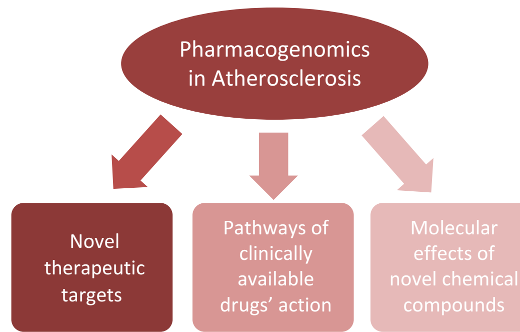

Fig. 2 Pharmacogenomics is making an increasingly significant contribution to the battle against atherosclerosis through the identification of novel therapeutic targets as well as the evaluation of the molecular mechanisms of action and pathways responsible for adverse effects in established and novel atheroprotective drugs

inhibitors also increase NO production and induce vasodilatation, leading to lower blood pressure and slower atherosclerosis progression [66]. Several lines of evidence support the involvement of additional mechanisms in the protective effects of ACE inhibitors, including actions on inflammatory cells and arterial SMCs [67]. Meanwhile, common adverse events of ACE inhibitors include dry cough, hypertension, hyperkalemia, renal dysfunction, dizziness, fatigue, and nausea [68].

Despite the advances towards a better understanding of the pathophysiology of CVD and the significant therapeutic advances over the past 50 years [69], numerous limitations persist, relating to the failure in the ability of lipid-lowering drugs to decrease the cardiovascular risk [16–18], the interindividual variation in drug response [11], and adverse drug effects [46]. Extensive efforts are geared towards the discovery of novel therapeutic targets and approaches preventing atherosclerosis development, inhibiting its progression and increasing HDL-C levels and/or HDL function [1, 12, 13, 16, 70]. Clinical trials are testing the novel atheroprotective HDL-targeted therapeutic strategies to determine their suitability for clinical practice [16], while the majority of these therapies are currently under preclinical evaluation in animal models (Fig. 1) [12, 16, 71–73].

In order to delineate the specific molecular mechanisms mediating the effect of approved or novel atheroprotective drugs, as well as to discover new therapeutic targets, pharmacogenomics approaches are playing a central role (Fig. 2). Through the global, unbiased investigation of the genome in different pathological settings or post-treatment, valuable information of clinical relevance is emerging. This information will contribute towards the faster transition of novel treatments to the clinical practice, but also the optimization of available atheroprotective drugs.

Herein, representative examples demonstrating the contribution of pharmacogenomics in the development and characterization of atheroprotective treatments, with an emphasis on HDL-based novel therapies, are presented.

2 Identification of Novel Therapeutic Targets Through Pharmacogenomics

2.1 Atherogenesis

Atherosclerosis is a complex pathological process that progresses over a prolonged period of time and varies considerably in severity and ultimate effect to the patient. It involves several different cell types and is characterized by perturbation of numerous cellular processes. To achieve an improved clinical outcome, new therapies need to be developed, that will be more effective in reversing atherosclerotic damage, will target simultaneously multiple underlying pathologies, ranging from foam cell formation to plaque instability, and will be administered earlier to the individuals at risk. Through the in-depth characterization of molecular pathogenesis, pharmacogenomics is aiming to unveil promising new therapeutic targets. Highlights of such representative efforts are presented herein.

The process of atherogenesis is generally understood to begin with the infiltration of the vessel wall by monocytes, which, at a later stage, differentiate into macrophages, take up oxidized LDL and are transformed into foam cells. This process results in the formation of "fatty streaks" across the vessel wall, which mediate chronic vascular inflammation and can later develop into atherosclerotic lesions. It is unclear when this process starts and how it is first induced. Systemic low-grade inflammation, caused by smoking, metabolic syndrome, autoimmune disease, or an infection, is hypothesized to be a decisive initiating stimulus.

To investigate these very early steps of atherosclerosis, low-grade inflammation was induced in humans and global gene expression changes in circulating monocytes were investigated [74]. The most promising finding was the upregulation of C3aR1, a gene encoding complement component 3a receptor 1, a G-protein-coupled membrane receptor, found in monocytes, macrophages, and ECs. The binding of C3, a protein involved in innate immunity, to this receptor is highly ligand specific and triggers a wide range of inflammatory and immune effects [75]. Interestingly, C3aR1 had been found overexpressed in advanced compared to intermediate atherosclerotic lesions [76]. Protein levels are also increased in human coronary plaques but not in healthy coronary intima. Additionally, signaling via C3aR1 has been shown to promote plaque instability [77]. As a result, the observed upregulation of C3aR1 in circulating monocytes, after the induction of low-grade inflammation, was hypothesized to have a pro-atherogenic effect, leading to atherogenesis [74]. This is further supported by a study demonstrating that double knockout mouse

models for apolipoprotein E (ApoE) and C3ar1 (C3ar1−/− and ApoE−/−) present with decreased atherosclerotic lesion sizes [78], compared to ApoE−/− mice. Therefore, it is interesting to speculate that targeting of C3aR1 could have a therapeutic potential.

The infiltration of the vessel wall by monocytes and/or macrophages is a decisive step towards foam-cell formation and early atherogenesis. The observed increased expression of Spp1, better known as osteopontin, in a genomic study with a focus in early atherogenesis, may be of significance in this process. This upregulation of osteopontin was detected in atherosclerosis-prone regions, in comparison to atherosclerosis-resistant regions, of ApoE−/− mice. In this particular study, gene expression profiling was conducted preceding the formation of atherosclerotic plaques, with an aim to study early atherogenesis [79]. The Spp1 gene encodes a non-collagenous matricellular protein, which, in its soluble form, has been shown to interact with cell surface integrins to regulate cell adhesion, migration and proliferation [80]. In atherosclerosis, osteopontin plays a decisive role in foam cell formation, since it is expressed by macrophages and acts as a potent macrophage-chemotactic stimulus, regulating macrophage infiltration and retention at sites of chronic inflammation [81]. Osteopontin transgenic mice fed a high-cholesterol diet develop significantly larger atherosclerotic "fatty streaks" lesions, than wild-type controls with the same diet [82, 83]. Furthermore, osteopontin overexpression in transgenic mice has been linked to SMC proliferation and thickening of the media and intima [84]. Additionally, osteopontin deficiency has been found to reduce atherosclerosis in ApoE−/− and LDL receptor deficient (Ldlr−/−) mice [85, 86]. Consequently, osteopontin suppression is considered to have some therapeutic merit [87].

The accumulation of cholesterol in the periphery is a decisive step in the formation of foam cells and early atherogenesis. It can be the result of reduced reverse transport of cholesterol from the vessel wall to the liver; a process mediated by HDL. For this reason, reduced levels of HDL are considered an important early clinical indicator of increased risk of coronary CVD and stroke, as they reflect a decreased capacity for clearing accumulated cholesterol, and thus a propensity towards foam cell formation. The marked upregulation of phospholipid transfer protein (PLTP), observed in a genomic study, of advanced human atherosclerotic lesions, may also be a key in early atherogenesis [88]. PLTP is a transfer protein involved in the transfer of phospholipids from TG-rich lipoproteins into HDL. As a result upregulation of PLTP leads to reduced HDL and attenuated reverse cholesterol transport (RCT), which would be expected to enhance atherogenesis [89, 90]. Elevated expression of PLTP has also been deemed "atherogenic," in studies involving ApoE−/− and Ldlr−/− mice [91, 92]. Additionally, two single nucleotide polymorphisms (SNPs) of PLTP (rs378114 and rs6065904), that are associated with decreased PLTP

transcription and activity in humans, have been linked to an increase in HDL particles and a decreased risk of CVD [93]. Interestingly, PLTP deficiency in mice significantly reduces the extent of atherosclerosis [94]. Based on this overall evidence, PLTP inhibition also, is thought to constitute a possible athero-protective target [95].

In parallel to the uptake of LDL by macrophages, foam cell formation can result from the uptake of platelets [96]. App, a protein stored in platelet granules, was found significantly upregulated in atherosclerotic-prone regions of ApoE−/− mice, fed a regular diet (right before they developed atherosclerotic lesions), in comparison to atherosclerosis-resistant regions, in a genomic study examining the molecular changes occurring during atherogenesis [79]. Since App has been reported to be essential for the inflammatory activation of macrophages, after platelet uptake [97], it is interesting to speculate that the upregulation of App could be responsible for maintaining active inflammation in the vessel wall, which would result in increased chemotaxis of more monocytes, the subsequent formation of more foam cells and the spread of atherogenesis. Preventing the expression of App may thus have atheroprotective effects and may attenuate the exasperation of inflammation in the progression of atherosclerosis.

2.2 Exacerbation of Inflammation

The progression of “fatty streaks” into advanced, clinically detectable, atherosclerotic plaques is a long process that may take decades in humans. Local chronic inflammation of the vascular wall is an important accelerator, increasing vascular permeability to macrophages and facilitating their endothelial infiltration. Since the main trigger of inflammation is foam cells themselves, the inflammatory response can be the mediator of a positive feedback loop, where foam cells attract more macrophages, stimulate their proliferation, and thus increase their numbers rapidly. As a result, a marked acceleration of vascular thickening takes place; with inflammation-induced apoptosis and proliferation of SMCs accompanying the accumulation of macrophages. The identification of molecular targets against these inflammatory processes may prove valuable towards blocking the progression to advanced atherosclerosis.

In search of such a target, gene expression profiling was conducted on human coronary atherosclerotic plaques, of various grades of severity (histologically graded I to V), from nondiabetic as well as diabetic patients. The latter group experienced a more rapid progression to advanced atherosclerosis [98]. The genes found differentially expressed via microarrays, among the different grades of severity (in both nondiabetic and diabetic patients), were subsequently combined with bibliography-derived atherosclerosis networks. This led to the identification of multiple genes involved in the progression of atherosclerosis. Of particular interest are

interleukin-27 (IL-27) and interleukin-6 (IL-6), which were upregulated in advanced atherosclerotic lesions, and appeared to have a central role in nondiabetic and in diabetic atherosclerotic patients, respectively. Both of them are known activators of the JAK/STAT pathway, which plays a decisive role in the progression of inflammation, and is associated with a plethora of autoimmune and inflammatory pathologies. Further analysis of the samples identified multiple pieces of evidence for the activation of JAK/STAT signaling pathway, with many members of the pathway itself or known downstream targets, being differentially expressed. These findings were confirmed in a separate microarray study of human coronary and carotid plaques, which presented with JAK/STAT activation in advanced atherosclerotic lesions, and increased circulating levels of IL-6 [88]. Subsequent studies reinforced the significance of IL-6 and IL-6-induced activation of JAK/STAT in atherosclerosis. In particular, oxidized LDL has been shown to enhance the expression of IL-6, among other inflammatory cytokines [99], while increased concentrations of exogenous IL-6 were found to enhance atherosclerosis in ApoE−/− mice [100]. Additionally, plasma levels of IL-6 are higher in patients with unstable angina and are predictive of the clinical outcome in patients with acute coronary syndrome [101]. It has therefore been suggested that targeting IL-6 could have a significant therapeutic potential.

Interestingly, the observed increase in IL-6 circulating levels was attributed, by one of the teams, to the observed downregulation of the bone morphogenetic protein receptor, type II (BMPR2), since BMPR2 loss-of-function mutations have been associated with a dramatic increase in IL-6 levels [102, 103]. The detected downregulation of several collagen genes was consistent with these findings, since the IL-6-induced activation of JAK/STAT has been shown to decrease the expression of collagen and other ECM structural proteins [104]. These gene expression changes would be anticipated to lead to ECM structure modifications, contribute to loss of physiological intercommunication between the components of the blood vessel wall, and thus contribute to SMC dedifferentiation and proliferation. Speculating that downregulation of BMPR2 was the initial step in accelerating atherosclerosis, followed by IL-6 upregulation, JAK/STAT activation, ECM degradation, and SMC proliferation, it was proposed that targeting BMPR2 could have a therapeutic potential in atherosclerotic patients.

The importance of the JAK/STAT pathway in atherosclerosis progression is also emerging from animal model studies, such as the global gene expression analysis of ApoE−/− mice [105]. Among the multiple significant changes, the authors placed particular emphasis on the upregulation of oncostatin M (Osm), a known JAK/STAT activator, and its receptor (Osmr). Osm is a cytokine shown to induce production of IL-6, among other inflammatory

cytokines [106]. Consistently with a role in the aggravation of atherosclerosis, OSM also upregulates matrix metallopeptidase 3 (MMP-3) and ABCA1, which are involved in ECM breakdown and regulation of cholesterol efflux, respectively [106, 107]. Consequently, Osm and its receptor may represent yet another alternative worth exploring for its atheroprotective potential.

Additional research in the regulation of inflammation, in the advanced atherosclerotic setting, demonstrated significant changes in chemokine pathways. For example, the genomic analysis of advanced atherosclerotic lesions, in ApoE−/− mice, unveiled multiple upregulated chemokine genes, among which the monocyte chemoattractant proteins 1 and 5 (Mcp-1 and Mcp-5) were of particular interest [108]. Chemokines are small cytokines that induce monocyte and T-lymphocyte migration, and thus facilitate vascular infiltration, with Mcp-1 being a main facilitator of atherogenesis [109–111]. Specifically, Mcp-1 has been shown to act as a link between inflammation and the degradation of ECM, by inducing expression of matrix metalloproteinases, and thus contributing to plaque instability [112]. Interestingly, MCP-1 upregulation has since been attributed to oxidized LDL and found increased in the presence of IL-6 [113]. The potential of chemokine targeting in treating atherosclerosis was further demonstrated [108] through the administration of a monoclonal antibody (11K2), designed to inhibit Mcp-1 and Mcp-5 in ApoE−/− mice. As a result, plaque size was significantly reduced, inflammatory cell content decreased, and ECM content increased, thus stabilizing the existing plaques and reducing their size. Since then, a number of studies have demonstrated a similar therapeutic atheroprotective potential of Mcp-1 inhibition [113–116].

The function of inflammatory mediators appears to be indirectly regulated by the function of pro-protein convertases subtilisin/kexin (PCSK) enzymes, which are known to cleave inflammatory cytokines, matrix metallopeptidases and integrins into mature end-products [117–119]. A genomic study identified FURIN as the primary PCSK that is dysregulated and significantly over-expressed in lymphocytes and macrophages of advanced human atherosclerotic plaques [120]. Two of the main known targets of FURIN, namely, BAFF/TNFSF13B and APRIL/TNFSF13, were also found consistently upregulated. Both of them are pro-inflammatory TNF-superfamily cytokines that increase inflammatory response and have been implicated in atherosclerosis [121]. Consequently, targeted inhibition of FURIN could be another promising therapeutic strategy against atherosclerosis [120, 122].

2.3 Instability of the Atherosclerotic Plaque

Coronary CVD mortality is mainly attributed to the sudden rupture of advanced atherosclerotic plaques and the subsequent, rapid thrombosis, vascular occlusion, and ischemia. Identifying molecular signatures of instability to prevent plaque rupture would be an effective approach towards reducing CVD mortality.

Aiming to determine the molecular mechanisms of atherosclerotic plaque instability and depict suitable therapeutic targets, Ijäs et al. [123], compared the gene expression profiles of symptomatic and asymptomatic carotid plaques from each of four patients. The distinction was made according to features such as the degree of vessel stenosis, prior symptoms, as well as plaque characteristics such as ulceration, inflammatory cell infiltration, and a thin fibrous cap. Thirty-three genes were found differentially expressed intraindividually. Among them CD163 and heme oxygenase (decycling) 1 (HO-1) were upregulated in the symptomatic plaques of these 4 patients, as well as in a subsequent set of atherosclerotic plaque samples from 40 patients. Both of these genes are involved in iron–heme homeostasis and are expressed in response to plaque instability-induced, intra-plaque hemorrhage, as part of a cellular repair mechanism for iron clearance. Their expression correlates with tissue iron content. However, symptomatic plaques did not differ from asymptomatic plaques in iron deposits or macroscopic hemorrhages. It was therefore hypothesized that symptomatic plaques exhibit a stronger molecular response to micro-hemorrhages, in comparison to asymptomatic plaques, because of potential differences in cellular composition or intraindividual genetic predisposition. Consistently with this hypothesis, the induction of both CD163 and HO-1 would be more pronounced in symptomatic compared to asymptomatic plaques. This hypothesis may be highly significant, since both of these molecules destabilize advanced atheromas. In particular, it has been demonstrated that certain CD163 genetic variants are associated with increased vascular complications in atherosclerosis [124]. Additionally, HO-1 is known to prevent proliferation of vascular SMCs and endothelial cells after vascular injury [125]. Therefore, the greater-than-average upregulation CD163 and HO-1, as a means of vascular repair after microscopic hemorrhages, may constitute a hallmark and ultimate cause of plaque instability and therefore an appealing target for therapeutic intervention.

The loss of normal vascular repair capacity is considered another causative mechanism of plaque instability, as shown through global gene expression comparisons between fibrous caps of plaques and healthy adjacent intima, derived from human carotid samples. This comparison in gene expression revealed a characteristic downregulation of the regulator of G-protein signaling 5 (RGS5) in the fibrous cap of plaques [126]. This gene is a distinguishing marker of arteries versus veins, which has been implicated in blood vessel formation and vascular development [127, 128]. It has been shown to inactivate contractile and trophic G-protein coupled receptor (GPCR) signals by activating their intrinsic GTPase activity [129]. Since vasoactive molecules, such as angiotensin, endothelin, norepinephrine, sphingosine, act via GPCRs, the loss of appropriate signaling would be significant and could

lead to cap pathology and subsequently to plaque instability. Restoring RGS5 function could therefore have a stabilizing effect to the atherosclerotic plaque.

More recently, Lee et al. [130] published a study focusing on the gene expression signature of macrophages associated with plaque rapture. Samples were collected from human carotid endarterectomy, designated as stable or ruptured, and the macrophage-rich regions were isolated utilizing laser micro-dissection. Genomic analysis revealed that the PPAR/adipocytokine signaling pathway was the most highly upregulated in ruptured plaques. Of particular interest were two members of this pathway, namely, fatty acid binding protein 4, adipocyte (FABP4), and leptin, which were upregulated by ninefold and fivefold, respectively. FABP4 has been shown to attenuate cholesterol efflux by inhibiting the ABCA1 pathway, to regulate inflammatory response via the JNK pathway and to be an essential bridge between lipid toxicity (evident in advanced plaques) and endoplasmic reticulum stress [131, 132]. High gene expression levels of plaque FABP4 and increased FABP4 plasma protein levels in atherosclerosis patients are associated with plaque instability and plaque-related symptoms [133, 134], while a genetic polymorphism in the FABP4 promoter region, which reduces gene expression, has been shown to reduce the risk of CVD [135]. Importantly, macrophages from FABP4 knockout animals produce less pro-inflammatory cytokines [136]. Furthermore, double knockout mice for ApoE and Fabp4 appear to be protected from atherosclerosis, in contrast to ApoE knockout mice, which exhibit hypercholesterolemia and develop atherosclerosis [137]. Chemical inhibition of Fabp4 in the latter using BMS309403 markedly reduced atheroma macrophage foam cell formation [138]. In parallel to FABP4, the upregulation of leptin may also be contributing to plaque rupture. Hyperleptinemia is an independent risk factor for coronary artery disease and acute myocardial infarction [139, 140]. Furthermore, leptin is a macrophage chemoattractant and has been proposed to cause endothelial dysfunction by the uncoupling of endothelial nitric oxide synthase (eNOS) and the subsequent oxidative stress [140–142]. Interestingly, macrophages from mice deficient in leptin (ob/ob mice) display decreased cholesterol accumulation [143]. However, the exact mechanism of autocrine/paracrine mode of function of macrophage-derived leptin remains unknown and may be the key in fully understanding the role of leptin in atherosclerosis. For all the aforementioned reasons, it was suggested that FABP4 and leptin can serve as biomarkers of increased atherosclerosis severity and plaque rupture risk. As a consequence, Lee et al. proposed the downregulation of the PPAR/adipocytokine signaling pathway, as a novel therapeutic strategy [130].

In summary, pharmacogenomics has proven to be a valuable tool for the characterization of key molecular pathways implicated in the different stages of atherogenesis as well as disease progression.

Within these pathways, specific molecules have emerged as particularly promising therapeutic targets, with several of them being currently investigated in depth (Fig. 2). At the same time, it is important to note a few important limitations encountered in pharmacogenomic studies: parameters such as the spatial and temporal origin of the isolated vascular tissue, the exact cellular source of mRNA and the selected controls for comparison purposes can vary considerably across studies. This complexity gives rise to seemingly heterogeneous or even conflicting data.

The harmonization of strategic designs and the careful control for as many variables as possible in these complex high-throughput studies could significantly increase the amount of meaningful and clinically valuable conclusions.

3 Evaluation of Established Atheroprotective Drugs by Pharmacogenomics

3.1 Applications of Pharmacogenomics in Delineating the Therapeutic Molecular Mechanisms of Established Atheroprotective Drugs

Although the therapeutic effect of atheroprotective drugs used in clinic today is well established, their molecular mechanisms of action are often partially understood. This becomes a significant limitation in resolving cases of adverse drugs reactions or reduced efficiency. Pharmacogenomics is playing a central role in the characterization of the global molecular pathways affected by atheroprotective drugs (Fig. 2).

Pharmacogenomic studies have been performed on a range of different cultured cells that participate in the formation of atherosclerotic lesion including ECs, SMCs, monocytes, and macrophages [144], as well as animal models [145], atherosclerotic lesion samples and blood-derived cells from patients with hyperlipidemia or coronary artery disease [144]. Representative examples of such studies and their contribution towards improving atheroprotective treatments are presented in the following sections.

3.1.1 Pharmacogenomics of Lipid-Lowering Drugs

Statins

The Lipid-Lowering Effect of Statins

Statins can exert their therapeutic effects both through lipid-lowering-dependent and independent pathways (Fig. 3). Two representative examples of pharmacogenomic studies analyzing the lipid-lowering effects of statins are presented in the following section. Global gene expression analysis of coronary artery SMCs treated with pitavastatin (16 h) revealed downregulation of genes involved in the cholesterol biosynthesis pathway including the rate limiting enzyme HMG-CoA reductase and the HMG-CoA synthetase 1. Additionally, multiple genes associated with cholesterol uptake, such as the scavenger receptor class B member 1 (SR-B1) were upregulated, with the exception of LDLR, which was downregulated [146]. SR-B1 is an HDL receptor in the liver that mediates selectively the uptake of cholesteryl ester from HDL [147], while LDLR is a LDL receptor that is involved in receptor-mediated endocytosis of LDL [148]. Similar effects were observed following lovastatin treatment of two human

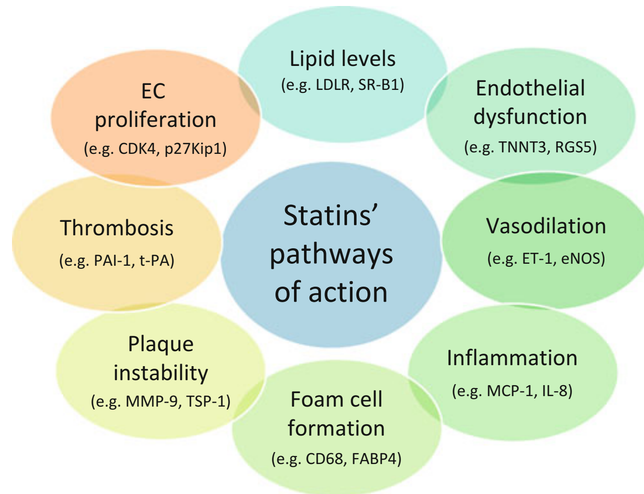

Fig. 3 The molecular mechanisms of statin atheroprotection are slowly being unraveled through pharmacogenomics

hepatocellular carcinoma cells line (HepG2 and SK-HEP-1) where both SR-B1 and LDLR, as well as the sterol regulatory element binding transcription factor 1 (SREBF-2), were upregulated [149]. SREBF-2 activates the transcription of HMG-CoA reductase [150], HMG-CoA synthetase [151], and LDLR [148, 152]. The upregulation of LDLR and SREBF-2 could facilitate cholesterol uptake to maintain the cellular cholesterol homeostasis [149]. In conclusion, statins appear to lower cellular cholesterol levels by modulating the expression of cholesterol biosynthesis genes. As a feedback response to the cellular cholesterol-lowering effects, statins upregulate genes involved in cholesterol uptake, and thus, they maintain cellular cholesterol homeostasis.

Lipid-Lowering-Independent Effects of Statins

Aside from their effect on lipid-lowering pathways, statins have been reported to affect most of the molecular mechanisms implicated in atherosclerosis (Fig. 3). Global gene expression profiling has been valuable towards elucidating this association.

Statins and Endothelial Dysfunction

Endothelial barrier dysfunction is a primary event in the pathogenesis of atherosclerosis [1, 153]. Statins can affect the expression of genes regulating endothelial cytoskeleton reorganization and thus have a beneficial effect on EC barrier function [154, 155]. Prolonged simvastatin treatment (16 h) on human pulmonary artery ECs induced differential expression of genes involved in endothelial barrier regulation, including the caldesmon and the integrin β4 subunit, suggesting its beneficial effect on EC barrier function [154]. Additionally, prolonged

pravastatin treatment (6 week) of apoE deficient mice, fed with a hypercholesterolemic diet, upregulated genes associated with cytoskeleton organization in atherosclerotic aortic lesions. The most upregulated genes were troponin T3 (TNNT3), actin a1, tubulin a1, regulator of Rgs5, stathmin-like 2, and myosin light chain kinase [155]. Such studies led to the conclusion that prolonged statin treatment has EC barrier-protective properties by attenuating EC barrier disruption and paracellular gap formation [154]. The vascular protective cholesterol-independent effect of the chronic statin treatment is largely dependent on its influence on the cytoskeleton reorganization in the vascular wall, which is needed for the atherosclerotic process involving the mediating cell adhesion, migration, and proliferation [155].

Statins and Vasodilation

The impairment of vascular endothelium-dependent vasodilation, through disturbance of the L-arginine/NO pathway, plays a central role in the pathogenesis of atherosclerosis. Impaired endothelium-mediated vasodilation has been demonstrated to be an early marker of atherosclerosis that occurs long before the formation of atherosclerotic plaque [1, 153, 156]. Several gene expression profiling studies have indicated that statins can induce expression alterations in genes regulating vascular tone. For example, atorvastatin or pitavastatin treatment of cultured human umbilical vein ECs (HUVECs) reduced the vasoconstrictive factor endothelin-1 (ET-1) [157–159], while upregulating the vasodilator factor eNOS [157]. Consequently, statins may influence vascular constriction leading to vasodilation by modulating the expression of endothelial vasoactive factors.

Statins and Inflammation

Numerous studies have focused on the effect of statins on inflammation, a critical process that contributes to both the onset and progression of atherosclerosis [99]. Microarray studies at both in vitro [157, 158] and animal model levels [160] have shown an overall inhibitory effect of statins on inflammatory signaling pathways. For examples, pitavastatin or atorvastatin treatment suppressed the mRNA expression of interleukin-8 (IL-8) and MCP-1 in cultured HUVECs [157]. These two pro-inflammatory cytokines are highly expressed in the atherosclerotic lesions and mediate the recruitment of monocytes in the arterial wall [161, 162]. Pitavastatin or atorvastatin treatment in HUVECs also downregulated pentraxin 3 (PTX3) [158], whose expression increases in advanced atherosclerotic plaques [163]. Furthermore, these two statins reduced the expression of the cysteine-rich angiogenic inducer 61 (Cyr61) and the connective tissue growth factor (CTGF) in human coronary arterial SMCs and HUVECs, adding to the downregulation of PTX3 in HUVECs, and thus further contributing to the suppression of inflammatory progress in vascular cells [158].

At the animal model level, hepatic gene expression profiles of apoE3Leiden mice, an established atherosclerosis model, showed that rosuvastatin affected both the IL-1 and MIF (cytokine macrophage migration inhibitory factor) inflammatory signaling pathways, via key regulators including IFN-γ, TGFb, IL-1, TNF-a, MIF, and IL-6. These inflammatory processes were mediated by C/EBP, SP1, ERK1/2, and JNK [160]. The pro-inflammatory cytokine MIF mediates monocyte and T cell recruitment to the site of the injury by engaging its receptors, CXCR2 and CXCR4, respectively [164]. Additionally, both MIF and IL-1β cytokines stimulate the monocyte recruitment to the vascular wall by inducing endothelial expression of adhesion molecules (VCAM-1 and ICAM-1) [162, 164–166].

The anti-inflammatory action of statins was further demonstrated in humans, using blood-derived cells from coronary artery disease or hyperlipidemic patients and gene expression analysis [167–169]. The chemokine IL-1β and its receptor antagonist, IL-1Ra, were downregulated in PBMCs (peripheral blood mononuclear cells) isolated from coronary artery disease patients receiving atorvastatin and/or simvastatin treatment [167], consistently with animal studies. Further evidence of an anti-inflammatory effect of statins emerges from the PBMC analysis of patients with primary hyperlipidemia, where multiple pro-inflammatory genes were downregulated early following atorvastatin treatment (24 h and/or 36 h after the start of treatment), before any detectable changes in circulating lipid levels. These genes included prostaglandin (PG) G/H synthase and the TxA2 (thromboxane A2) receptor [168]. Interestingly, in agreement with reports that statins induce a transient pro-inflammatory response in monocytes in vitro [170], several pro-inflammatory genes were upregulated soon after the start of treatment (12 h) in the same study. Many of these genes are downstream targets of the interferon (IFN)-a pathway including STAT-1 and the interferon stimulated gene (ISG) 54K gene [168]. The anti-inflammatory effect of atorvastatin has also been demonstrated in patients with familial combined hyperlipidemia (FCH) using microarray profiling of peripheral blood monocytes. Particular emphasis was placed on the upregulation of IL-1R2 (Interleukin 1 receptor, type II) [169], which is a non-signaling decoy receptor that negatively regulates the activity of IL-1 [171]. In order to investigate the contribution of IL-1R2 to the atherosclerotic process, the authors continued studies using THP-1 macrophages exposed to pro-atherogenic stimuli and human atherosclerotic lesions as well as qPCR analysis [171]. The results showed a decrease in the IL-1R2 expression of these cells and in human atherosclerotic lesions, suggesting that under atherogenic conditions it possibly facilitates IL-1 signaling and contributes to the atherosclerosis development [171].

The anti-inflammatory effect of statins has also been demonstrated in peripheral leukocytes from healthy volunteers after acute treatment with rosuvastatin (72 h) using gene expression analysis, where the expression of the inflammation-related genes, IL8RB (Interleukin 8 receptor, alpha), IL8RB (Interleukin 8 receptor, beta), and IL-1R2, was significantly downregulated [172]. Particular emphasis was placed on the downregulation of IL8RB [172], whose strong expression in macrophages of atherosclerotic lesion is essential for their retention to the lesion [173]. It therefore appears that the IL-8/IL8RB signaling plays a key role not only in monocyte recruitment to the arterial wall [161, 162] but also in macrophage accumulation in atherosclerotic lesions [173].

In summary, statins have anti-inflammatory properties mediated by the regulation of the T cell and monocyte recruitment to the intima of the arterial wall as well as the macrophage accumulation in atherosclerotic lesions, all of which are crucial events in early atherogenesis.

Statins and Foam Cell Formation

The intimal macrophages of early atherosclerotic lesions accumulate oxLDL, and thus become foam cells which may undergo necrosis, forming the lipid core of the advanced atherosclerotic plaques [1]. A pharmacogenomic study has deciphered the molecular effect of atorvastatin on foam cell formation process. The authors compared the gene expression profile in human THP-1 macrophages, a well-known model of foam cell formation, treated with oxLDL plus atorvastatin with that of cells exposed to oxLDL alone. Co-incubation with atorvastatin decreased the mRNA levels of CD68, FABP4, and APOE, compared to treatment with oxLDL alone [174]. FABP4 and CD68 can modulate the conversion of macrophages to foam cells and thus promote atherosclerosis development [137, 175–177]. FABP4 has been shown to attenuate cholesterol efflux by inhibiting the ABCA1 pathway [134]. Macrophages lacking FABP4 accumulate less cholesterol esters when they are exposed to modified lipoproteins. It is therefore believed that macrophage FABP4 expression promotes foam cell formation [137]. These findings suggest that the downregulation of FABP4 in macrophages treated with oxLDL and atorvastatin could possibly contribute to the decrease in cholesterol ester accumulation in these cells [174]. The scavenger receptor CD68 plays an important role in oxLDL uptake by activated macrophages in vitro, and thus, it could contribute to foam cell formation in atherosclerotic lesions [176]. Therefore, the decreased accumulation of lipoprotein-derived cholesterol in macrophages treated with oxLDL and atorvastatin could also be attributed to the downregulation of CD68 [174]. The downregulation of APOE, a gene that stimulates reverse cholesterol transport [178], which was observed in macrophages treated with oxLDL and atorvastatin,

could simply reflect the beneficial effect of atorvastatin, that is, the decrease in macrophage cholesterol levels [174]. In brief, statins have a direct atheroprotective effect on macrophages partly by limiting foam cell formation.

Statins and Plaque Instability

Several pharmacogenomic studies have reported the molecular effect of statins on the stability of the atherosclerotic plaque. Deposition of ECM and wound repair processes stabilize the cap during advanced atherosclerosis [179]. The beneficial effect of atorvastatin on plaque stability was demonstrated by the downregulation of matrix metalloproteinase 9 (MMP-9) in human THP-1 macrophages treated with oxLDL plus atorvastatin compared to treatment with oxLDL alone [174]. MMP-9 is a proteolytic enzyme that digests and weakens the plaque cap leading to the rupture of vulnerable atherosclerotic plaques [180]. The combined atorvastatin-mediated downregulation of thrombospondin-1 (TSP-1) and other wound healing factors, namely, CTGF and CYR61 [179, 181, 182], observed in primary HUVECs, suggest that atorvastatin possibly alters ECM deposition, wound repair, and tissue remodeling [159]. Meanwhile, the proliferation of vascular SMCs promotes the formation of mature and unstable atherosclerotic plaques [1]. Atorvastatin or pitavastatin treatment appears to have an inhibitory effect on the proliferation of human coronary artery SMCs (8 and 24 h after treatment). A pharmacogenomic study associated this effect with the down-regulation of cyclin B, H, CDK4, and p55cdc and the induction of p21 Waf1/Cip1, a potent inhibitor of CDK2, all of which are cell cycle regulators [158]. Consequently, statins appear to exert their plaque stabilizing properties through inhibition of vascular SMC proliferation and promotion of wound repair.

Statins and Thrombosis

The rupture of the most unstable atherosclerotic plaques leads to thrombosis [1]. Several pharmacogenomic studies have deciphered the molecular effect of statins on thrombosis. Atorvastatin or pitavastatin treatment of HUVECs reduced the mRNA levels of plasminogen activator inhibitor-1 (PAI-1) and PTX3 [157, 158], whereas it increased the mRNA levels of both thrombomodulin [157] and tissue plasminogen activator (t-PA) [158, 159]. PAI-1 expression was also reduced in human coronary SMCs treated with atorvastatin or pitavastatin, whereas the expression of thrombomodulin was strongly induced in those cells [158]. PAI-1 is the principal inhibitor of t-PA and urokinase (uPA) and hence of endogenous fibrinolysis [183], while PTX3 plays a role in thrombogenesis via in vitro upregulation of tissue factor (TF), which is a key player in thrombus formation after plaque rupture [163]. Thrombomodulin is an important anti-coagulation glycoprotein on the surface of ECs [184]. Consistent findings were observed in the study of PBMCs from atorvastatin treated patients with primary

hyperlipidemia, where multiple components of the von Willebrand factor receptor (GPIb and IX) and both subunits of GPIIbIIIa (integrins aIIb and bIII) were downregulated [168]. The von Willebrand factor receptor mediates platelet adhesion to the subendothelium [185], while GPIIbIIIa is a platelet receptor involved in platelet aggregation and thrombus formation [186]. Overall, statins appear to reduce thrombosis by enhancing profibrinolysis, while reducing platelet aggregation and coagulation.

Statins and EC Proliferation

In addition to changes in the proliferation of SMCs, changes have also been observed in the proliferation of ECs, although these are not well defined [158, 187, 188]. Endothelial progenitor cell (EPC) proliferation contributes to re-endothelialization, which impairs neointima formation after vascular injury [189], and preserves the integrity of the endothelium [190]. Genomic studies have shown that the expression of cell cycle-promoting proteins (e.g., cyclins A, D, F) increases at day 4 of atorvastatin treatment in EPCs, supporting the notion of increased proliferation capacity. Additionally, a cell cycle inhibitor, p27Kip1, is downregulated, possibly facilitating cell cycle progression and hence preventing replicative senescence [187]. Many genes associated with cell cycle and/or growth were regulated by atorvastatin or pitavastatin treatment (8 and 24 h after the start of treatment) of cultured HUVECs. Cyclin B was downregulated at 8 h of atorvastatin or pitavastatin treatment, whereas it was upregulated at 24 h. Meanwhile, cyclin H and CDK4, two cell cycle-promoting genes, were downregulated at 8 and 24 h of atorvastatin or pitavastatin treatment [158]. Additionally, simvastatin treatment (24–48 h after the start of treatment) of human coronary artery ECs (HCAECs) reduced the expression of 13 out 18 (>70 %) cell cycle/proliferation genes (e.g., CDC25B and ITGB4). The overall inhibitory effect of simvastatin on HCAEC growth possibly reflects its beneficial inhibition of neointima formation in the atherosclerotic artery stenosis [188]. These seemingly contradictory findings highlight the importance of methodically investigating different EC types, different drugs of the same family and different treatment time courses.

Overall, statins appear to affect EC proliferation; however, the fine details remain to be defined before they can be exploited in new drug design.

Fibrates

The Lipid-Lowering Effect of Fibrates

Several pharmacogenomic studies, mainly in animal models, have characterized the molecular mechanisms contributing to the lipid-lowering effect of fibrates. Hepatic transcription profiling of clofibrate or gemfibrozil-treated WT rats demonstrated increased expression of many genes involved in beta-oxidation, as well as FFA and cholesterol synthesis, including fatty acyl-Coenzyme A oxidase [45, 191, 192], acetyl-Coenzyme A acetyltransferase

[45, 192], carnitine palmitoyl transferase I, II, and fatty acid desaturase I [192]. Fatty acid transport (CD36) [191, 192] and hydroxylation (CYP4A14) genes were also overexpressed [192]. Importantly, these molecular changes have been reproduced at the in vitro level, in clofibrate- or gemfibrozil-treated primary cultured hepatocytes [192].

Fenofibrate treatment of WT mice (C57BL/6N and CD-1) induced the hepatic expression of genes involved in fatty acid beta-oxidation and lipid metabolism, such as enoyl-CoA hydratase/hydroxyacyl-CoA dehydrogenase and thiolase, in a PPARα-dependent manner [34]. Global hepatic transcriptional profiling in a fenofibrate-treated human ApoA-I transgenic mouse model demonstrated upregulation of genes involved in phospholipid biosynthesis (e.g., Mogat1, Dgkh, Pitpnm1, Chpt1, and Chka), lipid hydrolysis, HDL biogenesis and maturation (e.g., Abca1, Lcat, Abcg1, Pltp), lipolysis (e.g., Lpl, Pnliprp1 and Mgll), fatty acid transport, beta-oxidation (e.g., Ehhadh), and synthesis of unsaturated and long-chain fatty (e.g., Scd-1, Elovl3). Importantly, in silico analysis of the upregulated Esrgg (estrogen receptor-related gamma) gene in this study indicated that it could mediate the activation of a specific subset of fenofibrate's target-genes that control lipid and lipoprotein metabolism [193]. Similar results were obtained for mouse (WT, male C57/BL6) primary hepatocytes treated with fenofibrate or bezafibrate.

The gene expression profiles showed increased expression of genes involved in fatty acid beta-oxidation, such as the acyl-coA synthetase, which ligates CoA to a free fatty acid and thus catalyzes the precursor step to beta-oxidation, and three members of the long chain acyl CoA synthetase family (Acsl1, Acsl4, and Acsl5) [35].

In conclusion, fibrates exert their lipid-lowering effects through activation of a broad range of lipid metabolism pathways. Some of these could be selectively targeted by future drugs.

Lipid-Lowering-Independent Effects of Fibrates

Several lines of evidence indicate that beyond their lipid-lowering effect, fibrates also have an anti-inflammatory effect, mediated in part by PPARalpha [42]. The anti-inflammatory effect of fenofibrate has been shown through several in vitro studies. Fenofibrate treatment of HUVECs induced the expression of GDF15 (growth differentiation factor 15) in a PPARa-independent manner [194]. This gene encodes the macrophage inhibitory cytokine 1 (MIC-1), which is a divergent member of the TGF-beta superfamily, and has numerous effects, including the regulation of inflammatory pathways. The expression of GDF15 is induced rapidly by IL-1, TNF-a, and TGFb in macrophages, thereby limiting macrophage activation at late phase and inflammation [195]. Numerous transcriptome profiling studies, in animal models, have also been performed. Fenofibrate treatment of apoE3Leiden mice showed a suppressive effect on hepatic inflammatory pathways including IL-1 signaling

and MIF signaling, via key regulators, such as IFN-γ, TGFb, IL-1, TNF-a, MIF, and IL-6 [160]. Clofibrate or gemfibrozil treatment of WT male Sprague–Dawley rats decreased the hepatic mRNA levels of genes associated with the immune response (e.g., interleukin 6 signal transducer, cathepsin S, and cathepsin C) [192]. In the same rat model, significant downregulation was observed in blood coagulation genes, including coagulation factor 5 and fibrinogen alpha polypeptide [192]. The coagulation factor 5 gene encodes an essential cofactor of the blood coagulation cascade, while the fibrinogen alpha polypeptide is a component of fibrinogen, a glycoprotein that regulates blood clot formation and platelet aggregation. Interestingly, the aforementioned expression changes were not observed in primary rat hepatocytes treated with clofibrate or gemfibrozil [192].

Overall, fibrates have anti-inflammatory properties mediated through the downregulation of by suppressing the expression of inflammatory cytokines and thus the inhibition of monocyte recruitment and macrophage activation. These anti-inflammatory actions are largely, but not exclusively, PPARalpha-mediated. Additionally, molecular evidence for the fibrates' antithrombotic effect is emerging.

Nicotinic Acid

Nicotinic acid has been used as a hypolipidemic drug for more than five decades. However, the mechanisms underlying its lipid-lowering effect remained poorly understood for a long time. The study by Choi et al. [57] was one of the several, shedding light on these molecular mechanisms, by analyzing the effect of NA infusion on the gene expression profile of tissues of WT male Wistar rats, such as skeletal and cardiac muscles, liver, and adipose tissue. NA induced Akt- or FOXO-1-dependent expression changes in all the tissues studied. Of interest was a group of genes involved in energy metabolism whose expression was altered exclusively in adipose tissue, presumably, because of stimulation of the NA receptor in this tissue. Among those changes was the downregulation of PEPCK1 (Phosphoenolpyruvate carboxykinase 1), whose expression in adipocytes may be important for FFA release from these cells. These results suggested the existence of NA-activated pathways by which NA may alter gene expression exclusively in adipocytes, in vivo [57, 196]. LPL, a major regulator of circulating lipids, was significantly upregulated exclusively in skeletal muscle treated with NA. Increased LPL expression in this tissue would be anticipated to lower plasma lipid levels by increasing the rate of VLDL removal. NA also induced the hepatic expression of the Abca1 [57], which is a major player in the biogenesis of HDL [197]. The upregulation of Abca1 plays an important role in the NA-driven increase in plasma HDL levels [57]. These findings indicated that NA exerts its lipid-lowering effect not only by suppressing lipolysis

in adipose tissue, and thus decreasing plasma FFA levels, but also by upregulating gene expression in a number of the other tissues, including the liver and skeletal muscles.

Although NA initially reduces plasma FFA levels after chronic administration this effect is diminished and FFA plasma levels return to the initial levels [55, 56]. This represents a significant limitation of NA. Oh et al. [198] used whole genome analysis of adipose tissue of WT male Wistar rats following 24 h continuous NA infusion to reveal downregulation of PLIN1 (lipid droplet-associated protein perilipin), AdPLA (adipose-specific phospholipase A2), NA receptor, and PDE3B (phosphodiesterase-3B), all of which suppress lipolysis in adipocytes. The significant downregulation of adipocyte lipolysis was proposed to increase plasma FFA levels. Additionally, several key TG synthesis enzymes were downregulated, including 1-acylglycerol-3-phosphate *O*-acyltransferase (AGPAT), diacylglycerol *O*-acyltransferase-1 and -2 (DGAT1, DGAT2), glycerol-3-phosphate dehydrogenase (GPD1), and phosphoenolpyruvate carboxykinase-1 (PCK1) [198]. These results suggest that decreased TG synthesis or FFA reesterification in adipocytes could contribute, at least in part, to the observed rebound of plasma FFA levels [198].

In summary, chronic NA treatment could increase lipolysis and decrease TG synthesis in adipocytes leading to the subsequent increase in plasma FFA levels. Future studies are warranted to determine whether similar changes occur in humans to account for FFA rebound during chronic NA treatment.

Ezetimibe

Ezetimibe inhibits intestinal cholesterol absorption and thus reduces serum LDL cholesterol [199]. Additionally, it affects hepatic lipid metabolism [14]. At the pharmacogenomic level, it has been shown to increase the expression of both SREBP-2 (sterol regulatory element binding transcription factor 2) and SHP in the liver of C57BL/6 mice fed a high-fat diet. SREBP-2 controls cholesterol homeostasis, while SHP is involved in hepatic cholesterol metabolism. On the contrary, SREBP-1c, which regulates genes involved in sterol synthesis, was downregulated. Consistently with these alterations, genes involved in fatty acid and TG biosynthesis, such as acetyl-CoA carboxylase (ACC), an enzyme that catalyzes the rate-limiting step in the synthesis of fatty acid, and stearoyl-CoA desaturase (SCD-1), were downregulated. Apart from CYP7A1, all of the cholesterol homeostasis and bile acid biosynthesis related genes, including HMG-CoA synthase, HMG-CoA reductase, and LDLR, were upregulated. On the contrary, ezetimibe decreased the expression of carnitine palmitoyltransferase 1A (CPT-1), a key enzyme involved in fatty acid beta-oxidation [14].

In brief, ezetimibe suppresses fatty acid and TG synthesis, and may thus reverse the dyslipidemia in high-fat-diet-induced obese mice via a pathway involving SHP and SREBP-1c.

3.1.2 Pharmacogenomics of ACE-Inhibitors

Captopril, an ACE inhibitor, has atheroprotective actions in vivo, as proven in both animal models [200, 201] and patients [202]. Pharmacogenomics has only been applied to a limited extent in the evaluation of ACE inhibitors. A whole-genome expression profiling study of aortic tissue from captopril-treated ApoE−/− mice (C57BL/6J) pointed to molecular mechanisms contributing to the inhibitory effect of captopril on EC dysfunction. Specifically, captopril prevented the atherosclerosis-induced downregulation of aortic intima genes, such as the small proline-rich protein 3 (Sprr3) [200], which is considered to strengthen the aortic intima against biomechanical stress [203]. The captopril-induced upregulation of Sppr3 is consistent with the notion of captopril treatment protecting the aortic intima against reactive oxygen species (ROS)-mediated damage [200].

In the same mouse model significant underexpression was observed in genes regulating the recruitment of pro-inflammatory cells to the aortic tissue, such as Cd8a, Cd8b, Cd4, Cd28, and Ccr9. The specific ligand of Ccr9, Ccl25, was also downregulated [200, 201]. The Ccr9 receptor is expressed in various immune cells, while the Ccl25 ligand is expressed by plaque-resident cells. Consequently, the Ccl25-Ccr9 inflammatory pathway is involved in the formation of atherosclerosis plaque [204], and targeting it may prevent atherosclerotic plaque formation [201].

In brief, these findings shed light on the atheroprotective effects of captopril, which remained poorly understood over a long period of time. Specifically, captopril appears to limit atherosclerosis development by preventing EC dysfunction and inhibiting pro-inflammatory cell recruitment into the intima.

These examples as a whole, demonstrate the value of genomic studies in pinpointing the molecular mechanism implicated in the therapeutic effects of widely used atheroprotective treatments (Fig. 2).

3.2 Application of Pharmacogenomics in Characterizing the Mechanisms of Adverse Effects of Established Drugs

Pharmacogenomic investigations, through the global view of the transcriptomic changes can further provide a valuable insight into the molecular mechanisms responsible for the possible adverse effects of approved atheroprotective drugs (Fig. 2), and thus facilitates their improvement [167, 169]. Representative examples of such studies are presented in the following section, with an emphasis on lipid-lowering drugs.

3.2.1 Statins

Among the adverse effects of statins, rhabdomyolysis is considered to be a rare but significant one [30]. Global gene expression analysis of two skeletal muscle cell lines (differentiated rat L6 myotubes and a human skeletal muscle cell line) treated with statins

(atorvastatin, cerivastatin, and pitavastatin) revealed a significant upregulation in four cholesterol biosynthesis related genes, namely, HMG-CoA synthase 1, HMG-CoA reductase, farnesyl diphosphate synthase, and isopentenyl-diphosphate delta isomerase, in both cell lines. This profound upregulation was not observed in pitavastatin-treated coronary artery SMCs in same study. The upregulation of SREBP-2 and LDLR in statin-treated human skeletal muscle cells supports the notion that statins inhibit the cholesterol biosynthesis pathway [146]. However, it appears that the effects of statins on skeletal muscle are not mediated only through the SREBP pathway, since numerous other human skeletal muscle genes present with altered expression, such as the Kruppel-like zing finger transcriptional factor (KLF2) [146].

In brief, statins effectively inhibit cholesterol biosynthesis pathway in skeletal muscle by inducing the expression of enzymes associated with cholesterol production, which may be related to the pathogenesis of muscle damage in statin therapy. This effect appears to be largely mediated by the SREBP pathway. Further study of the mechanism of rhabdomyolysis using gene expression analysis is necessary for the safe use of statins.

3.2.2 Fibrates

Fibrates can lead to a number of adverse effects, including the rare but severe hepatic carcinogenesis [45]. Numerous pharmacogenomic studies have analyzed the expression changes induced by fibrates at in vitro and animal model levels. Specifically, bezafibrate or fenofibrate treatment in mouse (WT, male C57/BL6) primary hepatocytes revealed expression changes in genes associated with production of ROS (e.g., Aldh3a2, Apoc2, Cd36, and Slc25a10) and hepatic disorders [35]. At the animal model level, fenofibrate-treated rats (WT, male F344/N slc) presented with upregulation of cell cycle-related genes (e.g., Chek1, Cdc25a, and Ccdn1), in parallel to apoptosis- (e.g., Casp11 and Trp53inp1) and metabolism-related genes. Additionally, significant upregulation was observed in DNA repair-related genes (e.g., Aco, Cyp4a1, Cat, Yc2, Gpx2, Apex1, Xrcc5, Mgmt, Mlh1, Gadd45a, and Nbn), along with hepatic ROS production in fenofibrate-treated rats. These results provide evidence of a direct or indirect relationship between oxidative stress and fenofibrate treatment [205]. Similar results were obtained when treating WT, male Sprague–Dawley rats with clofibrate or gemfibrozil, suggesting an overall promotion of hepatocellular proliferation and attenuation of apoptosis [191, 192]. Additionally, significant alterations were observed in stress response genes (e.g., heat shock 27 kDa protein 1, hypoxia inducible gene 1, hypoxia up-regulated 1) suggesting an oxidative stress-dependent mechanism of hepatic injury, which could mediate clofibrate- or gemfibrozil-induced carcinogenesis [191, 192].

Such studies led to the conclusion that fibrates through regulation of ROS production-related genes can increase hepatic ROS production and thus cause oxidative damage, which could mediate carcinogenesis.

4 Evaluation of Novel Drugs by Pharmacogenomics

The area of HDL cholesterol metabolism and function holds considerable promise in atheroprotection. Novel HDL-directed therapeutic interventions mainly target LXR (liver X receptor), ABCA1, apoA-I, CEPT (cholesteryl ester transfer protein), and EL (endothelial lipase) [12, 16, 70]. The contribution of pharmacogenomics in the development of such drugs has not only been proven to be important (Fig. 2) but been required by the international drug regulatory bodies. Representative examples are presented below.

4.1 LXR Agonists and ApoA-I Mimetic Peptides

Promotion of the RCT, a process that mediates the cholesterol efflux from lipid-loaded macrophages, is a primary target of new HDL-based therapies. The HDL-target strategies and chemical compounds aiming to promote RCT, involve LXR agonists and apoA-I mimetic peptides [16, 70].

4.1.1 LXR Agonists

Many studies have demonstrated that the synthetic LXR agonist administration can substantially stimulate RCT in vivo, inhibit the progression of atherosclerosis, and even promote atherosclerotic lesion regression in murine models without actually raising plasma levels of HDL-C [206–209]. Specifically, the LXR agonist GW3965 has been shown to enhance cholesterol efflux from macrophage and promote RCT in three different mouse models, WT C57BL/6 mice, LDLR/apobec-1 double knockout mice, and human apoB/CEPT double transgenic mice, despite having little effect on plasma HDL-C levels [206]. Additionally, both the LXR agonist LXR-623 and T0901317 have been shown to inhibit the development of atherosclerosis in LDLR or apoE deficient mice [207, 208], while T0901317 induced regression of atherosclerosis and stabilization of established atherosclerotic lesions in macrophage-selective LXR-deficient mice, without significantly affecting plasma HDL-C levels [209, 210].

Synthetic LXR agonists including LXRα/β agonists (namely, LXR-623, T0901317, GW3965, GW6340, AZ876, and ATI-111) act by inducing the LXR-mediated transcription of ABCA1 and ABCG1 via high-affinity interactions with LXRα/β receptors [211]. These two ATP-binding cassette transporters promote cholesterol efflux from and macrophages to lipid-poor apoA-I [212] and mature HDL [213], respectively.

Even though LXR agonists have been viewed as an attractive novel therapeutic approach for atherosclerosis, some non-selective

LXR agonists have been shown to induce lipogenesis and hypertriglyceridemia in mice through the induction of hepatic lipogenic gene expression [211, 214]. Although the LXR-623 agonist which reached phase I trials was discontinued due to adverse nervous system-related effects [215], others LXR agonists (T091317, GW3965, AZ876, ATI-111, GW6340) are currently under pre-clinical evaluation in animal models [16].

The Lipid Metabolism-Related Effect of LXR Agonists

Several pharmacogenomic studies have demonstrated the lipid metabolism-related effects of the LXR agonists, T0901317 and GW3965. For example, treatment of HUVECs with T0901317 led to upregulation of lipid metabolism-associated genes including ABCA-1, CETP, SR-B1, EL, LPL, and LDLR [216], while the same agonist in pooled human umbilical and artery ECs not only increased the expression of ABCA-1 but upregulated additional LXR target-genes, such as ABCG1 and SREBP1c [217]. Animal studies such as on the high-cholesterol diet ApoE−/− mice, reproduced these findings, revealing T0901317-induced hepatic overexpression of lipid metabolism-associated genes including the LXR target-genes (e.g., LXRa and ABCA1) [218]. Similarly, the LXR agonist GW3965 induced the expression of LXR targets genes (e.g., Abca1) in cultured LPS-induced murine peritoneal macrophages [218], and increased basal lipolysis in vitro in human and murine adipocytes by downregulating the expression of lipolysis-regulating proteins, such as the lipid droplet-associated proteins (PLIN1, CGI-58, CIDEC) and the hormone-sensitive lipase (HSL) [219].

Such studies led to the conclusion that the LXR agonists, T0901317 and GW3965, regulate cholesterol efflux pathway via the upregulation of RCT-related genes. Additionally, these agonists have a profound effect on lipolytic signaling pathways in adipocytes through the downregulation of several lipolytic genes.

LXR Agonists and Inflammation

Several transcription profiling studies performed in vitro or in animal models, have indicated an anti-inflammatory effect of T0901317 and GW3965. For example, the effect of T0901317 or GW3965 on the inflammatory gene expression in HUVECs and human umbilical artery ECs (HUAECs) treated with LPS, an inflammatory stimuli that triggers endothelial activation, after pre-incubation with T0901317 or GW3965, was determined. Both LXR agonists reduced the LPS-dependent upregulation of inflammatory markers and mediators in a LXR-dependent manner, including adhesion molecules (e.g., ICAM-1, VCAM-1, SELE) and chemokines (IL-8, IL-1α, CCL2, CXCL1, CXCL2, CXCL11) [217]. Consistent findings were observed in the study of cultured LPS-induced murine peritoneal macrophages treated with GW3965, where the expression of inflammatory mediators such as IL-1β, IL-6, G-CSF (granulocyte colony-stimulating factor),

MCP-1, MCP-3, MIP-1 (macrophage inflammatory protein-1β), IP-10 (interferon-inducible protein-10), iNOS (inducible nitric oxide synthase), and COX2 was suppressed [218].

In accordance with the aforementioned in vitro findings, the anti-inflammatory effect of T0901317 was further demonstrated in ApoE−/− mice fed a high-cholesterol diet by inhibiting the hepatic expression of several pro-inflammatory cytokines, such IL-1α, IL-6, and IL-7, with the exception of TNF which was upregulated [220]. Similar results were obtained for apoE3Leiden mice treated with T0901317, where inflammatory pathways, such as the IL-1, MIF, and IL-6, were suppressed [160].

Overall, the LXR agonists, T0901317 and GW3965, are negative regulators of inflammation, by suppressing genes involved in T lymphocyte and monocyte recruitment.

LXR Agonists and Plaque Instability

In vitro and animal model studies have demonstrated the inhibitory effect of GW3965 on the expression of MMP-9 [218], a macrophage-derived contributor to plaque rapture [221]. Specifically, gene expression profiling of activated macrophages treated with GW3965 revealed decreased expression of MMP-9 [218]. The same study also demonstrated a downregulation of MMP-9 expression in the aortas of GW3965-treated atherosclerotic mice [218]. Such findings led to the conclusion that the reduced atherosclerotic lesion formation in both Ldlr−/− and ApoE−/− mice treated with GW3965 [208] could be achieved, at least partially through the regulation of MMP-9 expression [218].

In brief, the local action of GW3965 on macrophages within the artery wall contributes to its plaque stabilizing properties, and thus to its beneficial effect of on plaque development, through regulation of MMP-9 expression.

4.1.2 ApoA-I Mimetic Peptides

In parallel to LXR agonists, apoA-I mimetic peptides also promote RCT. Specifically, they have been reported to facilitate cholesterol efflux from macrophages and decrease atherosclerosis in apoE null mice fed a Western diet [13, 16, 222–224]. ApoA-I mimetics are small synthetic peptides that mimic the amphipathic α-helix of apoA-I and thus its functionality [225]. Several apoA-I mimetic peptides are currently under preclinical trials and clinical development (namely, D-4F, L-4F, 5F, 6F, 7F, 5A, ATI-5261, and ETC642). The apoA-I mimetic peptides, D-4F and L-4F, showed great promise in early human trials [226] leading to a phase I/II study in humans with high-risk CVD [227].

Several lines of evidence have indicated that some of the apoA-I mimetic peptides have anti-inflammatory properties, including 5F, 5A, 6F, and 4F [228–231]. However, phamacogenomics have only been applied to a limited extent in the

evaluation of these mimetics. For example, microarray analysis of monocyte-derived macrophages pretreated with 4F (7 days) led to a better understanding of its global anti-inflammatory action, at the molecular level. Pretreatment with 4F, followed by the addition of LPS (18 h), attenuated the LPS-induced upregulation of genes encoding Toll-like receptor (TLR) family members (TLR1, 2, and 6) and adaptor proteins in the MyD88-dependent (CD14, MyD88, TRAF6, IRAKA4, IKBKB) and MyD88-independent (IRF3, TBK1, TICAM1) pathways and downstream signaling intermediates [232]. TLRs initiate signals that activate innate immune responses. Increased activation of TLR-induced responses may lead to atherosclerosis [233]. In summary, the ability of 4F to downregulate pro-inflammatory genes of the TLR pathway in macrophages may be serving as the basis for its atheroprotective effect.

4.2 CEPT Inhibitors

CEPT inhibitors (torcetrapib, dalcetrapib, anacetrapib and evacetrapib) indirectly increase HDL-C levels through modulation of HDL metabolism [18, 234–236]. CEPT inhibitors act by reducing the activity of CEPT, an enzyme that mediates the exchange of cholesteryl esters from mature HDL into apoB-containing lipoproteins, such as chylomicrons, VLDL, and LDL [237]. Two CEPT inhibitors, anacetrapib and evacetrapib, are in phase III clinical trials [235, 236], while dalcetrapib and torcetrapib have previously been tested and rejected. Specifically, the dal-OUTCOMES phase trial III of dalcetrapib in patients with a recent coronary syndrome showed no benefit on cardiovascular outcome and was terminated [234]. Torcetrapib was the first CEPT inhibitor to be used in a large-scale clinical trial (ILLUMINATE), involving patients at high risk for coronary events, which was discontinued however, due to increased mortality and adverse effects [18].

Pharmacogenomics has only been applied to a limited extent in the evaluation of CEPT inhibitors. However, these studies proved to be valuable in determining the molecular basis of the undesirable effects of torcetrapib, ultimately leading to its discontinuation. Specifically, the evaluation of torcetrapib by microarrays demonstrated alterations in the IL-2 Receptor Beta chain, the PDGFR beta (Platelet-Derived Growth Factor Receptor Beta), the HGFR (Hepatocyte Growth Factor Receptor), and the ErbB1 signaling pathways. Torcetrapib mainly influenced these pathways via upregulation of CBL, SOCS1, JAK1, JUN, TGFR2, and EXOSC6 [238]. T cell activation mediated by IL-2 in the arterial vessel can induce apoptosis of vascular SMCs and facilitate the plaque formation [239]. Similarly, PDGFR beta signaling pathway could favor plaque formation by promoting the migration and proliferation of cultured human aortic SMCs [238]. HGF contributed to the pathogenesis of atherosclerosis by regulating the proliferation and migration of vascular ECs and SMCs [240]. The activation of ErbB1 receptor mediated by heparin binding

epidermal growth factor (HB-EGF) regulates vasoconstriction, which facilitated the formation of atherosclerotic plaque [241]. These results provide unique insights into the adverse effects of torcetrapib.

4.3 Endothelial Lipase Inhibitors

EL inhibition by boronic acid inhibitors and selective sulfonylfuran urea, may represent another promising strategy for increasing plasma HDL-C and apoA-I levels as well as reducing apoA-I catabolism [12, 13, 16, 70]. Endothelial lipase inhibitors act by reducing the activity of EL, an enzyme that preferentially hydrolyzes phospholipids within HDL [242]. Although EL inactivation was expected to be atheroprotective by raising HDL-C, conflicting findings exist. Targeted inactivation of EL increased plasma HDL-C levels and inhibited atherosclerosis in ApoE−/− mice [243]. On the other hand, it has been demonstrated that targeted inactivation of EL increased plasma HDL-C level, but resulted in an unexpected substantial increase of small dense LDL, a potentially atherogenic mechanism [244]. These findings initiated the synthesis of selective sulfonylurea urea inhibitors of EL. Recently, many of the new EL inhibitors synthesized from boronic acid have been evaluated for potency against EL. The EL inhibitors are currently under preclinical evaluation in animal models [16].

Pharmacogenomics has only been applied to a limited extent in the evaluation of EL inhibition. Specifically, microarray analysis of human THP-1 macrophages subjected to lentivirus-mediated RNA interference to suppress EL expression, revealed significant downregulated pro-inflammatory genes including IL-1β, IL-6, IL-8, and TNF-a, whereas the anti-inflammatory gene, TGFb1, was upregulated [242]. These results suggest that EL suppression can reduce pro-inflammatory cytokine secretion from macrophages.

Overall, pharmacogenomic studies play an increasing important role in the molecular characterization of the therapeutic potential of novel chemical compounds, as well as the determination of undesirable/possibly detrimental effects (Fig. 2).

5 Conclusions

The discovery of novel therapeutic approaches against atherosclerosis, the primary cause of death worldwide, represents an area of intensive research. Despite the benefits of clinically available therapies, the presence of residual cardiovascular risk in patients treated with lipid-lowering drugs is a critical problem that needs to be overcome. Pharmacogenomics is greatly contributing to the identification of novel therapeutic targets. Specific networks, pathways or even specific genes implicated in the different stages of atherogenesis as well as disease progression are uncovered and

proposed for targeting. In parallel, through the molecular dissection of current atheroprotective drug action and adverse effects, a valuable insight is acquired on desirable and undesirable, respectively, pathways of pharmacological intervention. Novel atheroprotective compounds can be pharmacogenomically assessed during early stage of drug development to ensure more effective and safer action at the molecular level. The increasing use of pharmacogenomics in basic research, clinical research, and ultimately clinical applications is promising to increase therapeutic options and quality of life, while reducing mortality and healthcare costs. The results obtained to date suggest that this is starting to materialize.

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Chapter 12

Management of Side Effects in the Personalized Medicine Era: Chemotherapy-Induced Peripheral Neuropathy

Paola Alberti and G. Cavaletti

Abstract

Pharmacogenomics has been establishing itself as a powerful tool to predict individual response to treatment, in order to personalize therapy management; this field has been explored in particular in Oncology. Not only efficacy on the malignant disease has been investigated, but also the possibility to predict adverse effects due to drug administration. Chemotherapy-Induced Neurotoxicity (CIPN) is one of those. This potentially severe and long-lasting/permanent side effect of commonly administered anticancer drugs can severely impair Quality of Life (QoL) in a large cohort of long survival patients. So far, a pharmacogenomics-based approach in CIPN regard has been quite delusive, making a methodological improvement warranted in this field of interest: even the most refined genetic analysis cannot be effective if not applied correctly. Here, we try to devise why it is so, suggesting how THE "bench-side" (Pharmacogenomics) might benefit from and should cooperate with THE "bed-side" (Clinimetrics), in order to make genetic profiling effective if applied to CIPN.

Key words Pharmacogenomics, Chemotherapy-induced peripheral neuropathy, Personalized medicine

1 Introduction: Pharmacogenomics and CIPN—What and Why?

1.1 The Identikit

Pharmacogenomics could be a powerful tool to predict individual response to therapy on the basis of interindividual genetics differences. It could be employed to define a genetic signature that can predict either efficacy either adverse effect of a given treatment. In respect to genetic variability, Single Nucleotide Polymorphisms (SNPs) account for more than 90 % of genetic variations in the human genome; remaining alterations are due to insertions and deletions, tandem repeats, and microsatellites [1].

A genetic profile on the basis of SNPs would be more than useful for Chemotherapy-Induced Peripheral Neurotoxicity (CIPN) risk stratification, prior to start treatment. CIPN is a potentially severe and long-lasting side effect of commonly employed anticancer drugs: platinum compounds, taxanes, proteasome inhibitors, vinca alkaloids, and epothilones; they are used every day to treat

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the "big killers": breast, colorectal, and lung cancer, and multiple myeloma [2]. CIPN influences chemotherapy administration: it can lead to dose reduction or even discontinuation for higher grade of neurological toxicity. Quality of Life (QoL) can be even severely impaired in a large population of cancer patients due to CIPN symptoms/signs. Affected subjects experience mainly sensory alterations at limb extremities (hypoesthesia, paraesthesia, neuropathic pain) and, less frequently, mild distal limb weakness [2].

1.2 Issues in CIPN

One of the main still unmet clinical and scientific needs in this field is the lack of a gold standard for CIPN diagnosis and graduation [3]. As a consequence, in clinical trials designed so far, there was a difficulty in appropriate endpoint(s) selection; moreover, the absence of precise data on incidence and prevalence of CIPN, due to this lack, made study design less solid than would be required. That could partially explain why no preventive or curative strategy for CIPN has been found as efficacious.

Thus, identifying patient in high/low risk to develop neuropathy, thanks to pharmacogenomics, is an even more urgent matter. But these limitations also reflect themselves on pharmacogenomics, when applied to CIPN: no clear gold standard to define CIPN presence and severity means a potentially poor population stratification before to proceed to pharmacogenomics analysis. Even more importantly, identification of target gene(s) is a crucial issue in any toxicogenomic analysis. For what regards CIPN most studies selected gene targets on the basis of mechanistic hypothesis mainly relevant to cancer cells, instead of genes involved in neurons and glial cells.

2 CIPN and Pharmacogenomics So Far

Principal Pharmacogenomics findings are reported in Table 1.

2.1 The "Old Fashioned" Approach

Gene target selection in the vast majority of studies reported in literature was performed mainly looking for pathways related to cancer cells. Therefore, genes that have a role in drug disposition, metabolism, and detoxification, DNA repair, and cancer-cell resistance have been studied much more extensively than ones directly related to the peripheral nervous system; to give a general idea, principal genes analysed were (*see* Table 1 for details):

- *GSTP1 gene:* it is part of the Glutathione S-transferases, a family of enzymes that catalyse conjugation of many hydrophobic and electrophilic compounds with reduced glutathione, covering an important role in detoxification [4–6]. See further, for more details.
- *GSTM1 and GSTM3 genes*: they are part of the μ class of glutathione S-transferases crucial for detoxification through

Table 1
An overview of pharmacogenomics findings in chemotherapy-induced neurotoxicity (CIPN)

Author (year) (reference)	Neoplasm (n)	CT regimen	Previous neurotoxic CT	Ethnic origin	Target studied for toxic effect	Neurological assessment	Association with CIPN	Number of patients with CIPN used for comparison
Antonacopoulou (2010) [55]	Colorectal (55)	FOLFOX-4	CT naive	Not stated	ITGB3 Leu59Pro	TNSc	Association with CIPN severity for T/T genotype ($p = 0.044$ vs. C/T and C/C)	C/C = 3; T/T = 18; C/T = 13 (any grade)
Argyriou (2013) [54]	Colorectal (200)	FOLFOX-4, FOLFOX-6, XELOX	CT naïve	Caucasian	SCN10Ars12632942, SCN10A-rs6800541, SCN9A-rs6746030, and SCN4A-rs2302237	TNSc, NCI-CTC v 3.0, 11-items specific Oxaliplatin Acute Toxicity Questionnaire	SCN4A-rs2302237 predictive of acute and chronic CIPN	169 (acute CIPN), 145 (chronic CIPN)
Baldwin (2012) [44]	Breast (855)	Paclitatexl 175 mg/m^2 every 2 weeks for 4 or 6 cycles	CT naïve	Caucasian, African American, Asian	*GWAS***	NCI-CTC v 2.0	EPHA5 rs7349683 C>T and FGD4 rs10771973 G>A are associated with an increased probability of developing grade 2 or greater sensory peripheral neuropathy	See reference for details
Basso (2010) [56]	Colorectal (40)	FOLFOX-4, FOLFOX-6	CT naive and not CT naïve (but neurotoxicity naïve)	Caucasian	CAG motif of SK3	Neurophysiological examination, acute toxicity symptoms scale	A short CAG repeats allele (13–15 repeats) associated to higher incidence of severe acute OXA-induced neurotoxicity	28 (11, severe)

(continued)

Table 1 (continued)

Author (year) (reference)	Neoplasm (n)	CT regimen	Previous neurotoxic CT	Ethnic origin	Target studied for toxic effect	Neurological assessment	Association with CIPN	Number of patients with CIPN used for comparison
Bergmann (2011) [57]	Ovarian (119)	Paclitaxel (175 mg/m^2); carboplatin AUC 5 or 6	Not stated	Not stated	ABCB1 Ser893Ala/Thr CYP2C8*3 (exon 3) CYP3A5*3	NCI-CTC (version not specified)	None	Sensory neuropathy: grade 1 = 58, grade 2 = 20, grade 3 = 3; neuropathy (ataxia): grade 1 = 25, grade 2 = 9, grade 3 = 1
Booton (2006) [40]	NSCLC (118)	Docetaxel (75 mg/m^2) with or without cisplatin (50 mg/m^2) or carboplatin AUC 6, every 3 weeks, to a maximum of four cycles	CT naive	Not stated	GSTP1 Ile105Val	NCI-CTC v2	None	Not reported
Boige (2010) [35]	Colorectal (346)	FOLFOX-6	CT naive and previous CT patients (no previous neurotoxic CT)	Not stated	GSTP1 Ile105Val GSTM1 deletion ERCC1 Asn118Asn	NCI-CTC v 2	None	Grades 2/3/4 = 111 (first-line treatment); grades 2/3/4 = 112 (secondline treatment); grades 2/3/4 = 31 (third-line treatment

Broyl (2010) [58]	Multiple myeloma (329 for gene expression, 369 for SNP analysis)	Bortezomib (1.3 mg/m^2) on days 1, 4, 8, 11; vincristine (0.4 mg) on days 1–4	CT naive	Not stated	See reference 16 for complete list of genes and SNPs	NCI-CTC v3	40 Genes differentially expressed in early vs. late onset CIPN in bortezomib-treated or vincristine-treated patients[a]; 59 SNPs associated with bortezomib or vincristine CIPN[a]	Bortezomib: CIPN after one cycle grade 2–4 = 20, CIPN after 2–3 cycles = 63; vincristine: CIPN after one cycle grade 2–4 = 11, CIPN after 2–3 cycles = 17
Caponigro (2009) [59]	Colorectal (12)	FOLFOX-4 plus bortezomib in escalating dose (1.0, 1.3, 1.6 mg/m^2)	CT naive and previous CT patients (no oxaliplatin based CT)	Not stated	Not stated	NCI-CTC v 3	None	Total = 12
Chang (2009) [60]	Breast (121)	Paclitaxel (175 mg/m^2) every 3 weeks	CT naive and previous CT patients (no previous neurotoxic CT)	Not stated	ABCB1 Ser893Ala/Thr	NCI-CTC v 3	None	Grade 3 = 12
Chen (2010) [61]	Colorectal (166)	FOLFOX-4	CT naive	Asian	GSTP1 Ile105Val ERCC1 Asn118Asn	NCI-CTC (version not specified)	Significant association with higher incidence of grade 3–4 CIPN for GSTP1 Ile105Val G/G + A/G vs. A/A ($p = 0.02$ after eight cycles and $p < 0.01$ after 12 cycles)	GSTP1 Ile105Val: A/A = 116, A/G and G/G = 33 (grade 0–2 after eight cycles); A/A = 9, A/G and G/G = 8 (grade 3–4 after eight cycles); A/A = 107, A/G and G/G = 26 (grade 0–2 after 12 cycles); A/A = 18, A/G and G/G = 15 (grade 3–4 after 12 cycles)

(continued)

Table 1 (continued)

Author (year) (reference)	Neoplasm (n)	CT regimen	Previous neurotoxic CT	Ethnic origin	Target studied for toxic effect	Neurological assessment	Association with CIPN	Number of patients with CIPN used for comparison
Cho (2010) [43]	B-cell lymphoma (94)	R-CHOP	CT naive	Asian	GSTP1 Ile105Val GSTM1 deletion	NCI-CTC v 3	None	Grade 3/4 = 2
Favis (2011) [62]	Multiple myeloma (351)	Bortezomib (1.3 mg/m^2 on days 1, 4, 8, 11)	CT naive	Not stated	See reference 21 for complete list of genes and SNPs	NCI-CTC (version not specified)	Five genes differentially expressed in patients with earlier onset of CIPN[b]	Total number with peripheral sensory neuropathy = 227
Gamelin (2007) [31]	Colorectal (135)	FOLFOX-4	CT naive or previous CT patients (no oxaliplatin-based CT)	White European	AGXT Pro11Leu AGXT Ile340Met GSTP1 Ile105Val	NCI-CTC v 1 and oxaliplatin specific scale	Significant association ($p < 0.001$) with CIPN severity for AGXT Pro11Leu C/T and T/T vs. C/C, and for AGXT Ile340Met A/G and G/G vs. A/A	AGXT Pro11Leu: C/C = 42, C/T and T/T = 12 (grade 1); C/C = 4, C/T and T/T = 14 (grade 2); C/C = 0, C/T and T/T = 6 (grade 3) AGXT Ile340Met: A/A = 41, A/G and G/G = 12 (grade 1); A/A = 4, A/G and G/G = 13 (grade 2); A/A = 0, A/G and G/G = 6 (grade 3)
Goekkurt (2009) [25]	Gastric (134)	FLO or FLP	Neurotoxic CT was an exclusion criterion	Not stated	ERCC1 Asn118Asn *GSTM1* deletion GSTP1 Ile105Val	Oxaliplatin specific scale	Significant association with higher incidence of grade 3–4 CIPN for GSTP1 Ile105Val A/A vs. A/G or G/G ($p = 0 \cdot 028$)	GSTP1 Ile105Val: A/A = 54, A/G = 46, G/G = 20 (grade 0–2); A/A = 10, A/G = 0, G/G = 2 (grade 3–4)

Green (2009) [63]	Ovarian (30), peritoneal (5), uterus (1), cervix (1), uncertain (1)	Paclitaxel (175 mg/m^2), carboplatin AUC 5 or 6	Not stated	White European	ABCB1 Ser893Ala/Thr CYP2C8*3 (exon 3)	NCI-CTC v 2, N score, and A self-created questionnaire	Patient heterozygous for CYP2C8*3 had higher risk of motor neuropathy ($p = 0.034$)	Not stated
Hertz (2012) [64]	Breast (109)	Paclitaxel Weekly (80–90 mg/m^2) or Q2 Weeks (175 mg/m^2) or Q3 Weeks (175 mg/m^2) or Q2.5 Weeks (175 mg/m^2)	CT naive	Caucasian African-American, other	CYP1B1*3 (rs1056836, 4326C[G), CYP2C8*3 (rs11572080, 416G[A and rs10509681, 1196A[G), CYP3A4*1B (rs2740574, -392A[G), CYP3A5*3C (rs776746, 6986A[G), and ABCB1*2 (rs1045642, 3435C[T, rs2032582, 2677G[T/A, and rs1128503, 1236C[T)	NCI-CTC v 4.0	CYP2C8*3 Variant revealed a trend toward increased neuropathy; no associations for other markers	12
Hong (2011) [26]	Colorectal (52)	Oxaliplatin 85 mg/m^2 every 2 weeks	CT naive and previous CT patients	Asian	ERCC1 Asn118Asn GSTP1 Ile105Val AGXT Ile340Met	NCI-CTC v 3	Significant association ($p = 0.03$) with higher incidence of grades 2–3 CIPN for GSTP1 Ile105Val A/G or G/G vs. A/A	GSTP1 Ile105Val: A/G and G/G = 5, A/A = 3 (grades 2–4)

(continued)

Table 1 (continued)

Author (year) (reference)	Neoplasm (n)	CT regimen	Previous neurotoxic CT	Ethnic origin	Target studied for toxic effect	Neurological assessment	Association with CIPN	Number of patients with CIPN used for comparison
Inada (2010) [28]	Colorectal (51)	FOLFOX-6	CT naive	Asian	ERCC1 Asn118Asn; GSTP1 Ile105Val	NCI-CTC v 3	Grade 1 CIPN developed earlier in patients with ERCC1 Asn118Asn C/T and T/T vs. C/C ($p = 0.016$) and in those with GSTP1 Ile105Val A/A than in those with A/G and G/G ($p = 0.032$), but no increased risk of grade 2 or higher CIPN was reported	ERCC1 Asn118Asn: C/C = 20, C/T and T/T = 16 (grade 1); C/C = 7, C/T and T/T = 8 (grade 2–3) GSTP1 Ile105Val: A/A = 27, A/G and G/G = 9 (grade 1); A/A = 11, A/G and G/G = 4 (grade 2–3)
Isla (2004) [65]	NSCLC (62)	75 mg/m^2 of both docetaxel and cisplatin every 3 weeks	Not stated	Not stated	ERCC1 Asn118Asn	WHO	None	36 Patients developed grade 2–4 neurological toxic effect
Johnson (2011) [66]	Multiple myeloma (1,495)	Vincristine (0.4 mg) on days 1–4; thalidomide (100, 200, or 400 mg)	Not stated	Not stated	See reference 28 for complete list of genes and SNPs	NCI-CTC v 2 (assessed only in induction phase)	Five SNPs were cross-validated in two different series of thalidomide-treated patients and nine SNPs were cross-validated in two different series vincristine-treated patients[a]	Significant association with grade ≥2 CIPN (number of patients not reported but overall patients with CIPN = 446)

Kanai (2010)[c] [36]	Colorectal (82)	Modified FOLFOX-6	CT naive and previous CT patients (no oxaliplatin-based CT)	Asian	GSTP1 Ile105Val AGXT Pro11Leu AGXT Ile340Met	Oxaliplatin specific scale	None	Grade 1 = 38, grade 2 = 43, grade 3 = 1
Keam (2008) [32]	Gastric (73)	Modified FOLFOX-6	CT naive and previous CT patients	Not stated	GSTP1 Ile105Val ERCC1 Asn118Asn	NCI-CTC v 3	None	Grade 1/2 = 12, grade 3/4 = 1
Kim (2009)[a] [41]	Ovarian (118)	Paclitaxel (175 mg/m^2), carboplatin AUC 5, docetaxel (75 mg/m^2), cisplatin (75 mg/m^2)	CT naive	Asian	GSTP1 Ile105Val *ERCC1* 8092C→A *GSTM1* deletion	NCI-CTC v 2	Higher rate of grade 3/4 sensory or motor CIPN for *ERCC1* 8092C→A C/C genotype vs. C/A or A/A ($p = 0.019$)	Grade 3/4 = 18
Leandro-Garcia (2013) [67]	Ovary, fallopian tube, peritoneum, lung, uterus, breast (144)	Paclitaxel 175 mg/m^2 + carboplatin 5–6 AUC, every 21 days	CT naïve and not CT naïve (6)	Caucasian	GWAS**	NCI-CTC v 2	EPHA5-rs7349683 and XKR4-rs4737264 detect risk of neuropathy	See reference for details
Lecomte (2006) [22]	Colorectal (59), pancreas (4), stomach (2)	FOLFOX-4, FOLFOX-6, FOLFOX-7, GEMOX, TOMOX	CT naive and pretreated patients (no previous neurotoxic CT)	White European (59), African (4), Asian (1)	GSTP1 Ile105Val; *GSTM1* deletion	Oxaliplatin specific scale	Significant association with CIPN grade 3 severity for GSTP1 Ile105Val A/A vs. A/G and G/G ($p = 0.02$)	GSTP1 Ile105Val: A/A = 13 (grade 3), A/G and G/G = 2 (grade 3)

(continued)

Table 1 (continued)

Author (year) (reference)	Neoplasm (n)	CT regimen	Previous neurotoxic CT	Ethnic origin	Target studied for toxic effect	Neurological assessment	Association with CIPN	Number of patients with CIPN used for comparison
Leskela (2011) [68]	Lung (39), breast (38), ovarian (24), uterus (6), head and neck (4), other (7)	Several paclitaxel schemes (80 or 90 mg/m^2 every week, 150 or 175 mg/m^2 every 21 days)	CT naive and previous CT patients	White European	CYP2C8*3 CYP2C8 haplotype C CYP3A5*3 ABCB1 Ser893Ala/Thr	NCI-CTC v 2	Significant association for increased risk of CIPN with polymorphisms CYP2C8*3 ($p = 0.049$) and reduced risk for CYP2C8 haplotype C ($p = 0.049$) and CYP3A5*3 ($p = 0.010$)	Sensory neuropathy: grade 1 = 17, grade 2 = 44, grade 3 = 14; motor neuropathy: grade 1 = 7, grade 2 = 5, grade 3 = 2
Li (2010) [27]	Gastric (85)	FOLFOX-4	CT naive	Not stated	GSTP1 Ile105Val	NCI-CTC v 2	More severe CIPN severity for GSTP1 Ile105Val A/A vs. A/G and G/G ($p = 0.005$)	Grade 1 = 29, grade 2 = 14, grade 3 = 12
Marsh (2007) [42]	Ovarian (914)	Carboplatin AUC 5, paclitaxel (175 mg/m^2), docetaxel (75 mg/m^2)	CT naive and previous CT patients	Not stated	CYP2C8*3 CYP3A5*5 ABCB1 Ser893Ala/Thr GSTP1 Ile105Val ERCC1 Asn118Asn	NCI-CTC v 2	None	Grade 0–1 = 710, grade 2–4 = 204
McLeod (2010) [29]	Colorectal (520)	FOLFOX-4, IROX	CT naive and previous CT patients	White European (450), black (36), Asian (9), Hispanic (16), other (9)	GSTP1 Ile105Val ABCB1 Ser893Ala/Thr ERCC1 Asn118Asn CYP3A5*3	NCI-CTC version 2 (adapted for paresthesias)	T/T genotype was more likely to discontinue FOLFOX treatment ($p = 0.01$); IROX (but not FOLFOX) patients with T/T genotype had more grade 3–4 CIPN ($p = 0.003$)	FOLFOX discontinuation rate 24 % vs. 10 %; 8/43 vs. 0/54 IROX patients (grade 3–4)

Mir (2009) [39]	Breast (16), non-small-cell cancer (14), prostate (16), other (12)	Breast (16), non-small-cell cancer (14), prostate (16), other (12)	Not stated	Not stated	GSTM1 deletion GSTP1 Ile105Val	NCI-CTC version 2	Association ($p = 0.03$ with univariate, $p = 0.01$ with multivariate analysis) with increased incidence of grade 2 or higher CIPN for GSTP1 Ile105Val A/A or A/G vs. G/G	GSTP1 Ile105Val: A/A = 17, A/G and G/G = 29 (grade 0–1), A/A = 8, A/G and G/G = 2 (grade 2 or higher)
Oldenburg (2007) [24]	Testicular (238)	Cisplatin combined with bleomycin, etoposide, or vinblastine (median cumulative dose 397 mg/m^2)	Different lines of treatment (up to four), neurotoxic effects undetermined	Not stated	GSTM1 deletion GSTP1 Ile105Val	SCIN	More severe long-term CIPN for GSTP1 Ile105Val A/A ($p = 0.012$) or A/G ($p = 0.003$) vs. G/G	GSTP1 Ile105Val: A/A = 23 moderate and 16 severe CIPN, A/G = 29 moderate and 20 severe CIPN, A/A = 10 moderate without severe CIPN
Oguri (2013) [37]	Colorectal (70)	FOLFOX-6	CT naïve and not CT naive (not stated)	Asian	ERCC1 C118T rs11615, GSTP1 Ile105Val rs1695, TAC1, FOXC1, ITGA1, ACYP2, DLEU7, BTG4, CAMK2N1, FARS2	NCI-CTC v 3.0	Association for severity for A/G and G/G genotypes of rs17140129 in FARS2, ERCC1 C118T rs11615 related to time of onset, rs10486003 C/C genotype related to shorter onset, NEGATIVE association for GSTP1 Ile105Val rs1695	48
Parè (2008) [33]	Colorectal (126)	FOLFOX-4	CT naive	Not stated	GSTP1 Ile105Val	Oxaliplatin specific scale	None	Grade 1 = 42, grade 2 = 66, grade 3 = 5

(continued)

Table 1 (continued)

Author (year) (reference)	Neoplasm (n)	CT regimen	Previous neurotoxic CT	Ethnic origin	Target studied for toxic effect	Neurological assessment	Association with CIPN	Number of patients with CIPN used for comparison
Rizzo (2010) [69]	Breast (95)	Paclitaxel (80 mg/m^2) weekly, docetaxel (75 or 100 mg/m^2) every 3 weeks	CT naive and previous CT patients	White European	ABCB1 Ser893Ala/Thr CYP2C8*3	NCI-CTC v 3	None	Grade 1 = 1, grade 2 = 6
Ruzzo (2007) [23]	Colorectal (166)	FOLFOX-4	CT naive and previous CT patients	Not stated	ERCC1 Asn118Asn GSTP1 Ile105Val *GSTM1* deletion	Oxaliplatin specific scale	Significant association with CIPN severity GSTP1 Ile105Val G/G > A/G > A/A ($p < 0.001$)	GSTP1 Ile105Val: A/A = 49, A/G = 27, G/G = 2 (grade 1–2); A/A = 4, A/G = 5, G/G = 8 (grade 3)
Seo (2009) [34]	Gastric (94)	Modified FOLFOX	CT naive and previous CT patients	Not stated	ERCC1 Asn118Asn GSTP1 Ile105Val *GSTM1* deletion	NCI-CTC (version not specified)	None	Grade 3/4 = 12
Sissung (2006) [70]	Advanced solid (26)	Paclitaxel	Not stated	Not stated	ABCB1 Ser893Ala/Thr	Not stated	None	22 Patients (not reported CIPN incidence, severity, and type)
Sissung (2008) [71]	Prostate (73)	Docetaxel (30 mg/m^2), thalidomide (200 mg daily)	Not stated	Not stated	ABCB1 Ser893Ala/Thr	Not stated	More rapid onset of CIPN only in patients co-treated with docetaxel and thalidomide ABCB1 Ser893Ala/Thr G/T and G/A and T/T vs. G/G ($p = 0.007$)	73 Patients (not reported CIPN incidence, severity, and type)

Stoehlmacher (2002)[a] [7]	Colorectal (107)	Oxaliplatin (130 mg/m^2) every 2 weeks	Previous CT patients (excluded neurotoxic CT)	White European (77), Hispanic (14), African (5), Asian (11)	GSTP1 Ile105Val GSTM1 deletion GSTT1 deletion	Not stated	None	10 Patients with grade 3–4 (not indicated lower grades)
Won (2011) [38]	Colorectal (247)	FOLFOX or XELOX	CT naïve and not CT naïve (FOLFOX-4)	Asian	*GWAS***	NCI-CTC v 3.0	Five SNPs rs10486003 for TAC1, rs2338 for FOXC1 and GMDS, rs830884 for ITGA1, PELO, rs843748 for ACYP2 and TSPYL6, and rs797519 for DLEU7) were significant in a multiple regression analysis ($p<0.05$). See reference for details	39 (41 %) and 85 (34 %) the discovery set and the validation set, respectively
Zarate (2010) [8]	Colorectal (60)	Oxaliplatin (85 mg/m^2) every 2 weeks	CT naive	Not stated	ERCC1 Asn118Asn GSTP1 Ile105Val GSTM1 deletion	NCI-CTC (version not specified)	None	Grade 1 = 25, grade 2 = 15, grade 3 = 1

AUC area under the curve, *CIPN* chemotherapy-induced peripheral neurotoxicity, *CT* chemotherapy, *GWAS*** Genome-Wide Association Study, *FLO* fluorouracil, leucovorin, oxaliplatin, *FLP* fuorouracil, leucovorin, cisplatin, *FOLFOX* leucovorin, fluorouracil, oxaliplatin, *GEMOX* gemcitabine, oxaliplatin, *IROX* irinotecan, oxaliplatin, *NCI-CTC* National Cancer Institute common toxicity criteria, *NSCLC* non-small-cell lung cancer, *R-CHOP* rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone, *SCIN* scale for chemotherapy-induced neurotoxicity, *SNPs* single nucleotide polymorphisms, *TNSc* total neuropathy score, clinical version, *XELOX* capecitabine, oxaliplatin, *TOMOX* raltitrexed, oxaliplatin

[a]Retrospective study

[b]None of the reported associations refers to other targets described in this table

[c]Leu11 allele was not found in this population of Japanese patients

glutathione conjugation of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins, and products of oxidative stress. Genetic variations can change an individual's susceptibility to carcinogens and toxins and affect toxic effects and effectiveness of specific drugs [4–8].

- *ERCC1 gene*: Excision repair cross-complementing group 1 (ERCC1) is part of the nucleotide excision-repair pathway and is required for repair of DNA lesions, such as those induced by ultraviolet light or formed by electrophilic compounds including cisplatin. Polymorphisms that alter expression of ERCC1 might have a role in carcinogenesis, and this gene has been investigated extensively for its role in cancer cell resistance to platinum drugs [9].
- *AGXT gene*: Alanine-glyoxylate aminotransferase (AGXT) prevents accumulation of glyoxylate in the cytosol by converting it into glycolate, which is subsequently metabolised into oxalate by lactate dehydrogenase [10]. Because of this role in oxalate metabolism, the AGTX Ile340Met polymorphism (rs4426527; NP_000021.1) has been investigated in two studies of patients with colorectal cancer treated with oxaliplatin (*see* Table 1).
- *ABCB1 gene*: they are part of the ATP-binding cassette proteins; they transport various molecules across extracellular and intracellular membranes. The membrane-associated protein ABCB1 (also known as P-gp or MDR1) is part of the MDR/TAP subfamily that reduces drug accumulation in multidrug resistant cells [11–13]. Paclitaxel and docetaxel are known substrates of ABCB1-mediated efflux from cancer cells [14], and the ABCB1 Ser893Ala and Ser893Thr SNPs (rs2032582; NP_000918.2) have been investigated in patients treated with taxanes.
- *CYP2C8 and CYP3A5 genes*: The cytochrome P450 superfamily is a large group of enzymes that are involved in organic substances and xenobiotics oxidation. They are the major enzymes of drug metabolism and bioactivation, accounting for about 75 % of total metabolic reactions [14, 15]. Although no clear data are available for their distribution and activity in the peripheral nervous system, their possible contribution to development of CIPN due to altered pharmacokinetics of taxanes has been hypothesised; in particular the enzymes CYP2C8 and CYP3A5 help to eliminate paclitaxel through successive hydroxylation reactions [16].
- *ITGB3 gene*: Integrin B3 (ITGB3) belongs to the large family of integrins, which are integral cell-surface proteins composed of an α and β chain and known to participate in cell adhesion and cell-surface-mediated signalling. The ITGB3 Leu59Pro

polymorphism (rs5918; NP_000203.2) has been associated with different activation of the MAPK3 and MAPK1 subgroup of mitogen-activated protein kinases, and reduced activation of MAPK3 and MAPK1 has been seen in in-vitro models of neurotoxic effects of platinum drugs [17].

Studies reported in literature regarding these most represented targets are quite conflicting (*see* Table 1): no conclusive findings can be inferred for any of them. We focus our attention here on GSTP1, as an extensive example of how and why data are still inconclusive.

2.2 The Example of GSTP1 Gene "Saga"

Glutathione S-transferases are a family of enzymes that catalyse conjugation of many hydrophobic and electrophilic compounds with reduced glutathione: they are of primary importance in detoxification. GSTP1 (glutathione S-transferase P1) belongs to the π class and plays a part in detoxification of platinum drugs [4–6]. A SNP in GSTP1 (562A→G; rs1695; NM_000852.3) causing substitution of isoleucine for valine diminishes the enzyme's activity, whereas homozygous deletion of the entire gene abolishes its action; this might be relevant to CIPN due to the role of oxidative stress in this disorder's onset and course [18–21]. The GSTP1 Ile105Val SNP (rs1695; NP_000843.1) has been investigated in relation to peripheral neurotoxicity of platinum drugs in 26 studies. In eleven of these, a positive association with CIPN was reported; instead, in the remaining 15 studies, no correlative evidence was recorded. In 2006, Lecomte and colleagues [22] described a cohort of white European, African, and Asian patients with colorectal, pancreatic, or gastric cancer, who were receiving treatment with oxaliplatin-based chemotherapy. In these individuals, a significant association was noted between occurrence of the A/A genotype and grade 3 CIPN, assessed with an oxaliplatin-specific scale. The number of patients available for comparison was very low, and the result was soon challenged by findings of a study undertaken in a larger cohort of patients affected by colorectal cancer and treated with oxaliplatin. The result of Lecomte's work was made more unclear when, in another study [23], a correlation with CIPN severity was established, but with the G rather than the A allele. Subsequently, Oldenburg and coworkers [24] used a symptom questionnaire to identify an association between more severe long-term CIPN and the A/A or A/G versus G/G genotype. In a study done in 2009 in 134 patients with gastric cancer, the A/A genotype was associated significantly with grade 3 CIPN, although only 12 patients were eventually available for comparison [25]. In 2010, a significant association was described in three independent studies between the A/A genotype and either more severe CIPN [26, 27] or earlier onset of grade 1 CIPN scored with the National Cancer Institute's common toxicity criteria (NCI-CTC)

without any effect on development of more severe CIPN grades [28]; however, as in previous work, only a few patients were available for comparison. In the largest study reported so far in individuals with colorectal cancer treated with oxaliplatin, McLeod and colleagues [29] studied two GSTP1 SNPs—Ile105Val and Ala114Val (rs1138272; NP_000843.1 [590C→T, NM_000852.3]). They stated that the T/T genotype was a predictor for more frequent discontinuation of FOLFOX (leucovorin, fluorouracil, oxaliplatin) and for more severe CIPN after treatment with irinotecan and oxaliplatin (but, rather surprisingly, not FOLFOX). Instead Hong and collaborators [26] observed a more pronounced risk for grade 2 oxaliplatin related neurotoxicity for A/A genotype (NCI-CTC was applied for grading). The Ala114Val SNP was not associated with CIPN in a study by Khrunin and coworkers [30]. In disagreement with these positive results, negative findings of an investigation of the GSTP1 Ile105Val SNP in patients treated with a platinum drug [31] were replicated in several subsequent studies [32–34]. In 2010, four independent clinical trials undertaken in individuals affected either by colorectal cancer and treated with oxaliplatin-based chemotherapy [8, 35, 36] or by ovarian cancer and treated with cisplatin [30] also had negative results. These prospective data accorded with those described in a previous retrospective study done in a mixed population of white European, Hispanic, Asian, and African individuals with colorectal cancer treated with oxaliplatin [7]. Also Oguri and colleagues [37] in 2013 reported a negative association for GSTP1 Ile105Val rs1695 polymorphism with oxaliplatin related CIPN in a cohort of Asian patients. A Genome-Wide Association Study (GWAS) performed by Won and colleagues [38] in a population of 247 patients treated with Oxaliplatin showed that rs1695 in GSTP1 had no association with Oxaliplatin-related CIPN. Rates of the GSTP1 Ile105Val polymorphism were also investigated in patients treated with docetaxel [39], taxane and platinum regimens [40–42], and vincristine [43]. In these studies, the A/A genotype was associated positively with a higher incidence of NCI-CTC grade 2 CIPN only in docetaxel-treated patients, whereas no association was reported in those treated with taxane and carboplatin regimens or with vincristine.

In summary GSTP1 example embodies why definitive inferences cannot be inferred from data reported in literature up to now. This quite confusing picture, so well depicted for GSTP1, can be extended to all candidate genes yet enlisted. Several reasons might explain these findings.

The first one is related to *CIPN assessment and population selection/data collection*. As it was yet said, there is still not a consensus on the best method(s) to measure neurological side effects due to chemotherapy. Different selected neurological endpoints

were applied among studies reported here: comparison among different works is quite difficult as a consequence. In the major part NCI-CTC was "the" outcome measure: it is well known that it is a poor instrument to graduate CIPN correctly, not containing a detailed neurological examination [3]. Another important issue is that some studies applied self-created scale (without a previous proper validation). Also, incidence, type and severity of other risk factors for peripheral nerve damage were rarely assessed (i.e., diabetes, alcohol intake, preexistent neuropathy due to other causes than CT); moreover, several studies included a mixed population at baseline (CT naïve and not CT naïve), without a clear basal neurological evaluation before starting CT, even if the previous CT regimen was a neurotoxic one. Rarely precise data on the actual cumulative dose administered were recorded, being it difficult to ascertain if cohorts in different studies were exposed to comparable amounts of the neurotoxic drug. Another aspect to be pointed out is that even if sample size is quite considerable at baseline, in many studies, the subgroup of patients with CIPN was remarkably sparse, making possible that statistics were underpowered.

Secondly, this review makes clear that a shift in the *gene selection strategy* is crucial to obtain valuable results from pharmacogenomics. Genes related to neurons would be the right choice to be employed in regard of a specific side effects as CIPN is. Apart from delusive data about genes such as GSTP1, first inferences from GWAS support this point of view; it is becoming clear that factors contributing to the function and repair of peripheral nerves are more important than alterations in pharmacokinetics for determining genetic susceptibility to this toxicity. An extensive example of this was given by Baldwin and colleagues in 2012 [44] in a GWAS aimed to identify new loci for paclitaxel related CIPN in a cohort of breast cancer patients. In their work it is interesting to note out that previously tested target genes were not associated with neuropathy development; differently, it was observed a positive association with a marker related to neurons. It is the case of FGD4: it encodes for the protein FGD1-related Factin binding protein (Frabin), and previous studies have shown specific point mutations in FGD4 can cause the congenital peripheral neuropathy CMT (CMT4H) [45–48]. Frabin is a guanine nucleotide exchange factor for cdc42, a Rho-GTPase that regulates cellular morphogenesis, including myelination [45]. The observed association between the FGD4 SNP rs10771973 and paclitaxel-induced sensory peripheral neuropathy is consistent with the hypothesis that common FGD4 polymorphisms subtly affect the development and/or maintenance of Schwann cell function. In this case, carriers of common FGD4 polymorphisms would have preexisting subclinical abnormalities and a predisposition for toxicity [49]. Alternatively, Authors suggested that FGD4 polymorphisms could lead to

impaired repair processes such as Schwann cell remyelination and/or axonal regeneration after paclitaxel exposure. Obviously more extensive studies are needed but this observation confirms that maybe we should move towards such target as this.

2.3 A "New" Approach

Even if it is true CIPN measurement is still a matter of debat, recently, the CI-PeriNomS [50] study, was published trying to find answers to this need. CI-PeriNomS objective was testing different outcome measures to elect the best method(s) among them; several were tested in a large population of patients affected by a clinically stable CIPN. It was aimed to establish a sound clinimetric approach to this matter of interest. First validity and reliability findings were obtained. In particular we strongly suggest to take into consideration one of the tool tested and found as a good one: the Total Neuropathy Score scale, clinical version (TNSc®) [53]. Apart from being valid and reliable, it is a scale that either comprehends a neurological examination, in contrast with NCI-CTC [51], either is simple and rapid enough to be applied in an everyday setting, in contrast with other neurological scales tested as mIss (modified Incat Sensory Score® [52]) which is way too complex for a routine setting; i.e., TNSc® seems an appropriate instrument applicable even in large cohorts of patients to be correctly described for a further pharmacogenomics analysis. In CI-PeriNomS also Patient Reported Outcome measures (PRO) and pain assessment were suggested for a full CIPN evaluation; these tools enable to encompass the wholeness of this phenomenon: since it consists mainly of sensory alterations, "subjective" point of view and pain evaluation cannot be underestimated. They should also be integrated to precisely describe the study population.

These new possible strategies suggested here are partially embodied in a work recently published by Argyriou and colleagues aimed to identify single nucleotide polymorphisms of voltage-gated sodium channels (SCNAs) genes [54]: it was selected on the basis of a possible sodium channel disfunction in oxaliplatin neuropathy development. The TNSc® was used to graduate neuropathy. A total of 200 patients with CRC were genotyped. SCN4A-rs2302237 emerged as being predictive of the clinical severity of neuropathy. The results of the study need to be further confirmed, as authors state; however, it is a convincing example of the methodology that should be applied: a thoughtful selection of genes of interest and a careful, yet simple, valid, and reproducible neurological assessment of subjects.

3 Future Perspectives: Cross talk Between "Bench" and "Bed" Side

From the overview here presented, it can be concluded that for future studies a golden rule could be proposed: the "bench" side (pharmacogenomics) should be employed rigorously as rigorously

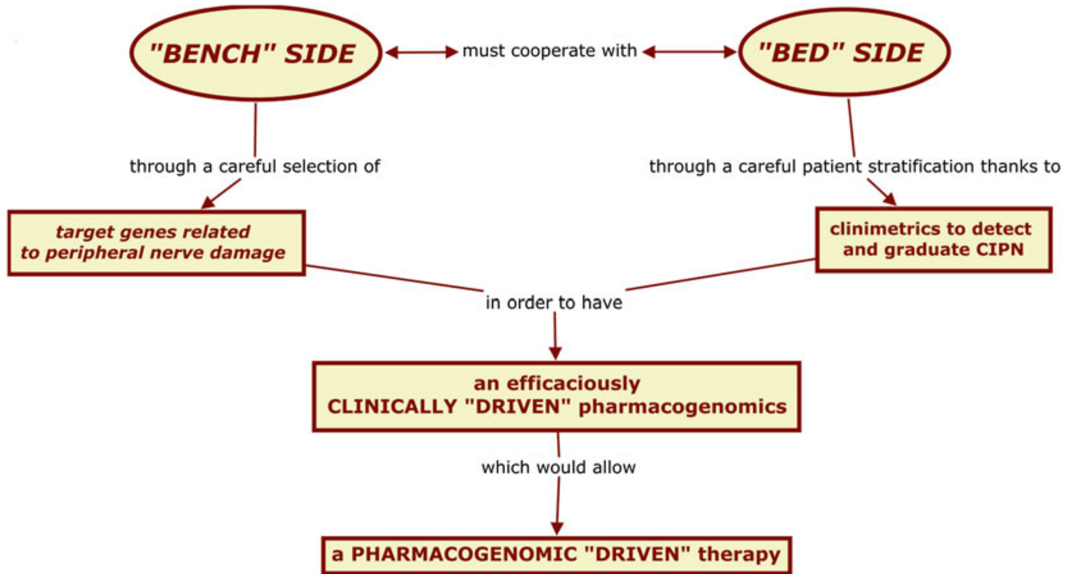

Fig. 1 Cross talk between "bench" and "bed" side in CIPN

the "bed" side (clinimetrics) should be managed (*see* Fig. 1): these two "sides" should cooperate. As a methodological strategy it could be proposed as follows:

- *Patients should be evaluated in a refined way.* Tools like TNSc® are to be applied. Population should be clearly stratified at baseline for preexisting and/or coexistent risk factors for neuropathy development. Precise actual cumulative dose data should be analysed. Sample size should consider the real amount of patients that have developed neurological signs/symptoms. Moreover, patient reported outcome measures (PRO) and pain assessment should be considered.
- *Genes related to peripheral nervous system are to be investigated.* Mechanisms of peripheral nerve damage in CIPN are not yet completely understood; however, hints from preclinical animal models could be considered as a guidance. GWAS might also be helpful to identify new potential predictors of neuropathy development.

In conclusion, a very powerful instrument as pharmacogenomics, being it delusive so far, is the confirmation that an appropriate clinimetric approach to CIPN is warranted, i.e., appropriate, valid, reliable, and feasible clinical instrument(s) for its measure are needed. That would probably be the key to build up a strategy to contrast an adverse effect that negatively impacts on QoL in a large population of long survival cancer patients.

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Chapter 13

Pharmacogenomics of Alzheimer's Disease: Novel Therapeutic Strategies for Drug Development

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Abstract

Alzheimer's disease (AD) is a major problem of health and disability, with a relevant economic impact on our society. Despite important advances in pathogenesis, diagnosis, and treatment, its primary causes still remain elusive, accurate biomarkers are not well characterized, and the available pharmacological treatments are not cost-effective. As a complex disorder, AD is a polygenic and multifactorial clinical entity in which hundreds of defective genes distributed across the human genome may contribute to its pathogenesis. Diverse environmental factors, cerebrovascular dysfunction, and epigenetic phenomena, together with structural and functional genomic dysfunctions, lead to amyloid deposition, neurofibrillary tangle formation, and premature neuronal death, the major neuropathological hallmarks of AD. Future perspectives for the global management of AD predict that genomics and proteomics may help in the search for reliable biomarkers. In practical terms, the therapeutic response to conventional drugs (cholinesterase inhibitors, multifactorial strategies) is genotype-specific. Genomic factors potentially involved in AD pharmacogenomics include at least five categories of gene clusters: (1) genes associated with disease pathogenesis; (2) genes associated with the mechanism of action of drugs; (3) genes associated with drug metabolism (phase I and II reactions); (4) genes associated with drug transporters; and (5) pleiotropic genes involved in multifaceted cascades and metabolic reactions. The implementation of pharmacogenomic strategies will contribute to optimize drug development and therapeutics in AD and related disorders.

Key words Alzheimer's disease, *ABCB1*, *APOE*, Biomarkers, Genetics, Genomics, *CYP2C9*, *CYP2C19*, *CYP2D6*, Pathogenesis, Pharmacogenomics, Therapeutic strategies

1 Introduction

Since the identification of its pathogenic features by Alois Alzheimer in 1906, over 90,000 papers have been published on Alzheimer's disease (AD) to date (2.5 million references to cancer since 1818; 1.6 million references to cardiovascular disorders since 1927; 1.01 million to central nervous system (CNS) disorders since 1893) [1]. The number of people affected by dementia is becoming a public and socioeconomic concern in many countries all over the world,

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independently of the economic condition of the society in question. The growth of the elderly population is a common phenomenon in both developed and developing countries, bringing about future challenges in terms of health policy and disability rates. In the USA, death rates for the leading causes of death are heart disease ($200.2 \times 100{,}000$), cancer ($180.7 \times 100{,}000$), and stroke ($43.6 \times 100{,}000$). AD is the fifth leading cause of death in people older than 65 years of age, representing 71,600 deaths/year. AD affects approximately 5.4 million individuals in the USA and is estimated to affect up to 16 million by 2050 [2]. Disability caused by senility and dementia affects $9.2 \times 1{,}000$ in the population aged 65–74 years, $33.5 \times 1{,}000$ in those within the 75–84 range, and $83.4 \times 1{,}000$ in the population over 85 years of age [3, 4]. In countries with low and middle income, dementia makes the largest contribution to disability, with a median population-attributable prevalence fraction of 25.1 %, followed by stroke (11.4 %), limb impairment (10.5 %), arthritis (9.9 %), depression (8.3 %), eyesight problems (6.8 %), and gastrointestinal impairments (6.5 %) [5]. In Western countries, AD is the most prevalent form of dementia (45–60 %), followed by vascular dementia (VD) (30–40 %), and mixed dementia (10–20 %), which in people older than 85 years of age may account for over 80 % of the cases.

The different forms of dementia pose several challenges to our society and the scientific community: (1) they represent an epidemiological problem, and a socioeconomic, psychological, and family burden; (2) most of them have an obscure/complex pathogenesis; (3) their diagnosis is not easy and lacks specific biomarkers; and (4) their treatment is difficult and inefficient.

In terms of economic burden, approximately 10–20 % of direct costs are associated with pharmacological treatment, with a gradual increase in parallel with the severity of the disease. A Canadian study [6] shows that the mean total cost to treat patients with very mild AD is $367 per month, compared with $4,063 per month for patients with severe or very severe AD. Only 20–30 % of patients with dementia respond appropriately to conventional drugs, and the onset of adverse drug reactions imposes the additional administration of other drugs to neutralize side effects, thus multiplying the initial cost of the pharmacological treatment and the health risk for the patients [7]. Wimo et al. [8] studied the economic impact of dementia in Europe within the EU-funded Eurocode project and found that the total cost of dementia in the EU27 in 2008 was estimated to be €160 billion (€22,000 per demented patient per year), of which 56 % were costs of informal care. The corresponding costs for the whole of Europe were €177 billion. Informal caregiver costs were the largest cost component accounting for about half to just over 60 % of total societal costs, depending on country in Europe and AD severity [9].

In addition to the problem of direct and indirect costs for the management of dementia, there is an alarming abuse of inappropriate psychotropic drug consumption worldwide. Antipsychotic medications are taken by over 30 % of elderly patients with dementia [10] and conventional antipsychotics are associated with a higher risk of all-cause mortality among nursing home residents [11]. Abuse, misuse, self-prescription, and uncontrolled medical prescription of CNS drugs are becoming major problems with unpredictable consequences for brain health. The pharmacological management of dementia is an issue of special concern due to the polymedication required to modulate the symptomatic complexity of dementia where cognitive decline, behavioral changes, and psychomotor deterioration coexist. In parallel, a growing body of fresh knowledge on the pathogenesis of dementia, together with data on neurogenomics and pharmacogenomics of CNS disorders, is emerging in recent times. The incorporation of this new armamentarium of molecular pathology and genomic medicine into daily medical practice, together with educational programs for the correct use of drugs, must help to: (1) understand AD pathogenesis, (2) establish an early diagnosis, and (3) optimize therapeutics either as a preventive strategy or as a formal symptomatic treatment [7, 12].

2 Toward a Personalized Medicine of Dementia and Neurodegenerative Disorders

Common features of neurodegenerative disorders include the following: (1) polygenic/complex disorders in which genetic, epigenetic, and environmental factors are involved; (2) deterioration of higher activities of the CNS; (3) multifactorial dysfunctions in several brain circuits; and (4) accumulation of toxic proteins in the nervous tissue. For instance, the neuropathological hallmarks of AD (amyloid deposition in senile plaques, neurofibrillary tangle formation, and neuronal loss) are but the phenotypic expression of a pathogenic process in which different gene clusters and their products are potentially involved [7, 12].

A large number of the genes which form the structural architecture of the human genome are expressed in the brain in a time-dependent manner along the lifespan. The cellular complexity of the CNS (with 10^3 different cell types) and synapses (with each of the 10^{11} neurons in the brain having around 10^3–10^4 synapses with a complex multiprotein structure integrated by 10^3 different proteins) requires a very powerful technology for gene expression profiling, which is still in its very early stages and is not devoid of technical obstacles and limitations [13]. Transcripts of 16,896 genes have been measured in different CNS regions. Each region possesses its own unique transcriptome fingerprint which is

independent of age, gender, and energy intake. Less than 10 % of genes are affected by age, diet, or gender, with most of these changes occurring between middle and old age. Gender and energy restriction have robust influences on the hippocampal transcriptome of middle-aged animals. Prominent functional groups of age- and energy-sensitive genes are those encoding proteins involved in DNA damage responses, mitochondrial and proteasome functions, cell fate determination, and synaptic vesicle trafficking [14].

The introduction of novel procedures into an integral genomic medicine protocol for CNS disorders and dementia is an imperative requirement in drug development and in clinical practice in order to improve diagnostic accuracy and to optimize therapeutics. Personalized strategies, adapted to the complexity of each case, are essential to depict a clinical profile based on specific biomarkers correlating with individual genomic profiles [7, 15].

Our understanding of the pathophysiology of CNS disorders and dementia has advanced dramatically during the last 30 years, especially in terms of their molecular pathogenesis and genetics. The drug treatment of CNS disorders has also made remarkable strides, with the introduction of many new drugs for the treatment of schizophrenia, depression, anxiety, epilepsy, Parkinson's disease, and AD, among many other quantitatively and qualitatively important neuropsychiatric disorders. Improvement in terms of clinical outcome, however, has fallen short of expectations, with up to one third of the patients continuing to experience clinical relapse or unacceptable medication-related side effects in spite of efforts to identify optimal treatment regimes with one or more drugs. Potential reasons to explain this historical setback might be that: (1) the molecular pathology of most CNS disorders is still poorly understood; (2) drug targets are inappropriate, not fitting into the real etiology of the disease; (3) most treatments are symptomatic, but not anti-pathogenic; (4) the genetic component of most CNS disorders is poorly defined; and (5) the understanding of genome–drug interactions is very limited [7, 12].

The optimization of CNS therapeutics requires the establishment of new postulates regarding (1) the costs of medicines, (2) the assessment of protocols for multifactorial treatment in chronic disorders, (3) the implementation of novel therapeutics addressing causative factors, and (4) the setting up of pharmacogenomic strategies for drug development [12]. Personalized therapeutics based on individual genomic profiles implies the characterization of five types of gene clusters: (1) genes associated with disease pathogenesis; (2) genes associated with the mechanism of action of drugs; (3) genes associated with drug metabolism (phase I and II reactions); (4) genes associated with drug transporters; and (5) pleiotropic genes involved in multifaceted cascades and metabolic reactions [16].

3 Genomics of Alzheimer's Disease

Over 3,000 genes distributed across the human genome have been screened for association with AD during the past 30 years [17].

In the Alzgene database [18] there are 695 genes potentially associated with AD, of which the top ten are (in decreasing order of importance): *APOE* (19q13.2), *BIN1* (2q14), *CLU* (8p21–p12), *ABCA7* (19p13.3), *CR1* (1q32), *PICALM* (11q14), *MS4A6A* (11q12.1), *CD33* (19q13.3), *MS4A4E* (11q12.2), and *CD2AP* (6p12). Potentially defective genes associated with AD represent about 1.39 % (35,252.69 kb) of the human genome, which is integrated by 36,505 genes (3,095,677.41 kb). The highest number of AD-related defective genes concentrates on chromosomes 10 (5.41 %; 7,337.83 kb), 21 (4.76 %; 2,289,15 kb), 7 (1.62 %; 2,584.26 kb), 2 (1.56 %; 3,799.67 kb), 19 (1.45 %; 854.54 kb), 9 (1.42 %; 2,010.62 kb), 15 (1.23 %; 1,264.4 kb), 17 (1.19 %; 970.16 kb), 12 (1.17 %; 1,559.9 kb), and 6 (1.15 %; 1,968.22 kb), with the highest proportion (related to the total number of genes mapped on a single chromosome) located on chromosome 10 and the lowest on chromosome Y [19] (*see* Figs. 1, 2, and 3).

The genetic and epigenetic defects identified in AD can be classified into four major categories: Mendelian mutations, susceptibility single-nucleotide polymorphisms (SNPs), mitochondrial DNA (mtDNA) mutations, and epigenetic changes. Mendelian mutations affect genes directly linked to AD, including 32 mutations in the amyloid beta precursor protein (*APP*) gene (21q21) (*AD1*); 165 mutations in the presenilin 1 (*PSEN1*) gene (14q24.3) (*AD3*); and 12 mutations in the presenilin 2 (*PSEN2*) gene (1q31–q42) (*AD4*) [17–22]. *PSEN1* and *PSEN2* are important determinants of γ-secretase activity responsible for proteolytic cleavage of APP and NOTCH receptor proteins. Mendelian mutations are very rare in AD (1:1,000). Mutations in exons 16 and 17 of the *APP* gene appear with a frequency of 0.30 % and 0.78 %, respectively, in AD patients. Likewise, *PSEN1*, *PSEN2*, and microtubule-associated protein Tau (*MAPT*) (17q21.1) mutations are present in less than 2 % of the cases. Mutations in these genes confer specific phenotypic profiles to patients with dementia: amyloidogenic pathology associated with *APP*, *PSEN1,* and *PSEN2* mutations and tauopathy associated with *MAPT* mutations representing the two major pathogenic hypotheses for AD [17–23].

Ten novel private pathogenic copy number variations (CNVs) in ten early-onset familial Alzheimer's disease (EO-FAD) families overlapping a set of genes (*A2BP1*, *ABAT*, *CDH2*, *CRMP1*, *DMRT1*, *EPHA5*, *EPHA6*, *ERMP1*, *EVC*, *EVC2*, *FLJ35024*, and *VLDLR*) have also been identified [24].

Multiple polymorphic risk variants can increase neuronal vulnerability to premature death (*see* Table 1).

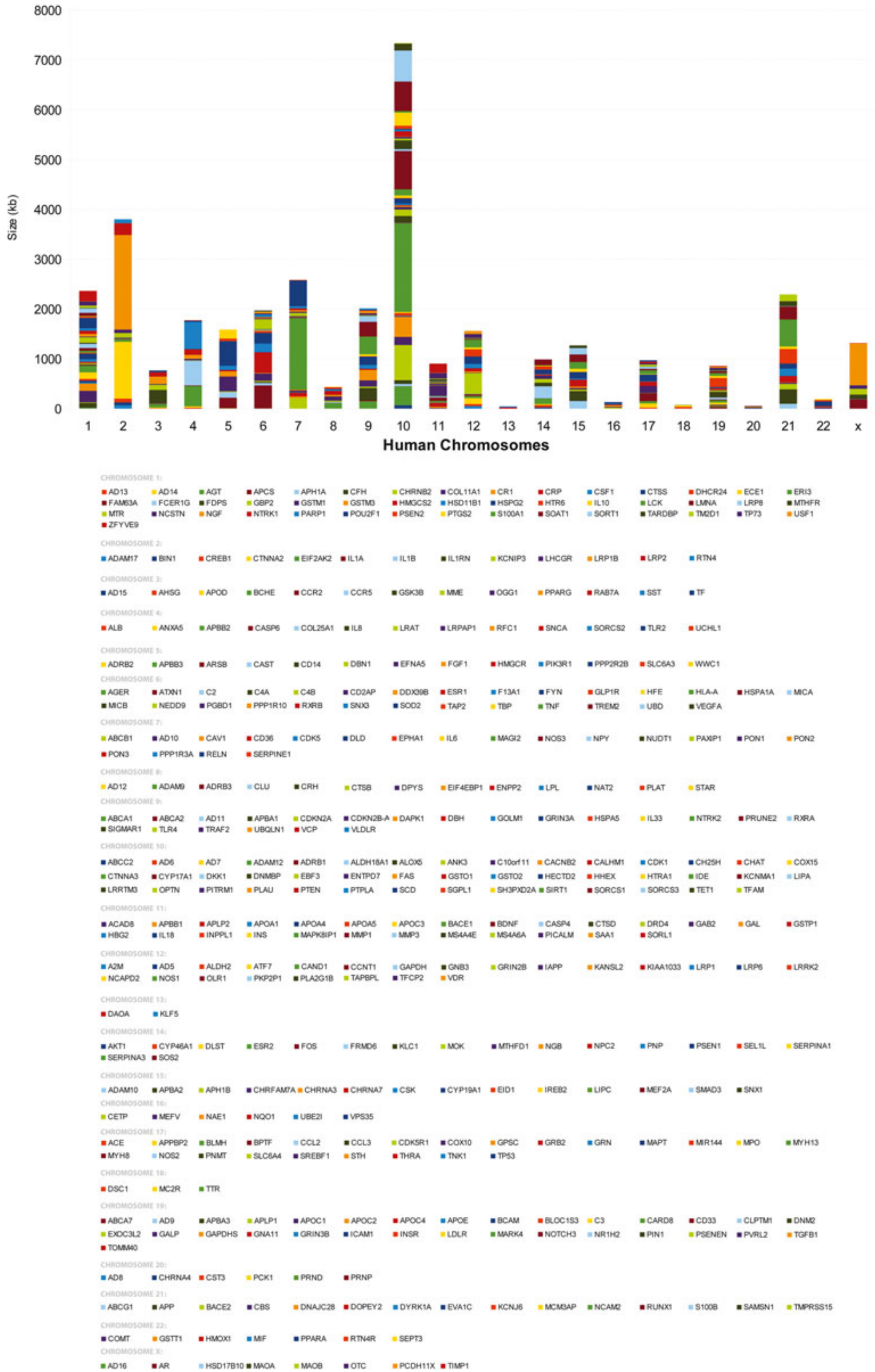

Fig. 1 Chromosomal distribution of Alzheimer's disease-related genes in the human genome

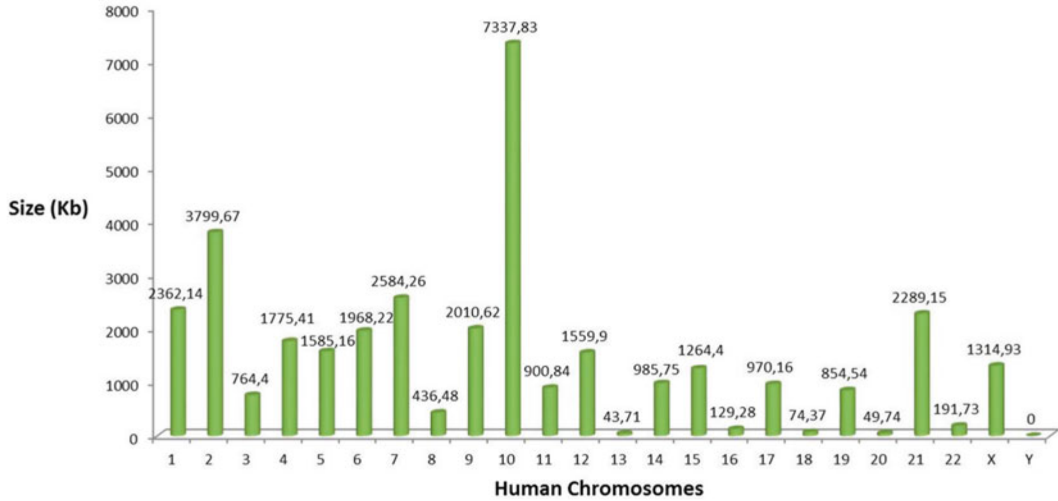

Fig. 2 Chromosomal distribution of Alzheimer's disease-related genes by size (kb)

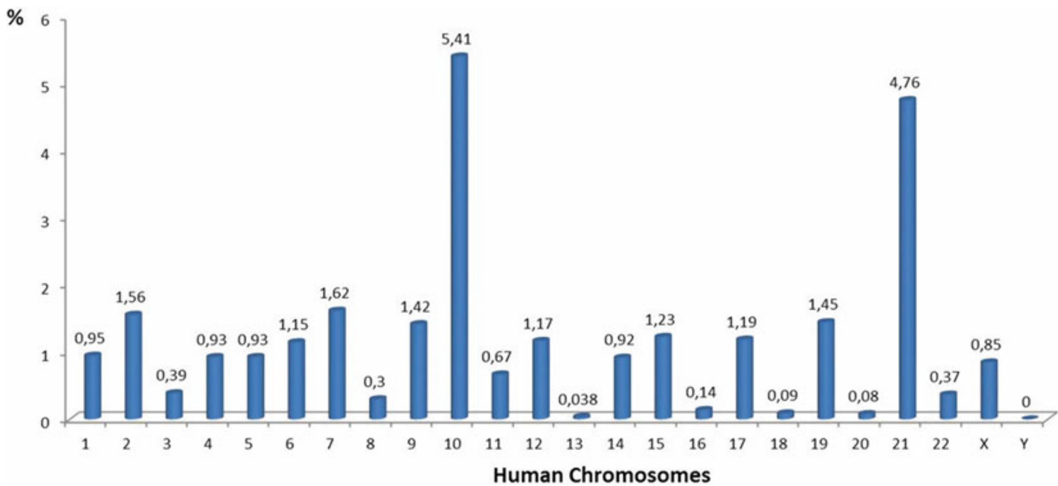

Fig. 3 Percentual distribution of Alzheimer's disease-related genes in the human genome

Among these susceptibility genes, the apolipoprotein E (*APOE*) gene (19q13.2) (*AD2*) is the most prevalent as a risk factor for AD, especially in those subjects harboring the *APOE-4* allele (*see* Fig. 4), whereas carriers of the *APOE-2* allele might be protected against dementia.

Polymorphic variants in other genes (GRB-associated binding protein 2 (*GAB2*) [25], *TLR9* rs187084 variant homozygote GG [26], *LRRK2* R1628P variant [27]) might be protective, as well.

APOE-related pathogenic mechanisms are also associated with brain aging and with the neuropathological hallmarks of AD [17, 28]. mtDNA damage may also contribute to increase brain vulnerability and neurodegeneration [29, 30].

Table 1
Selected genes potentially associated with Alzheimer's disease

Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
1p13.1	52.32	NGF	Nerve growth factor (beta polypeptide)	162030	Hereditary sensory and autonomic neuropathy type V, allergic rhinitis
1p13.3	5.95	GSTM1	Glutathione S-transferase mu 1	138350	Cancer
1p13.3	7.11	GSTM3	Glutathione S-transferase mu 3 (brain)	138390	Cancer
1p13.3	20.38	CSF1	Colony stimulating factor 1 (macrophage)	120420	
1p13–p12	20.94	HMGCS2	3-Hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	600234	HMG-CoA synthase-2 deficiency
1p21	232.03	COL11A1	Collagen, type XI, alpha 1	120280	Fibrochondrogenesis, Marshall syndrome, Stickler syndrome type II, lumbar disc herniation
1p21.3–p13.1	88.38	SORT1	Sortilin 1	602458	
1p22.2	18.49	GBP2	Guanylate binding protein 2, interferon-inducible	600412	
1p31.3	44.38	TM2D1	TM2 domain containing 1	610080	
1p32	134.20	ERI3	ERI1 exoribonuclease family member 3	609917	
1p32.3	37.62	DHCR24	24-Dehydrocholesterol reductase	606418	Desmosterolosis
1p32.3	204.31	ZFYVE9	Zinc finger, FYVE domain containing 9	603755	
1p34	85.79	LRP8	Low-density lipoprotein receptor-related protein 8, apolipoprotein e receptor	602600	Myocardial infarction, major depressive disorder
1p34.3	34.93	LCK	Lymphocyte-specific protein tyrosine kinase	153390	SCID due to LCK deficiency
1p36.1	128.30	ECE1	Endothelin converting enzyme 1	600423	Hirschsprung disease, cardiac defects, autonomic dysfunction, essential hypertension

1p36.13–q31.3	3.81	APH1A	APH1A gamma secretase subunit provided	607629	
1p36.1–p34	115.01	HSPG2	Heparan sulfate proteoglycan 2	142461	Dyssegmental dysplasia Silverman–Handmaker type, Schwartz–Jampel syndrome type 1, tardive dyskinesia
1p36.22	12.87	TARDBP	TAR DNA-binding protein	605078	Amyotrophic lateral sclerosis 10 with or without FTD, frontotemporal lobar degeneration TARDBP-related
1p36.3	83.64	TP73	Tumor protein p73	601990	Neuroblastoma
1p36.3	20.37	MTHFR	Methylenetetrahydrofolate reductase (NAD(P)H)	607093	Homocystinuria due to MTHFR deficiency, neural tube defects, schizophrenia, thromboembolism, occlusive vascular disease, colon cancer, acute leukemia
1p36–p35	14.28	HTR6	5-Hydroxytryptamine (serotonin) receptor 6, G protein-coupled	601109	
1q21	35.76	CTSS	Cathepsin S	116845	
1q21	N/A	AD13	Alzheimer's disease 13	611152	
1q21	3.64	S100A1	S100 calcium binding protein A1	176940	Cardiomyopathies
1q21.3	11.55	FAM63A	Family with sequence similarity 63, member A	N/A	
1q21.3	12.10	CHRNB2	Cholinergic receptor, nicotinic, beta 2 (neuronal)	118507	Nocturnal frontal lobe epilepsy 3
1q21–q22	66.10	NTRK1	Neurotrophic tyrosine kinase, receptor, type 1	191315	Insensitivity to pain with anhidrosis, medullary thyroid carcinoma, self-mutilating behavior, mental retardation
1q21–q23	1.05	APCS	Amyloid P component, serum	104770	Secondary amyloidosis

(continued)

Table 1
(continued)

Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
1q22	57.51	LMNA	Lamin A/C	150330	Emery–Dreifuss muscular dystrophy 2, Emery–Dreifuss muscular dystrophy 3, familial partial lipodystrophy 2, muscular dystrophy, limb girdle muscular dystrophy type 1B, dilated cardiomyopathy 1A, Charcot–Marie–Tooth disease type 2B1, Hutchinson–Gilford progeria syndrome, heart-hand syndrome of Slovenian type, Malouf syndrome, mandibuloacral dysplasia, lethal restrictive dermopathy
1q22	11.92	FDPS	Farnesyl diphosphate synthase	134629	
1q22–q23	15.68	NCSTN	Nicastrin	605254	Acne inversa 1
1q22–q23	6.72	USF1	Upstream transcription factor 1	191523	Hyperlipidemia
1q23	3.95	FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	147139	
1q23.2	2.30	CRP	C-reactive protein, pentraxin-related provided	123260	
1q24.2	206.52	POU2F1	POU class 2 homeobox 1	164175	
1q25	64.97	SOAT1	Sterol O-acyltransferase 1	102642	
1q25	N/A	AD14	Alzheimer's disease 14	611154	
1q25.2–q25.3	8.62	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	600262	
1q31–q32	4.89	IL10	Interleukin 10	124092	Rheumatoid arthritis

1q31–q42	25.53	PSEN2	Presenilin 2 (Alzheimer disease 4)	600759	Dilated cardiomyopathy 1 V
1q32	95.63	CFH	Complement factor H	134370	Hemolytic-uremic syndrome, chronic hypocomplemententemic nephropathy, basal laminar drusen, complement factor H deficiency, macular degeneration 4
1q32	145.64	CR1	Complement component (3b/4b) receptor 1 (Knops blood group)	120620	CR1 deficiency, systemic lupus erythematosus
1q32–q41	48.77	HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	600713	Cortisone reductase deficiency 2, obesity, insulin resistance
1q41–q42	47.41	PARP1	Poly (ADP-ribose) polymerase 1	173870	Xeroderma pigmentosum, Fanconi anemia, diabetes type I
1q42.2	12.07	AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	106150	Renal tubular dysgenesis, nonfamilial structural atrial fibrillation, inflammatory bowel disease, essential hypertension, preeclampsia
1q43	108.70	MTR	5-Methyltetrahydrofolate-homocysteine methyltransferase	156570	Methylcobalamin deficiency type cblG, neural tube defects
2p12–p11.1	1,140	CTNNA2	Catenin (cadherin-associated protein), alpha 2	114025	
2p16.3	78.41	RTN4	Reticulon 4	604475	
2p21	68.97	LHCGR	Luteinizing hormone/ choriogonadotropin receptor	152790	Leydig cell adenoma with precocious puberty, Leydig cell hypoplasia with hypergonadotropic hypogonadism, Leydig cell hypoplasia with pseudohermaphroditism, female luteinizing hormone resistance, male precocious puberty
2p22–p21	51.91	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	176871	

(continued)

Table 1
(continued)

Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
2p25	66.53	ADAM17	ADAM metallopeptidase domain 17	603639	Neonatal inflammatory skin and bowel disease
2q14	11.48	IL1A	Interleukin 1, alpha	147760	Rheumatoid arthritis
2q14	7.02	IL1B	Interleukin 1, beta	147720	
2q14	59.31	BIN1	Bridging integrator 1	601248	Centronuclear myopathy
2q14.2	16.12	IL1RN	Interleukin 1 receptor antagonist	147679	Interleukin 1 receptor antagonist deficiency, microvascular complications of diabetes 4
2q21.1	88.75	KCNIP3	Kv channel interacting protein 3, calsenilin	604662	
2q21.2	1,900	LRP1B	Low-density lipoprotein receptor-related protein 1B	608766	
2q24–q31	235.50	LRP2	Low-density lipoprotein receptor-related protein 2	600073	Donnai–Barrow syndrome, facio-oculoacoustico-renal syndrome
2q34	75.67	CREB1	cAMP responsive element binding protein 1	123810	Angiomatoid fibrous histiocytoma
3p21.31	7.18	CCR2	Chemokine (C-C motif) receptor 2	601267	
3p21.31	6.07	CCR5	Chemokine (C-C motif) receptor 5 (gene/pseudogene)	601373	Insulin-dependent diabetes mellitus 22
3p25	146.51	PPARG	Peroxisome proliferator-activated receptor gamma	601487	Carotid intimal medial thickness 1, insulin resistance, lipodystrophy 3, obesity, diabetes type 2, cancer
3p26.2	16.73	OGG1	8-Oxoguanine DNA glycosylase	601982	Renal cell carcinoma

3q13	133.67	CASR	Calcium-sensing receptor	601199	Hypercalciuric hypercalcemia, hyperparathyroidism neonatal, autosomal dominant hypocalcemia with Bartter syndrome, hypocalciuric hypercalcemia type I, epilepsy idiopathic generalized susceptibility
3q13.3	272.47	GSK3B	Glycogen synthase kinase 3 beta	605004	Parkinson disease
3q21.3	88.66	RAB7A	RAB7A, member RAS oncogene family	602298	Charcot–Marie–Tooth disease type 2B
3q22.1	32.87	TF	Transferrin	190000	Atransferrinemia
3q22–q24	N/A	AD15	Alzheimer's disease 15	611155	
3q25.2	104.08	MME	Membrane metallo-endopeptidase	120520	Membranous glomerulonephritis, neutral endopeptidase deficiency
3q26.1–q26.2	64.56	BCHE	Butyrylcholinesterase	177400	
3q26.2-qter	15.50	APOD	Apolipoprotein D	107740	
3q27	8.26	AHSG	Alpha-2-HS-glycoprotein	138680	
3q28	1.51	SST	Somatostatin	182450	
4p13	404.59	APBB2	Amyloid beta (A4) precursor protein-binding, family B, member 2	602710	
4p14	11.55	UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	191342	Parkinson disease 5
4p14–p13	78.93	RFC1	Replication factor C (activator 1) 1, 145 kDa	102579	
4p16.1	550.19	SORCS2	Sortilin-related VPS10 domain containing receptor 2	606284	
4p16.3	28.90	LRPAP1	Low-density lipoprotein receptor-related protein associated protein 1	104225	

(continued)

Table 1 (continued)

Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
4q12	154.71	CLOCK	Clock circadian regulator	601851	
4q13.3	17.16	ALB	Albumin	103600	Analbuminemia, dysalbuminemic hyperthyroxinemia, dysalbuminemic hyperzincemia
4q13–q21	3.21	IL8	Interleukin 8	146930	Bronchiolitis
4q21	114.20	SNCA	Synuclein, alpha (non A4 component of amyloid precursor)	163890	Lewy body dementia, Parkinson disease 1, Parkinson disease 4
4q22	183.50	ABCG2	ATP-binding cassette, subfamily G (WHITE), member 2	603756	
4q25	491.92	COL25A1	Collagen, type XXV, alpha 1	610004	
4q25	14.85	CASP6	Caspase 6, apoptosis-related cysteine peptidase	601532	
4q27	29.00	ANXA5	Annexin A5	131230	Recurrent pregnancy loss
4q32	21.80	TLR2	Toll-like receptor 2	603028	Colorectal cancer
4q32.1	9.11	LRAT	Lecithin retinol acyltransferase (phosphatidylcholine–retinol O-acyltransferase)	604863	Leber congenital amaurosis 14, retinal dystrophy, retinitis pigmentosa
5p15.3	52.64	SLC6A3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	126455	Epilepsy, parkinsonism-dystonia, attention-deficit hyperactivity disorder, Parkinson disease
5q13.1	86.07	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	171833	Agammaglobulinemia 7, insulin resistance
5q13.3–q14	24.93	HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase	142910	

5q14.1	209.33	ARSB	Arylsulfatase B	611542	Mucopolysaccharidosis type VI (Maroteaux–Lamy)
5q15	112.65	CAST	Calpastatin	114090	
5q21	294.01	EFNA5	Ephrin-A5	601535	
5q31	105.89	FGF1	Fibroblast growth factor 1 (acidic)	131220	
5q31	6.34	APBB3	Amyloid beta (A4) precursor protein-binding, family B, member 3	602711	
5q31.1	1.97	CD14	CD14 molecule	158120	
5q31–q32	2.04	ADRB2	Adrenoceptor beta 2, surface	109690	Nocturnal asthma, obesity, diabetes type 2
5q32	491.97	PPP2R2B	Protein phosphatase 2, regulatory subunit B, beta	604325	Spinocerebellar ataxia 12
5q34	180.24	WWC1	WW and C2 domain containing 1	610533	
5q35.3	17.08	DBN1	Drebrin 1	126660	
6p12	16.28	VEGFA	Vascular endothelial growth factor A	192240	Microvascular complications of diabetes 1
6p12	149.48	CD2AP	CD2-associated protein	604241	Focal segmental glomerulosclerosis 3
6p21	38.96	GLP1R	Glucagon-like peptide 1 receptor	138032	
6p21.1	4.68	TREM2	Triggering receptor expressed on myeloid cells 2	605086	Nasu-Hakola disease
6p21.3	7.96	HFE	Hemochromatosis	613609	Hemochromatosis, microvascular complications of diabetes 7, porphyria cutanea tarda, porphyria variegata
6p21.3	4.31	UBD	Ubiquitin D	606050	
6p21.3	3.42	HLA-A	Major histocompatibility complex, class I, A	142800	

(continued)

Table 1 (continued)

Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
6p21.3	12.26	DDX39B	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39B	142560	Rheumatoid arthritis
6p21.3	2.77	TNF	Tumor necrosis factor	191160	Asthma, vascular dementia, migraine without aura, insulin resistance, cancer
6p21.3	2.43	HSPA1A	Heat shock 70 kDa protein 1A	140550	
6p21.3	16.91	PPP1R10	Protein phosphatase 1, regulatory subunit 10	603771	
6p21.3	3.36	AGER	Advanced glycosylation end product-specific receptor	600214	Diabetes
6p21.3	16.94	TAP2	Transporter 2, ATP-binding cassette, subfamily B (MDR/TAP)	170261	Bare lymphocyte syndrome type I due to TAP2 deficiency, Wegener-like granulomatosis, ankylosing spondylitis, insulin-dependent diabetes mellitus, celiac disease
6p21.3	47.89	C2	Complement component 2	613927	C2 deficiency
6p21.3	20.62	C4B	Complement component 4B (Chido blood group)	120820	C4B deficiency, systemic lupus erythematosus
6p21.3	20.62	C4A	Complement component 4A (Rodgers blood group)	120810	C4A deficiency, systemic lupus erythematosus, type I diabetes mellitus
6p21.3	13.05	MICB	MHC class I polypeptide-related sequence B	602436	
6p21.3	7.11	RXRB	Retinoid X receptor, beta	180246	
6p21.33	11.72	MICA	MHC class I polypeptide-related sequence A	600169	
6p22.1	21.01	PGBD1	PiggyBac transposable element derived 1	N/A	

6p23	462.38	ATXN1	Ataxin 1	601556	Spinocerebellar ataxia 1
6p25.3–p24.3	176.61	F13A1	Coagulation factor XIII, A1 polypeptide	134570	Factor XIIIA deficiency
6p25–p24	199.05	NEDD9	Neural precursor cell expressed, developmentally downregulated 9	602265	Cancer metastasis
6q21	213.12	FYN	FYN oncogene related to SRC, FGR, YES	137025	
6q21	49.75	SNX3	Sorting nexin 3	605930	
6q25.1	412.78	ESR1	Estrogen receptor 1	133430	Breast cancer, atherosclerosis, migraine, myocardial infarction, endometrial cancer, osteoporosis
6q25.3	14.21	SOD2	Superoxide dismutase 2, mitochondrial	147460	Microvascular complications of diabetes 6, cardiomyopathy, premature aging, sporadic motor neuron disease, cancer
6q27	18.54	TBP	TATA box binding protein	600075	Spinocerebellar ataxia 17, Parkinson disease
7p15.1	7.68	NPY	Neuropeptide Y	162640	Elevated cholesterol levels, higher alcohol consumption, metabolic diseases, cardiovascular diseases
7p21	4.86	IL6	Interleukin 6 (interferon, beta 2)	147620	Crohn disease-associated growth failure, diabetes, intracranial hemorrhage in brain cerebrovascular malformations, Kaposi sarcoma, rheumatoid arthritis
7p22	8.92	NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	600312	
7q11.2	77.09	CD36	CD36 molecule (thrombospondin receptor)	173510	Platelet glycoprotein IV deficiency, macrothrombocytopenia, coronary heart disease
7q21	1,440	MAGI2	Membrane-associated guanylate kinase, WW, and PDZ domain containing 2	606382	

(continued)

Table 1 (continued)

Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
7q21.12	209.46	ABCB1	ATP-binding cassette, subfamily B (MDR/TAP), member 1	171050	Inflammatory bowel disease 13,
7q21.3	26.22	PON1	Paraoxonase 1	168820	Coronary artery disease, coronary artery spasm 2, microvascular complications of diabetes 5
7q21.3	36.50	PON3	Paraoxonase 3	602720	
7q21.3	30.21	PON2	Paraoxonase 2	602447	Coronary artery disease, diabetes
7q22	517.73	RELN	Reelin	600514	Lissencephaly 2 (Norman–Roberts type)
7q22.1	12.18	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	173360	Plasminogen activator inhibitor-1 deficiency, thrombophilia
7q31.1	42.20	PPP1R3A	Protein phosphatase 1, regulatory subunit 3A	600917	Insulin resistance
7q31.1	36.40	CAV1	Caveolin 1, caveolae protein, 22 kDa	601047	Lipodystrophy type 3
7q31–q32	30.06	DLD	Dihydrolipoamide dehydrogenase	238331	Dihydrolipoamide dehydrogenase deficiency, maple syrup urine disease
7q34	17.78	EPHA1	EPH receptor A1	179610	Cancer
7q36	23.54	NOS3	Nitric oxide synthase 3 (endothelial cell)	163729	Coronary artery spasm 1, hypertension, ischemic stroke, placental abruption
7q36	4.15	CDK5	Cyclin-dependent kinase 5	123831	
7q36	59.28	PAXIP1	PAX interacting (with transcription-activation domain) protein 1	608254	
7q36	N/A	AD10	Alzheimer's disease 10	609636	
8p11.2	8.38	STAR	Steroidogenic acute regulatory protein	600617	Lipoid adrenal hyperplasia

8p11.22	108.28	ADAM9	ADAM metallopeptidase domain 9	602713	Cone-rod dystrophy 9
8p12	3.67	ADRB3	Adrenoceptor beta 3	109691	Obesity
8p12	32.96	PLAT	Plasminogen activator, tissue	173370	Hyperfibrinolysis, thrombophilia
8p12	29.86	EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1	602223	
8p12–q22	N/A	AD12	Alzheimer's disease 12	611073	
8p21–p12	17.90	CLU	Clusterin	185430	Neoplasms
8p22	9.97	NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)	612182	Cancer
8p22	28.19	LPL	Lipoprotein lipase	609708	Hyperlipidemia, lipoprotein lipase deficiency
8p22	25.61	CTSB	Cathepsin B	116810	Esophageal adenocarcinoma, neoplasms
8q13	2.24	CRH	Corticotropin-releasing hormone	122560	
8q22	87.63	DPYS	Dihydropyrimidinase	613326	Dihydropyrimidinuria
8q24.1	81.79	ENPP2	Ectonucleotide pyrophosphatase/ phosphodiesterase 2	601060	
9p13.3	3.05	SIGMAR1	Sigma non-opioid intracellular receptor 1	601978	Amyotrophic lateral sclerosis 16
9p13.3	16.68	VCP	Valosin-containing protein	601023	Amyotrophic lateral sclerosis 14 with or without frontotemporal dementia, inclusion body myopathy with early-onset Paget disease and frontotemporal dementia
9p21	26.74	CDKN2A	Cyclin-dependent kinase inhibitor 2A	600160	Melanoma and neural system tumor syndrome, orolaryngeal cancer, pancreatic cancer, cutaneous malignant melanoma 2

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Table 1
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Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
9p21.3	126.31	CDKN2B-AS1	CDKN2B antisense RNA 1	613149	Cardiovascular diseases, cancer, intracranial aneurysm, type-2 diabetes, periodontitis, endometriosis, frailty in the elderly, glaucoma
9p24	32.69	VLDLR	Very-low-density lipoprotein receptor	192977	Cerebellar hypoplasia and mental retardation with or without quadrupedal locomotion 1
9p24.1	42.20	IL33	Interleukin 33	608678	
9q13–q21.1	244.83	APBA1	Amyloid beta (A4) precursor protein-binding, family A, member 1	602414	
9q21.2	294.71	PRUNE2	Prune homolog 2 (Drosophila)	610691	
9q21.2–q21.3	48.29	UBQLN1	Ubiquilin 1	605046	Parkinson disease
9q21.33	210.79	DAPK1	Death-associated protein kinase 1	600831	
9q21.33	74.06	GOLM1	Golgi membrane protein 1	606804	
9q22.1	355.04	NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	600456	Obesity, mood disorders
9q22.1	N/A	AD11	Alzheimer's disease 11	609790	
9q31.1	169.23	GRIN3A	Glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	606650	
9q31.1	147.25	ABCA1	ATP-binding cassette, subfamily A (ABC1), member 1	600046	HDL deficiency type 2, Tangier disease, coronary artery disease
9q33.1	13.32	TLR4	Toll-like receptor 4	603030	Colorectal cancer, macular degeneration
9q33.3	6.54	HSPA5	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	138120	

9q34	22.98	DBH	Dopamine beta-hydroxylase (dopamine beta-monooxygenase)	609312	Dopamine beta-hydroxylase deficiency
9q34	40.10	TRAF2	TNF receptor-associated factor 2	601895	
9q34	21.69	ABCA2	ATP-binding cassette, subfamily A (ABC1), member 2	600047	
9q34.3	114.12	RXRA	Retinoid X receptor, alpha	180245	
10	51.74	ENTPD7	Ectonucleoside triphosphate diphosphohydrolase 7	N/A	
10p12	401.08	CACNB2	Calcium channel, voltage-dependent, beta 2 subunit	600003	Brugada syndrome 4
10p12.31	161.34	C10orf112	Chromosome 10 open reading frame 112	N/A	
10p13	38.20	OPTN	Optineurin	602432	Amyotrophic lateral sclerosis 12, glaucoma
10p13	N/A	AD7	Alzheimer's disease 7	606187	
10p14–p13	27.42	PTPLA	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	610467	
10p15.2	35.12	PITRM1	Pitrilysin metallopeptidase 1	N/A	
10q	N/A	AD6	Alzheimer's disease 6	605526	
10q11.2	71.94	ALOX5	Arachidonate 5-lipoxygenase	152390	Atherosclerosis, cancer
10q11.2	56.01	CHAT	Choline O-acetyltransferase	118490	Myasthenic syndrome associated with episodic apnea
10q11.2	3.38	DKK1	Dickkopf WNT signaling pathway inhibitor 1	605189	
10q21	14.09	TFAM	Transcription factor A, mitochondrial	600438	
10q21	707.23	ANK3	Ankyrin 3, node of Ranvier (ankyrin G)	600465	
10q21	134.12	TET1	Tet methylcytosine dioxygenase 1	607790	

(continued)

Table 1
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Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
10q21	65.24	SGPL1	Sphingosine-1-phosphate lyase 1	603729	
10q21.1	16.52	CDK1	Cyclin-dependent kinase 1	116940	
10q21.3	175.08	LRRTM3	Leucine-rich repeat transmembrane neuronal 3	610869	
10q21.3	33.72	SIRT1	Sirtuin 1	604479	
10q22.2	1,780	CTNNA3	Catenin (cadherin-associated protein), alpha 3	607667	
10q22.2	6.40	PLAU	Plasminogen activator, urokinase	191840	Quebec platelet disorder
10q22.3	768.22	KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	600150	Generalized epilepsy and paroxysmal dyskinesia
10q23	1.38	CH25H	Cholesterol 25-hydroxylase	604551	
10q23.2-q23.3	38.34	LIPA	Lipase A, lysosomal acid, cholesterol esterase	613497	Cholesteryl ester storage disease, Wolman disease
10q23.3	105.34	PTEN	Phosphatase and tensin homolog	601728	Bannayan–Riley–Ruvalcaba syndrome, Cowden syndrome 1, endometrial carcinoma, Lhermitte–Duclos syndrome, macrocephaly/autism syndrome, malignant melanoma, PTEN hamartoma tumor syndrome, squamous cell carcinoma, thyroid carcinoma, VATER association with macrocephaly and ventriculomegaly, glioma, meningioma, prostate cancer
10q23.32	104.42	HECTD2	HECT domain containing E3 ubiquitin protein ligase 2	N/A	

10q23.33	5.73	HHEX	Hematopoietically expressed homeobox	604420	
10q23-q25	122.41	IDE	Insulin-degrading enzyme	146680	Type 2 diabetes mellitus
10q23-q25	624.13	SORCS3	Sortilin-related VPS10 domain containing receptor 3	606285	
10q23-q25	591.05	SORCS1	Sortilin-related VPS10 domain containing receptor 1	606283	
10q24	23.92	COX15	Cytochrome c oxidase assembly homolog 15 (yeast)	603646	Cardioencephalomyopathy due to cytochrome c oxidase deficiency 2, Leigh syndrome due to cytochrome c oxidase deficiency
10q24	69.20	ABCC2	ATP-binding cassette, subfamily C (CFTR/MRP), member 2	601107	Dubin–Johnson syndrome
10q24.1	25.25	FAS	Fas cell surface death receptor	134637	Autoimmune lymphoproliferative syndrome type IA, squamous cell carcinoma, autoimmune lymphoproliferative syndrome
10q24.2	134.34	DNMBP	Dynamin binding protein	611282	
10q24.3	50.88	ALDH18A1	Aldehyde dehydrogenase 18 family, member A1	138250	Cutis laxa type IIIA, hyperammonemia, hypoornithinemia, hypocitrullinemia, hypoargininemia, hypoprolinemia, cataracts, connective tissue diseases
10q24.3	7.00	CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	609300	17,20-lyase deficiency, 17-alpha-hydroxylase/17,20-lyase deficiency, pseudohermaphroditism, adrenal hyperplasia
10q24.31	17.82	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	604031	
10q24.33	261.38	SH3PXD2A	SH3 and PX domains 2A	N/A	
10q24.33	5.50	CALHM1	Calcium homeostasis modulator 1	612234	

(continued)

Table 1
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Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
10q25.1	13.27	GSTO1	Glutathione S-transferase omega 1	605482	
10q25.1	30.55	GSTO2	Glutathione S-transferase omega 2	612314	
10q25.3	2.86	ADRB1	Adrenoceptor beta 1	109630	Heart failure
10q26.3	128.60	EBF3	Early B-cell factor 3	607407	Glioblastoma multiforme, gastric carcinoma
10q26.3	53.38	HTRA1	HtrA serine peptidase 1	602194	CARASIL syndrome, macular degeneration
10q26.3	374.23	ADAM12	ADAM metallopeptidase domain 12	602714	
11p11.2	20.97	MAPK8IP1	Mitogen-activated protein kinase 8 interacting protein 1	604641	Noninsulin-dependent diabetes mellitus
11p13	67.17	BDNF	Brain-derived neurotrophic factor	113505	Central hypoventilation syndrome, anorexia nervosa, bulimia nervosa, memory impairment, obsessive-compulsive disorder
11p15	24.29	APBB1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	602709	
11p15.1	3.72	SAA1	Serum amyloid A1	104750	Atherosclerosis, rheumatoid arthritis, Crohn’s disease, neoplasms
11p15.5	3.40	DRD4	Dopamine receptor D4	126452	Autonomic nervous system dysfunction, novelty seeking personality, attention deficit-hyperactivity disorder
11p15.5	11.24	CTSD	Cathepsin D	116840	Breast cancer, neuronal ceroid lipofuscinosis type 10
11p15.5	1.43	INS	Insulin	176730	Insulin-dependent diabetes mellitus type 2, permanent neonatal diabetes mellitus, diabetes mellitus type 1, familial hyperproinsulinemia with or without diabetes

11p15.5	1.59	HBG2	Hemoglobin, gamma G	142250	Transient neonatal cyanosis
11q12	36.96	MS4A4A	Membrane-spanning 4-domains, subfamily A, member 4A	606547	
11q12.1	13.06	MS4A6A	Membrane-spanning 4-domains, subfamily A, member 6A	606548	
11q12.2	41.84	MS4A4E	Membrane-spanning 4-domains, subfamily A, member 4E	608401	
11q13	3.06	GSTP1	Glutathione S-transferase pi 1	134660	Cancer
11q13	14.31	INPPL1	Inositol polyphosphate phosphatase-like 1	600829	Opsismodysplasia, breast cancer
11q13.3	6.66	GAL	Galanin/GMAP prepropeptide	137035	
11q14	112.71	PICALM	Phosphatidylinositol binding clathrin assembly protein	603025	Acute myeloid leukemia, T-cell acute lymphoblastic leukaemia, malignant lymphomas
11q14.1	202.53	GAB2	GRB2-associated binding protein 2	606203	
11q22.2–q22.3	25.73	CASP4	Caspase 4, apoptosis-related cysteine peptidase	602664	
11q22.2–q22.3	20.87	IL18	Interleukin 18 (interferon-gamma-inducing factor)	600953	
11q22.3	8.33	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	120353	Epidermolysis bullosa dystrophica, arthritis, chronic obstructive pulmonary disease
11q22.3	7.82	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	185250	Coronary heart disease, arthritis
11q23	3.05	APOA5	Apolipoprotein A-V	606368	Hyperchylomicronemia, hypertriglyceridemia, hyperlipoproteinemia type V
11q23	2.59	APOA4	Apolipoprotein A-IV	107690	

(continued)

Table 1 (continued)

Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
11q23.2–q23.3	30.57	BACE1	Beta-site APP-cleaving enzyme 1	604252	
11q23.2–q24.2	181.56	SORL1	Sortilin-related receptor, L(DLR class) A repeats containing	602005	
11q23.3	3.16	APOC3	Apolipoprotein C-III	107720	Hyperalphalipoproteinemia 2, hypertriglyceridemia
11q23–q24	1.87	APOA1	Apolipoprotein A-I	107680	Amyloidosis, combined ApoA-I and apoC-III deficiency, corneal clouding, hypoalphalipoproteinemia, Tangier disease, systemic non-neuropathic amyloidosis
11q24	74.99	APLP2	Amyloid beta (A4) precursor-like protein 2	104776	
11q25	12.32	ACAD8	Acyl-CoA dehydrogenase family, member 8	604773	Isobutyryl-CoA dehydrogenase deficiency
12p11.23–q13.12	N/A	AD5	Alzheimer's disease 5	602096	
12p12	418.61	GRIN2B	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	138252	Mental retardation
12p12.1	7.11	IAPP	Islet amyloid polypeptide	147940	Diabetes type 2
12p13	4.55	PKP2P1	Plakophilin 2 pseudogene 1	602861	Arrhythmogenic right ventricular dysplasia 9
12p13	3.95	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	138400	
12p13	7.18	GNB3	Guanine nucleotide binding protein (G protein), beta polypeptide 3	139130	Essential hypertension, obesity
12p13.2	150.85	LRP6	Low-density lipoprotein receptor-related protein 6	603507	Coronary artery disease

12p13.2–p12.3	13.89	OLR1	Oxidized low-density lipoprotein (lectin-like) receptor 1	602601	Myocardial infarction, atherosclerosis
12p13.3	37.84	NCAPD2	Non-SMC condensin I complex, subunit D2	N/A	
12p13.31	10.31	TAPBPL	TAP binding protein-like	607081	
12p13.31	48.26	A2M	Alpha-2-macroglobulin	103950	Alpha-2-macroglobulin deficiency
12q12	144.28	LRRK2	Leucine-rich repeat kinase 2	609007	Parkinson disease 8
12q13	79.39	TFCP2	Transcription factor CP2	189889	
12q13	118.56	ATF7	Activating transcription factor 7	606371	
12q13.11	63.49	VDR	Vitamin D (1,25-dihydroxyvitamin D3) receptor	601769	Involutional osteoporosis
12q13.11	29.04	KANSL2	KAT8 regulatory NSL complex subunit 2	N/A	
12q13.11	28.54	CCNT1	Cyclin T1	143055	Neoplasms
12q13.3	84.86	LRP1	Low-density lipoprotein receptor-related protein 1	107770	
12q14	45.33	CAND1	Cullin-associated and neddylation-dissociated 1	607727	
12q23-q24.1	5.68	PLA2G1B	Phospholipase A2, group IB (pancreas)	172410	
12q24.11	61.42	KIAA1033	KIAA1033	N/A	
12q24.2	43.10	ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	100650	Esophageal cancer alcohol-related
12q24.2–q24.31	153.66	NOS1	Nitric oxide synthase 1	163731	Stroke
13q14–q21	85.20	HTR2A	5-Hydroxytryptamine (serotonin) receptor 2A, G protein-coupled	182135	Susceptibility to alcohol dependence, anorexia nervosa, major depressive disorder in response to citalopram therapy, obsessive-compulsive disorder, schizophrenia, seasonal affective disorder

(continued)

Table 1
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Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
13q14.3	103.19	ATP7B	ATPase, Cu++ transporting, beta polypeptide	606882	Wilson disease
13q22.1	18.54	KLF5	Kruppel-like factor 5 (intestinal)	602903	
13q34	25.17	DAOA	D-Amino acid oxidase activator	607408	Schizophrenia, bipolar affective disorder
14q13.1	8.63	PNP	Purine nucleoside phosphorylase	164050	Immunodeficiency due to purine nucleoside phosphorylase deficiency
14q21	114.25	SOS2	Son of sevenless homolog 2 (Drosophila)	601247	
14q22.1	241.61	FRMD6	FERM domain containing 6	614555	
14q23.2	111.52	ESR2	Estrogen receptor 2 (ER beta)	601663	
14q24	71.97	MTHFD1	Methylenetetrahydrofolate dehydrogenase (NADP+dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	172460	Abruptio placentae, spina bifida folate-sensitive
14q24.3	87.26	PSEN1	Presenilin 1	104311	Familial acne inversa 3, dilated cardiomyopathy 1U, frontotemporal dementia, Pick disease
14q24.3	13.44	NPC2	Niemann–Pick disease, type C2	601015	Niemann–pick disease type C2
14q24.3	21.86	DLST	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	126063	
14q24.3	3.46	FOS	FBJ murine osteosarcoma viral oncogene homolog	164810	
14q24.3	5.82	NGB	Neuroglobin	605304	
14q31	62.31	SEL1L	Sel-1 suppressor of lin-12-like (*C. elegans*)	602329	
14q32	76.35	MOK	MOK protein kinase	605762	

14q32.1	13.95	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	107400	Emphysema due to AAT deficiency, emphysema-cirrhosis due to AAT deficiency, hemorrhagic diathesis due to antithrombin Pittsburgh, chronic obstructive pulmonary disease
14q32.1	11.68	SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	107280	Alpha-1-antichymotrypsin deficiency, occlusive cerebrovascular disease
14q32.1	42.88	CYP46A1	Cytochrome P450, family 46, subfamily A, polypeptide 1	604087	
14q32.3	72.36	KLC1	Kinesin light chain 1	600025	
14q32.32	26.40	AKT1	V-akt murine thymoma viral oncogene homolog 1	164730	Breast cancer, colorectal cancer, Cowden syndrome 6, ovarian cancer, proteus syndrome, schizophrenia
15q11–q12	196.68	APBA2	Amyloid beta (A4) precursor protein-binding, family A, member 2	602712	
15q13.1	32.42	CHRFAM7A	CHRNA7 (cholinergic receptor, nicotinic, alpha 7, exons 5–10) and FAM7A (family with sequence similarity 7A, exons A-E) fusion	609756	
15q14	139.70	CHRNA7	Cholinergic receptor, nicotinic, alpha 7 (neuronal)	118511	Schizophrenia, myoclonic epilepsy
15q21.1	130.54	CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	107910	Aromatase deficiency, aromatase excess syndrome
15q21.1	2.09	EID1	EP300 interacting inhibitor of differentiation 1	605894	
15q21–q23	136.90	LIPC	Lipase, hepatic	151670	Hepatic lipase deficiency, noninsulin-dependent diabetes mellitus
15q22	153.67	ADAM10	ADAM metallopeptidase domain 10	602192	
15q22.2	31.58	APH1B	APH1B gamma secretase subunit	607630	

(continued)

Table 1
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Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
15q22.31	48.35	SNX1	Sorting nexin 1	601272	
15q22.33	129.34	SMAD3	SMAD family member 3	603109	
15q24	28.24	CHRNA3	Cholinergic receptor, nicotinic, alpha 3 (neuronal)	118503	Lung cancer
15q24.1	21.11	CSK	C-src tyrosine kinase	124095	
15q25.1	63.28	IREB2	Iron-responsive element binding protein 2	147582	
15q26	150.50	MEF2A	Myocyte enhancer factor 2A	600660	Coronary artery disease 1 with myocardial infarction
16p13.3	17.87	UBE2I	Ubiquitin-conjugating enzyme E2I	601661	
16p13.3	14.60	MEFV	Mediterranean fever	608107	Familial Mediterranean fever
16q12	29.56	VPS35	Vacuolar protein sorting 35 homolog (*S. cerevisiae*)	601501	Parkinson disease 17
16q21	21.92	CETP	Cholesteryl ester transfer protein, plasma	118470	Hyperalphalipoproteinemia
16q22	28.10	NAE1	NEDD8 activating enzyme E1 subunit 1	603385	
16q22.1	17.23	NQO1	NAD(P)H dehydrogenase, quinone 1	125860	Tardive dyskinesia, cancer
17p11.2	25.66	SREBF1	Sterol regulatory element binding transcription factor 1	184756	
17p12	139.28	COX10	Cytochrome c oxidase assembly homolog 10 (yeast)	602125	Charcot–Marie–Tooth type 1A, hereditary neuropathy with liability to pressure palsies
17p13	72.14	MYH13	Myosin, heavy chain 13, skeletal muscle	603487	
17p13.1	8.80	TNK1	Tyrosine kinase, nonreceptor, 1	608076	

17p13.1	19.15	TP53	Tumor protein p53	191170	Adrenal cortical carcinoma, breast cancer, choroid plexus papilloma, colorectal cancer, hepatocellular carcinoma, Li–Fraumeni syndrome, nasopharyngeal carcinoma, osteosarcoma, pancreatic cancer, basal cell carcinoma 7, glioma
17p13.1	31.63	MYH8	Myosin, heavy chain 8, skeletal muscle, perinatal	160741	Carney complex variant, trismus-pseudocamptodactyly syndrome
17q	2.22	PNMT	Phenylethanolamine N-methyltransferase	171190	Essential hypertension
17q11.2	4.17	CDK5R1	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	603460	
17q11.2	43.97	BLMH	Bleomycin hydrolase	602403	
17q11.2	39.58	SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	182138	Sudden infant death syndrome, aggressive behavior, depression, obsessive-compulsive disorder
17q11.2	31.68	THRA	Thyroid hormone receptor, alpha	190120	Hypothyroidism nongoitrous 6
17q11.2	0,086	MIR144	microRNA 144	612070	
17q11.2-q12	43.76	NOS2	Nitric oxide synthase 2, inducible	163730	Hypertension
17q11.2-q12	1.93	CCL2	Chemokine (C-C motif) ligand 2	158105	Psoriasis, rheumatoid arthritis, atherosclerosis, spina bifida
17q12	1.91	CCL3	Chemokine (C-C motif) ligand 3	182283	Human immunodeficiency virus type 1
17q21.1	133.95	MAPT	Microtubule-associated protein tau	157140	Pick disease, frontotemporal dementia, cortico-basal degeneration, progressive supranuclear palsy, Parkinson disease, tauopathy, and respiratory failure

(continued)

Table 1
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Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
17q21.1	0,445	STH	Saitohin	607067	
17q21.32	7.98	GRN	Granulin	138945	Frontotemporal lobar degeneration with ubiquitin-positive inclusions, primary progressive aphasia, neuronal ceroid lipofuscinosis 11
17q21-q22	N/A	GPSC	Gliosis, familial progressive subcortical	N/A	
17q23.1	11.08	MPO	Myeloperoxidase	606989	Myeloperoxidase deficiency
17q23.2	83.06	APPBP2	Amyloid beta precursor protein (cytoplasmic tail) binding protein 2	605324	
17q23.3	21.32	ACE	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	106180	Renal tubular dysgenesis, benign serum increase of angiotensin I-converting enzyme, myocardial infarction, stroke, severe acute respiratory syndrome, microvascular complications of diabetes 3
17q24.3	158.72	BPTF	Bromodomain PHD finger transcription factor	601819	
17q24–q25	87.63	GRB2	Growth factor receptor-bound protein 2	108355	
18p11.2	33.49	MC2R	Melanocortin 2 receptor (adrenocorticotropic hormone)	607397	Glucocorticoid deficiency, due to ACTH unresponsiveness
18q12.1	33.62	DSC1	Desmocollin 1	125643	
18q12.1	7.26	TTR	Transthyretin	176300	Amyloidotic polyneuropathy, euthyroid hyperthyroxinaemia, amyloidotic vitreous opacities, cardiomyopathy, oculoleptomeningeal amyloidosis, meningocerebrovascular amyloidosis, carpal tunnel syndrome

19p13	14.48	PIN1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1	601052	Cancer
19p13.2	113.86	DNM2	Dynamin 2	602378	Axonal Charcot–Marie–Tooth disease type 2M, Charcot–Marie–Tooth disease type B, centronuclear myopathy
19p13.2	44.47	LDLR	Low-density lipoprotein receptor	606945	Familial hypercholesterolemia
19p13.2	N/A	AD9	Alzheimer's disease 9	608907	
19p13.2–p13.1	41.35	NOTCH3	Notch 3	600276	Cerebral arteriopathy with subcortical infarcts and leukoencephalopathy
19p13.3–13.2	42.82	C3	Complement component 3	120700	C3 deficiency, atypical hemolytic uremic syndrome, macular degeneration age-related 9
19p13.3	27.06	GNA11	Guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	139313	
19p13.3	25.47	ABCA7	ATP-binding cassette, subfamily A (ABC1), member 7	605414	
19p13.3	10.90	APBA3	Amyloid beta (A4) precursor protein-binding, family A, member 3	604262	
19p13.3	9.29	GRIN3B	Glutamate receptor, ionotropic, N-methyl-D-aspartate 3B	606651	
19p13.3–p13.2	181.75	INSR	Insulin receptor	147670	Diabetes mellitus insulin-resistant with acanthosis nigricans, hyperinsulinemic hypoglycemia 5, leprechaunism, Rabson–Mendenhall syndrome
19p13.3–p13.2	15.78	ICAM1	Intercellular adhesion molecule 1	147840	
19q13	12.47	TOMM40	Translocase of outer mitochondrial membrane 40 homolog (yeast)	608061	

(continued)

Table 1
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Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
19q13.1	23.02	TGFB1	Transforming growth factor, beta 1	190180	Camurati–Engelmann disease
19q13.1	11.30	APLP1	Amyloid beta (A4) precursor-like protein 1	104775	
19q13.12	11.91	GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	609169	
19q13.12	1.41	PSENEN	Presenilin enhancer 2 homolog (*C. elegans*)	607632	Acne inversa 2
19q13.2	43.09	PVRL2	Poliovirus receptor-related 2 (herpesvirus entry mediator B)	600798	Multiple sclerosis
19q13.2	3.61	APOE	Apolipoprotein E	107741	Hyperlipoproteinemia type III, lipoprotein glomerulopathy, sea-blue histiocyte disease, macular degeneration, myocardial infarction
19q13.2	4.69	APOC1	Apolipoprotein C-I	107710	
19q13.2	3.26	APOC4	Apolipoprotein C-IV	600745	Coronary artery disease
19q13.2	3.58	APOC2	Apolipoprotein C-II	608083	Hyperlipoproteinemia type Ib
19q13.2	12.36	BCAM	Basal cell adhesion molecule (Lutheran blood group)	612773	
19q13.3	38.76	CLPTM1	Cleft lip and palate associated transmembrane protein 1	612585	
19q13.3	14.94	CD33	CD33 molecule	159590	
19q13.3	6.61	NR1H2	Nuclear receptor subfamily 1, group H, member 2	600380	
19q13.3	54.03	MARK4	MAP/microtubule affinity-regulating kinase 4	606495	

19q13.32	21.59	EXOC3L2	Exocyst complex component 3-like 2	N/A	
19q13.32	3.06	BLOC1S3	Biogenesis of lysosomal organelles complex-1, subunit 3	609762	Hermansky–Pudlak syndrome 8
19q13.33	47.86	CARD8	Caspase recruitment domain family, member 8	609051	Rheumatoid arthritis
19q13.43	9.76	GALP	Galanin-like peptide	611178	Neuroblastic tumor
20p	N/A	AD8	Alzheimer's disease 8	607116	
20p11.21	4.28	CST3	Cystatin C	604312	Cerebral amyloid angiopathy, macular degeneration 11
20p13	15.44	PRNP	Prion protein	176640	Creutzfeldt–Jakob disease, fatal familial insomnia, Gerstmann–Straussler disease, Huntington disease-like 1, kuru, prion disease
20pter-p12	6.55	PRND	Prion protein 2 (dublet)	604263	
20q13.2–q13.3	18.09	CHRNA4	Cholinergic receptor, nicotinic, alpha 4 (neuronal)	118504	Nocturnal frontal lobe epilepsy type 1
20q13.31	5.38	PCK1	Phosphoenolpyruvate carboxykinase 1 (soluble)	614168	Cytosolic phosphoenolpyruvate carboxykinase deficiency
21q11	98.17	SAMSN1	SAM domain, SH3 domain, and nuclear localization signals 1	607978	
21q21.1	134.54	TMPRSS15	Transmembrane protease, serine 15	606635	Enterokinase deficiency
21q21.1	541.88	NCAM2	Neural cell adhesion molecule 2	602040	
21q21.3	290.59	APP	Amyloid beta (A4) precursor protein	104760	Cerebral amyloid angiopathy
21q22.1	291.96	KCNJ6	Potassium inwardly rectifying channel, subfamily J, member 6	600877	
21q22.11	102.95	EVA1C	Eva-1 homolog C (*C. elegans*)	N/A	

(continued)

Table 1
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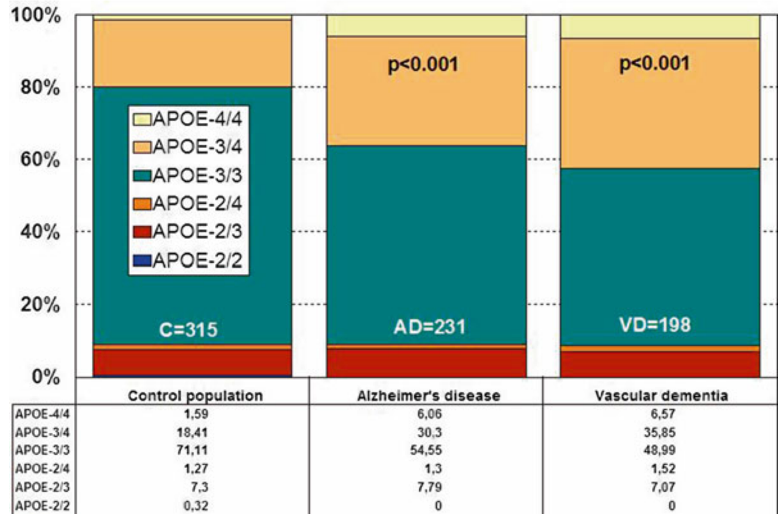
Locus	Size (kb)	Symbol	Title/gene	OMIM	Other related diseases
21q22.11	3.79	DNAJC28	DnaJ (Hsp40) homolog, subfamily C, member 28	N/A	
21q22.13	147.82	DYRK1A	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	600855	Down syndrome, mental retardation 7
21q22.2	129.73	DOPEY2	Dopey family member 2	604803	
21q22.3	261.50	RUNX1	Runt-related transcription factor 1	151385	Acute myeloid leukemia, platelet disorder with associated myeloid malignancy
21q22.3	108.80	BACE2	Beta-site APP-cleaving enzyme 2	605668	Down syndrome
21q22.3	97.56	ABCG1	ATP-binding cassette, subfamily G (WHITE), member 1	603076	
21q22.3	23.17	CBS	Cystathionine-beta-synthase	613381	Cystathionine beta-synthase deficiency, homocystinuria, hyperhomocysteinemic thrombosis
21q22.3	50.19	MCM3AP	Minichromosome maintenance complex component 3 associated protein	603294	
21q22.3	6.50	S100B	S100 calcium binding protein B	176990	Down syndrome, epilepsy, amyotrophic lateral sclerosis, melanoma, type I diabetes
22q11.21	28.24	COMT	Catechol-O-methyltransferase	116790	Panic disorder, schizophrenia
22q11.21	26.88	RTN4R	Reticulon 4 receptor	605566	Schizophrenia
22q11.23	0.845	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	153620	Rheumatoid arthritis
22q11.23	8.15	GSTT1	Glutathione S-transferase theta 1	600436	Carcinoma

22q13.1	13.15	HMOX1	Heme oxygenase (decycling) 1	141250	Heme oxygenase-1 deficiency, chronic obstructive pulmonary disease
22q13.2	21.30	SEPT3	Septin 3	608314	
22q13.31	93.16	PPARA	Peroxisome proliferator-activated receptor alpha	170998	Hyperapobetalipoproteinemia
Xp11.2	3.12	HSD17B10	Hydroxysteroid (17-beta) dehydrogenase 10	300256	17-Beta-hydroxysteroid dehydrogenase X deficiency, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency, mental retardation
Xp11.23	115.86	MAOB	Monoamine oxidase B	309860	
Xp11.3	91.92	MAOA	Monoamine oxidase A	309850	Brunner syndrome
Xp11.3–p11.23	4.50	TIMP1	TIMP metallopeptidase inhibitor 1	305370	
Xp21.1	68.97	OTC	Ornithine carbamoyltransferase	300461	Ornithine transcarbamylase deficiency, Duchenne muscular dystrophy
Xq12	186.59	AR	Androgen receptor	313700	Spinal and bulbar muscular atrophy of Kennedy, prostate cancer, complete androgen insensitivity, hypospadia type 1
Xq21.3	843.97	PCDH11X	Protocadherin 11 X-linked	300246	
Xq21.3	N/A	AD16	Alzheimer’s disease 16	300756	



	Control population	Alzheimer's disease	Vascular dementia
APOE-4/4	1,59	6,06	6,57
APOE-3/4	18,41	30,3	35,85
APOE-3/3	71,11	54,55	48,99
APOE-2/4	1,27	1,3	1,52
APOE-2/3	7,3	7,79	7,07
APOE-2/2	0,32	0	0

Fig. 4 Distribution and frequency of *APOE* genotypes in Alzheimer's disease and vascular dementia. (Adapted from ref. 19)

4 Pathogenic Events

The dual amyloidogenic-tauopathic theory of AD has dominated the pathogenic universe of AD-related neurodegeneration (and divided the research community as well) for the past 50 years, nourished by the presence of *APP*, *PSEN1*, *PSEN2*, and *MAPT* mutations in a very small number of cases with early-onset AD (EOAD); however, this theory does not explain in full AD pathogenesis, and consequently novel (or complementary) theories have been emerging during the past decades and in recent times. A summary of the pathogenic events in AD includes the following.

4.1 Genomic Defects

As a complex polygenic/multifactorial disorder, in which hundreds of polymorphic variants of risk might be involved (*see* Table 1) (*see* Fig. 3), AD fulfills the "golden rule" of complex disorders, according to which the larger the number of genetic defects distributed in the human genome, the earlier the onset of the disease and the poorer its therapeutic response to conventional treatments; and the smaller the number of pathogenic SNPs, the later the onset of the disease, and the better the therapeutic response to different pharmacological interventions [12, 17, 28, 31–35].

Genetic variation associated with different diseases interferes with microRNA-mediated regulation by creating, destroying, or modifying microRNA (miRNA) binding sites. miRNA-target variability is a ubiquitous phenomenon in the adult human brain, which may influence gene expression in physiological and pathological conditions. AD-related SNPs interfere with miRNA gene

regulation and affect AD susceptibility. The significant interactions include target SNPs present in seven genes related to AD prognosis with the miRNAs-miR-214, -23a & -23b, -486-3p, -30e*, -143, -128, -27a & -27b, -324-5p, and -422a. The dysregulated miRNA network contributes to the aberrant gene expression in AD [36–38].

4.2 Epigenetic Phenomena

Epigenetic factors have emerged as important mediators of development and aging, gene–gene and gene–environmental interactions, and the pathophysiology of complex disorders. Major epigenetic mechanisms (DNA methylation, histone modifications and chromatin remodeling, and noncoding RNA regulation) may contribute to AD pathology [37–39].

4.3 Cerebrovascular Dysfunction

Vascular and metabolic dysfunctions are key components in AD pathology throughout the course of the disease. Concurrent cerebrovascular disease is a common neuropathological finding in aged subjects with dementia, is more common in AD than in other neurodegenerative disorders, and lowers the threshold for dementia due to AD and α-synucleinopathies [40]. Global brain hypoperfusion, oxygen hypometabolism, and neurovascular decoupling present in patients with mild cognitive impairment (MCI) indicate that changes in cerebral hemodynamics occur early at a prodromal stage of AD [41]. Although common denominators between vascular and metabolic dysfunction are oxidative stress and Aβ [42], genetic factors and cardiovascular risk factors may also account for the cerebrovascular damage present in AD [43]. Inherited polymorphisms of the vascular susceptibility gene Ninjurin2 (*NINJ2*) are associated with AD risk [44]. Endothelial dysfunction has been implicated as a crucial event in the development of AD. Breakdown of the blood–brain barrier (BBB) as a result of disruption of tight junctions and transporters leads to increased leukocyte transmigration and is an early event in the pathology of many CNS disorders. BBB breakdown leads to neuroinflammation and oxidative stress, with mitochondrial dysfunction. The high concentration of mitochondria in cerebrovascular endothelial cells might account for the sensitivity of the BBB to oxidant stressors [45, 46]. BBB dysfunction may contribute to AD through a number of mechanisms that could be initiated in the presence or absence of Aβ pathology [47].

Chronic brain hypoperfusion may be sufficient to induce premature neuronal death and dementia in vulnerable subjects [17, 31, 32, 48–50]. *APOE*-related changes in cortical oxygenation and hemoglobin consumption are evident, as revealed by brain optical topography analysis, reflecting that *APOE-4* carriers exhibit deficient brain hemodynamics and a poorer panneocortical oxygenation than *APOE-3* or *APOE-2* carriers [19] (*see* Fig. 5).

Hypoperfusion in frontal, parietal, and temporal regions is a common finding in AD. White matter hyperintensities correlate

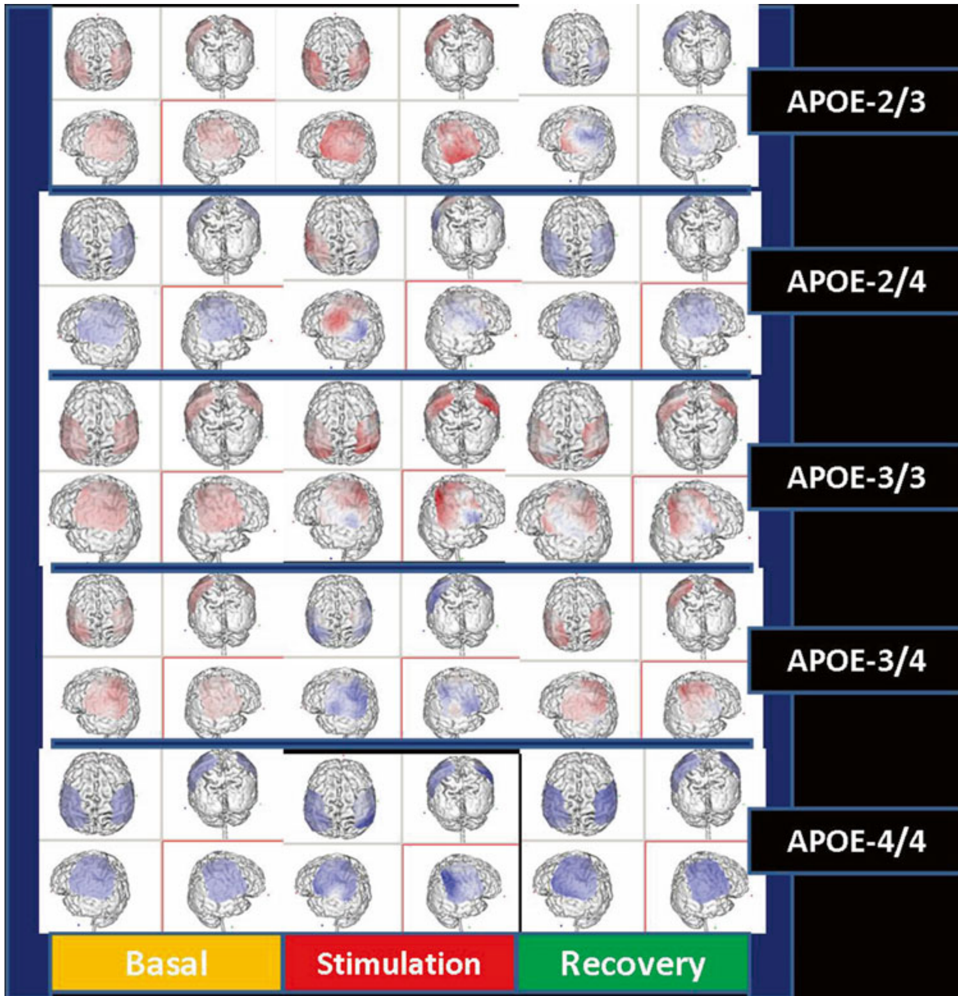


Fig. 5 *APOE*-related brain optical topography mapping in AD patients

with age and with disease severity [51]. Cerebral amyloid angiopathy (CAA) accounts for the majority of primary lobar intracerebral hemorrhages (ICH) among the elderly and represents the cause of 20 % of spontaneous ICHs in patients over 70 years of age. The basis for this disease process is the deposition and formation of eventually destructive amyloid plaques in the walls of brain vessels, predominantly arterial but not excluding venules and capillaries. CAA and CAA-associated microhemorrhages may also participate in the pathogenesis of AD [52]. Aβ deposition in asymptomatic elderly individuals is associated with lobar microhemorrhage (LMH). LMH is present in 30.8 % of AD, 35.7 % of MCI, and 19.1 % of controls [53]. Neurovascular dysfunction in AD leads to reduced clearance across the BBB and accumulation of neurotoxic Aβ peptides in the brain. The ABC transport protein P-glycoprotein

(P-gp, *ABCB1*) is involved in the export of Aβ from the brain into the blood. *P-gp*, *LRP1*, and *RAGE* mRNA expression is reduced in mice treated with $A\beta_{1-42}$. In addition to the age-related decrease in P-gp expression, $A\beta_{1-42}$ itself downregulates the expression of P-gp and other Aβ-transporters, which could exacerbate the intracerebral accumulation of Aβ and thereby accelerate neurodegeneration in AD and cerebral β-amyloid angiopathy [54].

Imfeld et al. [55] observed that the incidence rates of ischemic stroke for patients with AD, VD, or no dementia were 4.7/1,000 person-years (PYs), 12.8/1,000 PYs, and 5.1/1,000 PYs, respectively. Compared with dementia-free patients, the odds ratio of developing a transitory ischemic attack (TIA) for patients with AD treated with atypical antipsychotic drugs was 4.5. According to these results, patients with VD, but not AD, have a higher risk of developing an ischemic stroke than those without dementia. In patients with AD, but not VD, use of atypical antipsychotic drugs was associated with an increased risk of TIA.

4.4 Phenotypic Expression of Amyloid Deposits and Neurofibrillary Tangles (NFTs)

β-Amyloid deposits in senile and neuritic plaques and hyperphosphorylated tau proteins in NFTs are extracellular and intracellular expressions, respectively, of the AD neuropathological phenotype, together with selective neuronal loss in hippocampal and neocortical regions. Aβ plaque in the brain is the primary (postmortem) diagnostic criterion of AD. The main component of senile plaques is Aβ, a 39–43 amino acid peptide, generated by the proteolytic cleavage of APP by the action of β- and γ-secretases. Aβ is neurotoxic and the neurotoxicity of Aβ is related to its aggregation state [17, 19–21, 23].

4.5 Neuronal Apoptosis

Neuronal loss is a pathognomonic finding in AD and the final common path of multiple pathogenic mechanisms leading to neurodegeneration in dementia. Atrophy of the medial temporal lobe, especially the hippocampus and the parahippocampal gyrus, is considered to be the most predictive structural brain biomarker for AD. The medial and posterior parts of the parietal lobe seem to be preferentially affected, compared to the other parietal lobe parts [19].

4.6 Neurotransmitter Deficits

An imbalance of different neurotransmitters (glutamate, acetylcholine, noradrenaline, dopamine, serotonin, some neuropeptides) has been proposed as the neurobiological basis of behavioral symptoms in AD. Altered reuptake of neurotransmitters by vesicular glutamate transporters (VGLUTs), excitatory amino acid transporters (EAATs), the vesicular acetylcholine transporter (VAChT), the serotonin reuptake transporter (SERT), or the dopamine reuptake transporter (DAT) is involved in the neurotransmission imbalance in AD. Protein and mRNA levels of VGLUTs, EAAT1-3, VAChT, and SERT are reduced in AD [56].

4.7 Oxidative Stress

Oxidative damage is a classic pathogenic mechanism of neurodegeneration [46, 57, 58]. Oxidative damage is greater in brain tissue from patients with AD than age-matched controls. Tayler et al. [59] studied the timing of this damage in relation to other pathogenic processes in AD. Antioxidant capacity is elevated in AD and directly related to disease severity as indicated by Braak tangle stage and the amount of insoluble Aβ. Accumulation of Aβ has been shown in brain mitochondria of AD patients and of AD transgenic mouse models. The presence of Aβ in mitochondria leads to free radical generation and neuronal stress. A novel mitochondrial Aβ-degrading enzyme, presequence protease (PreP), has been identified in the mitochondrial matrix. hPreP activity is decreased in AD brains and in the mitochondrial matrix of AD transgenic mouse brains (Tg mAβPP and Tg mAβPP/ABAD). Mitochondrial fractions isolated from AD brains and Tg mAβPP mice have higher levels of 4-hydroxynonenal, an oxidative product. Activity of cytochrome c oxidase is significantly reduced in the AD mitochondria. Decreased PreP proteolytic activity, possibly due to enhanced reactive oxygen species (ROS) production, may contribute to Aβ accumulation in mitochondria leading to the mitochondrial toxicity and neuronal death in AD [60]. There is an age-dependent increase in oxidative stress markers, loss of lipid asymmetry, and Aβ production and amyloid deposition in the brain of APP/PS1 mice. Proteomic analysis of $APP^{NLh}/APP^{NLh} \times PS\text{-}1^{P246L}/PS\text{-}1^{P246L}$ human double mutant knock-in APP/PS-1 mice revealed specific targets of brain protein carbonylation in an age-dependent manner [61].

4.8 Cholesterol and Lipid Metabolism Dysfunction

Cholesterol seems to be intimately linked with the generation of amyloid plaques, which is central to the pathogenesis of AD. *APOE* variants are determinant in cholesterol metabolism and diverse forms of dyslipoproteinemia [12, 62, 63]. Cholesterol protects the Aβ-induced neuronal membrane disruption and inhibits β-sheet formation of Aβ on the lipid bilayer [64]. Jones et al. [65] found a significant overrepresentation of association signals in pathways related to cholesterol metabolism and the immune response in both of the two largest genome-wide association studies for late-onset Alzheimer's disease (LOAD). Intracellular lipid metabolism is perturbed in cardiovascular and neurodegenerative diseases with genetic and lifestyle components. Neural membranes contain several classes of glycerophospholipids (GPs) that not only constitute their backbone but also provide the membrane with a suitable environment, fluidity, and ion permeability. GP and GP-derived lipid mediators may be involved in AD pathology. Degradation of GPs by phospholipase A_2 can release two important brain polyunsaturated fatty acids (PUFAs), arachidonic acid and docosahexaenoic acid. Nonenzymatic and enzymatic oxidation of these PUFAs produces several lipid mediators, all closely associated with neuronal pathways involved in AD neurobiology [66].

4.9 Neuroinflammation and Immunopathology

Several genes associated with immune regulation and inflammation show polymorphic variants of risk in AD, and abnormal levels of diverse cytokines have been reported in the brain, cerebrospinal fluid (CSF) and plasma, of patients with AD [17, 63]. The activation of inflammatory cascades has been consistently demonstrated in the pathophysiology of AD. Reactive microglia are associated with Aβ deposit and clearance in AD. Resident microglia fail to trigger an effective phagocytic response to clear Aβ deposits although they mainly exist in an "activated" state. Oligomeric Aβ (oAβ) can induce more potent neurotoxicity when compared with fibrillar Aβ (fAβ). $A\beta_{(1-42)}$ fibrils, not $A\beta_{(1-42)}$ oligomers, increased the microglial phagocytosis [67]. Among several putative neuroinflammatory mechanisms, the TNF-α signaling system has a central role in this process. TNF-α levels are altered in serum and CSF in AD. Glial cells play important roles in local CNS inflammation. The miR-181 family is developmentally regulated and present in high amounts in astrocytes compared to neurons. Overexpression of miR-181c in cultured astrocytes results in increased cell death when exposed to lipopolysaccharide (LPS). miR-181 expression is altered by exposure to LPS in both wild-type and transgenic mice lacking both receptors for the inflammatory cytokine TNF-α. Knockdown of miR-181 enhanced LPS-induced production of proinflammatory cytokines (TNF-α, IL-6, IL-1β, IL-8) and HMGB1, while overexpression of miR-181 resulted in a significant increase in the expression of the anti-inflammatory cytokine IL-10 [68]. The abnormal production of inflammatory factors may accompany the progression from MCI to dementia. Abnormal activation of TNF-α signaling system, represented by increased expression of sTNFR1, is associated with a higher risk of progression from MCI to AD [69].

4.10 Neurotoxic Factors

Old and new theories suggest that different toxic agents, from metals (i.e., aluminum, copper, zinc, iron) to biotoxins and pesticides, might contribute to neurodegeneration. Dysfunctional homeostasis of transition metals is believed to play a role in the pathogenesis of AD [19]. Methylazoxymethanol (MAM), the genotoxic metabolite of the cycad azoxyglucoside cycasin, induces genetic alterations in bacteria, yeast, plants, insects, and mammalian cells, but adult nerve cells are thought to be unaffected. Kisby et al. [70] showed that the brains of adult C57BL6 wild-type mice treated with a single systemic dose of MAM acetate display DNA damage (O^6-methyldeoxyguanosine lesions, O^6-mG). MAM-treated mice lacking a functional gene encoding the DNA repair enzyme O^6-mG DNA methyltransferase (MGMT) showed elevated O^6-mG DNA damage. The DNA damage was linked to changes in the expression of genes in cell-signaling pathways associated with cancer, human neurodegenerative disease, and neurodevelopmental disorders.

4.11 Other Players

Many novel pathogenic mechanisms potentially involved in AD neurodegeneration have been proposed in recent times and the revival of some old hypotheses has also occurred. Examples of other pathogenic players in AD include the Ca^{2+} hypothesis [71], insulin resistance [72], nerve growth factor (NGF) imbalance [73], glycogen synthase kinase-3 (GSK-3), advanced glycation end products (AGEs) and their receptors (RAGE), the efflux transporter P-glycoprotein (P-gp), c-Abl tyrosine kinase [74], posttranscriptional protein alterations, compromising the proteasome system and the chaperon machinery (HSPB8-BAG3) [17, 63, 75], autophagy as a novel Aβ-generating pathway, hypocretin (orexin), cathepsin B [76], Nogo receptor proteins [77], adipocytokines and $CD34^{+}$ progenitor cells [78], CD147 [79], impairment of synaptic plasticity (PSD-95) [80], anomalies in neuronal cell division and apoptosis [81], stem cell factor, Serine-arginine protein kinases [82], Sirtuin deacetylases [83], telomere shortening [84], deficiency in repair of nuclear and mitochondrial DNA damage, and microRNAs [85]. The Ca^{2+} hypothesis focuses on the correlation between the dysfunction of brain Ca^{2+} homeostasis and the neurodegeneration process. An important contributing factor for AD might be the development of an unbalanced homeostasis of two signaling cations: calcium (Ca^{2+}) and zinc (Zn^{2+}). Both ions serve a critical role in the physiological functioning of the CNS, but their brain deregulation promotes amyloid-β dysmetabolism as well as tau phosphorylation. AD is also characterized by an altered glutamatergic activation, and glutamate can promote both Ca^{2+} and Zn^{2+} dyshomeostasis. The two cations can operate synergistically to promote the generation of free radicals that further intracellular Ca^{2+} and Zn^{2+} rises and set the stage for a self-perpetuating harmful loop. These phenomena might be some of the initial steps which activate the pathogenic cascade leading to AD [71].

Diabetic and prediabetic states, including insulin resistance, fasting hyperglycemia, and hyperinsulinemia, are associated with metabolic dysregulation which has been linked to increased risks of cognitive decline and AD. Metabolic dysregulation, especially insulin resistance, was associated with lower brain volumes and executive function in a large community-based cohort (Framingham Offspring Study) [72].

The neurotrophin NGF is essential for the maintenance and differentiation of basal forebrain cholinergic neurons. Alterations in NGF transport and signaling play a crucial role in sporadic AD neurodegeneration, leading to the hypothesis of "neurotrophic imbalance" as an upstream driver for sporadic AD [73].

The c-Abl tyrosine kinase participates in a variety of cellular functions, including regulation of the actin cytoskeleton, regulation of the cell cycle, and the apoptotic/cell cycle arrest response to stress; the Abl family of kinases also play a crucial role in development of the CNS. c-Abl activation has been reported in AD and

Parkinson's diseases and in mouse models and neuronal culture in response to amyloid β fibrils and oxidative stress. Overexpression of active c-Abl in adult mouse neurons results in neurodegeneration and neuroinflammation [74].

Posttranscriptional protein alterations, compromising the proteasome system and the chaperon machinery, are very relevant in neurodegeneration [63]. HSPB8 is a small heat shock protein that forms a complex with the co-chaperone BAG3. Overexpression of the HSPB8-BAG3 complex in cells stimulates autophagy and facilitates the clearance of mutated aggregation-prone proteins, whose accumulation is a hallmark of many neurodegenerative disorders. HSPB8-BAG3 could thus play a protective role in protein aggregation diseases and might be specifically upregulated in response to aggregate-prone protein-mediated toxicity. There is a strong upregulation of HSPB8 and a moderate upregulation of BAG3 specifically in astrocytes in the cerebral areas affected by neuronal damage and degeneration. The upregulation of HSPB8 and BAG3 may enhance the ability of astrocytes to clear aggregated proteins released from neurons and cellular debris; maintain the local tissue homeostasis and/or participate in the cytoskeletal remodeling that astrocytes undergo during astrogliosis [75].

Cathepsin B is suggested to be involved in Aβ processing. Plasma cathepsin B levels are higher in AD patients with no major changes in CSF [76].

Nogo receptor proteins (NgR1 to NgR3) regulate Aβ production via interaction with amyloid precursor protein (APP). A small region adjacent to the BACE1 cleavage site of APP mediates interaction of APP with Nogo receptor proteins. Increased interaction between Nogo receptor and APP reduces surface expression of APP and favors processing of APP by BACE1. When NgR2 was ablated in AD transgenic mice expressing Swedish APP and PS1ΔE9, amyloid deposition was clearly reduced. Downregulation of NgR expression might be a potential approach for inhibiting amyloid deposition in AD patients [77].

AD and atherosclerosis share common vascular risk factors such as arterial hypertension and hypercholesterolemia. Adipocytokines and $CD34^+$ progenitor cells are associated with the progression and prognosis of atherosclerotic diseases. Low plasma levels of leptin and increased numbers of $CD34^+$ progenitor cells are both associated with AD. Increased leptin plasma levels are associated with a reduced number of $CD34^+$ progenitor cells in AD patients. These findings point toward a combined involvement of leptin and $CD34^+$ progenitor cells in the pathogenesis of AD. Plasma levels of leptin and circulating $CD34^+$ progenitor cells could represent an important molecular link between atherosclerotic diseases and AD [78].

CD147, also known as basigin, EMMPRIN, neurothelin, TCSF, M6, HT7, OX47, or gp42, is a transmembrane

glycoprotein of the immunoglobulin superfamily. It is expressed in many neuronal and non-neuronal tissues including the hippocampus, prefrontal cortex thyroid, heart, early erythroid, amygdala, and placenta. This protein is involved in various cellular and biological functions, such as lymphocyte migration and maturation, tissue repair, cancer progression, T and B lymphocyte activation, and induction of extracellular matrix metalloproteinase. The CD147 protein interacts with other proteins such as cyclophilin A (CyPA), cyclophilin B (CyPB), sterol carrier protein (SCP), caveolin-1 and integrins, and can influence Aβ peptide levels [79].

Impairment of synaptic plasticity underlies memory dysfunction in AD. Molecules involved in this plasticity such as PSD-95, a major postsynaptic scaffold protein at excitatory synapses, may play an important role in AD pathogenesis. Either Aβ or tau can induce reduction of PSD-95 in excitatory synapses in hippocampus. This PSD-95 reduction is not an early event but occurs as the pathologies advance. Thus, the time-dependent PSD-95 reduction from synapses and accumulation in neuronal soma in transgenic mice and Hirano bodies in AD may mark postsynaptic degeneration that underlies long-term functional deficits [80].

Most cells undergo cell cycles up to 40–60 times in life, but neurons remain in a nondividing, nonreplicating phase. Neurons do not complete cell division, eventually entering apoptosis. Like cancer, AD may involve dysfunction in neuronal cell cycle reentry, leading to the development of the two-hit hypothesis of AD. The first hit is abnormal cell cycle reentry, which typically results in neuronal apoptosis and prevention of AD; with the second hit of chronic oxidative damage preventing apoptosis, neurons gain "immortality" analogous to tumor cells. According to Moh et al. [81], once both of these hits are activated, AD can develop and produce senile plaques and NFTs throughout brain tissue.

Telomere shortening represents one of the molecular causes of aging that limits the proliferative capacity of cells, including neural stem cells. Aged telomerase knockout mice with short telomeres (G3Terc$^{-/-}$) exhibit reduced dentate gyrus neurogenesis and loss of neurons in hippocampus and frontal cortex, associated with short-term memory deficit in comparison to mice with long telomere reserves (Terc$^{+/+}$). In contrast, telomere shortening improved the spatial learning ability of aging APP23 transgenic mice. Telomere shortening was also associated with an activation of microglia in aging amyloid-free brain. In APP23 transgenic mice, telomere shortening reduced both amyloid plaque pathology and reactive microgliosis. According to data reported by Rolyan et al. [84], telomere shortening, despite impairing adult neurogenesis and maintenance of post-mitotic neurons, can slow down the progression of amyloid plaque pathology in AD.

microRNA-146a (miRNA-146a) is an inducible, 22 nucleotide, small RNA overexpressed in AD brain. Upregulated

miRNA-146a targets several inflammation-related and membrane-associated messenger RNAs (mRNAs), including those encoding complement factor-H (CFH) and the interleukin-1 receptor-associated kinase-1 (IRAK-1), resulting in significant decreases in their expression. The most significant miRNA-146a-CFH changes are found in HMG cells, the "resident scavenging macrophages" of the brain [85].

Sirtuin enzymes are a family of highly conserved protein deacetylases that depend on nicotinamide adenine dinucleotide (NAD^+) for their activity. There are seven sirtuins in mammals and these proteins have been linked with caloric restriction and aging by modulating energy metabolism, genomic stability, and stress resistance. Modulation of sirtuin activity has been shown to impact the course of several aggregate-forming neurodegenerative disorders. Sirtuins can influence the progression of neurodegenerative disorders by modulating transcription factor activity and directly deacetylating proteotoxic species. Sirtuin deacetylases are also candidate targets for therapeutic intervention [83].

5 Biomarkers and Comorbidity

The phenotypic features of the disease represent the biomarkers to be used as diagnostic predictors and the expression of pathogenic events to be modified with an effective therapeutic intervention. Important differences have been found in the AD population as compared with healthy subjects in different biological parameters, including blood pressure, glucose, cholesterol and triglyceride levels, transaminase activity, hematological parameters, metabolic factors, thyroid function, brain hemodynamic parameters, and brain mapping activity [7, 31, 32, 63, 86–92]. These clinical differences indicate clear signs of comorbidity rather than typical features of AD. Blood pressure values, glucose levels, and cholesterol levels are higher in AD than in healthy elderly subjects. Approximately 20 % of AD patients are hypertensive, 25 % are diabetic, 50 % are hypercholesterolemic, and 23 % are hypertriglyceridemic. Over 25 % of the patients exhibit high GGT activity, 5–10 % show anemic conditions, 30–50 % show an abnormal cerebrovascular function characterized by poor brain perfusion, and over 60 % have an abnormal electroencephalographic pattern, especially in frontal, temporal, and parietal regions, as revealed by quantitative EEG (qEEG) or computerized mapping [7, 12, 63, 87].

Hypertension can lead to brain volume reduction, specifically in hippocampus, and may contribute to neurodegeneration [93].

Significant differences are currently seen between females and males, indicating the effect of gender on the phenotypic expression of the disease. In fact, the prevalence of dementia is 10–15 % higher in females than in males from 65 to 85 years of age. All these

parameters are highly relevant when treating AD patients because some of them reflect a concomitant pathology which also needs therapeutic consideration.

AD biomarkers can be differentiated within several categories: (1) neuropathological markers, (2) structural and functional neuroimaging markers, (3) neurophysiological markers (EEG, qEEG, brain mapping), (4) biochemical markers in body fluids (blood, urine, saliva, CSF), and (5) genomic markers (structural and functional genomics, proteomics, metabolomics).

5.1 Neuropathology

Plaques and tangles in the hippocampus and cortex are still considered the seminal findings in AD neuropathology, and conventional features to establish the boundary between amyloidopathies and tauopathies; however, both phenotypic markers are also present in normal brains, in over 60 % of cases with traumatic brain injury, and in many other brain disorders [94–96].

Steroid-responsive encephalopathies can be considered vasculitic or non-vasculitic. Clinical features are suggestive of Creutzfeldt–Jakob disease (CJD), dementia with Lewy bodies (DLB), and parkinsonism, but pathological examination revealed only AD-related findings without evidence of Lewy bodies or prion disease in most cases. AD is not diagnosed in life due to the atypical clinical features, lack of hippocampal atrophy on brain imaging, and a dramatic symptomatic response to steroids [97]. Some cases of new-variant CJD may also be misdiagnosed as AD.

The dentate gyrus is a major site of neuropathology in FTLD-TDP (frontotemporal lobar degeneration with transactive response DNA-binding protein of 43 kDa proteinopathy). Most laminae of the cerebral cortex are affected. GRN mutation cases are quantitatively different from sporadic cases while cases with associated hippocampal sclerosis and AD have increased densities of dystrophic neurites and abnormally enlarged neurons, respectively. There is little correlation between the subjective assessment of subtypes and the more objective quantitative data [98]. Atrophy of the corpus callosum in AD is independent of white matter lesions and may be associated with cognitive deterioration [99].

5.2 Structural and Functional Neuroimaging

Structural and functional neuroimaging techniques (MRI, fMRI, PET, SPECT) are essential diagnostic tools in dementia, though the specificity of the visual observations in degenerative forms of dementia is of doubtful value; however, these procedures are irreplaceable for differential diagnosis. Amyloid PET imaging is a novel diagnostic test that can detect in living humans amyloid-β deposition in the brain. The Amyloid Imaging Task Force of the Alzheimer's Association and Society for Nuclear Medicine and Molecular Imaging published appropriate use criteria for amyloid PET as an important tool for increasing the certainty of a diagnosis of AD in specific patient populations [100].

There is a characteristic regional impairment in AD that involves mainly the temporo-parietal association cortices, mesial temporal structures and, to a more variable degree, also the frontal association cortex. This pattern of functional impairment can provide a biomarker for the diagnosis of AD and other neurodegenerative dementias at the clinical stage of MCI, and for the monitoring of progression.

Lu et al. [101] used Tensor-based morphometry (TBM), a novel computational approach for visualizing longitudinal progression of brain atrophy, to determine whether cognitively intact elderly participants with the ε4 allele demonstrate greater volume reduction than those with the ε2 allele, and found that possession of the ε4 allele is associated with greater temporal and hippocampal volume reduction well before the onset of cognitive deficits.

Healthy young *APOE ε4* carriers have smaller hippocampal volumes than *APOE ε2* carriers. The difference in hippocampal morphology is cognitively/clinically silent in young adulthood, but could render *APOE ε4* carriers more prone to the later development of AD possibly due to lower reserve cognitive capacity [101]. LOAD patients have a selective parahippocampal white matter (WM) loss, while EOAD patients experience a more widespread pattern of posterior WM atrophy. The distinct regional distribution of WM atrophy reflects the topography of gray matter (GM) loss. *ApoE ε4* status is associated with a greater parahippocampal WM loss in AD. The greater WM atrophy in EOAD than LOAD fits with the evidence that EOAD is a more aggressive form of the disease [102]. Elderly normal *APOE ε2* (*APOE2*) carriers exhibit slower rates of hippocampal atrophy and memory decline compared to *APOE3/3* carriers and *APOE2* carriers have less Alzheimer pathology as reflected by CSF biomarkers [103].

FDG-PET is quantitatively more accurate than perfusion SPECT. Regional metabolic and blood flow changes are closely related to clinical symptoms, and most areas involved in these changes will also develop significant cortical atrophy. FDG-PET is complementary to amyloid PET, which targets a molecular marker that does not have a close relation to current symptoms. FDG-PET is expected to play an increasing role in diagnosing patients at an early stage of AD and in clinical trials of drugs aimed at preventing or delaying the onset of dementia [104]. Functional neuroimaging biomarkers are becoming popular with the introduction of novel tracers for brain amyloid deposits. Amyloid deposition causes severe damage to neurons many years before onset of dementia via a cascade of several downstream effects. Positron emission tomography (PET) tracers for amyloid plaque are desirable for early diagnosis of AD, particularly to enable preventative treatment once effective therapeutics is available. The amyloid imaging tracers flutemetamol, florbetapir, and florbetaben labeled with ^{18}F have been developed for PET. These tracers are currently undergoing

formal clinical trials to establish whether they can be used to accurately image fibrillary amyloid and to distinguish patients with AD from normal controls and those with other diseases that cause dementia [104].

[^{18}F]MK-3328 was identified as a promising PET tracer for in vivo quantification of amyloid plaques [105]. Fleisher et al. [106] characterized quantitative florbetapir ^{18}F positron emission tomographic (PET) measurements of fibrillar Aβ burden in a large clinical cohort of participants with probable AD or MCI and older healthy controls (OHCs) who differed in mean cortical florbetapir standard uptake value ratios (SUVRs), in percentage meeting levels of amyloid associated with AD by SUVR criteria (80.9 %, 40.0 %, and 20.7 %, respectively), and in percentage meeting SUVR criteria for the presence of any identifiable Aβ (85.3 %, 46.6 %, and 28.1 %, respectively). Among OHCs, the percentage of florbetapir positivity increased linearly by age decile. *APOE ε4* carriers had a higher mean cortical SUVR than did noncarriers. Wolk et al. [107] determined the correspondence of in vivo quantitative estimates of brain uptake of fluorine 18-labeled flutemetamol with immunohistochemical estimates of amyloid levels in patients who underwent previous biopsy.

5.3 Neurophysiology

There is a renewed interest for the use of computerized brain mapping as a diagnostic aid and as a monitoring tool in AD [108]. Electroencephalography (EEG) studies in AD show an attenuation of average power within the alpha band (7.5–13 Hz) and an increase in power in the theta band (4–7 Hz) [109]. It has also been reported that cortical sources of resting state EEG rhythms are abnormal in subjects with MCI [110]. *APOE* genotypes influence brain bioelectrical activity in AD. In general, *APOE-4* carriers tend to exhibit a slower EEG pattern from early stages [17, 19, 111]. Quantitative EEG (qEEG) separates between patients with amnestic MCI and patients in early stages of probable AD. Adding information about *Apo ε4* allele frequency slightly enhances diagnostic accuracy [112, 113].

5.4 Biochemistry of Body Fluids

Fluid biomarkers of AD currently provide indications of disease stage; however, they are not robust predictors of disease progression or treatment response, and most are measured in CSF, which limits their applicability [114]. Biomarkers of potential interest include CSF and peripheral levels of $A\beta_{42}$, protein tau, histamine, interleukins, and many other novel candidate markers in blood [7, 17, 32, 115–118].

The concentration of the 42 amino acid form of Aβ ($A\beta_{1-42}$) is reduced in the CSF from AD patients, which is believed to reflect the AD pathology with plaques in the brain acting as sinks. Novel C-truncated forms of Aβ ($A\beta_{1-14}$, $A\beta_{1-15}$, and $A\beta_{1-16}$) were identified in human CSF. The presence of these small peptides is consistent

with a catabolic APP cleavage pathway by β- followed by α-secretase. $A\beta_{1-14}$, $A\beta_{1-15}$, and $A\beta_{1-16}$ increase dose-dependently in response to γ-secretase inhibitor treatment while $A\beta_{1-42}$ levels are unchanged [119]. Kester et al. [120] investigated change over time in CSF levels of amyloid-beta 40 and 42 ($A\beta_{40}$ and $A\beta_{42}$), total tau (tau), tau phosphorylated at threonine 181 (ptau-181), isoprostane, neurofilaments heavy (NfH) and light (NfL). $A\beta_{42}$, tau, and tau phosphorylated at threonine 181, differentiated between diagnosis groups, whereas isoprostane, neurofilaments heavy, and NfL did not. In contrast, effects of follow-up time were only found for nonspecific CSF biomarkers: levels of NfL decreased, and levels of isoprostane, $A\beta_{40}$, and tau increased over time. An increase in isoprostane was associated with progression of MCI to AD, and with cognitive decline. Contrary to AD-specific markers, nonspecific CSF biomarkers show change over time which might be potentially used to monitor disease progression in AD. Soluble amyloid precursor proteins (sAPP) in CSF might also help to improve the identification of patients with incipient AD among patients with MCI [121]. Weight changes are common in aging and AD and postmortem findings suggest a relation between lower body mass index (BMI) and increased AD brain pathology. BMI is associated with higher core AD brain pathology as assessed by CSF-based biological markers of AD. Lower BMI is indicative of AD pathology [122]. Furthermore, diet may be a powerful environmental factor that modulates AD risk through its effects on CNS concentrations of $A\beta_{42}$, lipoproteins, oxidative stress, and insulin [123]. Lo et al. [124] delineated the trajectories of $A\beta_{42}$ level in CSF, fludeoxyglucose F18 (FDG) uptake using PET, and hippocampal volume using MRI and their relative associations with cognitive change at different stages in aging and AD. $A\beta_{42}$ level in CSF, FDG uptake, and hippocampal volume vary across different cognitive stages. The longitudinal patterns support a hypothetical sequence of AD pathology in which amyloid deposition is an early event before hypometabolism or hippocampal atrophy, suggesting that biomarker prediction for cognitive change is stage-dependent. The levels of Aβ and phosphorylated tau (p-tau) in CSF have been associated with the risk of progressing from normal cognition to onset of clinical symptoms during preclinical AD. The increased risk of progressing from normal cognition to symptom onset was associated with lower cognitive reserve, lower baseline Aβ, and higher baseline p-tau [125].

CSF Visinin-like protein-1 (VILIP-1), a calcium-mediated neuronal injury biomarker, has been described as a novel biomarker for AD. CSF VILIP-1 levels are increased in AD patients compared with both normal controls and DLB patients. CSF VILIP-1 levels positively correlate with t-tau and p-tau_{181P} and with α-synuclein. CSF VILIP-1 and VILIP-1/$A\beta_{1-42}$ levels show diagnostic accuracy to allow the detection and differential diagnosis of AD [126].

Major problems with CSF biomarkers such as Aβ_{1-42}, total tau, and phosphorylated tau are large variations in biomarker measurements among and within laboratories. The overall variability remains too high to allow assignment of universal biomarker cutoff values for a specific intended use [127].

5.5 Genomics, Proteomics, and Metabolomics

Structural markers are represented by SNPs in genes associated with AD (*see* Table 1), polygenic cluster analysis, and genome-wide studies (GWS). Functional markers attempt to correlate genetic defects with specific phenotypes (genotype–phenotype correlations). In proteomic studies, several candidate CSF protein biomarkers have been assessed in neuropathologically confirmed AD, non-demented (ND) elderly controls, and non-AD dementias (NADD). Markers selected included apolipoprotein A-1 (ApoA1), hemopexin (HPX), transthyretin (TTR), pigment epithelium-derived factor (PEDF), Aβ_{1-40}, Aβ_{1-42}, total tau, phosphorylated tau, α-1 acid glycoprotein (A1GP), haptoglobin, zinc α-2 glycoprotein (Z2GP), and apolipoprotein E (ApoE). The concentrations of Aβ_{1-42}, ApoA1, A1GP, ApoE, HPX, and Z2GP differed significantly among AD, ND, and NADD subjects. The CSF concentrations of these three markers distinguished AD from ND subjects with 84 % sensitivity and 72 % specificity, with 78 % of subjects correctly classified. By comparison, using Aβ_{1-42} alone gave 79 % sensitivity and 61 % specificity, with 68 % of subjects correctly classified. For the diagnostic discrimination of AD from NADD, only the concentration of Aβ_{1-42} was significantly related to diagnosis, with a sensitivity of 58 % and a specificity of 86 % [128]. Carrying the *APOE-ε4* allele was associated with a significant decrease in the CSF Aβ_{1-42} concentrations in middle-aged and older subjects. In AD, the Aβ_{1-42} levels are significantly lower in the *APOE ε4* carriers compared to the noncarriers. These findings demonstrate significant age effects on the CSF Aβ_{1-42} and pTau181 across lifespan, and also suggest that the decrease in Aβ_{1-42}, but not the increase in pTau181 CSF levels, is accelerated by the *APOE ε4* genotype in middle-aged and older adults with normal cognition [129]. Han et al. [130] carried out a genome-wide association study (GWAS) in order to better define the genetic backgrounds of normal cognition, MCI, and AD in terms of changes in CSF levels of Aβ_{1-42}, T-tau, and P-tau181P. CSF Aβ_{1-42} levels decreased with *APOE* gene dose for each subject group. T-tau levels tended to be higher among AD cases than among normal subjects. *CYP19A1* "aromatase" (rs2899472), *NCAM2*, and multiple SNPs located on chromosome 10 near the *ARL5B* gene demonstrated the strongest associations with Aβ_{1-42} in normal subjects. Two genes found to be near the top SNPs, *CYP19A1* (rs2899472) and *NCAM2* (rs1022442), have been reported as genetic factors related to the progression of AD. In AD subjects, *APOE ε2/ε3* and *ε2/ε4* genotypes were associated with elevated T-tau levels, and the *ε4/ε4*

genotype was associated with elevated T-tau and P-tau181P levels. Blood-based markers reflecting core pathological features of AD in presymptomatic individuals are likely to accelerate the development of disease-modifying treatments. Thambisetty et al. [131] performed a proteomic analysis to discover plasma proteins associated with brain Aβ burden in non-demented older individuals. A panel of 18 2DGE plasma protein spots effectively discriminated between individuals with high and low brain Aβ. Mass spectrometry identified these proteins, many of which have established roles in Aβ clearance, including a strong signal from ApoE. A strong association was observed between plasma ApoE concentration and Aβ burden in the medial temporal lobe. Targeted voxel-based analysis localized this association to the hippocampus and entorhinal cortex. *APOE ε4* carriers also showed greater Aβ levels in several brain regions relative to *ε4* noncarriers. Both peripheral concentration of ApoE protein and *APOE* genotype may be related to early neuropathological changes in brain regions vulnerable to AD pathology even in the non-demented elderly.

In a proteomic analysis of the hippocampus of transgenic animals with the E693Δ-APP mutation, Takano et al. [132] found alterations in 14 proteins: Actin cytoplasmic 1 (β-actin), heat shock cognate 71 kDa, γ-enolase, ATP synthase subunit β, tubulin β-2A chain, clathrin light chain B (clathrin), and dynamin-1 were increased, while heat shock-related 70 kDa protein 2, neurofilament light polypeptide (NFL), stress-induced phosphoprotein 2, 60 kDa heat shock protein (HSP60), α-internexin, protein kinase C and casein kinase substrate in neurons protein 1 (Pacsin 1), α-enolase, and β-actin were decreased. These proteins belong to cytoskeleton, chaperons, neurotransmission, energy supply, and signal transduction categories.

Metabolomic analysis can also help to differentiate different forms of dementia. Tsuruoka et al. [133] conducted a metabolomic analysis of serum and saliva obtained from patients with neurodegenerative dementias, including AD, frontotemporal lobe dementia (FTLD), and Lewy body disease (LBD), as well as from age-matched healthy controls, and found that six metabolites in serum (β-alanine, creatinine, hydroxyproline, glutamine, isocitrate, and cytidine) and two in saliva (arginine and tyrosine) were significantly different between dementias and controls. Forty-five metabolites in total were identified as candidate markers that could discriminate at least one pair of diagnostic groups from the healthy control group.

Transcriptome analysis of leukocytes from patients with MCI, AD, and controls by oligonucleotide microarray identified eight genes significantly associated with purine metabolism and the ABC transporters. The *ABCB1* gene exhibited significantly positive correlation with Mini-mental State Examination (MMSE) scores [134].

5.6 microRNAs

miRNAs belong to the class of noncoding regulatory RNA molecules of ~22 nt length and are now recognized to regulate ~60 % of all known genes through posttranscriptional gene silencing (RNA interference) (RNAi). miRNAs can be used as biomarkers to discriminate different disease forms, staging and progression, as well as prognosis [135]. A unique circulating 7-miRNA signature (hsa-let-7d-5p, hsa-let-7g-5p, hsa-miR-15b-5p, hsa-miR-142-3p, hsa-miR-191-5p, hsa-miR-301a-3p, and hsa-miR-545-3p) reported by Kumar et al. [135] in plasma could distinguish AD patients from normal controls with >95 % accuracy. Leidinger et al. [136] showed a novel miRNA-based signature for detecting AD from blood samples. Using this 12-miRNA signature, they differentiated between AD and controls with an accuracy of 93 %, a specificity of 95 %, and a sensitivity of 92 %. The differentiation of AD from other neurological diseases (MCI, multiple sclerosis, Parkinson disease, major depression, bipolar disorder, and schizophrenia) was possible with accuracies between 74 and 78 %. Alexandrov et al. [137] found increased levels of miRNA-9, miRNA-125b, miRNA-146a, and miRNA-155 in the CSF and brain tissue-derived extracellular fluid from patients with AD, suggesting that these miRNAs might be involved in the modulation or proliferation of miRNA-triggered pathogenic signaling in AD brains.

6 Therapeutic Strategies

Modern therapeutic strategies in AD are addressed to interfere with the main pathogenic mechanisms potentially involved in AD [7, 12, 17, 19, 31, 35, 63, 86–92] (*see* Table 2).

Since the early 1990s, the neuropharmacology of AD was dominated by acetylcholinesterase inhibitors, represented by tacrine, donepezil, rivastigmine, and galantamine [138–140]. Memantine, a partial NMDA antagonist, was introduced in the 2000s for the treatment of severe dementia [141]; and the first clinical trials with immunotherapy, to reduce amyloid burden in senile plaques, were withdrawn due to severe ADRs [142, 143]. After the initial promises of β- and γ-secretase inhibitors [144, 145] and novel vaccines [146, 147] devoid of severe side effects, during the past few years no relevant drug candidates have dazzled the scientific community for their capacity to halt disease progression; however, a large number of novel therapeutic strategies for the pharmacological treatment of AD have been postulated, with some apparent effects in preclinical studies (*see* Table 2).

Assuming that the best treatment for AD is neuronal death prevention prior to the onset of the disease, novel therapeutic options and future candidate drugs for AD might be a new generation of antiamyloid vaccines, such as DNA $A\beta_{42}$ trimer immunization [146] or vaccines developed with new immunogenic

Table 2
Experimental strategies for the pharmacological treatment of Alzheimer's disease

2-Phenylethynyl-butyltellurium
2,4-Bis(p-hydroxyphenyl)-2-butenal
3-(1H-indol-3-yl)propanehydrazide (JL418)
3-(3-Hydrazinylpropyl)-1H-indole (JL72)
3-Hydroxybutyrate methyl ester
3-N-Butylphthalide
5-Lipoxygenase inhibitors   Zileuton
AAD-2004 [2-hydroxy-5-[2-(4-trifluoromethylphenyl)-ethylaminobenzoic acid]
Adenosine A1 antagonists   8-Cyclopentyl-1,3-dipropylxanthine
AL-108
AMD3100 (CXCR4 antagonist)

Antiepileptic drugs
  Levetiracetam
  Topiramate
  Valproic acid

Antihistamines and Histamine H3 receptor antagonists and inverse agonists
  GSK239512
  Latrepirdine (Dimebon)
  MK-3134

Antihypertensive drugs
  Captopril
  Carvedilol
  Enalapril
  Nebivolol
  Perindopril
  Telmisartan

Antiparkinsonian agents
  Bromocriptine
  Dopamine
  Entacapone
  Levodopa
  Peroxide
  Pramipexole
  Rasagiline derivatives
    Lasdostigil
    m30
  Ropinirole
  Selegiline
  Zonisamide

(continued)

Table 2
(continued)

Antisense oligonucleotides
ApoE mimetic peptides   Ac-hE18A-NH2
Artemisinin
Arundic acid
Autophagy inducers   GTM-1

Aβ aggregation inhibitors
  Acteoside (Phenylethanoid Glycoside)
  Baicalein
  Bavachinin
  Bis-Styrylbenzene derivatives
    KMS80013
  Crocin
  cTfRMAb-ScFv
  Diphenylpropynone derivatives
  D-Trp-Aib
  DPP2s
    C1/C2
    P1/P2
    PA1/PA2
  Ferrocene tripeptide Gly-Pro-Arg conjugates
  Ferulic acid
  iAβ5p
  Isobavachalcone
  Novel β-sheet breakers (iAβ6)
  Polyoxometalate-peptide hybrid particles
  Sym-Triazines (TAE-1, TAE-2)

Aβ immunotherapy
  4AB1-15
  6Aβ15-T-Hc DNA chimeric vaccines
    AD01
    AD02
  AN-1792
  Anti-oligomeric monoclonal antibodies
  AV-1955
  Bapineuzumab
  CAD106
  EB-101
  Gammagard
  Genetic immunization (Aβ DNA vaccination)
    ICSM-18
    ICSM-35
  MER5101
  Mimovax

(continued)

Table 2
(continued)

p(Aβ3-10)10-C3d-p28.3 vaccine Ponezumab (PF-04360365) ScFv59 ScFv-h3D6 Solanezumab Tetravalent Aβ1–15 vaccine
β-Amyloid nontoxic conformers
β-Arrestin regulators
B6 peptide-modified PEG-PLA nanoparticles (B6-NP)
Benzimidazole-based glutaminyl cyclase inhibitors Benzimidazolyl-1,2,3-triazoles Benzimidazolyl-1,3,4-thiadiazoles
Biomarine derivatives *Astropectum polyacantus* *Conger conger* *Microsporum* sp. Neoechinulin A *Sardina pilchardus* *Trachurus trachurus*
Bis-chloroethylnitrosourea (BCNU, Carmustine)
Bis(propyl)-cognitin
Bone morphogenetic protein 9 (BMP-9)
Brain-penetrating angiotensin-converting enzyme (ACE) inhibitors Captopril Enalapril Perindopril
c-Jun N-terminal kinase (JNK) inhibitors
Caffeic acid and caffeic acid phenethyl ester
Calcineurin inhibitors FK506
Calcium channel blocker Dihydropyridines
Calpain inhibitors
Carnosine
Cathepsin B inhibitors CA-074Me
CB2 cannabinoid receptor agonists (JWH-133)
CCL2/MCP1 inhibitors Bindarit

(continued)

Table 2
(continued)

Cerebrolysin
Chaperones (small heat shock proteins, sHSPs; Hsp90 inhibitors and HSP inducers)
Chemokines

Cholinergic receptor agonists
  Muscarinic receptors
    Benzyl quinolone carboxylic acid analogs
    Cevimeline
    EUK1001
    Lanomeline
    Vedaclidine
  Nicotinic receptors
    Nicotinic analog ZY-1
    N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-6-chinolincarboxamide (EVP-5141)

Citidine-5-diphosphocholine (CDP-choline)
Coenzyme Q10
CopA3
CPPHA (N-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl] phenyl)-2-hydroxybenzamide)
Cyclin-dependent kinase 5/p25 (CDK5) inhibitors
Cyclodextrin-solubilized curcuminoids
Cyclooxygenase-1 and cyclooxygenase-2 inhibitors
Desferoxamine
Dihydrofuran-fused perhydrophenanthrenes
Docosahexaenoic acid (DHA, C22:6 n-3)
DYRK kinase inhibitors   Indirubin derivatives
E. coli protein toxin CNF1
Edaravone
Epigenetic drugs
Erythropoietin (EPO)

Estrogens
  17-β Estradiol
  Coumestrol
  Medroxyprogesterone
  Phyto-β-SERM
  Progesterone

(continued)

Table 2 (continued)

Fucoxanthin
Furan fatty acids
Galanin
Gene silencing (RNA interferente, iRNA)
Gene therapy
Glucagon-like peptide-1 (GLP-1) agonists Liraglutide Saxagliptin
Glucose-dependent insulinotropic polypeptide (GIP) analogs Incretin hormone analog D-Ala2GIP
Glucosylceramide
[Gly14]-Humanin
Glycogen synthase kinase-3β (GSK-3β) regulators Acetyl-2-carnitine AZD1080 CHIR 99021 CID 56840716/ML320 CID 5706819 Falcarindiol Gymnodimine
Growth hormone-releasing hormone (GHRH)
Helicase-primase inhibitor BAY 57-1293
Heterocyclic indazole derivatives
Histone deacetylase inhibitors Phenylbutyrate Quinazolin-4-one derivatives (E)-3-(2-Ethyl-7-fluoro-4-oxo-3-phenethyl-3,4-dihydroquinazolin-6-yl)-N-hydroxyacrylamide N-Hydroxy-3-(2-methyl-4-oxo-3-phenethyl-3,4-dihydro-quinazolin-7-yl)- acrylamide Suberoylanilide hydroxamic acid Valproic acid
Hydrogen sulfide
Icariin
IgG-single chain Fv fusion proteins
Imatinib methanesulfonate
Immunoglobulin

(continued)

Table 2
(continued)

Immunotherapy and treatment options for tauopathies Aminothienopyridazines Antisense oligonucleotides (Tau genetic ablation) c-Jun N-terminal kinase (JNKs) inhibitors Harmine (β-carboline alkaloid) Methylene blue NAP (davunetide) p38 MAP kinase inhibitors (CNI-1493) Phosphoprotein phosphatase 2A (PP2A) inhibitors Tau kinase inhibitors Diaminothiazoles Thiazolidinedione Troglitazone Pioglitazone Rosiglitazone
Indoleamine 2,3-dioxygenase (IDO) inhibitors Tryptoline derivatives
Inhibitors of the serum- and glucocorticoid-inducible-kinase 1 (SGK1)
Insulin-degrading enzyme inhibitors Peptide hydroxamate
Intransal insulin
Isoliquiritigenin derivatives
Kinin B1 receptor blockers SSR240612
Kynurenine 3-monooxygenase inhibitors
Latrepirdine
Leucettines
Lithium
Macrophage inflammatory protein-2 (MIP-2)
Melatonin and melatonin agonists Neu-P11
Memoquin
Metabotropic glutamate receptor 5 positive allosteric modulators CPPHA (N-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl)-2-hydroxybenzamide) NCFP(N-(4-chloro-2-((4-fluoro-1,3-dioxoisoindolin-2-yl)methyl)phenyl)- picolinamide)
Metalloendopeptidases
Metalloporphyrins

(continued)

Table 2 (continued)

Metallothioneinsin
Methyl 3,4-dihydroxybenzoate
Mibampator (LY451395)
Microglial modulators CHF5074
microRNAs (miRNAs)
Mifepristone
MLC601
Monoamine regulators
mTOR inhibitors Ropamycin
N-Substituted nipecotic acid derivatives
NADPH oxidase inhibitors (Apocynin)
NAP (Davunetide)
Natural compounds 3′-O-Methyl-epicatechin-5-O-β-glucuronide Acalypha wilkesiana var. Macafeana Geraniin Adhatoda vasica Alkaloids from the calabar bean (Physostigma venenosum) *Amberboa ramosa* Amberbin A Amberbin B Amberbin C Amberin *Angelica gigas* Decursinol *Apium graveolens* Apigenin *Arborvitae Seed* *Areca catechu* MA9701 *Azadirachta indica* *Bacopa monniera* Linn (Syn. Brahmi) *Baical skullcap* Baicalin Chrysin Scutellarin Baicalein Biochanin-A

(continued)

Table 2
(continued)

Buddleja davidii
Linarin
Bushenyisui formula
Caffeine
Camilla sinensis
Epi-gallocatechine-3-gallate (EGCG)
Cannabinoids (cannabidiol from Cannabis sativa)
Capsaicin
Carotenoids
β-Carotene
All-*trans* Retinoic acid
Crocin
Lycopene
Retinoic acid
Carthamus tinctorius L.
Hydroxysafflor yellow A
Cassia obtisufolia (Sicklepod)
Catechin (Green tea)
Catechin hydrate
Catharanthus roseus
Cholic acid
Chaetomium globosum JN711454
Adiantum capillus-veneris
Cistanche deserticota
Cistanche tubulosa
Glycosides
Citrus peel
Nobiletin
5-Demethylnobiletin
6-Demethoxynobiletin
6-Demethoxytangeretin
Sinensetin
Tangeretin
Cladonia macilenta
Biruloquinone
Clausena lansium
Clausenamide
Cnestis ferruginea
Amentoflavone
Cnidium monnieri
Osthole
Cochlospermum angolensis Welw
Ellagic acid
Cocoa
Coptidis rhizome
Tetrahydroisoquinoline alkaloids
Jatrorrhizine

(continued)

Table 2
(continued)

Coriandrum sativum var. *microcarpum* (Coriander)
Crocus sativus (Saffron)
Safranal
Cudrania cochinchinensis
Curcuma longa
Curcumin
Cyanidin 3-O-glucoside
Desmodium gangeticum (Sal Leaved Desmodium)
Diammonium glycyrrhizinate
Ecklonia cava
Emblica officinalis
Epigallocatechin-3-gallate
Erigeron annuus
Caffeic acid
Erythrina senegalensis
Evodiae fructus
Evodiamine
Ferula assafoetida
Flavonoids
Forsythia suspensa
Forsythiaside (3,4-dihydroxy-β-phenethyl-O-α-L-rhamnopyranosyl-(1 → 6)-4-O-caffeo yl-β-d-glucopyranoside, C29H36O15)
Fuzhisan, a Chinese herbal medicine
Galantamine from the snowdrop Galanthus woronowii
Gami-Chunghyuldan
Ganoderma lucidum
Garlic (*Allium sativum*)
Geranium thunbergii
Corilagin
Geraniin
Germinated brown rice
Ginger root extract
Ginkgo biloba (Ginkgo/Maidenhair tree)
Bilobalide
Biflavonoids
Amentoflavone
Ginkgetin
Isoginkgetin
Flavonoids
Isorhamnnetin
Kaempherol
Quercetin
Ginseng (*Panax* species)
Ginsenosides (saponins)
Panax ginseng
Rg1
Rg3
Panax notoginsen
Rd

(continued)

Table 2
(continued)

Panax quinquefolium
Pseudoginsenoside-F11
Glycyrrhiza uralensis
Liquirin
Grape seed polyphenolic extracts
Gynostemma pentaphyllum
Gypenosides
Hemidesmus indicus
2-Hydroxy-4-methoxybenzaldehyde
Hesperidin
Huannao Yicong recipe extract
Hunteria zeylanica (Apocynaceae)
Eburnamenine
Eburnamine
Eburnamonine
Geissoschizol
Huperzia serrata
Huperzine A
Hypericum perforatum
St. John's Wort
Hyperoside
Jasminoidin
Kampo medicine
Chotosan
Uncariae Uncis cum Ramulus
Yokukansan
Glycyrrhiza
Uncariae Uncis cum Ramulus
Kaixin San formulas
Karenia selliformis
Gymnodimine
Keampferol-3-O-rhamnoside
Lavandula pedunculata subsp. *lusitanica* (Chaytor) Franco
Camphor
Fenchone
Flavones
Luteonin
Apigenin
Hydroxycinnamic acids/Rosmarinic acid
Lemon balm (*Melissa officinalis*)
Lotus seedpod
Procyanidins
Luteolin
Lycium barbarum
Gouqi
Lycoris radiata
1,2-Di-O-acetyllycorine
1-O-acetyllycorine

(continued)

Table 2 (continued)

Magnolia officinalis
Magnolol
Melissa officinalis (Lemon Balm)
Mentha arvensis
Linarin
Monosialoangloside (GM-1 Ganglioside)
Moringa oleifera (Drumstick tree)
Naoerkang
Naringenin
Naringin
Natural compounds
Nicotine from nicotiana species
Nigella sativa
Thymoquinone
Nigella sativa Linn. seed
Oenanthe javanica (Japanese Parsley) (Falcarindiol)
Olive oil
Oleocanthal
Oleuropein aglycone
Orcein
Orobanche minor
Acteoside
Peganum harmala
Phellinus linteus mushroom
Phytoestrogens
Piper methysticum (Piperaceae)
Kavalactones
Piper nigrum
Pleiocarpa mutica (Annonaceae)
Kopsinine
Pleiocarpamine
Pleiocarpine
Plumula nelumbinis
Isoliensinine
Liensinine
Neferine
Polyalthia longifolia (Apocynaceae)
Darienine
Isooncodine
Liridonine
Noroliveroline
Oliveroline
Polyfothine
Polygala tenuifolia
Pomegranate polyphenols
Punicalagin
Ellagic acid
Pomelo peel
Naringin

(continued)

Table 2 (continued)

Psoraleae fructus derivatives
Bavachinin
Isobavachalcone
Punica granatum extracts
Qingxin kaiqiao formula
Quercetin-3-O-glucuronide
Radix morinda officinalis
Bajijiasu (β-D-fructofuranosyl (2-2) β-D-fructofuranosyl)
Radix puerariae
Puerarin
Rehmannia glutinosa
Rehmannia roots
Catalpol
Resveratrol and derivatives
Rhodiola crenulata
Salidroside
Rhodiola rosea
Rhodosin
Rhus parviflora
Aurones
Aurensidin
Sulfuretin
Aurone glicosides
Aurensidin 6-O-β-D glucopyranoside
Hovetrichoside C
Buflavonoid cupressu flavone
Flavonoid glycoside-quercetin-3-O-β-d galactopyranoside
Roccella tinctoria
Orcein
Saffron (*Crocus sativus*)
Salvia fructicosa
Salvia miltiorrhiza Bunge
Salvianolic acid A
Salvianolic acid B
Tanshinones
Tanshinone I
Tanshinone IIA
Salvia officinalis (garden sage, common sage)
Salvia sahendica
Salvia sclareoides
Rosmarinic acid
Salvia triloba
Schisandra chinensis (Trucz.) Baill (Schisandraceae)
Lignan-riched extract (ESP-806)
Schisandrone
Schisanhenol

(continued)

Table 2 (continued)

Scoparia dulcis
Scutellaria baicalensis
Baicalein
Securinega suffructicosa
Securinine
Sesamum indicum
Silybium marianum
Silymarin
Taxifolin
Sophora flavescens
Sphocardipine
Soybean isoflavones
Daidzein
Daidzin
Genistein
Genistin
Glycitein
Glyzitin
SuHeXiang Wan (SHXW)
KSOP1009
Sulforaphane
Syzygium aromaticum
Taxus chinensis
Sciadopitysin
Terminalia arjuna
Terminalia chebula
TongLuoJiuNao (Geniposide and Ginsenoside Rg1)
Tripterygium wilfordii Hook F
Triptolide
Turmeric
Valeriana amurensis roots and rhizomes
Germacrane-type sesquiterpenoids
Heishuixiecaoline A–C
Vanilla planifolia
4-Hydroxy-3-methoxybenzaldehyde (vanillin)
Vigna unguiculata
Walnut extract
Xanthoceras sorbifolia bunge
Xanthoceraside
Yizhi Jiannao
Yuzu (*Citrus junos* Tanaka)
Zhizi (*Fructus gardeniae*)
Crocetin
Crocin
Genipin
Geniposide
Zingiber officinalis

(continued)

Table 2
(continued)

Necrostatin-1
Neural cell adhesion molecule-derived mimetic peptides (FGL)
Neurosteroids
Neurotrophic factors Brain-derived neurotrophic factor (BDNF) Colony stimulating factor 3 (GCSF) Glial cell-derived neurotrophic factor (GDNF) Granulocyte colony stimulating factor (G-CSF) Nerve growth factor (NGF) Neurotrophic compound J147 Vascular endothelial growth factor (VEGF)
New cholinesterase inhibitors 1,3-Dihydroxyxanthone Mannich base derivatives 2-((Diethylamino)methyl)-1-hydroxy-3-(3-methylbut-2-enyloxy)-9H-xanthen-9-one 1,4-Substituted 4-(1H)-pyridylene-hydrazone-type inhibitors 2-(2-(4-Benzylpiperazin-1-yl)ethyl)isoindoline-1,3-dione derivatives 2-Benzoxazolinone derivatives 2H-Thiazolo [3,2-a]pyrimidines 5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinato Iron(III) Chloride 5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinato Iron(III) nitrosyl Chloride 5,6-Dimethoxybenzofuran-3-one derivatives 5H-thiazolo[3,2-a] pyrimidines 6-Chloro-pyridonepezils 7-Methoxytacrine-adamantylamine heterodimers Acetophenone derivatives Amberbins β-Naphtotacrines Benzimidazole derivatives 5IIc (ethyl 1-(3-(1H-imidazol-1-yl)propyl)-2-(4-nitrophenyl)-1H-benzo[d] imidazole-5-carboxylate) N-{2-[4-(1H-Benzimidazole-2-yl)phenoxy]ethyl}substituted amine derivatives Bis(9)-(-)-nor-meptazinol Diazapentacyclic analogs Donepezil-hydrazinonicotinamide hybrids Dual drugs (cholinesterase inhibitors + MAO inhibitors) Ladostigil NP-9 Huperzine A N′-2-(4-Benzylpiperidin-/piperazin-1-yl)acylhydrazone derivatives Phenothiazine cholinesterase inhibitors Racemic tetrahydrocurcuminoid dihydropyrimidinone analogs Tetrahydrobisdemethoxycurcumin-(THBDC-) Tetrahydrocurcumin (THC-) Tetrahydrodemethoxycurcumin (THDC-)

(continued)

Table 2
(continued)

Tacrine derivatives and hybrids 7-MEOTA (9-amino-7-methoxy-1,2,3,4-tetrahydroacridine Bis(7)tacrine dimer Cystamine-tacrine dimer Mercapto-tacrine hybrids Tacrine-ferulic acid hybrid Tacrine-ferulic acid-nitric oxide donor trihybrid Tacrine-flurbiprofen-nitrate trihybrids Tacrine-selegiline hybrids Tacrine-silibinin co-drug Tacrine organic nitrates Tetraphenylporphinesulfonate Thioflavin- and deferiprone-based molecules ZT-1
Nicotinamide riboside
Nicotine
NOSH-aspirin (NBS-1120)
NOX1/4 inhibitor GKT136901
Nuclear receptor agonists/Liver X Receptor (LXR) agonists 24S-Hydroxycholesterol 27-Hydroxycholesterol GW3965 T0901317
ω-3 Polyunsaturated fatty acids (n-3 PUFAs)
Oligonol (oligomerized lychee fruit-derived polyphenol)
P-Glycoprotein regulators
p38-alpha MAPK inhibitors
p75 Neurotrophin receptor (p75NTR) ligands LM11A-31
Palmitoylethanolamide
Peroxiredoxin 6
Peroxisome proliferator-activated receptor agonists PPARα agonist Ciglitazone Pioglitazone PPARγ agonists WY 14.643
Phenserine
Phenyl hydrazide J147

(continued)

Table 2
(continued)

Phosphodiesterase inhibitors
  Quinoline derivatives (PDE5i)
  Rolipram (PDE4i)
  S14 (PDE5i)
  Sildenafil (PDE5i)

Pituitary adenylate cyclase activating polypeptide
Plastoquinone antioxidant SkQ1
PN-1
p.p′-Methoxyl-diphenyl diselenide

Proteasome regulators
  Lithocholic acid derivatives
    3α-O-Pimeloyl-lithocholic acid methyl ester
    Isosteric isomer

Protein phosphatase methylesterase-1 inhibitors
Pyrrolo[3,2-e][1,2,4]triazolo[1,5-a]pyrimidine (SEN1176)

Retinoic acid receptor agonists
  Am80 (Tamibarotene)
  Acitretin

Rho kinase inhibitors   Fasudil
S-Nitrosoglutathione

Secretase inhibitors (β- and γ-secretase inhibitors and modulators)
  2-Amino-1,3-oxazine
  Avagacestat
  AZ-4217
  Biblycic thiazole-piperidine 14-S
  Compound VIa
  Corilagin
  Dot-siRNA nanocomplexes
  EVP-0015962
  Fused oxadiazepines
  Geraniin
  Ginsenosides
    CK
    F1
    R41
    Rh2
  Heparan sulfate hexa- to dodecasaccharides
  Hispidin-derived polyphenols
    Auricularia polytricha (wood ear mushroom)

(continued)

Table 2 (continued)

Hydroxyethylamine (HEA) BACE-1 inhibitors 2, 2-Dioxo-isothiochromanes Chroman-HEA derivatives Hydroxyethylene-based BACE-1 Inhibitors Iminopyrimidinone derivatives Phenylimino-2H-chromen-3-carboxamide derivatives Pyridazine and pyridine-derived γ-secretase modulators SCH 697466 Semagacestat SPI-1865 Spirocyclic BACE1 inhibitors Spirocyclic sulfamides Sulfonamide chalcones Tricyclic bispyran sulfone γ-secretase inhibitors
Serine palmitoyltransferase inhibitors
Serotonergic modulators Selective 5-HT_3 receptor antagonists Tropisetron Multiple 5-HT_3 partial agonists
Serrapeptase and nattokinase
Sigma-1 receptor antagonists BD1047 N,N-Dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride (NE-100)
Sirtuins
S-Nitrosoglutathione
Sodium fullerenolate
Sodium phenylbutyrate
Somatostatin receptor subtype-4 agonist NNC 26-9100
Sphingosine-1-phosphate receptor modulators FTY720 (Fingolimod) KRP203
Sphingosylphosphorylcholine
Statins and neostatins Atorvastatin Pitavastatin
Stem cell therapy
Stromal cell-derived factor-1α (SDF-1α)
Substituted 2-indolyl carbohydrazides (JL34, JL40, JL71, JL87, JL317, JL432, JL436)
Substituted 3-indolyl carbohydrazide JL344

(continued)

Table 2 (continued)

Sulfiredoxin-1
Sunifiram
Surfactin
Tart cherry extract and essential fatty acids
Tetrahydrohyperforin
Tetrahydrohyperforin
TNF inhibitors 2-(2,6-Dioxopiperidin-3-yl)phthalimidine EM-12 dithiocarbamates 3-Substituted 2,6-dioxopiperidines N-Substituted 3-(phthalimidin-2-yl)-2,6-dioxopiperidines Thalidomide
Transcription factor specificity protein 1 (Sp1) inhibitors Tolfenamic acid
Transglutaminase inhibitors
Trientine
Tyrosine kinase inhibitors Bosutinib Nilotinib
Ubidecarenone (Co. Q10 NLC).
Ubiquinone (coenzyme Q10)
Uridine prodrug PN401
Vitamins (A, B, C, D, E, K)
Zeolite

procedures [147], heterocyclic indazole derivatives [inhibitors of the serum- and glucocorticoid-inducible-kinase 1 (*SGK1*)] [148], NSAID-like compounds [149], neostatins [150], IgG-single chain Fv fusion proteins [151], Hsp90 inhibitors and HSP inducers [152], inhibitors of class I histone deacetylases [153], some phenolic compounds [154], agonists of the peroxisome proliferator-activated receptor gamma (*PPARγ*) [155], miRNA [156, 157], and gene silencing (RNAi) [158]. Current drug development for the treatment of AD is principally based on the amyloid cascade theory, and aims to reduce the levels of Aβ amyloid peptide in the brain. Some novel therapeutic strategies and candidate drugs postulated up to 2013 include the following (*see* Table 2).

6.1 New Cholinesterase Inhibitors

Novel cholinesterase inhibitors, cholinergic receptor agonists, and monoaminergic regulators have been developed in recent times [159–162]. Several *2-benzoxazolinone derivatives* have been proposed as potential cholinesterase inhibitors, with stronger activity than donepezil; and a series of *N-{2-[4-(1H-benzimidazole-2-yl) phenoxy]ethyl}substituted amine derivatives* were designed to assess cholinesterase inhibitor activities on both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) [163]. Two series of novel acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitors containing benzimidazole core structure were synthesized by a four-step reaction pathway starting from *4-fluoro-3-nitrobenzoic acid* as the basic compound [164].

Phenothiazine cholinesterase inhibitors. Synthetic derivatives of phenothiazine are well-tolerated drugs against a variety of human ailments from psychosis to cancer. A number of synthetic *N-10-carbonyl phenothiazine derivatives*, with cholinesterase inhibitory activity, were tested for interaction with a variety of neurotransmitter receptor systems. Phenothiazines can be prepared without significant neurotransmitter receptor interactions while retaining high potency as cholinesterase ligands for treatment of AD [165].

Tetraphenylporphinesulfonate (TPPS), *5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinato Iron^{III} Chloride* (FeTPPS), and *5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinato Iron^{III} nitrosyl Chloride* (FeNOTPPS) were investigated as candidate compounds for inhibition of acetylcholinesterase of *Drosophila melanogaster* [166].

Ladostigil. Ladostigil [(N-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate] is a dual acetylcholine-butyrylcholinesterase and brain-selective monoamine oxidase (MAO)-A and -B inhibitor. Ladostigil antagonizes scopolamine-induced impairment in spatial memory, prevents gliosis and oxidative-nitrative stress, reduces the deficits in episodic and spatial memory induced by intracerebroventricular injection of streptozotocin, and possesses potent antiapoptotic and neuroprotective activities in various neurodegenerative rat models. These neuroprotective activities involve regulation of APP processing, activation of protein kinase C and mitogen-activated protein kinase signaling pathways, inhibition of neuronal death markers, prevention of the fall in mitochondrial membrane potential, and upregulation of neurotrophic factors and antioxidative activity [167].

NP-9 is a monoamine oxidase B (MAO-B) and acetylcholinesterase (AChE) inhibitor. NP-9 inhibits AChE activity and Aβ aggregation, and protects against scopolamine and $A\beta_{1-42}$-induced memory impairments [168].

A series of novel *1,3-dihydroxyxanthone Mannich base derivatives* were synthesized, structure elucidated, and evaluated for anticholinesterase activity. Most of the target compounds exhibited moderate to good inhibitory activities with the IC_{50} values at micromole level concentration against both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Among them, 2-((diethylamino)methyl)-1-hydroxy-3-(3-methylbut-2-enyloxy)-9H-xanthen-9-one showed potent inhibitory activity against AChE and the best inhibitory activity against BuChE. The Mannich base derivatives were likely to bind to the active site (AS) and the peripheral anionic site (PAS) of cholinesterases [169].

Design, synthesis, and assessment of anticholinesterase activity of *2-(2-(4-Benzylpiperazin-1-yl)ethyl)isoindoline-1,3-dione derivatives* showed that some of these compounds can function as potential acetylcholinesterase inhibitors with a potency comparable to that of donepezil [170].

Diazapentacyclic analogs. Kumar et al. [171] synthesized and evaluated diazapentacyclic analogs for their acetylcholinesterase (AChE) inhibitory activity. The pentacyclic analogs were synthesized by one-pot three-component domino reactions in a microwave synthesizer. Most of the compounds exhibited moderate to good AChE inhibitory activity.

Studies of cholinesterase structure and the biological mechanisms of inhibition are necessary for effective drug development. Medicinal compounds like Ortho-7, Dibucaine, and HI-6 are predicted as good targets for modeled AChE and BChE proteins based on docking studies [172].

Huperzine A, isolated from the Chinese herb *Huperzia serrata* (Thunb) Trev, is a novel reversible and selective AChE inhibitor. *ZT-1* is a novel analog of huperzine A. ZT-1 is a prodrug that is rapidly absorbed and converted into huperzine A, and ZT-1 is well tolerated in healthy Chinese volunteers [173].

Arunkhamkaew et al. [174] reported the synthesis of *racemic tetrahydrocurcumin-* (THC-), *tetrahydrodemethoxycurcumin-* (THDC-), and *tetrahydrobisdemethoxycurcumin-* (THBDC) *dihydropyrimidinone (DHPM) analogs* utilizing the multicomponent Biginelli reaction in the presence of copper sulfate as a catalyst. THBDC-DHPM demonstrated the most potent inhibitory activity.

New *tacrine derivatives* have been reported [45], including: (a) *Bis(7)tacrine dimer*, which exhibited a 1,000-fold higher AChE inhibition potency, a double interaction with active and peripheral sites of AChE, and a better pharmacological profile consisting in the inhibition of the AChE-induced Aβ aggregation through interaction with its peripheral binding site (PAS), and in neuroprotective effects related to the interaction with β-secretase enzyme and NMDA and GABA receptors. (b) *Cystamine-tacrine dimer*,

endowed with a lower toxicity in comparison to bis(7)tacrine, able to inhibit AChE/BChE, self- and AChE-induced Aβ aggregation in the same range of the reference compound, exerting a neuroprotective action on the SH-SY5Y cell line against H_2O_2-induced oxidative injury. (c) *Tacrine-ferulic acid hybrid* as potent ChEIs which can block the PAS of the AChE. (d) *Tacrine-ferulic acid-nitric oxide* (NO) donor tri-hybrids showing potent multifunctional acetyl- and butyrylcholinesterase inhibition. (e) Nontoxic *tacrine-organic nitrates.* (f) *Tacrine-silibinin co-drug* showing high AChE and BChE inhibition, neuroprotective effects, lacking tacrine's hepatotoxicity in vitro and in vivo, with the same pro-cognitive effects in vivo as tacrine, being superior to the physical mixture of tacrine and silibinin in all these regards. (g) *Mercapto-tacrine hybrids* endowed with cholinesterase inhibition, long-term potentiation enhancement, neuroprotective activity, and less hepatotoxicity, and consequently good candidates for further studies directed toward the development of novel drugs for AD. Particularly interesting among all the tacrine derivatives is *7-MEOTA* (9-amino-7-methoxy-1,2,3,4-tetrahydroacridine), a potent, centrally active ChEI free of the serious side effects related to tacrine. In single-administration studies, 7-MEOTA was well tolerated, and thus further research efforts are currently aimed at improving its pharmacological profile. Fourteen new *N*-alkyl 7-MEOTA analog hydrochlorides, which were found to be less toxic than tacrine, were synthesized. Their activity in vitro on AChE and BChE showed inhibitory ability in μM scale. The inhibitory ability and selectivity index for *h*AChE of new compounds were compared to standards of THA, 7-MEOTA [45].

Chen et al. [175] synthesized *tacrine-flurbiprofen hybrids* and *tacrine-flurbiprofen-nitrate trihybrids.* These compounds displayed comparable or higher cholinesterase inhibitory activity relative to the bivalent hybrids, released NO, exerted blood vessel relaxative activity, and showed significant Aβ inhibitory effects.

The synthesis, toxicity, neuroprotection, and human acetylcholinesterase/butyrylcholinesterase inhibition properties of *β-naphthotacrines1–14* have been reported [176]. β-Naphthotacrines 1–14 showed lower toxicity than tacrine.

A series of *tacrine-coumarin hybrids* were designed, synthesized, and evaluated as multifunctional cholinesterase inhibitors against AD [177]. Most of them exhibited a significant ability to inhibit ChE and self-induced Aβ aggregation, and to act as metal chelators.

6-Chloro-pyridonepezils. 6-Chloro-pyridonepezils are chloropyridine-donepezil hybrids designed by combining the N-benzylpiperidine moiety present in donepezil with the 2-chloropyridine-3,5-dicarbonitrile heterocyclic ring system, both connected by a

polymethylene linker. 6-Chloro-pyridonepezils 1–8 were prepared by reaction of 2,6-dichloro-4-phenylpyridine-3, 5-dicarbonitrile [or 2,6-dichloropyridine-3,5-dicarbonitrile] with suitable 2-(1-benzylpiperidin-4-yl)alkylamines. These new compounds are cholinesterase inhibitors and some of them are potent hBChE inhibitors [178].

Novel *2H-thiazolo [3,2-a]pyrimidines* and *5H-thiazolo[3,2-a] pyrimidines* have been described [179]. The docking studies of 2H-thiazolo[3,2-a]pyrimidines and 5H-thiazolo[3,2-a] pyrimidines with human AChE have demonstrated that these ligands bind to the dual sites of the enzyme.

5,6-Dimethoxybenzofuran-3-one derivatives. Nadri et al. [180] described the synthesis of target compounds with dual acetylcholinesterase and butyrylcholinesterase inhibitory activities. Bayer-Villiger oxidation of 3,4-dimethoxybenzaldehyde furnished 3,4-dimethoxyphenol. The reaction of 3,4-dimethoxyphenol with chloroacetonitrile followed by treatment with HCl solution and then ring closure yielded the 5,6-dimethoxy benzofuranone. Condensation of the later compound with pyridine-4-carboxaldehyde and subsequent reaction with different benzyl halides afforded target compounds whose biological activity was measured using the standard Ellman's method.

A novel series of *tacrine-selegiline hybrids* for application as inhibitors of cholinesterase (AChE/BuChE) and monoamine oxidase (MAO-A/B) have also been synthesized [181].

Ibrahim et al. [182] isolated four sesquiterpene lactones from the ethyl acetate soluble fraction of *Amberboa ramosa*. One compound, Amberbin C, was found to be new while other three compounds, Amberin, Amberbin A, and Amberbin B, were previously reported. All compounds showed inhibitory activities against acetyl cholinesterase and butyryl cholinesterase.

A new series of *acetophenone derivatives* which possess alkylamine side chains were designed, synthesized, and assayed as acetylcholinesterase and butyrylcholinesterase inhibitors [183].

Based on a AChE inhibitor with a *1,4-substituted 4-(1H)-pyridylene-hydrazone* skeleton, Prinz et al. [184] generated a substance library for inhibition of AChE, BChE, and Aβ fibril formation. A bisnaphthyl-substituted compound was found to be the best overall inhibitor of AChE/BChE, with capacity for Aβ fibril destruction.

Donepezil-hydrazinonicotinamide hybrids. Zurek et al. [185] synthesized donepezil-hydrazinonicotinamide hybrids by condensation between indanone derivatives and the hydrazine nicotinated moiety, with acetylcholinesterase and butyrylcholinesterase inhibitory activity.

The ethyl acetate extract of the endophytic *Chaetomium globosum*, isolated from healthy leaves of Egyptian medicinal plant *Adiantum capillus-veneris*, collected from Saint Katherine Protectorate, Sinai, Egypt, showed strong antioxidant activity, potent anticancer activity, and promising butyrylcholinesterase inhibitory activity (>85 %) [186].

Other series of cholinesterase inhibitors recently characterized include *thioflavin- and deferiprone-based molecules* [187], *7-Methoxytacrine-adamantylamine heterodimers* [188], *N′-2-(4-benzylpiperidin-/piperazin-1-yl)acylhydrazone derivatives* [189], *and Bis(9)-(−)-nor-meptazinol* [190].

6.2 Muscarinic and Nicotinic Receptors

The restoration of neuronal signaling from the basal forebrain cholinergic system via the activation of the M1 muscarinic receptor has been proposed as a therapeutic strategy in AD. A number of non-selective M1 muscarinic agonists have shown positive effects on cognitive behaviors in AD patients, but were limited due to cholinergic adverse events thought to be mediated by the activation of the M2 to M5 subtypes. Quinoline carboxylic acids have been previously identified as highly selective M1 positive allosteric modulators with good pharmacokinetic and in vivo properties. A novel quinolizidinone carboxylic acid scaffold with 4-cyanopiperidines has been characterized [159]. A promising drug target under investigation to improve cognitive deficits in AD and other CNS disorders is the neuronal nicotinic alpha7 acetylcholine receptor (α7nAChR). The α7nAChR is a ligand-gated ion channel that has particularly high permeability to Ca^{2+} and is expressed in key brain regions involved in cognitive processes. The α7nAChRs are localized both presynaptically, where they can regulate neurotransmitter release, and postsynaptically where they can activate intracellular signaling cascades and influence downstream processes involved in learning and memory. Activation of the α7nAChR with small molecule agonists enhances long-term potentiation, an in vitro model of synaptic plasticity, and improves performance across multiple cognitive domains in rodents, monkeys, and humans [160].

α4β2-nAChR agonists have shown promising potential in preclinical cognition models of AD. One example is the *compound ZY-1*, a new nicotinic analog that activates α4β2-nAChR [191].

Other examples of cholinergic agonists are *N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-6-chinolincarboxamide (EVP-5141)*, a novel α7 nicotinic acetylcholine receptor agonist/serotonin 5-HT_3 receptor antagonist [192] or M1 muscarinic positive allosteric modulators/allosteric agonists such as *Benzyl Quinolone Carboxylic Acid* (BQCA) which are M1-selective over other muscarinic subtypes. Novel BQCA analogs augment ligand affinity for the receptor (pKB), intrinsic efficacy (τB), and both binding (α) and functional (β) cooperativity with acetylcholine [193].

The lack of selective muscarinic receptor ligands has for a long time limited the definition of therapeutic treatment based on muscarinic receptors as targets. Some muscarinic ligands such as *cevimeline* or *xanomeline* have been developed for the treatment of AD and other CNS disorders. Some muscarinic agonists (*vedaclidine*) have analgesic effects comparable to those produced by morphine or opiates [194].

The M1/M4-preferring muscarinic agonist xanomeline was found to have some benefit in the treatment of the memory impairment of AD, but side effects precluded further development. *EUK1001*, a fluorinated derivative of xanomeline, because of greater affinity for M1 muscarinic receptors, is likely to have a significantly better side effect profile than xanomeline. EUK1001 showed superiority to xanomeline with regard to attenuation of several AD-like neurodegenerative phenotypes in PS cDKO mice [161].

The arborvitae seed improves cognitive function and α7-nicotinic acetylcholine receptor (α7nAChR) protein expression in the hippocampus on AD model rats [195].

6.3 Monoaminergic Regulators

Damage to noradrenergic neurons in the locus coeruleus is a hallmark of AD and may contribute to disease progression. CNS noradrenaline (NA) levels in 5×FAD transgenic mice were increased using the NA precursor *L-threo-3,4-dihydroxyphenylserine* (L-DOPS). L-DOPS reduced astrocyte activation and Thioflavin-S staining; increased mRNA levels of neprilysin and insulin degrading enzyme, and of several neurotrophins; and increased brain-derived neurotrophic factor (BDNF) protein levels [162].

Selective 5-HT$_3$ receptor antagonists (Tropisetron). Tropisetron, a selective 5-HT$_3$ receptor antagonist, is conventionally used to counteract chemotherapy-induced emesis. Tropisetron protects against rat embolic stroke. In an Aβ rat model of AD, tropisetron diminished the elevated levels of TNF-α, COX-2, iNOS, NF-κB, active caspase 3, cytochrome c release, and calcineurin phosphatase activity and reversed cognitive deficit. Tropisetron was also found to be a potent inhibitor of calcineurin phosphatase activity. The selective 5-HT$_3$ receptor agonist m-Chlorophenyl-biguanide (mCPBG), when co-administered with tropisetron, completely reversed the pro-cognitive and antiapoptotic properties of tropisetron while it could only partially counteract the anti-inflammatory effects. mCPBG alone aggravated Aβ-induced injury [196].

6.4 Novel Compounds from Natural Sources

A myriad of natural compounds have been tested for the past 20 years in a frenetic search for agents with potential effects against AD neuropathology. Some of these compounds include alkaloids from the calabar bean (*Physostigma venenosum*); huperzine A from *Huperzia serrata*; galantamine from the snowdrop *Galanthus woronowii*; cannabinoids (cannabidiol from *Cannabis sativa*);

saffron (*Crocus sativus*); ginseng (*Panax* species); sage (*Salvia* species); lemon balm (*Melissa officinalis*); *Polygala tenuifolia*; nicotine from *Nicotiana* species [197]; grape seed polyphenolic extracts; Fuzhisan, a Chinese herbal medicine [198]; resveratrol [199]; xanthoceraside [200]; garlic (*Allium sativum*) [201]; linarin from *Mentha arvensis* and *Buddleja davidii* [202]; carotenoids such as retinoic acid, all trans retinoic acid, lycopene, and β-carotene [203]; curcumin from the rhizome of *Curcuma longa* [204]; plants of different origin such as Yizhi Jiannao, *Moringa oleifera* (Drumstick tree), *Ginkgo biloba* (Ginkgo/Maidenhair tree), *Cassia obtisufolia* (Sicklepod), *Desmodium gangeticum* (Sal Leaved Desmodium), *Melissa officinalis* (Lemon Balm), and *Salvia officinalis* (Garden sage, common sage) [205]; decursinol from the roots of *Angelica gigas* [206]; *Bacopa monniera* Linn (Syn. Brahmi); olive oil; phytoestrogens [207]; walnut extract [208]; *Erigeron annuus* leaf extracts; Epigallocatechin-3-gallate and luteolin [209]; the brown algae *Ecklonia cava* [210]; Gami-Chunghyuldan, a standardized multiherbal medicinal formula [211]; *Salvia* species [212]; *Punica granatum* extracts [213]; Naringenin [214]; Biochanin-A [215]; Caffeine [216]; Kampo medicine [217]; and multiple Flavonoids [218, 219] (*see* Table 2).

Gao et al. [220] classified traditional Chinese medicines for the treatment of AD in several categories: (a) Flavonoids: (i) Ginkgo flavonoids, the main constituents of *Ginkgo biloba* extracts (*quercetin*, *kaempherol*, *isorhamnnetin*), and biflavonoids (*ginkgetin*, *isoginkgetin*, *amentoflavone*); (ii) Soy isoflavones (*daidzin*, *daidzein*, *genistin*, *genistein*, *glycitin*, *glycitein*); (iii) Puerarin, an isoflavone glycoside extracted from species of the Leguminosae family, such as *Radix puerariae*; (iv) flavonoids of Baical Skullcap stem and leaf (*scutellarin*, *baicalin*, *chrysin*); (v) *Liquirin*, an extract from the root of *Glycyrrhiza uralensis* Fisch; (vi) *Apigenin*, a flavone from *Apium graveolens*; and (vii) other flavonoids, such as *Hyperoside*, a flavonol isolated from species of *Hypericum*, or *Rhodosin*, a flavonol obtained from the root of *Rhodiola rosea*; (b) Alkaloids: (i) *Huperzine A*, a cholinesterase inhibitor isolated from the Chinese herb *Huperzia serrate*; (ii) *Sphocardipine*, isolated from the root of *Sophora flavescens*; (iii) *Clausenamide*, isolated from the leaves of *Clausea lansium* (lour) Skeels (Rutaceae family); and (iv) other alkaloids, such as *Compound MA9701* (an arecoline analog), isolated from the seeds of *Areca catechu*, or *Securinine*, an alkaloid isolated from the leaves of *Securinega suffructicosa*; (c) Phenylpropanoids: (i) *Salvianolic acid B*, isolated from the root of *Salvia miltiorrhiza*; (ii) *Curcumin*, isolated from the root of *Curcuma longa*; (iii) *Schisandrone* and Schisanhenol, linans isolated from the fruit of *Schisandra chinensis*; and (iv) *Osthole*, a coumarin isolated from plants of the Umbelliferae family, such as *Cnidium monnieri*; (d) Triterpenoid saponins: (i) *Panax notoginseng saponins*, saponins of the dammarane type, the main

components of *Panax notoginseng*; (ii) *Ginsenoside*, isolated from *Panax ginseng* C.A. Mey; and (iii) *Gypenosides*, saponins isolated from *Gynostemma pentaphyllum*; and (e) Polysaccharides: (i) polysaccharides from *Cistanche deserticola*; (ii) polysaccharides from *Ganoderma lucidum*; and (iii) Oligosaccharides from *Rehmannia glutinosa*.

Flavonoids are one of the largest classes of phenylpropanoid-derived plant specialized metabolites, with 10,000 different members. They consist of two main groups, the 2-phenylchromans (flavonoids: flavanones, flavones, flavonols, flavan-3-ols, anthocyanidins) and the 3-phenylchromans (isoflavonoids: isoflavones, isoflavans, pterocarpans). Some flavonoids and their metabolites exhibit positive effects for disease therapy and chemoprevention [218, 219].

Aggregation of microtubule-associated protein tau into insoluble intracellular NFTs is a hallmark of AD and other neurodegenerative diseases, including progressive supranuclear palsy, argyrophilic grain disease, corticobasal degeneration, frontotemporal dementias with Parkinsonism linked to chromosome 17, and Pick's disease. Select *grape-seed polyphenol extracts* may interfere with the assembly of tau peptides into neurotoxic aggregates and attenuate the development of AD type tau neuropathology in the brain of TMHT mouse model of AD through mechanisms associated with attenuation of extracellular signal-receptor kinase 1/2 signaling in the brain [221].

Fuzhisan (FZS), a Chinese herbal medicine, may have a positive effect on cognition, behavioral functions, and regional cerebral metabolic rate of glucose consumption (rCMRglc) in mild-to-moderate AD patients [198].

Resveratrol is a natural compound found in grapes and red wine. Resveratrol seems to exert its neuroprotective role through inhibition of Aβ aggregation, by scavenging oxidants and exerting anti-inflammatory activities [199]. Resveratrol improves long-term memory formation and the LTP induction. These effects are blocked in SIRT1 mutant mice. Resveratrol effects might be mediated through reduced expressions of miR-134 and miR-124, which may in turn upregulate CREB levels to subsequently promote BDNF synthesis [222]. Resveratrol increased cell viability through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) colorimetric assays, reduced cell apoptosis, stabilized the intercellular Ca^{2+} homeostasis, attenuated $A\beta_{25-35}$ neurotoxicity, and reversed $A\beta_{25-35}$-suppressed silent information regulator 1 (SIRT1) activity, resulting in the downregulation of Rho-associated kinase 1 (ROCK1). These results indicate that resveratrol protects PC12 cells and inhibits the β-amyloid-induced cell apoptosis through the upregulation of SIRT1 [223].

A series of multitarget-directed resveratrol derivatives were designed and synthesized for the treatment of AD [224]. In vitro studies indicated that most of the target compounds exhibit significant inhibition of self-induced Aβ aggregation and Cu^{II}-induced $A\beta_{1-42}$ aggregation and acted as potential antioxidants and biometal chelators.

Novel resveratrol derivatives (*polyhydroxylated (E)-stilbenes*), synthesized by Mizoroki-Heck reactions, revealed potent butyrylcholinesterase-inhibiting properties [225].

Flavonoids inhibit Aβ and sAPPβ production by regulating BACE-1 expression and not by directly inhibiting BACE-1 activity. *Xanthoceraside* attenuates memory impairments through amelioration of oxidative stress and inflammatory responses induced by $A\beta_{25-35}$ [200]. Apart from its culinary use, garlic (*Allium sativum*) is being used to treat several ailments like cancer and diabetes. "Aged Garlic Extract" (AGE) and one of its active ingredients, *S-allyl-L-cysteine* (SAC), influence several pathological cascades related to the synaptic degeneration and neuroinflammatory pathways associated with AD [201].

Linarin, a naturally occurring flavanol glycoside derived from *Mentha arvensis* and *Buddleja davidii*, is known to have antiacetylcholinesterase effects. Linarin prevents $A\beta_{25-35}$-induced neurotoxicity through the activation of PI3K/Akt, which subsequently inhibits GSK-3β and upregulates Bcl-2 [202].

Nobiletin, a polymethoxylated flavone isolated from citrus peels, has the potential to improve cognitive dysfunction in patients with AD. Nobiletin activated extracellular signal-regulated kinase (ERK) signaling and subsequent cyclic AMP response element-dependent transcription. Kimura et al. [226] studied the effects of five nobiletin analogs, *6-demethoxynobiletin*, *tangeretin*, *5-demethylnobiletin*, *sinensetin*, and *6-demethoxytangeretin*, on ERK phosphorylation in PC12D cells. 6-Demethoxynobiletin markedly enhanced ERK phosphorylation in a concentration-dependent manner [226].

Baicalein, a flavonoid isolated from the roots of *Scutellaria baicalensis*, is known to modulate γ-aminobutyric acid (GABA) type A receptors. Baicalein reduces the production of Aβ by increasing APP α-processing. These effects are blocked by the GABAA antagonist bicuculline. AD mice treated daily with i.p. baicalein for 8 weeks showed enhanced APP α-secretase processing, reduced Aβ production, and reduced AD-like pathology together with improved cognitive performance [227].

Oleuropein. Transgenic AD mice supplemented with oleuropein aglycone (50 mg/kg of diet), the main polyphenol found in extra virgin olive oil, showed improvement in cognitive performance, reduced β-amyloid levels and plaque deposits, and an intense autophagic reaction [228].

Carotenoids play a pivotal role in the prevention of many degenerative diseases mediated by oxidative stress. Carotenoids like *retinoic acid*, *all trans retinoic acid*, *lycopene*, and *β-carotene* have been proposed as candidate compounds in prevention of AD symptoms primarily through inhibition of Aβ formation, deposition, and fibril formation either by reducing the levels of p35 or inhibiting corresponding enzymes [203].

TongLuoJiuNao (TLJN) (*Geniposide* and *Ginsenoside Rg1*). The effects of TongLuoJiuNao (TLJN), a traditional Chinese medicine preparation, against formaldehyde stress were studied in human neuroblastoma cells (SH-SY5Y cell line). Formaldehyde can induce misfolding and aggregation of Tau protein and β amyloid protein. TLJN and its main ingredients (geniposide and ginsenoside Rg1) rescued formaldehyde-injured cells, increased intracellular antioxidants (superoxide dismutase and glutathione peroxidase) and mRNA and protein levels of the antiapoptotic gene *Bcl-2*, and downregulated the apoptotic-related gene-*P53*, apoptotic executer-caspase 3, and apoptotic initiator-caspase 9 [229].

Huannao Yicong Recipe. Li et al. [230] studied the effects of Huannao Yicong Recipe (HNYCR) extract on learning and memory, as well as on the expressions of APP, beta-site APP-cleaving enzyme 1 (BACE1), presenilin-1 (PS-1), and Aβ in the hippocampal CA1 area of APP transgenic mice. The HNYCR extract improved learning and memory abilities in APP transgenic mice, and reduced the expressions of APP, BACE1, PS-1, and Aβ in the hippocampus.

Yuzu. Long-term oral consumption of yuzu (*Citrus junos* Tanaka) extract improves cognitive dysfunction and glucose homeostasis in β-amyloid-induced toxicity in rats. Yuzu treatment prevented β-amyloid accumulation and tau phosphorylation, attenuated hippocampal insulin signaling, and improved memory [231].

Plumula nelumbinis (*liensinine*, *isoliensinine*, and *neferine*). In a *Plumula nelumbinis* sample, three alkaloids (liensinine, isoliensinine, and neferine) have been detected with a strong BChE inhibition activity [232].

Catalpol. Zhang et al. [233] studied the neuroprotective effects of catalpol, an iridoid glycoside isolated from the fresh rehmannia roots, on the cholinergic system and inflammatory cytokines in the senescent mouse brain induced by D-galactose. Acetylcholinesterase (AChE) activity increased in senescent mouse brain and choline acetyltransferase (ChAT) decreased in the basal forebrain of senescent mouse. Muscarinic acetylcholine receptor M1 (mAChR1) expression declined and the levels of tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and advanced glycation end products

(AGEs) increased in senescent mouse brain. Catalpol reversed all these neurochemical changes, indicating that catalpol can exert protective effects on senescent mouse brain induced by D-galactose.

Cnestis ferruginea (*Amentoflavone*). *Cnestis ferruginea* Vahl ex DC (Connaraceae) (CF) is used in traditional African medicine in the management of CNS disorders. Ishola et al. [234] studied the effects of *C. ferruginea* and its active constituent amentoflavone (CF-2) on memory, oxidative stress, and acetylcholinesterase (AChE) activity in scopolamine-induced amnesia. Oral administration of CF and CF-2 significantly prevented scopolamine-induced memory impairment, inhibited AChE, and enhanced antioxidant enzyme activity in the brain.

Tanshinones. Tanshinones extracted from Chinese herb Danshen (*Salvia Miltiorrhiza* Bunge) were traditionally used as anti-inflammation and cerebrovascular drugs due to their antioxidation and antiacetylcholinesterase effects. Tanshinones protect neuronal cells. Tanshinone I (TS1) and tanshinone IIA (TS2), the two major components in the Danshen herb, inhibit the aggregation and toxicity of $A\beta_{1-42}$. Both TS1 and TS2 exhibit different inhibitory abilities to prevent unseeded amyloid fibril formation and to disaggregate preformed amyloid fibrils, in which TS1 shows better inhibitory potency than TS2 [235].

Acalypha wilkesiana var. macafeana hort. Din et al. [236] studied the properties of *Acalypha wilkesiana* var. *macafeana hort*, used to heal wounds in Malaysian traditional medicine. *Acalypha wilkesiana* var. *macafeana hort.* protected human hepatocellular liver carcinoma (HepG2) cells exposed to tert-butylhydroperoxide, and protected cells against oxidative injuries, showing potent antioxidant and cytoprotective activities. These effects might be exerted by *geraniin* [236].

Curcumin. Curcumin (*Diferuloylmethane*) is a phytochemical compound extracted from the rhizome of *Curcuma Longa*. Curcumin is a constituent of the ancient herbal medicine Jiawei-Xiaoyaosan that has been used for dyspepsia, stress, and mood disorders [237]. It is the pigment responsible for the characteristic yellow color of Indian curry. Curcumin, as well as some other polyphenols, strongly induces heme oxygenase 1 and Phase II detoxification enzymes in neurons and protects neurons against different modes of oxidative challenge. Curcumin reduces the activity of NADH dehydrogenase (complex I), succinic dehydrogenase (complex II), and cytochrome oxidase (Complex IV) in the brain and normalizes the activities of these mitochondrial complexes in aluminum-treated rats [204]. Curcumin protects human neuroblastoma SK-N-SH cells against acrolein toxicity. The addition of

curcumin restores the expression of γ-glutamylcysteine synthetase, ROS, and reactive nitrogen species levels but has no effect on the decrease of glutathione (GSH) and on the elevation of protein carbonyls. Acrolein induces the activity of Nrf2, NF-κB, and Sirt1, and these activations can be prevented by the presence of curcumin. Acrolein also induces a decrease of the pAkt, which can be counteracted by curcumin [238].

Hoppe et al. [239] investigated possible mechanisms involved in curcumin protection against $A\beta_{1-42}$-induced cognitive impairment and developed curcumin-loaded lipid-core nanocapsules in an attempt to improve the neuroprotective effect of this polyphenol. $A\beta_{1-42}$-infused animals showed a significant impairment on learning-memory ability, which was paralleled by a significant decrease in hippocampal synaptophysin levels. Animals exhibited activated astrocytes and microglial cells, as well as disturbance in BDNF expression and Akt/GSK-3β signaling pathway, beyond tau hyperphosphorylation. Administration of curcumin was effective in preventing behavioral impairments, neuroinflammation, tau hyperphosphorylation as well as cell signaling disturbances triggered by Aβ in vivo.

Turmeric. Turmeric possesses multiple medicinal uses. Curcuminoids, a mixture of curcumin, demethoxycurcumin, and bisdemethoxycurcumin, are constituents of turmeric, and curcumin is the most important constituent of the curcuminoid mixture that contributes to the pharmacological profile of parent curcuminoid mixture or turmeric [240]. Hishikawa et al. [241] reported a significant improvement of behavioral symptoms in AD patients with turmeric treatment.

Crocus sativus (Saffron, Safranal). Saffron (*Crocus sativus*) and its active metabolite safranal display neuropharmacological effects with anxiolytic, anticonvulsant, and neuroprotective activity. Safranal decreases quinolinic acid-induced lipid peroxidation and oxidative DNA damage, and prevents the decrease of hippocampal thiol redox and antioxidant status produced by quinolinic acid [242].

Traditional Korean herbs. Extracts of the roots of *Scutellaria baicalensis* Georgi (Labiatae) have been widely used to relieve fever related to bacterial infection and inflammatory diseases in traditional Korean medicine and have been reported to be effective in brain diseases. Administration of *Scutellaria baicalensis* mitigated alterations of hippocampal MAPK signaling by chronic cerebral infusion and microglial activation by chronic LPS infusion. Acetylcholinesterase (AChE) inhibition, modification of monoamines, antiamyloid aggregation effect, and antioxidant activity have been reported to be induced by extracts of a few plants of

different origin like Yizhi Jiannao, *Moringa oleifera* (Drumstick tree), *Ginkgo biloba* (Ginkgo/Maidenhair tree), *Cassia obtisufolia* (Sicklepod), *Desmodium gangeticum* (Sal Leaved Desmodium), *Melissa officinalis* (Lemon Balm), and *Salvia officinalis* (Garden sage, common sage) [205].

Decursinol is a major coumarin derived from the roots of *Angelica gigas* and has various pharmacological effects against inflammation, angiogenesis, nociceptive pain, and AD [206].

Gouqi (*Lycium barbarum*). Zhang et al. [243] examined the effects of Gouqi (*Lycium barbarum*) on the learning and memory abilities of an APP/PS1 double transgenic mouse model of AD. Oral administration of Gouqi extracts at 10 mg/kg improved the performance of the APP/PS1 mice in the learning and the memory retrieval phases of the Morris maze and the levels of $A\beta_{1-42}$ in hippocampal tissue were reduced.

Walnuts are rich in components that have antioxidant and anti-inflammatory properties. The inhibition of in vitro fibrillization of synthetic Aβ and solubilization of preformed fAβ by walnut extract have been reported. Walnut extract reduces Aβ-mediated cell death, membrane damage, DNA damage, and generation of ROS in a concentration-dependent manner [208].

Erigeron annuus leaf extracts containing *caffeic acid* as an active compound have antioxidative and neuroprotective effects on neuronal cells.

Epigallocatechin-3-gallate. The polyphenol epi-gallocatechine-3-gallate (EGCG), the main polyphenol in *Camilla sinensis*, binds directly to a large number of proteins that are involved in protein misfolding diseases and inhibits their fibrillization. Instead, it promotes the formation of stable, spherical aggregates. These spherical aggregates are not cytotoxic, have a lower β-sheet content than fibrils, and do not catalyze fibril formation. Correspondingly, epigallocatechine-3-gallate remodels amyloid fibrils into aggregates with the same properties [244]. Epigallocatechin-3-gallate and luteolin were identified as top mitochondrial restorative compounds from in vitro screening. EGCG treatment restored mitochondrial respiratory rates, MMP, ROS production, and ATP levels by 50–85 % in mitochondria isolated from the hippocampus, cortex, and striatum [209]. Short-term treatment with EGCG significantly attenuated the neurotoxicity of $A\beta_{1-42}$, reduced the number of apoptotic cells, decreased ROS generation, and downregulated caspase-3 levels after treatment with 25-μM $A\beta_{1-42}$. EGCG markedly strengthened activation of the α7 nicotinic acetylcholine receptor (α7 nAChR) signaling cascade as well as its downstream pathway signaling molecules phosphatidylinositol 3-kinase (PI3K) and Akt, subsequently leading to suppression of Bcl-2 downregulation in Aβ-treated neurons. Administration of α7nAChR

antagonist methyllycaconitine to neuronal cultures significantly attenuated the neuroprotection of EGCG against Aβ-induced neurotoxicity, thus presenting new evidence that the α7nAChR activity together with PI3K/Akt transduction signaling may contribute to the molecular mechanism underlying the neuroprotective effects of EGCG against Aβ-induced cell death [245].

TNF-α/c-Jun N-terminal kinase (JNK) signaling plays a central role in serine phosphorylation of insulin receptor substrate-1 (IRS-1). (−)-Epigallocatechin-3-gallate attenuates peripheral insulin resistance by reducing IRS-1 signaling blockage. EGCG ameliorates the impaired learning and memory in APP/PS1 mice, reduces IRS-1pS636 levels, decreases $A\beta_{42}$ levels in the hippocampus, inhibits TNF-α/JNK signaling, and increases the phosphorylation of Akt and glycogen synthase kinase-3β in the hippocampus [246].

Silymarin derivatives (Taxifolin). Silymarin, the seed extract of *Silybium marianum*, has preventive effects against AD. Sato et al. [247] isolated (+)-taxifolin from silymarin as an inhibitor of aggregation of $A\beta_{42}$.

The brown algae *Ecklonia cava* is known for its antioxidant and anti-inflammatory functions. The butanol extract of *E. cava* reduces Aβ secretion from HEK293 cells expressing APP with Swedish mutation and increases soluble APPα and C-terminal fragment-α (CTFα), of which activity is similar to BACE (β-site of APP cleaving enzyme) inhibitors. The extract inhibits Aβ oligomerization, particularly midsize oligomer formation, and protects primary cortical neurons from various Aβ-induced cell deaths [210].

Gami-Chunghyuldan (GCD), a standardized multiherbal medicinal formula, improves Aβ-induced memory impairment and reduces neuronal cell death, astrogliosis, and microgliosis in the hippocampus. GCD also prevents Aβ-triggered synaptic disruption and cholinergic fiber loss [211].

Derivatives of *Orcein*, which is a phenoxazine dye that can be isolated from the lichen *Roccella tinctoria*, accelerate fibril formation of the amyloid-beta peptide. These compounds deplete oligomeric and protofibrillar forms of the peptide [244].

Bajijiasu. Chen et al. [248] studied the protective effect of Bajijiasu (β-D-fructofuranosyl (2-2) β-D-fructofuranosyl), a dimeric fructose isolated from the Chinese herb radix *Morinda officinalis*, on Aβ-induced neurotoxicity in pheochromocytoma (PC12) cells. Bajijiasu reversed the reduction in cell viability induced by exposure to $A\beta_{25-35}$, reduced $A\beta_{25-35}$-induced toxicity, decreased the accumulation of intracellular ROS and the lipid peroxidation product malondialdehyde, upregulated expression of glutathione reductase and superoxide dismutase, prevented depolarization of the mitochondrial membrane potential (Ψm), and blocked

$A\beta_{25-35}$-induced increases in $[Ca^{2+}]i$. Bajijiasu also reversed $A\beta_{25-35}$-induced changes in the expression levels of p21, CDK4, E2F1, Bax, NF-κB p65, and caspase-3.

Nam and Lee [249] studied the chemical constituents of *Zhizi* (*Fructus Gardeniae*) and their antiamnesic effect in a mouse model of AD. Activity-guided fractionation of the total extracts resulted in the isolation of two glycosides, *geniposide* and *crocin* from the n-butanol fraction and *genipin* and *crocetin* from the ethylacetate fraction. The n-butanol fraction showed the strongest AChE inhibition (43.4 %) and also exhibited efficacy in an experimental model of amnesia. Geniposide showed a 22.8 % AChE inhibitory activity and a potent ameliorating effect on scopolamine-induced memory impairment in amnesic mice.

In European folk medicine, Salvia species have traditionally been used to enhance memory. *Salvia fruticosa* extracts exhibit acetylcholinesterase and butyrylcholinesterase activity [212].

Punica granatum extracts show antioxidant and neuronal protective effects against oxidative stress-induced cytotoxicity in PC12 cells and inhibit neuronal cell death caused by Aβ-induced oxidative stress and Aβ-induced learning and memory deficit [213].

Azadirachta indica extracts showed cognition enhancement, antidepressant, and antianxiety properties [250].

Naringin has been isolated from Pomelo peel (a *Citrus* species). In the APPswe/PS1dE9 transgenic mouse model of AD, naringin enhanced the autophosphorylation of CaMKII, increased the phosphorylation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptor at a CaMKII-dependent site, and improved long-term learning and memory ability [251].

Naringenin can improve learning and memory in rat models of AD through oxidative stress regulation by increasing SOD activity and reduction of $A\beta_{40}$ and $A\beta_{42}$ expression, and phosphorylation of tau, as well [214].

Xanthoceraside is a triterpenoid saponin extracted from the fruit husks of *Xanthoceras sorbifolia* Bunge with biological properties to reverse the cognitive deficits observed in several AD animal models. Xanthoceraside treatment rescued memory deficits, as well as IR and IGF-1R protein expression levels in STZ animals, and reversed the decreased phosphorylation of CREB induced by STZ [252].

Biochanin-A is a potent phytoconstituent which has been used in India as an antitumor, a dopaminergic neuron protective agent, an antioxidant, an anticholinergic and a neuroprotectant with antioxidant properties, anticholinesterase activity, and neurotransmitter (noradrenalin, dopamine) enhancing capacity [215].

Caffeine (0.75–1.5 mg/day) improves memory impairment and the expression of brain neurotrophic derived factor (BNDF) and TrkB in PS1/APP double transgenic mouse models [216].

Kampo. Clinical evidence has demonstrated the potential usefulness of traditional herbal formulations called Kampo medicines, such as *chotosan* (CTS) and *yokukansan* (YKS), in dementia. Ingredients of *Uncariae Uncis cum Ramulus*, a medicinal herb included in CTS and YKS, may play an important role in the actions of these formulae in dementia patients [217].

Yokukansan, a traditional Japanese (Kampo) medicine, composed of seven medicinal herbs, has been traditionally used to treat neurosis, insomnia, night crying, and irritability in children, and also to improve behavioral symptoms in dementia [253]. Yokukansan ameliorates Aβ oligomer-induced neuronal damage and suppresses DNA fragmentation caused by Aβ oligomers and Aβ oligomer-induced activation of caspase-3. Among the seven constituents of yokukansan, Glycyrrhiza and Uncaria Hook suppressed Aβ oligomer-induced neuronal damage, DNA fragmentation, karyopyknosis, and caspase-3 activation to almost the same extent as yokukansan [253].

Okada and Okada [254] studied the properties of 15 plants, nine medical herbs (Japanese honeywort, luffa, rapeseed, Chinese colza, potherb mustard, Japanese radish, bitter melon, red shiso, corn, and kaiware radish), and six general commercial plants (common bean, komatsuna, Qing geng cai, bell pepper, kale, and lettuce). The aqueous extracts showed antioxidant activities. Intracellular ROS accumulation resulting from Aβ treatment was reduced when cells were treated with some extracts. Kale, bitter melon, kaiware radish, red shiso, and corn inhibited tumor necrosis factor-alpha secretion in Aβ-stimulated neurons and all samples, except Japanese honeywort, showed enhancement of cell survival.

Quercetin-3-O-glucuronide. The brain-targeted polyphenol metabolite, quercetin-3-O-glucuronide, from red wine reduces the generation of Aβ peptides by primary neuron cultures generated from the Tg2576 AD mouse model. Quercetin-3-O-glucuronide is also capable of interfering with the initial protein–protein interaction of $A\beta_{1-40}$ and $A\beta_{1-42}$ that is necessary for the formation of neurotoxic oligomeric Aβ species [255].

3′-O-Methyl-epicatechin-5-O-β-glucuronide. Glucuronidated and/or methylated metabolites of the proanthocyanidin (PA) monomer (−)-epicatechin are detected in both blood and brain of rodents fed with a monomeric grape seed PA extract. Glucuronosyl transferases of the UGT1A and UGT2B families glucuronidate epicatechin or 3′-O-methyl epicatechin in vitro. UGT1A9 is the most efficient, producing epicatechin 3′-O-glucuronide as the major product. Incubation of UGT1A9 with 3′-O-methyl-epicatechin resulted in two major products, one of which was identified as 3′-O-methyl-epicatechin 5-O-glucuronide, a major metabolite found in blood and brain [256]. 3′-O-methyl-epicatechin-5-O-β-glucuronide (3′-O-Me-EC-Gluc), one of the

proanthocyanidin (PAC) metabolites identified in the brain following epicatechin treatment in monomeric form, promotes basal synaptic transmission and long-term potentiation at physiologically relevant concentrations in hippocampus slices through mechanisms associated with cAMP response element binding protein (CREB) signaling [257].

Lavandula pedunculata subsp. *lusitanica (Chaytor) Franco.*The essential oil and polar extracts of *Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco collected in south Portugal contain *camphor* (40.6 %) and *fenchone* (38.0 %), with *hydroxycinnamic acids* (*3-O-caffeoylquinic*, *4-O-caffeoylquinic*, *5-O-caffeoylquinic*, and *rosmarinic acids*) and flavones (luteolin and apigenin) in the polar extracts. Rosmarinic acid is the main compound in most of them. The bioactive compounds from *L. pedunculata* polar extracts acts as free-radical scavengers, Fe^{2+} chelators, and inhibitors of malondialdehyde production, and the essential oil is an active acetylcholinesterase inhibitor [258].

Cochlospermum angolensis Welw. Extracts (Ellagic Acid). Cochlospermum angolensis Welw. bark is a medicinal plant used for the treatment of hepatic diseases and for the prophylaxis of malaria. Ferreres et al. [259] characterized eight compounds from the extracts of this plant. Hydromethanolic extract was richer in *methyl ellagic acid* and its derivatives, while aqueous extract had higher amounts of *ellagic acid* and its derivatives. Methyl ellagic acid pentoside isomer and ellagic acid were the major compounds in the two extracts, respectively. Both extracts and ellagic acid showed potent antioxidant activity.

Cocoa peptide 13L (DNYDNSAGKWWVT). A bioactive peptide, 13L (DNYDNSAGKWWVT), was obtained from a hydrolyzed cocoa by-product by chromatography. Peptide 13L showed antioxidant activity in the wild-type strain (N2) of *Caenorhabditis elegans* and produced a significant delay in body paralysis in strain CL4176, after $A\beta_{1-42}$ peptide induction [260].

Cudrania cochinchinensis. The ethanol/water extracts of *Cudrania cochinchinensis* and the purified isolated components effectively reduced the nitric oxide production and iNOS expression stimulated by IFN-γ combined with fAβ, and decreased Aβ deposition [261].

Salvia sclareoides (Rosmarinic acid). Salvia sclareoides extracts showed acetylcholinesterase (AChE) inhibitory activity. Rosmarinic acid is present in the extracts where *luteolin 4′-O-glucoside*, *luteolin 3′,7-di-O-glucoside*, and *luteolin 7-O-(6′-O-acetylglucoside)* were also identified. Rosmarinic acid is the only explicit binder for AChE [262].

Ethyl acetate extract of germinated brown rice. The ethyl acetate extract of germinated brown rice (GBR) had higher total phenolic content and antioxidant capacity compared to brown rice (BR). The protection of human SH-SY5Y neuronal cells by the GBR extract was linked to its ability to induce transcriptional changes in antioxidant (*SOD1*, *SOD2*, and catalase) and apoptotic (*AKT*, *NF-Kβ*, *ERK1/2*, *JNK*, *p53*, and *p38 MAPK*) genes [263].

Taxus chinensis (Sciadopitysin). Five taxane diterpenoids derived from the 95 % ethanol extract of *Taxus chinensis* were tested for the inhibitory activities on Aβ aggregation. *Sciadopitysin* was found to exhibit the highest potency against Aβ aggregation and fibril formation, increased SH-SY5Y cell viability, and demonstrated neuroprotection against Aβ protein-induced damage in primary cortical neurons [264].

Ginkgo biloba. *Ginkgo biloba* leaf extracts (GLEs) have been currently used for the treatment of dementia, tinnitus, vertigo, and peripheral arterial disease. Preclinical studies revealed potential interactions of GLEs with other drugs causing inhibition and induction of metabolic enzymes and transporters. In humans, higher doses than the recommended ones (240 mg/day) may lead to a weak induction of the CYP2C19-mediated omeprazole 5-hydroxylation and a weak inhibition of the CYP3A4-mediated midazolam 1′-hydroxylation, respectively. Also, the regular intake of a poorly characterized GLE at a dose of 360 μmg/day slightly increased the bioavailability of talinolol, a substrate of P-glycoprotein and various organic anion-transporting polypeptides. According to data reported by Unger [265], the intake of the standardized GLE, EGb 761, together with synthetic drugs appears to be safe as long as daily doses up to 240 μmg are consumed.

Bilobalide. Bilobalide (BB) is one of the major active compounds extracted from *Ginkgo biloba* leaves. BB protects against learning and memory impairments induced by $A\beta_{25-35}$, attenuates the neuronal damage and apoptosis in frontal cortex and hippocampus, and inhibits TNF-α and $A\beta_{1-40}$ expression [266].

Green tea catechin. Transgenic mice treated with green tea catechin (GTC), a radical scavenger, exhibited decreases in behavioral impairment, $A\beta_{-42}$ production, APP-C99/89 expression, γ-secretase component and Wnt protein levels, γ-secretase activity, and MAPK activation. In contrast, the levels of APP-C83 protein and enzyme activities (α-secretase, neprilysin, and Pin1) were elevated in the GTC-treated mice, together with a decrease in the levels of total cholesterol and low-density lipoprotein cholesterol, whereas the level of high-density lipoprotein cholesterol increased [267].

Ginseng and Ginsenosides. Ginseng is one of the most widely used herbal medicines in humans [268]. Ginseng, the root of the *Panax*

ginseng, has been a popular and widely used traditional herbal medicine in Korea, China, and Japan for thousands of years. Ginseng- or ginsenoside-mediated neuroprotective mechanisms involve homeostasis regulation, and anti-inflammatory, antioxidant, antiapoptotic, and immune-stimulatory activities [269]. Kim et al. [270] examined the effect of fermented ginseng (FG) on memory impairment and Aβ reduction in models of AD in vitro and in vivo. After 8 h incubation with the FG extract, the level of soluble $A\beta_{42}$ was reduced. FG extract treatment resulted in a significant recovery of memory function in animal models. Brain soluble $A\beta_{42}$ levels measured from the cerebral cortex of transgenic mice were significantly reduced by the FG extract treatment.

(20S)-Rg3, a triterpene natural compound known as ginsenoside, reduced Aβ levels in cultured primary neurons and in the brains of a mouse model of AD. The (20S)-Rg3 treatment induced a decrease in the association of presenilin 1 (PS1) fragments with lipid rafts where catalytic components of the γ-secretase complex are enriched. The Aβ-lowering activity of (20S)-Rg3 directly correlated with increased activity of phosphatidylinositol 4-kinase IIα (PI4KIIα), a lipid kinase that mediates the rate-limiting step in phosphatidylinositol 4,5-bisphosphate synthesis. PI4KIIα overexpression recapitulated the effects of (20S)-Rg3, whereas reduced expression of PI4KIIα abolished the Aβ-reducing activity of (20S)-Rg3 in neurons [271].

Ginsenoside Rg1, one of the major active ingredients isolated from *Panax Ginseng*, has neuroprotective effects in animal models with memory impairment. Intracerebroventricular (i.c.v.) okadaic acid (OKA), a potent phosphatase inhibitor, induced memory impairment, decrease of phospho-tau and phospho-GSK3β, and formation of β-amyloid deposits in the brain of treated rats. All these changes were reversed by Rg1 [272].

Ginsenoside Rd, one of the principal active ingredients of *Panax notoginseng*, inhibits okadaic acid-induced tau phosphorylation in vivo and in vitro, inhibits tau phosphorylation at multiple sites in Aβ-treated cultured cortical neurons, reduces Aβ-induced increased expression of glycogen synthase kinase 3beta (GSK-3β), and enhances the activity of protein phosphatase 2A (PP-2A), a key phosphatase involved in tau dephosphorylation [273].

Pseudoginsenoside-F11 (PF11) is a component of *Panax quinquefolium* (American ginseng) with positive effects to antagonize the learning and memory deficits induced by scopolamine, morphine, and methamphetamine in mice. In APP/PS1 mice, PF11 8 inhibited the expressions of APP and $A\beta_{1-40}$ in the cortex and hippocampus, restored the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), decreased the production of malondialdehyde (MDA) in the cortex, and downregulated the expressions of JNK 2, p53, and cleaved caspase 3 in the hippocampus [274].

TongLuoJiuNao. The herbal medicine TongLuoJiuNao (TLJN) contains Ginsenoside Rg1 and Geniposide. TLJN has been used to treat patients with cerebral ischemic stroke and VD. In APP Swedish mutant transgenic mice (APP23), TLJN decreased Aβ production and deposition, downregulated the levels and activity of β-secretase 1 (BACE1) protein as well as the expression levels of γ-secretase complex components PS1, nicastrin, and anterior pharynx-defective 1 (APH1) but not presenilin enhancer 2 (PEN2), indicating that TLJN displays an inhibitory effect on the amyloidogenic APP processing by downregulating the cleavage enzymes BACE1 and γ-secretase [275].

Arabic traditional plants from Egypt. Ali et al. [276] tested 20 different Arabic traditional plants from Egypt. *Adhatoda vasica* and *Peganum harmala* showed inhibitory effect on AChE. *Ferula assafoetida*, *Syzygium aromaticum*, and *Zingiber officinalis* showed activity against COX-1 enzyme. Potent radical scavenging activity was demonstrated in *Terminalia chebula*, *T. arjuna*, and *Emblica officinalis* extracts.

Magnolol is a bioactivator extracted from *Magnolia officinalis* with protective effects on cholinergic neurons. Magnolol restores the impaired abilities of learning and memory induced by scopolamine, and AChE, NOS, and SOD activities in mice [277].

Plant-derived alkaloids. Naaz et al. [278] studied 13 plant-derived alkaloids, namely *pleiocarpine*, *kopsinine*, *pleiocarpamine* (from *Pleiocarpa mutica*, family: Annonaceae), *oliveroline*, *noroliveroline*, *liridonine*, *isooncodine*, *polyfothine*, *darienine* (from *Polyalthia longifolia*, family: Apocynaceae) and *eburnamine*, *eburnamonine*, *eburnamenine*, and *geissoschizol* (from *Hunteria zeylanica*, family: Apocynaceae), for their anticholinergic action through docking with acetylcholinesterase (AChE) as a target. Among the alkaloids, pleiocarpine showed promising anticholinergic potential, while its amino derivative showed about sixfold higher anticholinergic potential than pleiocarpine. Pleiocarpine and its amino derivative were found to be better inhibitors of AChE, as compared to commonly used drugs tacrine and rivastigmine.

Fragrant plant extracts. Kundu and Mitra [279] explored fragrant plant extracts that are traditionally used in flavoring foods, such as *Hemidesmus indicus* and *Vanilla planifolia*, as possible sources for AChEI. Root and pod extracts of *H. indicus* and *V. planifolia*, respectively, produce fragrant phenolic compounds, *2-hydroxy-4-methoxybenzaldehyde* (MBALD) and *4-hydroxy-3-methoxybenzaldehyde* (*vanillin*). These methoxybenzaldehydes were shown to have inhibitory potential against acetylcholinesterase (AChE). Vanillin is a more efficient inhibitor than MBALD.

Triptolide, isolated from the herb *Tripterygium wilfordii Hook F*, has anti-inflammatory and immunosuppressive activities, and

can also alleviate degeneration of dendritic spines in hippocampal neurons [280].

Phellinus linteus (PL) is a mushroom that has long been used as a folk medicine in China. PL decreases ROS formation in HepG2 cells, reduces tacrine-induced ROS production, disrupts ΔΨm, forms 8-OHdG in mitochondrial DNA, and induces cytotoxicity in HepG2 cells [281].

Lycoris radiata. Xin et al. [282] used the unique amyloid β-expressing transgenic *C. elegans* CL4176, which exhibits paralysis when human $A\beta_{1-42}$ is induced, to study two natural benzylphenethylamine alkaloids isolated from *Lycoris radiata* (L' Her.) Herb, *galanthamine* and *haemanthidine*, and their synthetic derivatives *1,2-Di-O-acetyllycorine* and *1-O-acetyllycorine* for their antiparalysis effects. These Lycoris compounds effectively delay the paralysis of CL4176 worms upon temperature upshift, and prolong the lives of these transgenic worms. Lycoris compounds were shown to significantly inhibit the gene expression of ace-1 and ace-2. Additionally, the Lycoris compounds may modulate inflammatory and stress-related gene expressions to combat the Aβ-toxicity in *C. elegans*.

Nigella sativa Linn. *Nigella sativa* Linn. seed (NS) has positive modulation effects on memory impairments, prevents hippocampal pyramidal cell loss, and enhances consolidation of recall capability of stored information and spatial memory [283].

Salvianolic acid A (Sal A) is a polyphenolic derivative, isolated from *Salvia miltorrhiza* Bunge. Sal A significantly inhibits amyloid beta self-aggregation and disaggregates preformed fibrils, reduces metal-induced aggregation through chelating metal ions, and blocks the formation of ROS in SH-SY5Y cells [284].

Salvianolic acid B (Sal B) ameliorated the $A\beta_{25-35}$ peptide-induced memory impairment, reduced the number of activated microglia and astrocytes that were observed during the inflammatory reaction after the administration of the $A\beta_{25-35}$ peptide, reduced inducible nitric oxide synthase and cyclooxygenase-2 expression levels and thiobarbituric acid reactive substances, which were increased by the administration of the $A\beta_{25-35}$ peptide, and rescued the $A\beta_{25-35}$ peptide-induced decrease of choline acetyltransferase and BDNF protein levels in an AD mouse model [285].

St. John's Wort (Hypericum perforatum). The adenosine triphosphate-binding cassette transport protein P-glycoprotein (ABCB1) is involved in the export of β-amyloid from the brain into the blood, and there is evidence that age-associated deficits in cerebral P-glycoprotein content may be involved in AD pathogenesis. P-glycoprotein function and expression can be pharmacologically induced by a variety of compounds including extracts of *Hypericum perforatum* (St. John's Wort). C57BL/6 J-APP/

$PS1^{+/-}$ mice receiving St. John's Wort extract showed significant reductions of parenchymal $A\beta_{1-40}$ and $A\beta_{1-42}$ accumulation, and moderate increases in cerebrovascular P-glycoprotein expression. According to these data reported by Brenn et al. [286], the induction of cerebrovascular P-glycoprotein may be a novel therapeutic strategy to protect the brain from β-amyloid accumulation.

Cistanche tubulosa Glycoside. Guo et al. [287] studied the efficacy and safety of *Cistanche tubulosa* glycoside capsules in 18 patients with AD for 48 weeks in China. No obvious aggravation of cognitive function was observed while independent living ability and overall conditions remained stable throughout the study.

Coriandrum sativum L. Coriandrum sativum L. (coriander) belongs to the Apiaceae family and is cultivated worldwide for its nutritional value and medicinal properties (relief of pain, anxiety, flatulence, loss of appetite, and convulsions). The effects of inhaled coriander volatile oil (1–3 %) extracted from *Coriandrum sativum* var. *microcarpum* on spatial memory performance were assessed in an $A\beta_{1-42}$ rat model of AD. Exposure to coriander volatile oil improved spatial memory, increased superoxide dismutase (SOD), lactate dehydrogenase (LDH), and decreased glutathione peroxidase (GPX) specific activities along with an elevation of malondialdehyde (MDA) level [288].

Sulforaphane. Sulforaphane (SUL) is an isothiocyanate in cruciferous vegetables. $A\beta_{25-35}$-induced cytotoxicity and apoptotic characteristics such as activation of c-JNK, dissipation of mitochondrial membrane potential, altered expression of Bcl-2 family proteins, and DNA fragmentation were effectively attenuated by SUL pretreatment in SH-SY5Y cells. The antiapoptotic activity of SUL seemed to be mediated by inhibition of intracellular accumulation of ROS and oxidative damages. SUL exerted antioxidant potential by upregulating expression of antioxidant enzymes including γ-glutamylcysteine ligase, NAD(P)H:quinone oxidoreductase-1, and heme oxygenase-1 via activation of NF-E2-related factor 2(Nrf2). The protective effect of SUL against $A\beta_{25-35}$-induced apoptotic cell death was abolished by siRNA of Nrf2 [289].

Hesperidin. Hesperidin, a bioactive flavonoid compound, inhibits $A\beta_{25-35}$-induced apoptosis by reversing Aβ-induced mitochondrial dysfunction, including the mitochondrial permeability transition pore opening, intracellular free calcium increase, and ROS production. Hesperidin can decrease the level of voltage-dependent anion channel 1 (VDAC1) phosphorylation through inhibiting the activity of the glycogen synthase kinase-3b and increase the level of hexokinase I in mitochondria, preventing release of cytochrome c from mitochondria. Hesperidin also inhibits the mitochondria-dependent downstream caspase-mediated apoptotic pathway, such as that involving caspase-9 and caspase-3. Hesperidin can protect

Aβ-induced neurotoxicity via theVDAC1-regulated mitochondrial apoptotic pathway [290].

Thymoquinone. Thymoquinone (TQ) is the main constituent of the oil extracted from *Nigella sativa* seeds, with antioxidant and anti-inflammatory effects. Treatment with TQ efficiently attenuates $A\beta_{1-42}$-induced neurotoxicity, inhibits the mitochondrial membrane potential depolarization and ROS generation caused by $A\beta_{1-42}$, restores synaptic vesicle recycling inhibition, partially reverses the loss of spontaneous firing activity, and inhibits $A\beta_{1-42}$ aggregation in vitro [291].

Arctigenin, a natural product from *Arctium lappa* (L.), inhibits Aβ production by suppressing β-site APP cleavage enzyme 1 expression and promotes Aβ clearance by enhancing autophagy through AKT/mTOR signaling inhibition and AMPK/Raptor pathway activation [292].

Flavonoid-based CDK5/p25 inhibitors. Rhus parviflora (Anacardiaceae) is an indigenous medicinal shrub found in South Asia with flavonoid-rich edible fruit. Shrestha et al. [293] examined flavonoid derivatives of *R. parviflora* fruit with CDK5/p25 inhibition activity. The *aurones*, *sulfuretin*, and *aureusidin*, the *aurone glycoside*, *aureusidin-6-O-β-d-glucopyranoside*, and *hovetrichoside C*, the *flavonoid glycoside*, *quercetin-3-O-β-d-galactopyranoside*, and the *biflavonoid*, *cupressuflavone*, had the potential to inhibit CDK5/p25.

Soy isoflavone and *Genistein*. Genistein, a main active ingredient of soybean isoflavone, has been shown to have neuroprotective effects by antagonizing oxidative damage induced by Aβ. The increased mitochondrial ROS accumulation in C6 cells induced by Aβ was reversed by genistein. The levels of 8-OHdG in C6 cells and mtDNA deletion were decreased and genistein upregulated the mRNA and protein expression of OGG1 [294].

Soy isoflavone improves the impairment of learning and memory of rats induced by $A\beta_{1-42}$, maintains Aβ homeostasis in brain, and regulates the disordered expressions of RAGE/LRP-1 and restrain RAGE related NF-κB and inflammatory cytokines activation in neurovascular structure [295].

Pomegranate polyphenols. Pomegranate extracts attenuate microgliosis and Aβ plaque deposition in APP/PS1 mice. Polyphenol components of pomegranate extract, *punicalagin* and *ellagic acid*, decrease Aβ-stimulated TNF-α secretion by murine microglia [296].

Blend of black chokeberry and lemon juice. The phytochemical composition, antioxidant capacity (scavenging of DPPH, superoxide and hydroxyl radicals, and hypochlorous acid), and inhibitory activity against cholinesterase of the new blend integrating black

chokeberry and lemon juice were investigated by Gironés-Vilaplana et al. [297]. The chokeberry concentrate is rich in cyanidin-glycosides, quercetin derivatives, and 3-O-caffeoylquinic acid; and lemon juice contains flavones, flavanones, quercetin derivates, and hydroxycinnamic acids. The new drink showed antioxidant effects and inhibited acetylcholinesterase and butyrylcholinesterase.

Combination of Chinese herb active components. The combination of Chinese herb active components (baicalin, jasminoidin, and cholic acid) (CBJC) has shown positive effects in rats treated with ibotenic acid. The expression levels of 19 genes in the forebrain were significantly influenced by CBJC; approximately 60 % of these genes were related to neuroprotection and neurogenesis, whereas others were related to antioxidation, protein degradation, cholesterol metabolism, stress response, angiogenesis, and apoptosis [298].

Other plants and natural products. Other plants, herbal derivatives, and traditional medicine formulas screened for anti-AD properties include the following: *Scoparia dulcis*, *Catharanthus roseus*, *Sesamum indicum*, *Erythrina senegalensis*, and *Vigna unguiculata* have been used as traditional medicines for the treatment of AD in certain cultures [299]; *Salvia triloba* L. and *Piper nigrum* extracts ameliorate neuroinflammatory insults in an AD rat model [300]; Evodiamine, a bioactive indole alkaloid obtained from *Evodiae fructus* [301]; *Bushenyisui formula* [302]; *Hydroxysafflor yellow A* (HSYA), a major active chemical component isolated from *Carthamus tinctorius* L. [303]; *kavalactones* isolated from *Piper methysticum* (Piperaceae) [304]; *Oleocanthal*, a phenolic component of extra-virgin olive oil [305]; *Biruloquinone*, an acetylcholinesterase inhibitor produced by the lichen-forming fungus *Cladonia macilenta* [306]; *Catechin hydrate*, a natural flavonoid with potential antioxidant and anti-inflammatory properties [307]; *Oligomeric procyanidins* of lotus seedpod which inhibit AGE product formation [308]; *Salidroside*, the major active ingredient of *Rhodiola crenulata*, with antioxidant, neuroprotective and anti-inflammatory effects [309]; the traditional Chinese medicinal ginger root extract [310]; *Kaixin San formulas* [311]; the lignan-enriched extract of *Schisandra chinensis* fruits (*ESP-806*) (*Schisandra chinensis* (Trucz.) Baill.) (Schisandraceae) [312]; *Forsythiaside* (3,4-dihydroxy-β-phenethyl-O-α-L-rhamnopyranosyl-(1 → 6)-4-O-caffeo yl-β-d-glucopyranoside, C29H36O15), isolated from air-dried fruits of *Forsythia suspensa*, with antioxidant, anti-bacterial, and anti-inflammatory activities [313]; *Naoerkang* [314]; *Tong Luo Jiu Nao* [315]; *qingxin kaiqiao formula* [316]; *Diammonium glycyrrhizinate* [317]; *Cyanidin 3-O-glucoside* [318]; *Keampferol-3-O-rhamnoside* [319]; New germacrane-type sesquiterpenoids, *heishuixiecaoline A-C*, from fractions of *Valeriana amurensis* roots and rhizomes [320]; *Jatrorrhizine*, a novel tetrahydroisoquinoline alkaloid originally extracted from the Chinese

herb *Coptidis rhizome* [321]; *SuHeXiang Wan* (SHXW), a Chinese traditional medicine, and modified SHXW, KSOP1009, with c-Jun N-terminal kinase (JNK) inhibitory activity and antiapoptotic effects [322]; *Monosialoanglioside* [323]; and *Salvia sahendica extract*, an endemic plant of Iran [324].

6.5 Immunotherapy and Treatment Options for Tauopathies

Since Aβ immunotherapy presents a limited clearance effect of tau aggregates in dystrophic neurites, the development of an alternative therapy that directly targets pathological tau has become crucial [325–328]. Increased levels of tau oligomers have been observed in the early stage of AD, prior to the detection of NFTs formed by aggregation and accumulation of the microtubule-associated protein tau [329]. Several approaches have been taken to treat AD by targeting tau, such as (1) the inhibition of tau hyperphosphorylation, by using a kinase inhibitor of soluble aggregated tau formation, also to prevent related motor deficits [325]; (2) activation of the proteolytic pathway, by the degrading action of calpain [330] and puromycin-sensitive aminopeptidase [331]; (3) the stabilization of microtubules, treating tauopathies by functionally binding and stabilizing microtubules with MT-binding protein tau [332] and paclitaxel, a drug proven effective in restoring the affected axonal transport and motor impairments [333]; and (4) tau clearance by immunotherapy; in this case, the tau active vaccination uses phosphorylated antigens of tau fragments associated with NFTs [334], which results in an efficient reduction of both soluble and insoluble tau active fragments, reducing phosphorylated NFTs in the AD-like mouse brains [325].

Both active and passive immunizations targeting disease-related tau epitopes successfully reduce tau aggregates in vivo and slow or prevent behavioral impairments in mouse models of tauopathy. Pathological tau protein is found in AD and related tauopathies. The protein is hyperphosphorylated and/or mutated which leads to aggregation and neurotoxicity. Because cognitive functions correlate well with the degree of tau pathology, clearing these aggregates might be a promising therapeutic approach [326]. A novel series of 2-aminothiazoles with strong protection in an AD model comprising tau-induced neuronal toxicity has been disclosed [327].

An alternative approach is to develop pharmaceuticals to enhance the activity of the principal phospho-tau phosphatase, phosphoprotein phosphatase 2A (PP2A). The activity of protein phosptase-2A (PP2A) is decreased in AD brains. Nicotinamide mononucleotide adenylyltransferase 2 (Nmnat2) is a key enzyme involved in energy metabolism and its gene expression level is reduced in AD brain specimens. The mRNA and protein levels of Nmnat2 were decreased with a simultaneous elevation of p-Tyr307-PP2A and tau phosphorylation in Tg2576 mice. Simultaneous inhibition of PP2A by okadaic acid abolished the Nmnat2-induced

tau dephosphorylation. Overexpression of Nmnat2 could activate PP2A with attenuation of tau phosphorylation, whereas downregulation of Nmnat2 by shRNA inhibited PP2A with tau hyperphosphorylation at multiple AD-associated sites. Nmnat2 affects tau phosphorylation by regulating PP2A activity, suggesting that Nmnat2 may serve as a potential target in arresting AD-like tau pathologies [335].

A number of different chemotypes have been reported to lead to enhanced PP2A activity. Some of these compounds appear to act directly as allosteric activators of PP2A, while others act indirectly by inhibiting the binding of PP2A inhibitors or by altering posttranslational modifications that act in turn to regulate PP2A activity toward phospho-tau [328].

The genetic ablation of tau substantially reduces hyperexcitability in AD mouse lines, induced seizure models and genetic in vivo models of epilepsy. These data demonstrate that tau is an important regulator of network excitability. Devos et al. [336] identified antisense oligonucleotides that selectively decrease endogenous tau expression throughout the entire mouse CNS—brain and spinal cord tissue, interstitial fluid, and CSF—while having no effect on baseline motor or cognitive behavior. In chemically induced seizure models, mice with reduced tau protein had less severe seizures than control mice.

The neurotoxic potential of tau immunotherapy has been reported, specifically of full-length unphosphorylated-tau vaccine under a CNS-proinflammatory milieu [induced by emulsification in complete Freund's adjuvant (CFA) and pertussis toxin (PT)] in young wild-type (WT) mice. A paralytic disease is evident in the phos-tau-immunized adult NFT mice, developing progressively to 26.7 % with the number of injections. The WT mice were even more prone to develop neuroinflammation following phos-tau immunization, affecting 75 % of the immunized mice. Anti-phos-tau antibodies, detected in the serum of immunized mice, partially correlated with the neuroinflammation in WT mice. Repeated phos-tau immunizations in the frame of a proinflammatory milieu may be encephalitogenic to tangle mice, and more robustly to WT mice, indicating that the safety of phos-tau immunotherapy is questionable, according to data reported by Rozenstein-Tsalkovich et al. [337].

Protein tau aggregates into NFTs when it is hyperphosphorylated. The amino acid sequence included in the third repeat (R3) of the microtubule-binding region is suspected to be the main factor for tau aggregation. Ikura and Ito [338] synthesized a 31-residue oligopeptide, corresponding to the R3 region, characterized its aggregation propensity under various conditions, and investigated the function of FK506-binding protein (FKBP) 12, which is known to accumulate in NFTs in vivo, on aggregation of the R3 peptide, and found that FKBP12 completely prevented the peptide from aggregating.

Targeting Tau kinases represents a potential therapeutic approach. Small molecules in the diaminothiazole class are potent Tau kinase inhibitors that target CDK5 and GSK3β [339].

In P301L animals treated with MC1, a conformational monoclonal antibody specific for PHF-tau, the rate of development of tau pathology is effectively reduced, while injecting DA31, a high-affinity tau sequence antibody, does not exert such benefit. MC1 appears superior to DA31 in overall effects, suggesting that specificity is more important than affinity in therapeutic applications. The survival rate was not improved when immunizing with either MC1 or PHF1, a high-affinity phospho-tau antibody efficacious in reducing pathological tau [340].

In a *Drosophila* model of tauopathy in which abnormal human tau mediates neuronal dysfunction characterized by microtubule destabilization, axonal transport disruption, synaptic defects and behavioral impairments, the microtubule-stabilizing drug, NAPVSIPQ (NAP) (davunetide), prevents as well as reverses these phenotypes even after they have become established [341].

The peroxisome proliferator-activated receptor gamma (PPARγ) agonists thiazolidinediones (TZDs) are prescribed for the treatment of type 2 diabetes mellitus. These compounds may also have a beneficial effect on neurodegenerative disorders. Troglitazone, a parent TZD drug, inhibits tau phosphorylation. Treatment with troglitazone decreased tau-Thr^{231} phosphorylation and p35, the specific activator of cyclin-dependent kinase 5 (CDK5), in a dose- and time-dependent manner. Troglitazone also decreased CDK5 enzymatic activity, and ectopic expression of p25, the cleaved and more active form of p35, restored the troglitazone-induced decrease in tau-Thr^{231} phosphorylation. Treatment with either MG-132, a reversible proteasome inhibitor, or lactacystin, a specific and irreversible 26S proteasome inhibitor, significantly reversed the observed inhibitory effects of troglitazone. GW9662, a specific and irreversible PPARγ antagonist, did not alter the observed inhibitory effects. Similar results were also found when other TZD drugs, pioglitazone and rosiglitazone, were used. Treatment with various inhibitors revealed that troglitazone-induced inhibitions of tau-Thr^{231} phosphorylation and p35 expression were not mediated by glycogen synthase kinase 3β, protein kinase A, and protein phosphatase 2A signaling pathways. TZDs repressed tau-Thr^{231} phosphorylation via the inhibition of CDK5 activity, which was mediated by the proteasomal degradation of p35 and a PPARγ-independent signaling pathway [342].

Aminothienopyridazines and methylene blue. Aminothienopyridazine (ATPZ) compounds inhibit Tau fibrillization. Active ATPZs were found to promote the oxidation of the two cysteine residues within 4-R Tau by a redox cycling mechanism, resulting in the formation of a disulfide-containing compact monomer that

was refractory to fibrillization. The ATPZs facilitated intermolecular disulfide formation between 3-R Tau monomers, leading to dimers that were capable of fibrillization. The ATPZs also caused cysteine oxidation in molecules unrelated to Tau. Methylene blue, an inhibitor of Tau fibrillization, caused a similar oxidation of cysteines in Tau [343].

6.6 Aβ Immunotherapy

Immunotherapy targeting Aβ holds great promise for reducing Aβ in the brain, and novel vaccines against Aβ are in development. There are two main modalities of immunotherapy for AD: (1) passive immunotherapy, with the administration of monoclonal Aβ-specific antibodies [344], and (2) active immunization with the $Aβ_{42}$ antigen [345, 346] or Aβ-conjugated synthetic fragments bound to a carrier protein, thus avoiding potential problems associated with mounting a T-cell response directly against Aβ [347]. A new approach by delivering $Aβ_{42}$ in a novel immunogen-adjuvant manner consisting of sphingosine-1-phosphate (S1P)-containing liposomes, administered to APP/PS1 transgenic mice before and after the detection of AD-like pathology in the brain, has recently been developed [147, 348]. The results from this novel vaccine (EB101) indicate that active immunization significantly prevents and reverses the progression of AD-like pathology and also clears prototypal neuropathological hallmarks in transgenic mice. This new approach strongly induced T-cell, B-cell, and microglial immune response activation, avoiding the Th1 inflammatory reaction [348].

The rationale for amyloid immunotherapy in AD [349, 350] is based on the following assumptions: (1) β-amyloid plaques and their aggregated, proto-fibrillar and oligomeric precursors contain immunologic neo-epitopes that are absent from the full-length APP, as well as from its soluble proteolytic derivatives restricted to the brain tissue; consequently, β-amyloid-based immunotherapies designed to selectively target pathologic neo-epitopes present on Aβ oligomers, protofibrils or fibrils, should not cause autoimmune disease in unaffected tissues throughout the organism. (2) β-amyloid buildup precedes neurodegeneration and functional loss, and the prevention of its formation or its removal can be expected to result in the slowing or the prevention of neurodegeneration. (3) β-amyloid can cause the formation of NFTs in vivo and in vitro; the removal of β-amyloid, or the prevention of its buildup, bears the potential not only to correct β-amyloid-related toxicity but also to prevent the formation of NFTs. (4) Conformational changes of endogenously occurring proteins and the formation of insoluble aggregates are commonly associated with neurodegeneration and brain disease, so the removal or prevention of these pathologic protein aggregates is also a therapeutic goal in the principle of immunotherapy. (5) Immunotherapy works in experimental animals and in initial clinical trials: both active immunization

and passive antibody transfer consistently reduce brain β-amyloid load, improve β-amyloid-related memory impairments, and protect neurons against degeneration in many independent experiments using different mouse and primate models [348].

Preclinical studies have shown clear evidence that Aβ immunization therapy provides protection and reverses the pathological effects of AD in transgenic mouse models [351]. This strategy seems to improve cognition performance [352] after $A\beta_{42}$ immunization, in addition to causing an effective reduction in Aβ pathology. A recent immunization study has proven that a fragment of the Aβ peptide bound to polylysines activated the immune response that results in the diminishing of AD-like pathology in APP transgenic mice. This report reinforces the notion that the immune-conjugate approach is an effective means of Aβ immunotherapy and also that the entire Aβ peptide is not necessary for its efficacy, and is in accordance with the hypothesis that specific antibodies directed against the amino-terminal and/or central region of the amyloid peptide provide beneficial protection against amyloid pathology. Passive immunization studies have also been conducted with promising experimental results, showing that a humoral response alone, without Aβ cellular response, is sufficient to reduce the β-amyloid burden and to reverse memory deficits [353].

The soluble prefibrillar Aβ-aggregates are the prime toxic agents in AD; however, different Aβ aggregate species are described and this promiscuity of potential targets represents a major obstacle for immunization. Passive immunotherapy with monoclonal antibodies (mAbs) against Aβ has been clinically tested and the two most advanced mAbs, *Bapineuzumab* and *Solanezumab*, targeting an N-terminal or central epitope, respectively, failed to meet their target of improving cognition. Intravenous polyclonal immunoglobulins (IVIG) appear to target different conformational epitopes with promising effects on cognitive stabilization. To target the whole spectrum of Aβ-aggregates might be a more efficient strategy than to focus on a single aggregate species for immunization [354].

Among the drugs and vaccines currently under development to treat the pathological effects of AD, *bapinezumab*, *solanezumab*, *ponezumab*, *CAD106*, and *EB101* are the most promising ones [355]. Solanezumab is a monoclonal antibody raised against $A\beta_{13-28}$, recognizing an epitope in the core of the amyloid peptide, binding selectively to soluble Aβ and with low affinity for the fAβ form [356], presenting fewer adverse events than bapineuzumab, which binds to Aβ amyloid plaques more strongly than soluble Aβ [355]. There are a few other monoclonal antibodies against Aβ that present properties different from those of bapineuzumab, such as (1) PF-04360365, which specifically targets the free carboxy-terminus of $A\beta_{1-40}$; (2) MABT5102A, which binds with equally high affinity to Aβ monomers, oligomers, and fibrils; (3) GSK933776A, which

targets the N-terminus of Aβ. Specific anti-Aβ antibodies are present in pooled preparations of intravenous immunoglobulin (IVIg or IGIV), which has already been approved by the FDA for the treatment of a variety of other neurological aspects. Current results from these studies have shown that IVIg treatment may also be an efficacious alternative approach in the treatment of AD neuropathologies [348, 357].

Ponezumab is a humanized anti-Aβ monoclonal antibody. A 2-h infusion of 0.1–10 mg/kg ponezumab was well tolerated in subjects with mild-to-moderate AD. Plasma Aβ increased with dose, and CSF Aβ increased at the highest dose, suggesting that intravenous ponezumab alters central Aβ levels [358].

Avoiding both the strong Th1 effects of QS-21 adjuvant and the T-cell epitopes at the C-terminus of Aβ, CAD106 consists of a short N-terminal fragment of Aβ attached to a virus-like particle, with no additional adjuvant [359]. This therapeutic agent is currently in phase II trials. Affiris is testing two short 6-amino-peptides (AD01, AD02), administered with aluminum hydroxide as adjuvant, that mimic the free N-terminus of Aβ and therefore cause cross-reactivity with the native peptide in phase I trials [360]. In terms of prevention and therapeutic treatment approach, the EB101 vaccine showed for the first time the effectiveness of combining a liposomal immunogen-adjuvant with an Aβ antigen to induce an effective immunological response combined with an anti-inflammatory effect in preclinical studies using APP/PS1 transgenic mice [147, 348]. The EB101 vaccine immunization process has shown a marked positive effect as a preventive and therapeutic treatment, reducing amyloidosis-induced inflammation as an effective Th2 immunomodulator. Moreover, this vaccine proved to stimulate innate immunity and enable effective phagocytosis to clear amyloid and NFTs, among the major hallmarks of AD-like neuropathology observed. A few other vaccines are currently under development, and recent studies have opened new perspectives in the immunization approach to AD pathology, in particular, the gene-gun-mediated genetic immunization with $A\beta_{42}$ gene [361], showing that self-tolerance can be broken in order to produce a humoral response to the $A\beta_{42}$ peptide with minimal cellular response.

Immunization against Aβ has been associated with meningoencephalitis due to activation of inflammatory T-cells. With the aim of producing an immunogenic vaccine without this side effect, Wiessner et al. [362] designed CAD106 comprising $A\beta_{1-6}$ coupled to the virus-like particle Qβ. Immunization with this vaccine did not activate Aβ-specific T-cells and no evidence for increased microhemorrhages or inflammatory reactions in amyloid-containing brain was observed.

Nojima et al. [363] developed a new edible vaccine: rice expressing GFP-Aβ_{42}. Aβ rice had therapeutic effects in the Tg2576 AD model mice.

Kou et al. [364] evaluated the efficacy and safety of anti-Aβ single-chain antibody (scFv59) delivery via recombinant adeno-associated virus (rAAV) on reducing Aβ deposits in an AD mouse model (TgAβPPswe/PS1dE9). Immunoreactive Aβ deposits were reduced in the hippocampus. Aβ_{42} levels in CSF tended to increase and the A$\beta_{40:42}$ ratio decreased in CSF, suggesting that Aβ_{42} was relocated from the parenchyma to CSF. Hemorrhages associated with a focal increase in blood vessel amyloid were found in the brain. While immunotherapy has great potential for clearing cerebral Aβ, caution for cerebrovascular effects should be exercised when rAAV-mediated anti-Aβ immunotherapy is applied.

A role for PrP in the toxic effect of oligomeric forms of Aβ, implicated in AD, has been suggested. PrP is required for the plasticity-impairing effects of ex vivo material from human AD brain and standardized Aβ-derived diffusible ligand (ADDL) preparations disrupt hippocampal synaptic plasticity in a PrP-dependent manner. Antibodies directed to the principal PrP/Aβ-binding site and to PrP helix-1 are able to block Aβ binding to PrP. Two monoclonal antibodies directed to these regions, ICSM-35 and ICSM-18, were shown to block the Aβ-mediated disruption of synaptic plasticity validating these antibodies as candidate therapeutics for AD either individually or in combination [365].

Active immunizations using DNA which codes for the protein against which the immune response will be directed (genetic immunizations) provide additional safety as the immune response in DNA immunizations differs from the response elicited by peptide immunizations [366]. Aβ DNA epitope vaccines have been proposed with optimism possibly combined with a prime boost regime in either very early AD, or preferably in preclinical stage individuals identified by validated AD biomarkers [367].

Yu et al. [368] described the immunological characterization and protective effect of DNA epitope chimeric vaccines using six copies of Aβ_{1-15} fused with PADRE or toxin-derived carriers. These naked 6Aβ15-T-Hc chimeric DNA vaccines induce robust anti-Aβ antibodies that could recognize Aβ oligomers and inhibit Aβ oligomer-mediated neurotoxicity, reducing cerebral Aβ load and Aβ oligomers, and improving cognitive function in AD mice, with no stimulation of Aβ-specific T-cell responses.

The single-chain variable fragment scFv-h3D6 may prevent in vitro toxicity induced by Aβ by withdrawing Aβ oligomers from the amyloid pathway. Studies in vivo in the triple-transgenic 3×Tg-AD mouse model revealed that scFv-h3D6 decreases Aβ oligomers in the cortex and olfactory bulb after treatment, but not in the hippocampus and cerebellum, and restores normal levels of both apoJ and apoE in the cortex [369].

Complement component C5-derived C5a locally generated in the brain may protect against glutamate-induced neuronal apoptosis and Aβ toxicity. C5a influences upstream signal transduction pathways associated with cAMP-response element-binding protein (CREB) activation, in which alterations of CREB levels are associated with cognitive deterioration in AD. Application of hrC5a in brain slices from Tg2576 mice significantly improves deficits in long-term potentiation, while this effect is blocked by a specific AMPA receptor antagonist. Low-dose human intravenous immunoglobulin (IVIG) treatment improves synaptic plasticity and cognitive function through C5a-mediated induction of the CREB/CEBP pathway by passing Aβ toxicity [370].

Evans et al. [371] reported humoral and cellular immune responses elicited in response to a novel DNA epitope-based vaccine (AV-1955) delivered to rhesus macaques using the TriGrid electroporation device. AV-1955 generates long-term, potent anti-Aβ antibodies and cellular immune responses specific to foreign T-helper epitopes but not to self-Aβ.

Guo et al. [372] constructed a plasmid DNA vaccine encoding ten repeats of $A\beta_{3-10}$ and three copies of C3d-p28 as a molecular adjuvant and administered it intramuscularly in 12-month-old female Tg-APPswe/PSEN1dE9 mice. Therapeutic immunization with p($A\beta_{3-10}$)10-C3d-p28.3 stimulated a Th2 immune response that elicited therapeutic levels of anti-Aβ antibodies and improved cognitive function. The vaccine reduced the cerebral Aβ burden and astrocytosis without increasing the incidence of microhemorrhage.

Gammagard IVIg is a therapeutic approach to treat AD currently in phase 3 clinical trials. Sudduth et al. [373] compared IVIg, mouse-pooled IgG, and the anti-Aβ antibody 6E10 injected intracranially into the frontal cortex and hippocampus of 7-month-old APP/PS1 mice. IVIg and pooled mouse IgG both significantly reduced Aβ deposition to the same degree as the 6E10 anti-Aβ antibody. Neuroinflammatory profiles were significantly altered by the antibody treatments. APP/PS1 transgenic mice at 7 months of age typically exhibit an M2a inflammatory phenotype. All antibody treatments stimulated an M2b response, yet anti-Aβ antibody was a more rapid change. The IVIg and pooled mouse IgG may act as immune modulators and this immune modulation is responsible for the reductions in amyloid pathology.

Guan et al. [374] investigated whether $4A\beta_{1-15}$ (four tandem repeats of GPGPG-linked $A\beta_{1-15}$ sequences) had therapeutic effects in the APP/PS1 transgenic mice model of AD. The anti-Aβ antibody concentrations were increased, bound to AD plaques, reduced Aβ pathology and levels of intracerebral Aβ, increased serum Aβ levels, and improved memory. Immunization via $4A\beta_{1-15}$ (mainly of the IgG1 Class) might induce a non-inflammatory Th2 reaction; however, analysis of MHC Class II and CD45 revealed that microglial cells were in a less activated state.

The conversion of Aβ from a physiological soluble monomeric form into insoluble fibrillar conformation is an important event. The most toxic form of Aβ is oligomers, which is the intermediate step during the conversion of monomeric form to fibrillar form. There are at least two types of oligomers: oligomers that are immunologically related to fibrils and those that are not. In transgenic AD animal models, both active and passive anti-Aβ immunotherapies improve cognitive function and clear the parenchymal accumulation of amyloid plaques in the brain. Anti-oligomeric monoclonal antibodies significantly reduce the amyloid load and improve cognition. The clearance of amyloid load correlated with reduced tau hyperphosphorylation and improvement in cognition [375].

MER5101 is a novel conjugate of $A\beta_{1-15}$ peptide (a B-cell epitope fragment) conjugated to an immunogenic carrier protein, diphtheria toxoid (DT), and formulated in a nanoparticular emulsion-based adjuvant. This novel vaccine induced high anti-Aβ antibody levels in both vaccinated APPswe/PS1ΔE9 Tg and Wt mice. Antibody isotypes were mainly IgG1 and IgG2b, suggesting a Th2-biased response. Reductions in cerebral Aβ plaque burden, accompanied by attenuated microglial activation and increased synaptic density, were observed in MER5101-vaccinated APPswe/PS1ΔE9 Tg mice compared with Tg adjuvant controls. MER5101-immunized APPswe/PS1ΔE9 Tg mice showed improvement of cognitive deficits [376].

Using an AD mouse model (Tg2576), Davtyan et al. [377] tested the immunogenicity and efficacy of clinical grade Lu AF20513 vaccine. Lu AF20513 induces robust "non-self" T-cell responses and the production of anti-Aβ antibodies that reduce AD-like pathology in the brains of Tg2576 mice without inducing microglial activation and enhancing astrocytosis or CAA. A single immunization with Lu AF20513 induced strong humoral immunity in mice with preexisting memory T-helper cells.

6.7 Aβ Breakers and Aggregation Inhibitors

Aβ aggregation is a key factor in the development of AD. Several Aβ aggregation inhibitors (polyphenols, short peptides, monomer- or oligomer-specific antibodies) have been identified, and some of them have been tested in clinical trials for treating protein misfolding diseases [378]. Antiamyloid antibodies (AAA) are under development as new therapeutics that disaggregate the amyloid plaque AD. Since AAAs are large molecule drugs that do not cross the BBB, an AAA was reengineered for receptor-mediated transport across the BBB via the endogenous BBB transferrin receptor (TfR) [379]. A single-chain Fv (ScFv) antibody form of an AAA was fused to the carboxyl terminus of each heavy chain of a chimeric monoclonal antibody (MAb) against the mouse TfR, and this produced a tetravalent bi-specific antibody designated the

cTfRMAb-ScFv fusion protein. Fusion protein treatment caused a 57 and 61 % reduction in amyloid plaque in the cortex and hippocampus, respectively. No increase in plasma immunoreactive Aβ amyloid peptide, and no cerebral microhemorrhage, was observed [379].

Hybrid molecules composed of an aromatic moiety and the α-aminoisobutyric acid β-sheet breaker elements may act as inhibitors of amyloid fibril formation. The D-Trp-Aib was shown to be an effective inhibitor of the formation of β-amyloid fibrils and oligomers both in vitro and in vivo [380].

Among *Psoraleae fructus* derivatives from China, *isobavachalcone* significantly inhibits both oligomerization and fibrillization of $A\beta_{42}$, whereas *bavachinin* inhibits fibrillization and leads to off-pathway aggregation [381].

A diphenylpropynone derivative, *DPP2*, targets metal-associated amyloid-β (metal-Aβ) species and control Aβ aggregation reactivity in vitro; however, its cytotoxicity has limited further biological applications. Liu et al. [382] characterized a series of small molecules (C1/C2, P1/P2, and PA1/PA2) as structurally modified DPP2 analogs. Structural variations adjacent to the metal binding site of DPP2 could govern different metal binding properties, interactions with Aβ and metal-Aβ species, reactivity toward metal-free and metal-induced Aβ aggregation, and cytotoxicity of the compounds, establishing a structure–reactivity–cytotoxicity relationship.

Kurisu et al. [383] studied the effects of *acteoside*, isolated from *Orobanche minor*, and its derivatives on the aggregation of a 42-mer amyloid β protein ($A\beta_{42}$). Acteoside strongly inhibited the aggregation of $A\beta_{42}$ in a dose-dependent manner.

Crocin is a carotenoid from the stigma of the saffron flower with many medicinal properties, including antioxidant effects. Crocin has the ability to prevent amyloid formation. The antiamyloidogenic effect of crocin may be exerted not only by the inhibition of Aβ amyloid formation but also by the disruption of amyloid aggregate [384].

Ferulic acid (FA) is a phenolic compound that inhibits $A\beta_{42}$ fibril-induced neurotoxicity in SH-SY5Y cells. FA inhibits the formation of the β-sheets that are required for the $A\beta_{42}$ monomer-to-oligomer transition but accelerated the $A\beta_{42}$ oligomer-to-fibril transition. FA may inhibit the aggregation of $A\beta_{42}$ oligomers by blocking the hydrogen bond with the forming β-sheets [385].

Li et al. [386] reported a novel strategy for the self-assembly of *polyoxometalate-peptide* (POM@P) hybrid particles as bifunctional Aβ inhibitors. The two-in-one bifunctional POM@P nanoparticles show an enhanced inhibition effect on amyloid aggregation in mouse CSF.

An antiamyloidogenic bis-styrylbenzene derivative, *KMS80013*, with low toxicity and hERG channel inhibition showed cognitive efficacy in an acute AD mouse model [387].

iAβ5p and sym-triazines (TAE-1, TAE-2). Two novel compounds (TAE-1 and TAE-2) containing a sym-triazine scaffold with acetylcholine-like substitutions were examined for inhibition of Aβ fibril formation in the presence of $A\beta_{1-42}$. The results showed comparable activity to that of the pentapeptide-based fibrillogenesis inhibitor iAβ5p. In addition to destabilization of $A\beta_{1-42}$ assemblies by TAE-1 and TAE-2, sym-triazine inhibition of acetylcholinesterase (AChE) activity was observed in cytosol extracted from differentiated human SH-SY5Y neuronal cells. The sym-triazine derivatives promoted beneficial effects on human neurons, upregulating expression of synaptophysin, a synaptic marker protein, and MAP2, a neuronal differentiation marker [388].

Ferrocene tripeptide Gly-Pro-Arg conjugates. The conjugates of ferrocene and Gly-Pro-Arg (GPR) tripeptide, Boc-Gly-Pro-Arg(NO_2)-Fca-OMe and Fc-Gly-Pro-Arg-OMe were synthesized and employed to inhibit $A\beta_{(1-42)}$ fibrillogenesis and to disaggregate preformed Aβ fibrils [389].

6.8 Secretase Inhibitors (β- and γ-Secretase Inhibitors and Modulators)

6.8.1 β-Secretase Inhibitors

The β-secretase, or β-site APP cleaving enzyme 1 (BACE1; also called Asp2, memapsin 2), is the enzyme that initiates the generation of Aβ. BACE1 is an attractive drug target for lowering cerebral levels of amyloid β for the treatment or prevention of AD [390]. Hoffman-La Roche and Siena Biotech's patent application WO2012156284 describes *1,3-Oxazines* as BACE1 and/or BACE2 inhibitors, including 65 BACE1 and 20 BACE2 inhibitors [391]. A series of amides (*2-amino-1,3-oxazine*) bearing a variety of amidine head groups were investigated as BACE1 inhibitors with respect to inhibitory activity in a BACE1 enzyme as well as a cell-based assay [392, 393]. Eketjäll et al. [394] reported the discovery and the pharmacokinetic and pharmacodynamic properties of BACE1 inhibitor *AZ-4217*. Central efficacy of BACE1 inhibition was observed after a single dose in C57BL/6 mice, guinea pigs, and in an APP transgenic mouse model of cerebral amyloidosis (Tg2576). The novel *compound VIa* (a non-peptidic BACE1 inhibitor) exhibits potent inhibitory effects and selectivity against the aspartic proteases BACE1, BACE2, cathepsin D, and renin. In cellular assays, VIa moderately reduces Aβ production inhibiting the β-cleavage of amyloid-β protein precursor (AβPP), and increases the production of sAβPPα. In vivo, the oral administration of VIa resulted in a significant decrease in $A\beta_{1-40}$ and $A\beta_{1-42}$ in the blood of a mouse model of AD by 17.5–72.44 % and 14.5–80.32 %, respectively [395]. Descamps et al. [396] reported the identification of novel AβPP-selective BACE inhibitors (ASBI), particularly a bioflavonoid nutritional supplement acting as an ASBI in cell models. Increasing brain levels of this bioflavonoid through a prodrug approach leads to reduction of $A\beta_{42}$ in an AD model.

Heparan sulfate (HS) may function as an inhibitor of the β-site cleaving enzyme β-secretase (BACE1), with 6-O-sulfation identified as a key requirement. Schwörer et al. [397] introduced a novel generic synthetic approach to HS oligosaccharides applied to production of a library of 16 hexa- to dodecasaccharides targeted at BACE1 inhibition, yielding a number of potent non-anticoagulant BACE1 inhibitors.

The structure-activity relationship of the prime region of conformationally restricted *hydroxyethylamine* (HEA) BACE inhibitors was described by Ng et al. [398]. Variation of the P1′ region provided selectivity over Cat-D with a series of *2,2-dioxo-isothiochromanes* and optimization of the P2′ substituent of *chromane-HEA* with polar substituents provided improvements in the compound's in vitro permeability.

Ginsenosides, a key component of *Panax ginseng*, are effective against AD. Karpagam et al. [399] reported that *ginsenosides CK*, *F1*, *Rh1*, and *Rh2* are potential BACE1 inhibitors from *Panax ginseng*.

Bennett et al. [400] investigated the capacity of several medicinal mushroom species—*Auricularia polytricha* (wood ear mushroom), *Agaricus bisporus* (button mushroom), *Flammulina velutipes* (winter or enoki mushroom), and *Lentinus edodes* (shiitake mushroom)—in the regulation of BACE1. Only BACE1 inhibitory species were detected in unprocessed and processed forms of *A. polytricha*, whereas the dominant extracted species from *A. bisporus*, *F. velutipes*, and *L. edodese* were activators of BACE1. Inhibitory species were attributed to *hispidin-derived polyphenols*, whereas activating species were attributed to soluble polysaccharides and possibly low-mass Maillard products produced during processing.

Other novel BACE-1 inhibitors with a hydroxyethylene central core have been developed [401], as well as *spirocyclic BACE1 inhibitors* [402], *phenylimino-2H-chromen-3-carboxamide derivatives* [403], *iminopyrimidinone derivatives* [404], or *sulfonamide chalcones* [405].

The EtOAc soluble fraction of *Geranium thunbergii* showed significant β-secretase inhibitory activity. Two compounds, *geraniin* and *corilagin*, isolated from the most active EtOAc fraction of *G. thunbergii* exhibited predominant inhibition against β-secretase. Both compounds did not show significant inhibition against α-secretase and other serine proteases, including trypsin and chymotrypsin [406].

A more sophisticated alternative for inhibition of BACE1 is delivery of quantum Dot-siRNA nanocomplexes for *BACE1* gene silencing [407].

6.8.2 γ-Secretase Inhibitors

γ-Secretase is an aspartyl intramembrane protease composed of presenilin, Nicastrin, Aph1, and Pen2 with 19 transmembrane domains. γ-Secretase cleaves the APP to release Aβ peptides.

γ-Secretase also cleaves Notch and other type I membrane proteins. γ-Secretase inhibitors (GSIs) have been developed and tested in clinical trials which have shown adverse effects of GSIs that are potentially linked with nondiscriminatory inhibition of Notch signaling, overall APP processing, and other substrate cleavages. γ-Secretase modulators (GSMs) originally derived from nonsteroidal anti-inflammatory drugs (NSAIDs) target γ-secretase activity to lower levels of $A\beta_{42}$ production without blocking the overall processing of γ-secretase substrates. Second-generation GSMs, including NSAID-derived carboxylic acid and non-NSAID-derived heterocyclic chemotypes, as well as natural product-derived GSMs have been developed [408, 409]. Since γ-secretase targets many different substrates, selective inhibition of its cleavage of APP is believed to be critical in order to avoid undesirable side effects. γ-Secretase modulator (GSM) shifts the cleavage site on APP and production of amyloidogenic to non-amyloidogenic Aβ fragments. A pyridazine and a pyridine-derived GSM (GSM-C and GSM-D) reduced $A\beta_{40}$ and $A\beta_{42}$ productions, increased shorter Aβ fragments, and had little effect on Notch signaling [409]. Potent, orally bioavailable γ-secretase inhibitors (GSIs) have been developed and tested in humans with AD and cancer. Compounds referred to as γ-secretase modulators (GSMs) remain in development as AD therapeutics. GSMs do not inhibit γ-secretase, but modulate γ-secretase processivity and thereby shift the profile of the secreted Aβ peptides produced [410]. γ-Secretase modulators (GSMs) selectively lower $A\beta_{42}$ production without affecting total Aβ levels or the formation of γ-secretase substrate intracellular domains such as APP intracellular domain and Notch intracellular domain. Several secretase inhibitors have been developed over the past 10 years with poor results [411].

Semagacestat is a small-molecule γ-secretase inhibitor that was developed as a potential treatment for AD. Doody et al. [412] conducted a double-blind, placebo-controlled trial in which 1,537 patients with probable AD underwent randomization to receive 100 mg of semagacestat, 140 mg of semagacestat, or placebo daily. The trial was terminated before completion on the basis of a recommendation by the data and safety monitoring board. The ADAS-cog scores and ADCS-ADL scores worsened in all three groups. Patients treated with semagacestat lost more weight and had more skin cancers and infections. Laboratory abnormalities included reduced levels of lymphocytes, T cells, immunoglobulins, albumin, total protein, and uric acid and elevated levels of eosinophils, monocytes, and cholesterol; the urine pH was also elevated.

Avagacestat (BMS-708163) is a GSI developed for selective inhibition of APP over Notch cleavage. Avagacestat inhibition of APP and Notch cleavage was evaluated in cell culture by measuring levels of Aβ and human Notch proteins. Avagacestat reduces CSF Aβ levels without causing Notch-related toxicities [413].

Oehlrich et al. [414] described the evolution of amide 3 into conformationally restricted *bicyclic triazolo-piperidine 14-S* as a γ-secretase modulator with high in vitro and in vivo potency against $A\beta_{42}$ peptide, reduced lipophilicity and enhanced brain free fraction compared to the previous series.

Modulation of the γ-secretase enzyme is a promising therapeutic approach for the treatment of AD. The *compound SPI-1865* exhibits potency in the nM range in vitro and is selective for lowering $A\beta_{42}$ and $A\beta_{38}$ while sparing $A\beta_{40}$ and total Aβ levels. Brain $A\beta_{42}$ and $A\beta_{38}$ levels were decreased upon treatment with SPI-1865, with no effect on $A\beta_{40}$ in the Tg2576 mice [415].

Hyde et al. [416] described the in vivo characterization of the novel γ-secretase inhibitor SCH 697466 in rodents. Although *SCH 697466* was effective at lowering Aβ, Notch-related side effects in the intestine and thymus were observed following subchronic administration at doses that provided sustained and complete lowering of Aβ.

Structure-activity relationship studies of *tricyclic bispyran sulfone* γ-secretase inhibitors [417] and the discovery of *fused oxadiazepines* [418] and *EVP-0015962* [419] as γ-secretase modulators have been reported.

6.9 Statins

Statins are widely prescribed for their cholesterol lowering ability [150]. By competitive inhibition of hydroxymethyl co-enzyme A-reductase, statins reduce the production of cholesterol and isoprenoid intermediates, including geranylgeranyl and farnesyl pyrophosphate. These isoprenoids modify small GTPase molecules that are essential in numerous cell-signaling pathways, including vesicular trafficking and inflammation. Statins reduce the production of Aβ by disrupting secretase enzyme function and by reducing neuroinflammation. Atorvastatin and pitavastatin reduce the level of oxidative stress, as revealed by the presence of 4-HNE and AGE, in AD mouse brains, and improve insulin signaling and LDL-R/ApoE systems, as well as the serum adiponectin/leptin balance [420]. Statins, secondary of their anti-hypercholesterolemic, plieotropic and anti-inflammatory effects, are being investigated for a potential therapeutic role in AD, though clinical studies are contradictory [150, 421].

6.10 Phosphodiesterase Inhibitors and PDE Gene Silencing

Phosphodiesterase-4 (PDE4) inhibitors enhance memory, increase hippocampal neurogenesis, and reverse amyloid-β (Aβ)-induced memory deficits. PDE4 inhibitors such as *rolipram* may reverse Aβ-induced memory deficits at least in part via the attenuation of neuronal inflammation and apoptosis mediated by cAMP/CREB signaling [422].

Phosphodiesterase-5 (PDE5) inhibitors prevent the breakdown of cGMP and are currently studied as a possible target for cognitive enhancement. Tg APP/PS1 mice and age-matched

wild-type (WT) mice treated with PDE5 inhibitor *sildenafil* and the inhibitor of cGMP-dependent protein kinase Rp-8-Br-PET-cGMPS showed that sildenafil restores cognitive deficits in Tg APP/PS1 mice by the regulation of PKG/pCREB signaling, anti-inflammatory response, and reduction of Aβ levels [423]. Quinoline-based, CNS-permeant PDE5Is have potential for AD therapeutics [424].

Phosphodiesterase 7 (PDE7) regulates the inflammatory response through the cyclic adenosine monophosphate signaling cascade. The effects of the PDE7 inhibitor *S14* were studied in a mouse model of AD. APP/Ps1 mice treated with S14 showed attenuation in behavioral impairment, decreased brain Aβ deposition, enhanced astrocyte-mediated Aβ degradation, and decreased tau phosphorylation. These effects appear to be mediated via the cyclic adenosine monophosphate/cyclic adenosine monophosphate response element-binding protein signaling pathway, and inactivation of glycogen synthase kinase (GSK)3 [425].

Long-form PDE4D knockdown by lentiviral RNA construct containing a specific microRNA/miRNA-mir hairpin structure (4DshR) reversed memory impairment caused by amyloid-β_{1-42} ($A\beta_{42}$) in mice. Microinfusions of lentiviruses resulted in downregulated expression of PDE4D4 and 4D5 proteins and reversed $A\beta_{42}$-induced cAMP decline and memory deficits, with a concomitant increase of pCREB and BDNF and a decrease of IL-1β, TNF-α, and NF-κB (p65) in the hippocampus of $A\beta_{42}$-challenged mice [426].

6.11 Protein Phosphatase Methylesterase-1 Inhibitors

The serine hydrolase protein phosphatase methylesterase-1 (PME-1) regulates the methylesterification state of protein phosphatase 2A (PP2A) and has been implicated in cancer and AD. A remarkably potent and selective class of aza-β-lactam (ABL) PME-1 inhibitors has been characterized. The optimized compound, *AMZ30*, selectively inactivates PME-1 and reduces the demethylated form of PP2A in living cells [427].

6.12 Metalloendopeptidases (Nardilysin, N-Arginine Dibasic Convertase)

The metalloendopeptidase nardilysin (N-arginine dibasic convertase; NRDc) enhances α-cleavage of APP, which results in the decreased generation of Aβ in vitro. The neuron-specific overexpression of NRDc prevents Aβ deposition in the AD mouse model. The activity of α-secretase in the mouse brain was enhanced by the overexpression of NRDc, and was reduced by the deletion of NRDc. NRDc controls Aβ formation through the regulation of α-secretase [428].

6.13 Histone Deacetylase Inhibitors

Histone deacetylase (HDAC) inhibitors ameliorate a wide range of neurologic and psychiatric disorders in experimental models. *Suberoylanilide hydroxamic acid* (SAHA) was the first HDAC inhibitor approved by the FDA for the sole use of cancer therapy. SAHA also showed neurotrophic and neuroprotective effects in

animal models of neurodegenerative diseases [429]. Novel *quinazolin-4-one derivatives*, each containing a hydroxamic acid moiety, were designed and synthesized by Yu et al. [430]. These compounds are selective HDAC6 inhibitors. *(E)-3-(2-Ethyl-7-fluoro-4-oxo-3-phenethyl-3,4-dihydroquinazolin-6-yl)-N-hydroxyacrylamide* is the most potent HDAC6 inhibitor. In vitro, these compounds induced neurite outgrowth and growth-associated protein 43 expression, enhancing the synaptic activities of PC12 and SH-SY5Y neuronal cells. Some HDAC6 inhibitors decreased zinc-mediated β-amyloid aggregation in vitro. *N-Hydroxy-3-(2-methyl-4-oxo-3-phenethyl-3,4-dihydro-quinazolin-7-yl)-acrylamide* inhibits HDAC6, does not affect human ether-a-go-go-related membrane channel activity or cytochrome P450 activity in vitro, and improves learning-based performances of mice with β-amyloid-induced hippocampal lesions [430]. SAHA is an inhibitor of histone deacetylases (HDACs) used for the treatment of cutaneous T-cell lymphoma (CTCL). SAHA treatment enhances basal excitatory but not inhibitory synaptic function. Presynaptic release probability and intrinsic neuronal excitability were unaffected suggesting SAHA treatment selectively enhanced postsynaptic excitatory function. Long-term potentiation of excitatory synapses was augmented, while long-term depression (LTD) was impaired in SAHA-treated slices. In vivo SAHA treatment did not rescue memory deficits in the Tg2576 mouse model of AD. SAHA is a substrate of the BBB efflux transporters Pgp and Bcrp1, thus probably contributing to its limited brain availability and lack of behavioral effects following peripheral delivery [431].

4-Phenylbutyrate (PBA) is a histone deacetylase (HDAC) inhibitor and a chemical chaperone which has been shown to decrease tau phosphorylation, and to restore dendritic spine density in hippocampal CA1 pyramidal neurons of Tg2576 mice [432].

6.14 mTOR Inhibitors

The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that can sense environmental stimuli such as growth factors, energy state, and nutrients. Dysregulation of mTOR signaling pathway is also associated with a number of human diseases [433]. Upregulation of mTOR signaling pathway is thought to play an important role in major pathological processes of AD. The mTOR inhibitors may ameliorate the AD-like pathology and consequently the application of mTOR inhibitors may be of potential value as an innovative therapeutic strategy for AD [434].

mTOR represents a family of serine-threonine protein kinase called mammalian target of rapamycin. In mammalian cells mTOR is present in two distinct heteromeric protein complexes commonly referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), involved in the control of a wide variety of cellular processes. mTOR is crucial for synaptic plasticity,

learning, memory, and brain control of food uptake. Activation of the mTOR pathway is involved in neuronal development, dendrite development, and spine morphogenesis. mTOR also participates in the mechanism of PI3K/Akt-induced upregulation of glutamate transporter 1, GLT1, that is linked to several neuronal disorders such as stroke, AD, and amyotrophic lateral sclerosis. mTOR modulators display anticancer and immunosuppressant effects, and also exhibit neuroprotective properties [433]. Chronic administration of the target-of-rapamycin (TOR) inhibitor *rapamycin*, which extends lifespan and delays aging, halts the progression of AD-like disease in transgenic human hAPP mice modeling AD when administered before disease onset. Chronic reduction of TOR activity by rapamycin treatment started after disease onset restored cerebral blood flow (CBF) and brain vascular density, reduced CAA and microhemorrhages, decreased amyloid burden, and improved cognitive function in symptomatic hAPP AD mice. Like acetylcholine (ACh), a potent vasodilator, acute rapamycin treatment induced the phosphorylation of endothelial nitric oxide (NO) synthase (eNOS) and NO release in brain endothelium. Administration of the NOS inhibitor L-NG-Nitroarginine methyl ester reversed vasodilation as well as the protective effects of rapamycin on CBF and vasculature integrity, indicating that rapamycin preserves vascular density and CBF in AD mouse brains through NOS activation [435]. Rapamycin treatment resulted in a significant reduction in cortical tau tangles, less tau hyperphosphorylation, and lowered levels of insoluble tau in the forebrain. The favorable effect of rapamycin on tau pathology was paralleled by a qualitative reduction in astrogliosis. Accumulation of the autophagy associated proteins p62 and LC3 in aged tangle-bearing P301S mice was also lowered upon rapamycin treatment [436].

6.15 Peroxisome Proliferator-Activated Receptor Agonists

The activation of the retinoid X receptor, which dimerizes with peroxisome proliferator-activated receptors (PPARs), leads to an enhanced clearance of Aβ from the brain of a transgenic mouse model of AD, with increased expression of ApoE and its main transporters. Both a PPARγ agonist (*ciglitazone*) and a PPARα agonist (*WY 14.643*) are able to protect neurons by modulating mitochondrial fusion and fission, leading to a better response of neurons to oxidative stress, suggesting that a PPAR-based therapy could act simultaneously in different cellular components [437].

The peroxisome proliferator-activated receptor gamma agonist *pioglitazone* normalized neurometabolic and neurovascular coupling responses to sensory stimulation, and reduced cortical astroglial and hippocampal microglial activation in bitransgenic A/T mice that overexpress a mutated human amyloid precursor protein (APPSwe,Ind) and a constitutively active form of transforming growth factor-β1 (TGF-β1) [438].

6.16 P-Glycoprotein Regulators

The efflux transporter P-glycoprotein (P-gp) has been proposed as a potential therapeutic target for AD [439]. Approximately 10–35 % decrease in ^{125}I-A$\beta_{(1-40)}$ intracellular accumulation was observed in cells treated with rifampicin, dexamethasone, caffeine, verapamil, hyperforin, β-estradiol, and pentylenetetrazole, drugs known to induce P-gp expression [439].

6.17 Nuclear Receptor Agonists/Liver X Receptor Agonists

Liver X receptors (LXRs) are nuclear receptors involved in the regulation of lipid metabolism and inflammatory responses in the CNS [440]. LXR agonists induce the transcriptional activity of LXR target genes, attenuating the imbalance of cholesterol metabolism and overactivation of microglia and astrocytes in inflammation [441].

The development of liver X receptor agonists has been an area of interest for over a decade, given the critical role of those receptors in cholesterol metabolism, glucose homeostasis, inflammation, innate immunity, and lipogenesis. Potential indications include atherosclerosis, diabetes, inflammation, AD, and cancer [442, 443].

Nuclear receptors are attractive targets for the treatment of AD due to their ability to facilitate degradation of Aβ, affect microglial activation, and suppress the inflammatory milieu of the brain. Liver X receptor agonists have proven difficult to move into clinical trials, as long-term treatment results in hepatic steatosis. PPAR-γ activation remains a promising avenue for the treatment for AD. The synthetic Liver X receptor (LXR) activator *T0901317* has been reported to exert neuroprotective effect in AD. T0901317 decreases membrane cholesterol levels, reduces BACE1 expression and activity as well as β-secretase cleaved C-terminal fragment (β-CTF) levels in vivo and in vitro, and enhances the expression of ATP-binding membrane cassette transport protein A1 (ABCA1), as well. Inhibition of ABCA1 abrogates the effects of T0901317 on membrane cholesterol levels and β-secretase activity. Addition of LXR antagonist reverses the effect of T0901317 on ABCA1 mRNA expression, membrane cholesterol levels, and β-secretase activity. Activation of LXR may decrease BACE1 expression and activity through a pathway associated with ABCA1-mediated reduction in membrane cholesterol levels [440]. The clinical potential of many LXR agonists is limited because of their nonselective actions on LXRα/β, which lead to undesired hepatic lipogenesis via LXRα-dependent pathways. ABCA1 upregulators were identified by Hu et al. [444] from a series of flavonoids and were found to preferentially activate LXRβ and upregulate expression of ABCA1 and apoE in different cell lines. These compounds facilitate intracellular Aβ clearance in Aβ-loaded BV2 cells. Compound 19 reduced total brain Aβ and plaque burden in APP/PS1 double transgenic mice, associated with elevated ABCA1 and ApoE expression.

GW3965 treatment restores memory in WT but not ApoE−/− mice, and suppresses elevated $A\beta_{(40)}$ and $A\beta_{(42)}$ levels and axonal damage via both ApoE-dependent and ApoE-independent pathways [445].

6.18 Glycogen Synthase Kinase-3β (GSK-3β) Regulators

The serine/threonine kinase glycogen synthase kinase-3 beta (GSK3B) is a known master regulator for several cellular pathways that include insulin signaling and glycogen synthesis, neurotrophic factor signaling, Wnt signaling, neurotransmitter signaling, and microtubule dynamics. This enzyme has been implicated in AD pathology. There are many GSK3b inhibitors, of which *CHIR 99021* is considered most potent and selective. A library of over 320,000 compounds was screened against human GSK3b. Among the inhibitors identified, CID 5706819 and CID 56840716/ML320 are leading compounds with high potency and selectivity [446]. Glycogen synthase kinase-3β (GSK-3β) plays a crucial role in memory deficits and tau hyperphosphorylation. Intraventricular co-injection of wortmannin and GF-109203X (WT/GFX) can induce tau hyperphosphorylation and memory impairment in rats through activation of GSK-3. Feeding the rats with *acetyl-L-carnitine* for 2 weeks rescues the WT/GFX-induced spatial memory retention impairment antagonizing GSK-3β activation independent of Akt, PKCζ, and Erk1/2. Acetyl-L-carnitine also arrests microtubule-associated protein tau hyperphosphorylation at multiple AD sites in vivo and in vitro, and enhances the expression of several memory-associated proteins including c-Fos, synapsin I (SynI) in rat hippocampus [447].

Falcarindiol, isolated from Japanese parsley (*Oenanthe javanica*), is an inhibitor of GSK-3β. Falcarindiol inhibits GSK-3β in an ATP-noncompetitive manner, suppresses gene expression of glucose-6-phosphatase (G6Pase) in rat hepatoma H4IIE cells, and protects mouse neuroblastoma HT22 cells from glutamate-induced oxidative cell death [448].

Shi et al. [449] investigated the neuroprotective mechanism of combination extract of Renshen (*Panax ginseng*), Yinyanghuo (Herba *Epimedii brevicornus*), Yuanzhi (*Radix palygalae*), and Jianghuang (Rhizoma *Curcumae longae*) (GEPT) in APPV7171 transgenic mice where GEPT decreased the level of GSK-3β expression in the brain cortex.

Noh et al. [450] reported two novel GSK-3 inhibitors showing good activity and pharmacokinetic (PK) profiles. These GSK-3 inhibitors reduced the Aβ-oligomer-induced neuronal toxicity, decreased the phosphorylated tau at pThr231, pSer396, pThr181, and pSer202, and inhibited the GSK-3 activity against Aβ-oligomer-induced toxicity. In the B6;129-Psen1(tm1Mpm) Tg(APPSwe, tauP301L)1Lfa/Mmjax model of AD, oral administration of one of these GSK-3 inhibitors showed improved short-term memory and a decrease in hippocampal phosphorylated tau (Ser396) levels.

AZD1080, a potent and selective GSK3 inhibitor, inhibits tau phosphorylation in cells expressing human tau and in intact rat brain. AZD1080 reverses MK-801-induced deficits, measured by long-term potentiation in hippocampal slices and in a cognitive test in mice [451].

6.19 Cyclin-Dependent Kinase 5/p25 (CDK5) Inhibitors

Cyclin-dependent kinase 5 (cdk5) is a serine/threonine kinase involved in AD pathogenesis. Ginsenoside-derived phosphorylated peptides (PKpTPKKAKKV) have been developed as effective CDK5 inhibitors [452].

6.20 Antihistamines and Histamine H_3 Receptor Inverse Agonists

Histamine alterations have been reported in the brain, CSF, and peripheral blood of AD patients [117, 453–455]. Enhancement of histaminergic neurotransmission or histaminergic plus cholinergic neurotransmission may represent novel strategies for improving cognition in AD [456]. The effects of a novel histamine H_3 receptor inverse agonist (*MK-3134*), an acetylcholinesterase inhibitor (donepezil), and their combination in attenuating the cognitive impairment associated with scopolamine have been evaluated. Exploratory analyses provided evidence for cognitive improvement through inverse agonism of the H_3 histamine receptor and for cooperation between human cholinergic and histaminergic neurotransmitter systems [457]. The histamine H_3 receptor plays a critical role in the negative neuromodulation of neurotransmitters involved in cognitive function. H_3 receptor antagonists/inverse agonists have been shown to exert pro-cognitive effects in preclinical models. *GSK239512* is a potent and selective H_3 receptor antagonist developed for the treatment of cognitive dysfunction in neurodegenerative disorders. Nathan et al. [458] examined the safety, tolerability, pharmacokinetics, and pro-cognitive effects of oral GSK239512 in patients with mild-to-moderate AD using ascending dose titration regimens. GSK239512 displayed a satisfactory level of tolerability, with evidence for positive effects on attention and memory.

Latrepirdine (Dimebon) is a retired nonselective antihistamine drug currently being investigated as a therapeutic agent for AD. Dimebon is bioavailable in the brains of mice following oral administration. TgCRND8 AD mice chronically treated with dimebon exhibited a trend of improvement in spatial memory function without affecting the levels of total Aβ as well as soluble oligomeric Aβ in the brain [459]. Latrepirdine stimulates mTOR- and ATG5-dependent autophagy, leading to the reduction of intracellular levels of APP metabolites, including Aβ. Chronic latrepirdine administration resulted in increased levels of the biomarkers thought to correlate with autophagy activation in the brains of TgCRND8 (APP K670M, N671L, V717F) or wild-type mice, and treatment was associated with abrogation of behavioral deficit, reduction in Aβ neuropathology, and prevention of autophagic failure among TgCRND8 mice [460].

6.21 Estrogens

Estrogen plays important neurotrophic and neuroprotective roles in the brain by activating estrogen receptors (ERs). Disruption of normal estrogen signaling can leave neurons vulnerable to a variety of insults, including β-amyloid peptide. Aroclor1254 (A1254), a polychlorinated biphenyl with anti-estrogenic properties, abolishes the neuroprotective activity of estrogen against Aβ toxicity, and attenuates the suppressive effect of estrogen on Aβ-induced tau phosphorylation and JNK activation. The effects of A1254 on the neuroprotective effects of estrogen in Aβ toxicity are very similar to the effects of the estrogen receptor antagonist ICI182,780 [461].

$A\beta_{1-42}$ induces a decrease in phosphorylation of CREB at Ser133 (CREB pS133) and causes a transient upregulation of the inhibitory GSK-3β phosphorylation at Ser9 (GSK-3β pS9), followed by downregulation of GSK-3β pS9. Pretreatment with 17β-estradiol protects against $A\beta_{1-42}$-induced changes of CREB. The protective role of 17β-estradiol against $A\beta_{(1-42)}$-induced downregulation of CREB pS133 is abolished by the mitogen-activated protein kinase (MAPK) pathway inhibitor U0126. 17β-Estradiol also prolongs the upregulation of GSK-3β pS9 and this effect is abrogated by the PKA inhibitor H-89, AKT inhibitor LY294002, and MAPK inhibitor U0126, indicating that the protection of 17β-estradiol on CREB is MAPK-dependent, but its effect on GSK-3β integrates several pathways [462].

Recent studies have proposed that beneficial effects of estrogen on AD are directly linked to its ability to reduce amyloid-β peptides and tau aggregates; however, despite high expectations, large clinical trials with postmenopausal women indicated that the beneficial effects of estrogen therapies were insignificant and, in fact, elicited adverse effects [463, 464].

Steroids may influence Aβ production. Jung et al. [465] screened 170 steroids at 10 μM for effects on $A\beta_{42}$ secreted from human APP-overexpressing Chinese hamster ovary cells. Many acidic steroids lowered $A\beta_{42}$, whereas many nonacidic steroids actually raised $A\beta_{42}$. $A\beta_{42}$-lowering steroids were GSMs and $A\beta_{42}$-raising steroids were inverse GSMs. The most potent steroid GSM identified was 5β-cholanic acid (equipotent to its endogenous analog lithocholic acid), and the most potent inverse GSM identified was 4-androsten-3-one-17β-carboxylic acid ethyl ester. Both estrogen and progesterone are weak inverse GSMs with further complex effects on APP processing. Certain endogenous steroids may have the potential to act as GSMs and other steroids may play a role in modulating Aβ production acting as γ-secretase modulators [465].

Phytoestrogens have been proposed as potential alternatives to estrogen replacement therapy and as candidate drugs in AD. A novel phytoestrogenic formulation, referred to as the *phyto-β-SERM* formulation, exhibits an 83-fold binding selectivity for the estrogen receptor subtype β (ERβ) over ERα. The phyto-β-SERM formulation is neuroprotective and promotes estrogenic

mechanisms in the brain while devoid of feminizing activity in the periphery. When initiated prior to the appearance of AD pathology, a 9-month dietary supplementation with the phyto-β-SERM formulation promoted physical health, prolonged survival, improved spatial recognition memory, and attenuated amyloid-β deposition and plaque formation in the brains of treated AD mice [466].

6.22 Kynurenine 3-Monooxygenase Inhibitors

Metabolites in the kynurenine pathway, generated by tryptophan degradation, are thought to play an important role in neurodegenerative disorders, such as AD and Huntington's disease, where glutamate receptor-mediated excitotoxicity and free radical formation have been correlated with decreased levels of the neuroprotective metabolite kynurenic acid. Inhibition of kynurenine 3-monooxygenase (KMO), an enzyme in the eukaryotic tryptophan catabolic pathway, leads to amelioration of Huntington's-disease-relevant phenotypes in yeast, fruitfly, and mouse models, as well as in a mouse model of AD [467]. KMO is a flavin adenine dinucleotide (FAD)-dependent monooxygenase and is located in the outer mitochondrial membrane where it converts l-kynurenine to 3-hydroxykynurenine. Zwilling et al. [468] described the synthesis and characterization of *JM6*, a small-molecule prodrug inhibitor of kynurenine 3-monooxygenase (KMO) which inhibits KMO in the blood, increasing kynurenic acid levels and reducing extracellular glutamate in the brain. In AD transgenic models, JM6 prevents spatial memory deficits, anxiety-related behavior, and synaptic loss. JM6 also extends lifespan, prevents synaptic loss, and decreases microglial activation in a mouse model of Huntington's disease.

6.23 Chaperones (Small Heat Shock Proteins, sHSPs)

Small heat shock proteins (sHsps) are molecular chaperones that protect cells from cytotoxic effects of protein misfolding and aggregation. HspB1, an sHsp commonly associated with senile plaques in AD, prevents the toxic effects of Aβ aggregates in vitro. Substoichiometric amounts of human HspB1 (Hsp27) abolish the toxicity of Aβ oligomers on N2a (mouse neuroblastoma) cells. HspB1 sequesters toxic Aβ oligomers and converts them into large nontoxic aggregates. Sequestration of oligomers by HspB1 constitutes a novel cytoprotective mechanism of proteostasis [469]. Hsp70 chaperones are increasingly becoming identified as targets for therapeutic intervention in cancer and neurodegenerative diseases. Hsp70 (heat shock protein 70 kDa) chaperones are key to cellular protein homeostasis. They also have the ability to inhibit tumor apoptosis and contribute to aberrant accumulation of hyperphosphorylated tau in neuronal cells affected by tauopathies. The action of the potent anticancer compound MKT-077 (1-ethyl-2-[[3-ethyl-5-(3-methylbenzothiazolin-2-yliden)]-4-oxothiazolidin-2-ylidenemethyl] pyridinium chloride) occurs through a differential

interaction with Hsp70 allosteric states. This type of compound is referred as an "allosteric drug" [470]. BRICHOS is a chaperone domain that, during biosynthesis, binds to precursor protein regions with high β-sheet propensities, thereby preventing them from amyloid formation. Recombinant BRICHOS domains from Bri2 and proSP-C have been found to efficiently prevent SP-C, the amyloid β-peptide associated with AD, and medin, found in aortic amyloid, from forming amyloid fibrils. BRICHOS domain might be harnessed in therapeutic strategies against amyloid diseases [471].

The glucose-regulated protein 78 (GRP78), also known as BiP, is the endoplasmatic reticulum (ER) homolog of HSP70, which plays a dual role in the ER by controlling protein folding, in order to prevent aggregation, and by regulating the signaling of the unfolded protein response (UPR). The expression levels and activity of GRP78 are altered with age raising the question of whether the lack of GRP78 could be a predisposing factor for many neurodegenerative disorders associated with age. GRP78 induction or upregulation in animal models of neurodegeneration has recently been made with the help of pharmacological BiP protein Inducer X (BIX) and GRP78 cDNA delivery via adeno-associated virus (AAV) vectors [472].

YM-01 is an allosteric promoter of triage functions of the most abundant variant of the heat shock protein 70 (Hsp70) family in the brain, heat shock cognate 70 protein (Hsc70). YM-01 reduces Tau levels in vitro and ex vivo. Overexpression of heat shock protein 40 (DNAJB2), an Hsp70 co-chaperone, suppresses YM-01 activity. In contrast to its effects in pathogenic tauopathy models, YM-01 had little activity in ex vivo brain slices from normal, wild-type mice unless microtubules were disrupted, suggesting that Hsc70 acts preferentially on abnormal pools of free tau. YM-01 also increases long-term potentiation in tau transgenic brain slices [473].

Hsp27 belongs to the small heat shock protein family, which are ATP-independent chaperones, with antiapoptotic and antioxidant activities. Hsp27 binds nonnative proteins and inhibits the aggregation of incorrectly folded proteins maintaining them in a refolding-competent state. Tóth et al. [474] generated transgenic mice overexpressing human Hsp27 protein and crossed with APPswe/PS1dE9 mouse strain, and found that the impaired spatial learning and long-term potentiation present in the AD model mice were rescued by Hsp27 overexpression. Amyloid plaques were also reduced in the brain of APPswe/PS1dE9/Hsp27 animals compared to AD model mice.

6.24 ApoE Mimetic Peptides

Handattu et al. [475] studied the effects of an ApoE mimetic peptide (Ac-hE18A-NH2) on amyloid-β pathology. Ac-hE18A-NH2 treatment improved cognition with a concomitant decrease in amyloid plaque deposition and reduced activated microglia and

astrocytes, and increased brain ApoE levels in APP/PS1ΔE9 mice. Oligomeric Aβ_{42} (oAβ_{42}) and oxidized PAPC (ox-PAPC) inhibited secretion of ApoE in the human astrocyte cell line U251, and this effect was ameliorated in the presence of Ac-hE18A-NH2. This mimetic peptide also increased Aβ_{42} uptake in a cell line of human macrophages.

6.25 Glucagon-Like Peptide-1 (GLP-1) Agonists

It has been proposed that glucagon-like peptide-1 (GLP-1) treatment may be useful in AD. GLP-1 receptors are expressed in areas of the brain important to memory and learning, and GLP-1 has growth-factor-like properties similar to insulin. GLP-1 and longer-lasting analogs have been shown to have both neuroprotective and neurotrophic effects, and to protect synaptic activity in the brain from Aβ toxicity [476]. *Saxagliptin* is a dipeptidyl peptidase-4 (DPP-4) inhibitor which increases the level of GLP-1. Saxagliptin reduces Aβ, tau phosphorylation, and inflammatory markers and improves hippocampal GLP-1 and memory retention in a streptozotocin (STZ)-induced rat model of AD [477].

Liraglutide, a GLP-1 agonist and a new anti-diabetic drug, can promote brain insulin signaling and inhibit tau hyperphosphorylation in the brains of type 2 diabetic rats. Subcutaneous liraglutide decreased CSF insulin, hyperphosphorylation of tau at AD-relevant phosphorylation sites, and decreased phosphorylation of protein kinase B (AKT) and glycogen synthase kinase-3β (GSK-3β) in the brain, which indicated decreased insulin signaling leading to over-activation of GSK-3β, a major tau kinase, in type 2 diabetic rats [478]. The GLP-1 analog liraglutide has been shown to increase cell proliferation in an AD mouse model. Acute treatment with liraglutide showed an increase in cell proliferation in APP/PS1 mice, immature neurons were increased in both acute and chronic treated animals, and newly generated cells differentiated into mature neurons [479].

6.26 Glucose-Dependent Insulinotropic Polypeptide (GIP) Analogs (Incretin Hormone Analog D-Ala2GIP)

Glucose-dependent insulinotropic polypeptide (GIP) regulates insulin signaling by facilitating insulin release, modulates neurotransmitter release and LTP formation, and protects synapses from the detrimental effects of β-amyloid fragments on LTP formation, and cell proliferation of progenitor cells in the dentate gyrus. Faivre and Hölscher [480] studied the potential therapeutic effect of the new long-lasting incretin hormone analog D-Ala2GIP on APPswe/PS1detaE9 transgenic mice. D-Ala2GIP improved memory in WT mice and rescued the cognitive decline of APP/PS1 mice, prevented deterioration of synaptic function in the dentate gyrus and cortex, facilitated synaptic plasticity in APP/PS1 and WT mice, and reduced the inflammatory response in microglia and the number of amyloid plaques in the cortex.

6.27 Chemokines, Macrophage Inflammatory Protein-2 (MIP-2) and Stromal Cell-Derived Factor-1α (SDF-1α)

Raman et al. [481] investigated the ability of the chemokines, macrophage inflammatory protein-2 (MIP-2) and stromal cell-derived factor-1α (SDF-1α), the respective ligands for chemokine receptors CXCR2 and CXCR4, to suppress Aβ-induced neurotoxicity in vitro and in vivo. Pretreatment with MIP-2 or SDF-1α significantly protected neurons from Aβ-induced dendritic regression and apoptosis in vitro through activation of Akt, ERK1/2 and maintenance of metalloproteinase ADAM17, especially with SDF-1α. The Aβ-induced morphometric changes of neurons and increase in biomarkers of oxidative damage, F_2-isoprostanes, were significantly inhibited by pretreatment with the chemokines MIP-2 or SDF-1α. MIP-2 or SDF-1α was able to suppress the aberrant mislocalization of p21-activated kinase (PAK), one of the proteins involved in the maintenance of dendritic spines. MIP-2 also protected neurons against Aβ neurotoxicity in $CXCR2^{-/-}$ mice, potentially through observed upregulation of CXCR1 mRNA.

6.28 Nonsteroidal Anti-inflammatory Drugs and Cyclooxygenase-1 and Cyclooxygenase-2 Inhibitors

Cyclooxygenase-1 and/or cyclooxygenase-2 inhibitors significantly increased the survival of pyramidal neurons. Both cyclooxygenase-1 and cyclooxygenase-2 isoforms, but not cyclooxygenase-3, are involved in the progression of neuronal damage [482]. The Alzheimer's Disease Anti-inflammatory Prevention Trial Follow-up Study (ADAPT-FS) was designed to evaluate the efficacy of naproxen and celecoxib for the primary prevention of AD several years after cessation of treatment in ADAPT. The results obtained during a follow-up of approximately 7 years do not support the hypothesis that celecoxib or naproxen prevents AD in adults with a family history of dementia [483].

6.29 Bone Morphogenetic Protein 9 (BMP-9)

Bone morphogenetic protein (BMP) 9 is a cholinergic differentiating factor during development both in vivo and in vitro. Cholinergic projection from the septum to the hippocampus is crucial for normal cognitive function and degeneration of cells and nerve fibers within the septohippocampal pathway contributes to the pathophysiology of AD. BMP9 administration can prevent lesion-evoked impairment of the cholinergic septohippocampal neurons in adult mice and, by inducing NGF, establishes a trophic environment for these cells [484].

6.30 Vitamin C

Oxidative stress is suggested to play a major role in the pathogenesis of AD. Vitamin C has been regarded as the most important antioxidant in neural tissue. Vitamin C decreases β-amyloid generation and acetylcholinesterase activity and prevents endothelial dysfunction by regulating nitric oxide. Clinical trials with different antioxidants, including vitamin C, yielded confusing results [485].

6.31 Vitamin D

Poor vitamin D nutrition is linked with dementia [486]. There is an association of reduced plasma 25-hydroxyvitamin D with

increased risk of AD and VD [487]. Vitamin D supports nerve transmission and synaptic plasticity, and vitamin D receptors are expressed in the hippocampus [488]. Nasal insulin acutely improves cognition and vitamin D upregulates insulin receptor expression and enhances insulin action. Dursun et al. [489] studied the expression of the vitamin D receptor (VDR), 25-hydroxyvitamin D_3 24-hydroxylase (an accelerator of vitamin D catabolism), and the L-type voltage-sensitive calcium channel A1C (LVSCC-A1C) in hippocampal neurons in response to β-amyloid. β-Amyloid suppressed the expression of VDR mRNA and induced the expression of 24OHase and LVSCC-A1C mRNAs, indicating that Aβ may disrupt the vitamin D-VDR pathway and cause defective utilization of vitamin D by suppressing the level of the VDR and elevating the level of 24OHase.

6.32 Vitamin E, Ubiquinone (Coenzyme Q10), and Ubidecarenone (Co. Q10 NLC)

Nanostructured lipid carriers (NLC) have been developed from mixtures of solid lipid and spatially incompatible liquid lipid by a solvent diffusion method. Glyceryl distearate and Glyceryl behenate were chosen as solid lipid and Glyceryl triacetate used as liquid lipid. Ubidecarenone used as model drug was incorporated into the NLC. The antioxidant activity of the Ubidecarenone (Co. Q10 NLC) was more effective than the Ubidecarenone (Coenzyme Q10) solution form on DPPH scavenging, anti-lipid peroxidation, and memory improvement in scopolamine-induced amnesia [490].

Coenzyme Q10 was found to affect the phosphatidylinositol 3-kinase (PI3K) pathway. CoQ10 can restore amyloid β $A\beta_{25-35}$ oligomer-inhibited proliferation of NSCs. CoQ10 treatment increased the expression levels of p85α PI3K, phosphorylated Akt (Ser473), phosphorylated glycogen synthase kinase-3β (Ser9), and heat shock transcription factor, which are proteins related to the PI3K pathway in $A\beta_{25-35}$ oligomers-treated NSCs. The effects of CoQ10 on the proliferation of NSCs inhibited by $A\beta_{25-35}$ oligomers were almost completely blocked by the PI3K inhibitor LY294002, indicating that CoQ10 restores $A\beta_{25-35}$ oligomer-inhibited proliferation of NSCs by activating the PI3K pathway [491].

6.33 Retinoids and Retinoid Acid Receptor Agonists

Retinoic acid (RA) is a vitamin A metabolite and the RA receptor (RAR) is a transcription factor. Vitamin A/RA administration improves memory in AD mouse models. A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) was identified as a key molecule in RA-mediated anti-AD mechanisms. The RAR agonist *Am80* (*Tamibarotene*) improved memory, reduced ADAM10 mRNA and protein expression in the hippocampus of 13-month-old SAMP8 mice, and restored the expression levels of Hes5 and Ki67, whereas APP and Aβ levels remained unchanged, indicating that these effects might be regulated by activation of the

hippocampal ADAM10-Notch-Hes5 proliferative pathway [492]. Retinoic acid (RA)-elicited signaling has been shown to play critical roles in development, organogenesis, and the immune response. RA regulates expression of AD-related genes and attenuates amyloid pathology in a transgenic mouse model. RA can suppress the production of Aβ through direct inhibition of γ-secretase activity. RA-induced inhibition of γ-secretase activity was found to be mediated through significant activation of extracellular signal-regulated kinases (ERK1/2). Treatment of cells with the specific ERK inhibitor PD98059 completely abolished RA-mediated inhibition of γ-secretase. RA inhibits γ-secretase through nuclear retinoic acid receptor-α (RARα) and retinoid X receptor-α (RXRα) [493].

ADAM10 (a disintegrin and metalloproteinase 10) acts as the main physiological α-secretase. Enzymatic activity of the α-secretase prevents the formation of toxic Aβ peptides and promotes the secretion of a neurotrophic and neuroprotective amyloid precursor protein fragment (APPs-α) by cleaving the APP within its Aβ sequence. Retinoids and synthetic retinoids such as *acitretin* increase ADAM10 expression and activity. Acitretin and tamibarotene are both potent activators of ADAM10 promoter activity. Acitretin crosses the murine BBB and its level in the mouse brain is not reduced by P-gp [494].

6.34 Peroxiredoxin 6

The antioxidant peroxiredoxin 6 (Prdx 6) protects against $A\beta_{25-35}$-induced neurotoxicity in rat PC12 cells. Treatment of PC12 cells with $A\beta_{25-35}$ resulted in a dose- and time-dependent cytotoxicity that was associated with increased accumulation of intracellular ROS and mitochondria-mediated apoptotic cell death, including activation of caspase 3/9, inactivation of poly ADP-ribosyl polymerase (PARP), and dysregulation of Bcl-2 and Bax. This apoptotic signaling machinery was markedly attenuated in PC12 cells that overexpress wild-type Prdx 6, but not in cells that overexpress the C47S catalytic mutant of Prdx 6 [495].

6.35 ω-3 Polyunsaturated Fatty Acids (n-3 PUFAs) and Docosahexaenoic Acid (DHA, C22:6 n-3)

Omega-3 PUFAs are essential unsaturated fatty acids obtained from food sources or from supplements. Amongst nutritionally important polyunsaturated n-3 fatty acids, *α-linolenic acid* (ALA), *eicosapentaenoic acid* (EPA), and *docosahexaenoic acid* (DHA) are highly concentrated in the brain and have antioxidative stress, anti-inflammatory and antiapoptotic effects. The exposure to n-3 fatty acids enhances adult hippocampal neurogenesis associated with cognitive and behavioral processes, promotes synaptic plasticity by increasing long-term potentiation, and modulates synaptic protein expression to stimulate the dendritic arborization and new spine formation [496].

Samieri et al. [497] investigated the association between dietary fat and plasma concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in elderly persons. Plasma

EPA increased with frequency of fish consumption, alcohol intake, and female gender, and decreased with intensive consumption of n-6 oils. The positive association between fish consumption and plasma DHA was highly significant whatever the *APOE* genotype but stronger in *APOE4* noncarriers than in carriers. Plasma DHA increased significantly with age in *APOE4* noncarriers only. According to these results, dietary habits, gender, and *APOE4* genotype should be considered when designing interventions to increase n-3 PUFA blood levels in older people. Some effects of ω3 FAs might be caused by downregulation and resolution of inflammation. EPA increases the levels of BDNF, and DHA decreases the levels of TNF-α. Both DHA and EPA decrease the proinflammatory M1 markers CD40 and CD86, and DHA has a stimulatory effect on the anti-inflammatory M2 marker CD206 [498]. Some authors have proposed the use of PUFAs to prevent age-related cognitive decline and AD [499].

Conjugated linoleic acid (CLA), an eighteen-carbon unsaturated fatty acid, was discovered as a μ-calpain-specific inhibitor. Hyperactivated μ-calpain enhances the accumulation of β-amyloid peptide by increasing the expression level of β-secretase (BACE1) and induces hyperphosphorylation of tau along with the formation of NFTs by mediating p35 cleavage into p25CLA, shows neuroprotective effects against neurotoxins such as H_2O_2 and $Aβ_{1–42}$ in SH-SY5Y cells, and inhibits Aβ oligomerization/fibrillation and Aβ-induced Zona Occludens-1 degradation. CLA decreased the levels of proapoptotic proteins, p35 conversion to p25 and tau phosphorylation [500].

Furan fatty acids are present in fish oils and marine organisms. Teixeira et al. [501] reported that treatment of rat brain C6 astroglioma cells with furan fatty acid F6 prior to exposure to hydrogen peroxide shows a strong protective effect of F6 against cell death resulting from oxidative stress. Brain cells might be rescued by F6 scavenging radicals elicited by lipid peroxidation within the cell membrane. Oxidative processes outside the cell membrane, such as protein carbonylation, are not affected by F6.

DHA and Coenzyme Q10. Coenzyme Q10 (CoQ10) is an essential cofactor involved in the mitochondrial electron transport chain. Zinc toxicity also affects cellular energy production by decreasing oxygen consumption rate (OCR) and ATP turnover in human neuronal cells, which can be restored by the neuroprotective effect of docosahexaenoic acid (DHA). DHA is specifically neuroprotective against zinc-triggered mitochondrial dysfunction, and CoQ10 has shown to be protective against both Aβ- and zinc-induced alterations in mitochondrial function [502].

6.36 Sphingosylphosphorylcholine

Sphingosylphosphorylcholine (SPC), a choline-containing sphingolipid, shows suppressive effect on Aβ production in PC12 cells which stably express Swedish mutant of amyloid precursor protein

(APPsw). SPC reduces the accumulation of Aβ and the expression of BACE1. Phosphocholine (PC) or other lysophospholipids, such as lysophosphatidylcholine (LPC), lysophosphatidic acid (LPA), and sphingosyl-1-phosphate (S1P), do not alter BACE1 expression. Downregulatory effect of SPC on BACE1 expression appears to be mediated by NF-κB which is known to suppress the *trans-activation* of BACE1 promoter in PC12 cells. The nuclear translocation of NF-κB is enhanced by SPC treatment. The catalytic activities of BACE1 and BACE2 are dose-dependently inhibited by SPC [503].

6.37 Sphingosine-1-Phosphate Receptor Modulator FTY720 (Fingolimod)

Sphingosine-1-phosphate (S1P) is a pluripotent lipophilic mediator working as a ligand for G-protein coupled S1P receptors (S1PR), which is currently highlighted as a therapeutic target for autoimmune diseases including relapsing forms of multiple sclerosis. Sphingosine-related compounds, *FTY720* and *KRP203* known as S1PR modulators, are phosphorylated by sphingosine kinase 2 (SphK2) to yield the active metabolites FTY720-P and KRP203-P, which work as functional antagonists for S1PRs. FTY720 and KRP203 decreased production of Aβ in cultured neuronal cells [504]. The sphingosine-1-phosphate receptor modulator FTY720 (Fingolimod) prevents lymphocytes from contributing to an autoimmune reaction and has been approved for multiple sclerosis treatment. Chronic administration of FTY720 prevents spatial learning and memory impairment in AD rats [505]. Fingolimod phosphate (FTY720-P) protects neurons against oligomeric amyloid β-induced neurotoxicity. Treatment with FTY720-P enhanced the expression of BDNF in neurons. Blocking BDNF-TrkB signaling with a BDNF scavenger, TrkB inhibitor, or ERK1/2 inhibitor almost completely ablated these neuroprotective effects, indicating that the neuroprotective effects of FTY720-P are mediated by upregulated neuronal BDNF levels [506].

6.38 Cathepsin B Inhibitors

Upregulation of the lysosomal system has been suggested to contribute to the pathogenesis of AD. Okadaic acid (OA), a protein phosphatase-2A inhibitor, increases tau phosphorylation, β-amyloid deposition, and neuronal cell death. While inhibition of cathepsin D and L failed to protect neurons from OA-induced cell death, CA074-Me, a cathepsin B inhibitor, conferred a protective effect. CA-074Me reduced APP accumulation and α-spectrin cleavage, similar to the effect of calpain inhibition [507].

6.39 Kinin B1 Receptor Blockers (SSR240612)

The inducible kinin B1 receptor (B1R) contributes to pathogenic neuroinflammation induced by Aβ. B1R protein levels are increased in APP mouse hippocampus and, prominently, in reactive astrocytes surrounding Aβ plaques. In APP mice, B1R antagonism with SSR240612 improves spatial learning, memory, and normalized protein levels of the memory-related early gene Egr-1 in the dentate gyrus of the hippocampus. B1R antagonism

restored sensory-evoked CBF responses, endothelium-dependent dilations, and normalized cerebrovascular protein levels of endothelial nitric oxide synthase and B2R. SSR240612 reduces microglial activation, brain levels of soluble $A\beta_{1-42}$, diffuse and dense-core Aβ plaques, and increases protein levels of the Aβ brain efflux transporter lipoprotein receptor-related protein-1 in cerebral microvessels [508].

6.40 Rho Kinase Inhibitors (Fasudil)

Fasudil is a Rho kinase inhibitor and has been reported to have neuroprotective effects. Administration of fasudil ameliorates spatial learning and memory impairment, attenuates neuronal loss, and neuronal injury induced by $A\beta_{1-42}$, and inhibits IL-1β and TNF-α production and NF-κB activation in the rat brain [509–511].

6.41 Transcription Factor Specificity Protein 1 (Sp1) Inhibitors (Tolfenamic Acid)

Tolfenamic acid lowers the levels of APP and Aβ when administered to C57BL/6 mice by lowering their transcriptional regulator specificity protein 1 (SP1). Treatment with tolfenamic acid attenuates long-term memory and working memory deficits in hemizygous R1.40 transgenic mice. Cognitive enhancement was accompanied by reduction in the levels of the SP1 protein, followed by lowering both the mRNA and the protein levels of APP and subsequent Aβ levels [510].

6.42 TNF Inhibitors (2-(2,6-Dioxopiperidin-3-yl)phthalimidine EM-12 Dithiocarbamates, N-Substituted 3-(Phthalimidin-2-yl)-2,6-dioxopiperidines, 3-Substituted 2,6-dioxopiperidines)

Eight novel 2-(2,6-dioxopiperidin-3-yl)phthalimidine EM-12 dithiocarbamates 9 and 10, N-substituted 3-(phthalimidin-2-yl)-2,6-dioxopiperidines 11–14, and 3-substituted 2,6-dioxopiperidines 16 and 18 were synthesized as tumor necrosis factor-α (TNF-α) synthesis inhibitors. The pharmacological focus of these compounds is toward the development of well-tolerated agents to ameliorate neuroinflammation in AD, Parkinson's disease, and other neurodegenerative/inflammatory disorders [512].

Thalidomide is a TNFα inhibitor which has been found to have abilities against tumor growth, angiogenesis, and inflammation. Inhibition of TNFα reduces amyloid-associated pathology, prevents neuron loss, and improves cognition. Genetic inhibition of TNFα/TNF receptor signal transduction downregulates β amyloid cleavage enzyme 1 (BACE1) activity, reduces Aβ generation, and improves learning and memory deficits. Thalidomide decreases the activation of both astrocytes and microglia, reduces Aβ load and plaque formation, and decreases BACE1 level and activity in APP23 mice [513].

6.43 Pyrrolo[3,2-e][1,2,4]triazolo[1,5-a]pyrimidine (SEN1176)

SEN1176 is a novel pyrrolo[3,2-e][1,2,4]triazolo[1,5-a]pyrimidine with anti-neuroinflammatory effects which suppresses $A\beta_{1-42}$-induced macrophage production of nitric oxide, TNF-α, IL-1β, and IL-6 in a dose-dependent fashion and alleviates chronic neuroinflammatory processes related to brain Aβ deposition [514].

6.44 Calcium Channel Blockers

Disturbance of the intracellular calcium homeostasis is involved in the pathogenesis of neurodegeneration and probably in the extracellular accumulation of Aβ. Calcium channel blockade attenuates Aβ-induced neuronal damage. Several calcium channel blockers have been tested in dementia with poor results. In some clinical trials, nimodipine and nilvadipine showed some positive results on cognition, whereas other calcium channel blockers failed [515].

6.45 Leucettines

Leucettines, a family of marine sponge-derived 2-aminoimidazolone alkaloids, are potent inhibitors of DYRKs (dual-specificity, tyrosine phosphorylation-regulated kinases) and CLKs (cdc2-like kinases), with potential therapeutic effect in AD [516].

6.46 Intranasal Insulin

AD has been associated with decreased CSF levels of insulin in combination with decreased insulin sensitivity (insulin resistance) in the brain. Attenuated receptor expression of insulin and insulin-like growth factor, enhanced serine phosphorylation of insulin receptor substrate-1, and impaired transport of insulin across the BBB are pathogenic events underlying brain insulin resistance. Some clinical trials have demonstrated that intranasal insulin improves memory performance of patients with AD and MCI [517].

6.47 Nicotine

Nicotine has been shown to speed attentional reorienting in cued target detection tasks. *APOE-4* carriers might show greater sensitivity to the cognitive effects of nicotine. Subjects harboring the *APOE-4* allele showed decreased extrastriate activation, and enhanced effects of nicotine on reorienting in right middle frontal regions. Evans et al. [518] reported that drug by genotype interactions were present in hippocampal and anterior cingulate regions.

6.48 Antihypertensive Agents

Antihypertensive agents, particularly centrally acting *ACE inhibitors* (*perindopril*, *enalapril*) and renin-aldosterone-angiotensin system-targeting drugs [519] which cross the BBB, are associated with a reduced rate of cognitive decline [520]. The effects of ACE inhibitors on AD may be different depending on ApoE genotype [521]. Angiotensin receptor blockers (ARBs) have cognitive protective effects that are related to their ability to decrease production and oligomerization and increase degradation of Aβ and their vascular effects (improving BBB, restoring endothelial function, decreasing inflammation, and increasing cerebral blood flow) [522]. The use of diuretics, angiotensin-1 receptor blockers (ARB), angiotensin-converting enzyme inhibitors (ACE-I), calcium channel blockers (CCB), or β-blockers (BB) was associated with a reduced risk of AD in participants with normal cognition or MCI. Diuretic use was reported by 15.6 %, ARB 6.1 %, ACE-I 15.1 %, CCB 14.8 %, and BB 20.5 %. In cases with MCI, only diuretic use was associated with decreased risk [523]. Angiotensin-converting enzyme inhibitors (ACE-I) have beneficial effects on endothelial

dysfunction. Human umbilical vein ECs (HUVECs) treated with sera from AD patients enter in an apoptotic process, and enalapril suppresses the induction of apoptosis by AD serum [524]. The angiotensin II AT1 receptor is a significant source of brain ROS, and AD patients have an increased brain angiotensin-converting enzyme (ACE) level, which could account for an excessive angiotensin-dependent AT1-induced ROS generation. The centrally active ACE inhibitor *captopril* normalized the excessive hippocampal ACE activity of AD transgenic mice. The neuroprotective profile triggered by captopril was accompanied by reduced amyloidogenic processing of APP, and decreased hippocampal ROS, which is known to enhance Aβ generation by increased activation of β- and γ-secretases [525].

Telmisartan is an angiotensin II type 1 receptor blocker with peroxisome proliferator-activated receptor γ-stimulating activity. Li et al. [526] studied the effects of telmisartan vs. amlodipine on the levels of $A\beta_{1-42}$, interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and cognition in elderly hypertensive patients with AD. After 6 months the patients displayed significantly higher $A\beta_{1-42}$ and greatly lower levels of IL-1β and TNF-α in the telmisartan group versus the amlodipine group. At 24 weeks, the patients in the telmisartan group had better mini-mental state examination (MMSE) and Alzheimer's disease assessment scale-cognitive subscale (ADAS-cog) scales scores than those taking amlodipine.

Carvedilol, a nonselective β-adrenergic receptor blocker, attenuates brain oligomeric β-amyloid content and cognitive deterioration in AD mouse models [527].

Nebivolol is a selective β1 adrenergic receptor antagonist with nitric oxide-mediated vasodilatory properties. Nebivolol modulates amyloid-β protein precursor processing in vitro and in vivo. Nebivolol is brain bioavailable and can be readily detected in the brain following 3 weeks of treatment at a dose of 1 mg/kg/day. Chronic nebivolol treatment of Tg2576 mice with amyloid neuropathology reduced brain amyloid content but failed to improve cognitive function [528].

6.49 NADPH Oxidase Inhibitors (Apocynin)

The NADPH oxidase inhibitors, apocynin and dextromethorphan (DM), were tested for their capacity to reduce learning deficits and neuropathology in transgenic mice overexpressing human amyloid precursor protein with the Swedish and London mutations (*$hAPP^{751}SL$*). Apocynin and DM ameliorate Aβ-induced extracellular superoxide production and neurotoxicity, but both failed to affect learning and memory tasks or synaptic density in $hAPP^{751}$ SL mice [529].

6.50 Surfactin

Microglial-mediated neuroinflammation and neurotoxicity are involved in the pathogenesis of AD. Regulation of microglial activation and suppression of neurotoxic proinflammatory molecules may provide a potential therapeutic approach for the treatment of

AD. Park et al. [530] investigated the effects of surfactin, a surfactant from *Bacillus subtilis*, on oligomeric Aβ-induced microglial activation and neurotoxicity. Surfactin suppressed expression of MMP-9, iNOS, and COX-2, as well as production of ROS, NO, PGE2, TNF-α, IL-1β, IL-6, and MCP-1 in Aβ-stimulated BV-2 microglial cells. Surfactin also inhibited Aβ-induced nuclear translocation and activation of NF-κB as well as phosphorylation of JNK and p38 MAPK, and protected hippocampal HT22 cells from indirect neuronal toxicity mediated by Aβ-treated microglial cells.

6.51 Lithium

Scopolamine causes spatial learning and memory deficits that involved activation of glycogen synthase kinase-3β (GSK-3β) and impairments of dendrite arborization and spine formation/maturation associated with alterations of AMPAR, Homer1, and CREB. Pretreatment by intraperitoneal injection of lithium, an inhibitor of GSK-3, for 1 week prevented the synaptic changes and the learning and memory deficits induced by scopolamine. Lithium treatment also activated cholineacetyltransferase and inhibited acetylcholinesterase, which might have also contributed to the improved memory [531].

6.52 Uridine Prodrug PN401

Uridine prodrug PN401 shows neuroprotective effects in models of Parkinson's disease, Huntington's disease, and AD. The effects of PN401 treatment were tested in the Tg2576 and Tg2576 X P301L (TAPP) mouse models of AD. Treatment with PN401 reduced impairments in the Tg2576 mice in contextual fear conditioning and novel object recognition. In the TAPP mice, PN401 reduced the impairments in novel object recognition and social transmission of food preference. PN401 also improved motor behavior and reduced anxiety-like behavior in the TAPP mice. TAPP mouse hippocampal tau phosphorylation and lipid peroxidation were reduced by PN401 treatment [532].

6.53 3-N-Butylphthalide

dl-3-n-Butylphthalide (NBP) has been reported to attenuate astroglial activation and exert neuroprotective effects in AD transgenic mice. NBP inhibited the Aβ-induced activation of astrocytes and the upregulation of proinflammatory molecules. NBP suppressed Aβ-induced IκBα degradation and nuclear factor-κB (NF-κB) translocation, suggesting that NBP attenuates Aβ-induced activation of astrocytes and neuroinflammation via inhibition of the NF-κB signaling pathway [533].

6.54 Methylene Blue

Methylene blue (MB), a member of the phenothiazinium family, has been used to treat a variety of human conditions and has beneficial effects on the CNS of rodents. Paban et al. [534] studied whether chronic MB treatment taken after (therapeutic effect) or before (preventive effect) the onset of β-amyloid pathology influences cognition in a transgenic mouse model (APP/PS1) and found that oral or intraperitoneal MB injection protected mice

from cognitive impairments in a variety of social, learning, and exploratory tasks. Immunoreactive β-amyloid deposition was significantly reduced in the hippocampus and adjacent cortex in MB-treated transgenic mice. Methylene blue protects HT22 hippocampal cell death induced by serum deprivation, accompanied by induction of macroautophagy. 5′-Adenosine monophosphate-activated protein kinase (AMPK) signaling, but not inhibition of mammalian target of rapamycin signaling, is activated after methylene blue treatment in a dose-dependent manner. Methylene blue-induced neuroprotection is mediated by macroautophagy through activation of AMPK signaling [535]. Methylene blue can also attenuate superoxide production by functioning as an alternative mitochondrial electron transfer carrier and as a regenerable antioxidant in mitochondria [536]. This molecule has also been proposed as a potential therapy for Friedreich's ataxia [537].

6.55 Metalloporphyrins

Metalloporphyrins, characterized by a redox-active transitional metal coordinated to a cyclic porphyrin core ligand, mitigate oxidative/nitrosative stress in biological systems. Side-chain substitutions tune redox properties of metalloporphyrins to act as potent superoxide dismutase mimetics, peroxynitrite decomposition catalysts, and redox regulators of transcription factor function. Metalloporphyrins are efficacious in AD models [538].

6.56 Imatinib Methanesulfonate

Imatinib methanesulfonate salt (IM), known to interfere with the interaction between γ-secretase and the γ-secretase activating protein (GSAP), decreases the cleavage of peripheral APP into Aβ. Because IM poorly penetrates the BBB, co-administration of IM with LPS would decrease peripheral production of Aβ in the presence of LPS-induced inflammation, leading to a decrease in Aβ accumulation in the hippocampus. Peripheral IM treatment eliminates hippocampal Aβ elevation that follows LPS-induced peripheral inflammation [539].

6.57 Nicotinamide Adenine Dinucleotide

Nicotinamide adenine dinucleotide (NAD^+), a coenzyme involved in redox activities in the mitochondrial electron transport chain, is a key regulator of the lifespan-extending effects, and the activation of NAD^+ expression may decrease Aβ toxicity in AD. Nicotinamide riboside (NR), a NAD^+ precursor, promotes peroxisome proliferator-activated receptor-γ coactivator 1 (PGC)-1α expression in the brain. PGC-1α is a crucial regulator of Aβ generation by modulating β-secretase (BACE1) degradation. NR treatment in an AD mouse model could attenuate Aβ toxicity through the activation of PGC-1α-mediated BACE1 degradation. Dietary treatment with NR might benefit AD cognitive function and synaptic plasticity, in part by promoting PGC-1α-mediated

BACE1 ubiquitination and degradation, thus preventing Aβ production in the brain [540].

Using titration with buthionine sulfoximine, an inhibitor of γ-glutamyl cysteine synthetase (GCL), Ghosh et al. [541] observed that GSH depletion increased neuronal death of 3×Tg-AD cultured neurons at increasing rates across the age span, whereas non-Tg neurons were resistant to GSH depletion until old age. They targeted for neuroprotection activation of the redox-sensitive transcription factor, nuclear erythroid-related factor 2 (Nrf2) by 18 alpha glycyrrhetinic acid to stimulate GSH synthesis through GCL. This balanced stimulation of a number of redox enzymes restored the lower levels of Nrf2 and GCL seen in 3×Tg-AD neurons compared with those of non-Tg neurons and promoted translocation of Nrf2 to the nucleus. The combination of the Nrf2 activator with the NADH precursor, nicotinamide, increased neuron survival against amyloid β stress in an additive manner. According to these results, the dual neuroprotective treatment with nicotinamide and an Nrf2 inducer might indicate that these age-related and AD-related changes are reversible.

6.58 Calpain Inhibitors

E-64 is an epoxide-containing natural product identified as a potent nonselective, calpain inhibitor, with demonstrated efficacy in animal models of AD. Several E-64 derivatives have been developed [542]. Hyperactivation of the calcium-dependent cysteine protease calpain 1 (Cal1) is implicated as a primary or secondary pathological event in a wide range of illnesses and in neurodegenerative states, including AD.

6.59 Growth Hormone-Releasing Hormone (GHRH)

Growth hormone-releasing hormone (GHRH) displays neurotrophic activity and has cognition-enhancing effects [543, 544]. Friedman et al. [545] examined the neurochemical effects of GHRH in a subset of participants from the parent trial in a randomized, double-blind, placebo-controlled substudy of a larger trial. Participants self-administered daily subcutaneous injections of *tesamorelin*, a stabilized analog of human GHRH, or placebo 30 min before bedtime for 20 weeks. Brain levels of glutamate, inhibitory transmitters γ-aminobutyric acid (GABA) and N-acetylaspartylglutamate (NAAG), and myo-inositol (MI), an osmolyte linked to AD in humans, were measured in three $2\times2\times2\text{-cm}^3$ left-sided brain regions (dorsolateral frontal, posterior cingulate, and posterior parietal). After 20 weeks of GHRH administration, GABA levels were increased in all brain regions, NAAG levels were increased in the dorsolateral frontal cortex, and MI levels were decreased in the posterior cingulate. These effects were similar in adults with MCI and older adults with normal cognitive function. No changes in the brain levels of glutamate were

observed. In the posterior cingulate, treatment-related changes in serum insulin-like growth factor 1 were positively correlated with changes in GABA and tended to be negatively correlated with MI. Consistent with the results of the parent trial, a favorable treatment effect on cognition was observed in substudy participants.

6.60 Galanin

The neuropeptide galanin (GAL) and its receptors are overexpressed in degenerating brain regions in AD. The effects of Aβ, galanin, galanin receptor 1 agonist M617, and galanin receptor 2 agonist AR-M1896 on spatial memory were tested by Li et al. [546] in an AD rat model. Galanin administration was effective in improving the spatial memory and decreasing hippocampal Aβ levels after intracerebroventricular injection of Aβ. AR-M1896 rather than M617 mimics effects of galanin. GAL and GALR2 mRNA and protein levels increased significantly in hippocampus after Aβ administration, while GALR1 mRNA and protein levels did not change. GAL, AR-M1896, and M617 administration did not show significant effect on GAL, GalR1, and GalR2 mRNA and protein levels in hippocampus after Aβ administration. Galanin receptor 2, but not receptor 1, is involved in the protective effects against spatial memory impairment and hippocampal Aβ aggregation.

6.61 Somatostatin Receptor Subtype-4 Agonist NNC 26-9100

Somatostatin receptor subtype-4 (SSTR4) agonists have been proposed to reduce Aβ levels in the brain via enhancement of enzymatic degradation. Sandoval et al. [547] evaluated the effect of selective SSTR4 agonist NNC 26-9100 on the changes in learning and soluble $A\beta_{42}$ oligomer brain content with and without co-administration of the M13-metalloproteinase family enzyme-inhibitor phosphoramidon, using the senescence-accelerated mouse prone-8 (SAMP8) model. NNC 26-9100 treatment improved learning, which was blocked by phosphoramidon. NNC 26-9100 decreased total soluble $A\beta_{42}$, an effect which was blocked by phosphoramidon. NNC 26-9100 decreased the $A\beta_{42}$ trimeric (12 kDa) form within the extracellular and intracellular fractions, and produced a band-split effect of the $A\beta_{42}$ hexameric (25 kDa) form within the extracellular fraction. These effects were also blocked by phosphoramidon. Subsequent evaluation of NNC 26-9100 in APPswe Tg2576 transgenic mice showed a similar learning improvement and corresponding reduction in soluble $A\beta_{42}$ oligomers within extracellular, intracellular, and membrane fractions. NNC 26-9100 reduces soluble $A\beta_{42}$ oligomers and enhances learning through a phosphoramidon-sensitive metalloproteinase-dependent mechanism.

6.62 Melatonin and Melatonin Agonists

Melatonin is involved in modulating learning and memory processing, and also exerts neuroprotection against Aβ-induced injury [548]. *Neu-P11* (*piromelatine*, *N-(2-(5-methoxy-1H-indol-3-yl) ethyl)-4-oxo-4H-pyran-2-carboxamide*) is a novel melatonin (MT1/

MT2) receptor agonist and a serotonin 5-HT1A/1D receptor agonist recently developed for the treatment of insomnia. Neu-P11 enhanced object recognition memory and attenuated cellular loss and cognitive impairment in the rat AD model [549].

6.63 Calcineurin Inhibitors

Dendritic spine alteration is mediated by calcineurin activation, a calcium-dependent phosphatase involved in synapse signaling. Acute treatment of young and plaque-free transgenic mice with the calcineurin inhibitor *FK506* leads to a complete rescue of long-term depression and postsynaptic density composition [550].

6.64 Tyrosine Kinase Inhibitors (Nilotinib, Bosutinib)

Tyrosine kinase inhibitors (TKIs) are effective therapies for leukemia. AD animals have high levels of insoluble parkin and decreased parkin–beclin-1 interaction, while peripheral administration of TKIs, including nilotinib and bosutinib, increases soluble parkin leading to amyloid clearance and cognitive improvement. Blocking beclin-1 expression with shRNA or parkin deletion prevents tyrosine kinase (TK) inhibition-induced amyloid clearance, suggesting that functional parkin–beclin-1 interaction mediates amyloid degradation. Bosutinib and nilotinib increase parkin–beclin-1 interaction, resulting in protein deposition in the lysosome. Decreased parkin solubility impedes parkin–beclin-1 interaction and amyloid clearance [551].

6.65 p38-Alpha MAPK Inhibitors

p38α Mitogen-activated protein kinase (MAPK) has been implicated in senile plaque accumulation, NFT formation, tau phosphorylation, and inflammation. p38α MAPK pathway is activated by a dual phosphorylation at Thr180 and Tyr182 residues. Drug design of p38α MAPK inhibitors is mainly focused on small molecules that compete for adenosine triphosphate in the catalytic site. Pinsetta et al. [552] characterized 13 compounds that meet the criteria of potent inhibitors.

6.66 Antiepileptic Drugs

Valproic acid exerts protective effects in AD transgenic models, acting as a histone deacetylase inhibitor. *Topiramate* and *levetiracetam* also inhibit histone deacetylase activity in vivo. Topiramate and levetiracetam alleviate behavioral deficits and reduce amyloid plaques in APPswe/PS1dE9 transgenic mice, increase Aβ clearance and upregulate Aβ transport and autophagic degradation, inhibit Aβ generation and suppress γ-secretase activity, inhibit GSK-3β activation and increase AMPK/Akt activation [553].

6.67 Antiparkinsonian Agents

Neurotoxic oligomeric assemblies of aggregated Aβ and α-synuclein (αS) represents a pathogenic event in AD and Lewy body diseases (LBD). Ono et al. [554] studied the effects of antiparkinsonian agents (dopamine, levodopa, trihexyphenidyl, selegiline, zonisamide, bromocriptine, peroxide, ropinirole, pramipexole, and entacapone) on the in vitro oligomer formation of $A\beta_{40}$, $A\beta_{42}$, and αS.

The antiparkinsonian agents (except trihexyphenidyl) inhibited both Aβ and αS oligomer formations, with dopamine, levodopa, pramipexole, and entacapone displaying the strongest in vitro activity.

Rasagiline derivatives (Ladostigil and M30). Using the pharmacophore of the antiparkinsonian drug rasagiline (N-propagrgyl-1-R-aminoindan) a series of novel multifunctional neuroprotective drugs have been developed including (1) [TV-3326 (N-propargyl-3R-aminoindan-5yl)-ethyl methylcarbamate)], with both cholinesterase-butyrylesterase and brain selective monoamine-oxidase (MAO) A/B inhibitory activities and (2) the iron chelator-radical scavenging-brain selective monoamine oxidase (MAO) A/B inhibitor M30 possessing the neuroprotective and neurorescuing propargyl moiety of rasagiline. These series of drugs have the ability of regulating and processing APP since APP and alpha-synuclein are metaloproteins (iron-regulated proteins), with an iron responsive element 5′ UTR mRNA similar to transferring and ferritin. Ladostigil is a dual acetyl and butyrylcholinesterase inhibitor which also inhibits MAO-A and -B in the brain. The propargylamine moiety of ladostigil confers neuroprotective activity against cytotoxicity induced by ischemia and peroxynitrite in cultured neuronal cells. The multi-target iron chelator M30 has all the properties of ladostigil and similar neuroprotective activity to ladostigil, but is not a ChE inhibitor. According to Youdim [555], the neurorestorative activity is related to the ability of the drug to activate hypoxia-inducing factor (HIF) which induces the production of BDNF, VEGF, erythropoietin, and glia-derived neurotrophic factor (GDNF).

6.68 Adenosine A1 Antagonists (8-Cyclopentyl-1,3-dipropylxanthine)

The adrenergic A1 pathway might be involved in cognitive impairment in AD. Acute treatment with A1 antagonists appears to improve behavioral deficits in rodent models of memory and behavioral impairment. Vollert et al. [556] studied the effects of the chronic administration of 8-cyclopentyl-1,3-dipropylxanthine, a potent and selective adenosine A1 antagonist, on memory deficits found in aged APPswe/PS1dE9 mice and found that this compound did not improve memory in the APPswe/PS1dE9 mouse model, resulting in reduced exploratory behavior, suggestive of reduced anxiety, and a worsening of long-term memory in nontransgenic mice.

6.69 Glutaminyl Cyclase Inhibitors

Glutaminyl cyclase (hQC) represents a new potential target for the treatment of AD, since inhibition of hQC prevents the formation of Aβ3(pE)-40,42-species. Novel molecules containing benzimidazole as the metal binding group connected to 1,3,4-oxadiazole as the central scaffold were identified. Benzimidazolyl-1,3,4-thiadiazoles and -1,2,3-triazoles display inhibitory potency in the nano-molar range [557].

6.70 5-Lipoxygenase Inhibitors (Zileuton)

5-Lipoxygenase (5LO) is upregulated in AD, and plays an active role in the development of brain amyloidosis in the APP transgenic mice. APP transgenic mice treated with Zileuton, a specific 5LO inhibitor, showed memory improvement reduction of Aβ levels and deposition, secondary to a downregulation of the γ-secretase pathway. Zileuton-treated mice had a reduction in tau phosphorylation, secondary to a decreased activation of the cdk5 kinase [558].

6.71 Artemisinin

The antimalarial drug artemisinin extenuates amyloidogenesis and neuroinflammation in APPswe/PS1dE9 transgenic mice via inhibition of nuclear factor-κB and NLRP3 inflammasome activation [559].

6.72 Sodium Phenylbutyrate

Sodium phenylbutyrate (NaPB), a Food and Drug Administration-approved oral medication for hyperammonemia, induces astrocytic BDNF and NT-3 expression via the protein kinase C (PKC)-cAMP-response element-binding protein (CREB) pathway. NaPB treatment increased the direct association between PKC and CREB followed by phosphorylation of CREB (Ser^{133}) and induction of DNA binding and transcriptional activation of CREB. NaPB increased the levels of BDNF and NT-3 in the CNS and improved spatial learning and memory in a mouse model of AD [560].

6.73 E. coli Protein Toxin CNF1

Transgenic mice homozygous for human *ApoE4* gene show decreased levels of ATP, increased inflammatory cytokines level, and accumulation of β-amyloid in the brain. A single administration of the bacterial *E. coli* protein toxin CNF1 to aged *apoE4* mice induced a strong amelioration of both spatial and emotional memory deficits and favored the cell energy restore through an increment of ATP content, accompanied by a modulation of cerebral Rho and Rac1 activity. CNF1 decreased the levels of β-amyloid accumulation and interleukin-1β expression in the hippocampus [561].

6.74 Necrostatin-1

Qinli et al. [562] studied the preventive effect of necrostatin-1 (Nec-1) on neural cell death induced by aluminum (Al). Al-exposed primary cultures of newborn mouse cortical cells were separately treated with 3-methylamphetamine (3-MA), benzyloxycarbonylvalyl-alanyl-aspartic acid (O-methyl)-fluoromethylketone (zVAD-fmk), and Nec-1. The cell viabilities inhibited by Al were enhanced by 3-MA, zVAD-fmk, and Nec-1. Administration of Nec-1 on Al-treated mice significantly improved learning and memory retention in the Morris water maze task, decreased the neural cell death, and inhibited the expression of AD-related proteins in the mouse brain.

6.75 Plastoquinone Antioxidant SkQ1

Intraperitoneal injection of mitochondria-targeted plastoquinone derivative SkQ1 at very low concentrations (250 nmol/kg body weight) prevents the deleterious effect of Aβ on LTP. In vivo and in vitro injection of SkQ1 compensates for Aβ-induced oxidative damage of long-term synaptic plasticity in the hippocampus [563].

6.76 bis-Chloroethylnitrosourea (BCNU, Carmustine)

bis-Chloroethylnitrosourea (BCNU or carmustine) is an effective Aβ-reducing compound. BCNU decreased normalized levels of Aβ in CHO cells compared to a control group treated with butyl amine, a structural derivative of BCNU. Soluble amyloid precursor protein α (sAPPα) levels were increased to 167 % at 0.5 μM, 186 % at 1 μM, 204 % at 5 μM, and 152 % at 10 μM compared to untreated cells. BCNU reduced Aβ generation independent of secretases which were not altered. Levels of transforming growth factor beta (TGFβ) were increased. Cell culture results were confirmed in vivo after chronic administration of BCNU at 0.5 mg/kg which led to the reduction of $Aβ_{40}$ by 75 % and amyloid plaque burden by 81 %. Conversely, the levels of sAPPα were increased by 45 %, according to data reported by Hayes et al. [564].

6.77 Sunifiram

Sunifiram is a novel pyrrolidone nootropic drug structurally related to piracetam. Sunifiram is known to enhance cognitive function, N-methyl-D-aspartate receptor (NMDAR)-dependent synaptic function in the hippocampal CA1 region, and NMDAR-dependent long-term potentiation. The enhancement of LTP by sunifiram treatment was inhibited by 7-chloro-kynurenic acid (7-ClKN), an antagonist for glycine-binding site of NMDAR, but not by ifenprodil, an inhibitor for polyamine site of NMDAR. The enhancement of LTP by sunifiram was associated with an increase in phosphorylation of α-amino-3-hydroxy-5-methylisozazole-4-propionate receptor (AMPAR) through activation of calcium/calmodulin-dependent protein kinase II (CaMKII) and an increase in phosphorylation of NMDAR through activation of protein kinase Cα (PKCα). Sunifiram treatments at 1–1,000 nM increased the slope of field excitatory postsynaptic potentials (fEPSPs) in a dose-dependent manner. The enhancement was associated with an increase in phosphorylation of AMPAR receptor through activation of CaMKII. Sunifiram treatments increased PKCα (Ser-657) and Src family (Tyr-416) activities with the same bell-shaped dose–response curve as that of LTP peaking at 10 nM. The increase in phosphorylation of PKCα (Ser-657) and Src (Tyr-416) induced by sunifiram was inhibited by 7-ClKN treatment. The LTP enhancement by sunifiram was significantly inhibited by PP2, a Src family inhibitor. Sunifiram stimulates the glycine-binding site of NMDAR with concomitant PKCα activation through Src kinase. Enhancement of PKCα activity triggers to potentiate hippocampal LTP through CaMKII activation [565].

6.78 Hydrogen Sulfide

Fan et al. [566] studied the neuroprotective effects of hydrogen sulfide (H_2S) on neuroinflammation in rats with $Aβ_{1–40}$ hippocampal injection. NaHS (a donor of H_2S) treatment rescued neuronal cell death. H_2S suppressed the release of TNF-α, IL-1β, and IL-6 in the hippocampus. H_2S inhibited the upregulation of COX-2 and the activation of NF-κB in the hippocampus, indicating that H_2S suppresses neuroinflammation via inhibition of the NF-κB activation pathway in the Aβ-induced rat model.

6.79 N-Substituted Nipecotic Acid Derivatives

γ-Amino butyric acid (GABA) is the major inhibitory neurotransmitter in the CNS. It has been suggested that a possibility to enhance GABA levels in the synaptic cleft is to inhibit mGAT1, one of the four known plasma membrane-bound GABA transporters, responsible for the removal of GABA from the synaptic cleft after a neuronal impulse. Some lipophilic derivatives of nipecotic acid such as tiagabine, an antiepileptic drug, are known to inhibit the uptake of mGAT1. Quandt et al. [567] synthesized new N-substituted nipecotic acid derivatives with a vinyl ether spacer and an unsymmetrical bis-aromatic residue, which carries fluorine substituents at various positions of the aromatic ring system. These new compounds were characterized as mGAT1 inhibitors.

6.80 NOSH-Aspirin (NBS-1120)

Several SH-donors including H_2S-releasing aspirin (S-ASA) exhibited anti-inflammatory and neuroprotective activity against toxins released by activated microglia and astrocytes. McGeer's group has reported that NOSH-ASA, an NO- and H_2 S-releasing hybrid of aspirin, has a significantly greater anti-inflammatory and neuroprotective effect than S-ASA or NO-ASA [568].

6.81 Helicase-Primase Inhibitor BAY 57-1293

Herpes simplex virus type 1 (HSV1) infection of cultured cells causes the formation of Aβ and abnormal tau (P-tau). The formation of P-tau, but not of Aβ, depends on viral DNA replication, and three antiviral agents that inhibit HSV1 DNA replication, including acyclovir (ACV), were found to reduce greatly the level of Aβ as well as P-tau, the former probably through prevention of viral spread. HSV1 DNA is active in the brain of AD patients. It has been postulated that HSV1 DNA, in combination with APOE-4 might play a role in AD, via Aβ and P-tau production. Wozniak et al. [569] compared the efficacy of ACV with that of the antiviral BAY 57-1293 and found that BAY 57-1293 is more efficient than ACV in inhibiting HSV1 replication and in decreasing Aβ and P-tau formation.

6.82 Methyl 3,4-Dihydroxybenzoate

Methyl 3,4-dihydroxybenzoate (MDHB) is a phenolic acid compound, reported to have antioxidative and neurotrophic effects. Pretreatment of primary cortical neurons with MDHB suppressed $A\beta_{25-35}$-induced cellular toxicity and increased the level of Bcl-2, decreased the level of Bax, and inhibited the activation of caspase-9 and caspase-3 [570].

6.83 3-Hydroxybutyrate Methyl Ester

A degradation product of microbially synthesized polyhydroxybutyrate (PHB), 3-hydroxybutyrate (3HB), can be an alternative to glucose during sustained hypoglycemia. The 3HB derivative 3-hydroxybutyrate methyl ester (HBME) is used by cells as an alternative to glucose. HBME inhibited cell apoptosis under glucose deprivation, rescued activities of mitochondrial respiratory

chain complexes, and decreased the generation of ROS. Meanwhile, HBME stabilized the mitochondrial membrane potential. HBME improved the spatial learning and working memory of mice and reduced amyloid-β deposition in mouse brains [571].

6.84 Trientine

Triethylene tetramine dihydrochloride (trientine), a Cu^{II}-selective chelator, is a commonly used treatment for Wilson's disease to decrease accumulated copper, and thereby decreases oxidative stress. In APP/PS1 double transgenic AD mice trientine reduced the level of advanced glycation end products (AGEs), and decreased Aβ deposition and synapse loss. Trientine blocked the receptor for AGEs (RAGE), downregulated β-site APP cleaving enzyme 1 (BACE1), inhibited amyloidogenic APP cleavage, and subsequently reduced Aβ levels. Trientine might mitigate amyloidosis in AD by inhibiting the RAGE/NF-κB/BACE1 pathway [572].

6.85 Sigma-1 Receptor Antagonist NE-100

The selective sigma-1 receptor antagonist, N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride (NE-100), suppresses ischemia-induced neuronal cell death in the murine hippocampus. NE-100 protected the ER stress-induced cell death of murine hippocampal HT22 cells, but not the oxidative stress-induced cell death. Another sigma-1 receptor antagonist (BD1047) did not suppress ER stress-induced cell death. NE-100 attenuates the upregulation of C/EBP homologous protein (CHOP) induced by ER stress and upregulates the expression of both the 50-kDa activating transcription factor 6 (p50ATF6) and the 78-kDa glucose-regulated protein (GRP78). NE-100 does not affect the expression of phosphorylated eukaryotic initiation factor 2α (p-eIF2α) nor splicing of X-box-binding protein 1 (XBP-1), suggesting that NE-100 suppresses ER stress-induced cell death via CHOP expression by the upregulation of GRP78 through the ATF6 pathway [573].

6.86 AAD-2004 [2-Hydroxy-5-[2-(4-trifluoromethylphenyl)-ethylaminobenzoic Acid]

The compound AAD-2004 [2-hydroxy-5-[2-(4-trifluoromethylphenyl)-ethylaminobenzoic acid] has antioxidant and anti-inflammatory properties, reducing the accumulation of lipid peroxidation in the brain of Tg-betaCTF99/B6 mice, a murine AD model developed to display age-dependent neuronal loss and neuritic atrophy in the brain. AAD-2004 suppressed neuronal loss and neuritic atrophy, and partially reversed depleted expression of calbindin in the brain of Tg-beta-CTF99/B6 [574].

6.87 NOX1/4 Inhibitor GKT136901

NADPH oxidases (NOX), catalyzing the reduction of molecular oxygen to form the superoxide radical anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are involved in stroke, diabetes, atherosclerosis, and chronic neurodegenerative diseases. GKT136901 is a novel NOX-1/4 inhibitor with potential application in the areas of

diabetic nephropathy, stroke, or neurodegeneration. GKT136901 did not interact with nitric oxide ($NO^{\cdot}$), $O_2^{\cdot -}$, or hydroxyl radicals ($OH^{\cdot}$), but it acted as selective scavenger of peroxynitrite (PON). GKT136901 prevented tyrosine nitration and di-tyrosine-dependent dimer formation of α-synuclein by PON and was degraded when exposed to PON [575].

6.88 Glucosylceramide

Glucosylceramide shows memory-enhancing activity after 3-month treatment in aged mice (C56BL/6). Long-term treatment of glucosylceramide decreased the expression of iNOS and COX-2 in the brain of aged mice. The LPS-induced mRNA level of iNOS, COX-2, IL-1β, and TNF-α was reduced by the acute treatment with glucosylceramide in adult mice [576].

6.89 Erythropoietin (EPO)

Erythropoietin (EPO) promotes neurogenesis and neuroprotection. Maurice et al. [577] studied the effects of two formulations of EPO (rHu-EPO, and a low sialic form, Neuro-EPO) on $A\beta_{25-35}$ peptide toxicity. rHu-EPO and Neuro-EPO led to a significant prevention of $A\beta_{25-35}$-induced learning deficits. Both EPO formulations prevented the induction of lipid peroxidation in the hippocampus and the $A\beta_{25-35}$-induced increase in Bax level, TNFα, and IL-1β production and decrease in Akt activation.

6.90 Carnosine

Carnosine is an endogenous dipeptide present in the CNS, with multi-protective homeostatic functions, acting as an intracellular pH buffering molecule, Zn/Cu ion chelator, antioxidant, and anti-crosslinking agent. Carnosine is effective against $A\beta_{1-42}$ aggregation [578, 579].

6.91 Memoquin

Memoquin is a quinone-bearing polyamine compound which acts as an acetylcholinesterase and β-secretase-1 inhibitor, and also possesses antiamyloid and antioxidant properties [580].

6.92 Hydroxysafflor Yellow A

Homocystine may induce Aβ accumulation, synaptic dysfunction, and memory impairment. Lu et al. [581] reported that Hydroxysafflor Yellow A (HSYA) attenuates Aβ accumulation, improves synaptic function, and reverses homocysteine-induced cognitive impairment.

6.93 Recombinant Soluble Neprilysin

Neprilysin (NEP), a plasma membrane glycoprotein of the neutral zinc metalloendopeptidase family, is a major Aβ-degrading enzyme in the brain. Viral vectors have been used to express NEP for reduction of Aβ deposition. Park et al. [582] produced recombinant soluble NEP from insect cells using an NEP expression vector, which was administered by intracerebral injection into AD mice, resulting in significantly reduced accumulation of Aβ and improved behavioral performance.

6.94 Autophagy Inducers

Autophagy is a major intracellular degeneration pathway involved in the elimination and recycling of damaged organelles and long-lived proteins by lysosomes. Neurodegeneration-related pathogenic mechanisms may disturb autophagic activity, which is associated with misfolded protein aggregate accumulation. Maturation of autophago-lysosomes and their retrograde trafficking are perturbed in AD, causing a massive concentration of autophagy elements along degenerating neurites [583]. Autophagy operates in mammalian target of rapamycin (mTOR)-dependent pathway or mTOR-independent pathway to keep the neuronal homeostasis [584]. Autophagy has been regarded as a potential therapeutic target for AD. A novel autophagy inducer known as *GTM-1* has been identified. GTM-1 exhibits dual activities, such as autophagy induction and antagonism against Aβ-oligomer toxicity. GTM-1 modulates autophagy in an Akt-independent and mTOR-independent manner. GTM-1 enhances autophagy clearance and reverses the downregulation of autophagy flux by thapsigargin and asparagine. GTM-1 attenuates Aβ pathology and ameliorates cognitive deficits in AD mice [585].

A subpopulation of oligodendroglial precursor cells (NG2 cells) represent a new cell type that can clear β-amyloid peptides in the AD transgenic mice and in NG2 cell line. NG2 cells are recruited and clustered around the amyloid plaque in the APPswe/PS1dE9 mice. NG2 cell line and primary NG2 cells engulf β-amyloid peptides through the mechanisms of endocytosis. $A\beta_{42}$ internalization by NG2 cells is mediated by actin-dependent macropinocytosis. β-Amyloid peptides stimulate the autophagic pathway in NG2 cells. β-Amyloid peptides in NG2 cells are transported to lysosomes and degraded by autophagy [586].

6.95 CCL2/MCP1 Inhibitors (Bindarit)

CCL2, also known as monocyte chemotactic protein-1 (MCP-1), is associated with neuroinflammation. CCL2 levels are higher in the CSF of patients with AD. Bindarit, a CCL2 synthesis inhibitor, protects neurons against both $A\beta_{25-35}$ and $A\beta_{1-42}$-induced toxicity in primary mixed neural cultures. Bindarit reversed cell death induced by Aβ in a dose-dependent manner and reduced the transcription and release of CCL2 by astrocytes after Aβ treatment. Astroglial activation and CCL2 release were induced by ATP released by damaged neurons through interaction with P2X7 receptors present on astrocytes. Astroglial CCL2 probably interacts with neuronal CCR2 contributing to enhance the toxic activity of Aβ, and bindarit might be able to disconnect this neuroglial interaction [587].

6.96 Serrapeptase and Nattokinase

Serrapeptase (SP) and nattokinase (NK) are proteolytic enzymes of the serine protease family. It has been hypothesized that SP and NK might modulate certain factors that are associated with AD pathophysiology. Oral administration of SP or NK in a rat model of AD daily for 45 days resulted in a significant decrease in brain

AchE activity, TGF-β, Fas, and IL-6 levels. SP and NK also increased BDNF and IGF-1 levels and the expression levels of *ADAM9* and *ADAM10* genes in brain [588].

6.97 Microglial Modulators (CHF5074)

Ross et al. [589] evaluated the safety, tolerability, pharmacokinetics, and pharmacodynamics of CHF5074, a new microglial modulator, in a 12-week, double-blind, placebo-controlled, parallel groups, ascending dose study involving 96 MCI patients. No significant differences between treatment groups were found in neuropsychological tests but a positive dose–response trend was found on executive function in *APOE4* carriers. This study shows that CHF5074 is well tolerated in MCI patients after a 12-week titrated treatment up to 600 mg/day and dose-dependently affects CNS biomarkers of neuroinflammation.

6.98 Dihydrofuran-Fused Perhydrophenanthrenes

Sugimoto et al. [590] synthesized various dihydrofuran-fused perhydrophenanthrenes via o-quinodimethane chemistry for AD treatment.

6.99 Neurotrophic Factors

A range of neurotrophic factors and growth peptide factors derived from activity-dependent neurotrophic factor/activity-dependent neuroprotective protein has been suggested to restore neuronal function, improve behavioral deficits, and prolong the survival in animal models of AD and ALS [591]. Glial cell-derived neurotrophic factor (GDNF), NGF, and BDNF are important for the survival, maintenance, and regeneration of specific neuronal populations [592]. Disruption of fast axonal transport (FAT) is an early pathological event in AD. Soluble amyloid-β oligomers (AβOs) act as proximal neurotoxins in AD, impair organelle transport, and stimulate hyperphosphorylation of tau. AβOs reduce vesicular transport of BDNF in hippocampal neurons from both wild-type and tau knockout mice, indicating that tau is not required for transport disruption. Inhibition of calcineurin (CaN), a calcium-dependent phosphatase implicated in AD pathogenesis, rescues BDNF transport. Inhibition of protein phosphatase 1 (PP1) and glycogen synthase kinase 3β (GSK3β), downstream targets of CaN, prevents BDNF transport defects induced by AβOs [593]. In addition to conventional neurotrophic factors, such as NGF or BDNF, other trophic factors might become beneficial in AD. There is a substantial accumulation of $Aβ_{40}$ and $Aβ_{42}$ in the brain arterioles of AD patients and of transgenic mice. Purified $Aβ_{40}$ and $Aβ_{42}$ exhibited vascular regression activity and vessel density is reversely correlated with numbers and sizes of amyloid plaques. Vascular cells undergo cellular apoptosis in AD brains. *VEGF* prevents Aβ-induced endothelial apoptosis in vitro, and neuronal expression of VEGF in transgenic mice restores memory [594]. *GCSF* is an endogenous neuronal hematopoietic factor that displays robust in vitro and

in vivo neuroprotective activity. Prakash et al. [595] evaluated the effect of GCSF on Aβ-induced memory loss in an AD model of rats. Improvement in memory by GCSF was coupled with marked reduction of lipid peroxidation, acetylcholinesterase levels, and a significant increase in antioxidant enzymes as well as total RNA expression in the brain. GCSF also increased progenitor cells (iPSCs) and surface marker $CD34^+$ in the brain, inducing neurogenesis. The neurotrophic molecule J147 has the ability to rescue cognitive deficits in correlation with induction of the neurotrophic factors NGF and BDNF as well as several BDNF-responsive proteins which are important for learning and memory [596].

6.100 Neural Cell Adhesion Molecule-Derived Mimetic Peptide (FGL)

FG-Loop (FGL), a neural cell adhesion molecule-derived peptide that corresponds to its second fibronectin type III module, has been shown to provide neuroprotection against a range of cellular insults. FGL improves memory and alleviates the deleterious effects on CA1 pyramidal cells induced by $A\beta_{25-35}$ injection. These effects might be due to inactivation of GSK3β [597].

6.101 CopA3

The antibacterial peptide, CopA3 (a D-type disulfide dimer peptide, LLCIALRKK), inhibits LPS-induced macrophage activation and also has anticancer activity in leukemia cells. CopA3 increased cell proliferation by up to 31 ± 2 % in human neuroblastoma SH-SY5Y cells, and up to 29 ± 2 % in neural stem cells isolated from neonatal mouse brains. In both cell types, CopA3 also inhibited the apoptosis and viability losses caused by 6-hydroxy dopamine and okadaic acid. The p27Kip1 protein was markedly degraded in CopA3-treated SH-SY5Y cells. Conversely, an adenovirus expressing p27Kip1 significantly inhibited the antiapoptotic effects of CopA3 against 6-hydroxy dopamine- and okadaic acid-induced apoptosis, and decreased the neurotropic effects of CopA3. CopA3-mediated protein degradation of p27Kip1 may be the main mechanism through which CopA3 exerts neuroprotective and neurotropic effects [598].

6.102 Biomarine Derivatives

Different marine proteins, peptides, and lipoproteins exhibit neuroprotective effects [31, 63, 90–92, 599]. Thao et al. [600] described the inhibitory effect of crude extracts and steroids isolated from the starfish *Astropecten polyacanthus* on proinflammatory cytokines (IL-12 p40, IL-6), and TNF-α production in LPS-stimulated bone marrow-derived dendritic cells. *Neoechinulin A* is an indole alkaloid isolated from marine-derived *Microsporum* sp. which suppresses amyloid-β oligomer-induced microglia activation and protects neurons against inflammatory reactions [601]. Various lipoproteins extracted from marine species such as *Sardina Pilchardus*, *Trachurus trachurus*, *Scomber scombrus*, or *Conger conger* have demonstrated immunoprotective, anticancer, and antiatherosclerotic properties [31, 63, 90–92].

6.103 Icariin

Zhang et al. [602] investigated the effects of icariin (ICA) on the content of Aβ and the expression of neurotrophic factors in the brain of mitochondrial deficiency model rats. Chronic infusion of sodium azide by minipump induced a decrease in the activity of mitochondrial cytochrome C oxidase, an increase in the content of Aβ, and a marked decline in the expression of NGF, BDNF, and its receptor TrkB in the brain of rats. Intragastric administration of ICA ameliorated all these abnormalities in the model rats, increasing mitochondrial activity, inhibiting Aβ production, and enhancing the expression of neurotrophic factors in the brain of model rats induced by sodium azide.

6.104 Isoliquiritigenin Derivatives

A series of new isoliquiritigenin (ISL) derivatives were synthesized and evaluated as dual inhibitors for Aβ aggregation and 5-lipoxygenase (5-LO) [603].

6.105 Transferrin-Like Peptides

B6 peptide was discovered as a substitute for transferrin, and was conjugated with PEG-PLA nanoparticles (NP) with the aim of enhancing the delivery of neuroprotective drugs across the BBB for the treatment of AD. B6-modified NP (*B6-NP*) exhibited significantly higher accumulation in brain capillary endothelial cells via lipid raft-mediated and clathrin-mediated endocytosis. Administration of B6-NP encapsulated neuroprotective peptide—NAPVSIPQ (NAP)—to AD mouse models showed amelioration in learning impairments, cholinergic disruption, and loss of hippocampal neurons [604].

6.106 *β*-Arrestins Regulators

β-Arrestins represent a small family of G protein-coupled receptors (GPCRs) regulators, which provide modulating effects by facilitating desensitization and internalization of GPCRs. β-Arrestin levels correlate with Aβ pathology in AD brains. β-Arrestins might enhance the activity of γ-secretase via interacting with anterior pharynx-defective 1 subunit, which increases Aβ production and contributes to AD pathogenesis. Aβ-induced internalization of β_2-adrenergic receptor internalization and loss of dendritic spine in neurons were proven to be mediated by β-arrestins. Deletion of β-arrestins markedly attenuates AD pathology [605].

6.107 Combination Therapy

Combination therapy, integrating several drugs in a pharmacologic protocol, is the commonest strategy in the treatment of AD, since AD patients usually require medication for memory disorders, behavioral changes, and concomitant pathologies [12, 15, 19, 31, 35, 63, 86–92]. In the past, the most frequent combination was a nootropic with a neuroprotectant and/or a memory enhancer; at present, the combination of a cholinesterase inhibitor with memantine is becoming very common in some countries [606]. The combination of a cholinesterase inhibitor (ChEI) and memantine has been shown to yield positive results [607] and significantly delay admission

to nursing homes as compared to treatment with a ChEI alone [608]. In France, overall costs are 98,609€ for ChEIs alone and 90,268€ for combination therapy, representing cost savings of 8,341€ for the Health Care System. According to Touchon et al. [608] combination therapy with memantine and a ChEI is a cost-saving alternative compared to ChEI alone as it is associated with lower cost and increased QALYs from both a societal and a healthcare perspective.

6.108 Antisense Oligonucleotides

Antisense oligonucleotides can act through numerous mechanisms to decrease or increase total protein levels, preferentially shift splicing patterns, and inhibit miRNA, all at the level of the RNA molecule. In neurodegenerative mouse models, antisense oligonucleotides specifically target the detrimental transgenes to rescue disease-associated phenotypes in vivo [609]. Reduction of Aβ, using an antisense oligonucleotide (AO) directed against the Aβ region of APP, reduced oxidative stress-mediated damage and prevented or reverted cognitive deficits in senescence-accelerated prone mice (SAMP8). Aged SAMP8 treated by AO directed against PS-1, a component of the γ-secretase complex, showed improvement in learning and memory deficits and reduction in Aβ-mediated oxidative stress [610].

6.109 Gene Therapy

Both genetic inactivation and pharmacological inhibition of the cholesteryl ester synthetic enzyme acyl-CoA:cholesterol acyltransferase 1 (ACAT1) have shown benefit in mouse models of AD. Murphy et al. [611] tested the potential therapeutic applications of adeno-associated virus (AAV)-mediated Acat1 gene knockdown in AD mice. Acat1-targeting AAV delivered to the brains of AD mice decreased the levels of brain amyloid-β and full-length human amyloid precursor protein (hAPP), to levels similar to complete genetic ablation of Acat1. Expression of long-lasting synaptic plasticity and long-term memory requires protein synthesis, which can be repressed by phosphorylation of eukaryotic initiation factor 2 α-subunit (eIF2α) [612]. Elevated phosphorylation of eIF2α is present in the brains of AD patients and transgenic mice. Ma et al. [612] tested whether suppressing eIF2α kinases could alleviate synaptic plasticity and memory deficits in AD model mice. Genetic deletion of eIF2α kinase PERK prevented enhanced phosphorylation of eIF2α and deficits in protein synthesis, synaptic plasticity, and spatial memory in APP/PSEN1 mutant mice. Deletion of another eIF2α kinase, GCN2, prevented impairments of synaptic plasticity and defects in spatial memory. PERK and GCN2 might be potential therapeutic targets for AD.

6.110 Nontoxic Conformers of Amyloid β

The 42-mer amyloid β-protein ($A\beta_{42}$) oligomers cause neurotoxicity and cognitive impairment in AD. Izuo et al. [613] identified the toxic conformer of $A\beta_{42}$ with a turn at positions 22–23 ("toxic" turn) to form oligomers and to induce toxicity in rat primary neurons, along with the nontoxic conformer with a turn at positions

25–26. G25P-Aβ_{42} and E22V-Aβ_{42} are nontoxic mutants that disfavor the "toxic" turn. G25P-Aβ_{42} and E22V-Aβ_{42} suppressed the neurotoxicity and aggregation of Aβ_{42} as well as the formation of the toxic conformer. The nontoxic mutants of Aβ_{42} without the "toxic" turn could prevent the propagation process of the toxic conformer of Aβ_{42} resulting in suppression of the formation of the toxic oligomers.

6.111 microRNAs (miRNAs) and Gene Silencing

miRNAs exert regulatory control over mRNA stability and translation and may contribute to local and activity-dependent posttranscriptional control of synapse-associated mRNAs. miRNAs are small noncoding RNA regulators of protein synthesis that are essential for normal brain development and function. Their profiles are significantly altered in AD. miR-9 and -181c are downregulated by Aβ in hippocampal cultures. The Aβ precursor protein APP itself is a target of miRNA regulation. The 3′ untranslated regions (3′ UTRs) of TGFBI, TRIM2, SIRT1, and BTBD3 are repressed by miR-9 and -181c, either alone or in combination [19]. miRNA are integral components of the APP regulatory framework and potential targets for future AD therapeutics. Cohen et al. [614] found a developmentally and activity-regulated miRNA (miR-485) that controls dendritic spine number and synapse formation in an activity-dependent homeostatic manner. Many plasticity-associated genes contain predicted miR-485 binding sites. The presynaptic protein SV2A is a target of miR-485. miR-485 negatively regulates dendritic spine density, postsynaptic density 95 (PSD-95) clustering, and surface expression of GluR2. miR-485 overexpression reduced spontaneous synaptic responses and transmitter release. miRNA-485 and the presynaptic protein SV2A regulate homeostatic plasticity and CNS development, and their dysfunction might have possible implications in AD.

RNA interference (RNAi) technology may potentially be able to control AD, inhibiting the protein expression of specific genes by activating a sequence-specific RNA degradation process [615]. Deficits of protein phosphatase-2A (PP2A) play a role in tau hyperphosphorylation, amyloid overproduction, and synaptic loss in AD. PP2A is inactivated by the inhibitor-2 of PP2A (I_2^{PP2A}). It has been postulated that in vivo silencing I_2^{PP2A} may rescue PP2A and mitigate AD-related neurodegeneration. By infusion of lentivirus-shRNA targeting I_2^{PP2A} (LV-siI_2^{PP2A}) into hippocampus and frontal cortex of 11-month-old Tg2576 mice, Liu et al. [616] showed that expression of LV-siI_2^{PP2A} decreased the elevated I_2^{PP2A} in both mRNA and protein levels. Silencing I_2^{PP2A} induced a long-lasting attenuation of amyloidogenesis in Tg2576 mice with inhibition of APP hyperphosphorylation and β-secretase activity. Inhibition of PP2A abolished the antiamyloidogenic effects of I_2^{PP2A} silencing. Silencing I_2^{PP2A} also improved learning and memory of Tg2576 mice.

miR-26a plays a role in the CNS. The overexpression of miR-26a was hypothesized to significantly enhance synaptic plasticity

and regulate neuronal morphogenesis. The number and distribution of neurites was markedly increased by miR-26a. Inhibition of miR-26a function attenuated neuronal outgrowth. Phosphatase and tensin homolog (PTEN) was identified as a direct target of miR-26a in this process. The growth of neurites was consistently suppressed by PTEN overexpression. miR-26a promoted neurite outgrowth via the suppression of PTEN expression, indicating that miR-26a is important in neuronal development and morphogenesis [617].

An adenovirus vector expressing small interfering RNA (siRNA) against the sphK1 gene (sphK1-siRNA) was designed by Zhang et al. [618], and the effects of sphK1-siRNA on the APP/PS1 mouse four weeks after treatment with sphK1-siRNA hippocampal injection were examined. Aβ load in transfected mice was accelerated in vivo, with significant aggravation of the learning and memory ability.

The P2X7 receptor (P2X7R) is an ATP-gated cation channel that promotes microglia activation. Inhibiting P2X7R indirectly reduces the rate of Aβ-induced neurodegeneration by suppressing secretion of inflammatory factors from activated microglia. The application of RNA interference to silence P2X7R in microglial cells in vitro increased microglial phagocytosis of $A\beta_{1-42}$. Increased phagocytic activity was dependent on decreasing the rate of interleukin-1β release from microglia and required inhibition of the COX-2 pathway [619].

Excellent results have been reported with RNAi therapy in transthyretin amyloidosis [620]. Transthyretin amyloidosis is produced by deposition of hepatocyte-derived transthyretin amyloid in peripheral nerves and the cardiovascular system. Coelho et al. [620] identified a potent antitransthyretin small interfering RNA which was encapsulated in lipid nanoparticles, generating ALN-TTR01 and ALN-TTR02. ALN-TTR01 suppressed transthyretin levels by 38 % at day 7, and ALN-TTR02 suppressed transthyretin levels by 56.6–67.1 % at 28 days. These effects were shown to be RNAi-mediated.

6.112 Epigenetic Drugs

There is increasing evidence suggesting that epigenetic changes in gene expression underlie neurodegeneration and CNS disorders [621]. Epigenetic drugs (histone deacetylase (HDAC) inhibitors, and drugs targeting DNA methylation) reverse epigenetic changes in gene expression and may open future avenues in AD therapeutics [622, 623].

6.113 Stem Cell Therapy

Cell therapy is a potential therapeutic approach for AD. Tricyclodecan-9-yl-xanthogenate (D609) was used to induce human mesenchymal stem cells isolated from Wharton jelly of the umbilical cord (HUMSCs) to differentiate into neuron-like cells

(HUMSC-NCs). Transplantation of HUMSC-NCs into AβPP/PS1 mice improved the cognitive function, increased synapsin I level, and significantly reduced Aβ deposition. The beneficial effects were associated with "alternatively activated" microglia (M2-like microglia). In the mice transplanted with HUMSC-NCs, M2-like microglial activation was significantly increased, and the expression of anti-inflammatory cytokine associated with M2-like microglia, interleukin-4 (IL-4), was also increased, whereas the expression of proinflammatory cytokines associated with classic microglia (M1-like microglia), including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), was significantly reduced. The expression of Aβ-degrading factors, insulin-degrading enzyme (IDE) and neprilysin (NEP), was increased substantially in the mice treated with HUMSC-NCs [624].

Zidon et al. [625] examined the effects of intra-hippocampal transplantation of neural precursor cells (NPCs) with transgenic overexpression of IL-1 receptor antagonist (IL-1raTG) on memory functioning and neurogenesis in a murine model of AD (Tg2576 mice). 12-Month-old Tg2576 mice exhibited increased mRNA expression of hippocampal IL-1β, along with severe disturbances in hippocampal-dependent contextual and spatial memory as well as in neurogenesis. Transplantation of IL-1raTG NPCs 1 month before the neurobehavioral testing completely rescued these disturbances and increased the number of endogenous hippocampal cells expressing the plasticity-related molecule BDNF.

Kim et al. [626] examined human placenta amniotic membrane-derived mesenchymal stem cells (AMSCs), which have potent immunomodulatory and paracrine effects in a Tg2576 (APPswe) transgenic mouse model of AD. AMSCs secreted high levels of transforming growth factor-β under in vitro inflammatory environment conditions. APPswe mice showed evidence of improved spatial learning, which significantly correlated with the observation of fewer Aβ plaques in brain. The number of ED1-positive phagocytic microglial cells associated with Aβ plaques was higher in AMSC-injected mice than in phosphate-buffered saline-injected mice, and the level of Aβ-degrading enzymes (matrix metallopeptidase-9 and insulin-degrading enzyme) was also significantly higher. The level of proinflammatory cytokines, interleukin-1 and tumor necrosis factor-α, was lower and that of anti-inflammatory cytokines, interleukin-10 and transforming growth factor-β, was higher in AMSC-injected mice.

6.114 Miscellaneous Strategies

Many other experimental strategies are under development in the global fight against AD. It is impossible to predict which of them will provide in the future some benefit to patients with AD. It is very likely that the vast majority of successful studies at the preclinical level will become a scientific frustration when they reach the stage of formal clinical trials. In addition to the proposals postulated in Subheading 6.1–6.113, many other candidate substances and

therapeutic procedures enrich the pipeline of potential AD therapeutics (with important pharmacogenetic repercussions) including (*see* Table 2): Sodium fullerenolate [627], Transglutaminase inhibitors [628], AL-108 [629], Metallothioneinsin [630], Phenyl hydrazide J147 [631], Sirtuins [632], c-Jun N-terminal kinase (JNK) inhibitors [633, 634], MLC601 [635], Tetrahydrohyperforin (IDN5706), a semi-synthetic derivative of hyperforin [636, 637], Substituted 2-indolyl carbohydrazides (JL34, JL40, JL71, JL87, JL317, JL432, JL436), Substituted 3-indolyl carbohydrazide JL344, 3-(3-hydrazinylpropyl)-1H-indole (JL72), and 3-(1H-indol-3-yl) propanehydrazide (JL418) [638], Tart cherry extract and essential fatty acids [639], Zeolite [640], p,p'-methoxyl-diphenyl diselenide [641], Sulfiredoxin-1 [642], 2-Phenylethynyl-butyltellurium [643], Fucoxanthin [644], Edaravone [645, 646], p75 neurotrophin receptor (p75(NTR) ligands (LM11A-31) [647], [Gly14]-humanin [648], Oligonol (oligomerized lychee fruit-derived polyphenol) [649], Cyclodextrin-solubilized curcuminoids [650], Serine palmitoyltransferase inhibitors [651], Novel β-sheet breakers (iAβ6) [652], CB2 cannabinoid receptor agonists (JWH-133) [653], Arundic acid [654], PN-1 [655], Cerebrolysin [656, 657], Neurosteroids [658], Insulin-degrading enzyme (IDE) inhibitors [659], Levetiracetam [660], Citidine-5-diphosphocholine or citicoline (CDP-choline) [12, 15, 31, 63, 86–92, 661], Palmitoylethanolamide [662], Phenserine [663], Immunoglobulin [664], P-glycoprotein stimulators (benzopyrane derivatives) [665], Bis(propyl)-cognitin (an uncompetitive N-methyl-d-aspartate receptor antagonist, similar to memantine) [666], CPPHA (N-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl)-2-hydroxybenzamide) and/or NCFP (N-(4-chloro-2-((4-fluoro-1,3-dioxoisoindolin-2-yl) methyl)phenyl)picolinamide) related compounds (novel metabotropic glutamate receptor 5 positive allosteric modulators) [667], Capsaicin (a specific TRPV1 agonist) [668], Indoleamine 2,3-dioxygenase (IDO) inhibitors (tryptoline derivatives) [669], DYRK kinase inhibitors (indirubins) [670], Proteasome regulators (lithocholic acid derivatives: 3α-O-pimeloyl-lithocholic acid methyl ester and its isosteric isomer) [671], Caffeic acid and caffeic acid phenethyl ester [672], the glucocorticoid receptor antagonist mifepristone (RU486) [673], Vitamin K_3 analogs [674], the novel pyrrolidone nootropic drug Sunifiram [675], the anti-inflammatory and antiamyloidogenic small molecule, 2,4-bis(p-hydroxyphenyl)-2-butenal(HPB242) [676], the iron chelator Desferrioxamine (DFO) [677], Mibampator (LY451395) (an amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor potentiator, for agitation/aggression in AD, with negative results in preliminary clinical trials) [678], and S-Nitrosoglutathione [679], among many others.

7 Pharmacogenomics

AD patients may take 6–12 different drugs/day for the treatment of dementia-related symptoms, including memory decline (conventional anti-dementia drugs, neuroprotectants), behavioral changes (antidepressants, neuroleptics, sedatives, hypnotics), and functional decline, or for the treatment of concomitant pathologies (epilepsy, cardiovascular and cerebrovascular disorders, parkinsonism, hypertension, dyslipidemia, anemia, arthrosis, etc.). Over 20 % of dementia patients are current users of cardiovascular drugs. A high-throughput screening study assessed 1,600 FDA-approved drugs for their ability to modulate Aβ activity; 559 drugs of the 1,600 had no effect on APP processing or were toxic to neurons at the testing concentration, while 800 drugs could reduce Aβ content over 10 % in primary neurons derived from Tg2576 mice, among which 184 drugs were able to reduce Aβ content greater than 30 %; 241 drugs could potentially promote Aβ accumulation including 26 drugs that could increase the level of Aβ greater than 30 % [680]. The co-administration of several drugs may cause side effects and adverse drug reactions in over 60 % of AD patients, who in 2–10 % of the cases require hospitalization. Montastruc et al. [681] assessed the prevalence of potentially inappropriate medication (PIM) in French patients with mild-to-moderate AD. 46.8 % of the patients had at least one PIM. "Cerebral vasodilators" were the most widely used class of PIM, accounting for 24.0 % of all prescriptions, followed by atropinic drugs and long half-life benzodiazepines. Atropinic drugs were associated with cholinesterase inhibitors in 16 % of patients. In over 20 % of the patients, behavioral deterioration and psychomotor function can be severely altered by polypharmacy. The principal causes of these iatrogenic effects are (1) the inappropriate combination of drugs, and (2) the genomic background of the patient, responsible for his/her pharmacogenomic outcome.

Pharmacogenomics account for 30–90 % variability in pharmacokinetics and pharmacodynamics. The genes involved in the pharmacogenomic response to drugs in AD fall into five major categories: (1) genes associated with AD pathogenesis and neurodegeneration (*APP*, *PSEN1*, *PSEN2*, *MAPT*, *PRNP*, *APOE*, and others); (2) genes associated with the mechanism of action of drugs (enzymes, receptors, transmitters, messengers); (3) genes associated with drug metabolism (phase I (*CYPs*) and phase II reactions (*UGTs*, *NATs*)); (4) genes associated with drug transporters (*ABCs*, *SLCs*); and (5) pleiotropic genes involved in multifaceted cascades and metabolic reactions (*APOs*, *ILs*, *MTHFR*, *ACE*, *AGT*, *NOS*, etc.) [16, 19].

7.1 Pathogenic Genes

In over 100 clinical trials for dementia, *APOE* has been used as the only gene of reference for the pharmacogenomics of AD [7, 12, 15, 17, 19, 28, 35, 63, 86–92]. Several studies indicate that the presence of the *APOE-4* allele differentially affects the quality and extent of drug responsiveness in AD patients treated with cholinergic enhancers (tacrine, donepezil, galantamine, rivastigmine), neuroprotective compounds (nootropics), endogenous nucleotides (CDP-choline), immunotrophins (anapsos), neurotrophic factors (cerebrolysin), rosiglitazone, or combination therapies [682–684]; however, controversial results are frequently found due to methodological problems, study design, and patient recruitment in clinical trials. The major conclusion in most studies is that *APOE-4* carriers are the worst responders to conventional treatments. When *APOE* and *CYP2D6* genotypes are integrated in bigenic clusters and the *APOE+CYP2D6*-related therapeutic response to a combination therapy is analyzed in AD patients, it becomes clear that the presence of the *APOE-4/4* genotype is able to convert pure *CYP2D6*1/*1* extensive metabolizers into full poor responders to conventional treatments, indicating the existence of a powerful influence of the *APOE-4* homozygous genotype on the drug-metabolizing capacity of pure *CYP2D6* extensive metabolizers. In addition, a clear accumulation of *APOE-4/4* genotypes is observed among *CYP2D6* poor and ultra-rapid metabolizers [12].

Different *APP* and *PSEN1* and *PSEN2* mutations may also modify the therapeutic response to drugs acting on the amyloid cascade [685].

7.2 Genes Involved in the Mechanism of Action of CNS Drugs

Most genes associated with the mechanism of action of CNS drugs encode receptors, enzymes, and neurotransmitters on which psychotropic drugs act as ligands (agonists, antagonists), enzyme modulators (substrates, inhibitors, inducers), or neurotransmitter regulators (releasers, reuptake inhibitors) [16]. In the case of conventional anti-dementia drugs, tacrine, donepezil, rivastigmine, and galantamine are cholinesterase inhibitors; and memantine is a partial NMDA antagonist (*see* Table 3).

7.3 Genes Involved in Drug Metabolism

Drug metabolism includes phase I reactions (i.e., oxidation, reduction, hydrolysis) and phase II conjugation reactions (i.e., acetylation, glucuronidation, sulphation, methylation) (*see* Table 4).

The principal enzymes with polymorphic variants involved in phase I reactions are the following: Cytochrome P450 monooxygenases (CYP3A4/5/7, CYP2E1, CYP2D6, CYP2C19, CYP2C9, CYP2C8, CYP2B6, CYP2A6, CYP1B1, CYP1A1/2), epoxide hydrolase, esterases, NQO1 (NADPH-quinone oxidoreductase), DPD (dihydropyrimidine dehydrogenase), ADH (alcohol dehydrogenase), and ALDH (aldehyde dehydrogenase); and major enzymes involved in phase II reactions include UGTs (uridine 5′-triphosphate glucuronosyl transferases), TPMT (thiopurine

Table 3
Pharmacogenomics of conventional drugs for the treatment of Alzheimer's disease

Donepezil	
Category	Anti-dementia agent; cholinesterase inhibitor
Mechanism	Centrally active, reversible acetylcholinesterase inhibitor; increases the acetylcholine available for synaptic transmission in the CNS
Genes Pathogenic	APOE, CHAT
Mechanistic	CHAT, ACHE, BCHE
Metabolism	
Substrate	CYP2D6 (major), CYP3A4 (major), UGTs ACHE
Inhibitor	ACHE, BCHE
Transporter	ABCB1
Galantamine	
Category	Anti-dementia agent; cholinesterase inhibitor
Mechanism	Reversible and competitive acetylcholinesterase inhibition leading to an increased concentration of acetylcholine at cholinergic synapses; modulates nicotinic acetylcholine receptor; may increase glutamate and serotonin levels
Genes Pathogenic	APOE, APP
Mechanistic	ACHE, BCHE, CHRNA4, CHRNA7, CHRNB2
Metabolism	
Substrate	CYP2D6 (major), CYP3A4 (major), UGT1A1
Inhibitor	ACHE, BCHE
Memantine	
Category	Anti-dementia drug; N-methyl-D-aspartate receptor antagonist
Mechanism	Binds preferentially to NMDA receptor-operated cation channels; may act by blocking actions of glutamate, mediated in part by NMDA receptors; antagonist: GRIN2A, GRIN2B, GRIN3A, HTR3A, CHRFAM7A
Genes Pathogenic	APOE, PSEN1, MAPT
Mechanistic	GRIN2A, GRIN2B, GRIN3A, HTR3A, CHRFAM7A, FOS, HOMER1, DLGAP1
Metabolism	
Inhibitor	CYP1A2 (weak), CYP2A6 (weak), CYP2B6 (strong), CYP2C9 (weak), CYP2C19 (weak), CYP2D6 (strong), CYP2E1 (weak), CYP3A4 (weak), NR1I2
Transporter	NR1I2 rs1523130 CT/TT
Pleiotropic	APOE, MAPT, MT-TK, PSEN1
Rivastigmine	
Category	Anti-dementia agent; cholinesterase inhibitor
Mechanism	Increases acetylcholine in CNS through reversible inhibition of its hydrolysis by cholinesterase
Genes Pathogenic	APOE, APP, CHAT
Mechanistic	ACHE, BCHE, CHAT, CHRNA4, CHRNB2
Metabolism	
Inhibitor	ACHE, BCHE
Pleiotropic	APOE, MAPT

(continued)

Table 3 (continued)

Tacrine	
Category	Anti-dementia agent; cholinesterase inhibitor
Mechanism	Elevates acetylcholine in cerebral cortex by slowing degradation of acetylcholine
Genes Pathogenic	APOE
Mechanistic	ACHE, BCHE, CHRNA4, CHRNB2
Metabolism	
Substrate	CYP1A2 (major), CYP2D6 (minor), CYP3A4 (major)
Inhibitor	ACHE, BCHE, CYP1A2 (weak)
Transporter	SCN1A
Pleiotropic	APOE, MTHFR, CES1, LEPR, GSTM1, GSTT1

Source: R. Cacabelos (Ed.). World Guide for Drug Use and Pharmacogenomics (ref. 687)

ADH1A: Alcohol dehydrogenase 1A (class I), alpha polypeptide; *AADAC*: Arylacetamide deacetylase; *AANAT*: aralkylamine N-acetyltransferase; *ACSL1*: Acyl-CoA synthetase long-chain family member 1; *ACSL3*: Acyl-CoA synthetase long-chain family member 3; *ACSL4*: Acyl-CoA synthetase long-chain family member 4; *ACSM1*: Acyl-CoA synthetase medium-chain family member 1; *ACSM2B*: Acyl-CoA synthetase medium-chain family member 2B; *ACSM3*: Acyl-CoA synthetase medium-chain family, member 3; *ADH1B*: Alcohol dehydrogenase 1B (class I), beta polypeptide; *ADH1C*: Alcohol dehydrogenase 1C (class I), gamma polypeptide; *ADH4*: Alcohol dehydrogenase 4 (class II), pi polypeptide; *ADH5*: Alcohol dehydrogenase 5 (class III), chi polypeptide; *ADH6*: Alcohol dehydrogenase 6 (class V); *ADH7*: Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide; *ADHFE1*: Alcohol dehydrogenase, iron containing, 1; *AGXT*: Alanine-glyoxylate aminotransferase; *AKR1A1*: Aldo-keto reductase family 1, member A1 (aldehyde reductase); *AKR1B1*: Aldo-keto reductase family 1, member B1 (aldose reductase); *AKR1C1*: Aldo-keto reductase family 1, member C1; *AKR1D1*: Aldo-keto reductase family 1, member D1; *ALDH1A1*: Aldehyde dehydrogenase 1 family, member A1; *ALDH1A2*: Aldehyde dehydrogenase family 1, subfamily A2; *ALDH1A3*: Aldehyde dehydrogenase family 1, subfamily A3; *ALDH1B1*: Aldehyde dehydrogenase 1 family, member B1; *ALDH2*: Aldehyde dehydrogenase 2 family (mitochondrial); *ALDH3A1*: Aldehyde dehydrogenase 3 family, member A1; *ALDH3A2*: Aldehyde dehydrogenase 3 family, member A2; *ALDH3B1*: Aldehyde dehydrogenase 3 family, member B1; *ALDH3B2*: Aldehyde dehydrogenase 3 family, member B2; *ALDH4A1*: Aldehyde dehydrogenase 4 family, member A1; *ALDH5A1*: Aldehyde dehydrogenase 5 family, member A1; *ALDH6A1*: Aldehyde dehydrogenase 6 family, member A1; *ALDH7A1*: Aldehyde dehydrogenase 7 family, member A1; *ALDH8A1*: Aldehyde dehydrogenase 8 family, member A1; *ALDH9A1*: Aldehyde dehydrogenase 9 family, member A1; *AOX1*: Aldehyde oxidase 1; *AS3MT*: Arsenic (+3 oxidation state) methyltransferase; *ASMT*: Acetylserotonin O-methyltransferase; *BAAT*: Bile acid CoA: amino acid N-acyltransferase (glycine N-choloyltransferase); *CBR1*: Carbonyl reductase 1; *CBR3*: Carbonyl reductase 3; *CBR4*: Carbonyl reductase 4; *CCBL1*: Cysteine conjugate-beta lyase, cytoplasmic; *CDA*: Cytidine deaminase; *CEL*: Carboxyl ester lipase; *CES1*: Carboxylesterase 1; *CES1P1*: Carboxylesterase 1 pseudogene 1; *CES2*: Carboxylesterase 2; *CES3*: Carboxylesterase 3; *CES5A*: Carboxylesterase 5A; *CHST1*: Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1; *CHST2*: Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2; *CHST3*: Carbohydrate (chondroitin 6) sulfotransferase 3; *CHST4*: Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 4; *CHST5*: Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5; *CHST6*: Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6; *CHST7*: Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7; *CHST8*: Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8; *CHST9*: Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9; *CHST10*: Carbohydrate sulfotransferase 10; *CHST11*: Carbohydrate (chondroitin 4) sulfotransferase 11; *CHST12*: Carbohydrate (chondroitin 4) sulfotransferase 12; *CHST13*: Carbohydrate (chondroitin 4) sulfotransferase 13; *COMT*: Catechol-O-methyltransferase; *CYB5R3*: Cytochrome b5 reductase 3; *CYP1A1*: Cytochrome P450, family 1, subfamily A, polypeptide 1; *CYP1A2*: Cytochrome P450, family 1, subfamily A, polypeptide 2; *CYP1B1*: Cytochrome P450, family 1, subfamily B, polypeptide 1; *CYP2A6*: Cytochrome P450, family 2, subfamily A, polypeptide 6; *CYP2A7*: Cytochrome P450, family 2, subfamily A, polypeptide 7; *CYP2A13*: Cytochrome P450, family 2, subfamily A, polypeptide 13; *CYP2B6*: Cytochrome P450, family 2, subfamily B, polypeptide 6; *CYP2C8*: Cytochrome P450, family 2, subfamily C, polypeptide 8; *CYP2C9*: Cytochrome P450, family 2, subfamily C, polypeptide 9; *CYP2C18*: Cytochrome P450, family 2, subfamily C, polypeptide 18; *CYP2C19*: Cytochrome P450, family 2, subfamily C, polypeptide 19; *CYP2D6*: Cytochrome P450, family 2, subfamily D, polypeptide 6; *CYP2D7P1*: Cytochrome P450, family 2, subfamily D, polypeptide 7 pseudogene 1; *CYP2E1*:

(continued)

Table 3 (continued)

Cytochrome P450, family 2, subfamily E, polypeptide 1; *CYP2F1*: Cytochrome P450, family 2, subfamily F, polypeptide 1; *CYP2J2*: Cytochrome P450, family 2, subfamily J, polypeptide 2; *CYP2R1*: Cytochrome P450, family 2, subfamily R, polypeptide 1; *CYP2S1*: Cytochrome P450, family 2, subfamily S, polypeptide 1; *CYP2W1*: Cytochrome P450, family 2, subfamily W, polypeptide 1; *CYP3A4*: Cytochrome P450, family 3, subfamily A, polypeptide 4; *CYP3A5*: Cytochrome P450, family 3, subfamily A, polypeptide 5; *CYP3A7*: Cytochrome P450, family 3, subfamily A, polypeptide 7; *CYP3A43*: Cytochrome P450, family 3, subfamily A, polypeptide 43; *CYP4A11*: Cytochrome P450, family 4, subfamily A, polypeptide 11; *CYP4A22*: Cytochrome P450, family 4, subfamily A, polypeptide 22; *CYP4B1*: Cytochrome P450, family 4, subfamily B, polypeptide 1; *CYP4F2*: Cytochrome P450, family 4, subfamily F, polypeptide 2; *CYP4F3*: Cytochrome P450, family 4, subfamily F, polypeptide 3; *CYP4F8*: Cytochrome P450, family 4, subfamily F, polypeptide 8; *CYP4F11*: Cytochrome P450, family 4, subfamily F, polypeptide 11; *CYP4F12*: Cytochrome P450, family 4, subfamily F, polypeptide 12; *CYP4Z1*: Cytochrome P450, family 4, subfamily Z, polypeptide 1; *CYP7A1*: Cytochrome P450, family 7, subfamily A, polypeptide 1; *CYP7B1*: Cytochrome P450, family 7, subfamily B, polypeptide 1; *CYP8B1*: Cytochrome P450, family 8, subfamily B, polypeptide 1; *CYP11A1*: Cytochrome P450, family 11, subfamily A, polypeptide 1; *CYP11B1*: Cytochrome P450, family 11, subfamily B, polypeptide 1; *CYP11B2*: Cytochrome P450, family 11, subfamily B, polypeptide 2; *CYP17A1*: Cytochrome P450, family 17, subfamily A, polypeptide 1; *CYP19A1*: Cytochrome P450, family 19, subfamily A, polypeptide 1; *CYP20A1*: Cytochrome P450, family 20, subfamily A, polypeptide 1; *CYP21A2*: Cytochrome P450, family 21, subfamily A, polypeptide 2; *CYP24A1*: Cytochrome P450, family 24, subfamily A, polypeptide 1; *CYP26A1*: Cytochrome P450, family 26, subfamily A, polypeptide 1; *CYP26B1*: Cytochrome P450, family 26, subfamily B, polypeptide 1; *CYP26C1*: Cytochrome P450, family 26, subfamily C, polypeptide 1; *CYP27A1*: Cytochrome P450, family 27, subfamily A, polypeptide 1; *CYP27B1*: Cytochrome P450, family 27, subfamily B, polypeptide 1; *CYP39A1*: Cytochrome P450, family 39, subfamily A, polypeptide 1; *CYP46A1*: Cytochrome P450, family 46, subfamily A, polypeptide 1; *CYP51A1*: Cytochrome P450, family 51, subfamily A, polypeptide 1; *DDOST*: Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit (non-catalytic); *DHRS1*: Dehydrogenase/reductase (SDR family) member 1; *DHRS2*: Dehydrogenase/reductase (SDR family) member 2; *DHRS3*: Dehydrogenase/reductase (SDR family) member 3; *DHRS4*: Dehydrogenase/reductase (SDR family) member 4; *DHRS7*: Dehydrogenase/reductase (SDR family) member 7; *DHRS9*: Dehydrogenase/reductase (SDR family) member 9; *DHRS12*: Dehydrogenase/reductase (SDR family) member 12; *DHRS13*: Dehydrogenase/reductase (SDR family) member 13; *DHRSX*: Dehydrogenase/reductase (SDR family) X-linked; *DLGAP1:* discs, large (Drosophila) homolog-associated protein 1; *DPEP1*: Dipeptidase 1 (renal); *DPYD*: Dihydropyrimidine dehydrogenase; *EPHX1*: Epoxide hydrolase 1, microsomal (xenobiotic); *EPHX2*: Epoxide hydrolase 2, microsomal (xenobiotic); *ESD*: Esterase D; *FMO1*: Flavin containing monooxygenase 1; *FMO2*: Flavin containing monooxygenase 2; *FMO3*: Flavin containing monooxygenase 3; *FMO4*: Flavin containing monooxygenase 4; *FMO5*: Flavin containing monooxygenase 5; *FMO6P*: Flavin containing monooxygenase 6 pseudogene; *FOS:* FBJ murine osteosarcoma viral oncogene homolog; *GAL3ST1*: Galactose-3-O-sulfotransferase 1; *GAMT*: Guanidinoacetate N-methyltransferase; *GLRX*: Glutaredoxin (thioltransferase); *GLYAT*: Glycine-N-acyltransferase; *GNMT*: Glycine N-methyltransferase; *GPX1*: Glutathione peroxidase 1; *GPX2*: Glutathione peroxidase 2 (gastrointestinal); *GPX3*: Glutathione peroxidase 3 (plasma); *GPX4*: Glutathione peroxidase 4; *GPX5*: Glutathione peroxidase 5; *GPX6*: Glutathione peroxidase 6 (olfactory); *GPX7*: Glutathione peroxidase 7; *GSR*: Glutathione reductase; *GSTA1*: Glutathione S-transferase alpha 1; *GSTA2*: Glutathione S-transferase alpha 2; *GSTA3*: Glutathione S-transferase alpha 3; *GSTA4*: Glutathione S-transferase alpha 4; *GSTA5*: Glutathione S-transferase alpha 5; *GSTCD*: Glutathione S-transferase, C-terminal domain containing; *GSTK1*: Glutathione S-transferase kappa 1; *GSTM1*: Glutathione S-transferase mu 1; *GSTM2*: Glutathione S-transferase mu 2 (muscle); *GSTM3*: Glutathione S-transferase mu 3 (brain); *GSTM4*: Glutathione S-transferase mu 4; *GSTM5*: Glutathione S-transferase mu 5; *GSTO1*: Glutathione S-transferase omega 1; *GSTO2*: Glutathione S-transferase omega 2; *GSTP1*: Glutathione S-transferase pi 1; *GSTT1*: Glutathione S-transferase theta 1; *GSTT2*: Glutathione S-transferase theta 2; *GSTZ1*: Glutathione S-transferase zeta 1; *GZMA*: Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3; *GZMB*: Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1); *HNMT*: Histamine N-methyltransferase; *HOMER1*: homer homolog 1 (Drosophila); *HSD11B1*: Hydroxysteroid (11-beta) dehydrogenase 1; *HSD17B10*: Hydroxysteroid (17-beta) dehydrogenase 10; *HSD17B11*: Hydroxysteroid (17-beta) dehydrogenase 11; *HSD17B14*: Hydroxysteroid (17-beta) dehydrogenase 14; *INMT*: Indolethylamine N-methyltransferase; *MAOA*: Monoamine oxidase A; *MAOB*: monoamine oxidase B; *METAP1*: Methionyl aminopeptidase 1; *MGST1*: Microsomal glutathione S-transferase 1; *MGST2*: Microsomal glutathione S-transferase 1; *MGST3*: Microsomal glutathione S-transferase 3; *NAA20*: N(alpha)-acetyltransferase 20, NatB catalytic subunit; *NAT1*: N-acetyltransferase 1 (arylamine N-acetyltransferase); *NAT2*: N-acetyltransferase 2 (arylamine N-acetyltransferase); *NNMT*: Nicotinamide N-methyltransferase; *NQO1*: NAD(P)H dehydrogenase, quinone 1; *NQO2*: NAD(P)H dehydrogenase, quinone 2; *NR1I2*: nuclear receptor subfamily 1, group

(continued)

Table 3 (continued)

I, member 2; *PNMT*: Phenylethanolamine N-methyltransferase; *PON1*: Paraoxonase 1; *PON2*: Paraoxonase 2; *PON3*: Paraoxonase 3; *POR*: P450 (cytochrome) oxidoreductase; *PTGES*: Prostaglandin E synthase; *PTGS1*: Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase); *PTGS2*: Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase); *SAT1*: Spermidine/spermine N1-acetyltransferase 1; *SMOX*: Spermine oxidase; *SOD1*: Superoxide dismutase 1, soluble; *SOD2*: Superoxide dismutase 2, mitochondrial; *SULT1A1*: Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1; *SULT1A2*: Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2; *SULT1A3*: Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3; *SULT1B1*: Sulfotransferase family, cytosolic, 1B, member 1; *SULT1C1*: Sulfotransferase family, cytosolic, 1C, member 1; *SULT1C2*: Sulfotransferase family, cytosolic, 1C, member 2; *SULT1C3*: Sulfotransferase family, cytosolic, 1C, member 3; *SULT1C4*: Sulfotransferase family, cytosolic, 1C, member 4; *SULT1E1*: Sulfotransferase family 1E, estrogen-preferring, member 1; *SULT2A1*: Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1; *SULT2B1*: Sulfotransferase family, cytosolic, 2B, member 1; *SULT4A1*: Sulfotransferase family 4A, member 1; *SULT6B1*: sulfotransferase family, cytosolic, 6B, member 1; *TBXAS1*: Thromboxane A synthase 1 (platelet); *TPMT*: Thiopurine S-methyltransferase; *TST*: Thiopurine S-methyltransferase; *UCHL1*: Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase); *UCHL3*: Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase); *UGT1A1*: UDP glucuronosyltransferase 1 family, polypeptide A1; *UGT1A3*: UDP glucuronosyltransferase 1 family, polypeptide A3; *UGT1A4*: UDP glucuronosyltransferase 1 family, polypeptide A4; *UGT1A5*: UDP glucuronosyltransferase 1 family, polypeptide A5; *UGT1A6*: UDP glucuronosyltransferase 1 family, polypeptide A6; *UGT1A7*: UDP glucuronosyltransferase 1 family, polypeptide A7; *UGT1A8*: UDP glucuronosyltransferase 1 family, polypeptide A8; *UGT1A9*: UDP glucuronosyltransferase 1 family, polypeptide A9; *UGT1A10*: UDP glucuronosyltransferase 1 family, polypeptide A10; *UGT2A1*: UDP glucuronosyltransferase 2 family, polypeptide A1, complex locus; *UGT2A3*: UDP glucuronosyltransferase 2 family, polypeptide A3; *UGT2B10*: UDP glucuronosyltransferase 2 family, polypeptide B10; *UGT2B11*: UDP glucuronosyltransferase 2 family, polypeptide B11; *UGT2B15*: UDP glucuronosyltransferase 2 family, polypeptide B15; *UGT2B17*: UDP glucuronosyltransferase 2 family, polypeptide B17; *UGT2B28*: UDP glucuronosyltransferase 2 family, polypeptide B28; *UGT2B4*: UDP glucuronosyltransferase 2 family, polypeptide B4; *UGT2B7*: UDP glucuronosyltransferase 2 family, polypeptide B7; *UGT3A1*: UDP glycosyltransferase 3 family, polypeptide A1; *UGT8*: UDP glycosyltransferase 8; *XDH*: Xanthine dehydrogenase

methyltransferase), COMT (catechol-O-methyltransferase), HMT (histamine methyl-transferase), STs (sulfotransferases), GST-A (glutathione S-transferase A), GST-P, GST-T, GST-M, NAT1 (N-acetyltransferase 1), NAT2, and others (*see* Table 4). Among these enzymes, CYP2D6, CYP2C9, CYP2C19, and CYP3A4/5 are the most relevant in the pharmacogenetics of CNS drugs [15, 16] (*see* Table 3). Approximately, 18 % of neuroleptics are major substrates of CYP1A2 enzymes, 40 % of CYP2D6, and 23 % of CYP3A4; 24 % of antidepressants are major substrates of CYP1A2 enzymes, 5 % of CYP2B6, 38 % of CYP2C19, 85 % of CYP2D6, and 38 % of CYP3A4; 7 % of benzodiazepines are major substrates of CYP2C19 enzymes, 20 % of CYP2D6, and 95 % of CYP3A4 [15, 16]. Most CYP enzymes exhibit ontogenic-, age-, sex-, circadian-, and ethnic-related differences [16, 686].

In dementia, as in any other CNS disorders, CYP genomics is a very important issue since in practice over 90 % of patients with dementia are daily consumers of psychotropics. Furthermore, some acetylcholinesterase inhibitors (the most prescribed anti-dementia drugs worldwide) are metabolized via CYP enzymes (*see* Table 3). Most CYP enzymes display highly significant ethnic differences, indicating that the enzymatic capacity of these proteins

Table 4
Drug metabolism-related genes

Phase I enzymes			
Cluster	**Gene**	**Name**	**Locus**
Alcohol dehydrogenases	*ADH1A*	Alcohol dehydrogenase 1A (class I), alpha polypeptide	4q23
	ADH1B	Alcohol dehydrogenase 1B (class I), beta polypeptide	4q23
	ADH1C	Alcohol dehydrogenase 1C (class I), gamma polypeptide	4q23
	ADH4	Alcohol dehydrogenase 4 (class II), pi polypeptide	4q22
	ADH5	Alcohol dehydrogenase 5 (class III), chi polypeptide	4q23
	ADH6	Alcohol dehydrogenase 6 (class V)	4q23
	ADH7	Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	4q23–q24
	ADHFE1	Alcohol dehydrogenase, iron containing, 1	8q13.1
Aldehyde dehydrogenases	*ALDH1A1*	Aldehyde dehydrogenase 1 family, member A1	9q21.13
	ALDH1A2	Aldehyde dehydrogenase 1 family, member A2	15q21.3
	ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	15q26.3
	ALDH1B1	Aldehyde dehydrogenase 1 family, member B1	9p11.1
	ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	12q24.2
	ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	17p11.2
	ALDH3A2	Aldehyde dehydrogenase 3 family, member A2	17p11.2
	ALDH3B1	Aldehyde dehydrogenase 3 family, member B1	11q13
	ALDH3B2	Aldehyde dehydrogenase 3 family, member B2	11q13
	ALDH4A1	Aldehyde dehydrogenase 4 family, member A1	1p36
	ALDH5A1	Aldehyde dehydrogenase 5 family, member A1	6p22
	ALDH6A1	Aldehyde dehydrogenase 6 family, member A1	14q24.3
	ALDH7A1	Aldehyde dehydrogenase 7 family, member A1	5q31
	ALDH8A1	Aldehyde dehydrogenase 8 family, member A1	6q23.2
	ALDH9A1	Aldehyde dehydrogenase 9 family, member A1	1q23.1
	AOX1	Aldehyde oxidase 1	2q33
Aldo-keto reductases	*AKR1A1*	Aldo-keto reductase family 1, member A1 (aldehyde reductase)	1p33–p32
	AKR1B1	Aldo-keto reductase family 1, member B1 (aldose reductase)	7q35
	AKR1C1	Aldo-keto reductase family 1, member C1	10p15–p14
	AKR1D1	Aldo-keto reductase family 1, member D1	7q32–q33
Amine oxidases	*MAOA*	Monoamine oxidase A	Xp11.3
	MAOB	Monoamine oxidase B	Xp11.23
	SMOX	Spermine oxidase	20p13
Carbonyl reductases	*CBR1*	Carbonyl reductase 1	21q22.13
	CBR3	Carbonyl reductase 3	21q22.2
	CBR4	Carbonyl reductase 4	4q32.3
Cytidine deaminase	*CDA*	Cytidine deaminase	1p36.2–p35
Cytochrome P450 family	*CYP1A1*	Cytochrome P450, family 1, subfamily A, polypeptide 1	15q24.1
	CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2	15q24.1
	CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	2p22.2
	CYP2A6	Cytochrome P450, family 2, subfamily A, polypeptide 6	19q13.2
	CYP2A7	Cytochrome P450, family 2, subfamily A, polypeptide 7	19q13.2
	CYP2A13	Cytochrome P450, family 2, subfamily A, polypeptide 13	19q13.2
	CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6	19q13.2
	CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8	10q23.33
	CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9	10q24
	CYP2C18	Cytochrome P450, family 2, subfamily C, polypeptide 18	10q24

(continued)

Table 4 (continued)

Phase I enzymes			
Cluster	**Gene**	**Name**	**Locus**
	CYP2C19	Cytochrome P450, family 2, subfamily C, polypeptide 19	10q24
	CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6	22q13.1
	CYP2D7P1	Cytochrome P450, family 2, subfamily D, polypeptide 7 pseudogene 1	22q13
	CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	10q26.3
	CYP2F1	Cytochrome P450, family 2, subfamily F, polypeptide 1	19q13.2
	CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	1p31.3–p31.2
	CYP2R1	Cytochrome P450, family 2, subfamily R, polypeptide 1	11p15.2
	CYP2S1	Cytochrome P450, family 2, subfamily S, polypeptide 1	19q13.1
	CYP2W1	Cytochrome P450, family 2, subfamily W, polypeptide 1	7p22.3
	CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4	7q21.1
	CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5	7q21.1
	CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	7q21–q22.1
	CYP3A43	Cytochrome P450, family 3, subfamily A, polypeptide 43	7q21.1
	CYP4A11	Cytochrome P450, family 4, subfamily A, polypeptide 11	1p33
	CYP4A22	Cytochrome P450, family 4, subfamily A, polypeptide 22	1p33
	CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1	1p34–p12
	CYP4F2	Cytochrome P450, family 4, subfamily F, polypeptide 2	19p13.12
	CYP4F3	Cytochrome P450, family 4, subfamily F, polypeptide 3	19p13.2
	CYP4F8	Cytochrome P450, family 4, subfamily F, polypeptide 8	19p13.1
	CYP4F11	Cytochrome P450, family 4, subfamily F, polypeptide 11	19p13.1
	CYP4F12	Cytochrome P450, family 4, subfamily F, polypeptide 12	19p13.1
	CYP4Z1	Cytochrome P450, family 4, subfamily Z, polypeptide 1	1p33
	CYP7A1	Cytochrome P450, family 7, subfamily A, polypeptide 1	8q11–q12
	CYP7B1	Cytochrome P450, family 7, subfamily B, polypeptide 1	8q21.3
	CYP8B1	Cytochrome P450, family 8, subfamily B, polypeptide 1	3p22.1
	CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	15q23–q24
	CYP11B1	Cytochrome P450, family 11, subfamily B, polypeptide 1	8q21
	CYP11B2	Cytochrome P450, family 11, subfamily B, polypeptide 2	8q21–q22
	CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	10q24.3
	CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	15q21.1
	CYP20A1	Cytochrome P450, family 20, subfamily A, polypeptide 1	2q33.2
	CYP21A2	Cytochrome P450, family 21, subfamily A, polypeptide 2	6p21.3
	CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1	20q13
	CYP26A1	Cytochrome P450, family 26, subfamily A, polypeptide 1	10q23–q24
	CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1	2p13.2
	CYP26C1	Cytochrome P450, family 26, subfamily C, polypeptide 1	10q23.33
	CYP27A1	Cytochrome P450, family 27, subfamily A, polypeptide 1	2q33-qter
	CYP27B1	Cytochrome P450, family 27, subfamily B, polypeptide 1	12q13.1–q13.3
	CYP39A1	Cytochrome P450, family 39, subfamily A, polypeptide 1	6p21.1–p11.2
	CYP46A1	Cytochrome P450, family 46, subfamily A, polypeptide 1	14q32.1
	CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1	14q32.1
	POR	P450 (cytochrome) oxidoreductase	7q11.2
	TBXAS1	Thromboxane A synthase 1 (platelet)	7q34–q35
Cytochrome b5 reductase	*CYB5R3*	Cytochrome b5 reductase 3	22q13.2
Dihydroprimidine dehydrogenase	*DPYD*	Dihydropyrimidine dehydrogenase	1p22

(continued)

Table 4 (continued)

Phase I enzymes			
Cluster	**Gene**	**Name**	**Locus**
Esterases	*AADAC*	Arylacetamide deacetylase	3q25.1
	CEL	Carboxyl ester lipase	9q34.3
	CES1	Carboxylesterase 1	16q22.2
	CES1P1	Carboxylesterase 1 pseudogene 1	16q12.2
	CES2	Carboxylesterase 2	16q22.1
	CES3	Carboxylesterase 3	16q22.1
	CES5A	Carboxylesterase 5A	16q12.2
	ESD	Esterase D	13q14.1–q14.2
	GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	5q11–q12
	GZMB	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	14q11.2
	PON1	Paraoxonase 1	7q21.3
	PON2	Paraoxonase 2	7q21.3
	PON3	Paraoxonase 3	7q21.3
	UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	4p14
	UCHL3	Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	13q22.2
Epoxidases	*EPHX1*	Epoxide hydrolase 1, microsomal (xenobiotic)	1q42.1
	EPHX2	Epoxide hydrolase 2, microsomal (xenobiotic)	8p21
Flavin containing monooxygenases	*FMO1*	Flavin containing monooxygenase 1	1q24.3
	FMO2	Flavin containing monooxygenase 2	1q24.3
	FMO3	Flavin containing monooxygenase 3	1q24.3
	FMO4	Flavin containing monooxygenase 4	1q24.3
	FMO6	Flavin containing monooxygenase 5	1q21.1
	FMO6P	Flavin containing monooxygenase 6 pseudogene	1q24.3
Glutathione reductase/ peroxidases	*GPX1*	Glutathione peroxidase 1	3p21.3
	GPX2	Glutathione peroxidase 2 (gastrointestinal)	14q24.1
	GPX3	Glutathione peroxidase 3 (plasma)	5q23
	GPX4	Glutathione peroxidase 4	19p13.3
	GPX5	Glutathione peroxidase 5	6p22.1
	GPX6	Glutathione peroxidase 6 (olfactory)	6p22.1
	GPX7	Glutathione peroxidase 7	1p32
	GSR	Glutathione reductase	8p21.1
Peptidases	*DPEP1*	Dipeptidase 1 (renal)	16q24.3
	METAP1	Methionyl aminopeptidase 1	4q23
Prostaglandin-endoperoxide synthases	*PTGS1*	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	9q32–q33.3
	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1q25.2–q25.3

(continued)

Table 4
(continued)

Phase I enzymes			
Cluster	**Gene**	**Name**	**Locus**
Short-chain dehydrogenases/ reductases	*DHRS1*	Dehydrogenase/reductase (SDR family) member 1	14q12
	DHRS2	Dehydrogenase/reductase (SDR family) member 2	14q11.2
	DHRS3	Dehydrogenase/reductase (SDR family) member 3	1p36.1
	DHRS4	Dehydrogenase/reductase (SDR family) member 4	14q11.2
	DHRS7	Dehydrogenase/reductase (SDR family) member 7	14q23.1
	DHRS9	Dehydrogenase/reductase (SDR family) member 9	2q31.1
	DHRS12	Dehydrogenase/reductase (SDR family) member 12	13q14.3
	DHRS13	Dehydrogenase/reductase (SDR family) member 13	17q11.2
	DHRSX	Dehydrogenase/reductase (SDR family) X-linked	Xp22.33; Yp11.2
	HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	1q32–q41
	HSD17B10	Hydroxysteroid (17-beta) dehydrogenase 10	Xp11.2
	HSD17B11	Hydroxysteroid (17-beta) dehydrogenase 11	4q22.1
	HSD17B14	Hydroxysteroid (17-beta) dehydrogenase 14	19q13.33
Superoxide dismutase	*SOD1*	Superoxide dismutase 1, soluble	21q22.11
	SOD2	Superoxide dismutase 2, mitochondrial	6q25.3
Xanthine dehydrogenase	*XDH*	Xanthine dehydrogenase	2p23.1
Phase II enzymes			
Amino acid transferases	*AGXT*	Alanine-glyoxylate aminotransferase	2q37.3
	BAAT	Bile acid CoA: amino acid N-acyltransferase (glycine N-choloyltransferase)	9q22.3
	CCBL1	Cysteine conjugate-beta lyase, cytoplasmic	9q34.11
Dehydrogenases	*NQO1*	NAD(P)H dehydrogenase, quinone 1	16q22.1
	NQO2	NAD(P)H dehydrogenase, quinone 2	6p25.2
	XDH	Xanthine dehydrogenase	2p23.1
Esterases	*CES1*	Carboxylesterase 1	16q22.2
	CES1P1	Carboxylesterase 1 pseudogene 1	16q12.2
	CES2	Carboxylesterase 2	16q22.1
	CES3	Carboxylesterase 3	16q22.1
	CES5A	Carboxylesterase 5A	16q12.2
Glucuronosyl transferases	*DDOST*	Dolichyl-diphosphooligosaccharid--rotein glycosyltransferase subunit (non-catalytic)	1p36.1
	UGT1A1	UDP glucuronosyltransferase 1 family, polypeptide A1	2q37
	UGT1A3	UDP glucuronosyltransferase 1 family, polypeptide A3	2q37
	UGT1A4	UDP glucuronosyltransferase 1 family, polypeptide A4	2q37
	UGT1A5	UDP glucuronosyltransferase 1 family, polypeptide A5	2q37
	UGT1A6	UDP glucuronosyltransferase 1 family, polypeptide A6	2q37
	UGT1A7	UDP glucuronosyltransferase 1 family, polypeptide A7	2q37
	UGT1A8	UDP glucuronosyltransferase 1 family, polypeptide A8	2q37
	UGT1A9	UDP glucuronosyltransferase 1 family, polypeptide A9	2q37
	UGT1A10	UDP glucuronosyltransferase 1 family, polypeptide A10	2q37
	UGT2A1	UDP glucuronosyltransferase 2 family, polypeptide A1, complex locus	4q13
	UGT2A3	UDP glucuronosyltransferase 2 family, polypeptide A3	4q13.2
	UGT2B4	UDP glucuronosyltransferase 2 family, polypeptide B4	4q13
	UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	4q13
	UGT2B10	UDP glucuronosyltransferase 2 family, polypeptide B10	4q13.2

(continued)

Table 4 (continued)

Phase I enzymes			
Cluster	**Gene**	**Name**	**Locus**
	UGT2B11	UDP glucuronosyltransferase 2 family, polypeptide B11	4q13.2
	UGT2B15	UDP glucuronosyltransferase 2 family, polypeptide B15	4q13
	UGT2B17	UDP glucuronosyltransferase 2 family, polypeptide B17	4q13
	UGT2B28	UDP glucuronosyltransferase 2 family, polypeptide B28	4q13.2
	UGT3A1	UDP glycosyltransferase 3 family, polypeptide A1	5p13.2
	UGT8	UDP glycosyltransferase 8	4q26
Glutathione transferases	*GSTA1*	Glutathione S-transferase alpha 1	6p12.1
	GSTA2	Glutathione S-transferase alpha 2	6p12.1
	GSTA3	Glutathione S-transferase alpha 3	6p12.1
	GSTA4	Glutathione S-transferase alpha 4	6p12.1
	GSTA5	Glutathione S-transferase alpha 5	6p12.2
	GSTK1	Glutathione S-transferase kappa 1	7q34
	GSTM1	Glutathione S-transferase mu 1	1p13.3
	GSTM2	Glutathione S-transferase mu 2 (muscle)	1p13.3
	GSTM3	Glutathione S-transferase mu 3 (brain)	1p13.3
	GSTM4	Glutathione S-transferase mu 4	1p13.3
	GSTM5	Glutathione S-transferase mu 5	1p13.3
	GSTO1	Glutathione S-transferase omega 1	10q25.1
	GSTO2	Glutathione S-transferase omega 2	10q25.1
	GSTP1	Glutathione S-transferase pi 1	11q13
	GSTT1	Glutathione S-transferase theta 1	22q11.23
	GSTT2	Glutathione S-transferase theta 2	22q11.23
	GSTZ1	Glutathione S-transferase zeta 1	14q24.3
	GSTCD	Glutathione S-transferase, C-terminal domain containing	4q24
	MGST1	Microsomal glutathione S-transferase 1	12p12.3–p12.1
	MGST2	Microsomal glutathione S-transferase 2	4q28.3
	MGST3	Microsomal glutathione S-transferase 3	1q23
	PTGES	Prostaglandin E synthase	9q34.3
Methyl transferases	*AS3MT*	Arsenic (+3 oxidation state) methyltransferase	10q24.32
	ASMT	Acetylserotonin O-methyltransferase	Xp22.3/Yp11.3
	COMT	Catechol-O-methyltransferase	22q11.21
	GNMT	Glycine N-methyltransferase	6p12
	GAMT	Guanidinoacetate N-methyltransferase	19p13.3
	HNMT	Histamine N-methyltransferase	2q22.1
	INMT	Indolethylamine N-methyltransferase	7p14.3
	NNMT	Nicotinamide N-methyltransferase	11q23.1
	PNMT	Phenylethanolamine N-methyltransferase	17q
	TPMT	Thiopurine S-methyltransferase	6p22.3
N-Acetyl transferases	*ACSL1*	Acyl-CoA synthetase long-chain family member 1	4q35
	ACSL3	Acyl-CoA synthetase long-chain family member 3	2q34–q35
	ACSL4	Acyl-CoA synthetase long-chain family member 4	Xq22.3–q23
	ACSM1	Acyl-CoA synthetase medium-chain family member 1	16p12.3
	ACSM2B	Acyl-CoA synthetase medium-chain family member 2B	16p12.3
	ACSM3	Acyl-CoA synthetase medium-chain family, member 3	16p13.11
	AANAT	Aralkylamine N-acetyltransferase	17q25
	GLYAT	Glycine-N-acyltransferase	11q12.1
	NAA20	N(Alpha)-acetyltransferase 20, NatB catalytic subunit	20p11.23
	NAT1	N-Acetyltransferase 1 (arylamine N-acetyltransferase)	8p22
	NAT2	N-Acetyltransferase 2 (arylamine N-acetyltransferase)	8p22
	SAT1	Spermidine/spermine N1-acetyltransferase 1	Xp22.1

(continued)

Table 4 (continued)

Phase I enzymes			
Cluster	**Gene**	**Name**	**Locus**
Thioltransferase	*GLRX*	Glutaredoxin (thioltransferase)	5q14
Sulfotransferases	*CHST2*	Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2	3q24
	CHST3	Carbohydrate (chondroitin 6) sulfotransferase 3	10q22.1
	CHST4	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 4	16q22.2
	CHST5	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5	16q22.3
	CHST6	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6	16q22
	CHST7	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7	Xp11.23
	CHST8	Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8	19q13.1
	CHST9	Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9	18q11.2
	CHST10	Carbohydrate sulfotransferase 10	2q11.2
	CHST11	Carbohydrate (chondroitin 4) sulfotransferase 11	12q
	CHST12	Carbohydrate (chondroitin 4) sulfotransferase 12	7p22
	CHST13	Carbohydrate (chondroitin 4) sulfotransferase 13	3q21.3
	GAL3ST1	Galactose-3-O-sulfotransferase 1	22q12.2
	SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	16p12.1
	SULT1A2	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	16p12.1
	SULT1A3)	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3	16p11.2
	SULT1B1	Sulfotransferase family, cytosolic, 1B, member 1	4q13.3
	SULT1C1	Sulfotransferase family, cytosolic, 1C, member 1	2q12.3
	SULT1C2	Sulfotransferase family, cytosolic, 1C, member 2	2q12.3
	SULT1C3	Sulfotransferase family, cytosolic, 1C, member 3	2q12.3
	SULT1C4	Sulfotransferase family, cytosolic, 1C, member 4	2q12.3
	SULT1E1	Sulfotransferase family 1E, estrogen-preferring, member 1	4q13.1
	SULT2A1	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	19q13.3
	SULT2B1	Sulfotransferase family, cytosolic, 2B, member 1	19q13.3
	SULT4A1	Sulfotransferase family 4A, member 1	22q13.2
	SULT6B1	Sulfotransferase family, cytosolic, 6B, member 1	2p22.2
	CHST1	Thiopurine S-methyltransferase (TST), Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	11p11.2

varies depending upon the polymorphic variants present in their coding CYP genes. The practical consequence of this genetic variation is that the same drug can be differentially metabolized according to the genetic profile of each subject, and that knowing the pharmacogenomic profile of an individual, his/her pharmacodynamic response is potentially predictable. This is the cornerstone of pharmacogenetics. In this regard, the *CYP2D6*, *CYP2C19*, *CYP2C9*, and *CYP3A4/5* genes and their respective protein products deserve special consideration.

7.3.1 CYP2D6

CYP2D6 is a 4.38 kb gene with nine exons mapped on 22q13.2. Four RNA transcripts of 1,190–1,684 bp are expressed in the brain, liver, spleen, and reproductive system where four major

proteins of 48–55 kDa (439–494 aa) are identified. This protein is a transport enzyme of the cytochrome P450 subfamily IID or multigenic cytochrome P450 superfamily of mixed-function monooxygenases. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids. This protein localizes to the endoplasmic reticulum and is known to metabolize as many as 25 % of commonly prescribed drugs and over 60 % of current psychotropics. Its substrates include debrisoquine, an adrenergic-blocking drug; sparteine and propafenone, both anti-arrhythmic drugs; and amitriptyline, an antidepressant. The gene is highly polymorphic in the population. There are 141 *CYP2D6* allelic variants of which -100C>T, -1,023C>T, -1,659G>A, -1,707delT, -1,846G>A, -2,549delA, -2,613–2,615delAGA, -2,850C>T, -2,988G>A, and -3,183G>A represent the ten most important variants [687–689]. Different alleles result in the extensive, intermediate, poor, and ultra-rapid metabolizer phenotypes, characterized by normal, intermediate, decreased, and multiplied ability to metabolize the enzyme's substrates, respectively. The hepatic cytochrome P450 system is responsible for the first phase in the metabolism and elimination of numerous endogenous and exogenous molecules and ingested chemicals. P450 enzymes convert these substances into electrophilic intermediates which are then conjugated by phase II enzymes (e.g., UDP glucuronosyltransferases, N-acetyltransferases) to hydrophilic derivatives that can be excreted. According to the database of the World Guide for Drug Use and Pharmacogenomics [687], 982 drugs are *CYP2D6*-related: 371 drugs are substrates, over 300 drugs are inhibitors, and 18 drugs are CYP2D6 inducers.

In healthy subjects, extensive metabolizers (EMs) account for 55.71 % of the population, whereas intermediate metabolizers (IMs) account for 34.7 %, poor metabolizers (PMs) 2.28 %, and ultra-rapid metabolizers (UMs) 7.31 %. Remarkable interethnic differences exist in the frequency of the PM and UM phenotypes among different societies all over the world [690–692]. On average, approximately 6.28 % of the world population belongs to the PM category. Europeans (7.86 %), Polynesians (7.27 %), and Africans (6.73 %) exhibit the highest rate of PMs, whereas Orientals (0.94 %) show the lowest rate [690]. The frequency of PMs among Middle Eastern populations, Asians, and Americans is in the range of 2–3 %. *CYP2D6* gene duplications are relatively infrequent among Northern Europeans, but in East Africa the frequency of alleles with duplication of *CYP2D6* is as high as 29 % [693]. In Europe, there is a North–South gradient in the frequency of PMs (6–12 % of PMs in Southern European countries, and 2–3 % PMs in Northern latitudes) [16].

In AD, EMs, IMs, PMs, and UMs are 56.38 %, 27.66 %, 7.45 %, and 8.51 %, respectively, and in VD, 52.81 %, 34.83 %, 6.74 %, and 5.62 %, respectively (*see* Fig. 6).

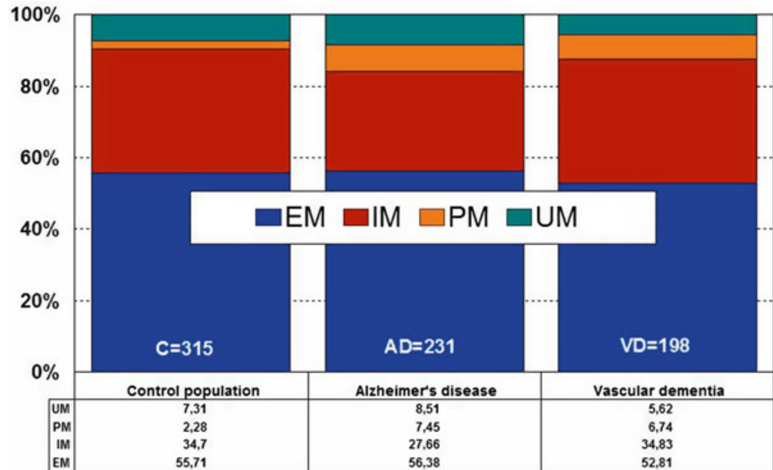


Fig. 6 Distribution and frequency of *CYP2D6* phenotypes in Alzheimer's disease and vascular dementia. EM: Extensive Metabolizers; IM: Intermediate Metabolizers; PM: Poor Metabolizer; UM: Ultra-Rapid Metabolizer. (Adapted from ref. 19)

There is an accumulation of AD-related genes of risk in PMs and UMs. EMs and IMs are the best responders, and PMs and UMs are the worst responders to a combination therapy with cholinesterase inhibitors, neuroprotectants, and vasoactive substances. The pharmacogenetic response in AD appears to be dependent upon the networking activity of genes involved in drug metabolism and genes involved in AD pathogenesis [7, 12, 15, 17, 19, 28, 35, 59].

7.3.2 CYP2C9

CYP2C9 is a gene (50.71 kb) with nine exons mapped on 10q24. An RNA transcript of 1,860 bp is mainly expressed in hepatocytes where a protein of 55.63 kDa (490 aa) can be identified. Over 600 drugs are *CYP2C9*-related, 311 acting as substrates (177 are major substrates, 134 are minor substrates), 375 as inhibitors (92 weak, 181 moderate, and 102 strong inhibitors), and 41 as inducers of the CYP2C9 enzyme [687]. There are 481 *CYP2C9* SNPs. By phenotypes (*see* Fig. 7), in the control population, PMs represent 7.04 %, IMs 32.39 %, and EMs 60.56 %. In AD, PMs, IMs, and EMs are 6.45 %, 37.64 %, and 55.91 %, respectively, and in VD are 3.61 %, 28.92 %, and 67.47 %, respectively [19] (*see* Fig. 7).

7.3.3 CYP2C19

CYP2C19 is a gene (90.21 kb) with nine exons mapped on 10q24.1q24.3. RNA transcripts of 1,901, 2,395, and 1,417 bp are expressed in liver cells where a protein of 55.93 kDa (490 aa) is identified. Nearly 500 drugs are *CYP2C19*-related, 281 acting as substrates (151 are major substrates, 130 are minor substrates), 263 as inhibitors (72 weak, 127 moderate, and 64 strong inhibitors), and 23 as inducers of the CYP2C19 enzyme [687]. About 541

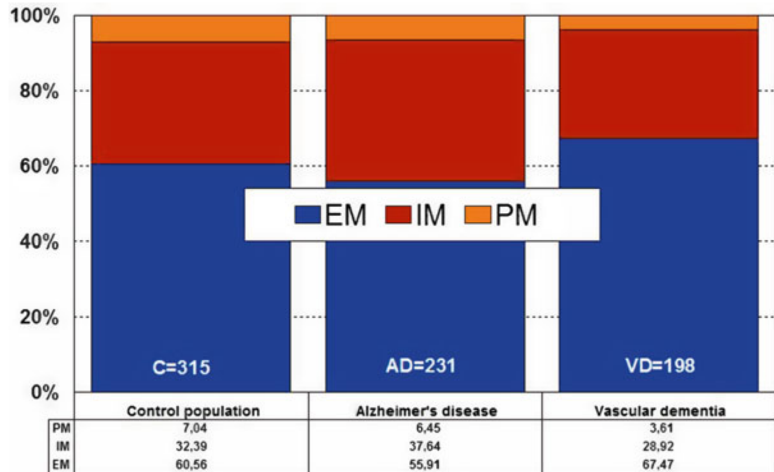


Fig. 7 Distribution and frequency of *CYP2C9* phenotypes in Alzheimer's disease and vascular dementia. EM: Extensive Metabolizer; IM: Intermediate Metabolizer; PM: Poor Metabolizer. (Adapted from ref. 19)

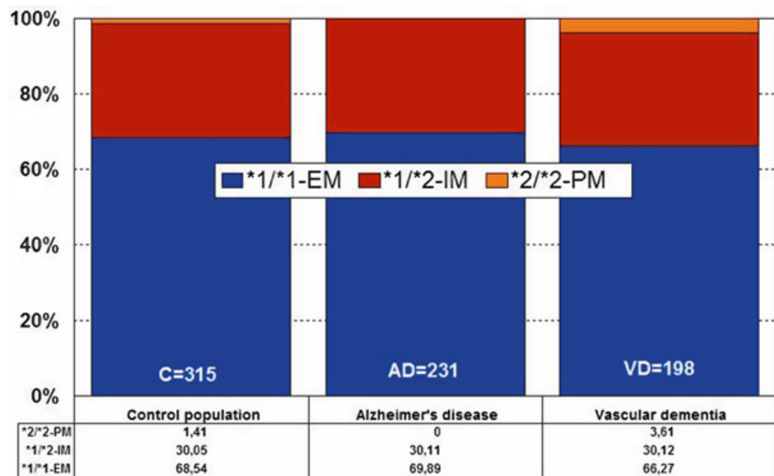


Fig. 8 Distribution and frequency of *CYP2C19* pheno-genotypes in Alzheimer's disease and vascular dementia. EM: Extensive Metabolizer; IM: Intermediate Metabolizer; PM: Poor Metabolizer. (Adapted from ref. 19)

SNPs have been detected in the *CYP2C19* gene. The frequencies of the three major *CYP2C19* geno-phenotypes in the control population are *CYP2C19-*1/*1*-EMs 68.54 %, *CYP2C19-*1/*2-IMs* 30.05 %, and *CYP2C19-*2/*2*-PMs 1.41 %. EMs, IMs, and PMs account for 69.89 %, 30.11 %, and 0 %, respectively, in AD, and 66.27 %, 30.12 %, and 3.61 %, respectively, in VD [19] (*see* Fig. 8).

7.3.4 CYP3A4/5

CYP3A4 is a gene (27.2 kb) with 13 exons mapped on 7q21.1. RNA transcripts of 2,153, 651, 564, 2,318 and 2,519 bp are

expressed in intestine, liver, prostate, and other tissues where four protein variants of 57.34 kDa (503 aa), 17.29 kDa (153 aa), 40.39 kDa (353 aa), and 47.99 kDa (420 aa) are identified. The human *CYP3A* locus contains the three *CYP3A* genes (*CYP3A4*, *CYP3A5*, and *CYP3A7*), three pseudogenes, as well as a novel *CYP3A* gene termed *CYP3A43*. The gene encodes a putative protein with between 71.5 and 75.8 % identity to the other CYP3A proteins. The predominant hepatic form is CYP3A4, but CYP3A5 contributes significantly to the total liver CYP3A activity. This enzyme metabolizes over 1,900 drugs, 1,033 acting as substrates (897 are major substrates, 136 are minor substrates), 696 as inhibitors (118 weak, 437 moderate, and 141 strong inhibitors), and 241 as inducers of the CYP3A4 enzyme [687]. About 347 SNPs have been identified in the *CYP3A4* gene (*CYP3A4*1A*: Wild-type), 25 of which are of clinical relevance. Concerning *CYP3A4/5* polymorphisms in AD, 82.75 % of the cases are EMs (*CYP3A5*3/*3*), 15.88 % are IMs (*CYP3A5*1/*3*), and 1.37 % are UMs (*CYP3A5*1/*1*). Unlike other human P450s (*CYP2D6*, *CYP2C19*) there is no evidence of a "null" allele for *CYP3A4* [687].

7.3.5 CYP Clustering

The construction of a genetic map integrating the most prevalent *CYP2D6*+ *CYP2C19*+ *CYP2C9* polymorphic variants in a trigenic cluster yields 82 different haplotype-like profiles. The most frequent trigenic genotypes in the AD population are **1*1-*1*1-*1*1* (25.70 %), **1*1-*1*2-*1*2* (10.66 %), **1*1-*1*1-*1*1* (10.45 %), **1*4-*1*1-*1*1* (8.09 %), **1*4-*1*2-*1*1* (4.91 %), **1*4-*1*1-*1*2* (4.65 %), and **1*1-*1*3-*1*3* (4.33 %). These 82 trigenic genotypes represent 36 different pharmacogenetic phenotypes. According to these trigenic clusters, only 26.51 % of the patients show a pure 3EM phenotype, 15.29 % are 2EM1IM, 2.04 % are pure 3IM, 0 % are pure 3 PM, and 0 % are 1UM2PM (the worst possible phenotype). This implies that only one-quarter of the population processes normally the drugs which are metabolized via CYP2D6, CYP2C9, and CYP2C19 (approximately 60 % of the drugs of current use) [12]. Taking into consideration the data available, it might be inferred that at least 20–30 % of the AD population may exhibit an abnormal metabolism of cholinesterase inhibitors and/or other drugs which undergo oxidation via *CYP2D6*-related enzymes. Approximately 50 % of this population cluster would show an ultra-rapid metabolism, requiring higher doses of cholinesterase inhibitors in order to reach a therapeutic threshold, whereas the other 50 % of the cluster would exhibit a poor metabolism, displaying potential adverse events at low doses. If we take into account that approximately 60–70 % of therapeutic outcomes depend upon pharmacogenomic criteria (e.g., pathogenic mechanisms associated with AD-related genes), it can be postulated that pharmacogenetic and pharmacogenomic factors

are responsible for 75–85 % of the therapeutic response (efficacy) in AD patients treated with conventional drugs [12, 15, 17, 19, 28, 35, 63, 86–92].

7.4 Genes Encoding Drug Transporters

ABC genes, especially *ABCB1* (ATP-binding cassette, subfamily B, member 1; P-glycoprotein-1, P-gp1; Multidrug Resistance 1, MDR1) (7q21.12), *ABCC1* (9q31.1), *ABCG2* (White1) (21q22.3), and other genes of this family encode proteins which are essential for drug metabolism and transport. The multidrug efflux transporters P-gp, multidrug-resistance associated protein 4 (MRP4), and breast cancer resistance protein (BCRP), located on endothelial cells lining brain vasculature, play important roles in limiting movement of substances into and enhancing their efflux from the brain. Transporters also cooperate with Phase I/Phase II metabolism enzymes by eliminating drug metabolites. Their major features are their capacity to recognize drugs belonging to unrelated pharmacological classes, and their redundancy, by which a single molecule can act as a substrate for different transporters. This ensures an efficient neuroprotection against xenobiotic invasions. The pharmacological induction of ABC gene expression is a mechanism of drug interaction, which may affect substrates of the upregulated transporter, and overexpression of MDR transporters confers resistance to anticancer agents and CNS drugs [694, 695].

Aberrant cholesterol trafficking and accumulation may contribute to the early onset of AD. Several ATP-binding cassette (ABC) transporters, such as ABCA1, ABCG1, ABCG5, and ABCG8, have been shown to play important roles in the regulation of cellular cholesterol homeostasis by mediating cholesterol efflux. Mutations in ABC transporters influence pathogenesis and therapeutics of brain disorders [696].

Genome-wide significance in fully adjusted models was observed for a SNP in ABCA7 (rs115550680, allele = G; frequency, 0.09 cases and 0.06 controls), which is in linkage disequilibrium with SNPs associated with AD in Europeans. The effect size for the SNP in ABCA7 was comparable with that of the APOE ϵ4-determining SNP rs429358 (allele = C; frequency, 0.30 cases and 0.18 controls) [697].

7.4.1 ABCB1

ABCB1 (ATP-binding cassette, subfamily B (MDR/TAP), member 1; Doxorubicin resistance; Multidrug resistance 1; Multidrug resistance protein 1; P glycoprotein 1; P glycoprotein 1/multiple drug resistance 1; P-Glycoprotein 1; P-glycoprotein-1/multiple drug resistance-1; P-gp) is probably the most important drug transporter in the brain. The *ABCB1* gene maps on 7q21.12 spanning 209.39 kb (29 Exons) with the structure of a P-glycoprotein and a Y-box sequence 5′-CTGATTGG-3′ in its cis-regulatory elements. Several transcripts/variants (ABCB1-001: 4,645 bp. ABCB1-002: 3,602 bp. ABCB1-003: 461 bp. ABCB1-004:

582 bp. ABCB1-005: 555 bp. ABCB1-006: 913 bp. ABCB1-007: 1,864 bp. ABCB1-008: 642 bp. ABCB1-009: 787 bp. ABCB1-010: 539 bp. ABCB1-201: 345 bp) are highly expressed in adrenal gland, BBB, brain, kidney, liver, placenta, small intestine, and uterus, and low expression is present in many other tissues. These transcripts encode a protein (ABCB1-001: 141.48 kDa; 1,280 aa. ABCB1-002: 5.89 kDa; 51 aa. ABCB1-003: 5.68 kDa; 48 aa. ABCB1-201: 2.52 kDa; 22 aa) of the ATP-binding cassette superfamily, subfamily B (MDR/TAP) with two ATP binding and two transmembrane (2TM) domains (2×6 segments), acting as a transport carrier and a lipid translocase of broad specificity. This is a large transmembrane protein which is an integral part of the BBB and functions as a drug-transport pump transporting a variety of drugs from the brain back into the blood. Functions of this protein include the following: ABC transporter, traffic ATPase, energy-dependent efflux pump responsible for decreased drug accumulation in multidrug-resistant cells; potentially implicated in cholesterol transport; may maintain neural stem/progenitor cells in an undifferentiated state; and could be a neural stem/progenitor marker.

About 1,630 *ABCB1* variants have been identified [687]. Of interest, *ABCB1* has approximately 116 polymorphic sites in Caucasians and 127 in African-Americans with a minor allele frequency greater than 5 %. Some of the most commonly studied variants are 1,236C>T, 2,677G>A/T, and 3,435C>T and the most commonly studied haplotype involves the 1,236, 2,677, and 3,435 (TTT) SNPs and three intronic SNPs (intron 9, intron 13, intron 14) named *ABCB1*13.* There are many other *ABCB1* variants such as -129C>T (5′-UTR), 61A>G (Asn21Asp), and 1,199G>A (Ser400Asn) that have been studied in vivo and in vitro. To date, there is no clear consensus on the impact of any of these variants on drug disposition, response, or toxicity.

Variants of the *ABCB1* gene have been associated with a diverse number of diseases and with a great variety of drugs, natural products, and endogenous agents (*see* Table 5) [687].

Over 1,270 drugs have been reported to be associated with the Abcb1 transporter protein (P-gp), of which 490 are substrates, 618 are inhibitors, 182 are inducers, and 269 additional compounds which belong to different pharmacological categories of products with potential Abcb1 interaction [687].

ATP-binding cassette (ABC) transporters, which are localized on the surface of brain endothelial cells of the BBB and brain parenchyma, may contribute to the pathogenesis of AD. ABC transporters including ABCB1 (P-glycoprotein, P-gp), ABCG2 (breast cancer resistant protein, BCRP), ABCC1 (multidrug resistance protein 1, MRP1), and the cholesterol transporter ABCA1 play important roles in the pathogenesis of AD and Aβ peptide deposition inside the brain [698–703]. Decreased clearance of

Table 5
ABCB1-related drugs (adapted from R. Cacabelos, World Guide for Drug Use and Pharmacogenomics, ref. 687)

ABCB1-related drugs
(+)-Catechin; 1-Cyclohexyl-4-(4-arylcyclohexyl)piperazines; 1-d-Ribofuranosylbenzimidazole; 1-Naphthylisothiocyanate; 1-Phenyl-2-decanoylamino-3-morpholino-1-propanol; 1,2-Diarylimidazole-4-carboxamides; 1,3,5-Triphenyl-2-pyrazolines; 1,4-bis-[2-(3,5-Dichloropyridyloxy)]benzene (TCPOBOP); 1,4-Dihydropyridines; 1,9-Pyrazoloanthrone (SP600125); 2-Acetylaminofluorene; 2-Arylthiazolidine-4-carboxylic acid amides (ATCAA); 2-Chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide; 2-Deoxy-D-glucose; 2-Fluoro N^{10}-substituted acridones; 2n-Propylquinoline; 2-Phenyl-5-(pyrrolidin-1-yl)-1-(3,4,5-trimethoxybenzyl)-1H-benzimidazole; 2-Pyridylphenyl amides (CJB 090 [N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)butyl)-4-(pyridine-2-yl) benzamide hydrochloride] and PG 01037 [N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)-trans-but-2-enyl)-4-(pyridine-2-yl)benzamide hydrochloride]); (2R)-Anti-5-{3-[4-(10,11-difluoromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride (LY335979); 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD); 2,4-Dinitrophenyl-S-glutathione; 2,5-Diaryl-2,3-dihydro-1,3,4-oxadiazoline analogs of combretastatin-A4; 3-Aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP; Triapine); 3-Benzyloxyfluorenes; 3-Dinitrobenzene; 3-O-Sulfate conjugate of 17α-ethinylestradiol; 3′R,4′R-Disubstituted-2′,2′-dimethyldihydropyrano[2,3-f]chromone (DSP) analogs; 3α,6α,7α,12α-Tetrahydroxy-cholanoyl taurine; 3β-Acetyl tormentic acid; 4-Ethyl 5-(3, 4-methylenedioxyphenyl)-2E,4E-pentadienoic acid piperidide (PA-1); 4′-Geranyloxyferulic acid; 4-Oxo-4,5,6,7-tetrahydro-1H-indole-3-carboxylic acid (4-methylaminomethyl-phenyl)-amide (GABA partial agonist); 4-Substituted methoxybenzoyl-aryl-thiazole; 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid; 4,11-bis[(2-Aminoethyl)amino]anthra[2,3-b]furan-5,10-diones; 4β-Anilino-podophyllotoxin derivatives; 5-Aza-2′-deoxycytidine; 5-Bromotetrandrine; 5-Cyclohexylindolyl-2′-deoxyribose (non-natural nucleoside); 5′-Fluorosulfonylbenzoyl 5′-adenosine; 5-Fluorouracil; 5,5-Diphenylbarbituric acid; 6-[(2S,4R,6E)-4-Methyl-2-(methylamino)-3-oxo-6-octenoic acid] cyclosporine D (PSC833); 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; 6-Mercaptopurine; 6-Prenylchrysin; 6-Thioguanine; 6,7-Dimethoxy-2-{3-[4-[^{11}C]methoxy-3,4-dihydro-2H-naphthalen-(1E)-ylidene]-propyl}-1,2,3,4-tetrahydro-isoquinoline; 6,7-Dimethoxy-2-(6-methoxy-naphthalen-2-ylmethyl)-1,2,3,4-tetrahydroisoquinoline (MC80); 6,6,8-Triethyldesmosdumotin B; 7-Xylosyl-10-deacetylpaclitaxel; 8-Azidoadenosine 5′-triphosphate; 8-Prenylnaringenin; 9-β-D-Arabinofuranosylguanine; 16α-Carbonitrile; 17-N-Allylamino-17-demethoxygeldanamycin; 20S-Ginsenoside Rh2; 23-Hydroxybetulinic acid from *Pulsatilla chinensis* (Bunge) Regel; [^{11}C]XR9576; [^{11}C]Methyl 4-((4-(2-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl) ethyl)phenyl)amino-carbonyl)-2-(quinoline-2-carbonylamino)benzoate; Abacavir; Abacavir and Lamivudine; Abacavir, Lamivudine and Zidovudine; Abamectin; ABT-737; ABT-263; ABT-773; *Acanthopanax senticosus* HARMS extract; Acenocoumarol; Acetaminophen; Acetochlor; Actinomycin D; Acylcarnitines (lauroylcarnitine, palmitoylcarnitine); Acylphloroglucinols; Adjudin; Adriamycin; African potato (*Hypoxis hemerocallidea*); AG1478 (Tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline); Alachlor; Albendazole; Albendazole sulfoxide; Aldosterone; Alfentanil; Aliskiren; Alkylbenzene sulfonate; Alkylphenols; Allyl methyl disulfide; alpha-Methyldigoxin; alpha-Tocotrienol; Altanserin; Ambrisentan; Ametantrone; Amiloride; Aminoguanidine; Amiodarone; Amitriptyline; Amlodipine; Amodiaquine; Amoxicillin; Amprenavir; Amrubicinol; Amurensin G; Amyloid-β; Amyloid-β peptides; AN 204; AN 215; AN 238; Angiopep-2; Angiotensin receptor type 1 blockers; Anidulafungin; Annexin A1; Anthelmintics; Antheraxanthin; Anthocyanins; Anthocyanidins; Anthracyclines; Anthrafurandiones; Anthranilamide modulators; Anthraquinone; Antibody-maytansinoid conjugates; Anticonvulsants; Antifouling biocides; Antimuscarinic agents; Antiplatelet agents; Antipsychotics; Antiretrovirals; Apatinib (YN968D1); Apicidin; Apigenin; Apixaban; Apolipoprotein E; Apurinic/apyrimidinic (AP) endonuclease (APE1/Ref-1); Aranidipine; Aripiprazole; Arsenic; Arsenic trioxide; Arsenite; Artemisinin; Artesunate; Artesunic acid;

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Table 5
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Aryl hydrocarbon receptors; ASA; Asparaginase (colaspase); Aspirin; Astemizole; AT80; Atazanavir; Atorvastatin; Atovaquone; Aurones; Auxin transport inhibitors (1-Naphthoxyacetic acids; Avermectins; Averolimus; Axitinib; Azathioprine; AZD5672 (N-(1-{(3R)-3-(3,5-difluorophenyl)-3-[4-methanesulfonylphenyl]propyl}piperidin-4-yl)-N-ethyl-2-[4-methanesulfonylphenyl]acetamide); Azelastine; Azidopine; Azithromycin; Azole antifungal agents; Aβ oligomerization inhibitors; BACE-1 inhibitors; Baicalein; Baicalin; Barbituric acid; Barnidipine; Bazedoxifene; Beauvericin; Bendamustine; Benidipine; Benzo(a)pyrene; Benzo[a]quinolizin-4-ones; Benzo(k)fluoranthene; Benzo[a]quinolizin-4-ones; Benzocaine; Bepidil; Bepotastine; Berberine; Bergamottin; Bergaptol; Beryllium fluoride; beta-Acetyldigoxin; beta-Tocotrienol; Betamethasone; Betti-base derivatives of tylosin (N-tylosil-1-alpha-amino-(3-bromophenyl)-methyl-2-naphthol); Bevacizumab; BIBF 1120; Biochanin A; Biricodar; bis(4-Fluorobenzyl)trisulfide; bis(12)-Hupyridone; Bisantrene; Bisbibenzyl derivatives; BMK-152; BMS-310705; BMS-690514; BMS-690514; Bodipy-FL-forskolin; Bodipy-FL-vinblastine; Boiogito; Bortezomib; Bosentan; Bromocriptine; Bromotetrandrine; Bromperidol; *Brugia malayi*; Buagafuran; Budesonide; Bunitrolol; Buprenorphine; Bupropion; Butyrates; Butyric acid; Byakangelicol; Cabazitaxel; Cabergoline; Cadmium; Calcein AM; Calcineurin inhibitors; Calcitriol (1α,25-dihydroxyvitamin D_3); Calcium antagonists; Calcium channel blockers; Calicheamicin-γ1; Camptothecin; Candesartan-Cilexetil; Capsaicin; Capsanthin; Capsorubin; CAR agonists; CAR antagonists; Carbamazepine; *Carpobrotus edulis*; Carbon tetrachloride; Carboplatin; Carminomycin I; Carmustine; Carnosic acid; Carnosol; Carotenoids; *Carpobrotus edulis*; Carvedilol; Caspofungin; Catechins from green tea; Caveolin-1; CBT-1; CD44; Celecoxib; Celiprolol; Cepharanthine; Ceramide; Cerivastatin; Cetirizine; Cetuximab; Changweiqing; Chargrilled meat diet; Chelerythrine; Chiral polychlorinated biphenyls; Chlorambucil; Chlorcyclizine; Chlordiazepoxide; Chloro benztropine analogs; Chloroform; Chloroquine; Chlorpheniramine; Chlorpromazine; Chlorpyrifos; Chrysene; Chrysin; Cilostazol; Cimetidine; Cinchonine; Cineole; Ciprofloxacin; Cisapride; Cisplatin; Citalopram; Citral; CJX1 cpd; CJY compound; CJZ3 (lomerizine derivative); Clarithromycin; Class III antiarrhythmic agents; Clerodane-type diterpenoid from *Sindora sumatrana*; Clopidogrel; Clorsulon; Closantel; Clotrimazole; Clozapine; Cnidiadin; Codeine; Coelenterazine; Colchicine; Colistin; Combretastatin-A4 (*Combretum caffrum*); Common sage; Common valerian; Concanavalin A; Copper N-(2-hydroxyacetophenone) glycinate; Copper pyrithione; Coptisine; Corticoids; Corticosteroids; Cortisol; C-Phycocyanin; Cremophor EL; Crocidolite asbestos; *Crossostephium chinense*-related flavonoids (selagin, apometzgerin, tricetin-3′,4′,5′-trimethylether, quercetagetin-3,6,7-trimethylether, hispidulin, quercetagetin); *Curcuma heyneana*; *Curcuma longa*; *Curcuma* zedoaria; Curcumenol; Curcumin; Curcumin analogs; Curcuminoids; Cyanidin; Cyanidin-3-galactoside; Cyanidin-3-glucoside; Cyanidin-3-rutinoside; Cyclin-dependent kinase inhibitors; Cyclooxygenase-2; Cyclophosphamide; Cyclosporine; Cyclosporine A; Cyproheptadine; Cytarabine; Cytisine; Cytokines; Cytotoxic ribonuclease PE5; D-α-Tocopheryl poly(ethylene glycol) 1000 succinate (TPGS); D,L-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; Dabigatran; Dactinomycin; Daidzein; Danshen extract (*Salvia miltiorrhiza* Bunge); Danshensu (3-(3, 4-dihydroxyphenyl) lactic acid); Danusertib; Dapagliflozin (BMS-512148); Darifenacin; Darunavir; Darusentan; Dasatinib; Daunorubicin; DDT; Debrisoquine; Deferoxamine (DFO); Delavirdine; Delphinidin; Delphinidin-3-glucoside; Deoxyglucose; Deoxyribozyme; Desethylamiodarone; Desferioxamine; Desipramine; Desloratadine; Desmethoxyyangonin; Desmosdumotin B analogs; Desvenlafaxine; Dexamethasone; Dexlansoprazole; Dexloxiglumide; Dexniguldipine; Dexrazoxane; Dianicline; Diazinon; Diazoxide; Dibenzo(a,l) pyrene; Dichlofluanid; Dichlorodiphenyl dichloroethylene; Diclofenac; Dicloxacillin; Dietary ingredients; Dietary nucleotide supplements; Diethylhexyl phthalate; Diethylstilbestrol; Digitoxin; Digoxin; Dihydrokavain; Dihydromethysticin; Dihydro-β-agarofuran sesquiterpenes from *Celastrus vulcanicola*; Diltiazem; Diltiazem analogs; Dimesna; Dimethyl sulfoxide; Dimethylnitrosamine; Dioscin; Dipeptidylpeptidase-4 inhibitors (gliptins); Diphenylene iodonium;

Diprenorphine; Dipyridamole; *Dirofilaria immitis*; Disorazole C1 and A1; Disubstituted adamantyl derivatives; Disulfiram; D,L-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol and Tetrandrine; Docetaxel; Dofequidar fumarate; Domperidone; Dopamine D_3 receptor-selective fluorenyl-amides (NGB 2904 [N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)butyl)-9H-fluorene-2-carboxamide] fumarate) and JJC 4-077 [N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)-3-hydroxybutyl)-9H-fluorene-2-carboxamide hydrochloride]); Doramectin; Dox; Doxazosin; Doxepin; Doxercalciferol (1α-hydroxyvitamin D_2); Doxorubicin; Doxycycline; Dronedarone; Duloxetine; Dysadherin; (E)-3-(Pyridin-2-ylethynyl)cyclohex-2-enone O-2-(2-(18)F-fluoroethoxy)ethyl oxime; Ebastine; Ebselen; *Echinacea purpurea*; Echinocandin antifungal drugs; Efavirenz; Efonidipine; EGFR tyrosine kinase inhibitors; EL-35; Elacridar (GF120918); Eletriptan; ELR510444; Elvitegravir; Emetine; Emodin; Endosulfan; Endothelin-1 receptor antagonists (ambrisentan, darusentan, bosentan, sitaxentan); Endotoxins; Endoxifen; Enkephalin-Leu, Ala2-melphalan methyl ester; Enniatin; Enoxacin; Entecavir; Enzastaurin; Epiberberine; Epigallocatechin gallate; Epipodophyllotoxins; Epirubicin; Eprinomectin; Equol; Ergocalciferol; Eribulin mesylate; Eribulin analogs; Erlotinib; Erythromycin; Erythromycin estolate; Erythrosine; Escitalopram; Esomeprazole; Estradiol; Estrone; Etanercept; Ethylparaoxon; Etiracetam; Etoposide; Etravirine; *Euphorbia* factor L1; Euphorbiasteroid; Everolimus; Evodiamine; Exatecan mesylate (DX-8951f); Ezetimibe; ^{18}F-Fluoroethyl GF120918; Fangchinoline; Farnesiferol A (*Ferula persica*); Felbamate; Felodipine; Fenbendazole; Fenofibrate; Fentanyl; Ferric nitrilotriacetate; Ferrocene amino acid derivative (HUNI 068); Feselol; Fexofenadine; FG020326; Fisetin; FK506; Flavones from *Hippophae rhamnoides* L; Flavonoids; Flezelastine; Flubendazole; Flucloxacillin (floxacillin); Fluconazole; Fludarabine; Fluoroaluminum; Fluoroelacridar; Fluoropyrimidine; Fluorouracil; Fluoxetine; Fluphenazine; Flurouracil; Fluvastatin; Fluvoxamine; Folic acid; Food additives; Forskolin; Fosamprenavir; Fosphenytoin; FPEPIR regimen; *Fructus Schisandrae chinensis* derivatives; Fucidin; Furanocoumarin derivatives (byakangelicol, notopterol, rivulobirin A); Furanocoumarins (furocoumarins) (grapefruit juice, *Citrus paradisi* Macf.); Furoadhyperforin; Furohyperforin; Furohyperforin isomer 1; Furohyperforin isomer 2; GA2-50; Gabapentin; Galangin; Galbanic acid (*Ferula szowitsiana*); Galectin-1; Galectin-9; Gallic acid; Gallopamil; Gamitrinibs; gamma-Secretase inhibitors; gamma-Tocotrienol; Ganciclovir; Garlic; Gastrodin (*Gastrodia elata* Blume); Gatifloxacin; GDC-0449 (2-chloro-N-(4-chloro-3-(pyridin-2-yl)phenyl)-4-(methylsulfonyl)benzamide); Gefitinib; Gemcitabine; Gemfibrozil; Genipin; Genistein; Genz-123346; GF120918; Ginger (*Zingiber officinale* Roscoe) derivatives; *Ginkgo biloba*; *Ginkgo biloba* extract EGb761; Ginkgolides; Ginseng; Ginsenosides Rd; Ginsenosides; Gleevec; Glibenclamide; Glucocorticoids; Glucosylceramide synthase; Glucosylceramide synthase inhibitors; Glufosfamide; Glutathione; Glutoxime; Glyburide; Glycolytic pyruvate; *Glycyrrhiza inflata*; Goldenseal (*Hydrastis canadensis*); Gomisin A; Gramicidin D; Granisetron; Grapefruit juice; Grapefruit juice bioflavonoids; Green tea (catechins) (epicatechin gallate, epigallocatechin gallate); Grepafloxacin; Guanfacine; Guggulsterone; H 89; H1 Tetrandrine derivative; Haishengsu (*Tegillarca granosa* extract); Halichondrin B; Halogenated xanthene food dyes; Haloperidol; Hepatitis C virus protease inhibitor S5; HEPES buffer; Herbal medicines; Heroin; HhAntag691; Histone methyltransferase MLL1; HIV-protease inhibitors; HM30181; Hoechst 33342; Honokiol; *Hoodia gordonii* (oxypregnane steroidal glycoside P57AS3); Hoodigogenin A; Hop extracts; Hop-containing products; Horse chestnut; Hydantoin; Hydramethylnon; Hydrocinchonine; Hydrocortisone; Hydroxyurea; Hydroxyzine; Hyperforin; Hypericin; *Hypericum perforatum*; Hypoxia-inducible factor 1-α inhibitor YC-1; HZ08; [^{123}I]-4-(2-(bis(4-Fluorophenyl)methoxy)ethyl)-1-(4-iodobenzyl)piperidine; (^{125}I)Iodoarylazidoprazosin; I-387 (3-(1H-Indol-2-yl)phenyl)(3,4,5-trimethoxyphenyl) methanone; Ibogaine; Ibutilide; Icariin; Idarubicin; Ifosfamide; IL-2-granzyme A chimeric protein; Imatinib; Imazalil; Imipramine; Immunosuppressants; Indacaterol; Indinavir; Indole-3-carbinol; Indomethacin; Ingenol-3-angelate (PEP005); Iron chelate (iron N-(2-hydroxy acetophenone) glycinate); Iron Dextran Complex; Insulin; Interferon-α2b; Interleukin-2;Interleukin-6; Iodoacetate; Irinotecan; Iron chelate; Isosafrole; Itraconazole; Ivermectin; Ixabepilone; Jateorhizine; Jatrorrhizine; Josamycin; K02 (morpholine-urea-Phe-Hphe-vinylsulfone); KP018; K-2-11 (amphiphilic dihydropyridine antioxidant derivative); Kaempferol; Kampo medicines; Kansui;Kava kava (*Piper methysticum*);

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Kavain; Kavalactone pharmacophores; Kavalactones; Kawain; KBH-A42; Ketoconazole; KNG-I-322; KR66222; KR66223; KT 5720; Kuguacin J; L-754,394 (N-[2(R)-hydroxy-1(S)-indanyl]-5-[2(S)-(1, 1-dimethylethylaminocarbonyl)-4-[(furo[2, 3-b]pyridin-5-yl)methyl]piperazin-1-yl]-4(S)-hydroxy-2(R)-phenylmethy lpentanamide); LAAM; *Lactobacilli*; Lactosylceramide; Lamotrigine; Laniquidar; Lansoprazole; Lapachol [2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone]; Lapatinib; L-asparaginase; Lauroylcarnitine; L-Dopa; Lecithin; Leucovorin; Levamisole; Levetiracetam; Levobupivacaine; Levofloxacin; Levonorgestrel; Levosulpiride; Levothyroxine; Licorice drink (aqueous extract of *Glycyrrhiza glabra Fabaceae*); Licorice root (kanzo); Lidocaine; Ligustrazine; Limonene; Linagliptin; Lisinopril; Lisofylline; Lithocholic acid; L-NG-Nitroarginine methyl ester (L-NAME); Lomustine; Loperamide; Lopinavir; Loratadine; Losartan; Lovastatin; LP-261; LQB-118; LSN335984; Luciferin; Lumefantrine; Lutein; Luteolin; LY294002; Ly335979; Lycopene; Lipopolysaccharide; Macelignan; Macrocyclic lactones; Macrocyclic pyridyl polyoxazoles; Macrolide antibiotics (azithromycin, erythromycin, clarithromycin, roxithromycin, and telithromycin); MAD2; Mafosfamide; Magnetic Fe_3O_4 nanoparticles; Malvidin; Malvidin-3-galactoside; Malvidin-3-glucoside; Malvidin-3,5-diglucoside; Manidipine; Mannitol; Maprotiline; Maraviroc; Marchantin C; Marine sponge-derived sipholane triterpenoids (sipholenol A, sipholenone E, sipholenol L and siphonellinol D); MC18; MC266; MC80; MDL 100907; MDR modulating agents (MC89, MC70, PB28, IG9); Mebendazole; Mefloquine; Meglitinide; Meloxicam; Melphalan; Mesalazine; Mesna; Metalloprobes; Metazachlor; Metformin; Methadone; Methotrexate; Methoxymorpholinyl doxorubicin; Methylcholanthrene; Methylenedioxymethamphetamine (MDMA); Methylprednisolone; Methylxanthine derivatives (pentoxifylline and lisofylline); Methysticin; Metoclopramide; Metolachlor; Metoprolol; Metronidazole; Mibefradil; Micafungin; Miconazole; Midazolam; Mifepristone; Milbemycin compounds; Milk thistle (*Silybum marianum*); Miltefosine; Mirtazapine; Mistletoe; Mithramycin; Mitomycin; Mitomycin C; Mitoxantrone; MK-0731; MK-3207 (2-[(8R)-8-(3,5-Difluorophenyl)-10-oxo-6,9-diazaspiro[4.5]dec-9-yl]-N-[(2R)-2′-oxo-1,1′,2′,3-tetrahydrospiro[indene-2,3′-pyrrolo[2,3-b]pyridin]-5-yl]acetamide); MK-0731; MK571; MM80; Montelukast; Morin; Morphine; Morphine sulfate; Mosapride; Mycophenolate mofetil; Mycophenolic acid; Myricetin; N(α)-Boc-l-Asp(OBn)-l-Lys(Z)-OtBu (reversin 121); N-(3-Iodobenzyl)-adenosine-5′-N-methylcarboxamide (A3 adenosine receptor (A3AR) agonist); N-[(4R)-6-(4-Chlorophenyl)-7-(2,4-dichlorophenyl)-2,2-dimethyl-3,4-dihydro-2H-pyrano[2,3-b]pyridin-4-yl]-5-methyl-1H-pyrazole-3-carboxamide (MK-5596); N-(4-[2-(1,2,3,4-Tetrahydro-6,7-dimethoxy-2-isoquinolinyl) ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918); Nadolol; Naloxone; Naltrexone; Nanoparticles; Naringin; Naringenin; N-Arylalkyl-3,4-diaryl-substituted pyrrole-2,5-diones; N-Desmethyl imatinib; N-Desmethyl-loperamide; Naphthoquinone derivatives; Nefazodone; Neferine; Nelfinavir; Nemadectin; Nevirapine; Nicardipine; Nicotine; Nifedipine; Nifedipine analogs; Nifurtimox; Nigericin; Nilotinib; Nimesulide; Nisoldipine; Nitrendipine; Nitric oxide; Nitrofurantoin; Nitroxynil; Nizatidine; NK 104; N,N-bis(Alkanol)amine aryl esters; N,N-bis(Cyclohexanol)amine aryl esters; N,N-Diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine HCl (DPPE); Nobiletin; Nocardioazines; N-Octyl-O-sulfate chitosan (NOSC) micelles; Non-ionic surfactants (cremophor EL, cremophor RH 40, polysorbate 80, vitamin E TPGS 1000, pluronic PE 10300 and sucrose ester L-1695); Non-nucleoside reverse transcriptase inhibitors; NOR5; Norbuprenorphine; Nordihydroguaiaretic acid; Noroxycodone; Nortriptyline; Norverapamil; NS-398; NSC 613060; NSC77037; Notopterol; Nutlin-1; O-(4-Ethoxyl-butyl)-berbamine; OC144-093; OC144-193; *Oenothera paradoxa* defatted seed extract (pentagalloylglucose, gallic acid, (+)-catechin, procyanidin); Ofloxacin; Olanzapine; Olaparib; Omeprazole; Ondansetron; Opioid analgesics; Opioid receptor agonists; Opioids; Opioid receptor agonists; Oral anticoagulants (dabigatran, rivaroxaban, apixaban); Orange juice flavones (3,3′,4′,5,6,7,8-heptamethoxyflavone, 4′,5,6,7,8-pentamethoxyflavone (tangeretin), 3′,4′,5,6,7,8-hexamethoxyflavone (nobiletin)); Orange juice-Seville; Organochlorine insecticides; Orphan nuclear receptors; Oseltamivir; Oxaborole 6-carboxamides; Oxaliplatin; Oxcarbazepine; Oxfendazole; Oxybutynin; Oxycodone; Oxymatrine; P1075; Paclitaxel; Paliperidone; Palmatine;

Palmitoylcarnitine; Pantoprazole; Papyriferic acid derivatives; Parathion; Paroxetine; Patupilone; Pazopanib; PD98059; PEG-400; Pegylated phosphotidylethanolamine; Pelargonidin; Pelargonidin-3,5-diglucoside; Pentagalloylglucose; Pentazocine; Pentoxifylline; Peonidin; Peonidin-3-glucoside; Peptidomimetic inhibitors; Permethyl ningalin B analogs; Perphenazine; Pesticides; Petunidin; P-glycoprotein inhibitors; Phalloidine; Phenethyl isothiocyanate; Phenobarbital; Phenothiazines; Phenylcinnamides; Phenylsulfonylfuroxans; Phenytoin; Phloretin; Phloxine; Phosphatidylcholine; Phosphatidylinositol 3-kinase inhibitor GDC-0941 (2-(1H-Indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thieno[3,2-d] pyrimidine); Phosphines; Phycocyanin; Phytochemicals; Phytohormones (Indole-3-acetic acid; Brassinosteroid); Phytosterols; Pimozide; *Piper cubeba*; *Piper nigrum* fruit; Piperine (black pepper); Pirarubicin; Pitavastatin; PKC412; PKD2; Plant extracts; Plastochromanol 8; Plastoquinone-Decylrhodamine 19 conjugate (SkQR1); Platinum; Pluronic; Pluronic block copolymer p85; Pluronic F68 block copolymer; PNU-288034 (N-({(5S)-3-[4-(1,1-dioxidothiomorpholin-4-yl)-3,5-difluorophenyl]-2-oxo-1,3-oxazolidin-5-yl}methyl)acetamide); Podophyllotoxin derivatives (4-[4”-(2”, 2”, 6”, 6”-tetramethyl-l”-piperidinyloxy) amino]-4′-demethyl epipodophyllotoxin)(GP7)(YB-1EPN); Poly(ethylene glycol)-conjugated multi-walled carbon nanotubes; Poly[ethylene oxide]-poly[propylene oxide] [PEO-PPO] amphiphiles; Polyethyleneimine (PEI)/all-trans retinoic acid (ATRA) conjugates; Poly[ethylene oxide]-poly[propylene oxide] [PEO-PPO] amphiphiles; Polyinosinic/polycytidylic acid; Polyisohexylcyanoacrylate; Polymeric inhibitors; Polymethyoxyflavonoids (tangeretin, nobiletin, baicalein, wogonin, quercetin, epigallocatechin gallate); Polyphenols from *Mangifera indica* (mango stem bark extract) (mangiferin, norathyriol, catechin, gallic acid and quercetin); Posaconazole; Prasugrel; Pravastatin; Praziquantel; Prazosin; Prednisolone; Prednisone; Pregnenolone carbonitrile; Prenyloxycinnamic acids (Boropinic acid, 4′-Isopentenyloxy-p-coumaric acid); Prilocaine; Primaquine; Probenecid; Probiotics; Prochlorperazine; Procyanidin; Progesterone; Progesterone-adenine hybrids; Promethazine; Propafenone; Propiconazole; Propiverine; Propranolol; Proteasome inhibitor TP-110; Protoberberine alkaloids from *Coptidis Rhizoma* (berberine, palmatine, coptisine, epiberberine and jatrorrhizine); Proton pump inhibitors; Prucalopride; Prulifloxacin; PSC833 (valspodar); Psoralen; Puerarin (*Pueraria lobata*); Puromycin; PXR agonists; PXR antagonists; Pyrrolidine dithiocarbamate; Pyrrolo-1,5-benzoxazepines; Pyrrolopyrimidine; PZ-39 (N-(4-chlorophenyl)-2-[(6-{[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]amino}-1,3-benzothiazol-2-yl)sulfanyl]acetamide); Quaternary ammonium compounds; Quercetin; Quetiapine; Quinacrine; Quinidine; Quinidinium; Quinine; (R)-(-)-2-chloro-N-[1-^{11}C-propyl]n-propylnorapomorphine; Rab4; Rabeprazole; Rafoxanide; Raloxifene; Raltegravir; Ranitidine; Ranolazine; Rapamycin; Razoxane; Rebamipide; Red wine; Repaglinide; Reserpine; Resveratrol; Retinoid acid; Reversin-205; Rhei Rhizoma extract; Rhodamine 123 (6-amino-9-(2-methoxycarbonylphenyl)xanthen-3-ylidene]azanium chloride); *Rhodiola rosea*; Riccardin D; Rifampicin (rifampin); Rikkunshito; Riluzole; Risperidone; Ritonavir; Rivaroxaban; Rivulobirin A; Roflumilast; Romidepsin; Ropivacaine; Roscovitine; Rose Bengal; Rosemary phytochemicals (carnosic acid, carnosol, rosmarinic acid, ursolic acid); Rosiglitazone; Rosmarinic acid; Rostafuroxin; Rosuvastatin; Rotenone; Roxithromycin; RU486 (mifepristone); Rutin; S14506 (1-[2-(4-fluorobenzoylamino)ethyl]-4-(7-methoxy-naphthyl)piperazine); Sakuranetin; Salinomycin; Salvinorin A; Saquinavir; Saquinavir-NO; Saxagliptin; SB T-1214; SC236; Schisandra fruit; *Schisandra sphenanthera* extract (Wuzhi); Schisandrol A; Schisandrol B; Scillarenin; Scutellarein; Scutellarin; Scorpion alcoholic extraction; SCY-635; Sea-Nine 211; Seliciclib (R-roscovitine); Senkyu-cha-cho-san; Sertraline; Sesamin; Sesquiterpenes; Sesquiterpenes from *Celastraceae*; Sesquiterpene coumarins (*Ferula species*); Sestamibi; Siamois polyphenols (quercetin); Sigma-2 agonists; Sildenafil; Silodosin; Silvestrol; Silybin; Silybinin; Silymarin; Simvastatin; Sirolimus; Sitagliptin; Sitaxentan; Sitosterol; SJC-136; S-Methyl N,N-diethylthiocarbamate sulfone; S-Methyl N,N-diethylthiocarbamate sulfoxide; SN-38; Sodium arsenite; Sodium azide; Sodium nitroprusside; Sodium valproate; Sokei-kakketsu-to; Solifenacin; Sorafenib; Sorcin; SP1049C-doxorubicin-pluronics; SP600125; Sparfloxacin; Spinosad; Spironolactone; SR47063; St. John’s wort (*Hypericum perforatum*) (hyperforin, hypericin, quercetin); STAT3 decoy oligodeoxynucleotides; Statins; Staurosporine; Stearic acid-g-chitosan polymeric micelle;

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Stemona alkaloids (stemocurtisine, oxystemokerrine, stemofoline); Steroid carbamates (11-carbamic acid N,N-dibenzyl progesterone ester and 11-carbamic acid N,N-dibutyl progesterone ester); Steroid carbamates (11-carbamic acid N,N-dibenzyl progesterone ester and 11-carbamic acid N,N-dibutyl progesterone ester); Stigmasterol; Stilbenes; Streptozotocin; S-Trityl-l-cysteine derivatives; Succinimidyl ester (bodipy-FL)-verapamil; Sucralose (Splenda); Sufentanil; Sulfasalazine; Sulfisoxazole; Sulfonylurea receptor (ABCC8/9) ligands; Sulindac; Sunitinib; Syl611 (semisynthetic taxane derivative); Tacrolimus; Talinolol;Tamoxifen; Tandutinib; Tangeretin; Tariquidar; Tanshinones from *Salvia miltiorrhiza* (cryptotanshinone, dihydrotanshinone, tanshinone I, tanshinone IIA); Tariquidar (XR9576); Taurocholic acid; Tauromuricholic acid; Tauroursodeoxycholic acid; Taxanes; Taxol; Taxotere; Tea tree oil (terpinen-4-ol); Tebuconazole; Technetium ^{99m}Tc sestamibi; Tectochrysin; Telatinib; Telithromycin; Telmisartan; Temozolomide; Temsirolimus; Teniposide; Terameprocol; Terazosin; Terfenadine; Terpenic compounds from *Euphorbia lagascae* and *E. tuckeyana* methanolic extracts (21α-hydroxytaraxasterol, 21α-hydroxytaraxasterol acetate, 3β,30-dihydroxy-20(21)-taraxastene, 3β-hydroxy-20-taraxasten-30-al, stigmastane-3,6-dione, ergosterol peroxide); Tesmilifene; Testosterone; Tetrabenazine; Tetrachlorodibenzodioxin; Tetracycline; Tetrahydrobenzo[4′,5′]thieno[3′,2′:5,6]pyrido[4,3-d]pyrimidin-4(3H)-ones; Tetrahydrocannabinol; Tetrahydroisoquinoline-ethyl-phenylamine; Tetramethylpyrazine (*Ligusticum chuanxiong* Hort); Tetramethylrosamine; Tetrandrine; Thai plant extracts; Thalidomide; Thapsigargin; Thiabendazole; Thiazolyl blue; Thioacetamide; Thiodicarb; Thiophene amide derivatives; Thioridazine; Thymocytes; Ticagrelor; Tilidine; Tipifarnib; Tipranavir; Tocofersolan; Tocopherol (Vitamin E); Tocotrienols; Tolbutamide; Tolterodine; Tolvaptan; Tolylfluanid; Topiramate; Topotecan; Toremifene; TPGS; Trabectedin; TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) and TRAIL death receptor 5 (DR5); Tramadol; trans-3,5,3′,4′-Tetramethoxystilbene; Transkingdom RNAi; Trazodone; Tretinoin; Triamcinolone; Trichloroethylene; Triclabendazole; Triclabendazole sulfone; Triclabendazole sulfoxide; Trifluoperazine; Trifluopromazine; Trimethoprim; Trimipramine; Triptolide (*Tripterygium wilfordii*); [tris(1,10-Phenanthroline)lanthanum(III)]trithiocyanate (KP772; FFC24); Triterpenoid saponins; Trofosfamide; Troglitazone; Tropisetron; Trospium; Tryptanthrine; Tulathromycin; Tween-80; Tyrosine kinase inhibitors; U 0126; Udenafil; UIC2 antibody; UK-343,664; UK-369,003; Ursodeoxycholic acid; Ursodiol; Ursolic acid; Uvaol; Valerian; Valinomycin; Valproic acid; Valspodar (PSC-833); Vanadate; Varenicline; Vascular endothelial growth factor (VEGF); Vecuronium; Veliparib (ABT-888); Venlafaxine; Verapamil; Verlukast; Vicriviroc; Vinblastine; Vinca alkaloids; Vincristine; Vindesine; Vinorelbine; Violaxanthin; Vitamin A; Vitamin D; Vitamin D_3; Vitamin D_3 metabolite 1,25-dihydroxycholecalciferol (DHC); Vitamin E; Vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate); VMY-1-101; VMY-1-103; Voriconazole; VP-16; W232R suppressor; Warfarin; Wellsolve; Withaferin A;Wogonin (*Scutellaria baicalensis*); Xanthene food dyes (rose bengal, phroxine, amaranth, erythrosine B, allura red, new coccine, acid red, tartrazine, sunset yellow FCF, brilliant blue FCF, and indigo carmine); Xanthine derivatives; Xenobiotics; Ximelagatran; XR9576; Yangonin; YC-1(3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole); Yohimbine; Yokukansan; YQ36 (3-(1-methyl-1H-indol-3-yl)-1-phenyl-4-(1-(3-(piperidin-1-yl)propyl)-1H-pyrazolo[3,4-b]pyridine-3-yl)-1H-pyrrole-2,5-dione)(bisindolylmaleimide analog); Yttrium complex; Zampanolide; Zearalenone; Zhi Shi; Zidovudine; Zilongjin; Zinc (II) complex; Zosuquidar; α_{2C}-Adrenoceptor agonists; $\Delta^{5,6}\Delta^{12,13}$-Jatrophane diterpenes; Δ^{9}-Tetrahydrocannabinol

Substrates
2-Pyridylphenyl amides (CJB 090 [N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)butyl)-4-(pyridine-2-yl)benzamide hydrochloride]; 3α,6α,7α,12α-Tetrahydroxy-cholanoyl taurine; 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid; 5-Fluorouracil; 6,7-Dimethoxy-2-(6-methoxy-naphthalen-2-ylmethyl)-1,2,3,4-tetrahydroisoquinoline (MC80); 6,6,8-Triethyldesmosdumotin B; 7-Xylosyl-10-deacetylpaclitaxel; ABT-263; ABT-378; ABT-737; Acenocoumarol; Actinomycin D; Adjudin; Adriamycin; Albendazole; Aldosterone; Aliskiren; alpha-Methyldigoxin; Altanserin; Ambrisentan; Amiloride; Amiodarone; Amitriptyline; Amlodipine; Amoxicillin; Amprenavir; Amyloid-beta (Aβ) peptides; Anthracyclines; Antiplatelet agents; Apixaban; Aripiprazole; Artemisinin; Atazanavir; Atorvastatin; Atovaquone; Aurones; Averolimus; Axitinib; AZD5672 (N-(1-{(3R)-3-(3,5-difluorophenyl)-3-[4-methanesulfonylphenyl]propyl}piperidin-4-yl)-N-ethyl-2-[4-methanesulfonylphenyl]acetamide); Azelastine; Azithromycin; BACE-1 inhibitors; Barbituric acid; Bazedoxifene; Bendamustine; Berberine; beta-Acetyldigoxin; bis(12)-Hupyridone; Betamethasone; Bisantrene; BMS-310705; BMS-690514; Bodipy-FL-forskolin; Bodipy-FL-prazosin; Bodipy-FL-vinblastine; Bortezomib; Bosentan; Bromperidol; Buagafuran; Budesonide; Bunitrolol; Bupropion; Cabazitaxel; Cabergoline; Calcein-AM; Calcineurin inhibitors; Carbamazepine; Carboplatin; Carotenoids; Carvedilol; Celiprolol; Ceramide; Cetirizine; Chargrilled meat diet; Chloro benztropine analogs; Chloroquine; Chlorpromazine; Cimetidine; Ciprofloxacin; Cisplatin; Citalopram; Clarithromycin; Clopidogrel; Clorsulon; Closantel; Clozapine; Coelenterazine; Colchicine; Concanavalin A; Copper pyrithione; Coptisine; Corticoids; Corticosteroids; Cortisol; *Crossostephium chinense*-related flavonoids (selagin, apometzgerin, tricetin-3′,4′,5′-trimethylether, quercetagetin-3,6,7-trimethylether, hispidulin, quercetagetin); Curcumin; Cyclophosphamide; Cyclosporine; Cyclosporine A; Cytarabine; Cytokines; Dabigatran; Danshensu (3-(3, 4-dihydroxyphenyl) lactic acid); Dapagliflozin (BMS-512148); Darifenacin; Darunavir; Darusentan; Dasatinib; Daunorubicin; Debrisoquine; Deferoxamine (DFO); Delavirdine; Desloratadine; Desmethoxyyangonin; Dexamethasone; Dexloxiglumide; Diazinon; Diazoxide; Dichlofluanid; Dicloxacillin; Digitoxin; Digoxin; Dihydromethysticin; Diltiazem; Dimesna; Diprenorphine; Dipyridamole; Disorazole C1 and A1; Disubstituted adamantyl derivatives; Disulfiram; Docetaxel; Domperidone; Dopamine D_3 receptor-selective fluorenyl-amides (NGB 2904 [N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)butyl)-9H-fluorene-2-carboxamide] fumarate); Doramectin; Dox; Doxepin; Doxorubicin; Doxycycline; Dronedarone; Duloxetine; (E)-3-(Pyridin-2-ylethynyl)cyclohex-2-enone O-2-(2-(18)F-fluoroethoxy)ethyl oxime; Ebastine; Efavirenz; EGFR tyrosine kinase inhibitors; Eletriptan; Elvitegravir; Emetine; Endoxifen; Enoxacin; Entecavir; Epipodophyllotoxins; Epirubicin; Eprinomectin; Equol; Eribulin mesylate; Eribulin analogs; Erlotinib; Erythromycin; Escitalopram; Eslicarbazepine; Esomeprazole; Estradiol; Etoposide; Euphorbiasteroid; Everolimus; Exatecan mesylate (DX-8951f); Ezetimibe; Felbamate; Fentanyl; Fexofenadine; FG020326; FK506; Flavonoids; Flucloxacillin (floxacillin); Fluvastatin; Fluvoxamine; Fosamprenavir; Fosphenytoin; *Fructus Schisandrae chinensis* derivatives; GA2-50; Gabapentin; Ganciclovir; Gatifloxacin; gamma-Secretase inhibitors; Gefitinib; Gemcitabine; Genipin; Genistein; Genz-123346; Ginger (*Zingiber officinale* Roscoe) derivatives; *Ginkgo biloba*; *Ginkgo biloba* extract EGb761; Ginkgolides; Gleevec; Glibenclamide; Glucocorticoids; Glucosylceramide synthase inhibitors; Glufosfamide; Glyburide; Goldenseal (*Hydrastis canadensis*); Gomisin A; Gramicidin D; Granisetron; Grepafloxacin; Halichondrin B; Hepatitis C virus protease inhibitor S5; Heroin; Hoechst 33342; *Hoodia gordonii* (oxypregnane steroidal glycoside P57AS3); Hoodigogenin A; Hydrocortisone; [^{123}I]-4-(2-(bis(4-Fluorophenyl)methoxy)ethyl)-1-(4-iodobenzyl)piperidine; Icariin; Idarubicin; Ifosfamide; Imatinib; Indacaterol; Indinavir; Ingenol-3-angelate (PEP005); Irinotecan; Iron Dextran Complex; Itraconazole; Ivermectin; Ixabepilone; Jateorhizine; JJC 4-077 [N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)-3-hydroxybutyl)-9H-fluorene-2-carboxamide hydrochloride]); K02 (morpholine-urea-Phe-Hphe-vinylsulfone); Kaempferol; Kava kava (*Piper methysticum*); Ketoconazole; KNG-I-322 (desmosdumotin B derivative); KP018; KR66222; KR66223; Kuguacin J; LAAM; L-asparaginase; Lamotrigine; Lansoprazole; Lapatinib; L-Dopa; Levamisole; Levetiracetam; Levobupivacaine; Levofloxacin; Levosulpiride; Levothyroxine; Ligustrazine; Lisinopril; Lisofylline;

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Table 5
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Loperamide; Lopinavir; Loratadine; Losartan; Lovastatin; Luciferin; Macrocyclic lactones (ivermectin, eprinomectin, and moxidectin); Macrolide antibiotics (azithromycin, erythromycin, clarithromycin, roxithromycin and telithromycin); Mafosfamide; Malvidin; Malvidin-3-galactoside; Manidipine; Maraviroc; MC266; MDL 100907; Mebendazole; Mefloquine; Meglitinide; Melphalan; Mesalazine; Mesna; Metalloprobes; Metformin; Methadone; Methotrexate; Methoxymorpholinyl doxorubicin; Methylenedioxymethamphetamine (MDMA); Methylprednisolone; Methylxanthine derivatives (pentoxifylline and lisofylline); Methysticin; Mibefradil; Micafungin; Milk thistle (*Silybum marianum*); Mirtazapine; Mithramycin; Mitomycin; Mitomycin C; Mitoxantrone; MM80; Morphine sulfate; Moxidectin; Mycophenolate mofetil; N-(3-Iodobenzyl)-adenosine-5′-N-methylcarboxamide (A3 adenosine receptor (A3AR) agonist); N-[(4R)-6-(4-Chlorophenyl)-7-(2,4-dichlorophenyl)-2,2-dimethyl-3,4-dihydro-2H-pyrano[2,3-b]pyridin-4-yl]-5-methyl-1H-pyrazole-3-carboxamide (MK-5596); N,N-Diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine; N-Desmethyl imatinib; N-Desmethyl-loperamide; Nadolol; Nelfinavir; Nevirapine; Nicardipine; Nifedipine; Nilotinib; Nitrofurantoin; Nitroxynil; Nizatidine; Nortriptyline; NSC 613060; Nutlin-1; Olanzapine; Omeprazole; Ondansetron; Opioids; Opioid receptor agonists; Oral anticoagulants (dabigatran, rivaroxaban, apixaban); Oseltamivir; Oxaliplatin; Oxfendazole; Oxycodone; P1075; Paclitaxel; Paliperidone; Palmatine; Pantoprazole; Paroxetine; Pazopanib; Pentazocine; Pentoxifylline; Pesticides; Petunidin; PG 01037 [N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)-trans-but-2-enyl)-4-(pyridine-2-yl)benzamide hydrochloride]); Phenobarbital; Phenytoin; Phosphatidylinositol 3-kinase inhibitor GDC-0941 (2-(1H-indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thieno[3,2-d] pyrimidine); Piperine; Pirarubicin; Pitavastatin; Plant extracts; Plastoquinone-Decylrhodamine 19 conjugate (SkQR1); Platinum; PNU-288034 (N-({(5S)-3-[4-(1,1-dioxidothiomorpholin-4-yl)-3,5-difluorophenyl]-2-oxo-1,3-oxazolidin-5-yl}methyl)acetamide); Podophyllotoxin derivatives (4-[4”-(2”, 2”, 6”, 6”-tetramethyl-l”-piperidinyloxy) amino]-4′-demethyl epipodophyllotoxin)(GP7)(YB-1EPN); Prasugrel; Pravastatin; Praziquantel; Prazosin; Prednisolone; Prednisone; Probenecid; Prochlorperazine; Progesterone; Propafenone; Propranolol; Propiverine; Proton pump inhibitors; Prucalopride: Puromycin; Quaternary ammonium compounds; Quetiapine; Quinacrine; Quinidine; Quinine; (R)-(-)-2-Chloro-N-[1-^{11}C-propyl]n-propylnorapomorphine; Rabeprazole; Rafoxanide; Raloxifene; Raltegravir; Ranitidine; Ranolazine; Rapamycin; Resveratrol; Rhodamine 123; Rifampicin (rifampin); Riluzole; Risperidone; Ritonavir; Rivaroxaban; Romidepsin; Ropivacaine; Rosiglitazone; Rosuvastatin; Roxithromycin; S14506 (1-[2-(4-fluorobenzoylamino) ethyl]-4-(7-methoxy-naphthyl)piperazine);Salvinorin A; Saquinavir; Saquinavir-NO; Saxagliptin; Schisandra fruit; Schisandrol A; Scutellarein; Scutellarin; SCY-635; Seliciclib (R-roscovitine); Sea-Nine 211; Sertraline; Sestamibi; Silybin; Silodosin; Silvestrol; Simvastatin; Sirolimus; Sitagliptin; SN-38; Sodium arsenite; Sodium valproate; Solifenacin; Sorafenib; Sparfloxacin; Spinosad; SR47063; St. John’s wort (*Hypericum perforatum*); Succinimidyl ester (bodipy-FL)-verapamil; Sucralose (Splenda); Sulfasalazine; Sunitinib; Tacrolimus; Talinolol; Tamoxifen; Tandutinib; Tangeretin; Taxanes; Taxol; Taxotere; Telatinib; Telithromycin; Temsirolimus; Teniposide; Terfenadine; Tetracycline; Tetrandrine; Thiabendazole; Ticagrelor; Tilidine; Tipifarnib; Tocopherol (Vitamin E); Tocotrienols; Tolbutamide; Tolterodine; Tolvaptan; Tolylfluanid; Topiramate; Topotecan; Trabectedin; Tramadol; Triamcinolone; Triclabendazole; Triclabendazole sulfone; Triclabendazole sulfoxide; Trimipramine; Trofosfamide; Tropisetron; Trospium; Tyrosine kinase inhibitors; Udenafil; UK-343,664; UK-369,003; Ursodeoxycholic acid; Ursodiol; Valinomycin; Valproic acid; Valspodar; Vecuronium; Veliparib (ABT-888); Venlafaxine; Verapamil; Vicriviroc; Vinblastine; Vinca alkaloids; Vincristine; Vindesine; Vinflunine; Vinorelbine; Vitamin A; VP-16; Warfarin; Ximelagatran; Zearalenone; Zidovudine; α_{2C}-Adrenoceptor agonists

Inhibitors
(+)-Catechin; 1-Cyclohexyl-4-(4-arylcyclohexyl)piperazines; 1-d-Ribofuranosylbenzimidazole; 1,3,5-Triphenyl-2-pyrazolines; 1,4-Dihydropyridines; 2n-Propylquinoline; 2-Arylthiazolidine-4-carboxylic acid amides (ATCAA); (2R)-Anti-5-{3-[4-(10,11-Difluoromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride (LY335979); 2-Deoxy-D-glucose; 2,4-Dinitrophenyl-S-glutathione; 3-Benzyloxyfluorenes; 3′R,4′R-Disubstituted-2′,2′-dimethyldihydropyrano[2,3-f]chromone (DSP) analogs; (3-(1H-Indol-2-yl)phenyl)(1H-indol-2-yl)methanone; 4β-Anilino-podophyllotoxin derivatives; 4,11-bis[(2-Aminoethyl)amino]anthra[2,3-b]furan-5,10-diones; 4-Ethyl 5-(3,4-Methylenedioxyphenyl)-2E,4E-pentadienoic acid piperidide (PA-1); 4-Substituted methoxybenzoyl-aryl-thiazole; 4′-Geranyloxyferulic acid; 5-Aza-2′-deoxycytidine; 5-Bromotetrandrine; 5-Cyclohexylindolyl-2′-deoxyribose (non-natural nucleoside); 5′-Fluorosulfonylbenzoyl 5′-adenosine; 6-[(2S,4R,6E)-4-Methyl-2-(methylamino)-3-oxo-6-octenoic acid]cyclosporine D (PSC833); 6-Thioguanine; 6,7-Dimethoxy-2-{3-[4-[^{11}C]methoxy-3,4-dihydro-2H-naphthalen-(1E)-ylidene]-propyl}-1,2,3,4-tetrahydro-isoquinoline; 8-Prenylnaringenin; 20S-Ginsenoside Rh2; 23-Hydroxybetulinic acid from *Pulsatilla chinensis* (Bunge) Regel; A3 Adenosine receptor agonist 2-chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide; [^{11}C]XR9576; [^{11}C]Methyl 4-((4-(2-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl)phenyl)amino-carbonyl)-2-(quinoline-2-carbonylamino)benzoate; Abamectin; Acanthopanax senticosus HARMS extract; Acylphloroglucinols; AG1478 (Tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline); Alfentanil; Alkylbenzene sulfonate; Allyl methyl disulfide; Ametantrone; Amiloride; Amiodarone; Amitriptyline; Amlodipine; Amodiaquine; Amurensin G; Antheraxanthin; Anthrafurandiones; Anthranilamide modulators; Anthraquinone; Apatinib (YN968D1); Apigenin; Aranidipine; Artesunic acid; Astemizole; AT80; Atazanavir; Atorvastatin; Atovaquone; AZD5672 (N-(1-{(3R)-3-(3,5-difluorophenyl)-3-[4-methanesulfonylphenyl]propyl}piperidin-4-yl)-N-ethyl-2-[4-methanesulfonylphenyl]acetamide); Azelastine; Azidopine; Azithromycin; BACE-1 inhibitors; Baicalein; Barnidipine; Beauvericin; Benidipine; Benzo(a)quinolizin-4-ones; Benzocaine; Bepidil; Berberine; Bergamottin; Bergapten; Bergaptol; Betti-base derivatives of tylosin (N-tylosil-1-alpha-amino-(3-bromophenyl)-methyl-2-naphthol); Bevacizumab; BIBF 1120; Biochanin A; Biricodar; bis(4-Fluorobenzyl)trisulfide; Boiogito; Bromocriptine; Buprenorphine; Byakangelicol; Calcium antagonists; Calcium channel blockers; Candesartan-Cilexetil; Capsaicin; Capsanthin; Capsorubin; CAR antagonists; Carbamazepine; Carnosic acid; Carnosol; Carotenoids; *Carpobrotus edulis*; Carvedilol; Catechins from green tea; CBT-1; Celecoxib; Cetuximab; Changweiqing; Chelerythrine; Chloroquine; Chlorpromazine; Chrysin; Cimetidine; Cinchonine; Cineole; Cisapride; Citral; CJZ3 (lomerizine derivative); Clarithromycin; Class III antiarrhythmic agents; Clerodane-type diterpenoid from *Sindora sumatrana*; Clozapine; Common sage; Common valerian; Copper N-(2-hydroxyacetophenone) glycinate; Coptisine; Cortisol; C-Phycocyanin; Cremophor EL; Cremophor RH 40; *Curcuma heyneana*; *Curcuma longa*; *Curcuma zedoaria*; Curcumenol; Curcumin; Curcuminoids; Curcumin analogs; Cyanidin; Cyanidin-3-galactoside; Cyanidin-3-glucoside; Cyanidin-3-rutinoside; Cyclosporine; Cyclosporine A; Cyproheptadine; D-α-Tocopheryl poly(ethylene glycol) 1000 succinate (TPGS); Danshen extract (*Salvia miltiorrhiza* Bunge); Dapagliflozin (BMS-512148); Darifenacin; Darunavir; Dasatinib; Daunorubicin; Delavirdine; Delphinidin; Delphinidin-3-glucoside; Deoxyribozyme; Desethylamiodarone; Desferioxamine; Desipramine; Desmethoxyyangonin; Dexamethasone; Dexloxiglumide; Dexniguldipine; Dexrazoxane; Diazinon; Diclofenac; Dihydro-β-agarofuran sesquiterpenes from *Celastrus vulcanicola*; Dihydrokavain; Dihydromethysticin; Diltiazem; Diltiazem analogs; Dioscin; Diphenylene iodonium; Dipyridamole; Disulfiram; D,L-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol and Tetrandrine; Dofequidar fumarate; Doramectin; Doxazosin; Dronedarone; Duloxetine; Echinacea; Efonidipine; EL-35; Elacridar (GF120918); Elvitegravir; Emetine; Enniatin; Epiberberine; Epigallocatechin gallate; Eribulin mesylate; Erythromycin; Erythrosine; Escitalopram; Esomeprazole; Estradiol; Etanercept; Etravirine; *Euphorbia* factor L1; Euphorbiasteroid; Everolimus; ^{18}F-Fluoroethyl GF120918 and XR9576; Fangchinoline; Farnesiferol A (*Ferula persica*); Felodipine; Fenofibrate; Fentanyl; Ferrocene amino acid derivative (HUNI 068); Feselol; FG020326; Fisetin; FK506; Flavonoids; Flezelastine; Fludarabine; Fluoroelacridar; Fluoxetine; Fluphenazine; Fluvoxamine;

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Table 5
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Fucidin; Furanocoumarin derivatives (byakangelicol, notopterol, rivulobirin A); Furanocoumarins (furocoumarins) (grapefruit juice, *Citrus paradisi* Macf.); Furoadhyperforin; Furohyperforin; Furohyperforin isomer 1; Furohyperforin isomer 2; Gabapentin; Galbanic acid (*Ferula szowitsiana*); Gallic acid; Gallopamil; Gamitrinibs; Garlic; GDC-0449 (2-chloro-N-(4-chloro-3-(pyridin-2-yl)phenyl)-4-(methylsulfonyl)benzamide); Gefitinib; Gemfibrozil; Genipin; Genz-123346; GF120918; *Ginkgo biloba*; *Ginkgo biloba* extract EGb761; Ginkgolides; Ginseng; Ginsenosides Rd; Ginsenosides; Glucosylceramide synthase inhibitors; Glutoxime; Glyburide; *Glycyrrhiza inflata*; Goldenseal (*Hydrastis canadensis*); Gomisin A; Gramicidin D; Grapefruit juice; Green tea (catechins) (epicatechin gallate, epigallocatechin gallate); Guggulsterone; H1 Tetrandrine derivative; Halogenated xanthene food dyes; Haloperidol; Herbal medicines; HhAntag691; HM30181; Honokiol; Hop-containing products; Hop extracts; Horse chestnut; Hydantoin; Hydrocinchonine; Hydrocortisone; Hydroxyzine; Hyperforin; Hypericin; Hypoxia-inducible factor 1-α inhibitor YC-1; HZ08; I-387 (3-(1H-indol-2-yl)phenyl)(3,4,5-trimethoxyphenyl) methanone; Ibogaine; IL-2-granzyme A chimeric protein; Imatinib; Imipramine; Immunosuppressants (cyclosporine, everolimus, mycophenolate mofetil, sirolimus, tacrolimus); Indomethacin; Ingenol-3-angelate (PEP005); Interleukin-2; Iodoacetate; Irbesartan; Iron chelate (iron N-(2-hydroxy acetophenone) glycinate); Itraconazole; Ivermectin; Ixabepilone; Jatrorrhizine; Josamycin; K-2-11 (amphiphilic dihydropyridine antioxidant derivative); Kaempferol; Kampo medicines; Kansui; Kava kava (*Piper methysticum*); Kavalactones; Kavalactone pharmacophores; Kavain; Kawain; Ketoconazole; KNG-I-322 (desmosdumotin B derivative); Kuguacin J; Lamotrigine; Laniquidar; Lansoprazole; Lapachol [2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone]; Lapatinib; Lauroylcarnitine; Levetiracetam; Levothyroxine; Lidocaine; Licorice root (kanzo); Ligustrazine; Limonene; Lipopolysaccharide; Lithocholic acid; Loperamide; Lopinavir; Loratadine; Lovastatin; LP-261; LQB-118; LSN335984; Lutein; LY294002; Lycopene; Macelignan; Macrocyclic pyridyl polyoxazoles; Macrolide antibiotics (azithromycin, erythromycin, clarithromycin, roxithromycin and telithromycin); Magnetic Fe_3O_4 nanoparticles; Malvidin; Malvidin-3-galactoside; Malvidin-3-glucoside; Malvidin-3,5-diglucoside; Mangiferin; Maprotiline; Marchantin C; Marine sponge-derived sipholane triterpenoids (sipholenol A, sipholenone E, sipholenol L and siphonellinol D); MC18; MDR modulating agents (MC89, MC70, PB28, IG9); Mefloquine; Metformin; Methadone; Methysticin; Mibefradil; Micafungin; Miconazole; Midazolam; Mifepristone; Milbemycin compounds; Milk thistle (*Silybum marianum*); Myricetin; Mistletoe; Mitomycin C; MK-0731; MK571; Montelukast; Morin; Mosapride; Mycophenolate mofetil; N-(4-[2-(1,2,3,4-Tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918); N(α)-Boc-l-Asp(OBn)-l-Lys(Z)-OtBu (reversin 121); Nanoparticles; Naphthoquinone derivatives; N-Arylalkyl-3,4-diaryl-substituted pyrrole-2,5-diones; Naringin; Naringenin; N-Desmethyl-loperamide; Nefazodone (acute); Neferine; Nelfinavir; Nemadectin; Nicardipine; Nicotine; Nifedipine; Nigericin; Nilotinib; Nimesulide; Nisoldipine; Nitrendipine; Nitric oxide; N,N-bis(Alkanol)amine aryl esters; N,N-bis(Cyclohexanol)amine aryl esters; N,N-Diethyl-2-[4-(phenylmethyl) phenoxy]ethanamine HCl (DPPE); Nobiletin; Nocardioazines; N-Octyl-O-sulfate chitosan (NOSC) micelles; Non-ionic surfactants (cremophor EL, cremophor RH 40, polysorbate 80, vitamin E TPGS 1000, pluronic PE 10300 and sucrose ester L-1695); NOR5; Norathyriol; Norbuprenorphine; Norverapamil; Notopterol; NS-398; NSC77037; Nutlin-1; O-(4-Ethoxyl-butyl)-berbamine; OC144-093; OC144-193; *Oenothera paradoxa* defatted seed extract (pentagalloylglucose, gallic acid, (+)-catechin, procyanidin); Ofloxacin; Olanzapine; Olaparib; Omeprazole; Orange juice flavones (3,3′,4′,5,6,7,8-heptamethoxyflavone; 4′,5,6,7,8-pentamethoxyflavone (tangeretin); 3′,4′,5,6,7,8-hexamethoxyflavone (nobiletin)); Orange juice-Seville; Oxybutynin; Oxymatrine; Paliperidone;Palmatine; Palmitoylcarnitine; Pantoprazole; Papyriferic acid derivatives; Paroxetine; Patupilone; PD98059; PEG-400; Pegylated phosphotidylethanolamine; Peonidin; Peonidin-3-glucoside; Pelargonidin; Pelargonidin-3,5-diglucoside; Pentagalloylglucose; Peptidomimetic inhibitors; Petunidin; Phenobarbital; Phenothiazines; Phenylcinnamides; Phenylsulfonylfuroxans; Phenytoin; Phloretin; Phloxine; Phytosterols; Pimozide; *Piper cubeba*; *Piper nigrum* fruit; Piperine (black pepper); Pirarubicin;

Plant extracts; Pluronic; Pluronic F68 block copolymer; Pluronic PE 10300; Podophyllotoxin derivatives (4-[4”-(2”, 2”, 6”, 6”-tetramethyl-l”-piperidinyloxy) amino]-4′-demethyl epipodophyllotoxin) (GP7) (YB-1EPN); Poly(ethylene glycol)-conjugated multi-walled carbon nanotubes; Polyethyleneimine (PEI)/ all-trans retinoic acid (ATRA) conjugates; Poly[ethylene oxide]-poly[propylene oxide] [PEO-PPO] amphiphiles; Polyinosinic/polycytidylic acid; Polymethyoxyflavonoids (tangeretin, nobiletin, baicalein, wogonin, quercetin, epigallocatechin gallate);Polyphenols from *Mangifera indica* (mango stem bark extract) (mangiferin, norathyriol, catechin, gallic acid and quercetin); Polysorbate 80; Posaconazole; Pravastatin; Prazosin; Prednisolone; Prenyloxycinnamic acids (boropinic acid, 4′-isopentenyloxy-p-coumaric acid); Primaquine; Probenecid; Procyanidin; Progesterone; Progesterone-Adenine hybrids; Promethazine; Propafenone; Propranolol; Protoberberine alkaloids from *Coptidis Rhizoma* (berberine, palmatine, coptisine, epiberberine and jatrorrhizine); PSC833 (valspodar); Psoralen; Puerarin (*Pueraria lobata*); PXR antagonists; Pyrrolo-1,5-benzoxazepines; Pyrrolidine dithiocarbamate; Pyrrolopyrimidine; PZ-39 (N-(4-chlorophenyl)-2-[(6-{[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]amino}-1,3-benzothiazol-2-yl)sulfanyl]acetamide); Quercetin; Quetiapine; Quinacrine; Quinidine; Quinine; Rabeprazole; Ranitidine; Ranolazine; Rapamycin; Red wine; Reserpine; Reversin-205; Rhei Rhizoma extract; *Rhodiola rosea*; Riccardin D; Rikkunshito; Risperidone; Ritonavir; Rivulobirin A; Romidepsin; Roscovitine; Rose Bengal; Rosemary phytochemicals (carnosic acid, carnosol, rosmarinic acid, ursolic acid); Rosmarinic acid; Rosiglitazone; Rotenone; Roxithromycin; Rutin; RU486 (mifepristone); Sakuranetin; Salinomycin; Saquinavir; SC236; Schisandra fruit; *Schisandra sphenanthera* extract (wuzhi); Schisandrol A; Schisandrol B; Scillarenin; Scorpion alcoholic extraction; SCY-635; Senkyu-cha-cho-san; Sertraline; Sesamin; Sesquiterpenes; Sesquiterpenes from *Celastraceae*; Sesquiterpene coumarins (*Ferula* species); Siamois polyphenols (quercetin); Sigma-2 agonists; Sildenafil; Silybinin (silibinin); Silymarin; Simvastatin; Sirolimus; Sitosterol; S-Methyl N,N-diethylthiocarbamate sulfone; S-Methyl N,N-diethylthiocarbamate sulfoxide; Sodium nitroprusside; Sokei-kakketsu-to; Solifenacin; Sorafenib; SP600125; Spinosad; Spironolactone; STAT3 decoy oligodeoxynucleotides; Staurosporine; Stearic acid-g-chitosan polymeric micelle; Stemona alkaloids (stemocurtisine, oxystemokerrine, stemofoline); Steroid carbamates (11-carbamic acid N,N-dibenzyl progesterone ester and 11-carbamic acid N,N-dibutyl progesterone ester); Stigmasterol; St. John’s wort (*Hypericum perforatum*) (hyperforin, hypericin, quercetin); Streptozotocin; S-trityl-l-cysteine derivatives; Sucrose ester L-1695; Sufentanil; Sulindac; Sunitinib; Syl611 (semisynthetic taxane derivative); Tacrolimus; Talinolol; Tamoxifen; Tangeretin; Tanshinones from *Salvia miltiorrhiza* (cryptotanshinone, dihydrotanshinone, tanshinone I, tanshinone IIA);Tariquidar (XR9576); Tea tree oil (terpinen-4-ol); Telithromycin; Telmisartan; Temsirolimus; Terazosin; Terfenadine; Terpenic compounds from *Euphorbia lagascae* and *E. tuckeyana* methanolic extracts (21α-hydroxytaraxasterol, 21α-hydroxytaraxasterol acetate, 3β,30-dihydroxy-20(21)-taraxastene, 3β-hydroxy-20-taraxasten-30-al, stigmastane-3,6-dione, ergosterol peroxide); Tesmilifene; Testosterone; Tetrabenzine; Tetrahydrobenzo[4′,5′]thieno[3′,2′:5,6]pyrido[4,3-d]pyrimidin-4(3H)-ones; Tetrahydrocannabinol; Tetrahydroisoquinoline-ethyl-phenylamine; Tetramethylpyrazine (*Ligusticum chuanxiong* Hort); Tetrandrine; Thapsigargin; Thioridazine; Ticagrelor; Tilidine; Tipranavir; Tocofersolan; Tolterodine; Tolvaptan; Toremifene; TPGS; TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) and TRAIL death receptor 5 (DR5); Trifluoperazine; Trifluopromazine; Trimipramine; Triptolide (*Tripterygium wilfordii*); [tris(1,10-Phenanthroline)lanthanum(III)]trithiocyanate (KP772; FFC24); Triterpenoid saponins; Troglitazone; Trospium; Tryptanthrine; Tumor necrosis factor alpha; Tyrosine kinase inhibitors; Tween-80; UIC2 antibody; Ursolic acid; Uvaol; Valerian; Valinomycin; Valproic acid; Valspodar (PSC-833); Vanadate; Vascular endothelial growth factor (VEGF); Venlafaxine; Verapamil; Vicriviroc; Vinblastine; Violaxanthin; Vitamin E TPGS; Vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate); Withaferin A and siamois polyphenols (quercetin); Wogonin (*Scutellaria baicalensis*); Xanthene food dyes (rose bengal, phroxine, amaranth, erythrosine B, allura red, new coccine, acid red, tartrazine, sunset yellow FCF, brilliant blue FCF, and indigo carmine); Xanthine derivatives; XR9576; Yangonin; YC-1(3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole);Yokukansan; Yttrium complex; Zearalenone; Zilongjin; Zosuquidar; $\Delta^{5,6}\Delta^{12,13}$-Jatrophane diterpenes; Δ^{9}-Tetrahydrocannabinol

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Table 5
(continued)

Inducers
1,4-bis-[2-(3,5-Dichloropyridyloxy)]benzene (TCPOBOP); 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD); 5,5-Diphenylbarbituric acid; 6,7-Dimethoxy-2-(6-methoxy-naphthalen-2-ylmethyl)-1,2,3,4-tetrahydroisoquinoline (MC80); 9-β-s-Arabinofuranosylguanine; 16α-Carbonitrile; Acetaminophen; Acylphloroglucinols; Adriamycin; African potato (*Hypoxis hemerocallidea*); Aldosterone; Alkylphenols; Aminoguanidine; Amiodarone; Amitriptyline; Amprenavir; Amrubicinol; Anthracyclines; Apolipoprotein E; Artemisinin; ASA; Baicalein; Bromocriptine; Budesonide; Calcitriol (1α,25-dihydroxyvitamin D_3); Calicheamicin-γ1; Capsaicin; CAR agonists; Carbamazepine; Catechin; Catechins from green tea; Chargrilled meat diet; Chlorambucil; Cisplatin; Clotrimazole; Colchicine; Coptisine; Cortisol; Cyclosporine; Cytarabine; Daidzein; Darunavir; Daunorubicin; Delavirdine; Dexamethasone; Diclofenac; Dietary nucleotide supplements; Diltiazem; Doxercalciferol (1α-hydroxyvitamin D_2); Doxorubicin; Ebselen; Efavirenz; Emodin; Endothelin-1 receptor antagonists (ambrisentan, darusentan, bosentan, sitaxentan); Enzastaurin; Eprosartan; Erlotinib; Erythromycin; Ethylparaoxon; Etoposide; Fenbufen; Fexofenadine; Flucloxacillin (Floxacillin); Fluorouracil; Galangin; Gallic acid; Garlic; Genistein; *Ginkgo biloba*; *Ginkgo biloba* extract EGb761; Ginkgolides; Glibenclamide; Glucocorticoids; Glycolytic pyruvate; HIV-protease inhibitors; Hydroxyurea; Hyperforin; Hypericin; Imatinib; Indinavir; Indomethacin; Insulin; Interleukin-6; Kaempferol; Kava kava (*Piper methysticum*); Ketoconazole; *Lactobacilli*; Lapatinib; Levetiracetam; Levonorgestrel; Levothyroxine; Licorice drink (aqueous extract of *Glycyrrhiza glabra Fabaceae*); Lithocholic acid; L-NG-Nitroarginine methyl ester (L-NAME); Lopinavir; Mangiferin; Maraviroc; MC80; Meglitinide; Meloxicam; Mepirizole; Methotrexate; Methysticin; Metoclopramide; Metoprolol; Mitoxantrone; Morphine; N,N-Diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine; Nanoparticles; Naringenin; Nefazodone; Nelfinavir; Nevirapine; Nicardipine; Nifedipine; Nimesulide; Norathyriol; Olmesartan; Oxaliplatin; Oxcarbazepine; P1075; Paclitaxel; Pesticides; Phenobarbital; Phenothiazines; Phenytoin; Piperine; Plant extracts; Poly(ethylene glycol)-conjugated multi-walled carbon nanotubes; Polyphenols from *Mangifera indica* (mango stem bark extract) (mangiferin, norathyriol, catechin, gallic acid and quercetin); Praziquantel; Prazosin; Prednisolone; Prednisone; Probenecid; Probiotics; Proteasome inhibitor TP-110; PXR agonists; Quercetin; Reserpine; Resveratrol; Retinoid acid; Rifampicin (rifampin); Ritonavir; Rutin; Saquinavir; Sesamin; Sildenafil; Silvestrol; Silybin; SN-38; Spironolactone; SR47063; Stilbenes; St. John's wort (*Hypericum perforatum*); Sucralose (Splenda); Sulindac; Tacrolimus; Tamoxifen; Tangeretin; Tipranavir; Tocotrienols; Tolbutamide; Topiramate; trans-3,5,3′,4′-Tetramethoxystilbene; Trazodone; Tumor necrosis factor alpha; Ursodeoxycholic acid; Ursodiol; Valproic acid; Venlafaxine; Verapamil; Vicriviroc; Vinblastine; Vinca alkaloids; Vincristine; Vitamin A; Vitamin D; Vitamin D_3; Vitamin D_3 metabolite 1,25-dihydroxycholecalciferol (DHC); Vitamin E; Yohimbine; Zhi Shi

amyloid-β from the brain may lead to elevated amyloid-β levels. One of the clearance pathways of amyloid-β is transport across the BBB via efflux transporters. P-glycoprotein, an efflux pump highly expressed at the endothelial cells of the BBB, has been shown to transport amyloid-β. The P-glycoprotein transporter at the BBB is compromised in AD and decreased P-glycoprotein function may be involved in the pathogenesis of AD [702].

In addition to the age-related decrease of P-gp expression, $A\beta_{1-42}$ itself downregulates the expression of P-gp and other Aβ-transporters, which could exacerbate the intracerebral accumulation of Aβ and thereby accelerate neurodegeneration in AD and cerebral β-amyloid angiopathy [703]. Furthermore, amyloid efflux transporter expression at the BBB declines with aging in normal conditions [54], and expression of P-gp protein is significantly lower in hippocampal vessels of patients with AD compared to normal individuals [704].

The low-density lipoprotein receptor-related protein-1 (LRP-1) and the ATP-binding cassette (ABC) protein ABCB1 (P-glycoprotein) are involved in the efflux of Aβ across the BBB. Other ABC proteins, such as members of the G subfamily, are also involved in the BBB clearance of Aβ. ABCG2 and ABCG4 mediate the cellular efflux of $[^{3}H]A\beta_{1-40}$. Probucol inhibits the efflux of $[^{3}H]A\beta_{1-40}$ from HEK293-abcg4 cells. GF120918 (a dual inhibitor of Abcb1 and Abcg2) strongly enhances the uptake of $[^{3}H]A\beta_{1-40}$ by the brains of Abcb1-deficient mice, but not by the brains of Abcb1/Abcg2-deficient mice, suggesting that Abcg2 is involved in the transport of Aβ at the mouse BBB. Abcg4 acts in concert with Abcg2 to efflux Aβ from the brain across the BBB [705].

ATP-binding cassette subfamily G member 2 (ABCG2) is involved in amyloid-β transport and was found to be upregulated in AD brains. A functional polymorphism of the *ABCG2* gene (C421A; rs2231142) (*ABCG2* C/C genotype) was associated with AD in the Hungarian population. The *ABCG2* C/C genotype and the *APOE ε4* allele may also exert an interactive effect on AD risk [706].

Single-nucleotide polymorphisms in the *ABCB1* gene have been associated with altered P-glycoprotein expression and function. P-glycoprotein function at the BBB can be quantified in vivo using the P-glycoprotein substrate tracer (R)-$[^{11}C]$verapamil and PET. Three different kinds of imaging probes have been described to measure ABC transporters in vivo: (1) radiolabeled transporter substrates, (2) radiolabeled transporter inhibitors, and (3) radiolabeled prodrugs which are enzymatically converted into transporter substrates in the organ of interest [707]. Van Assema et al. [708] assessed the effects of C1236T, G2677T/A, and C3435T single-nucleotide polymorphisms in *ABCB1* on BBB P-glycoprotein function in healthy subjects and patients with AD. In healthy

controls, binding potential did not differ between subjects without and with one or more T present in C1236T, G2677T, and C3435T. In contrast, patients with AD with one or more T in C1236T, G2677T, and C3435T had significantly higher binding potential values than patients without a T. There was a relationship between binding potential and T dose in C1236T and G2677T. In AD patients, C1236T, G2677T/A, and C3435T SNPs may be related to changes in P-glycoprotein function at the BBB, and genetic variations in *ABCB1* might contribute to the progression of amyloid-β deposition in the brain. Kohen et al. [709] investigated a possible association between two common *ABCB1* polymorphisms, G2677T/A (Ala893Ser/Thr) and C3435T, AD, and CSF levels of Aβ, and no strong evidence for association was found. Frankfort et al. [710] studied *ABCB1* SNPs (C1236T in exon 12, G2677T/A in exon 21, and C3435T in exon 26) and inferred haplotypes in patients with dementia and age-matched non-demented control patients and found no differences between both groups; however, in a transcriptome analysis of leukocytes from patients with MCI, AD, as well as normal controls only the *ABCB1* gene exhibited significantly positive correlation with MMSE scores, representing a novel biomarker of AD [134].

Aβ transport (flux) across the BBB is thought to contribute to the pathogenesis of AD and elimination of toxic amyloid from the brain by immunotherapy, as well. Several BBB transporters have been implicated in Aβ exchange between brain parenchyma and the circulation, including efflux transporters P-glycoprotein/ABCB1 and BCRP/ABCG2. Deficiency of either of the two major efflux pumps, Abcb1 and Abcg2, implicated in Aβ trafficking across the BBB, results in increased accumulation of peripherally injected $A\beta_{1-40}$ in the brain [711].

The drug transporter ABCB1 directly transports Aβ from the brain into the blood circulation, whereas the cholesterol transporter ABCA1 neutralizes Aβ aggregation capacity in an Apolipoprotein E (ApoE)-dependent manner, facilitating Aβ subsequent elimination from the brain [712]. Cascorbi et al. [713] genotyped selected variants in *ABCA1*, *ABCA7*, *ABCB1*, *ABCC2*, and *ABCG2* in DNAs from brain tissue of 71 AD cases with Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropathological stages B/C and 81 controls. The novel *ABCA7* SNP, rs3752246, tended to be associated with AD. *ABCB1* variants were significantly less frequent in AD cases older than 65 years of age and among females. This association of *ABCB1* 2677G>T (rs2032582) was more pronounced in APOE4-negative cases. Only *ABCC2* 3972C>T (rs3740066) was significantly associated with AD risk.

Efflux transporter P-glycoprotein (P-gp) at the BBB restricts substrate compounds from entering the brain and may thus contribute to pharmacoresistance in CNS disorders, cancer, and brain

infections. PET has become a promising method to study the role of P-gp at the BBB. The first PET study of P-gp function was conducted in 1998, and during the past 15 years two main categories of P-gp PET tracers have been investigated: tracers that are substrates of P-gp efflux and tracers that are inhibitors of P-gp function [714].

The ABC transporter P-gp protects the brain from accumulation of lipophilic compounds by active efflux transport across the BBB. Müllauer et al. [715] investigated the suitability of the radiolabeled Pgp inhibitors [^{11}C]elacridar and [^{11}C]tariquidar to visualize P-gp density in rat brain with PET. The small P-gp binding signals obtained with [^{11}C]elacridar and [^{11}C]tariquidar limit the applicability of these tracers to measure cerebral P-gp density.

Molecular transporters that are expressed in brain, especially at the BBB, are therapeutic targets in the treatment of AD. Some ATP-binding cassette (ABC) transporters, particularly P-glycoprotein (ABCB1), MRP1 (ABCC1), and BCRP (ABCG2), have been implicated in the clearance of neurotoxic polypeptides that characteristically accumulate in the brain, such as Aβ peptides. A benzopyrane derivative with P-gp stimulating properties has been proposed as a candidate agent to decrease Aβ accumulation in AD [665]. Lipid transporters of the A-branch of ABC transporters are also potentially involved in AD pathogenesis. Induction of transporters via the activation of specific nuclear receptors may represent a novel approach to restoring diminished BBB function. Transporters in the brain capillary endothelium regulate the permeation of therapeutic compounds into the brain [716, 717].

Induction of the multidrug resistance protein 1 (MDR1)/P-glycoprotein (P-gp) by the vitamin D receptor (VDR) was investigated in isolated rat brain capillaries and rat (RBE4) and human (hCMEC/D3) brain microvessel endothelial cell lines. Incubation of isolated rat brain capillaries with the VDR ligand, 1α,25-dihydroxyvitamin D3 [1,25OH$_2$D$_3$], increased P-gp protein expression fourfold. Incubation with 1,25OH$_2$D$_3$ increased P-gp transport activity by 25–30 %. In RBE4 cells, Mdr1b mRNA was induced in a concentration-dependent manner by exposure to 1,25OH$_2$D$_3$. Concomitantly, P-gp protein expression increased 2.5-fold and was accompanied by a 20–35 % reduction in cellular accumulation of the P-gp substrates, rhodamine 6G (R6G), and HiLyte Fluor 488-labeled human amyloid-β 1–42 (hAβ$_{42}$). In hCMEC/D3 cells, exposure to 1,25OH$_2$D$_3$ increased MDR1 mRNA expression (40 %) and P-gp protein; and reduced cellular accumulation of R6G and hAβ$_{42}$ by 30 %. VDR activation upregulates Mdr1/MDR1 and P-gp protein in brain capillaries and microvascular endothelia, implicating a role for VDR in increasing the brain clearance of P-gp substrates, including hAβ$_{42}$ in AD [718].

Since P-gp prevents the entry of compounds into the brain by an active efflux mechanism at the BBB, inhibition of P-gp may help

to enhance drug penetration. New reversible inhibitors of P-gp have been developed. Some galantamine-like compounds inhibit the efflux of the fluorescent P-gp substrate rhodamine 123 in cancer cells that overexpress P-gp, and also inhibit the efflux of therapeutic substrates of P-gp, such as doxorubicin, daunomycin, and verapamil. These compounds modulate P-gp-mediated efflux by competing for the substrate binding sites [719]. Activation of the liver X receptors (LXRs) by natural or synthetic agonists decreases the amyloid burden and enhances cognitive function in transgenic murine models of AD. LXR activation may affect the transport of Aβ peptides across the BBB. LXR agonists (24S-hydroxycholesterol, 27-hydroxycholesterol, and T0901317) modulate the expression of target genes involved in cholesterol homeostasis (ABCA1) and promote cellular cholesterol efflux to apolipoprotein A-I and high-density lipoproteins. LXR stimulation increases the expression of the ABCB1 transporter, which restricts Aβ peptide influx [720].

It is also important that drugs for AD treatment optimize CNS penetration by minimizing hydrogen bond donors and reducing P-gp-mediated efflux [721–723]. The increase of P-glycoprotein expression and activity by a P-gp inducer could be an effective pharmacological strategy in slowing or halting the progression of AD. Approximately 10–35 % decrease in ^{124}I-Aβ$_{1–40}$ intracellular accumulation was observed in cells treated with rifampicin, dexamethasone, caffeine, verapamil, hyperforin, β-estradiol, and pentylenetetrazole (P-gp inducers) [439]. Perrone et al. [724] validated the new dye-probe β-amyloid (1–40) HiLyte Fluor™ TR-labeled (Ab-HiLyte) (Anaspec) P-gp mediated transport in the ex vivo rat everted gut sac assay by using MC18 or MC266, a fully characterized P-gp inhibitor and substrate, respectively, and compared it with the commonly used dye rhodamine, demonstrating that the new dye probe, Ab-HiLyte, could be a probe of choice to unequivocally distinguish between a P-gp substrate and an inhibitor.

Mehta et al. [717] assessed the impact of AD-associated BBB alterations on the uptake of therapeutics into the brain of triple transgenic (3×TG) AD mice. The brain uptake of passively diffusing markers, [^{3}H]diazepam and [^{3}H]propranolol, decreased 54–60 % in 3×TG mice, consistent with a 33.5 % increase in the thickness of the cerebrovascular basement membrane in 3×TG mice. Despite a 42.4 % reduction in P-gp expression in isolated brain microvessels from a subpopulation of 3×TG mice, the brain uptake of P-gp substrates ([^{3}H]digoxin, [^{3}H]loperamide, and [^{3}H]verapamil) was not different between genotypes, likely due to a compensatory thickening in the cerebrovascular basement membrane counteracting any reduced efflux of these lipophilic substrates.

7.4.2 Other Transporters

Also of importance for CNS pharmacogenomics are transporters encoded by genes of the solute carrier superfamily (*SLC*) and solute carrier organic (*SLCO*) transporter family, responsible for the

transport of multiple endogenous and exogenous compounds, including folate (*SLC19A1*), urea (*SLC14A1*, *SLC14A2*), monoamines (*SLC29A4*, *SLC22A3*), amino acids (*SLC1A5*, *SLC3A1*, *SLC7A3*, *SLC7A9*, *SLC38A1*, *SLC38A4*, *SLC38A5*, *SLC38A7*, *SLC43A2*, *SLC45A1*), nucleotides (*SLC29A2*, *SLC29A3*), fatty acids (*SLC27A1-6*), neurotransmitters (*SLC6A2* (noradrenaline transporter), *SLC6A3* (dopamine transporter), *SLC6A4* (serotonin transporter, SERT), *SLC6A5*, *SLC6A6*, *SLC6A9*, *SLC6A11*, *SLC6A12*, *SLC6A14*, *SLC6A15*, *SLC6A16*, *SLC6A17*, *SLC6A18*, *SLC6A19*), glutamate (*SLC1A6*, *SLC1A7*), and others [725]. Some organic anion transporters (OAT), which belong to the solute carrier (SLC) 22A family, are also expressed at the BBB, and regulate the excretion of endogenous and exogenous organic anions and cations [726]. The transport of amino acids and di- and tripeptides is mediated by a number of different transporter families, and the bulk of oligopeptide transport is attributable to the activity of members of the *SLC15A* superfamily (Peptide Transporters 1 and 2 [*SLC15A1* (PepT1) and *SLC15A2* (PepT2)], and Peptide/Histidine Transporters 1 and 2 [*SLC15A4* (PHT1) and *SLC15A3* (PHT2)]). ABC and SLC transporters expressed at the BBB may cooperate to regulate the passage of different molecules into the brain [727]. Polymorphic variants in ABC and SLC genes may also be associated with pathogenic events in CNS disorders and drug-related safety and efficacy complications [16, 725]. For instance, an important issue to be elucidated is the role of transporters in patients under chronic treatment with psychotropic drugs or exposed to general anesthesia. Chen et al. [728] studied the potential influence of endotracheal tube intubation general anesthesia (ETGA), intravenous injection general anesthesia (IVGA) or intramuscular injection general anesthesia (IMGA), and heavy sedation on dementia in Taiwan and found that individuals exposed to surgery under ETGA and those exposed to surgery under IVGA or IMGA were at significantly higher risk of dementia in a dose–response relationship, whereas surgery under heavy sedation was not associated with increased risk of dementia. Subjects who had received surgery under ETGA with comorbidities such as stroke, hypertension, diabetes mellitus, and atherosclerosis could have a potential relationship with dementia risk [733]. Interestingly, the anesthetics propofol and thiopental are associated with Aβ assembly and GM1 expression on the neuronal cell surface through the γ-aminobutyric acid A receptor, and both compounds have direct and indirect inhibitory effects on Aβ fibrillogenesis [729].

7.5 Pleiotropic Activity of APOE in Dementia

APOE is the prototypical paradigm of a pleiotropic gene with multifaceted activities in physiological and pathological conditions [17, 28]. ApoE is consistently associated with the amyloid plaque marker for AD. *APOE-4* may influence AD pathology interacting with APP metabolism and Aβ accumulation, enhancing

hyperphosphorylation of tau protein and NFT formation, reducing choline acetyltransferase activity, increasing oxidative processes, modifying inflammation-related neuroimmunotrophic activity and glial activation, altering lipid metabolism, lipid transport, and membrane biosynthesis in sprouting and synaptic remodeling, and inducing neuronal apoptosis [17, 28, 31, 32, 63].

Yu et al. [730] examined the genomic structure of *APOE* in search for properties that may contribute novel biological consequences to the risk of disease and identified one such element in the *ε2/ε3/ε4* allele-carrying 3′-exon of *APOE*. This exon is embedded in a well-defined CpG island that is highly methylated in human postmortem brain. This *APOE* CpG island exhibits transcriptional enhancer/silencer activity and differentially modulates expression of genes at the APOE locus in a cell type-, DNA methylation-, and *ε2/ε3/ε4* allele-specific manner. An *APOE4*-associated molecular pathway promotes LOAD. A set of candidate core regulatory mediators (APBA2, FYN, RNF219, and SV2A) encode known or novel modulators of LOAD-associated amyloid-β A4 precursor protein (APP) endocytosis and metabolism. A genetic variant within *RNF219* was found to affect amyloid deposition in human brain and LOAD [731].

To address the complex misfolding and aggregation that initiates the toxic cascade resulting in AD, Petrlova et al. [33] developed a 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid spin-labeled amyloid-β (Aβ) peptide to observe its isoform-dependent interaction with the ApoE protein. Oligomer binding involves the C-terminal domain of ApoE, with ApoE3 reporting a much greater response through this conformational marker. ApoE3 displays a higher affinity and capacity for the toxic Aβ oligomer. *ApoE* polymorphism and AD risk can largely be attributed to the reduced ability of ApoE4 to function as a clearance vehicle for the toxic form of Aβ. *MAPT* and *APOE* are involved in the pathogenic mechanisms of AD, and both *MAPT H1/H1* genotype and *APOE ε4* allele lead to a more rapid progression to dementia among MCI subjects, probably mediating an increased rate of amyloid-β and tau brain deposition.

APOE interacts with age to modify rate of decline in cognitive and brain changes in AD [732].

The distribution of *APOE* genotypes in the Iberian peninsula is as follows: *APOE-2/2* 0.32 %, *APOE-2/3* 7.3 %, *APOE-2/4* 1.27 %, *APOE-3/3* 71.11 %, *APOE-3/4* 18.41 %, and *APOE-4/4* 1.59 % [19] (*see* Fig. 4). These frequencies are very similar in Europe and in other Western societies. There is a clear accumulation of *APOE-4* carriers among patients with AD (*APOE-3/4* 30.30 %, *APOE-4/4* 6.06 %) and VD (*APOE-3/4* 35.85 %, *APOE-4/4* 6.57 %) as compared to controls (*see* Fig. 4). Different *APOE* genotypes confer specific phenotypic profiles to AD patients [15, 17, 28]. Some of these profiles may add risk or benefit when the

patients are treated with conventional drugs, and in many instances the clinical phenotype demands the administration of additional drugs which increase the complexity of therapeutic protocols. From studies designed to define *APOE*-related AD phenotypes [7, 12, 15, 17, 19, 28, 35, 63, 86–92], several conclusions can be drawn: (1) the age-at-onset is 5–10 years earlier in approximately 80 % of AD cases harboring the *APOE-4/4* genotype; (2) the serum levels of ApoE are lowest in *APOE-4/4*, intermediate in *APOE-3/3* and *APOE-3/4*, and highest in *APOE-2/3* and *APOE-2/4*; (3) serum cholesterol levels are higher in *APOE-4/4* than in the other genotypes; (4) HDL-cholesterol levels tend to be lower in *APOE-3* homozygotes than in *APOE-4* allele carriers; (5) LDL-cholesterol levels are systematically higher in *APOE-4/4* than in any other genotype; (6) triglyceride levels are significantly lower in *APOE-4/4*; (7) nitric oxide levels are slightly lower in *APOE-4/4*; (8) serum and CSF Aβ levels tend to differ between *APOE-4/4* and the other most frequent genotypes (*APOE-3/3*, *APOE-3/4*); (9) blood histamine levels are dramatically reduced in *APOE-4/4* as compared with the other genotypes; (10) brain atrophy and AD neuropathology is markedly increased in *APOE-4/4* > *APOE-3/4* > *APOE-3/3*; (11) brain mapping activity shows a significant increase in slow wave activity in *APOE-4/4* from early stages of the disease; (12) brain hemodynamics, as reflected by reduced brain blood flow velocity and increased pulsatility and resistance indices, is significantly worse in *APOE-4/4* (and in *APOE-4* carriers in general, as compared with *APOE-3* carriers); brain hypoperfusion and neocortical oxygenation are also more deficient in *APOE-4* carriers (*see* Fig. 5); (13) lymphocyte apoptosis is markedly enhanced in *APOE-4* carriers; (14) cognitive deterioration is faster in *APOE-4/4* patients than in carriers of any other *APOE* genotype; (15) in approximately 3–8 % of the AD cases, the presence of some dementia-related metabolic dysfunctions accumulates more in *APOE-4* carriers than in *APOE-3* carriers; (16) some behavioral disturbances, alterations in circadian rhythm patterns, and mood disorders are slightly more frequent in *APOE-4* carriers; (17) aortic and systemic atherosclerosis is also more frequent in *APOE-4* carriers; (18) liver metabolism and transaminase activity also differ in *APOE-4/4* with respect to other genotypes; (19) hypertension and other cardiovascular risk factors also accumulate in *APOE-4*; and (20) *APOE-4/4* carriers are the poorest responders to conventional drugs. These 20 major phenotypic features clearly illustrate the biological disadvantage of *APOE-4* homozygotes and the potential consequences that these patients may experience when they receive pharmacological treatment for AD and/or concomitant pathologies [7, 12, 15, 17, 19, 28, 35, 63, 86–92, 733].

APOE-4 genotype has been recommended as a potential inclusion or exclusion criterion in targeted clinical trials for AD and MCI resulting from AD, and has been implemented in trials of

immunotherapeutic agents. According to Kennedy et al., although samples enriched for *APOE-4* carriers in AD or MCI clinical trials showed slightly more cognitive impairment and greater decline using the number *APOE-4* alleles as an inclusion criterion most likely would not result in more efficient trials, and trials would take longer because fewer patients would be available. The *APOE* genotype could be useful, however, as an explanatory variable or covariate if warranted by a drug's action [734].

7.6 Pharmacogenomics of Anti-dementia Drugs

7.6.1 Donepezil

Donepezil is a centrally active, reversible acetylcholinesterase inhibitor which increases the acetylcholine available for synaptic transmission in the CNS. The therapeutic response of donepezil is influenced by pathogenic gene variants (*APOE*, *CHAT*), as well as mechanistic gene polymorphic variants of *CHAT*, *ACHE*, and *BCHE*. Donepezil is a major substrate of CYP2D6, CYP3A4, ACHE, and UGTs, inhibits ACHE and BCHE, and is transported by ABCB1 [16, 35, 63, 90, 91, 139, 687, 735, 736]. Most studies convey that *CYP2D6* variants affect donepezil efficacy and safety in AD [16, 35, 90, 91, 139, 687, 736]. The common variant rs1080985 of *CYP2D6* was found to be associated with poor response to donepezil [737, 738]. A high-throughput genetic analysis of *CYP2D6* polymorphisms discriminate responders/nonresponders of the *CYP2D6* allele **2A*. A higher frequency of mutated alleles in responder than in nonresponder patients (75.38 % vs. 43.48 %) was observed. The presence of a mutated allele of *CYP2D6* was associated with a response to CYP2D6-metabolized drugs [739]. In agreement with this criteria, in an Italian study 67 % of patients were responders and 33 % were nonresponders to donepezil treatment. A significantly higher frequency of gene variants conferring decreased or absent enzyme activity was observed in responder than in nonresponder patients (73.68 % vs. 36.84 %) [740]. Among Chinese patients, 58.3 % were responders and 41.7 % were nonresponders to donepezil treatment. AD patients with the mutant allele *CYP2D6*10* may respond better to donepezil than those with wild allele *CYP2D6*1*. A significantly higher frequency of patients with genotypes *CYP2D6*1/*10* and **10/*10* was found in responders than in nonresponders. Patients with *CYP2D6*1/*10* and **10/*10* genotypes had higher steady-state plasma concentrations of donepezil and improved cognition scores than those with *CYP2D6*1/*1* genotype [741]. However, in other studies, *CYP2D6*-PMs and UMs tend to be poor responders to conventional doses of donepezil as compared to EMs and IMs [7, 12, 15, 17, 19, 28, 63, 86–92, 742–744]. In contrast, a Polish group could not find any influence of the rs1080985 SNP on response to treatment with donepezil in AD [745].

Magliulo et al. [746] evaluated the impact of *CYP3A4* (**1B*, **3*, and **4*), *CYP3A5* (**2*, **3*, and **6*), and *ABCB1* (3,435C>T, 2,677G>T/A, and 1,236C>T) polymorphisms on donepezil

disposition and clinical outcome in 54 Italian AD patients. Three patients carried one detrimental *CYP3A4* allelic variant, and 12 carried one functional *CYP3A5*1* allele. No association was found between *CYP3A4* or *CYP3A5* genotypes and plasma donepezil concentrations, or between genotypes and clinical response. The most common *ABCB1* haplotypes were 1,236C/2,677G/3,435C (46 %) and 1,236T/2,677T/3,435T (41 %). Patients homozygous for the T/T/T haplotype had lower plasma donepezil concentration-to-dose ratios and better clinical response than patients with other genotypes.

7.6.2 Galantamine

Galantamine is a reversible and competitive acetylcholinesterase inhibitor leading to an increased concentration of acetylcholine at cholinergic synapses. This drug also modulates nicotinic acetylcholine receptors and may increase glutamate and serotonin levels. *APOE*, *APP*, *ACHE*, *BCHE*, *CHRNA4*, *CHRNA7*, *CHRNB2* variants may potentially influence galantamine efficacy and safety; it is a major substrate of CYP2D6, CYP3A4, and UGT1A1, and an inhibitor of ACHE and BCHE [687, 735, 736, 747–749]. Major metabolic pathways are glucuronidation, O-demethylation, N-demethylation, N-oxidation, and epimerization. In extensive metabolizers for CYP2D6, urinary metabolites resulting from O-demethylation represented 33.2 % of the dose compared with 5.2 % in poor metabolizers, which showed correspondingly higher urinary excretion of unchanged galantamine and its N-oxide. The glucuronide of O-desmethyl-galantamine represented up to 19 % of the plasma radioactivity in extensive metabolizers but could not be detected in poor metabolizers [750]. Galantamine is extensively metabolized by the enzymes CYP2D6 and CYP3A and is a substrate of the P-glycoprotein. Noetzli et al. [751] studied the relationship between genetic variants of *CYP2D6*, *CYP3A4/5*, and *ABCB1* with galantamine steady-state plasma concentrations. The *CYP2D6* genotype seemed to be an important determinant of galantamine pharmacokinetics, with CYP2D6 poor metabolizers presenting 45 and 61 % higher dose-adjusted galantamine plasma concentrations than heterozygous and homozygous CYP2D6 extensive metabolizers. However, Clarke et al. [752] were unable to make inferences about an association between CYP2D6 phenotype and galantamine responsiveness. The bioavailability of galantamine is increased by co-administration with paroxetine, ketoconazole, and erythromycin [753]. In healthy subjects and in AD patients, the co-administration of galantamine with ketoconazole (a CYP3A4 strong inhibitor) or paroxetine (a CYP2D6 strong inhibitor) leads to a 30 and 40 % increase, respectively, in galantamine exposure compared to galantamine given alone [754]. Galantamine can interact with foods which might alter its bioavailability and therapeutic effects. Zhai and Lu [755] reported

interaction between galantamine and capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide, CAP), a naturally occurring alkaloid extracted from the fruit of *Capsicum* plant family, which is a common ingredient in spicy foods. The pretreatment of rats with capsaicin resulted in a decrease in the $AUC_{0-\infty}$ of galantamine of about 49.70 % compared with the control group. After oral administration of galantamine (10 mg/kg), the apparent oral clearance of galantamine was raised by 2.05-fold by pretreatment with capsaicin, indicating that the chronic ingestion of high doses of capsaicin decreases the bioavailability of galantamine, at least in rats.

7.6.3 Rivastigmine

Rivastigmine is a cholinesterase inhibitor which increases acetylcholine in CNS through reversible inhibition of its hydrolysis by cholinesterase. *APOE*, *APP*, *CHAT*, *ACHE*, *BCHE*, *CHRNA4*, *CHRNB2*, and *MAPT* variants may affect its pharmacokinetics and pharmacodynamics. The hepatic cytochrome P-450 (CYP-450) system is not involved in the metabolism of rivastigmine [687, 735, 736, 753, 756]. Sonali et al. [757] studied the clinical effectiveness of *CYP2D6*, *CYP3A4*, *CYP2C9/19*, and *UGT* polymorphism on the steady-state plasma concentrations and therapeutic outcome of rivastigmine monotherapy and combination therapy in patients with AD in India. A significant allele frequency was observed for the *CYP2D6*3* polymorphism in patients under rivastigmine combination therapy (A>del: 0.50 AD/0.20 controls), *UGT2B7* (T: 0.17 AD/0.33 C), and *UGT1A9*5* (A = 0.58 AD/0.26 C). Poor metabolizer subjects of the *UGT2B7* polymorphism in patients under rivastigmine combination therapy have higher drug levels with a poor response to treatment.

7.6.4 Tacrine

Tacrine was the first FDA-approved anti-dementia drug. Its use was stopped due to hepatotoxicity. Tacrine is a cholinesterase inhibitor which elevates acetylcholine in cerebral cortex by slowing degradation of acetylcholine. *ACHE*, *BCHE*, *CHRNA4*, *CHRNB2*, *APOE*, *MTHFR*, *CES1*, *LEPR*, *GSTM1*, and *GSTT1* variants may affect its therapeutic and toxic effects. Tacrine is a major substrate of CYP1A2, and CYP3A4, a minor substrate of CYP2D6, and is transported via SCN1A. Tacrine is an inhibitor of ACHE, BCHE, and CYP1A2 [687]. Both tacrine and some tacrine hybrids may cause an induction of CYP1A1, 2B1, and 3A2 expression [758]. Tacrine is associated with transaminase elevation in up to 50 % of patients. The mechanism of tacrine-induced liver damage is influenced by genetic factors. The strongest association was found between alanine aminotransferase levels and three SNPs within ATP-binding cassette, subfamily B (MDR/TAP), member 4 (*ABCB4*) [759].

7.6.5 Memantine

Memantine is an N-Methyl-D-Aspartate (NMDA) receptor antagonist which binds preferentially to NMDA receptor-operated

cation channels; it may act by blocking actions of glutamate, mediated in part by NMDA receptors, and is also an antagonist of GRIN2A, GRIN2B, GRIN3A, HTR3A, CHRFAM7A. Several pathogenic (*APOE*, *PSEN1*, *MAPT*) and mechanistic gene variants (*GRIN2A*, *GRIN2B*, *GRIN3A*, *HTR3A*, *CHRFAM7A*, *c-Fos*, *Homer1b*, and *PSD-95*) may influence its therapeutic effects. Memantine is a strong inhibitor of CYP2B6 and CYP2D6, and a weak inhibitor of CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2E1, and CYP3A4 [687, 736, 760]. Memantine is beneficial for AD patients in terms of cognition and clinician's global impression; however, some memantine-related major side effects (somnolence, weight gain, confusion, hypertension, nervous system disorders, falling) [760] might be associated with pharmacogenetic factors. Micuda et al. [761] studied the drug interaction potential of memantine by elucidation of its inhibitory effects on cytochrome P450 enzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) using pooled human liver microsomes (HLM) and recombinant P450s. In HLM, memantine inhibited CYP2B6 and CYP2D6 activities, showed no appreciable effect on CYP1A2, CYP2E1, CYP2C9, or CYP3A4 activities, and decreased CYP2A6 and CYP2C19 activities. When co-administered with CYP2B6 substrates, over 65 % decrease in metabolism can be expected. Eap's group [762] investigated clinical and genetic factors influencing memantine disposition. A population pharmacokinetic study was performed including data from 108 patients recruited in a naturalistic setting. Patients were genotyped for common polymorphisms in renal cation transporters (*SLC22A1/2/5*, *SLC47A1*, *ABCB1*) and nuclear receptors (*NR1I2*, *NR1I3*, *RXR*, *PPAR*) involved in transporter expression. The average clearance was 5.2 L/h with a 27 % interindividual variability. Glomerular filtration rate and sex influenced memantine clearance. *NR1I2* rs1523130 was identified as the unique significant genetic covariate for memantine clearance, with carriers of the *NR1I2* rs1523130 CT/TT genotypes presenting a 16 % slower memantine elimination than carriers of the CC genotype.

Administration of NMDA receptor antagonists, such as ketamine and MK-801, may induce psychotic-like behaviors, and ketamine can exacerbate psychotic symptoms in patients with schizophrenia; in contrast, memantine, a noncompetitive NMDA receptor antagonist approved for AD, may potentially display antipsychotic effects. The molecular mechanisms by which these NMDA receptor antagonists cause different neurochemical, behavioral, and clinical effects are associated with differential expression of particular genes (Homer1a/Homer1b/PSD-95 signaling network), involved in glutamate-dependent synaptic plasticity, as well as in psychosis pathophysiology and treatment. Ketamine and MK-801 significantly induced the transcripts of immediate-early genes (Arc, c-fos, and Homer1a) in cortical regions, whereas they

reduced Homer1b and PSD-95 expression in cortical and striatal regions. Memantine did not increase Homer1a signal, whereas it induced c-fos in the somatosensory and in the medial agranular cortices, not affecting Homer1b and PSD-95 expression. When compared to ketamine and MK-801, memantine significantly increased the expression of c-fos, Homer1b, and PSD-95. Overall, ketamine and MK-801 prominently increased Homer1a/Homer1b expression ratio, whereas memantine elicited the opposite effect. According to de Bartolomeis et al. [763], these data may support the view that ketamine, MK-801, and memantine exert divergent effects on PSD transcripts, which may contribute to their partially different behavioral and clinical effects.

Martinelli-Boneschi et al. [764] conducted a genome-wide association study in a cohort of 176 Italian AD patients treated with cholinesterase inhibitors, classifying the patients into responders (positive, stable, or ≤1 worsening of MMSE score) and nonresponders (>3 points worsening in MMSE score) during a median follow-up of 0.85 years of treatment. Among the 48 SNPs screened, only two SNPs were associated with response to treatment: rs6720975A, and rs17798800A, an intergenic variant potentially acting as a *cis*-regulator of NBEA, an A kinase-anchoring protein playing a substantial role in the maturation of the nervous system.

8 Multifactorial Therapy

Some studies using a multifactorial therapy also indicate that diverse pharmacogenomic factors may influence efficacy and safety. In one of these studies [15, 91], patients with dementia received for 3 months a multifactorial therapy integrated by CDP-choline (500 mg/day, p.o.), Nicergoline (5 mg/day, p.o.), Sardilipin (E-SAR-94010) (LipoEsar®) (250 mg, t.i.d.), and Animon Complex® (2 capsules/day), a nutraceutical compound integrated by a purified extract of *Chenopodium quinoa* (250 mg), ferrous sulfate (38.1 mg equivalent to 14 mg of iron), folic acid (200 μg), and vitamin B_{12} (1 μg) per capsule (RGS: 26.06671/C). Patients with chronic deficiency of iron (<35 μg/mL), folic acid (<2.5 ng/mL), or vitamin B_{12} (<150 pg/mL) received an additional supplementation of iron (80 mg/day), folic acid (5 mg/day), and B complex vitamins (B_1, 15 mg/day; B_2, 15 mg/day; B_6, 10 mg/day; B_{12}, 10 μg/day; nicotinamide, 50 mg/day), respectively, to maintain stable levels of serum iron (50–150 μg/mL), folic acid (5–20 ng/mL), and vitamin B_{12} levels (500–1,000 pg/mL) in order to avoid the negative influence of all these metabolic factors on cognition. Patients with hypertension (>150/85 mmHg) received Enalapril (20 mg/day). The frequency of *APOE* genotypes was: *APOE-2/3*, 7.97 %; *APOE-2/4*, 1.18 %; *APOE-3/3*, 58.95 %; *APOE-3/4*, 27.32 %; and *APOE-4/4*, 4.58 %. Systolic and

diastolic blood pressure, cognitive function (as assessed by MMSE, 20.51 ± 6.51 vs. 21.45 ± 6.95, $p < 0.0000000001$; ADAS-Cog, 22.94 ± 13.87 vs. 21.23 ± 12.84, $p < 0.0001$; ADAS-Non-Cog, 5.26 ± 4.18 vs. 4.15 ± 3.63, $p < 0.0000000001$; ADAS-Total, 27.12 ± 16.93 vs. 24.28 ± 15.06, $p < 0.00009$), and mood (HAM-A, 11.35 ± 5.44 vs. 9.79 ± 4.33, $p < 0.0000000001$; HAM-D, 10.14 ± 5.23 vs. 8.59 ± 4.30, $p < 0.0000000001$) improved after treatment. Glucose levels did not change. Total cholesterol levels (224.78 ± 45.53 vs. 203.64 ± 39.69 mg/dL, $p < 0.0000000001$), HDL-cholesterol levels (54.11 ± 14.54 vs. 52.54 ± 14.86 mg/dL, $p < 0.0001$), and LDL-cholesterol levels (148.15 ± 39.13 vs. 128.89 ± 34.83 mg/dL, $p < 0.0000000001$) were significantly reduced. Folate (7.07 ± 3.61 vs. 18.14 ± 4.23 ng/mL, $p < 0.000000001$) and vitamin B_{12} levels (459.65 ± 205.80 vs. 689.78 ± 338.82 pg/mL, $p < 0.000000001$) also increased, and both TSH and T_4 levels remained unchanged after treatment. The response rate in terms of cognitive improvement was as follows: 59.74 % responders (RRs), 24.44 % nonresponders (NRs), and 15.82 % stable responders (SRs) (no change in MMSE score after 3 months of treatment). The response rate in cholesterol levels was very similar: 57.78 % RRs, 28.50 % NRs, and 13.72 % SRs [15].

8.1 APOE-Related Cognitive Function Changes

In this study, the basal MMSE score differed in *APOE-2/3* carriers with respect to *APOE-2/4* ($p < 0.02$), *APOE-3/4* ($p < 0.004$), and *APOE-4/4* carriers ($p < 0.0009$); in *APOE-3/3* vs. *APOE-3/4* ($p < 0.0005$), and *APOE-3/3* vs. *APOE-4/4* ($p < 0.002$). The best responders were *APOE-3/3* ($p < 0.0000000001$) > *APOE-3/4* ($p < 0.00001$) > *APOE-4/4* carriers ($p < 0.05$). Patients harboring the *APOE-2/3* and *APOE-2/4* genotypes did not show any significant improvement. The response rate by genotype was the following: *APOE-2/3*: 44.26 % RRs, 36.07 % NRs, 19.67 % SRs; *APOE-2/4*: 55.56 % RRs, 44.44 % NRs, 0.0 % SRs; *APOE-3/3*: 63.42 % RRs, 21.06 % NRs, 15.52 % SRs; *APOE-3/4*: 56.94 % RRs, 27.75 % NRs, 15.31 % SRs; *APOE-4/4*: 51.43 % RRs, 28.57 % NRs, 20.00 % SRs [15] (*see* Figs. 9 and 10).

8.2 APOE-Related Changes in Blood Pressure Values

Systolic blood pressure was significantly reduced in patients with the *APOE-3/3* ($p < 0.00007$) and *APOE-3/4* genotypes ($p < 0.01$), and diastolic blood pressure exhibited a similar pattern (*APOE-3/3*, $p < 0.005$; *APOE-3/4*, $p < 0.01$), with no changes in either systolic blood pressure or diastolic blood pressure in *APOE-2/3*, *APOE-2/4*, and *APOE-4/4* carriers [15].

8.3 APOE-Related Blood Lipid Response to Sardilipin (E-SAR-94010)

Basal cholesterol levels were significantly different in patients with the *APOE-2/3* genotype vs. *APOE-3/3* ($p < 0.007$), vs. *APOE-3/4* ($p < 0.001$), vs. *APOE-4/4* ($p < 0.00002$); *APOE-2/4* vs. *APOE-4/4* ($p < 0.01$); *APOE-3/3* vs. *APOE-4/4* ($p < 0.005$); and *APOE-3/4* vs. *APOE-4/4* ($p < 0.01$).

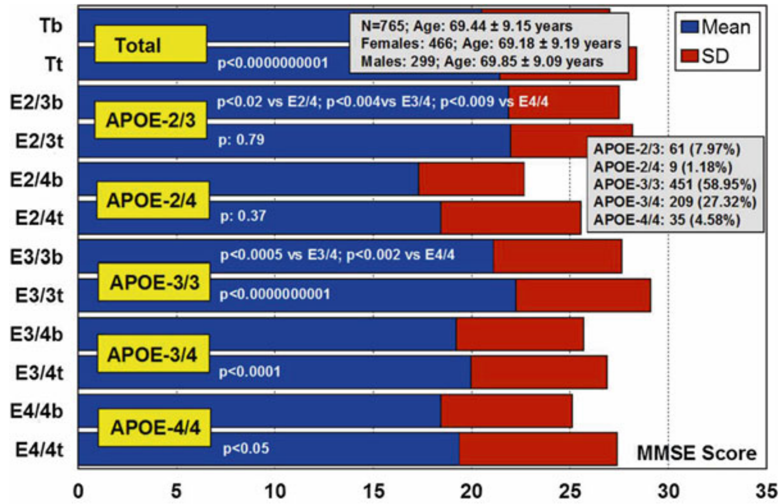

Fig. 9 APOE-related cognitive performance in response to a multifactorial therapy in patients with dementia. Tb: Basal MMSE score prior to treatment; Tt: MMSE score after 3 months of treatment in the total sample. E2/3b: Basal MMSE score in *APOE-2/3* carriers; E2/3t: MMSE score after treatment in *APOE-2/3* carriers; E2/4b: Basal MMSE score in *APOE-2/4* carriers; E2/4t: MMSE score after treatment in *APOE-2/4* carriers; E3/3b: Basal MMSE score in *APOE-3/3* carriers; E3/3t: MMSE score after treatment in *APOE-3/3* carriers; E3/4b: Basal MMSE score in *APOE-3/4* carriers; E3/4t: MMSE score after treatment in *APOE-3/4* carriers; E4/4b: Basal MMSE score in *APOE-4/4* carriers; E4/4: MMSE score after treatment in *APOE-4/4* carriers. (Adapted from ref. 15)

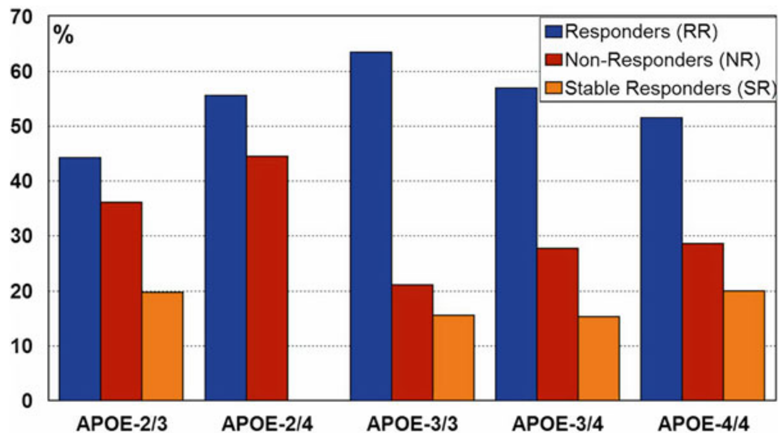

Fig. 10 APOE-related cognitive response rate in patients with dementia treated with a multifactorial therapy

The highest cholesterol levels were seen in *APOE-4/4*> *APOE-3/4*> *APOE-3/3*. All patients showed a clear reduction in cholesterol levels after treatment with Sardilipin. This was particularly significant in *APOE-3/3* ($p < 0.0000000001$) > *APOE-3/4* ($p < 0.00000008$) > *APOE-4/4* ($p < 0.002$) > *APOE-2/3* ($p < 0.02$) >

APOE-2/4 carriers (*p*: 0.26). The response rate by genotype was as follows: *APOE-2/3*: 63.93 % RRs, 29.51 % NRs, 6.56 % SRs; *APOE-2/4*: 44.44 % RRs, 22.22 % NRs, 33.34 % SRs; *APOE-3/3*: 54.32 % RRs, 28.16 % NRs, 17.52 % SRs; *APOE-3/4*: 53.59 % RRs, 31.58 % NRs, 14.83 % SRs; *APOE-4/4*: 65.71 % RRs, 20.00 % NRs, 14.29 % SRs [15].

HDL-cholesterol levels significantly decreased in *APOE-3/3* ($p < 0.001$) > *APOE-3/4* ($p < 0.05$), with no significant changes in patients with other genotypes. In contrast, LDL-cholesterol levels showed identical changes to those observed in total cholesterol, with similar differences among genotypes at baseline and almost identical decreased levels after treatment (*APOE-3/3*, $p > 0.0000000001$; >*APOE-3/4*, $p < 0.00001$; >*APOE-2/3*, $p < 0.0004$; >*APOE-4/4*, $p < 0.001$; >*APOE-2/4*, $p = 0.31$) [15].

Sardilipin (E-SAR-94010, LipoEsar®, LipoSea®) is a natural product extracted from the marine species *Sardina pilchardus*, by means of non-denaturing biotechnological procedures. The main chemical compounds of LipoEsar® are lipoproteins (60–80 %) whose micelle structure probably mimics that of physiological lipoproteins involved in lipid metabolism. In preclinical studies, sardilipin has been shown to be effective in (1) reducing blood cholesterol (CHO), triglyceride (TG), uric acid (UA), and glucose (Glu) levels, as well as liver alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activity; (2) enhancing immunological function by regulating both lymphocyte and microglia activity; (3) inducing antioxidant effects mediated by superoxide dismutase activity; and (4) improving cognitive function [15].

According to these results, it appears that the therapeutic response of patients with dyslipidemia to sardilipin is *APOE*-related. The best responders were patients with *APOE-3/3* > *APOE-3/4* > *APOE-4/4*. Patients with the other *APOE* genotypes (*2/2*, *2/3*, *2/4*) did not show any hypolipemic response to this novel compound. In patients with dementia, the effects of sardilipin were very similar to those observed in patients with chronic dyslipidemia, suggesting that the lipid-lowering properties of sardilipin are *APOE*-dependent [15] (*see* Fig. 11).

9 Future Perspective

To make AD a global health priority in the coming years, conceptual and procedural changes are needed on several grounds, such as (1) political, administrative, economic, legal, ethical, industrial, regulatory, and educational issues; (2) the implantation of novel biomarkers (genomics, proteomics, molecular neuroimaging) as diagnostic aids; (3) the introduction of innovative therapeutics; (4) the implementation of pharmacogenomics in clinical practice in

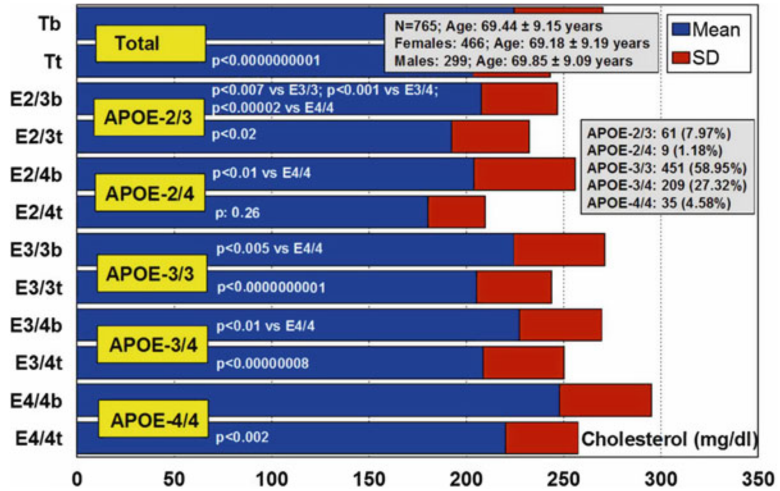

Fig. 11 APOE-related total cholesterol levels response to a multifactorial therapy in patients with dementia. Tb: Basal cholesterol levels prior to treatment; Tt: Cholesterol levels after 3 months of treatment in the total sample. E2/3b: Basal cholesterol levels in *APOE-2/3* carriers; E2/3t: Total cholesterol levels after treatment in *APOE-2/3* carriers; E2/4b: Basal cholesterol levels in *APOE-2/4* carriers; E2/4t: Total cholesterol levels after treatment in *APOE-2/4* carriers; E3/3b: Basal cholesterol levels in *APOE-3/3* carriers; E3/3t: Total cholesterol levels after treatment in *APOE-3/3* carriers; E3/4b: Basal cholesterol levels in *APOE-3/4* carriers; E3/4t: Total cholesterol levels after treatment in *APOE-3/4* carriers; E4/4b: Basal cholesterol levels in *APOE-4/4* carriers; E4/4: Total cholesterol levels after treatment in *APOE-4/4* carriers. (Adapted from ref. 15).

order to optimize therapeutics; and (5) the promotion of selective preventive plans for the population at risk.

There is a disharmony in the world concerning the interest of the public and governments toward dementia and its social, medical, and economic implications. The diagnosis and management of dementia is dissimilar in Europe, North America, Latin America, Asia, Africa, and Oceania. The economic/cultural status of each country (developed vs. developing), the particular epidemiology of aging and dementia in each latitude, national standards of education, health priorities (infectious diseases vs. degenerative diseases), and the quality and efficiency of the medical services are conditioning factors for investing (or not) national resources in dementia as a health priority. Educational programs, international guidelines, and consensus protocols for the management of dementia are necessary for a global harmonization of the subject, to speak the same conceptual language among societies and among professionals, and to improve cost-effectiveness ratios [765–768]. There are many legal (i.e., informed consent, lawsuit, testament, tutorship) and ethical issues (i.e., clinical trials, use of genetic information, institutionalization) which deserve more attention to humanize

the end of life in the very frail conditions under which demented patients survive. The updating of regulatory issues is also a matter of deep concern. Regulatory aspects of drug development are not universal, with notable peculiarities in the EU (EMEA), USA (FDA), and Japan (Koseisho). The costs of dementia cannot be fully assumed by over 60 % of the European population; therefore, the European authorities must take into account this circumstance when the new Health Reform is implemented in the coming years [8, 19].

Genomics, transcriptomics, proteomics, and metabolomics will revolutionize medicine in the next decades. Genetic testing is gaining acceptance among physicians and patients in different countries [768–771], although African Americans and Whites in the USA, Europeans, and Japanese differ notably in their knowledge, beliefs, and attitudes regarding genetic testing for AD [767, 771, 772]. The validation of protocols for genomic screening will contribute to introducing structural genomics, functional genomics, and proteomics as diagnostic aids and therapeutic targets [773].

An accurate diagnosis of AD demands the urgent introduction of reliable biomarkers into routine protocols at a reasonable price [116]. The proteomic analysis of levels of specific secreted cellular signaling proteins in CSF or plasma correlate with pathological changes in the AD brain and can thus be used as a biomarker procedure [774]. It is likely that the best biomarkers result from the combination of genomic, transcriptomic, and proteomic analyses of body fluids. The measurement of these biomarkers would correlate with brain imaging markers and cognitive performance [128–131]. New initiatives for the prevention of dementia (global vs. selective prevention) will also emerge [775], together with new insights into the role of nutrition and nutrigenomics in brain function and neurodegeneration [92, 776]. In terms of prevention, it must be taken into consideration that neuronal death and Aβ accumulation starts many years before the onset of the disease, and that preventive strategies should be selective to protect to the population at risk. For this purpose, accurate biomarkers are essential [777]; and surrogate markers are needed to facilitate primary prevention. In the coming years, sophisticated therapeutic approaches to AD and neurodegenerative disorders, such as miRNA, RNAi, stem cell therapy, or gene therapy, will be also postulated as evolving options [778, 779].

Without doubt, the maximum priority for the coming decade will be an intense search for novel therapeutic options in the form of both symptomatic treatments and preventive strategies (*see* Table 2). Past failures must be learned by researchers and the pharmaceutical industry in order to avoid unnecessary expenses in redundant trials which lead nowhere. Combination treatments require further evaluation and more sophisticated strategies than dual combinations [657, 780]. The administration of psychotropic

drugs to demented patients should be reduced and predicted with pharmacogenetic markers to minimize side effects, cerebrovascular risk, and cognitive deterioration.

Priority areas for pharmacogenetic research are the prediction of serious adverse reactions (ADRs) and the establishment of variation in efficacy [781]. Both requirements are necessary in CNS disorders and dementia, to cope with efficacy and safety issues associated with current psychotropics and anti-dementia drugs, and new CNS drugs as well.

Another important issue to take into account is the risk of inducing AD-like pathology in patients chronically treated with drugs for major problems of health, such as cardiovascular disorders, hypertension, diabetes, dyslipidemia, psychiatric disorders, or cancer, which require long-term treatments. For instance, reduced estrogens, after breast cancer treatment with the oral nonsteroidal aromatase inhibitor letrozole, are linked with declined cognitive abilities. Mitochondrial and synaptic structural deficits are exacerbated when letrozole therapy is combined with $A\beta_{1-42}$ treatment, indicating that letrozole may increase neuronal susceptibility to pathological insults, and explaining the increased prevalence of cognitive decline associated with aromatase inhibitor use [782]. Furthermore, CYP19 polymorphisms affect risk for AD in women, and risk alleles vary by ancestry informative markers (AIM)-defined ancestry. Risk for AD was associated with six SNPs (of 41 screened SNPs) in women of predominantly Caucasian AIMs-defined ancestry. Of these, two were also associated with decreased risk of AD in women of admixed/Hispanic AIMs ancestry. Two separate SNPs were found to be protective in women of predominantly African AIMs-based ancestry [783].

With regard to the future of pharmacogenomics as a practical discipline to efficiently optimize therapeutics, several issues should be addressed: (1) the education of physicians in medical genomics and pharmacogenomics is fundamental (less than 2 % of the members of the medical community are familiar with genomic science); (2) genomic screening of gene clusters involved in pharmacogenomic outcomes must become a clinical routine (without genetic testing there is no pharmacogenetics); (3) each patient must be a carrier of a pharmacogenetic card [784] indicating what kind of drugs he/she can take and which medications he/she should avoid; (4) Regulatory Agencies should request pharmacogenetic data from the pharmaceutical industry when applying for drug approval; (5) pharmacogenetic data must be incorporated into the patient information leaflet and the pharmaceutical vade mecum; and (6) new guidelines for daily praxis, such as that of the first World Guide for Drug Use and Pharmacogenomics [687], will facilitate the understanding of the relationship between drugs and genes (and vice versa) to make drug prescription a real personalized procedure.

10 Conclusions

AD is a major problem of health, with a high cost for our society. As a clinical entity, AD is a polygenic/complex disorder in which many different gene clusters may be involved. Most genes screened to date belong to different proteomic and metabolomic pathways potentially affecting AD pathogenesis, represented by accumulation of Aβ deposits in senile plaques, intracellular NFTs with hyperphosphorylated tau, and neuronal loss. The presence of the *APOE-4* allele of the apolipoprotein E gene seems to be a major risk factor for both degenerative and VD, and *APOE* variants are directly involved in AD pathogenesis at multiple levels. Specific biomarkers (structural and functional genomic markers, proteomic markers in body fluids, neuroimaging markers) are needed for an accurate diagnosis of AD. The present pharmacological treatment of AD with cholinesterase inhibitors (donepezil, rivastigmine, galantamine) and memantine is not cost-effective, and there is an overuse of psychotropic drugs in patients with dementia (which contribute to deteriorating cognitive and psychomotor functions). Old treatments addressed memory impairment; however, new treatments are oriented to halt disease progression by interfering with Aβ accumulation, NFT formation, oxidative stress, neuroinflammation, and cerebrovascular damage. Over the past few years diverse candidate drugs (*see* Table 2) have been investigated in AD models but no one has reached the market. Genomic factors potentially involved in AD pharmacogenomics include at least five categories of gene clusters: (1) genes associated with disease pathogenesis; (2) genes associated with the mechanism of action of drugs; (3) genes associated with drug metabolism (phase I and II reactions); (4) genes associated with drug transporters; and (5) pleiotropic genes involved in multifaceted cascades and metabolic reactions. Since only 25–30 % of the population is an extensive metabolizer for drugs which are metabolized via CYP2D6, CYP2C9, and CYP2C19 enzymes, it seems reasonable to incorporate pharmacogenomic procedures to optimize AD therapeutics, reducing ADRs and unnecessary costs. The therapeutic response to conventional drugs in patients with AD is genotype-specific, with *CYP2D6*-PMs, *CYP2D6*-UMs, and *APOE-4/4* carriers acting as the worst responders. *APOE* and *CYP2D6* may cooperate, as pleiotropic genes, in the metabolism of drugs and hepatic function.

By knowing the pharmacogenomic profiles of patients who require treatments with anti-dementia drugs and/or psychotropic drugs of current use, it might be possible to obtain some of the following benefits related to efficacy and safety issues: (1) to identify candidate patients with the ideal genomic profile to receive a particular drug; (2) to adapt the dose in over 90 % of the cases

according to the condition of EM, IM, PM, or UM (diminishing the occurrence of direct side effects in 30–50 % of the cases); (3) to reduce drug interactions by 30–50 % (avoiding the administration of inhibitors or inducers able to modify the normal enzymatic activity on a particular substrate); (4) to enhance efficacy; and (5) to eliminate unnecessary costs (>30 % of pharmaceutical direct costs) derived from the consequences of an inappropriate drug selection and the overmedication administered to mitigate ADRs [19].

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Chapter 14

Pharmacogenetics of Antipsychotic Treatment in Schizophrenia

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Abstract

Antipsychotics are the mainstay treatment for schizophrenia. There is large variability between individuals in their response to antipsychotics, both in efficacy and adverse effects of treatment. While the source of interindividual variability in antipsychotic response is not completely understood, genetics is a major contributing factor. The identification of pharmacogenetic markers that predict antipsychotic efficacy and adverse reactions is a growing area of research, and holds the potential to replace the current trial-and-error approach to treatment selection in schizophrenia with a personalized medicine approach.

In this chapter, we provide an overview of the current state of pharmacogenetics in schizophrenia treatment. The most promising pharmacogenetic findings are presented for both antipsychotic response and commonly studied adverse reactions. The application of pharmacogenetics to schizophrenia treatment is discussed, with an emphasis on the clinical utility of pharmacogenetic testing and directions for future research.

Key words Antipsychotics, Pharmacogenetics, Genetics, Schizophrenia, Response, Side effects

1 Introduction

Schizophrenia is a debilitating disorder affecting 1 % of the global population. It is a pervasive disease, affecting many aspects of mental function. Positive, negative, affective, and cognitive symptom clusters characterize schizophrenia (Table 1).

Antipsychotics are the current standard of care in schizophrenia management. There are two classes of antipsychotics: typical or first generation antipsychotics (FGAs), and atypical or second generation antipsychotics (SGAs). Both classes of antipsychotics block dopamine D2 receptors, and this dopaminergic antagonism is considered necessary and sufficient for antipsychotic action [1]. While there are no major differences in efficacy of FGAs and SGAs, their tolerability profiles are diverse. FGAs are more likely to cause extrapyramidal side effects (e.g., acute motor side effects, tardive dyskinesia) and increased prolactin secretion, while SGAs are generally more likely to cause marked weight gain. These differences in

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Table 1
Symptom clusters in schizophrenia

Symptom cluster	Clinical examples
Positive symptoms	Delusions Hallucinations Disorganized speech
Negative symptoms	Avolition Anhedonia Poverty of thought
Affective symptoms	Depression Anxiety Affective flattening
Cognitive symptoms	Attention Memory Executive function

Adapted with permission from [151]

adverse drug reactions are most likely because FGAs dissociate more slowly from D2 receptors, resulting in distortion of physiological dopaminergic transmission, and because SGAs modulate a variety of additional neurotransmitter systems (Table 2) [1].

The selection of appropriate antipsychotic treatment is often a "trial-and-error" procedure, with multiple failed trials required before achieving an acceptable balance between symptom management and tolerability of adverse effects. This is problematic because it increases the risk of adverse drug reactions and delays symptom management, worsening long-term treatment outcomes [2].

There is large variability between individuals in their response to and tolerability of antipsychotic treatment. Some patients enter complete remission following treatment with a particular antipsychotic, while others treated with the same antipsychotic show no response or experience common adverse effects such as tardive dyskinesia or weight gain. While environmental factors such as lifestyle habits (smoking, diet), demographics (sex, ethnicity), and health status (concurrent medications, illness onset and duration, medical comorbidities) contribute to the variability in both response and tolerability of antipsychotic treatment, there is also a clear genetic contribution to this variability [3–7].

Given the underlying genetic regulation of response to antipsychotic treatment, pharmacogenetics holds the potential to provide a robust rationale for treatment optimization. The goal of pharmacogenetics in schizophrenia is to replace the current trial-and-error treatment paradigm with a personalized medicine approach, allowing clinicians to map the right dose of the right drug to first-episode schizophrenia patients based on their genetic profile [8].

Table 2
Differences in pharmacology and side effect profiles of FGAs and SGAs

	First generation	Second generation
Metabolism	*CYP2D6* Chlorpromazine[a] Fluphenazine[a] Haloperidol[a] Perphenazine[a] Thioridazine[a] *CYP3A4* Haloperidol[a] Loxapine[a] Pimozide[a] *CYP1A2* Chlorpromazine[a] Loxapine[a] Perphenazine[a] Thioridazine[a] Thiothixene[a] Trifluoperazine[a]	*CYP2D6* Aripiprazole[a] Clozapine[b] Iloperidone[a] Olanzapine[b] Risperidone[a] *CYP3A4* Aripiprazole[a] Clozapine[a] Iloperidone[a] Lurasidone[b] Quetiapine[b] Risperidone[a] Ziprasidone[a] *CYP1A2* Clozapine[a] Olanzapine[a]
Site of action	*DRD2*: Higher affinity antagonists Variable effects on other receptors: serotonergic, adrenergic, histaminic, muscarinic	*DRD2*: Lower affinity antagonists *HTR2*: Higher affinity antagonists Variable effects on other receptors: adrenergic, histaminic, muscarinic
Common adverse reactions	Acute motor side effects Tardive dyskinesia Hyperprolactinemia	Weight gain and metabolic disturbances Sedation Agranulocytosis[c]

Information compiled from [152]
[a]Primary metabolism
[b]Secondary metabolism
[c]This adverse reaction is associated primarily with clozapine treatment and occurs in a minority of patients

In recent years much progress has been made in identifying genetic variants associated with antipsychotic response and adverse reactions to treatment. Initially, candidate gene studies were conducted to explore single nucleotide polymorphisms (SNPs) in pharmacokinetic genes affecting the bioavailability of antipsychotics (through absorption, distribution, metabolism, and excretion), with a particular emphasis on the cytochrome P450 enzymes. Considerable attention has also been given to studying pharmacodynamic genes affecting the mechanism of antipsychotic drug action (through neurotransmitter transporters or receptors). More recently, genome-wide association studies (GWAS) have identified variants in previously uninvestigated genes that are associated with antipsychotic response and adverse reactions [9–13].

The scope of the present chapter is to provide an overview of the current state of pharmacogenetics in schizophrenia treatment. The most promising pharmacogenetic findings will be presented for both antipsychotic response and the most commonly studied antipsychotic side effects. The application of pharmacogenetics to schizophrenia treatment will also be discussed, with an emphasis on the clinical utility of pharmacogenetic testing and directions for future research.

2 Pharmacogenetics of Antipsychotic Response

Response to antipsychotics is a complex phenotype likely involving several different genes, making pharmacogenetic studies in this area challenging. Despite these methodological challenges, a number of genetic variants have been consistently observed in association with antipsychotic treatment response, and preliminary efforts to integrate these pharmacogenetic findings into treatment selection for schizophrenia patients are now underway.

2.1 Pharmacokinetic Candidates in Antipsychotic Response

As most antipsychotic medications undergo extensive first-pass metabolism, drug metabolizing enzymes (DME) play an important role in patient response to antipsychotic treatment by determining drug *bioavailability* (*the fraction of the antipsychotic that reaches the systemic circulation and is available to act on its targets in the brain*). Some antipsychotics, such as clozapine and risperidone, also undergo *bioactivation* by DME resulting in the generation of active metabolites of the parent drug which may have toxic or therapeutic effects. DMEs are also important in antipsychotic *clearance* (*the ability of the body to eliminate the drug*). The cytochrome P450 (CYP) enzymes are the major enzymes influencing antipsychotic bioavailability, bioactivation, and clearance [14].

The genes encoding CYP enzymes are typically polymorphic, and their variation leads to decreased or elevated catalytic activity. Individual CYP genotypes contribute to various combinations of haplotypes commonly classified as "star (*) alleles," which are considered to be functionally "active," "decreased activity," or "inactive" in terms of catalytic activity. An individual's phenotype for a particular CYP enzyme is commonly classified as "poor metabolizer" (two inactive alleles), "intermediate metabolizer" (one inactive allele + one active or decreased activity allele, or two decreased activity alleles), "extensive/normal metabolizer" (two active alleles), or "ultra-rapid metabolizer" (gene duplication with no inactive or decreased activity alleles). Therefore, genetic variation in CYPs affects their catalytic activity, contributing to differences in the bioavailability, bioactivation, and clearance of antipsychotic drugs between patients and likely influencing their drug plasma levels or metabolite ratios and, consequently, their response and adverse effect profiles.

In addition to the CYP enzymes, drug transporters in the blood–brain barrier play an important role in the pharmacokinetics of antipsychotics by regulating their accumulation in the brain. Several antipsychotics are substrates of the P-glycoprotein transporter [15]. P-Glycoprotein is expressed ubiquitously by cells of the blood–brain barrier and acts as an efflux pump to remove antipsychotics from brain tissues. By influencing the transport efficacy of P-glycoprotein, polymorphisms in the P-glycoprotein gene *ABCB1* may lead to variability among schizophrenia patients in the accumulation of antipsychotics in their brains, thereby leading to differences in treatment response.

As antipsychotic drugs are metabolized primarily by CYP1A2, CYP2D6, and CYP3A4, with CYP2C19 partially involved in clozapine metabolism [16], the most promising findings in relation to these enzymes, in addition to the P-glycoprotein gene *ABCB1*, will be reviewed here (*see* Table 3 for a summary of pharmacokinetic findings). As a supplement to the findings presented here, the interested reader is referred to the comprehensive review by Ravyn et al. [17].

2.1.1 CYP2D6

As approximately 40 % of antipsychotics are major substrates for CYP2D6 [16], individual variability in antipsychotic efficacy may be influenced by *CYP2D6* genotype. With more than 100 identified allelic variants of *CYP2D6* conferring variable catalytic activity, *CYP2D6* genotype has a clear correlation with metabolic capacity. *CYP2D6*1*, **2*, **33*, and **35* are considered active alleles, *CYP2D6*9*, **10*, **17*, **29*, **36*, and **41* show decreased activity, and *CYP2D6*3-8*, **11-16*, **19-21*, **38*, **40*, and **42* are considered inactive [18].

CYP2D6 genotype strongly affects the bioavailability of many antipsychotics, and CYP2D6 poor metabolizers have higher levels of dose-corrected risperidone, haloperidol, aripiprazole, and perphenazine following antipsychotic treatment (reviewed by Ravyn et al. [17]). At the time of writing *CYP2D6* is the only FDA-approved pharmacogenetic test for antipsychotic medications, with *CYP2D6* testing recommended to guide dosage of aripiprazole, iloperidone, and pimozide [19]. While there is some evidence that *CYP2D6* genotype affects susceptibility to adverse drug reactions, it is not a reliable predictor of clinical response to antipsychotics. At least 14 studies have investigated the association between *CYP2D6* genotypes and antipsychotic efficacy, with few positive findings (reviewed by Ravyn et al. [17]).

2.1.2 CYP1A2

Approximately 18 % of antipsychotics are major substrates for CYP1A2 [16], suggesting *CYP1A2* genotype may be an important factor influencing antipsychotic bioavailability and response. There is an estimated tenfold to 20-fold variation in CYP1A2 activity between individuals in the population [20]. Although CYP1A2 is inducible by dietary and lifestyle factors such as caffeine and smoking, genetic

Table 3
Most robust pharmacogenetic findings for antipsychotic metabolism and response

Gene	Polymorphism	Findings	Reference
Pharmacokinetic genes			
CYP2D6	Poor metabolizer	Higher levels of dose-corrected antipsychotics	[17][a]
ABCB1	C3435T G2677T C1236T	Better treatment response for T allele carriers Better treatment response for T allele carriers Better treatment response for T allele carriers	[15][a]
Dopaminergic genes			
DRD2	−141C Ins/Del	Better treatment response for Ins allele carriers	[33][b]
DRD3	Ser9Gly	Better treatment response for Gly allele carriers	[38][b]
Serotonergic genes			
HTR1A	C-1019G	Greater improvement of negative symptoms for C allele carriers	[40–42]
HTR2A	T102C	Better treatment response for T allele carriers	[45][b]
HTR2C	Cys23Ser	Better treatment response for Ser allele carriers	[51][b]
5HTT	5HTTLPR L/S	Better treatment response for L allele carriers	[54–56]
Other genes			
COMT	Val108/158Met	Greater improvement of negative and cognitive symptoms for Met allele carriers	[63–65]
GNB3	C825T	Better treatment response for C allele carriers	[67–69]
BDNF	Val66Met	Better treatment response for Val allele carriers	[76, 77]
ZNF804A	rs1344706	Less improvement of positive symptoms for AA carriers	[83, 84]

[a]Findings described are from a systematic review
[b]Findings described are from a meta-analysis

factors are thought to account for the majority of variability in CYP1A2 activity in the population [21]. Over 20 *CYP1A2* alleles have been identified, but they have so far been associated with relatively small changes in enzymatic activity [18]. Further research is required to identify functional CYP1A2 variants.

CYP1A2 activity is highly correlated with olanzapine clearance [20]. However, few studies have investigated *CYP1A2* genotype in relation to antipsychotic response. One notable exception is the *CYP1A2**1F* (rs762551) allele, which is associated with increased CYP1A2 inducibility. While initial studies reported an association between *CYP1A2**1F* and serum olanzapine concentrations, further studies failed to replicate this finding (reviewed by Perera et al. [20]).

2.1.3 CYP3A4

CYP3A4 is considered the most abundant CYP in drug metabolism [22], and approximately 23 % of antipsychotics are major substrates for this enzyme suggesting *CYP3A4* genotype may play a key role in antipsychotic pharmacokinetics and treatment response. CYP3A4 activity shows at least 20-fold variation in the population, and like CYP1A2 the majority of this variation is due to genetic factors [23]. While over 20 alleles for *CYP3A4* have now been identified, most are very rare and do not appear to influence catalytic activity substantially [22], and the identification of additional variants in this gene is an important area for future research. As such, few *CYP3A4* alleles have been studied in relation to antipsychotic plasma levels or response, despite its important role in the metabolism of several antipsychotics including haloperidol and quetiapine. Worthy of mention is the *CYP3A4**1B* (rs2740574) polymorphism, which increases *CYP3A4* expression [24] and CYP3A4 catalytic activity [25]. *CYP3A4**1B* has shown preliminary association with response to antipsychotic treatment, with one study reporting that *CYP3A4**1B* carriers had better treatment outcomes compared to *CYP3A4**1A* homozygotes [26].

Notably, CYP3A4 activity can be altered by medications (e.g., CYP3A4 induction by carbamazepine), and CYP3A4 is generally recognized as an important contributor in drug–drug interactions.

2.1.4 ABCB1

There is evidence for P-glycoprotein mediated transport of many antipsychotics, including amisulpride, aripiprazole, olanzapine, and risperidone, at therapeutic concentrations [15]. Interestingly, there is a high degree of overlap in the substrates of P-glycoprotein and CYP3A4, and inhibitors of P-glycoprotein often also inhibit CYP3A4 [15]. The gene encoding P-glycoprotein, *ABCB1*, is highly polymorphic. Pharmacogenetic investigations have focused primarily on three SNPs in *ABCB1*: C3435T (rs1045642), G2677T/A (rs2032582) and C1236T (rs1128503). Unfortunately these SNPs do not provide a complete picture of the genetic variation in *ABCB1*, particularly because they are in significant linkage disequilibrium ($D' > 0.75$). Nevertheless, there is preliminary evidence that polymorphisms in *ABCB1* affect SGA treatment response. At least 15 studies have investigated C3435T, G2677T/A and C1236T polymorphisms in association with SGA response, and the rare alleles were associated with better treatment outcomes in the majority of studies (reviewed by Moons et al. [15]). The C3435T, G2677T/A and C1236T polymorphisms form a haplotype that has been associated with *ABCB1* gene expression and hepatic P-glycoprotein activity [27], as well as P-glycoprotein substrate specificity [28].

2.2 Pharmacodynamic Candidates in Antipsychotic Response

Another emphasis of pharmacogenetic research in schizophrenia has been the pharmacodynamic genes, which encode targets of antipsychotic drug action (including neurotransmitter receptors and signalling molecules). Variation in genes coding for the targets

of antipsychotic drugs may influence binding affinity and efficiency of signalling induction by antipsychotics, thereby contributing to variability in response to these drugs among patients.

All antipsychotics show high binding affinity for the dopamine D2 receptor, and as previously discussed, antagonism of this receptor is considered the primary requirement for antipsychotic drug efficacy [1]. Many antipsychotics, particularly SGAs, also act as antagonists at serotonergic receptors (primarily serotonin 2A and 2C) [29]. It has been suggested that negative and cognitive symptom improvement following antipsychotic treatment are the result of this serotonergic antagonism [29]. In line with this theory, the majority of variants associated with negative symptom improvement appear to be in serotonergic genes [30]. However, as relatively few studies have investigated positive and negative symptom response separately this requires further validation.

Given the apparently central role of the dopaminergic and serotonergic systems in antipsychotic efficacy, genes of these systems have been the primary focus of pharmacogenetic research in schizophrenia. A number of antipsychotics also show some affinity for receptors of the adrenergic, muscarinic, and histaminic systems. There has been some investigation of genes of these systems in association with antipsychotic response, but results lack independent replication or are inconsistent between studies. Given the vast amount of research in this area, only the most promising pharmacodynamic candidates will be presented here (*see* Table 3 for a summary). To supplement the findings presented here, the interested reader is referred to additional reviews of this topic by Blanc et al. [31] and Zhang and Malhotra [32].

2.2.1 DRD2

The dopamine D2 receptor (DRD2) plays a critical role in antipsychotic drug action, with DRD2 antagonism considered necessary and sufficient for antipsychotic drug efficacy [1]. As such, *DRD2* is an obvious candidate gene for association with antipsychotic response. There is evidence for a modest association of the −141C Ins/Del polymorphism (rs1799732) with antipsychotic response, with individuals carrying the Del allele showing modestly lower odds of treatment response (OR = 0.65, 95 % CI: 0.43–0.97) (meta-analysis by Zhang et al. [33]). The Del allele is associated with lower *DRD2* gene expression and decreased DRD2 protein density in the striatum [34]. The Taq1A (rs1800497) polymorphism of *DRD2* has also been extensively studied, but does not appear to have a robust impact on antipsychotic response despite evidence that the A1 allele is associated with reduced *DRD2* gene expression [33]. Other polymorphisms including A-241G (rs1799978), Ser311Cys (rs1801028), and Taq1B (rs1079597) have also been investigated in multiple studies, but with inconsistent results (reviewed by Zhang and Malhotra [32]).

2.2.2 DRD3

Most antipsychotics show similar affinity for the dopamine D3 receptor (DRD3) as for DRD2 [35], and DRD3 receptor numbers

increase in the striatum following antipsychotic treatment [36]. Together these observations suggest DRD3 may be an important site of antipsychotic drug action, which has led to extensive study of *DRD3* as a candidate gene influencing antipsychotic response. Studies have focused primarily on the Ser9Gly (rs6280) polymorphism. The Ser allele confers lower binding affinity and weaker response in D3-mediated signalling pathways [37]. Overall, the Ser9Gly polymorphism is weakly associated with antipsychotic response, with modestly lower odds of response for Ser allele carriers (OR = 0.82, 95 % CI: 0.65–1.04, meta-analysis by Hwang et al. [38]).

2.2.3 HTR1A

The serotonin 1A receptor (HTR1A) may influence antipsychotic drug response, particularly of negative and cognitive symptoms [39]. The C-1019G (rs6295) polymorphism has shown consistent association with antipsychotic response, particularly for SGAs, with G allele carriers showing significantly less negative symptom improvement in three independent samples [40–42]. This polymorphism is located in the promoter region of *HTR1A* and affects a transcription factor binding site such that the G allele is associated with increased *HTR1A* expression [43].

2.2.4 HTR2A

The serotonin 2A receptor (HTR2A) is considered to be critically involved in the pathophysiology of hallucinations, and is a major target of SGAs [44]. Three polymorphisms in the *HTR2A* gene have been extensively studied. The T102C (rs6313) polymorphism is weakly predictive of antipsychotic response, particularly for SGAs, with the T allele associated with modestly increased odds of response (OR = 1.36, 95 % CI: 1.08–1.72, meta-analysis by Arranz et al. [45]). Although the T102C polymorphism does not encode an amino acid change, the C allele has been associated with lower *HTR2A* expression resulting from suspected epigenetic mechanisms [46]. The A-1438G (rs6311) polymorphism, located in the promoter region of *HTR2A*, also appears to be associated with antipsychotic response, although results are inconsistent regarding the risk allele [31]. Given the significant linkage disequilibrium between T102C and A-1438G, it has been proposed that the latter may be the functional allele given its proximity to the *HTR2A* promoter and demonstrated association of the G allele with decreased promoter activity [47]. Finally, the His452Tyr (rs6314) polymorphism is robustly associated with antipsychotic response. The His allele, conferring increased signalling efficacy and binding affinity of HTR2A [48], is associated with greater odds of response to antipsychotics (OR = 1.69, 95 % CI: 1.14–2.52, meta-analysis by Arranz et al. [45]). Of interest, imprinting effects modulate transcription of *HTR2A* in males such that it is monoallelic or biallelic depending on the individual [49]. This could complicate the interpretation of *HTR2A* genotype in pharmacogenetic studies, making it difficult to ascertain whether observed associations are the result of genotype or expression profile.

2.2.5 *HTR2C*

The serotonin 2C receptor (HTR2C) is critically involved in mediating the effect of antipsychotics on negative symptoms and cognitive function [50]. The most studied *HTR2C* polymorphism is Cys23Ser (rs6318), which has shown suggestive association with clozapine response with Cys allele carriers showing poorer treatment response as reported in an early meta-analysis [51]). HTR2C receptors play a role in modulating brain noradrenergic activity, and there is some evidence that the Cys allele of Cys23Ser is associated with lower norepinephrine levels in the cerebrospinal fluid [52].

2.2.6 *5HTT*

The serotonin transporter gene (*5HTT* or *SLC6A4*) transports serotonin from synaptic clefts into presynaptic neurons, thus terminating the action of serotonin in the synapse. 5-HTTLPR L/S, an insertion/deletion polymorphism of 44 bp in the *5HTT* promoter, is referred to as long (L allele) when there is an insertion and short (S allele) when there is a deletion. Functionally, the S allele is associated with lower promoter activity, decreased *5HTT* expression, and decreased serotonin reuptake compared to the L allele [53]. This polymorphism is the most studied genetic variant in psychiatry. The S allele has shown association with poor response to clozapine and risperidone treatment in three independent samples [54–56]. However, an additional three studies have reported no association with clozapine response [57–59].

2.2.7 *COMT*

Catechol-O-methyltransferase (COMT) is the major metabolizer of dopamine, as well as norepinephrine and epinephrine. As all antipsychotics act on the dopaminergic system, variation in COMT may alter antipsychotic drug action. The Val108/158Met (rs4680) polymorphism is the result of a G to A transversion, resulting in threefold to fourfold lower enzyme activity for the Met/Met (A/A) genotype and subsequently higher levels of dopamine in the synapse due to less effective degradation [60]. The first investigation of Val108/158Met genotype in association with antipsychotic response by Illi et al. reported that the Met allele was associated with poorer response to FGA treatment [61]. However, this group failed to replicate their initial finding [62], and the Val allele has now shown association with poor response to clozapine and olanzapine, particularly for cognitive and negative symptoms, in three independent samples [63–65].

2.2.8 *GNB3*

The *GNB3* gene encodes the β-subunit 3 of G-proteins, and is involved in receptor signal transduction. As both dopaminergic and serotonergic receptors signal through G-protein coupled receptors, variation in *GNB3* may alter the efficacy of antipsychotic action at these receptors. Marker C825T (rs5443) in *GNB3* has shown weak association with overall symptom improvement following antipsychotic treatment. The T allele of C825T was associated

with lower odds of response to antipsychotics in three independent samples [67–69], but showed no association with response in two additional samples [70, 71]. The T allele is associated with the occurrence of a splice variant, in which 41 amino acids are deleted but the resulting enzyme (GNB3-s) confers enhanced activation of G-proteins [66].

2.2.9 BDNF

Brain-derived neurotrophic factor (BDNF) is a key regulator of neurotransmission and synaptic plasticity, and plays a major role in neurodevelopment and dynamic neuronal processes such as learning and memory (reviewed by [72]). Antipsychotics modulate long-term potentiation in the hippocampus, a process in which BDNF is intimately involved. Serum BDNF levels are lower than normal in drug-naïve schizophrenia patients, and antipsychotics alter BDNF levels, suggesting that variation in BDNF activity may influence antipsychotic drug action (reviewed by [73]). The most studied polymorphism in BDNF is Val66Met (rs6265), a functional variant for which the Met allele impairs BDNF mRNA targeting to dendritic cells, as well as BDNF protein packaging and secretion, leading to reduced synaptic plasticity [74, 75]. The Met allele has shown association with poorer treatment response in two independent samples [76, 77]. However, negative studies have also been reported [78, 79], and the role of Val66Met or additional *BDNF* variants in antipsychotic efficacy requires further research.

2.2.10 ZNF804A

The precise function of ZNF804A, a zinc-finger domain-containing protein, is an active area of research. *ZNF804A* appears to play a role in neurodevelopment, and has been implicated in the risk for schizophrenia [80]. Marker rs1344706 of *ZNF804A* is considered functional, with the A allele conferring increased *ZNF804A* expression and showing association with schizophrenia [80, 81]. Although an initial study reported no association between rs1344706 and antipsychotic response [82], two more recent studies have reported a significant association between the A allele and less improvement in positive symptoms [83, 84]. The apparent association between *ZNF804A* and antipsychotic efficacy may be the result of a direct role for this gene in antipsychotic response, the mechanism of which has not yet been elucidated. Alternatively, the observed association may be due to the role of *ZNF804A* as a biomarker for more severe forms of schizophrenia, which show greater treatment resistance.

2.3 Genome-Wide Association Studies (GWAS)

While candidate gene studies have provided an insight into the role of established pharmacokinetic and pharmacodynamic targets in antipsychotic treatment response (Table 3), these studies are restricted to our current limited understanding of antipsychotic mechanisms of action. An alternative approach is the use of genome-wide association studies (GWAS), which investigate the

association of variants across the entire genome with antipsychotic response using a hypothesis-free approach. The strongest associations observed in GWAS of antipsychotic response have been in previously uninvestigated genes or intergenic regions, shedding a new insight into genetic factors regulating antipsychotic response.

The first GWAS of antipsychotic response was conducted in a phase 3 randomized controlled trial of iloperidone, and reported an association between marker rs11851892 in *NPAS3* and improvement in total PANSS (non-G/G genotype showed OR = 2.74 for 20 % improvement in PANSS following treatment, $p = 1.1 \times 10^{-3}$) [9]. Ikeda et al. performed a GWAS of risperidone response in a Japanese population, and found a variant in *ATP2B2* was nominally associated with risperidone response ($p = 1.60 \times 10^{-5}$) [10]. A GWAS in the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) sample identified intergenic variants on chromosome 4 (rs17390445, rs11722719) to be associated with positive symptom improvement on ziprasidone, variants in *ANKS1B* (rs7968606) to be nominally associated with negative symptom improvement on olanzapine, and variants in *CNTNAP5* (rs17727261) and *TRPM1* (rs17815774) to be nominally associated with negative symptom improvement on risperidone [11]. A major strength of these GWAS is that they have investigated drug-specific response, unlike many previous candidate gene studies. However, the results of these GWAS should be interpreted cautiously as genome-wide significance ($<5 \times 10^{-8}$) was not achieved in any of the studies. Additionally, in the iloperidone study the patient population was ethnically heterogeneous, with inadequate correction for population stratification [9]. In order to validate the preliminary findings from these GWAS, replication in independent samples is required.

2.4 Summary of Pharmacogenetics of Antipsychotic Metabolism and Response

Regarding antipsychotic metabolism, *CYP2D6* plays an important and well-understood role. While numerous genetic variants have been studied in association with antipsychotic response, most have not been replicated or have not remained significant after pooling results across independent studies. There is now robust evidence that variation in *DRD2* and *HTR2A* are associated with antipsychotic response, although the effect sizes appear to be modest. New pharmacogenetic candidates for antipsychotic response have recently been identified by GWAS, providing promising directions for research in this area.

The challenge of identifying replicable pharmacogenetic variants in antipsychotic response is the result of a number of factors. Perhaps most importantly, antipsychotic response is complicated, multidimensional, and fluctuating, making it difficult to measure objectively. Most studies have relied on clinician-rated scales such as the Brief Psychiatric Rating Scale (BPRS), Positive and Negative Symptom Scale (PANSS), or Global Clinical Impressions Scale

(CGI) to define response, while others used overall remission or recovery. Even among those studies that used clinician-rated scales there was no standard criteria for follow-up time or response threshold, making comparisons across studies difficult. Furthermore, most studies have not assessed the response of positive and negative symptoms separately, instead reporting associations with overall symptom response. Current findings may therefore primarily reflect improvement in positive symptoms, which show greatest improvement clinically with most antipsychotics [30], leaving genetic predictors of negative symptom improvement relatively understudied. It is also worthy of mention that in many studies the samples consisted of chronic schizophrenia patients who had undergone previous antipsychotic treatment, and were either studied naturalistically or following the initiation of a new antipsychotic. Previous antipsychotic treatment may be an important confound when investigating pharmacokinetic variants, because the effect of genetic variation on DME activity have already been adjusted by dose titration. Another issue is that, with most samples being relatively small, studies were often underpowered to detect genetic associations with small to moderate effects on antipsychotic response. Additionally, although some studies have included patients treated with different antipsychotics it is still not clear whether pharmacogenetic associations will emerge as general, class-specific, or drug-specific. Finally, nongenetic factors influencing antipsychotic response such as diet, smoking habits, medical comorbidities, and patient compliance, have been inconsistently accounted for across studies.

These methodological differences account, at least in part, for the lack of replication observed across pharmacogenetic studies of antipsychotic response. Despite the challenges of conducting genetic studies in this area, a number of variants have shown consistent association with antipsychotic metabolism and response (Table 3).

3 Pharmacogenetics of Antipsychotic-Induced Side Effects

Patient compliance is a significant challenge in the treatment of schizophrenia, with an estimated non-compliance rate of 42 % [85]. Given that long-term outcomes are significantly improved with early symptom management [2], promoting compliance in first episode patients represents an opportunity to greatly improve clinical outcomes of schizophrenia. Harmful side effects of antipsychotic treatment are one of the strongest predictors of non-compliance reported by first episode schizophrenia patients [86]. Pharmacogenetic predictors of antipsychotic-induced side effects have the potential to allow clinicians to predict which patients will experience adverse effects when treated with a particular antipsychotic, based on their

genetic information. This information would provide a rational basis for treatment selection in a way that minimized side effects, thereby improving compliance.

With this goal of predicting adverse reactions in mind, a number of candidate gene studies have explored the association between particular genetic variants and adverse effects of antipsychotics. Some of the most studied adverse reactions are weight gain, tardive dyskinesia, and agranulocytosis. Genetic associations with these adverse effects appear to be more robust than for antipsychotic response, both with respect to pharmacokinetic and pharmacodynamic variants. This is likely because of the more objective nature of adverse drug reactions, in contrast to the previously discussed complexities of defining antipsychotic response. In the following section, the most promising genetic variants from candidate gene studies as well as GWAS will be discussed in relation to antipsychotic-induced weight gain, tardive dyskinesia, and agranulocytosis.

3.1 Antipsychotic-Induced Weight Gain

Weight gain is a common side effect of SGAs, with up to 20 % of patients gaining ≥7 % of their baseline weight within the first 6 weeks of treatment [87]. SGAs have varying propensities to cause weight gain, with clozapine and olanzapine associated with the greatest weight gain [88]. Antipsychotic-induced weight gain (AIWG) is a serious adverse effect clinically, as it increases the risk of medical comorbidities including metabolic syndrome, type 2 diabetes, and cardiovascular disease. In addition, the stigma associated with weight gain is a major contributing factor to patient non-compliance [88]. As such, pharmacogenetic studies have focused on identifying genetic variants predictive of severe AIWG, in order to prevent this treatment side effect. Genes involved in mediating AIWG include those involved in antipsychotic metabolism, neurotransmitter systems (particularly serotonergic), and appetite regulation. The most promising genetic findings for AIWG will be presented here. For supplementary information, the interested reader may wish to consult the recent review by Lett et al. on this topic [88].

3.1.1 HTR2C

The serontonin 2C (HTR2C) receptor is involved in regulation of food intake, with HTR2C antagonists causing increased food intake and weight gain. The C-759T (rs3813929) polymorphism of *HTR2C* influences risk for AIWG. In particular, the C allele was significantly associated with increased risk of weight gain in a meta-analysis of 12 independent samples (OR = 2.70; 95 % CI: 1.46–5.01) [89, 90]. This polymorphism, located in the promoter region of *HTR2C*, appears to affect transcription factor binding [91]. Interestingly, the C-759T polymorphism appears to have the greatest effect on early weight gain (OR = 5.40, 95 % CI: 2.08–14.01 in a meta-analysis of four first-episode samples) [90, 92].

3.1.2 *MC4R*

The melanocortin-4 receptor (*MC4R*) is the receptor for melanocyte-stimulating hormones (MSH), which regulate appetite and energy expenditure. Null mutations of *MC4R* are associated with obesity [93]. A GWAS of AIWG in a drug-naïve pediatric population found the A/A genotype of rs489693, a variant 190 kb downstream of the *MC4R* gene, to be associated with increased weight gain following SGA treatment [12]. Notably, the association between rs489693 and AIWG identified in the discovery cohort ($p = 2.80 \times 10^{-7}$) was replicated in three additional samples (meta-analysis across four samples: $p = 5.59 \times 10^{-12}$). The effect size was clinically of high relevance for all four samples, where patients homozygous for the A risk allele gained at least 3 kg more (or twice as much weight on average) than non-homozygotes. The association between this region downstream of *MC4R* was recently replicated in an additional independent sample, with the A allele of rs489693 associated with greater weight gain following antipsychotic treatment (the C allele of marker rs17782313 was also associated with greater weight gain) [94, 95]. The rs17782313 variant is in significant LD with rs489693, and has been previously identified in relation to weight gain in a GWAS of obesity [96].

3.1.3 *LEP*

Leptin is a peptide hormone secreted by adipose tissue, which acts on the hypothalamus to inhibit food intake and increase energy expenditure. This has made it an attractive candidate gene for pharmacogenetic studies of AIWG. In particular, the promoter polymorphism G-2548A (rs7799039) has been investigated in 14 studies. Although no meta-analysis has been conducted, results of these studies were reviewed by Brandl et al. [97]. The G allele has been associated with increased weight gain following antipsychotic treatment in the majority of studies, although some discrepancies suggest sex and age-specific effects of this variant may exist [97]. The G-2548A polymorphism appears to be functional, with the A/A genotype associated with high *LEP* expression [98].

3.1.4 *BDNF*

Brain derived neurotrophic factor (BDNF) plays a crucial role in regulating energy balance (reviewed by [99]). The low-activity Met allele of the Val66Met (rs6265) polymorphism in *BDNF* has shown association with higher body mass index (BMI) [100]. The Met allele has also been associated with less weight gain following antipsychotic treatment in two independent samples, and remained associated after correction for baseline weight [101, 102]. However, there was some discrepancy in the findings of these studies, as the genotypic effect of Met/Met was significant in the total sample in the first study [101], but only reached significance in male patients in the second study [102]. The most recent study of Val66Met in AIWG showed no significant association [77]. Further research is required to determine the relevance of Val66Met, or additional functional markers in BDNF, in association with AIWG.

3.2 Antipsychotic-Induced Tardive Dyskinesia

Tardive dyskinesia (TD) is a movement disorder characterized by repetitive and involuntary movements such as grimacing, rapid eye blinking, and lip smacking. TD is a potentially irreversible side effect experienced by an estimated 25 % of patients treated long-term with FGAs [103]. Presence of TD is most commonly defined using the Schooler and Kane criteria [104], while severity of TD is most commonly measured using the abnormal involuntary movement scale score (AIMS) [105].

Numerous pharmacogenetic studies have focused on identifying genetic predictors of TD, with the goal of predicting who will experience this adverse effect when treated with a particular antipsychotic. Genes implicated in susceptibility to TD include those involved in antipsychotic metabolism, neurotransmitter systems (particularly dopaminergic), and oxidative stress. The most promising susceptibility loci for TD will be described here. For additional information, the interested reader may wish to consult reviews by Lee and Kang [106] and Müller et al. [107].

3.2.1 *CYP2D6*

Over 20 studies have investigated *CYP2D6* genotype in association with TD, under the hypothesis that altered drug metabolism will result in unfavorable concentrations of antipsychotic plasma levels or metabolites. Several studies have observed greater risk of TD among CYP2D6 poor metabolizers, but no overall association was observed across 20 studies in a recent meta-analysis [108]. However, studies differed considerably in their design and sample size. When the meta-analysis was limited to prospective studies, *CYP2D6* poor or intermediate metabolizers had greater risk of TD compared to extensive metabolizers (OR = 2.08, 95 % CI: 1.21–3.57). Overall, it is likely that an individual's CYP2D6 metabolizer status has some effect on TD risk.

3.2.2 *DRD2*

The dopaminergic system has been an intense focus of pharmacogenetic research in relation to TD due to the role of dopaminergic signalling within the nigrostriatal system in motor control (reviewed by [109]). As the FGAs primarily responsible for causing TD are potent DRD2 antagonists, a number of studies have investigated polymorphisms in *DRD2* in association with TD. The Taq 1A (rs1800497) polymorphism, located approximately 10 kb downstream of *DRD2*, is robustly associated with TD. In a meta-analysis of 1,256 patients, A2 carriers showed significantly greater odds of developing TD compared to non-A2 carriers (OR = 1.30, 95 % CI: 1.09–1.55) [110]. The A2 allele is associated with increased *DRD2* gene expression [111, 112]. The −141 Ins/Del and Ser311Cys polymorphisms of *DRD2* have also been extensively studied, but do not appear to be associated with TD [110, 113].

3.2.3 *DRD3*

Due to the importance of dopaminergic signalling in TD development, and the suspected role of the dopamine D3 receptor (DRD3) in antipsychotic drug action, at least 20 studies have investigated

DRD3 in association with TD. Initial studies investigating the Ser9Gly (rs6280) polymorphism reported significant association with TD [114, 115]. Early meta-analysis of 12 independent samples suggested a modest effect of Ser9Gly on TD development, with the Gly allele associated with increased susceptibility to TD (OR=1.17, 95%CI: 1.01–1.37) [116]. However, a more recent meta-analysis did not show an overall effect of Ser9Gly genotype on TD development across 13 independent samples [117]. Overall, the biological importance of dopaminergic signalling in movement disorders suggests additional variants in *DRD3* may play an important role in TD development. Preliminary findings of a haplotype block in the 5′ region of *DRD3* in association with TD suggest investigating additional *DRD3* variants is an important direction for future research [118].

3.2.4 *HTR2A*

Serotonergic systems may modify susceptibility to TD by modulating nigrostriatal dopaminergic signalling. There is robust genetic evidence that the serotonin 2A receptor gene (*HTR2A*) is involved in susceptibility to TD. In particular, the C allele of the T102C (rs6313) polymorphism confers increased risk of developing TD following antipsychotic treatment (OR 1.64, 95 % CI: 1.17–2.32) (meta-analysis by Lerer et al. [119]). The His452Tyr polymorphism has also been extensively studied in relation to TD, but does not show an overall significant effect (meta-analysis by Lerer et al. [119]). As previously discussed, the imprinting effects of *HTR2A* should be kept in mind when considering the results of pharmacogenetic studies of this gene, as they complicate the interpretation of *HTR2A* genotype.

3.2.5 *COMT*

As the major metabolizer of dopamine, catechol-O-methyltransferase (COMT) is of interest in TD for its role in modulating dopaminergic signalling. The Val108/158Met (rs4680) polymorphism of *COMT* has been investigated as a risk variant for TD in at least seven studies. The Val allele has shown association with increased risk of TD in some studies, although meta-analysis suggests it is a robust finding only for female patients (OR=1.25, 95 % CI: 0.93–1.68, $p=0.019$) [120]. This sex-specific association parallels observations of sexually dimorphic phenotypes in COMT-deficient mice and sexually dimorphic effects of *COMT* genotype on psychiatric phenotypes, for which a mechanism involving estrogen-response elements in the *COMT* promoter region may be responsible (reviewed by [121]).

3.2.6 *HSPG2*

Marker rs2445142 in the heparan sulfate proteoglycan 2 (*HSPG2*) gene was first associated with TD in a GWAS of Japanese ancestry schizophrenia patients [13]. The association between rs2445142 and TD was replicated in two additional samples of patients with European and Jewish Israeli ancestry [122]. The mechanism of HSPG2 association with TD is unknown at present. *HSPG2* heterozygous knockout mice showed reduced vacuous chewing

movements following haloperidol-reserpine combination treatment in a mouse model of TD, suggesting that reduced HSPG2 activity protects against TD [13]. In accordance with this hypothesis, the G risk allele of rs2445142 is associated with higher *HSPG2* expression in human brain tissue [13].

3.2.7 *VMAT2*

The vesicular monoamine transporter 2 (*VMAT2*, or *SLC18A2*), which depletes synaptic dopamine and other monoamines, was first reported in association with TD in a candidate gene array study of the CATIE sample [123]. In this study, the T alleles of polymorphisms rs2619097 (OR 0.60, $p=0.0035$) and rs2015586 (OR 0.60, $p=0.0001$) were protective against TD. The role of *VMAT2* in TD was recently investigated in an additional independent sample, where a number of SNPs selected to tag variation across the *VMAT2* gene showed nominal association with TD [124]. The association of genetic variants in *VMAT2* with TD is of particular interest, as the VMAT2 inhibitor tetrabenazine has shown promise as a therapeutic for movement disorders such as Huntington's disease [125].

3.3 Clozapine-Induced Agranulocytosis

Agranulocytosis, a blood condition characterized by a decrease in the number of circulating neutrophils, greatly impairs the immune response and increases the risk of potentially lethal infection. Clozapine-induced agranulocytosis (CIA), commonly defined as an absolute neutrophil count of $<500\ \mu L^{-1}$, is a rare but potentially fatal adverse effect of clozapine treatment. The cumulative risk of CIA is 0.8–1.5 % within the first year of treatment [126]. While the mechanism of CIA is not clear, it may be the result of toxic clozapine metabolites or immunological processes damaging neutrophils and their bone marrow precursors [127, 128]. Although clozapine is the standard of care for treatment refractory schizophrenia [129], it is currently underutilized due to the fears and inconvenience surrounding the potential side effect of agranulocytosis [130]. Currently, clozapine may only be prescribed if two other antipsychotic treatments have failed. The identification of genetic predictors of CIA holds the potential to improve patient access to clozapine treatment by providing clinicians with a way to identify patients at highest risk of developing CIA, and use caution in clozapine prescription specifically for those patients.

Across the few pharmacogenetic studies of CIA that have been conducted, a variety of human leukocyte antigen (HLA) alleles have been implicated (reviewed by [90]). However, given the rare occurrence of CIA the sample sizes have been very small, and replication in independent samples is lacking. One of the more robust findings in CIA is the *HLA-DQB1* locus. Polymorphisms in *HLA-DQB1* have been associated with CIA across a number of studies [131–134]. Most notable is polymorphism G6672C (rs113332494). The C allele of G6672 showed very strong association with CIA across two independent samples (OR=16.9, 95 % CI: 3.57–109) [134].

Table 4
Most robust pharmacogenetic findings for adverse reactions to antipsychotics

Gene	Polymorphism	Findings	Reference
Antipsychotic-induced weight gain			
HTR2C	C-759T	Greater weight gain for C allele carriers	[90][b]
MC4R	rs489693	Greater weight gain for A allele carriers	[12][b]
LEP	G-2548A	Greater weight gain for G allele carriers	[97][a]
Antipsychotic-induced tardive dyskinesia			
CYP2D6	Poor or intermediate metabolizers	Increased risk of tardive dyskinesia	[108]
DRD2	Taq 1A	Increased risk of tardive dyskinesia for A2 allele carriers	[110][b]
HTR2A	T102C	Increased risk of tardive dyskinesia for C allele carriers	[119][b]
Clozapine-induced agranulocytosis			
HLA-DQB1	G6672C	Increased risk of agranulocytosis for C allele carriers	[134]

[a]Findings described are from a systematic review
[b]Findings described are from a meta-analysis

Other variants in addition to those in *HLA-DQB1* are likely important in conferring risk for CIA, and future research in this area is required. Recently the first whole-exome sequencing study was undertaken in CIA, identifying variants in *PPFIA4*, *USP43*, *ACTN1*, *PODNL1*, and *SPATS1* as putative novel candidate genes contributing to CIA [135]. Replication of these findings is required in independent samples. For supplementary information on genetic findings in CIA, the interested reader may wish to consult reviews of this topic by Chowdhury et al. [136] and Zhang and Malhotra [90].

3.4 Summary of Antipsychotic-Induced Adverse Effects

Overall, notable and robustly replicated findings have been observed in association with antipsychotic-induced adverse effects (Table 4), suggesting large effect sizes where the phenotype can be measured reliably such as for weight gain and agranulocytosis. For AIWG, there is robust evidence that variation in *HTR2C*, *MC4R*, and *LEP* are associated with moderate to large effect sizes. For TD, findings appear to be less robust with smaller effect sizes relative to AIWG and CIA, perhaps due to diagnostic limitations. Nevertheless, there is evidence for a modest effect of *CYP2D6*, *DRD2*, and *HTR2A* in TD susceptibility. Promising initial results for *HSPG2*, first discovered in association with TD in a GWAS and now replicated in two independent samples, illustrate the potential for scientifically

rigorous GWAS to identify novel genes for future investigations of antipsychotic tolerability. As for CIA, a number of classical HLA alleles have shown significant association in small samples, and are awaiting replication. One notable exception is marker G6672C (rs113332494), which has shown a very strong association with CIA in two independent samples. Some of the most promising pharmacogenetic findings in antipsychotic-induced adverse effects are summarized in Table 4.

4 Clinical Perspectives

The rationale for pharmacogenetics in schizophrenia has now been presented, along with the most promising pharmacogenetic findings. Preliminary efforts to translate these findings into the clinic are now under way. Some commercial pharmacogenetic tests have been developed and marketed. In this final section, some of the pharmacogenetic tests currently available for use in schizophrenia will be presented. Access to these tests will be discussed, with an emphasis on barriers to access. Finally, directions for future research in pharmacogenetics of schizophrenia will be presented.

4.1 Pharmacogenetic Testing in Schizophrenia Treatment

A number of commercially available pharmacogenetic tests with relevance to antipsychotics have been developed in recent years, and an overview of these tests is presented in this section. In addition to these commercial pharmacogenetic tests, a number of laboratories provide genetic testing for individual genetic variants implicated in antipsychotic efficacy and tolerability.

4.1.1 AmpliChip™ CYP450 Test

In 2005, the FDA approved the first-ever pharmacogenetic test, the AmpliChip™ CYP450 Test (Roche Molecular Systems, Inc.). The test genotypes up to 33 alleles in *CYP2D6* and three alleles in *CYP2C19* associated with different metabolizing phenotypes. CYP2D6 is a major enzyme involved in antipsychotic metabolism, making this a potentially useful test for the clinical management of schizophrenia. Medical laboratories may use the AmpliChip™ CYP450 genotyping platform and analysis software to test for the pharmacogenetic variants in this test, and obtain a report summarizing the patient's *CYP2D6* and *CYP2C19* genotype and predicted metabolizer phenotype. Preliminary findings suggest the CYP2D6 phenotype provided by the AmpliChip™ CYP450 test was a useful predictor of adverse reactions to risperidone treatment (OR = 3.1, 95 % CI: 1.4–7.0 for poor metabolizers) [137], but was not significantly associated with antipsychotic efficacy in a small pilot study [138]. Additionally, in a prospective study evaluating clinician impressions of the AmpliChip™ CYP450 test, psychiatrists had positive attitudes toward incorporating the test results in their clinical decision-making [139]. However, there has been no

update of the CYP alleles included in the AmpliChip™ since its initial development, and no large-scale studies evaluating its clinical utility.

4.1.2 DMET™ Plus Solution

The DMET (Drug-Metabolizing Enzymes and Transporters)™ Plus Solution (Affymetrix, Inc.) is one of the most comprehensive pharmacogenetic batteries available, providing coverage of 1,936 genetic variants across 231 genes in one assay. This test includes 95 % of the "Core ADME (*A*bsorption, *D*istribution, *M*etabolism, and *E*xcretion) Markers" (185 variants). The ADME markers were selected by an expert consortium to represent the most robustly implicated variants in drug metabolism across 32 genes (http://www.pharmaadme.org). The DMET™ Plus Solution is marketed as a flexible pharmacogenetic testing platform. Medical laboratories can use DMET™ Plus genotyping platforms to type the pharmacogenetic variants included in the test, and have the option to interpret the results themselves or purchase DMET™ Plus analysis software to determine CYP star allele classifications and predict metabolizer status. To the best of our knowledge, the DMET™ Plus Solution has not yet been evaluated for efficacy in improving clinical outcomes with psychotropic drugs.

4.1.3 GeneSight®

The GeneSight® (Assurex Health®) psychotropic test captures variation in 50 alleles across *CYP2D6*, *CYP2C19*, *CYP2C9*, *CYP1A2*, *5HTT*, and *HTR2A*. Clinicians can order the test, which is done by mailing a cheek swab from the patient to an accredited Assurex Health® laboratory. The results of the GeneSight® psychotropic test are used in an algorithm to categorize individuals as high, intermediate, or low risk for poor response and adverse side effects to 26 psychotropic medications, and these results are mailed back to the clinician. The test categorizations correlate with the efficacy of antidepressants [140, 141], but have not yet been studied in relation to antipsychotic efficacy.

4.1.4 Genecept™ Assay

The Genecept™ Assay (Genomind, L.L.C.) consists of a panel of variants in *CYP2D6*, *CYP2C19*, *CYP3A4*, *5HTT*, *HTR2C*, *DRD2*, *COMT*, *CACNA1C*, *ANK3*, and *MTHFR*. Clinicians order the test, and then mail the patient's saliva sample to Genomind, L.L.C. for genotyping and test interpretation. The clinician receives a report with the patient's test results and suggested therapeutic options. To the best of our knowledge, the clinical benefit of using the Genecept™ Assay to guide treatment decisions has not yet been evaluated.

4.1.5 PGxPredict: CLOZAPINE®

PGxPredict:CLOZAPINE® (PGx Health, Division of Clinical Data, Inc.) was made commercially available in 2007 for prediction of CIA. The test, which covers variation in polymorphism G6672C (rs113332494) of the *HLA-DQB1* gene, reportedly has 21 %

sensitivity and 98 % specificity for predicting CIA [134]. The test showed preliminary promise in guiding decisions surrounding clozapine rechallenge [142], but was discontinued due to the lack of clinical uptake.

4.2 Access to Pharmacogenetic Tests

Several companies offer genetic testing directly to consumers by mailing a kit to them for DNA collection, typically from saliva or blood samples. The DNA samples are then mailed to the company for processing, and results are provided directly to the consumer. Although such direct-to-consumer pharmacogenetic testing is available, evidence of its predictive ability is limited and there are ethical concerns surrounding the proper interpretation of results by the public without assistance from a health-care professional [143].

The other major avenue for access to pharmacogenetic testing is through health-care providers, who may order such tests in order to guide treatment decisions. However, whether the clinical benefit of pharmacogenetic testing in schizophrenia justifies the test costs is not yet clear, and is an area of active research. As a result, clinical expert consensus guidelines on the appropriate use, interpretation, and storage of pharmacogenetic data are still lacking for antipsychotic medications. Without appropriate clinical guidelines governing the use of pharmacogenetic tests in schizophrenia, insurance providers are reluctant to provide coverage for such testing. At the time of writing, pharmacogenetic tests for schizophrenia management are generally not covered by insurance, presenting a significant financial barrier to access. However, it is expected that improvements in this area will be made in the near future as clinical guidelines surrounding pharmacogenetic testing have generally been improving in recent years, partially due to the influence of organized consortiums such as the Clinical Pharmacogenetics Implementation Consortium (CPIC) of the National Institutes of Health's Pharmacogenomics Research Network (http://www.pgrn.org) [144] and the Pharmacogenomics Knowledge Base (PharmGKB, http://www.pharmgkb.org) [145].

4.3 Future directions

At this point, substantial evidence is available for a number of genetic variants in relation to antipsychotic efficacy and tolerability, and first commercial tests have been developed to incorporate pharmacogenetic information into clinical practice. However, insufficient validation of the clinical benefit of using pharmacogenetic tests to guide prescribing practices has delayed the development of clinical guidelines for their use and interpretation. The lack of clinical expert consensus guidelines surrounding pharmacogenetic tests in schizophrenia has contributed to poor clinical uptake and inconsistent patient reimbursement for testing. These barriers need to be addressed by appropriate policies governing pharmacogenetics in schizophrenia, but what threshold of evidence is required before such policies are implemented? Efforts to

develop clinical guidelines for pharmacogenetics by the Clinical Pharmacogenetics Implementation Consortium (CPIC) and others suggest the evidence threshold for clinical implementation can be met by (1) strong biological rationale for the gene–drug interaction, (2) replicated evidence that the genetic variant is linked to drug response or adverse reactions, and (3) noninferiority of using the pharmacogenetic test compared to ”treatment as usual” [144, 146, 147]. Currently, the first two levels of evidence are well established for a number of genetic variants with respect to antipsychotic response and tolerability. In order to translate these research advances into clinical practice, testing for these pharmacogenetic variants prior to prescribing antipsychotics must be proven no worse than current prescribing practices.

This final piece of evidence will require prospective assessment of the value of these genetic markers in predicting antipsychotic response and side effects. Randomized controlled trials (RCTs) evaluating pharmacogenetics-based prescribing were proposed in the past [148], but have yet to be fully realized. Notably, genotypic analysis was incorporated into the registration clinical trials for a novel antipsychotic, iloperidone [149]. One of the primary challenges has been that randomized controlled trials (RCTs), the traditional method of demonstrating clinical benefit of a new intervention, require the recruitment of large numbers of first-episode, treatment-naïve schizophrenia patients. Given the accumulation of biological evidence for a number of genetic predictors of antipsychotic response and side effects, delaying clinical implementation of pharmacogenetics until data from such large-scale RCTs is available may actually deprive schizophrenia patients of safer prescribing practices.

In light of this, “pragmatic clinical trials” (PCTs) were proposed as an alternative approach by Mrazek and Lerman [147]. PCTs are a more naturalistic version of the traditional RCT, conducted without the stringent inclusion and exclusion criteria and using the standard or least expensive treatment as the comparator rather than placebo [150]. Currently, a large-scale prospective study is being led by our group to evaluate the cost–benefit of pharmacogenetic testing in prescribing antipsychotic and antidepressant medications (www.im-pact.ca). The results of such prospective trials will facilitate the incorporation of pharmacogenetic predictors of antipsychotic response and tolerability into clinical practice in the future.

5 Conclusion

At the present time the use of *pharmacokinetic variants*, particularly within *CYP2D6*, appear to be clinically useful in providing rationale for antipsychotic dosing. Despite the demonstrated role of pharmacokinetic

variants in determining serum levels of antipsychotics, little data supports their clinical utility in predicting antipsychotic response. However, there is some data supporting an effect of CYP2D6 on antipsychotic-induced side effects such as tardive dyskinesia. The general lack of association observed between variants in the CYP enzymes and antipsychotic treatment outcomes may be a consequence of methodological limitations of studies. This should be further evaluated in studies with improved power and consistent definitions of outcome. Additional prospective studies would also be beneficial in assessing the association between pharmacokinetic variants and antipsychotic response, as the effects of these variants on drug metabolism can be clinically overcome to some extent by dose titration over time and may not be apparent in cross-sectional or retrospective studies. While pharmacokinetic variants are included in all commercially available pharmacogenetic tests, the predictive value of these tests in relation to antipsychotic efficacy and side effects requires further investigation.

A number of *pharmacodynamic variants* have shown robust association with both antipsychotic response and certain adverse effects, and may be useful in predicting therapeutic efficacy and tolerability. In particular, variants in *DRD2*, *HTR2A*, and *HTR2C* are modestly associated with antipsychotic efficacy. Variants in *HTR2C*, *MC4R* and *LEP* are robustly associated with AIWG, while variants in *DRD2* and *HTR2A* show modest association with susceptibility to TD. Finally, marker G6672C in *HLA-DQB1* is strongly associated with CIA. These pharmacodynamic variants are generally underrepresented in commercially available pharmacogenetic tests, which have typically focused on genotyping the CYP enzymes, although GeneSight® and Genecept™ include some pharmacodynamic markers.

In addition to the results of candidate gene studies, GWAS efforts have shown preliminary promise in identifying novel candidates for association with antipsychotic response and adverse effects. If replicated, these findings will further our understanding of the mechanism of antipsychotic action, potentially opening the door to the development of new pharmacological agents and stronger genetic predictors of antipsychotic efficacy and tolerability.

While the discovery of additional genetic variants is an exciting research avenue, there is already robust evidence for a number of variants in genes with a well-understood link to antipsychotic action. Implementation of personalized medicine approaches to schizophrenia treatment is in its early stages. Prospective trials evaluating the predictive value and clinical utility of established pharmacogenetic variants are required to expand the use of current research discoveries in clinical practice. We are confident that the information gleaned from such prospective trials will facilitate further clinical implementation of pharmacogenetics, bringing revolutionary changes in treatment selection for schizophrenia in the near future.

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Chapter 15

Pharmacogenetics of Addiction Therapy

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Abstract

Drug addiction is a serious relapsing disease that has high costs to society and to the individual addicts. Treatment of these addictions is still in its nascency, with only a few examples of successful therapies. Therapeutic response depends upon genetic, biological, social, and environmental components. A role for genetic makeup in the response to treatment has been shown for several addiction pharmacotherapies. For several addiction pharmacotherapies, response to treatment varies based on individual genetic makeup. In this chapter, we discuss the role of genetics in pharmacotherapies, specifically for cocaine, alcohol, and opioid dependences. The elucidation of the role of genetics should aid in the development of new treatments and increase the efficacy of existing treatments.

Key words Gene, Alcohol, Cocaine, Opioid, Addiction, Dependence, Abuse, Drug, Therapy, Polymorphism

1 Introduction

Addiction to illicit drugs such as cocaine and opioids, as well as to legal drugs such as alcohol, can lead to both physiological and social morbidities and have significant economic impact. It is estimated that in the USA alone over $122 billion per year is lost in productivity and time, and an additional $15 billion is incurred in health insurance costs due to drug-related behaviors [1]. In addition, drug addiction may lead to other risky behaviors, making comorbid drug use and addiction, emergency care, and HIV infection more likely [2–4]. Substance abuse has been identified as the number one health problem in the USA, and it results in more deaths, disabilities, and illnesses, and in higher costs to society than does any other preventable disease [5].

Twenty-two and a half million Americans over age 12 were illicit drug users in 2011, which represents 8.7 % of the population [1]. Of these, 16.7 million were addicted to alcohol only, 3.9

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million to illicit drugs, and 2.6 million with codependency to both alcohol and illicit drugs. Approximately 800,000 cocaine users were classified as having dependence or abuse. Although the use of cocaine is lower than that of alcohol or opiates, the numbers are still considerable. Cocaine use has decreased over the past 5 years. In 2006, there were 2.4 million cocaine users in the USA, decreasing to 1.4 million users in 2011, which translates to 0.5 % of the population using cocaine.

Alcohol, a legal drug in most of the world, is used by just over half of the adults in the USA. However, about a quarter of adults participated in binge drinking of five or more drinks on the same occasion in the last month, with nearly 40 % of young adults aged 18–25 engaging in binge drinking [1]. Alcoholism in the USA is slowly declining, with 16.7 million persons aged 12 or older having alcohol dependence or abuse compared to 18.9 million in 2006. However, these numbers still translate to a considerable proportion, 6.5 %, of the US population.

Opioid addiction in the USA has been increasing at alarming rates. Almost two million persons aged 12 or older have used pain relievers non-medically for the first time in the past year [1]. Fifty-five percent of these pain relievers were obtained from family and friends, with only 17 % having been prescribed by a doctor. In the treatment of chronic pain, opioid addiction develops in only about 3 % of those treated in general medical practice [6]. The overuse of these pain relievers may explain the upsurge in the number of heroin users that has increased from 373,000 individuals in 2007 to 620,000 in 2011.

Addiction develops in several stages; initiation of drug use, intermittent to regular use, and, finally, addiction and relapse [7]. Features of addiction are the development of dependence to the drug, such that there is a physiological need for the drug for the individual to function properly, the development of tolerance, whereby larger doses of the drug are required to achieve the same effect, and the development of withdrawal, symptoms that occur when a drug is discontinued. Addiction pharmacotherapy tries to reduce some of these features to attenuate craving and drug use, and to prevent relapse after drug use is terminated.

It is the intent of this review to present the evidence to date of the role of genetic variation in the pharmacological treatment of cocaine, alcohol, and opioid addiction. We will communicate these findings by first presenting the major neurochemical systems, dopaminergic, serotonergic, and opioidergic, and then by discussing the genes functioning in other physiological pathways. Within these sections we will discuss the genes for which genetic variants have been found to be associated with pharmacotherapeutic response for addiction treatment, with respect to three addictions: cocaine, alcohol, and opioids.

2 Pharmacogenetics

Pharmacogenetics is the study of genetic variation that affects treatment response, where "treatment response" may be defined in terms of treatment outcome or adverse effects. New technologies that consider the full genome have given rise to a newer term, pharmacogenomics. Only recently has pharmacogenetics been applied to the study of addiction treatment, and only a few examples of FDA-approved treatment regimens exist that are successful for alcoholism and opioid addiction. For cocaine addiction, no FDA-approved therapy currently exists.

A central goal of pharmacogenetic research focuses on drugs that target craving in order to promote abstinence and on understanding the mechanistic differences of drug addiction affecting individuals. Response to addiction pharmacotherapy is complex, depending upon genetic, biological, environmental, and social components. A substantial portion of the success rate of a therapy may depend upon the genetic makeup of those receiving the treatment. For psychiatric diseases in general, pharmacotherapies succeed in only 60–70 % of patients [8]. Evidence from a number of medical specialties demonstrates that the consideration of a patient's genetic makeup can improve the initial selection of medications [9–11]. The use of genetic information may increase both compliance and positive therapeutic response, as well as avoid dangerous side effects due to toxicity [9, 10, 12, 13].

3 Genetics

The human genome consists of 3.2 billion nucleotides of DNA. In the genome there are approximately 21,000 protein encoding genes, 9,600 long noncoding RNA "genes" (>200 nucleotides long), 8,800 small RNAs "genes", and 11,224 pseudogenes, which previously had been considered to be remnants of "dead" genes, but, in fact, may be active in specific cells [14]. These regions account for approximately 80 % of the genome. Over 38 million single nucleotide polymorphisms (SNPs), 1.4 million short insertions and deletions, and more than 14,000 large structural variants have been mapped [15]. The average person has from 250 to 300 loss-of-function variants in genes that have been annotated [16]. Of these, 50–100 previously have been found to be involved in the development of inherited diseases. In addition, in every person various regions of the genome may be deleted or duplicated. These regions can range from one to millions of nucleotides. It is estimated that approximately 0.4 % of the genome is different between any two unrelated individuals with respect to copy number, that is, the number of repeated or deleted regions. Additionally, functional

variation may also be introduced by mRNA splicing or via epigenetic modification processes, such as DNA methylation or histone modifications.

Genetic variation can regulate how a gene is expressed at a number of levels, including transcriptional regulation, mRNA splicing and stability, and protein translation, stability, and function (such as enzymatic activity or binding affinity). Alteration of these processes may affect the synthesis, metabolism, and transport of major neurotransmitters. Variants (or polymorphisms) in genes coding for components of pathways involved in substance use disorders may be responsible for the variation found a patient's response to pharmacotherapy for an addiction. These responses are likely to be dependent upon many genes (e.g., polygenic), but may also be oligogenic, where only a few genes play a major role. Data from studies on the genetic basis of response to pharmacotherapy may allow for the effective tailoring of therapy to the needs of the individual based on his or her genetic makeup.

4 Drugs of Abuse and Dopamine System

Central to the development of addiction is the reward pathway of the brain, which is mediated by the catecholamine neurotransmitter dopamine (DA). Natural reinforcers, such as copulation and food, produce a surge of dopamine release in the nucleus accumbens (NAc) of the brain from neurons originating in the ventral tegmental area (VTA) [17, 18]. Most drugs of abuse act either directly or indirectly on the dopaminergic reward system, increasing levels of dopamine in the NAc [19]. In the long term, drugs of abuse hijack the reward system so that the individual requires the drug of abuse for activation of their reward system [20].

Cocaine's mechanism of action is through the blockade of three major neurotransmitter system transporters, dopamine, serotonin, and norepinephrine transporters [21]. The blockade of these transporters raises synaptic levels of their corresponding neurotransmitter, and therefore increasing signaling. For example, cocaine binds to the dopamine transporter and leads to an inhibition of the removal of dopamine from synapses, increasing the availability of dopamine to bind to both presynaptic and postsynaptic receptors [22].

The impact alcohol and other drugs of abuse have on the mesolimbic dopamine pathways plays an important role in the development of craving and the loss of control over use of these substances [23]. It is not clear how alcohol increases dopamine levels, but it is believed to be through μ-opioid receptors in the mesolimbic system [24]. Alcohol most likely produces its reinforcing effects by stimulating the release of endogenous opioids that increase extracellular mesolimbic dopamine levels in the NAc [25–27]. Binding of the

endogenous opioid peptide β-endorphin, disinhibits the GABAergic interneurons in the VTA promoting the release of dopamine in the NAc [28]. Thus, both opioid receptor and dopamine receptor antagonists play critical roles in the investigation of moderating alcohol craving and stimulation. Opioids, on the other hand, directly bind to the μ-opioid receptors, causing disinhibition the GABAergic interneurons and the subsequent release of dopamine [29].

In this chapter, we discuss the genetics of several pharmacotherapies for the addictions. Some of these are FDA-approved therapies, while others are under investigation. The pharmacotherapies we explore are listed in Table 1 and the genetic variants are listed in Table 2.

Table 1
Drugs therapies discussed in pharmacogenetic studies

Pharmacotherapy	Addiction	Notes
Acamprosate	Alcohol	Acamprosate is a drug with a chemical structure similar to that of γ-aminobutyric acid (GABA) and acts as a partial agonist of *N*-methyl-D-aspartate (NMDA) receptors in the brain. Acamprosate has been used to treat alcoholism since 1989 in Europe and since 2004 in the USA. Alcohol exposure is thought to depress glutamatergic signaling which then rebounds after the cessation of alcohol use resulting in hyperstimulation. Acamprosate's effects include increasing taurine as well as in binding NMDA receptors, and therefore inhibiting the excitatory effects of alcohol withdrawal [227].
Bromocriptine	Alcohol	Bromocriptine is an ergot alkaloid and a dopamine receptor D2 agonist that inhibits prolactin release from the pituitary gland [228]. Bromocriptine is typically used to treat Parkinsonian syndrome as well as hyperprolactinaemia, growth hormone- and prolactin-related disorders such as menstrual disorders, infertility, and hypogonadism.
Buprenorphine	Opioid	Buprenorphine is a synthetic μ-opioid partial agonist synthesized in 1967 [229] and initially utilized as an analgesic [230]. It was not used as a maintenance treatment for opioid addiction until the mid-1980s. Studies have showed that buprenorphine's effects were longer-acting and that it had a lower potential for abuse than did morphine [231]. Suboxone is a combination of buprenorphine plus naloxone formulated to prevent misuse.
Cocaine vaccine	Cocaine	The cocaine vaccine consists of a cocaine derivative conjugated to cholera toxin [86]. Following a series of vaccinations with the vaccine, subjects produce anti-cocaine antibodies. The hypothesis is that immunization of treatment-seeking patients vaccinated with this vaccine will stimulate the production of anti-cocaine antibodies. When subjects who are abstinent relapse and take cocaine, the anti-cocaine antibodies will sequester the cocaine in the blood, thereby preventing a rapid surge of cocaine into the brain.

(continued)

Table 1 (continued)

Pharmacotherapy	Addiction	Notes
Disulfiram	Alcohol, cocaine	Disulfiram was initially synthesized as a reagent to vulcanize rubber. Disulfiram is approved for use in treating alcoholism [232]. A major metabolite of disulfiram is diethyldithiocarbamate, a copper chelator. The aldehyde dehydrogenase isozyme ALDH2 is an enzyme active in the ethanol metabolic pathway that uses copper as a cofactor, and is inhibited by disulfiram treatment [233]. When inhibited by disulfiram, the reduction in aldehyde dehydrogenase activity causes the accumulation of acetaldehyde, thus inducing nausea, vertigo, flushing, and other unpleasant effects after the consumption of alcohol. Disulfiram also inhibits other copper containing enzymes including dopamine β-hydroxylase (DβH), which is the enzyme that converts dopamine to norepinephrine. Therefore, disulfiram treatment increases dopamine levels due to an inhibition of DβH activity, with a concomitant decrease in central and peripheral norepinephrine levels [234, 235]. In addition to treating alcoholism, disulfiram has been shown to reduce cocaine use as well [236–238]. Its action may be through the inhibition of DβH as well as by the inhibition of plasma and microsomal carboxylesterases and plasma cholinesterase, enzymes that inactivate cocaine systemically [239]. In addition, disulfiram reduced subjective measures of IV administered cocaine-produced craving [240, 241]. Studies have found that in treatment of individuals with disulfiram who were dependent both on cocaine and alcohol, the use of both illicit drugs were reduced [242, 243].
Methadone	Opioid	Methadone was synthesized in the late 1937 by Bockmühl and Ehrhart [244], and first utilized experimentally to relieve opiate withdrawal in 1948 [245]. It is a synthetic μ-opioid receptor agonist that binds with high affinity, reduces opioid cravings, and can block the binding of other superimposed opioids [60]. Methadone is a synthetic opioid that is used in the pharmacotherapy of the addition to short-acting opiates such as heroin [246]. Due to its long half-life of approximately 22 h and efficacy, methadone is also using in the management of chronic pain. Methadone is a μ-opioid receptor agonist and a weak NMDA receptor antagonist. It is effective at reducing opioid withdrawal symptoms and at blocking the euphoric effects of heroin, morphine, and other opioids. Methadone is a racemic mixture of levomethadone and dextromethadone. Levomethadone is the selective μ-opioid receptor agonist while dextromethadone is a glutamatergic NMDA receptor antagonist [59]. Since glutamate is an excitatory neurotransmitter, NMDA antagonism may be partly involved in methadone's efficacy.
Naltrexone	Alcohol, opioid	Naltrexone is a μ-opioid receptor antagonist first synthesized in the 1960s. Naltrexone was approved by the FDA for the treatment of opioid addiction treatment in 1984 and alcohol addiction in 1994 [247]. Naltrexone blocks the euphoric effects of opioids by binding competitively to opioid receptors, but does little to curb craving for opioids. Because naltrexone is an opioid antagonist there is little risk of abuse or dependence given that it does not have intrinsic opiate effects and therefore is not reinforcing [248].

(continued)

Table 1
(continued)

Pharmacotherapy	Addiction	Notes
Olanzapine	Alcohol	Olanzapine is a second generation antipsychotic used to treat schizophrenia and mania related to bipolar disorder. Olanzapine binds neurotransmitter receptors of several classes including dopaminergic, adrenergic, and serotonergic receptors [249, 250].
Ondansetron	Alcohol	Ondansetron is a serotonin 5-HT_3 receptor antagonist, with low affinity for α1-adrenergic, 5-HT_{1B}, 5-HT_{1C}, and μ-opioid receptors [251]. It is used primarily to treat nausea and vomiting (antiemetic) following chemotherapy.
Tiapride	Alcohol	Tiapride is a dopamine receptor D2 and D3 antagonist. It is used to treat alcohol withdrawal syndrome where it has anxiolytic effects. Tiapride has been shown to reduce psychological stress, decrease drinking, and improve reintegration into society [252].

Table 2
Genetic variants involved in pharmacotherapy for the addictions

Gene	Product	Variant	Addiction	Pharmacotherapy
ABCB1	ATP-binding cassette, subfamily B, member 1	rs1128503, rs1045642, rs2032582	Opioids	Methadone
ADRA1A	α_{1A}-Adrenoceptor	rs1048101	Cocaine	Disulfiram
ANKK1	Ankyrin repeat and kinase domain-containing 1	rs1800497 (*Taq*IA)	Cocaine, alcohol, opioids	Disulfiram, naltrexone, methadone
ARRB2	Arrestin β-2	rs3786047, rs1045280, rs2036657	Opioids	Methadone
BDNF	Brain-derived neurotrophic factor	rs7127507, rs1967554, rs11030118, rs988748, rs2030324, rs11030119, rs2239622	Opioids	Methadone
COMT	Catechol-O-methyltransferase	rs4680 (Val158Met)	Methamphetamine	Modafinil
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6	Multiple (see [253])	Opioids	Methadone

(continued)

Table 2
(continued)

Gene	Product	Variant	Addiction	Pharmacotherapy
CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6	rs2279343, rs3745274	Opioids	Methadone
DBH	Dopamine β-hydroxylase	rs1611115 (C-1021T)	Cocaine	Disulfiram, cocaine vaccine
DRD2	Dopamine receptor D2	rs6277, rs1799978, rs6275	Cocaine, alcohol, opioids	Acamprosate, bromocriptine, disulfiram, methadone
DRD4	Dopamine receptor D4	Exon 3 VNTR	Alcohol	Olanzapine
GABRB2	γ-Aminobutyric acid β-2	rs3219151 (C+1412T)	Alcohol	Acamprosate, naltrexone
GABRA6	γ-Aminobutyric acid α-6	rs3219151 (T+1519C)	Alcohol	Acamprosate, naltrexone
GATA4	GATA-binding protein 4	rs13273672	Alcohol	Acamprosate
KCNJ6	Potassium inwardly rectifying, channel subfamily J, member 6	rs2070995	Opioids	Methadone
MTHFR	Methylenetetrahydrofolate reductase	rs1801133 (C677T)	Cocaine	Disulfiram
MYOCD	Myocardin	rs1714984	Opioids	Methadone
OPRD1	δ-Opioid receptor	rs678849	Opioids	Methadone, suboxone, buprenorphine
OPRK1	κ-Opioid receptor	rs6473797	Cocaine	Cocaine vaccine
OPRM1	μ-Opioid receptor	rs1799971 (A118G)	Alcohol	Naltrexone
SLC6A4	Serotonin transporter	5-HTTLPR VNTR	Alcohol	Ondansetron, sertraline
TPH2	Tryptophan hydroxylase 2	rs4290270	Cocaine	Disulfiram

5 Genes of the Dopaminergic System

5.1 Dopamine Receptor D2 (DRD2)/Ankyrin Repeat and Kinase Domain-Containing 1 (ANKK1) Genes

The *DRD2* and *ANKK1* genes are located approximately 10,000 nucleotides apart on chromosome 11q22-23. Variants in both genes have been found to be associated with several psychiatric diseases such as schizophrenia, as well as with substance abuse disorders, including alcohol, heroin, nicotine, cocaine, opioid, gambling, methamphetamine, and polysubstance addiction [30–37].

The *DRD2* gene encodes the G-protein coupled dopamine receptor D2, which is central to dopaminergic signaling in the brain. This gene can be alternatively spliced to produce two protein isoforms, designated the long and short forms of the receptor protein (D2L and D2S, respectively). The variant rs2283265 is a G→T transversion in an intron of the *DRD2* gene. The T allele of this variant has been shown to alter the ratio of D2L to D2S protein isoforms and is overrepresented in cocaine-addicted populations [38, 39]. The *DRD2* variant rs6277 is a synonymous (does not alter the amino acid coding) C to T transition in *DRD2*. The T allele has been shown to be associated with enhanced D2 receptor availability, altered mRNA folding, and reduced mRNA stability [40, 41]. Several variants in *DRD2* have been associated with psychiatric disorders such as schizophrenia and alcoholism [42, 43].

The *ANKK1* gene (also known as *receptor interacting protein 5* or *RIP5*) encodes the ankyrin repeat and kinase domain-containing 1 protein. The RIP serine/threonine kinase family is involved in activation of various cellular signaling pathways, including NF-KB, JNK, and apoptotic signaling [44]. The *ANKK1/DRD2 TaqI*A variant, also known as rs1800497, is a functional SNP located in the final exon of *ANKK1* that codes for a non-synonymous Glu→Lys (C→T) amino acid change in the C-terminus of the ANKK1 protein. Initially associated with alcohol addiction in 1990 by Blum et al. the *ANKK1/DRD2 TaqI*A variant has been one of the most-examined variants with regard to substance addiction [45]. The T (*Taq*IA1) allele of *ANKK1/DRD2* has also been found to be associated with reduced dopamine receptor D2 density [46] and with reduced opioid receptor binding [47]. The brains of cocaine-, opioid-, and alcohol-addicted individuals have shown reduced D2 receptor availability, and therefore providing a potential mechanism through which the gene variant may affect addictive behavior [48–50]. Another variant in *ANKK1* is rs7118900 that codes for an alanine to threonine (Ala239Thr) substitution creating a predicted phosphorylation site and is found to be in strong linkage disequilibrium (LD) with *ANKK1/DRD2 TaqI*A [51]. Cells transfected with the *ANKK1* rs7118900 Thr239 variant constructs tagged with green fluorescent protein (GFP) expressed greater levels than did constructs containing the Ala239 variant. The Thr239 constructs decreased expression when treated with the dopamine agonist apomorphine, while the Ala239 constructs increased expression. This finding provides a potential functional link for the *ANKK1* gene product to the dopaminergic system.

Disulfiram (Table 1) was tested in a cohort of cocaine and opioid codependent individuals as a pharmacotherapy for cocaine addiction in a placebo controlled clinical trial [52]. Patients were stabilized on methadone and the treatment group received 250 mg of disulfiram daily. Disulfiram pharmacotherapy decreased cocaine use as measured by urine cocaine metabolites, with no concomitant

difference in the amount of opioid use. Genetic analysis revealed that *ANKK1* rs1800497 T allele (*Taq*IA1) carriers showed reduced cocaine-positive urines during pharmacotherapy with disulfiram, while CC homozygous individuals, those carrying two copies of the same allele, showed no treatment response [53]. Additionally, *DRD2* rs2283265 T allele carriers showed a large reduction in cocaine-positive urines with disulfiram pharmacotherapy, while GG homozygous individuals displayed less reduction.

Neuroendocrine studies have suggested that alcoholics have reduced *DRD2* receptor sensitivity after a few months to several years of abstinence from alcohol [54, 55]. This may be reflective of why the dopamine receptor agonists bromocriptine and the D2 antagonist tiapride (Table 1) are efficacious in the treatment of alcoholism and alcohol withdrawal syndrome, respectively. The dopamine receptor D2 agonist bromocriptine has been shown to decrease alcohol craving and anxiety in *TaqI*A1 carrier alcoholics [56]. Lucht et al. showed that the AA genotype of rs71653615 in *DRD2* was found to be associated with higher doses of the selective dopamine receptor D2 antagonist tiapride that was required to treat alcohol withdrawal symptoms of alcohol-dependent Caucasians [57].

The pharmacogenetics of acamprosate, a NMDA receptor partial agonist, and naltrexone, a μ-opioid receptor antagonist (Table 1), were evaluated in a cohort of Dutch alcoholics [58]. Acamprosate was shown to have a greater effect on cue-induced craving than did naltrexone in alcoholic subjects homozygous for the *ANKK1/DRD2* rs1800497 *Taq*IA1 allele. However, naltrexone was more effective in subjects homozygous for the *Taq*IA2 allele. In heterozygous subjects (those carrying one copy of each allele), naltrexone and acamprosate were equally effective. The greater effectiveness of naltrexone in the *Taq*IA2 homozygous subjects may be related to the finding that individuals homozygous for the *Taq*IA2 allele exhibited higher [^{3}H]naloxone binding in the caudate nucleus indicative of having greater opioid receptor density [47].

Methadone, a mixture of levomethadone, a selective μ-opioid receptor agonist, and dextromethadone, a glutamatergic NMDA receptor antagonist [59] is an effective therapy for opioid addiction [60, 61]. In methadone maintenance therapy for opioid addiction, carriers of the *DRD2* rs6275 T allele were found to required higher methadone doses than did noncarriers and required longer periods of time to reach maximum methadone maintenance dose [62]. Another study illustrated that subjects carrying at least one copy of the *ANKK1/DRD2* rs1800497 *TaqI*A1 allele in the *ANKK1* gene were more likely to be in the methadone maintenance treatment "poor treatment" outcome group, which was composed of individuals who withdrew from the study or who continued use of heroin at least once weekly, compared to being in the "successful treatment" outcome group [37]. Additionally, *TaqI*A1 allele

carrier subjects used twice the amount of heroin in the previous year than did A2 homozygous subjects. Similarly, subjects with the *DRD2* rs6277 CC genotype were more likely to be non-responders to methadone maintenance therapy and their duration of opioid-free urines were shorter than did subjects who were T-allele carriers, but with no difference in the frequency of the *ANKK1/DRD2 TaqI*A variant with these measures [63]. Hung et al. examined a different *DRD2* variant in Han Chinese subjects and found that subjects carrying the rs1799978 G (-214A>G) allele required a lower methadone dose than did noncarriers [64].

5.2 Dopamine Receptor D4 (DRD4) Gene

The *DRD4* gene has a variable number tandem repeat (VNTR) located in exon 3, with the common alleles of two, four, and seven repeats [65]. The seven repeat appears to be the critical allele relevant to pharmacotherapy and alcohol consumption. Functionally, the *DRD4* seven tandem repeat allele codes for a dopamine receptor D4 that blunts intracellular forskolin-stimulated cyclic AMP (cAMP) response to dopamine relative to the receptor encoded by the two and four tandem repeat alleles [66].

The exon 3 *DRD4* VNTR has been shown to moderate the influence of alcohol on craving and related responses. Subjects who were carriers of the seven or longer repeat had a greater "urge to drink" and a lower "subjective high" following alcohol consumption than did those without this allele [67]. Although both genotype groups had similar decreases in craving at baseline for alcohol following olanzapine treatment, olanzapine only reduced craving after exposure to alcohol in subjects carrying a 7 or longer VNTR allele [68]. However, unlike the μ-opioid receptor antagonist naltrexone (see below), olanzapine had little to no effect on moderating alcohol's reinforcing effects [69].

5.3 Dopamine β-Hydroxylase (DBH) Gene

Dopamine β-hydroxylase (DβH) is the enzyme that metabolizes dopamine into norepinephrine (reviewed in [70, 71]). Norepinephrine modulates many behavioral, cognitive, and physiological functions [72, 73]. The catecholamine neurotransmitters dopamine and norepinephrine are stored in synaptic vesicles prior to release from the cell. It is within these vesicles that DβH is localized. Although most of the DβH is bound to the membrane of the vesicles, some DβH is free and is co-released with the catecholamines during synaptic transmission from neurons and into the blood from neurosecretory cells of the adrenal medulla [74]. The levels found in serum are highly correlated between sibs, but varies between unrelated subjects [75]. This variation has been found to be heritable in family and twin studies in both serum and CSF [76].

Although a number of polymorphisms have been studied in the *DBH* gene and have been found to be associated with DβH levels [77–80], one appears to be the primary functional variant. [81–83]. In a study examining 11 SNPs spanning the *DBH* locus,

the C-1021T variant was found to be most highly associated with DβH plasma levels [81]. The C-1021T variant (rs1611115) has been shown to be associated with decreased enzyme activity in plasma in several populations, including European Americans, African Americans, Japanese, and an Eastern Indian population, with the T allele being associated with decreased activity in all populations examined [82, 83]. This polymorphism accounted for 35–52 % of the variation in DβH levels. The rs1611115 variant has been shown to be associated with alcohol dependence in females [84], as well as with progression of heroin self-administration [85].

In the aforementioned trial of disulfiram for the reduction of cocaine use with cocaine and methadone codependent subjects the role of this *DBH* variant was studied [52]. When the disulfiram treatment group was stratified by *DBH* rs1611115 genotype, the CC genotype group with normal DβH expression reduced their cocaine use when treated with disulfiram, while those patients who were carriers of the low expressing T allele did not. It was suggested that the reduction in norepinephrine neurotransmission by disulfiram may not reduce the use of cocaine in those with the low DβH levels, which may have caused an upregulation of dopamine receptors.

Vaccines have been tested as a potential pharmacotherapy for cocaine addiction. A cocaine vaccine (Table 1) of succinyl norcocaine conjugated to the cholera toxin (TA-CD) was administered to cocaine-dependent individuals [86]. Individuals who produced adequate antibody levels showed reduced cocaine use. Genetic analysis showed that T allele carriers of variant rs1611115 (which has been shown to be associated with low DβH expression) reduced cocaine use with vaccine, while subjects with the CC genotype did not [87]. This may be related to the increase incidence of paranoia while using cocaine in subjects with the low expressing *DBH* genotype [80].

6 Opioidergic System Genes

6.1 μ-Opioid Receptor (OPRM1) Gene

The G protein-coupled μ-opioid receptor (MOP-r) mediates most opioid antagonists and is the receptor for morphine, methadone, and endogenous opioids, such as endomorphin and β-endorphin. The opioid receptor family functions in nociception, but also indirectly in the mesolimbic dopaminergic pathway, partly mediating the addictive process [88]. Opioids bind MOP-r and attenuate GABAergic inhibition of dopaminergic neurons [29], producing an increase in dopamine release at nerve terminals in the ventral striatum and medial prefrontal cortex by VTA neurons [89, 90].

OPRM1, the gene encoding MOP-r, is located on chromosome 6q25.2 and contains several functional variants that have been studied extensively in association with not only substance abuse and dependence [61, 91, 92], but also a variety of conditions, such as major depression, schizophrenia, and pain sensitivity

(reviewed in ref. 7). The most studied SNP in *OPRM1*, rs1799971, is located in the coding region at nucleotide 118. At this location there is an A to G non-synonymous transition that codes for an aspartic acid (Asp) instead of an asparagine (Asn) at position 40 in the N-terminus of the receptor [93, 94]. This substitution removes one of five highly conserved putative N-glycosylation sites from the N-terminal extracellular domain of the receptor. Approximately 30 % of Europeans and 60 % of Asians carry one or two copies of this allele, while being essentially absent in African Americans (http://www.ncbi.nlm.nih.gov/snp).

At the molecular level, the 118G allele of *OPRM1* rs1799971 encodes a receptor that binds the endogenous opioid peptide β-endorphin with three times the affinity than does the variant receptor encoded by the 118A allele [94]. However, the G allele leads to reduced mRNA and protein levels resulting in a net functional loss of *OPRM1* gene expression. In postmortem autopsy brain tissue of 118A/118G heterozygous individuals, the Asn40 mRNA encoded by the118A allele was about 1.5 times more prevalent than was the Asp40 mRNA encoded by the 118G allele [95]. In vitro cellular expression assays have shown that the 118G receptor allele produced lower cell-surface binding site availability than did the 118A receptor allele [96]. G-allele carriers of *OPRM1* have increased hypothalamic–pituitary–adrenal (HPA) axis response relative to those homozygous for the A allele under opioid receptor antagonism [97], enhanced cortisol response, and a reduced agonist effect of morphine-6-glucoronide [98].

In a sample of Swedish subjects, the odds ratio of being alcohol dependent was twofold greater in OPRM1 118G-allele carriers [99]; however, this finding was not replicated in two recent reports using European populations [100, 101]. Studies on the subjective effects of alcohol have shown that *OPRM1* 118G-allele carriers experience greater subjective feelings of intoxication, euphoria, and sensitivity to both the reinforcing and sedative effects of alcohol, and had a threefold increase in family history of alcoholism [102]. The *OPRM1* 118G variant has been found to be associated with heroin addiction in Asians [103].

Alcohol craving develops through repeated alcohol administration and intensifies over time. Alcohol cues, such as the sight and smell of alcohol, and the consumption of small priming doses of alcohol, elicit craving. Craving, in part, acts by prompting dopamine release as an incentive to continue drinking. A functional neuroimaging study found a greater hemodynamic (blood flow) response in mesocorticolimbic structures, including the VTA, following alcohol tastes in alcoholics compared to healthy volunteers [104]. A positron emission tomography (PET) study found that *OPRM1* 118G-allele carriers had stronger striatal dopamine response to intravenous alcohol administration compared to 118A-allele homozygous subjects as measured by the displacement

of the D2 receptor ligand [^{11}C]-raclopride [105]. These studies demonstrate the interconnectivity between the opioidergic and dopaminergic systems in response to alcohol consumption.

Naltrexone (Table 1), a μ-opioid receptor antagonist, targets the dopaminergic pathway by inhibiting μ-opioid receptors and disrupting the neurocascade that leads to striatal dopamine release [106]. After animal studies demonstrated the involvement of endogenous opioid system on the effect of alcohol, naltrexone was selected to be tested on alcohol dependent subjects in the hope of improving psychosocial rehabilitation [107, 108]. These studies showed the beneficial effects of naltrexone and were quickly replicated (e.g., [109]), which led to FDA approving this medication for treatment of alcoholism in 1983.

Pharmacotherapeutic trials have tested the effectiveness of naltrexone for treatment of alcoholism and found positive therapeutic results. Naltrexone has been shown to reduce the frequency of heavy drinking days, increase the time before first relapse, produce lower relapse rates, reduce the number of total drinking days, and lower the number of drinks per drinking episode in alcoholics, and to decrease the time lapse between first and second drinks among social drinkers [108–119]. However, other studies were unable to establish naltrexone's efficacy as a moderator of alcohol consumption [120, 121].

Although its method of action is not fully understood, naltrexone is widely accepted as one of the safest and most effective pharmacotherapies for alcohol dependence. Naltrexone blunts alcohol's reinforcing effects, including the "high" and the subjective positive stimulation following alcohol consumption and, in general, restricts the euphoria produced by alcohol [122–124]. This makes alcohol intoxication less satisfying and impedes the progression of drinking when delivered in combination with behavioral intervention [125], causing alcoholics to be less likely to resist relapse into heavy drinking.

Naltrexone has been shown to activate the HPA axis by increasing proopiomelanocortin (POMC) synthesis [126] and cortisol levels, which have been correlated with decreased craving for alcohol [127]. When the *OPRM1* A118G rs1799971 polymorphism was examined, 118G-allele carriers had higher cortisol concentrations both at baseline and after naloxone, a μ-opioid antagonist, treatment than did 118A/118A homozygous subjects [98]. 118G-allele carriers experienced more intense "highs" and greater positive stimulation following alcohol consumption, and experienced greater blunting of these subjective effects when treated with naltrexone than did AA homozygous subjects [128]. When given naltrexone prior to drinking, G-allele carrier subjects had lower levels of alcohol craving and more intense alcohol "highs" as their blood alcohol content increased. On the other hand, when 118A/118A homozygous subjects were given

naltrexone, alcohol cues produced greater craving with no effect in 118G-allele carriers [129].

Pharmacogenetic trials of naltrexone that examined the *OPRM1* A118G rs1799971 polymorphism demonstrated that G-allele carriers experience better clinical response and lower relapse rates than did AA homozygous patients when treated with naltrexone [130, 131]. Oslin et al. examined the C17T rs1799972 and A118G polymorphisms of *OPRM1*, and their association with treatment outcome of naltrexone in alcohol-dependent patients [130]. They found that individuals of European descent with at least one copy of the A118G G allele had better results (e.g., lower rates of relapse and a longer time before relapse into heavy drinking) when treated with naltrexone, than did those subjects who were homozygous for the A allele. However, no difference in long-term abstinence rates between genotype groups was found. The C17T variant had no effect on treatment response to naltrexone. Ray et al. showed that naltrexone blunted alcohol craving, but increased subjective intoxication in *OPRM1* rs1799971 G-allele carriers compared to AA homozygous subjects or placebo in a cohort of Asian Americans [132]. Setiawan et al. found that naltrexone blunted alcohol-induced euphoria both in women and in individuals with the G-allele of *OPRM1* A118G in a cohort of social drinkers [133]. Similarly, in a Korean cohort the A118G G-carriers had longer time until relapse as compared to AA homozygous subjects while on naltrexone [134]. However, studies have shown no pharmacogenetics effect of naltrexone, such as Coller et al., where naltrexone was found to be effective in reducing craving and alcohol use, but the A118G variant of *OPRM1* was not found to be a predictor of these effects [135]. Oroszi et al. showed that specific *OPRM1* haplotypes are found to be associated with good clinical outcome on naltrexone [136]. Specifically, 90 % of individuals with the diplotype (haplotype combination) AACCC/AGCCC of the variants: rs1074287, rs1799971, rs510769, rs524731, and rs1381376, respectively, had "good clinical outcome" as compared to subjects with other diplotypes that did not carry the rs1799971 G allele.

Clinical trials have established the efficacy of naltrexone as a pharmacotherapy in conjunction with standard treatment protocols. Anton et al. studied the clinical outcomes of patients treated with naltrexone or placebo [131]. All participants in that study received either standard medical management (MM) alone or the same along with combined behavioral intervention (CBI). In patients who received both MM and CBI, no gene by medication interactions was found. However, in the subjects who received MM without CBI, the *OPRM1* 118G-allele carriers treated with naltrexone had an increased percentage of days abstinent and an overall decrease in the percentage of drinking days relative to individuals receiving placebo. Within the MM without CBI group, patients who were carriers of the 118G allele had better results on

naltrexone than did those patients homozygous for the A allele. However, within the participants who were treated with placebo those homozygous for the A allele had better results than did those patients who were G-allele carriers.

6.2 δ-Opioid Receptor (OPRD1) Gene

The *OPRD1* gene is located on chromosome 1p36.1 and encodes the G-protein coupled δ-opioid receptor (DOP-r). Although MOP-r is the primary opioid receptor thought to function in the reinforcing effects of drugs of abuse, DOP-r can form heterodimers with the MOP-r to resulting in dopamine release in the nucleus accumbens [137–139]. *OPRD1* genetic variants have been shown to be associated with substance abuse and dependence. Variants and haplotypes of *OPRD1* were found to be associated with opioid, alcohol, and cocaine dependence in a case–control study [140]. Other studies have found other genetic variation in the *OPRD1* gene in association with heroin addiction vulnerability in Germans [141] and European Americans [142], and with cocaine addiction susceptibility in African Americans [143].

The DOP-r has also been shown to be involved in modulating the effects of other addictive substances, including ones that activate MOP-r, and functioning in nociceptive responses. Knockout mice with deletion of the *Oprd1* gene have shown DOP-r to be involved in mood states, such as anxiety or depression [144]. *Oprd1* knockout mice become physically dependent on morphine, but fail to develop tolerance to the drug [145, 146]. In addition, DOP-r is involved in response to cocaine. The selective DOP-r agonist [D-penicillamine^2, D-penicillamine^5] enkephalin (DPDPE) was unable to attenuate adenylyl cyclase activity in the NAc or caudate-putamen following chronic repeated cocaine administration, suggesting that cocaine impairs DOP-r activity [147].

A recent study examined the pharmacogenetics of treatment of opioid dependence with methadone or buprenorphine plus naloxone (Suboxone). It was found that African American subjects with the *OPRD1* rs678849 CC had fewer positive opioid urine drug screens genotype when treated with methadone than did individuals in the T-allele carrier group [143]. In contrast, the opposite outcome was observed when treated with buprenorphine plus naloxone. African American subjects carrying the rs678849 T allele had fewer opioid-positive urine screens than did those subjects with a CC genotype. This study demonstrates that pharmacological tailoring of treatments for opioid addiction in African Americans may be optimized by using methadone for CC subjects and buprenorphine for T-allele carrier patients.

6.3 κ-Opioid Receptor (OPRK1) Gene

OPRK1 encodes the κ-opioid receptor (KOP-r) and is located on chromosome 8q11.2. KOP-r is a G-protein coupled receptor involved in mood, motivation, reward, and cognitive function, for which dynorphin is its primary ligand [148]. Binding of dynorphin A [1–17], the endogenous ligand of KOP-r, decreases

basal and cocaine-induced striatal dopamine levels [90, 149, 150]. Upregulation of KOP-r following cocaine administration may occur to lower dopaminergic activity [151]. Pharmacological inactivation of KOP-r reduces response to stress and pain sensitivity [152, 153].

Several *OPRK1* variants have been found to be associated as protective or risk factors for cocaine addiction, while some have been found to be associated with comorbid cocaine and opioid addiction [8, 154]. The intronic variant rs6473797 of *OPRK1* is an A to G transition. Increased vulnerability to develop opioid dependence [142] and alcohol dependence [155] was observed with the G allele. Several other *OPRKI* variants and haplotypes have been found to be associated with alcohol dependence [140, 155].

In the previously mentioned clinical trial of a cocaine vaccine, individuals homozygous for the A allele of the *OPRK1* rs6473797 variant showed reduced cocaine use while individuals carrying the G allele did not [156]. This was hypothesized to be due to the blunting effects of the cocaine vaccine combined with a less efficient KOP-r coded for by the A allele, which reduced the rate of dopamine surge to a greater extent in the AA homozygous subjects than it did in the G allele carriers.

7 Serotonergic System Genes

7.1 Serotonin Transporter (SLC6A4) Gene

The serotonin transporter is encoded by the *SLC6A4* (*5-HTT*) gene located on chromosome 17q11.2, and has been the focus of pharmacogenetic studies of serotonergic medications. In the promoter region of this gene is a triallelic VNTR polymorphism, *5-HTTLPR*, which alters transcriptional activity [157]. The common forms of this polymorphism are either the long (L) version containing 16 repeats of 20–23 nucleotides, or a short (S) version containing 14 repeats. The L allele has higher transcriptional activity than does the S allele [157, 158]. Moreover, an A to G transition (rs25531) in the L VNTR of the *5-HTTLPR* codes for a G allele (L_G) with lower transcriptional activity and expression, similar to that of the S allele [159, 160]. In most recent reports, these two alleles (L_G and S) are collectively referred to as S′, while the higher activity L_A allele is referred to as L′. Current evidence suggests that the S' allele may make people more susceptible to the influence of the environment [161] and sensitivity to perceived limitations [162], with S′-allele carrier individuals being more sensitive than do those with the L′L′ genotype. It may be that certain genes, such as the *serotonin transporter* gene, may control the impact of the environment on a person, instead of directly influencing a psychiatric disease [163–165].

Selective serotonin reuptake inhibitors (SSRIs) and 5-HT$_3$ (serotonin) receptor antagonists present potential pharmacotherapeutic targets. These have been used to treat a wide range of

conditions, but show only moderate efficacy for treating alcoholism [166]. A moderating role of *5-HTTLPR* genotype has been demonstrated in behavioral therapy of cocaine addiction. Mannelli et al. showed that 12-week behaviorally oriented outpatient treatment program decreased alcohol use most in the LL genotype group, with only moderate reductions in the SS or LS genotype groups [167]. No difference among the genotype groups in the decrease of drug use was noted. In the study of the efficacy of disulfiram as a treatment of cocaine dependence, genetic analysis showed that S′-allele carrier subjects responded to disulfiram treatment (i.e., had fewer cocaine-positive urines over the course of the study), while the L′L′ homozygous subjects did not respond [168]. This may have been due to the increase in serotonin resulting from disulfiram pharmacotherapy that may have a greater impact on subjects with low levels of serotonin transporter [169–171].

Ondansetron (Table 1), a 5-HT_3 receptor antagonist, has shown positive results of reducing heavy drinking in early-onset alcoholics when used as a treatment of alcohol dependence. By prompting reductions in alcohol craving and helping maintain mood and emotional state, ondansetron aids in the reduction of heavy drinking [172]. Individuals who underwent treatment with ondansetron experienced significantly more abstinent days and less alcohol consumption relative to placebo individuals. When treated with ondansetron, Johnson et al. found *5-HTTLPR* LL homozygous patients had a better treatment response, as measured by the mean number of drinks per drinking day and percentage of days of total abstinence than did those with one or two S alleles [173]. Additionally, a SNP (rs1042173) in the 3′ untranslated region (3′UTR) of the serotonin transporter gene augments this effect. Individuals with a genotype pattern of LL *5-HTTLPR* and TT 3′UTR (rs1042173) drank less and had a higher percentage of days abstinent from drinking after ondansetron treatment than did those with any other combination of these genotypes.

A clinical trial testing the efficacy of sertraline, a selective serotonin reuptake inhibitor (SSRI, Table 1) was conducted on early-onset and late-onset alcoholics [174]. In the late-onset alcoholics, the *5-HTTLPR* L′L′ homozygous subjects responded to sertraline treatment by decreasing the number of days drinking per week compared with the S′-allele carrier subjects, while the early-onset alcoholics subjects with the L′L′ genotype increased the number of days drinking compared with the S′-allele carrier subjects. In both these aforementioned trials, the efficacy of both ondansetron and sertraline was moderated by the high-activity L′ allele. Moreover, after 3 months sertraline-treated L′L′ homozygous late-onset alcoholics had fewer drinking days than did placebo individuals [175]. However, in another study examining the efficacy of sertraline versus ondansetron, non-treatment seeking

alcohol-dependent *5-HTTLPR* LL individuals consumed less volume over a 45 min drinking period and had fewer drinks per drinking day on ondansetron as compared to *5-HTTLPR* S-allele carrier individuals, while sertraline showed no beneficial effect in either genotype group [176].

7.2 Tryptophan Hydroxylase (TPH2) Gene

Tryptophan hydroxylase is the rate-limiting enzyme in the production of serotonin [177] and is encoded by the *TPH2* and *TPH1* genes. *TPH2* is localized to chromosome 12q21.1 and is the major isoform expressed in the brain [178]. Genetic variation in *TPH2* has been found to be associated with several psychiatric conditions, including obsessive compulsive disorder, attention deficit hyperactivity disorder, personality traits and disorders, and emotional processing (reviewed in [179]). A genotype pattern of a synonymous variant 1125A>T (rs4290270) in exon 9 of *TPH2* and an intron 7 variant of *TPH1* was found to be associated with heroin addiction [180]. The *TPH2* variant 1125A>T (rs4290270) has been demonstrated to be a marker for allelic expression imbalance with the T allele being expressed at twice the level of the A allele [181]. Individuals with a TT genotype may produce more serotonin than do A-allele carrier subjects.

In the disulfiram pharmacotherapy study for cocaine addiction, genetic analysis showed that individuals carrying the *TPH2* rs4290270 A allele responded better to disulfiram compared to placebo than did TT homozygous individuals. Additionally, A carriers responded even better to disulfiram if they were also carrying an 5-*HTTLPR S′* allele [168]. Hence, individuals with the low-expressing *TPH2* and low-expressing *5-HTTLPR* variants responded better, presumably in response to disulfiram's effect of increasing serotonin levels. Thus, it appears that subjects with low serotonergic metabolism respond to disulfiram, while those with normal serotonergic metabolism do not.

8 Other Genes with Variants Associated with Pharmacotherapeutic Response

8.1 ATP-Binding Cassette, Subfamily B (MDR/TAP), Member 1 (ABCB1) Gene

Methadone is transported across the blood–brain barrier by the P-glycoprotein 170 (P-gp) methadone transporter [182, 183] encoded by the *ABCB1* gene located on chromosome 7q21.12 and is a member of the ATP-binding cassette (ABC) transporters. The *ABCB1* gene is a member of the MDR/TAP subfamily whose gene products are involved in multidrug resistance. Levran et al. showed that individuals with the TT genotype of the *ABCB1* rs1128503 (C1236T) variant and those with the TT–TT–TT genotype pattern of the *ABCB1* variants rs1045642, rs2032582, and rs1128503, respectively, require higher doses of methadone to achieve stabilization [184]. A study by Coller et al. investigated the

role several additional variants and haplotypes in *ABCB1* have in moderating methadone dose [185]. They reported that homozygous carriers of the AGCGC haplotype of five SNPs [A61G, G1199A, rs1128503 (C1236T), rs2032582 (G2677T), and rs1045642 (C3435T)] required higher doses of methadone than did noncarriers of this haplotype. Noncarriers of another haplotype, AGCTT, required higher methadone doses to achieve stabilization than did carriers of this haplotype. In contrast, a study of Han Chinese reported that subjects who were carriers of the T variant allele of rs1045642 (C3435T) had a higher likelihood of requiring a larger methadone dose than did noncarrier subjects [64]. In a multi-gene analysis, *OPRM1* 118A/118A homozygous subjects who were also homozygous for the *ABCB1* AGCGC ("wild-type") haplotype (defined above) or who were homozygous for the AGTTT haplotype required lower methadone doses and had higher plasma methadone concentrations (C_{trough}) to suppress withdrawal than did AGCGC/AGTTT diplotype subjects [186]. Conversely, those subjects with *ABCB1* AGCGC/AGTTT diplotype who were also *OPRM1* AA homozygous required a lower methadone dose and had lower plasma methadone concentrations than did *OPRM1* G-allele carriers.

8.2 α_{1A}-Adrenoceptor (ADRA1A) Gene

α-1-Adrenoceptors are members of the G-coupled protein receptor (GCPR) superfamily and regulate proliferation and growth through the activation of the phosphatidylinositol-calcium second messenger system. Previous studies have shown that the adrenergic system is involved in cocaine addiction, treatment and the development of cocaine-induced paranoia [52, 80, 87, 187]. In the disulfiram study for cocaine addiction, genetic analysis showed that T carriers of the rs1048101 (Arg347Cys) variant in exon 1 of the *ADRA1A* gene on chromosome 8q21.2 had a reduced number of cocaine-positive urines on disulfiram, while individuals with the CC genotype showed no treatment effect [188]. It is likely that the Arg allele of *ADRA1A* encodes an α_{1A}-adrenoceptor with reduced signaling efficiency and his may partly explain its role in the efficacy of disulfiram.

8.3 Brain-Derived Neurotrophic Factor (BDNF) and Nerve Growth Factor (Beta Polypeptide) (NGF) Genes

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors and is required for neuronal growth and differentiation. It is encoded by the *BDNF* gene located on chromosome 11p14.1. Variants in *BDNF* have been found to be associated with synaptic plasticity, hippocampal volume, TBI severity, and a number of psychiatric diseases, including schizophrenia [189–191]. In a study of the response of opioid addicts to methadone treatment, carriers of the *BDNF* haplotype CCGCCG (rs7127507, rs1967554, rs11030118, rs988748, rs2030324, and rs11030119) had poorer response to methadone maintenance treatment compared to individuals with other haplotypes [192].

The *nerve growth factor* (*beta polypeptide*) gene (*NGF*) located on chromosome 1p13.1 encodes a neurotrophic factor that is important in the differentiation and maintenance of several types of sympathetic and sensory neurons and that is critical to the sensation of pain [193]. Individuals homozygous for the rs2239622 A allele of *NGF* were found to require a lower mean daily methadone dose than did those individuals with the other genotypes [194].

8.4 β-Arrestin2 (ARRB2) Gene

Arrestin/beta-arrestin protein family members are involved in the agonist-mediated desensitization of G-protein-coupled receptors (GPCRs). GPCRs are a large family of receptors that signal ligand binding through the cell membrane. A member of the arrestin/beta-arrestin protein family is β-arrestin2, encoded by *ARRB2* on chromosome 17p13.2. *ARRB2* is expressed in many tissues, with high expression in the brain. The function of β-arrestin2 is to promote the desensitization and internalization of GPCRs, including the opioid receptors [195]. A study on the role *ARRB2* variants influence response to methadone treatment was conducted on a Swiss cohort. Heroin-dependent individuals homozygous for the *ARRB2* gene variant who had the rs3786047 AA, rs1045280 CC, or rs2036657 GG genotypes had poor response to methadone maintenance therapy [196].

8.5 Cytochrome P450, Family 2, Subfamily D, Polypeptide 6 (CYP2D6) and Subfamily B, Polypeptide 6 (CYP2B6) Genes

The cytochrome (CYP) P450 superfamily of enzymes is involved in the oxidation of many organic substances, including methadone. Methadone is primarily metabolized in the liver by CYP3A4 by N-demethylation to the inactive metabolite EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) [197]. Several other cytochrome P450s, including CYP2B6 and CYP2D6, are also involved in the metabolism of methadone.

The product of the *cytochrome P450*, *family 2*, *subfamily D*, *polypeptide 6* (*CYP2D6*) gene is an enzyme that metabolizes specific opioid drugs into their active state, such as codeine and methadone [198]. *CYP2D6* is a highly polymorphic gene located on chromosome 22q13.2 with at least 75 different alleles. Individuals can be categorized as poor (PM), extensive (EM), and ultrarapid (UM) metabolizers of drugs based on their number of functional *CYP2D6* alleles. Using this classification based on the *CYP2D6* genotyping, heroin-dependent patient satisfaction of methadone maintenance treatment was assessed in a Caucasian cohort [199]. Patients that were classified as PM or EM scored higher on the Verona Service Satisfaction Scale for methadone-treated opioid-dependent patients (VSSS-MT) than did the patients classified as UM. In addition, UM males were less satisfied than were UM females with methadone maintenance treatment on the Basic Intervention VSSS-MT subscale. Other studies have shown the involvement of specific variants in *CYP3A*, as well as *CYP2D6*, on methadone plasma levels [200].

The *cytochrome P450, family 2, subfamily B, polypeptide 6* gene (*CYP2B6*) located on chromosome 19q13.2 encodes a protein that is localized to the liver and metabolizes specific drugs, including methadone. Israeli former heroin addicts in methadone maintenance treatment who were homozygous for the *CYP2B6 6*6* genotype, which is defined by the variant alleles of SNPs 516G>T (rs3745274) and 785A>G (rs2279343), required lower mean methadone doses than did heterozygous or noncarrier individuals of the 6*6 genotype [201]. Related to this finding is another study that found an association of *CYP2B6 6*6* homozygous subjects with having higher methadone plasma levels [202]. Resequencing of the *CYP2B6* gene identified seven variants, including rs3745274 and rs2279343, that were found to be associated with (S)-methadone plasma levels, suggesting an association with reduced *CYP2B6* activity [203]. Additionally, Hung et al. demonstrated that individuals with the T allele of rs374274 (516 A→T) had a threefold higher chance of requiring lower methadone doses than did individuals homozygous for the G allele [64].

8.6 GATA Binding Protein 4 (GATA4) Gene

The transcription factor GATA-binding protein 4 encoded by the *GATA4* gene located on chromosome 8p23.1 is a zinc-finger transcription factor, which binds a GATA motif found in the promoter region of a variety of genes. GATA4 has been shown to regulate the expression of atrial natriuretic peptide (ANP) [204], which may play a major role in alcohol withdrawal and dependence [205]. A SNP in *GATA4*, rs13273672, has been found to be associated with ANP plasma concentration in alcohol dependent subjects, with the AA genotype group having higher levels of ANP than did the G-allele carrier genotype groups [206]. One study found an association with nominal significance of alcohol dependence with several *GATA4* variants, but not with the rs13273672 variant [207]. A genome-wide association study of alcohol dependence conducted with German alcoholics identified the *GATA4* rs13273672 as being 1 of 16 SNPs showing an association with nominal significance [208]. A follow-up study was conducted on this variant in a trial of acamprosate, naltrexone, or placebo for the treatment of alcohol dependence [206]. The *GATA4* rs13273672 variant was shown to be associated with relapse risk during the 90-day trial, with the GG genotype group relapsing sooner than did the AG genotype group, which relapsed sooner than did the AA group. Post-hoc analysis demonstrated this finding was due to those subjects treated with acamprosate. It was hypothesized that the *GATA4* rs13273672 variant would regulate ANP activity, which subsequently would interact with the glutamate system, since GABA receptors are a target of acamprosate's action.

8.7 Methylenetetrahydrofolate Reductase (MTHFR) Gene

Methylenetetrahydrofolate reductase (MTHFR) is encoded by the *MTHFR* gene located on chromosome 1p36.22. It is the rate-limiting enzyme in the folic acid metabolic cycle that is critical for the

production of metabolites for downstream DNA and protein methylation. The minor T allele of rs1801133 codes for an alanine to valine substitution that results in a thermolabile form of the MTHFR enzyme having 50–60 % the activity of the non-variant enzyme [209]. This variant has been found to be associated with vulnerability to develop spinal bifida, a condition in which the neural tube fails to close leaving neural tissue open to the environment [210, 211].

In the disulfiram pharmacogenetics of cocaine addiction study, T-allele carriers of the *MTHFR* rs1801133 C677T variant were shown to respond to disulfiram treatment, with no change in cocaine free urines in the placebo group with these genotypes [212]. Those subjects with two copies of the C allele had a poorer response to disulfiram than did those with one or two T alleles. Both disulfiram and cocaine have been shown to change the epigenetic landscape of the genome [213, 214]. Since MTHFR is a critical enzyme in the production of metabolites for DNA and protein methylation, it is possible that there is an interaction of these variants with epigenetics. The resulting effects on gene expression may cause the pharmacogenetic effect observed with disulfiram and *MTHFR*.

8.8 Myocardin (MYOCD) and Glutamate Receptor Metabotropic 6 (GRM6) Genes

Myocardin (encoded by the *MYOCD* gene on chromosome 17p12) is a transcription factor that functions in the cardiovascular system. It is expressed primarily early in development in cardiac muscle cells, where it is involved in the chromatin remodeling of SRF target genes [215]. The *GRM6* gene located on chromosome 5q35 encodes the metabotropic glutamate receptor 6 (mGuR6), a G-protein coupled receptor involved in glutamatergic signaling in the central nervous system. An intronic variant in *MYOCD*, rs1714984, intronic variant was previously been found as the top hit with the smallest *p*-value based on genotype frequency to be associated with heroin addiction vulnerability in a genome-wide association study of former heroin addicts and controls, with the AA genotype group at greatest risk [216]. In that same study, *GRM6* rs953741was identified as one of the top candidate gene variant being in association with heroin addiction, with the A allele conferring increased risk. A subsequent study was conducted using these SNPs to assess response to treatment in methadone-treated subjects [217]. They found that patients carrying the A allele at *MYCOD* rs1714984 had an increased risk of being nonresponders to methadone maintenance treatment if they were also carriers of the AG genotype at *GRM6* rs953741.

8.9 Potassium Inwardly Rectifying Channel, Subfamily J, Member 6 (KCNJ6) Gene

The *potassium inwardly rectifying channel*, *subfamily J*, *member 6* gene (*KCNJ6*, also known as *GIRK2*) is located on chromosome 21q22.13. It encodes a potassium channel that is regulated by G-protein coupled receptors and is important in opioid receptor signaling and analgesia [218]. *KCNJ6* is expressed in the substantia nigra (SN), as well as in the VTA [219]. Genetic variation in *KCNJ6* has been shown to be associated in alcoholism. Specifically,

the rs2836016 G allele was found to be associated with alcohol dependence in an adult and adolescent cohort. The GG homozygous subjects in the adolescent group had an increase in hazardous alcohol use, but only in those subjects who experienced early life stress [220]. Activation of μ-, δ-, or κ-opioid, or dopamine D2 receptors can open GIRK channels, inhibiting voltage-gated calcium channels and adenylyl cyclases [221, 222], as well as inhibiting neuronal activity [223].

Genetic variation in *KCNJ6* was examined in relation to the requirements for postoperative analgesics [224]. In patients who had undergone major open abdominal surgery, those with the AA genotype of rs2070995 were found to require a higher dose of equivalent oral morphine and had more frequent administration of analgesics. Another study reported on the involvement of this SNP in methadone treatment of former heroin addicts, chronic pain patients, and healthy volunteers [225]. The average and daily methadone dose during the first year of treatment was greater in former heroin subjects with the *KCNJ6* rs2070995 AA genotype than did in individuals with other genotypes as was a higher opioid dosing if chronic pain patients.

9 Conclusions

In this chapter, we have shown that the response to pharmacotherapy for addiction is genetically complex; that is, therapeutic response is a result of small influences of many genetic variants, as well as that of the environment. The research reviewed herein suggests that these influences may be additive and that the identification of more predictive genetic factors may help to tailor more effective pharmacotherapies.

Most of the genetic associations we have reviewed have not been replicated, which will be required to confirm and extend their findings. A number of confounding factors may be influencing the results. Differences in ethnic composition may alter the findings and ethnic stratification should be properly controlled. Many of these studies were conducted in cohorts with small sample sizes, which may have influenced their conclusions. Large sample sizes will be required to validate the role these variants have in pharmacotherapeutic response, especially for variants with low effect sizes or low allele frequencies. In addition, some genetic studies may not have been adequately powered to detect a genetic effect and, therefore, the results have appeared as negative findings.

Given the heterogeneity of drug abuse and dependence, the "one size fits all" strategy typically utilized in the treatment of these disorders has seen limited success. As demonstrated with the examples presented in this chapter, a significant proportion of the variability seen in drug addiction and dependence pharmacotherapy is

due to genetic heterogeneity. Similar to the effects that genetic variation has on the vulnerability to develop an addiction [226], genetic variation affects response to treatments for drug addiction, including reward and positive stimulation resulting from drug use.

A goal of personalized medicine is to match the patient to the most effective pharmacotherapy for their disease based on that person's genetic background. Further studies will need to be conducted to identify genetic differences in the pathways involved in the development, maintenance, and relapse of addiction in order to optimize treatment outcomes. Personalized medical treatment already has been incorporated into the care of cardiology and oncology patients to improve their efficacy. This success may be replicated with the creation of personalized treatments for cocaine, opioid, and alcohol addiction based on an individual's genetics, history, current physical condition, and other elements unique to that person [175]. Since the initial success of treatment plays a substantial role in compliance and retention, personalizing treatment based on genetic background should increase treatment efficacy, and therefore improving compliance as well. The findings from pharmacogenetic studies of treatments of drug addiction will, in the near future, better equip medical professionals with the knowledge to assign personalized treatment strategies to patients with substance use disorders, and therefore effecting better outcomes and greater treatment success.

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Chapter 16

Pharmacogenetics in Rheumatoid Arthritis

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Abstract

Rheumatoid arthritis (RA) is a systemic inflammatory arthritis leading to severe joint damage and associated with high morbidity and mortality. Disease-modifying antirheumatic drugs (DMARDs) are the mainstay of treatment in RA. DMARDs not only relieve the clinical signs and symptoms of RA but also inhibit the radiographic progression of disease. In the last decade, a new class of disease-modifying medications, the biologic agents, has been added to the existing spectrum of DMARDs in RA. However, patients' response to these agents is not uniform with considerable variability in both efficacy and toxicity. There are no reliable means of predicting an individual patient's response to a given DMARD prior to initiation of therapy. In this chapter, the current published literature on the pharmacogenetics of traditional DMARDS and the newer biologic DMARDs in RA is highlighted. Pharmacogenetics may help individualize drug therapy in patients with RA in the near future.

Key words Pharmacogenetics, Polymorphisms, Rheumatoid, Arthritis, Methotrexate, Azathioprine, Sulfasalazine, Tumor necrosis factor antagonists, Rituximab, Tocilizumab

1 Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory arthritis that occurs in about 1 % of the population worldwide. Untreated, RA is associated with joint destruction, disability, and increased mortality [1]. Early detection and therapy with disease-modifying antirheumatic drugs (DMARDs) is critical in preventing these sequelae of RA. With the advent of biologic DMARDs, which are effective but expensive therapies for RA, there has been a focus on developing methods including those based on pharmacogenetics to predict a priori, an individual patient's response to a given DMARD.

This chapter highlights some of the recent, major publications in the field of pharmacogenetics in RA and describes the implications of this field for future research and clinical care. The pharmacogenetics of three major non-biologic DMARDs, methotrexate (MTX), azathioprine, and sulfasalazine (SSZ), and three biologic DMARDs, the tumor necrosis factor antagonists, rituximab, and tocilizumab in RA, is reviewed.

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2 Pharmacogenetics of Drugs in RA

2.1 Pharmacogenetics of Methotrexate

For over the past two decades, MTX has been the first line DMARD in RA because of its well-established efficacy and safety [2–4]. However, the response among patients to MTX can be quite variable, ranging from 46 to 65 % [5, 6]. The exact mechanism of action of the drug in RA remains unclear; however, it is believed that MTX's effects in RA occur due to its effects on the intracellular folate and adenosine pathway.

MTX is actively transported into the cell by solute carrier 19A1 (SLC 19A1), also called reduced folate carrier 1 (RFC1) (Fig. 1). MTX is pumped out of the cell by members of the ATP binding cassette (ABC) family of transporters, also known as multidrug resistant transporters (MDRs), and multidrug resistance-associated

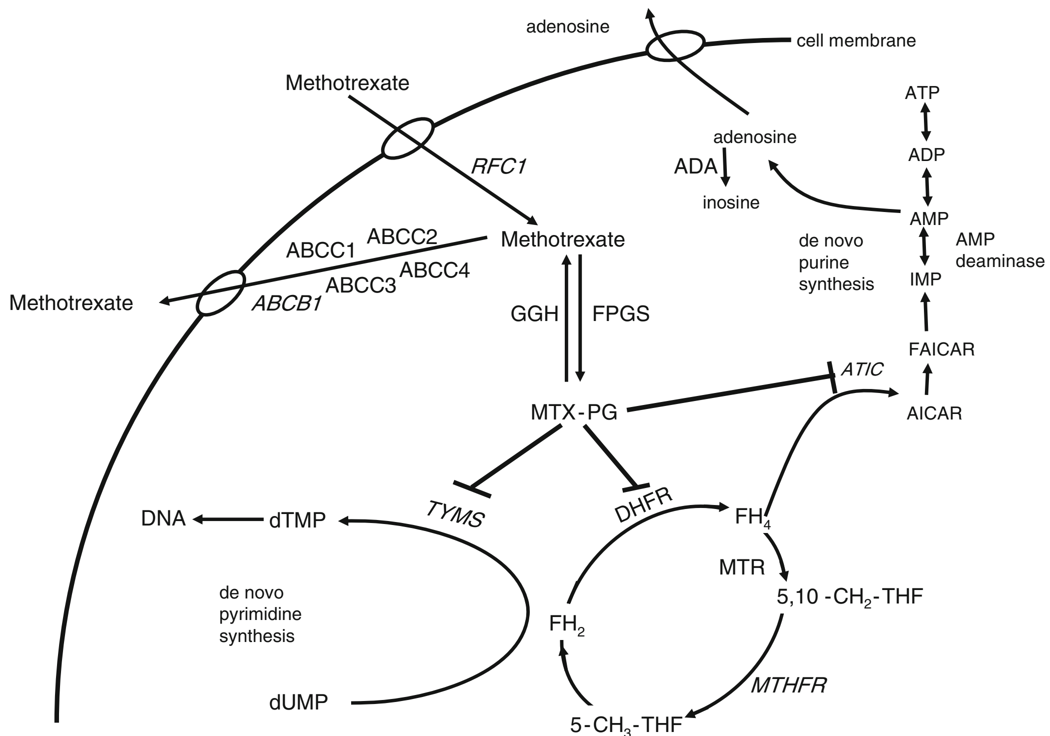

Fig. 1 Cellular pathway of methotrexate. *RFC1* reduced folate carrier 1, *ABCB1* ABCC1–4—ABC transporters, *GGH* γ-glutamyl hydrolase, *FPGS* folylpolyglutamyl synthase, *MTX-PG* methotrexate polyglutamate, *TYMS* thymidylate synthase, *dUMP* deoxyuridine monophosphate, *dTMP* deoxythymidine monophosphate, *DHFR* dihydrofolate reductase, *FH_2* dihydrofolate, *5-CH_3-THF* 5-methyl tetrahydrofolate, *MTHFR* methylene tetrahydrofolate reductase, *5,10-CH_2-THF* 5,10-methylene tetrahydrofolate, *MTR* methyl tetrahydrofolate reductase, *AICAR* aminoimidazole carboxamide ribonucleotide, *FAICAR* 10-formyl AICAR, *ATIC* AICAR transformylase, *IMP* inosine monophosphate, *AMP* adenosine monophosphate, *ADP* adenosine diphosphate, *ATP* adenosine triphosphate, *ADA* adenosine deaminase. *Italicized* genes have been targets of pharmacogenetic analyses in studies published so far. Reproduced from [148] with permission from John Wiley & Sons, Inc

proteins (MRPs) [7]. Intracellular MTX is polyglutamated by the enzyme folylpolyglutamyl synthase (FPGS). This process can be reversed by gamma glutamyl hydrolase (GGH). Polyglutamation of MTX ($MTXPG_n$) helps retain MTX within the cell preventing drug efflux by the ABC transporters. The ratio between GGH and FPGS may be a predictor of MTX treatment outcomes in human primary leukemia [8]. Decreased FPGS activity occurs in MTX-resistant cells [9–11]. MTXPGs inhibit dihydrofolate reductase (DHFR), which reduces dihydrofolate to tetrahydrofolate (THF) [12]. THF is converted to 5,10 methylene tetrahydrofolate (5,10-CH_2-THF) and subsequently to 5-methyl THF (5-CH_3-THF) by methylene tetrahydrofolate reductase (MTHFR). 5-Methyl THF is a biologically active folate cofactor that functions as a one carbon donor for many important cellular reactions, including the conversion of homocysteine to methionine [13]. MTXPGs also inhibit thymidylate synthase (TYMS), which converts deoxyuridylate to deoxythymidylate in the de novo pyrimidine synthetic pathway [14].

MTX also has several effects on the purine synthetic pathway. MTXPGs inhibit the enzyme aminoimidazole carboxamide ribonucleotide (AICAR) transformylase which in turn causes intracellular accumulation of AICAR. AICAR and its metabolites can then inhibit two enzymes in the adenosine pathway: adenosine deaminase (ADA) and adenosine monophosphate (AMP) deaminase which leads to intracellular accumulation of adenosine and adenine nucleotides. Subsequent dephosphorylation of these nucleotides results in increased extracellular concentrations of adenosine which is a powerful anti-inflammatory agent [15].

Polymorphisms in genes encoding MTX transporters and enzymes in the folate and adenosine pathways inhibited by MTX have been studied in RA patients.

2.1.1 Transporter Genes: RFC1/GGH/ABCB1

RFC1 transports MTX into the cell. Polymorphisms that inactivate the RFC1 enzyme or change the function of transcription factors leading to loss of RFC1 gene expression can alter MTX transport [16, 17]. The RFC1 gene is a 27 kb gene located on chromosome 21 (21q22.3). An 80G>A polymorphism leading to substitution of arginine for histidine at codon 27 in the first transmembrane domain (TMD1) of the RFC1 protein and a 61 base pair (bp) repeat polymorphism in the RFC1 promoter causing increased transcriptional activity of the gene have been described [18]. In a study by Dervieux et al., the effect of the G80A single nucleotide polymorphism (SNP) on response to MTX in 105 RA patients was examined. Patients within the top 25th percentile of MTX responders were identified using a visual analog scale (VAS) measuring the patients' and physicians' response to MTX. Patients homozygous for the RFC SNP 80A/A had a greater response to MTX compared to patients carrying the wild type 80G/G SNP. Patients homozygous for the A allele were three times more likely to be

within the top 25th percentile of MTX responders (confidence intervals (CI) 1.3–8.4; $p < 0.01$) compared to the patients with the wild type G allele. Thus, the RFC 80AA SNP may be a marker of increased response to MTX in RA [19].

SNPs exist in the GGH promoter that influences GGH expression [20] and MTX polyglutamation [21, 22]. A 452C>T SNP leading to decreased GGH activity and accumulation of intracellular long-chain MTX polyglutamates [22] and a 401C>T promoter polymorphism also altering intracellular MTXPG levels have been described [21]. There is a GGH 16T>C polymorphism whose functional effects are unknown. A Japanese study of patients with RA demonstrated that the presence of the RFC 80AA and GGH–401TT genotypes independently predicted MTXPG levels. Patients carrying the RFC 80AA genotype were 3.4-fold more likely to have MTXPG levels above the group median compared to patients with the 80GG or 80GA genotype (odds ratio (OR) 3.4, 95 % CI 1.4–8.4; $p = 0.007$). In contrast, patients with the GGH–401TT genotype were 4.8-fold (OR 4.8, 95 % CI 1.8–13.0; $p = 0.002$) more likely to have MTXPG below the study median compared to those who carried the GGH–401CC or CT genotype. Thus, both the GGH 401 C>T and RFC 80G>A SNPs influenced intracellular MTXPG levels and thereby may predict MTX response in RA [20, 22–24].

2.1.2 ABCB1

The ABCB1 gene is a 209 kb gene located on chromosome 7 (7q21.1). P-glycoprotein (P-gp) the product of the ABCB1 gene is a membrane transporter important in the transport of several drugs, including MTX. SNPs in the ABCB1 gene have been identified and their effects on P-gp expression studied [25]. The 3435C>T SNP is a synonymous SNP in exon 26 of the ABCB1 gene. It is often linked to a 2677G>T SNP in exon 21 which results in substitution of alanine in position 893 by serine or threonine [26, 27]. It is unclear whether variations in ABCB1 and/or P-gp expression impact MTX efflux from the cell. Although there is lack of published data to support that ABCB1 SNPs influence MTX cellular transport directly, some studies suggest that higher P-gp expression may mediate MTX resistance [28] while other studies do not support this [29, 30]. Considering the linkage of the two SNPs, haplotype analyses may be more helpful in examining the role of these genetic variants in influencing MTX's effects.

Ninety-two RA patients and ninety-seven healthy controls were genotyped for the 3435C>T polymorphism in one study. Patients who had active RA ($n = 62$) after 6 months of treatment with MTX (7.5–15 mg/week) and prednisone (5–10 mg daily) and those who responded after 6 months of the same treatment ($n = 30$) were classified as two groups and studied. The ABCB1 genotypes were distributed similarly among the RA patients and controls. Patients with the 3435CC and 3435CT genotypes were

more likely to have active RA compared to patients with the 3435TT genotype (OR 2.89, CI 0.87–9.7; $p < 0.05$). Thus, the presence of the 3435T allele seemed to be protective in that patients homozygous for this allele had less severe RA which was more responsive to MTX and prednisone [31].

2.1.3 MTHFR/ DHFR/ TYMS

The MTHFR gene is a 19 kb gene located on chromosome 1 (1p36.3). Of the several MTHFR polymorphisms that have been identified [32], two polymorphisms, the 677C>T and 1298A>C polymorphisms, have been well studied for their influence on MTX's clinical effects. The 677C>T polymorphism leads to an alanine to valine substitution in the codon at nucleotide 677 of the MTHFR gene [33]. This change leads to a thermolabile variant of MTHFR to be encoded with resultant decreased enzyme activity. The 1298A>C polymorphism causes a glutamine to alanine substitution in the codon at nucleotide 1298 and also leads to reduced MTHFR enzyme activity [34]. As MTHFR is important in the generation of 5-methyl THF (Fig. 1), which acts as the carbon donor for the remethylation of homocysteine to methionine, these two SNPs by reducing MTHFR activity can increase plasma homocysteine levels [35].

Elevated plasma homocysteine levels mediated by these two SNPs may exacerbate MTX's toxic effects. Several studies have evaluated the effects of these SNPs on MTX response. One study examined 105 patients with RA, 35 of whom were treated with MTX (7.5–15 mg/week), 34 with SSZ (2–3 g/day), and 36 with MTX and SSZ. All patients were genotyped for the 677C>T SNP and their plasma homocysteine levels measured. Patients on MTX had higher plasma homocysteine levels than those on SSZ alone, but those on both MTX and SSZ had the highest levels. Patients heterozygous for the 677C>T SNP had higher plasma homocysteine levels after 1 year than patients without the SNP. Patients homozygous for the SNP had a higher plasma homocysteine level at baseline which did not change significantly. Elevated plasma homocysteine levels (17 %, $p < 0.05$) were found in patients with gastrointestinal (GI) side effects from MTX, such as nausea, abdominal pain, and discomfort, compared to patients without side effects. Patients on MTX and SSZ had the highest homocysteine levels and the highest incidence of GI side effects. However, the presence of the C677T SNP was not directly associated with the occurrence of GI events. This study suggests that plasma homocysteine levels (exacerbated by the presence of the MTHFR 677C>T SNP) may influence the GI toxicity of MTX [36].

In another study 236 patients with RA on MTX were genotyped for the 677C>T SNP. MTX was initiated at 7.5 mg/week and titrated to a maximum dose of 25 mg/week. Patients were assessed for MTX toxicity and disease activity periodically. One hundred and twenty-two of two hundred and thirty-six patients (52 %) did

not have the SNP; nineteen patients (8 %) were homozygous, and ninety-five patients (40 %) heterozygous for the polymorphism. Patients who were homozygous and heterozygous for the 677C>T SNP had an increased risk of discontinuing MTX due to adverse events (relative risk (RR) 2.01, CI 1.09–3.70) particularly hepatotoxicity (RR 2.38, CI 1.06–5.34). This effect of the genotype on MTX toxicity was also evident in patients on folate supplementation in this study. However, there was no effect of the 677C>T genotype on MTX efficacy [37].

In a cross-sectional study 93 RA patients treated with MTX (average dose 11.9 mg/week) and 377 healthy controls were genotyped for the 677C>T and 1298A>C polymorphisms and assessed for RA disease activity and MTX toxicity. Serum folate and plasma homocysteine levels were measured. More RA patients carried the 1298CC genotype (24.7 %) than the controls (12.8 %) and this was statistically significant ($p < 0.001$). There were interesting effects of the 1298CC genotype on MTX toxicity but not efficacy. Homozygotes for the 1298C SNP appeared to be protected from MTX toxicity; 33 % did not experience toxicity, only 9.1 % had adverse reactions ($p = 0.035$). In contrast, patients with the AA genotype were five times more likely to develop toxicities than those with the CC genotype (OR 5.24, CI 1.38–20). Also, patients carrying the CC genotype had higher plasma homocysteine levels than patients with AA or AC genotype and this was not influenced by serum folate levels. The 677C>T polymorphism had no effects on MTX toxicity or efficacy in this study. This study suggests that homozygosity for the C1298 allele increases susceptibility to RA but also protects from MTX toxicity presumably via a homocysteine-dependent mechanism [38].

One hundred and six RA patients who had been treated with MTX were assessed retrospectively for MTX efficacy and toxicity. The MTX dose ranged from 2.5 to 12.5 mg/week. All patients were genotyped for the 677C>T and 1298A>C SNPs. As this was a retrospective study, no direct assessment of MTX efficacy (as measured by disease activity scores) was possible. However, patients carrying the 1298A>C polymorphism (homozygous or heterozygous) were more likely to be on lower doses of MTX compared to those without the polymorphism (RR 2.18, CI 1.17–4.06; $p < 0.05$). Patients carrying this polymorphism also showed improvement in their inflammatory markers such as ESR (erythrocyte sedimentation rate) and CRP (C reactive protein), suggesting clinical efficacy of MTX ($p < 0.05$). Such changes were not seen with the presence of the 677C>T polymorphism. The presence of the 677C>T polymorphism was associated with an increased likelihood of side effects from MTX (RR 1.25, CI 1.05–1.49, $p < 0.05$) but not with indicators of MTX efficacy such as lower doses of the drug or improvement in inflammatory markers. Thus, the 677C>T polymorphism appeared to be a marker for MTX toxicity and the 1298A>C polymorphism for MTX efficacy [39].

More studies have yielded inconsistent results. Three studies demonstrated an effect of the 677C>T polymorphism on MTX efficacy. However, results from these studies were conflicting with the T allele being a marker of both decreased and increased MTX efficacy in US [40] and Polish [41] cohorts respectively and the C allele a marker of increased MTX efficacy in a Dutch cohort [42]. Eight studies showed an effect of the T allele on MTX toxicity. Four of these studies examined Asian patients which included Japanese [39, 43], Korean [44], and Chinese [45]; others included Dutch [37], US [46, 47], and Spanish [48] cohorts. Two meta-analyses have also yielded disparate results. One meta-analysis found an association between the 677C>T polymorphism and MTX toxicity, but no such association for the 1298A>C variant [49]. However, another meta-analysis (which included 1,514 patients with RA) found no association between either of these polymorphisms and MTX toxicity and efficacy [50].

TYMS is an important enzyme in the de novo synthesis of pyrimidines. It converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) and is a direct target of polyglutamated MTX. The TYMS gene is a 15 kb gene located on chromosome 18 (18p11.32). A polymorphic tandem 28 bp repeat sequence has been described in the 5′ untranslated region (TSER) of the TYMS gene with a variable number of repeat elements [51]. This repeat element may function as an enhancer as in vitro studies have shown that TYMS mRNA expression and enzyme activity is increased with an increasing number of these repeat sequences [51–53]. Patients homozygous for the triple repeat allele (TSER*3/*3 or 3R) have higher TYMS mRNA expression compared to patients homozygous for the double repeat allele (TSER*2/*2 or 2R) [53, 54]. An additional G to C substitution within the 3R allele further diversifies 3R into 3RC and 3RG; the C allele abolishes a critical residue of the upstream stimulatory factor (USF)-binding site lowering TYMS activity in 3RC carriers [55]. Deletion of a 6 bp sequence at nucleotide 1494 in the 3′ untranslated region (3′UTR) of TYMS has also been described and may be associated with decreased TYMS mRNA stability and expression [56, 57].

One hundred and sixty-seven patients with RA, of whom one hundred and fifteen were treated with MTX were genotyped for the following polymorphisms—TYMS 5′UTR enhancer repeat (TSER), 3′ UTR deletion, MTHFR 677C>T, and 1298A>C. The mean weekly MTX dose in this study was 5.7 ± 2.3 mg. Information on MTX toxicity data was collected retrospectively. Both MTX treated and untreated groups displayed similar frequencies of these SNPs. The TYMS and MTHFR polymorphisms were not associated with toxicity, although a significant percentage (45 %) of patients on MTX experienced adverse effects. Weekly MTX dose (rather than standardized disease activity measures) was used as a

marker of efficacy in this study. A dose of >6 mg/week was considered indicative of less efficacy and <6 mg/week was considered indicative of greater efficacy. Homozygotes for the TSER*2 allele (TSER*2/*2) required lower doses of MTX (had greater efficacy) than homozygotes for the TSER*3 allele ($p = 0.033$). The TYMS 3′UTR deletion and MTHFR polymorphisms had no effects on MTX efficacy. The authors speculated that the repeat enhancer TSER*3 polymorphism by increasing TYMS mRNA expression may lead to decreased MTX efficacy. Based on the findings of this study, it also appeared that MTHFR polymorphisms did not influence MTX toxicity or efficacy [58].

DHFR reduces dihydrofolate to THF in the intracellular folate pathway. It is directly inhibited by polyglutamated MTX and encoded by the DHFR gene which is a 28 kb gene located on chromosome 5 (5q11.2–q13.2). DHFR gene polymorphisms have been studied in 205 MTX treated RA patients. MTX was started at 7.5 mg/week and increased to 15 mg/week (with folic acid supplementation) after 4 weeks based on response to the drug. MTX efficacy and toxicity (gastrointestinal side effects, elevated liver enzyme levels, skin and mucosal disorders, pneumonitis, and overall adverse drug events) were assessed periodically. Genotyping for the MTHFR 677C>T, MTHFR 1298A>C, DHFR –473G>A, DHFR 35289G>A and RFC 80 G>A SNPs was performed. At 6 months, patients carrying the MTHFR 1298AA and MTHFR 677CC (wild-type) genotypes showed a greater response to MTX compared to patients carrying the heterozygous or homogenous genotype (OR 2.3, CI 1.18–4.41 and OR 2.73, CI 1.03–7.26 respectively). Haplotype analysis for the MTHFR 1298A and 677C SNPs revealed that patients with two copies of the haplotype had greater improvement than those with one or no copies of the haplotype (OR 3.0, CI 1.4–6.4). Patients homozygous and heterozygous for the MTHFR 1298 SNP (MTHFR 1298AC+CC) had an increased number of overall adverse drug events at 3 and 6 months (OR 2.55, CI 1.20–5.41 and OR 2.5, CI 1.32–4.72, respectively) compared to those with other genotypes. The RFC and DHFR SNPs were not associated with MTX toxicity or efficacy. Thus, patients with the wild-type MTHFR alleles (MTHFR 1298AA and 677CC) responded better to MTX, while those with the 1298C allele had an increased risk for MTX toxicity [42].

Thus, based on the literature cited above (Table 1), the 677C>T SNP in MTHFR appears to have effects on MTX toxicity, presumably through its effects on homocysteine metabolism [36, 37, 39, 49] and on MTX efficacy [42]. The effects of the 1298A>C polymorphism on MTX are less clear, with data suggesting that it may increase [59] or decrease [42] patients' response to MTX and possibly protect them from MTX toxicity [38]. The seemingly inconsistent results of these studies may stem from the fact that these SNPs may have effects other than that on homocysteine

Table 1
Pharmacogenetics of methotrexate in RA

Gene	Role in MTX pathway	Polymorphism	Postulated effect of polymorphism	Clinical effects	References
RFC1	Active transport of MTX into cell	80G>A	Increased transcriptional activity of RFC1 gene; increased MTX entry into cell	Increased response to MTX	[19, 23]
ABCB1	MTX efflux from the cell	3435C>T	Increased MTX entry into cell	Increased response to MTX	[31]
MTHFR	Important in regeneration of reduced folate; indirectly inhibited by MTX	677C>T	Thermolabile MTHFR with decreased activity; increased plasma homocysteine	Increased GI side effects	[36]
				Increased hepatic toxicity, GI toxicity, alopecia, stomatitis and rash	[36, 37, 39, 42, 49]
				No effect on toxicity	[38]
				No effect on efficacy or toxicity	[50, 58]
MTHFR		1298A>C	Decreased MTHFR activity; increased plasma homocysteine	Increased MTX efficacy	[42]
				Increased susceptibility to RA; decreased MTX toxicity	[38]
				No effect on efficacy or toxicity	[49, 50, 58]
DHFR	Reduction of dihydrofolate to tetrahydrofolate	473G>A 35289G>A	Decreased DHFR activity	No effect on MTX efficacy or toxicity	[58]
ATIC	Conversion of AICAR to 10-formyl AICAR; directly inhibited by MTX	347C>G	Decreased ATIC activity; increased AICAR accumulation; increased adenosine	Increased MTX efficacy	[24, 40, 42, 46, 65]

(continued)

Table 1 (continued)

Gene	Role in MTX pathway	Polymorphism	Postulated effect of polymorphism	Clinical effects	References
TYMS	Conversion of dUMP to dTMP; directly inhibited by MTX	5′UTR 28-bp repeat (TSER)	Increased TYMS enzyme activity	Decreased MTX efficacy	[58]
		3′UTR 6-bp deletion	Decreased TYMS mRNA stability and expression	Increased MTX efficacy	[58]
GGH	Reversal of polyglutamation of MTX	452C>T	Decreased GGH activity, decreased accumulation of intracellular long-chain MTX polyglutamates	Increased MTX efficacy	[22]
		3 G/3 G genotype	Increased GGH activity	Decreased response to MTX and increased bone marrow toxicity	[64]

metabolism which may influence response to MTX. Some of these studies were retrospective which may also have led to inaccuracies in the assessment of MTX effects, particularly adverse effects. Although one of the studies [58] concluded that MTHFR SNPs did not affect MTX efficacy or toxicity, it is worth pointing out that the doses of MTX used in this study were small (6 mg/week) which may have masked the differences in MTX response between patient groups. Also, MTX efficacy was not assessed using standardized measures of disease activity in this study; rather, MTX dose was used as a surrogate marker of MTX efficacy. Ethnicity may have been another factor which influenced the results in this Japanese study [58].

2.1.4 *CCND1/ATIC*

G1–S-specific cyclin-D1 is a protein that in humans is encoded by the CCND1 gene, whose activity is required for cell cycle G1–S transition [60]. The 870A>G substitution resides at the mRNA splicing site [61]. The A allele preferentially encodes the transcript lacking the exon 5 leading to an increased level and longer half-life of cyclin D1 [62]. Elevated levels or expression of cyclin D1 protein (coded by the A allele) have been found in a variety of cancers, including breast cancer, head and neck cancer, non-small-cell lung cancer, and mantle cell lymphomas [63]. Hochhauser, D. et al.

showed in a human fibrosarcoma HT1080 cell line that CCND1 overexpression affected sensitivity to MTX [63].

One hundred and eighty-four RA patients treated with MTX were genotyped for selected polymorphisms in the GGH (−354G>T and 452C>T), CCND1 (870A>G), and TYMS (variable number of tandem repeats [VNTR], and G to C substitution of the triple repeat, 3R allele) genes. Based on the European League Against Rheumatism (EULAR, a standardized measure of disease activity in RA) response criteria, 146 RA patients (79.3 %) were classified as responders and 38 (20.7 %) as non-responders after 6 months of MTX therapy. There was no difference in the frequency of polymorphisms in the GGH and CCND1 genes or TYMS VNTRs between MTX responders and non-responders. However, when the TYMS gene was analyzed with respect to VNTRs and the 3R G to C substitution, a higher frequency of the 3 G/3 G genotype was found in MTX non-responders when compared to other genotypes; $p=0.02$, OR 5.4, 95 % CI 1.0–21.1 [64]. 8/184 patients developed bone marrow toxicity, and all eight patients carried the GGH-354GG genotype. The −354T allele has been shown to correlate with increased GGH gene expression [20]. Thus, the authors postulated that the −354GG genotype possibly results in reduced GGH levels and higher cellular concentrations of MTXPG, which may lead to increased toxicity and explain the observed association [64].

AICAR transformylase (ATIC) converts AICAR to 10-formyl AICAR and is directly inhibited by MTX (Fig. 1). This leads to accumulation of AICAR and adenosine, a purine with anti-inflammatory properties. Adenosine may be an important mediator of the anti-inflammatory effects of MTX [15]. The ATIC gene is a 37 kb gene located on chromosome 2 (2q35). The ATIC 347C>G SNP leads to a threonine to serine substitution in codon 2, which may cause a decrease in ATIC's enzymatic activity and affect AICAR accumulation and adenosine release. A study examined the combined effects of the ATIC 347C>G SNP, TSER*2, and RFC 80G>A polymorphism on MTX efficacy. One hundred and eight RA patients on MTX at a dose of 14 mg/week (range 5–25 mg/week) were examined. Red blood cell long-chain MTX polyglutamate (MTXPG) concentrations were measured, and a pharmacogenetic index was calculated from the sum of homozygous variant genotypes (RFC1 80AA, ATIC 347GG, TSER*2/*2). The allelic frequency for the ATIC 347G variant was 37 %. Patients were categorized as MTX responders or MTX non-responders using a visual analog scale (VAS). Eighteen patients who were carriers of the ATIC 347GG genotype had fewer swollen joints (1.9 ± 0.6 versus 4.5 ± 0.6 [$p=0.06$]) and a lower score for physician's assessment of patient's response to MTX (1.8 ± 0.3 versus 2.8 ± 0.2 [$p=0.02$]) compared to ninety patients who were carriers of the C allele (ATIC 347CC; $n=47$) or ATIC 347CG ($n=43$) genotype [24]. Among other studies, only one study demonstrated

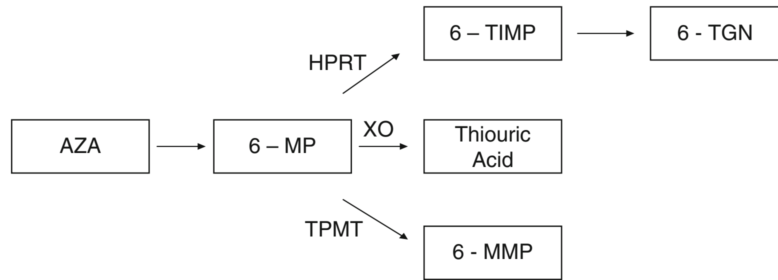

Fig. 2 Scheme of thiopurine drug metabolism. *TPMT* thiopurine methyltransferase, *XO* xanthine oxidase, *HPRT* hypoxanthine phosphoribosyl transferase, *6-TIMP* 6-thiosine monophosphate, *6-MMP* 6-methylmercaptopurine, *6-TGN* 6-thioguanine nucleotides, *AZA* Azathioprine, *6-MP* 6-Mercaptopurine

the ATIC 347C allele to be associated with MTX efficacy in a Dutch cohort [42]. Four studies showed an association of the ATIC 347G allele with MTX toxicity in US [40, 46], Dutch [42], and Slovenian cohorts [65].

2.2 Pharmacogenetics of Azathioprine

Azathioprine (AZA) is used in the treatment of several rheumatic diseases, including systemic lupus erythematous (SLE) and RA. About 10–30 % of RA patients discontinue AZA due to side effects [66]. AZA is a prodrug that after oral intake is converted into 6-mercaptopurine (6-MP) an active purine anti-metabolite which affects the purine de novo synthetic and salvage pathways (Fig. 2). 6-MP is converted by hypoxanthine phosphoribosyl transferase (HPRT) to cytotoxic thioguanine nucleotides (6-TGN) via the intermediary metabolite, 6-thio-inosine monophosphate (6-TIMP). Inosine monophosphate is phosphorylated to inosine triphosphate (ITP), a toxic metabolite, and this process can be reversed by inosine triphosphate pyrophosphatase (ITPase, encoded by *ITPA*). ITPase deficiency results in the accumulation of toxic ITP. ITPase-deficient individuals treated with AZA can develop toxicity because of accumulation of thio-ITP [67]. By a parallel pathway, 6-MP can be inactivated by thiopurine methyltransferase (TPMT) to 6-methylmercaptopurine (6-MMP) or by xanthine oxidase (XO) to thiouric acid (TU). Thus, a relative deficiency of TPMT leads to accumulation of cytotoxic TGN and significantly increased AZA toxicity. Common, significant toxicities of AZA are hematologic and gastrointestinal.

The TPMT gene is a 26 kb gene located on chromosome 6 (6p22.3). Allelic variants of this gene determine the level of TPMT activity in erythrocytes. TPMT activity in erythrocytes can be classified into high, intermediate, and low or no activity. Population studies have shown that approximately 90 % of the population has high activity, 10 % has intermediate activity, and 0.3 % has little to no activity [68]. Standard doses of AZA when given to patients

with low TPMT activity can lead to severe hematologic toxicity which may be fatal. 80–95 % of patients with low TPMT activity usually possess one of the three common allelic variants of the TPMT gene, TPMT*2, TPMT*3A, or TPMT*3C [69–71]. The frequencies of these allelic variants vary in different populations worldwide; thus, ethnicity influences the occurrence of these variants [72, 73]. Patients with low TPMT activity require lower AZA doses to avoid toxicities [74].

Sixty-eight patients with rheumatic disease on AZA (2–3 mg/kg/day), were genotyped for TPMT*2 and TPMT*3A alleles. All patients were assessed for side effects from AZA, such as leucopenia, liver function abnormalities, and gastrointestinal intolerance. Six (9 %) patients were heterozygous for TPMT*3A, of whom five discontinued AZA within 4 weeks of starting the medication due to hematologic toxicity [75]. In another study 40 RA patients on AZA (0.7–1.4 mg/kg/day) were genotyped for the TPMT alleles. Six out of forty patients discontinued AZA due to toxicity. Three of the six patients with severe gastrointestinal toxicity were heterozygous for the TPMT*3A allele while the remainder possessed the wild type TPMT allele. The association between the TPMT allele and AZA toxicity was significant ($p = 0.018$). Based on the results of this study, the positive predictive value for toxicity in a TPMT polymorphism carrier was 60 % [76].

Boonsrirat et al. reported the case of a SLE patient, who presented with pancytopenia, sepsis, typhlitis, and disseminated intravascular coagulopathy after a short period of AZA therapy. The patient had low TPMT activity due to the TPMT*3C genotype [77]. A recent meta-analysis of 67 studies examined whether patients with intermediate TPMT activity were at increased risk of myelosuppression when taking thiopurine medications. This meta-analysis included all primary studies of patients on a thiopurine medication that included either genotypic or phenotypic testing for TPMT activity, and reported cases of hematological adverse reactions. The search was not limited to a specific disease or condition. Patients with two *TPMT* variant alleles who were TPMT deficient had a substantial increase in their risk of myelotoxicity (86 % of deficient patients developed myelosuppression). Patients heterozygous (i.e., with one variant allele) for any of the *TPMT* variant alleles that led to intermediate TPMT activity, were also at high risk for drug-induced myelosuppression compared to those with wild-type alleles (OR 4.19, 95 % CI 3.20–5.48) [78].

In a prospective study of patients with Crohn's disease on AZA, dropouts during the first 2 weeks of AZA therapy due to adverse events (AEs) were significantly more frequent in carriers of the *ITPA 94C>A* allele [79]. In a more recent study of patients with inflammatory bowel disease, 40 of 160 patients on AZA were found to have decreased ITPA activity [below the lower limit of the reference range <122 μmol/(gHb h)]. ITPA activity was

measured in erythrocyte lysates by a high-performance liquid chromatography procedure based on conversion of ITP to IMP. ITPA activity was expressed as micromoles of IMP formed per gram of Hb per hour [μmol/(gHb h)]. Patients with decreased ITPA activity were separated into quartiles, resulting in three additional thresholds [37.3, 59.9, and 89.2 μmol/(gHb h)]. AEs were reported for 88 of the 160 patients (55 %). Patients with ITPA activity <89.2 μmol/(gHb h) developed leukopenia more often than did patients with higher enzyme activity (OR 3.27, 95 % CI: 1.21–8.82), whereas individuals with very low ITPA activity [<37.3 μmol/(gHb h)] demonstrated a higher incidence of elevated liver enzymes compared with those with ITPA activity >37.3 μmol/(gHb h) (OR 5.0, 95 % CI 1.35–18.57). The only significant association by genotyping was seen for the ITPA *94C>A* polymorphism and leukopenia [80].

In contrast, an earlier study failed to demonstrate a significant association between the presence of ITPA alleles and AZA related toxicity. In this study by Gearry et al., genotyping was performed for the 94C>A missense mutation in ITPA, TPMT*2, and TPMT*3 in 73 patients with inflammatory bowel disease who had side effects from AZA, and 74 patients who had tolerated AZA without adverse events. There was no significant difference in the frequency of the ITPA allele between patients who had experienced an adverse event versus those who had not (16/146 versus 16/148, $p = 0.56$). There was no association of the ITPA 94C>A polymorphism with adverse events such as rash or pancreatitis or flu-like symptoms [81].

The conversion of AZA to 6-MP was considered to be a non-enzymatic reaction until recently. Studies in the last few years have shown that glutathione S transferases (GSTs) may be involved in this conversion [82, 83]. A recent study demonstrated that patients with inflammatory bowel disease who carried the GST-M1 null genotype required a lower AZA dose to generate 6-TGN compared to those with the GST-M1 wild-type genotype who required higher doses of AZA. Other GST genotypes did not show a significant effect on AZA metabolism [84].

Other studies have examined the association between the activity of TPMT and other enzymes in the purine pathway and AZA toxicity. In one study, TPMT, HPRT, 5′-nucleotidase, and purine nucleoside phosphorylase activity in the red blood cells (RBC) of 33 RA patients on AZA (dose of approximately 2 mg/kg/day) and 66 controls was measured. Fourteen RA patients with low ($p = 0.004$) and seven patients with intermediate TPMT activity (RR 3.1) developed AZA toxicity when compared to patients with normal TPMT activity [66]. Another study measured TPMT activity in 3 RA patients who had experienced AZA-induced hematologic toxicity and 16 RA patients without AZA toxicity. Two patients with AZA-induced hematologic toxicity were TPMT

deficient, one partial and the other complete [85]. The patients were not genotyped in either of these studies.

Thus, both TPMT genotyping and measurement of TPMT activity in RBC have been studied in predicting and preventing AZA toxicity. Clearly, large, prospective studies are needed to validate the observations from the smaller studies described above (Table 2). There is some evidence for *ITPA* variants influencing AZA toxicity in inflammatory bowel disease, but there are no studies to date on this polymorphism in RA. Of note, TPMT genotyping is available to clinicians to screen patients prior to initiation of AZA and is the first commercially available assay for pharmacogenetic testing in rheumatology.

2.3 Pharmacogenetics of Sulfasalazine

SSZ is another DMARD often used in the treatment of RA. It is estimated that 20–30 % of RA patients on SSZ report adverse drug reactions. Adverse drug events of SSZ are gastrointestinal and hematologic.

SSZ is a combination of sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA). After ingestion, colonic bacteria split SSZ into these two compounds. 5-ASA remains in the large bowel while most of sulfapyridine is completely absorbed and undergoes acetylation, hydroxylation, and glucuronidation in the liver. Acetylation of sulfapyridine is carried out by the enzyme *N*-acetyltransferase 2 (NAT2) which acetylates sulfapyridine into *N*-acetylsulfapyridine. The NAT2 gene is a 9 kb gene located on chromosome 8 (8p22) and can be polymorphic. NAT2 gene polymorphisms may alter the acetylator phenotype of an individual (slow versus fast acetylator) and thus have effects on an individual's susceptibility to SSZ toxicity. Slow acetylators have been shown to be more prone to SSZ toxicity such as abdominal discomfort, nausea, rash, and headaches compared to fast acetylators [86, 87].

Two studies evaluated the effects of NAT2 polymorphisms on SSZ toxicity in RA patients. One retrospective study assessed 144 RA patients on SSZ at a dose range of 500–1,500 mg/day. NAT2 genotyping was carried out in all patients. Slow acetylators lacking the wild type NAT2*4 allele experienced adverse reactions more frequently (63 %) compared to fast acetylators with at least one NAT2*4 allele (8 %). This association between the NAT2 genotype and SSZ toxicity was clinically significant (OR 7.73, CI 3.54–16.86, $p < 0.001$). In fact, 25 % of the slow acetylators had to be hospitalized for their toxicities [59]. In the second study, 114 patients with inflammatory bowel or joint disease treated with SSZ (mean dose of 2 g/day) were studied. Patients were genotyped for five allelic variants, NAT2*5A, NAT2*5B, NAT2*5C, NAT2*6, and NAT2*7 (encoding slow acetylator status) and the wild type NAT2*4 allele (encoding rapid acetylator status). Twenty-seven of thirty-nine patients (69 %) who developed agranulocytosis within 3 months of starting treatment with SSZ were slow acetylators

Table 2
Pharmacogenetics of AZA in RA

Polymorphism	Amino acid change in enzyme	Population prevalence of polymorphism (%)	Effect of polymorphism on enzyme activity	Biochemical effect of polymorphism	Clinical effects	References
TPMT*2 238G>C	Alanine to proline	0.2–0.5	"Low to intermediate" due to enhanced degradation of enzyme	Decreased methylation of AZA to inactive compounds	Hematologic and GI toxicity	[75]
TPMT*3A 460G>A 71A>9G	Alanine to threonine and tyrosine to cystine respectively	3.2–5.7	As above	As above	Hematologic toxicity	[75, 76]
TPMT*3C 719A>G	Tyrosine to cystine	0.2–0.8	As above	As above	Hematologic toxicity	[77]

Table 3
Pharmacogenetics of SSZ in RA

Polymorphism	Effect of polymorphism	Biochemical changes associated with polymorphism	Clinical effects	References
NAT2*5A	Decreased activity of NAT2 enzyme leading to slow acetylation (slow acetylator)	Increased concentrations of SSZ intermediates due to slow acetylation	Agranulocytosis Fever, rash	[59] [88]
NAT2*5B	As above	As above	As above	As above
NAT2*5C	As above	As above	As above	As above [89]
NAT2*6	As above	As above	As above	As above
NAT2*7	As above	As above	As above	As above

compared to 34 of 75 patients (45 %) who received SSZ without a hematological adverse event (OR 2.7; $p=0.002$). Patients with SSZ-induced agranulocytosis had higher frequencies of the NAT2*6 alleles among other allelic variants (36 %) compared to patients without agranulocytosis (23 %) ($p=0.033$) [88].

In a more recent study, the authors performed two bioavailability studies under comparable conditions with 24 healthy subjects of both genders equally distributed. Plasma levels of sulfapyridine and acetylsulfapyridine were determined after oral intake of enteric coated formulations of SSZ (500 mg and 1,000 mg, respectively). NAT2 genotype was analyzed in parallel for all subjects. Compared to those with the rapid acetylator genotype, the apparent terminal elimination half-life of sulfapyridine as well as of acetylsulfapyridine was prolonged in those with the slow acetylator genotype. The strongest functional effect on enzyme activity was noticed in slow acetylators carrying the 341T>C polymorphism in NAT2 [89].

Thus, the acetylator status of a patient as determined by the NAT2 genotype appears to be an important determinant of the risk for SSZ toxicity based on the limited data published so far (Table 3). While more studies and data are clearly needed, this suggests that prospective screening of patients for the NAT2 genotype prior to initiation of SSZ may be a useful tool to prevent SSZ toxicity.

2.4 Pharmacogenetics of Biologics

2.4.1 Tumor Necrosis Factor Antagonists

The tumor necrosis factor (TNF-α) antagonists, a class of biological DMARDs have dramatically altered the treatment of RA in recent years. These agents not only ameliorate the signs and symptoms of RA but more importantly are highly effective in slowing the radiographic progression of disease [6, 90]. In spite of their

well-established efficacy, about 20–40 % of RA patients do not respond adequately to these agents [91, 92].

Five TNF antagonists are currently approved for the treatment of RA, etanercept (ETN), infliximab (INF), adalimumab (ADA), golimumab (GOL), and certolizumab pegol (CZP). ETN, a fusion protein of two identical chains of the recombinant human TNF receptor, p75, fused with the Fc portion of human IgG1, binds to soluble TNFα in vivo. INF, ADA, and GOL are all monoclonal antibodies to TNF-α; INF is chimeric while ADA and GOL are fully humanized. CZP is a human anti-TNF Fab fragment that is conjugated with polyethylene glycol.

Over the past few years several studies have attempted to define pharmacogenetic markers to predict response to anti-TNF therapy (Table 4). Some studies have used a candidate gene approach and have looked at genes in the TNF pathway, including genes for TNF-α, TNF receptors, and polymorphisms affecting signaling pathways downstream of the TNF receptors. Fc receptor polymorphisms and risk variants for RA have also been recently studied in this respect. Other studies have identified predictive variants from genome-wide association studies (GWAS) in large cohorts of patients treated with anti-TNF therapy.

TNF Gene Polymorphisms

The TNF family consisting of TNF-α and lymphotoxins A (LTA) and B (LTB) has vital functions in immune regulation. The TNF-α gene is located on chromosome 6 and lies within the human MHC III region (Fig. 3). The TNF locus is a 7 kb region where the TNF, LTA, and LTB genes are arranged in tandem and lie in close proximity to the HLA B and MHC III DR regions.

The most studied of TNF polymorphisms is the TNF–308 A>G SNP which is in the promoter region. The TNF–308A allele is associated with increased transcription and synthesis of TNF-α as compared to the TNF–308G allele. In one study of 59 RA patients treated with INF, patients without the A allele had improved Disease Activity Scores (DAS), a standardized measure of disease activity in RA, with use of INF (81 %) compared to patients with the A allele (42 %) ($p = 0.0009$) [93]. Cuchacovich et al. proposed an interesting explanation for the findings of the above study based on the results of their own study. In the study by Cuchacovich et al., 132 patients with RA were genotyped for the TNF −308 promoter polymorphism. From these 132 patients, ten patients with the TNF −308G/A and ten with the TNF −308G/G polymorphism were selected and received INF. Although both groups showed a similar response and demonstrated an increase in TNF-α levels after INF treatment, the increase in TNF-α levels correlated with the ACR50 response only in patients with the G/A polymorphism ($p < 0.03$). The authors postulated that the TNF −308 polymorphism influences response to INF by its effects on circulating TNF-α levels [94].

Table 4
Pharmacogenetics of TNF antagonists in RA

Genes/polymorphisms	Postulated effect of gene/ polymorphism	Clinical effects	References
TNF promoter −308A>G	May increase transcription of TNF-α gene May increase circulating TNF-α levels	Increased response to INF No effect on response to INF	[93, 97] [95, 96]
TNF promoter −238G>A	May increase transcription of TNF-α gene	No effect on response to ETN	[98]
TNF +489G/G	Intronic polymorphism—function unknown	No effect on response to ETN	[99]
TNFRSF1A −609, −580, −383	May affect ligand binding	No effect on response to ETN	[104]
TNFRSF1B 196T>G	May affect receptor shedding and ligand binding; may increase synthesis of IL-6	Increased response to INF, ETN No effect on response to ETN	[99] [104]
TNFRSF1B 676T>G	May affect membrane receptor shedding	Increased response to anti TNF	[102]
TNF microsatellites a, b, c, d and e	May influence production of TNF by PBMC, linked to TNF −308 SNP, increased susceptibility to RA	Specific TNFa/b haplotype associated with response to INF No effect on response to ETN	[108] [104]
HLA DR, DQ alleles	May affect response to TNF blockade and increase susceptibility to and severity of RA because of close proximity to TNF locus	No effect on response to INF Specific individual HLA DRB1 alleles and haplotypes markers of increased response to ETN	[108] [104]
MHC class I chain-related gene A transmembrane polymorphism	As above	No effect on response to INF	[108]
HLA microsatellites BAT2, D6S273, D6S2223	Haplotype may carry "response gene"	BAT2-D6S273 haplotype associated with increased response to INF	[108]
FcγRIIIA 158FF	Low affinity for IgG, affects antibody clearance	Increased response to anti-TNF therapy	[123–125]
FCGγIIA-131RR	Low affinity for IgG, affects antibody clearance	Increased response to IFN	[124]

(continued)

Table 4 (continued)

Genes/polymorphisms	Postulated effect of gene/polymorphism	Clinical effects	References
PTPRC rs10919563	RA susceptibility marker	Associated with good response to anti-TNF therapy	[110, 111]
Polymorphisms in MAP kinase pathways MAP3K1 rs96844, MAP3K14 rs4792847, MAP2K6 rs11656130	Affect signaling pathways in RA	Associated with good response to anti-TNF therapy	[113, 114]
AFF3 SNPs rs10865035 and rs1160542	RA susceptibility markers	Associated with good response to anti-TNF therapy	[117]
CD226 SNP rs763361	T and NK cell cytotoxicity	Associated with good response to anti-TNF therapy	[117]
IL10 promoter microsatellite polymorphism R3	Associated with IL10 secretion	Good response to ETN	[120]
–1087G>A in IL10 in combination with -308 in TNF	Associated with IL10 secretion	Good response to ETN	[119]
IL6 –174GG	Associated with IL6 levels	Good response to ETN	[121]

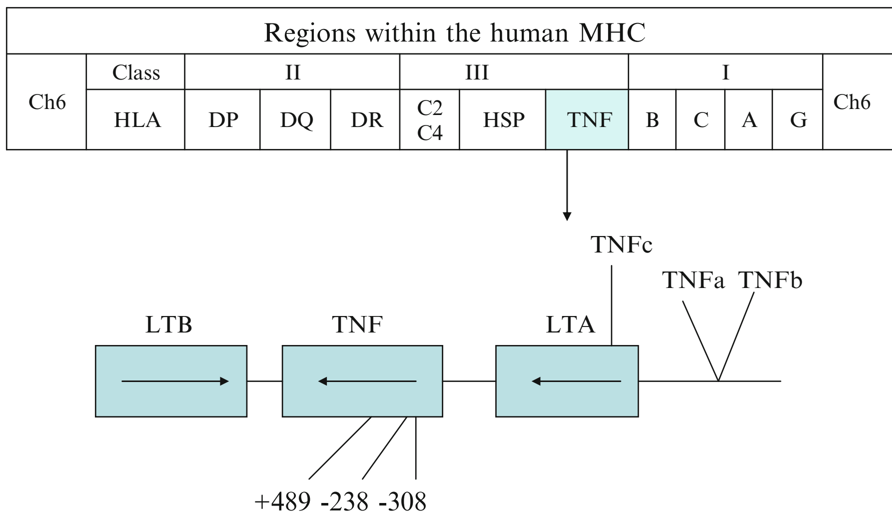

Fig. 3 TNF locus with some of the polymorphic sites known within the TNF locus. *C2, C4* complement C2, C4, *Ch* chromosome, *HLA* human leukocyte antigen, *HSP* heat shock protein, *LTA* lymphotoxin alpha, *LTB* lymphotoxin beta, *MHC* major histocompatibility complex, *TNF* tumor necrosis factor. Reproduced from [149] with permission from Future Medicine Ltd

Over the past decade, more than a dozen studies have looked at the effects of this polymorphism on anti-TNF therapy in RA. Three recent meta-analyses analyzed these studies but yielded mixed results. The meta-analyses by Lee et al. and Pavy et al. did not find a significant association between the TNF −308 polymorphism and response to anti-TNF therapy in RA. Lee et al. suggested that the initial positive studies were due to small sample sizes and therefore could not be replicated in larger cohorts [95, 96]. Zeng et al. in their meta-analysis of 15 studies with a total of 2,127 patients with RA did however report an improved response to anti-TNF therapy in patients with the −308G allele [97].

The −238G>A SNP is also in the promoter region and has possible effects on TNF-α production. Maxwell et al. found a poor response to IFN but not ETN with the GA genotype at TNF-238 rs361525 ($p = 0.028$, $n = 40$) [98]. Other TNF gene polymorphisms including the intronic SNP +489 and the promoter region SNP −857C>T are associated with severe RA, but no clear association has emerged between these SNPs and response to anti-TNF therapy [99].

TNF Receptor Polymorphisms

Polymorphisms in the TNF-α receptors also appear to be important in determining response to anti-TNF therapy. Soluble TNF-α binds to two transmembrane receptors; p55, also known as CD 120a or TNF receptor type 1 (TNFRSF1A) and p75, also known as CD 120b or TNF receptor type 2 (TNFRSF1B). Local production of soluble TNFRs and their upregulation is important in the modulation of TNF-α activity in RA joints. The TNFRSF1B gene is located on chromosome 1 and has ten exons and nine introns [100]. A SNP has been described in exon 6 of the TNFRSF1B gene, a single base substitution at codon 196 (T to G) that leads to a nonconservative amino acid substitution, methionine to arginine [101]. The TNFRSF1B 196T>G polymorphism was studied in 175 RA patients for its effects on response to anti-TNF therapy. Of the 175 patients, 66 were treated with either ETN or INF and their response to treatment assessed using the DAS. Of the 66 patients on TNF antagonist therapy, 38 had the TT, 22 had the TG, and 6 had the GG genotypes. Patients with severe RA carried the GG genotype more often (6.4 %) than those in the mild to moderate group (3.1 %). Patients carrying the TT genotype had a better response to therapy over 24 weeks compared to the patients with the TG or GG genotype as measured by the DAS with the greatest difference seen at 12 weeks (OR 5.1, CI 1.3–19.96, $p = 0.03$) [99]. Another polymorphism in the TNFRSF1B gene, 676T>G in exon 6 that results in an amino acid change from methionine to arginine has been described. The 676TT genotype is associated with a better response to anti-TNF therapy as compared to the 676TG genotype at 3 months (OR 3.78, 95 % CI 1.07–13.31) and 12 months (OR 4.30, 95 % CI 1.16–15.99) in RA [102].

Some studies could only verify this association in patients who were anti-CCP positive [102, 103], while a study of 457 RA patients by Criswell et al. could not confirm this association at all [104].

MHC Gene Polymorphisms, TNF, and MHC Microsatellites

The close proximity of the "TNF locus" to the HLA B and HLA DR genes (MHC genes) on chromosome 6 and the fact that there is a strong link between specific HLA DRB1 alleles (also called the shared epitope alleles) and susceptibility to RA and its severity [105], make it likely that MHC gene polymorphisms may influence response to anti-TNF agents. Some microsatellite haplotypes have been previously associated with susceptibility to RA or linked to TNF promoter region SNPs [106, 107]. In one study, 78 RA patients treated with INF were genotyped for HLA-DRB1, HLA-DQA1, HLA-DQB1, MHC class I chain related gene A (MICA) transmembrane polymorphism alleles, microsatellites TNFa–e, D6S273, HLA-B associated transcript 2 (BATS2), and D6S2223. None of the alleles influenced response to INF including the TNFa/b microsatellites (linked to the TNF −308 promoter polymorphism), implying that this TNF promoter variant may not be important in determining response to INF. However, there were some interesting associations observed between certain microsatellite haplotypes and response. Among the microsatellite haplotypes, the D6S273_4/BAT2_2 pair was a marker of the INF responder group, both among patients and when compared to controls (46 % versus 11 % in non-responders; $p = 0.001$; 46 % in responders versus 17 % in controls; $p = 0.00002$) indicating that this microsatellite pair may occur on the haplotype that carries the "response gene" or each microsatellite allele could serve as a marker of a "response gene" in proximity. The frequency of one of the TNFa/b haplotypes was increased in responders compared to non-responders (41 % versus 16 % in non-responders; $p = 0.01$). Thus, some microsatellite haplotypes were associated with response to INF in this study; single alleles did not reveal similar associations [108].

In a second study, patients were genotyped for specific HLA-DRB1 alleles, i.e., the shared epitope (SE) alleles and categorized as having 0, 1, or 2 copies of the SE. Four hundred and fifty-seven patients with early active RA (duration of ≤3 years) treated with MTX or ETN were genotyped and response to therapy measured by ACR50 response rates after a year of treatment. SNPs at positions −308, −238, and +488 of the TNF gene and +249, +365, and +720 of the LTA gene were also examined. (These six LTA-TNF SNPs mark haplotypes spanning the "TNF locus" region.) Five TNF microsatellites (TNF a through e), SNPs in TNFRSF1A at positions −609, −580, −383 and the 196T/G polymorphism in TNFRSF1B were also examined. As the Fc receptor (FcR) pathway appears important in the degradation of ETN–TNF complexes, three FcR polymorphisms were also examined. The number of HLA-DRB1 (SE) alleles correlated with response to treatment.

Patients with 2 copies of the SE alleles had a better response to ETN compared to those with 0 or 1 copy of the allele (OR 4.3, 95 % CI 1.8–10.3). Haplotypes defined by the 6 LTA—TNF SNPs and DRB1 alleles were deduced for the 16 most common DRB1 alleles in a subset of 224 Caucasian patients. Among 448 haplotypes thus examined, two haplotypes HLA-DRB1 *0101-GGGAGG and HLA-DRB1 *0404-GGAAGG strongly correlated with response (76 % and 61 % ACR50 response at 12 months, respectively). Thus, the number of copies of HLA-DRB1 SE alleles inherited and specific haplotypes spanning the HLA-DRB1 region and SNPs in the LTA–TNF region may be associated with response to ETN, at least in the Caucasian population [104].

RA Risk Variants

There are more than 30 risk alleles associated with susceptibility to RA [109]. Among the RA risk variants studied so far, protein tyrosine phosphatase receptor type C (PTPRC) variants have the strongest association with response to anti-TNF therapy. The PTPRC gene encodes a CD45 antigen and is a known RA susceptibility marker. In a large cohort of 1,283 patients with RA, those who had the rs10919563 SNP in the PTPRC gene, had a better EULAR response to TNF inhibitors as compared to patients without the polymorphism ($p = 0.0001$) [110]. Plant et al. reported similar findings in a large cohort of 1,115 RA patients from the UK who were tested for 29 SNPs known to be RA susceptibility variants. The rs10919563 SNP in the PTPRC gene was associated with improved response to anti-TNF therapy (OR 0.62, 95 % CI 0.40–0.95; $p = 0.03$) [111]. Despite the strength of the associations found in these studies, a subsequent study by Krintel et al. was unable to replicate these findings [112].

Mitogen activated protein kinases (MAPKs) are crucial to several signaling pathways in RA. Bowes et al. reported two SNPs rs96844 in MAP3K1 and rs4792847 in MAP3K14 to be nominally associated with response to anti-TNF therapy in a UK cohort of 642 RA patients ($p < 0.05$). However, these associations could not be confirmed in a validation cohort of 428 patients with RA in the same study [113]. In another large cohort of 1,102 RA patients, polymorphisms in 11 genes in the MAPK pathway were investigated. Seven polymorphisms in five genes in the MAPK signaling pathways were associated with an improved DAS28 response to IFN and ADA therapy, but not to ETN. One polymorphism rs11656130 in MAP2K6 was associated with a good EULAR response [114]. Tan et al. genotyped 1,012 RA patients and identified two more SNPs in susceptibility genes associated with response to anti-TNF therapy. The AFF3 gene which encodes nuclear transcription activators in lymphoid tissue has been identified as a RA susceptibility marker [115]. The G allele of two SNPs, rs10865035 and rs1160542 in AFF3 gene was associated with response to anti-TNF therapy [118]. CD226 is a membrane protein

on the surface of haematopoietic cells which is involved in T and NK cell cytotoxicity. The SNP rs763361 in the CD226 gene results in a glycine to serine substitution and is a RA risk variant [116]. This SNP was also associated with response to anti-TNF therapy [117].

Cytokines, Toll-Like Receptors, and Signaling Pathways

Cytokines play an important role in the pathogenesis of RA. Thus far, polymorphisms in interleukin (IL)1β, IL1 receptor antagonist (ILRA), IL6, IL10, and tumor growth factor (TGF) β have been studied in relation to anti-TNF therapy. While no polymorphisms in individual cytokine genes have been significantly associated with response [118], one study found that a SNP −1087G>A in the IL10 gene in combination with the −308 SNP in the TNF gene was associated with a good response to ETN. The same study reported that the combination of the A2 allele in intron 2 of the ILRA gene and a rare +915C allele in codon 25 of the TGFB1 gene correlated with a poor response to ETN therapy. The +915C allele which is a rare allele was found significantly more often in combination with the A2 allele in the ILRA gene in non-responders to ETN ($p < 0.05$) [119]. The IL10 promoter microsatellite polymorphism R3 allele and the R3-G9 haplotype were associated with a good response, whereas the G13 allele and the haplotype R2-G13 were associated with moderate to no response to ETN [120].

In a small study, 77 patients with RA were genotyped for the 174G>C polymorphism in the IL6 gene. The 174G>C polymorphism influences IL6 levels. After 12 months of ETN therapy more patients with the IL6 −174GG (95.7 %) genotype had an improvement in disease activity by DAS as compared to those with the GC (75.6 %) or CC (44.4 %) genotype ($p = 0.006$) [121].

Another study by Potter et al. looked at genotypes in the TLR (toll-like receptor) and NFkB (nuclear factor kappa B, a transcription factor) pathways to predict response to anti-TNF therapy in RA. A total of 187 SNPs in these pathways were studied in 909 patients with RA. Twelve SNPs in nine genes showed a nominal association with treatment response in patients on anti-TNF therapy [122].

Fcγ Receptor Variants

Most anti-TNF agents are antibodies and therefore have a Fc component to them (except CZB which is a pegylated Fab fragment). The Fc component of these antibodies attaches to the Fc gamma receptor (Fc**γ**R) on various cells. Polymorphisms in the Fc**γ**R gene affect the avidity and strength of Fc binding. Since binding of the Fc fragment to Fc**γ**R is a mechanism for antibody clearance, polymorphisms in the Fc**γ**R gene may potentially influence the efficacy of the anti-TNF drugs. Two polymorphisms, Fc**γ**RIIIA F158V and Fc**γ**RIIA R131H, have been studied in this respect. The Fc**γ**RIIIA 158FF genotype has a lower affinity for IgG1. In a small study of 30 patients with RA, the 158 FF variant was found in greater frequency amongst patients who had a very good

response to anti-TNF agents [123]. In a study by Canete et al. of 98 individuals with RA, those who were homozygous for the low affinity FCγRIIIA 158FF genotype had a better ACR50 (American College of Rheumatology 50, another standardized measure of disease activity in RA) and EULAR responses at week 6 of IFN therapy. Other individuals with the low affinity variant FCGγIIA 131RR also had better ACR20 responses at week 30 of INF treatment [124]. Similar results were seen in a study of 33 Japanese patients with RA [125]. However, this finding could not be replicated in a subsequent larger study of 282 Swedish RA patients treated with INF or ETN [126].

GWAS Studies

Several genome-wide association studies (GWAS) have been done to assess markers of response to anti-TNF therapy in RA. The first GWAS study in this regard by Liu et al. looked at a small sample of 89 patients and reported 16 SNPs in nine genes that were associated with response to anti-TNF therapy [127]. This finding was not confirmed by subsequent studies. Plant et al. looked at a larger cohort of 566 patients and found seven loci by multi stage GWAS that were associated with response to anti-TNF therapy. The strongest association was for SNP rs17301249, mapping to the eyes absent homolog 4 (EYA4) gene on chromosome 6. EYA4 is a co-transcription factor associated with expression of interferon β and CXCL10. Another SNP rs1532269 mapped to the PDZ domain-containing protein 2 (PDZD2) gene which is associated with insulin secretion. (*PDZ* is an acronym combining the first letters of three proteins—Postsynaptic density protein, Drosophila disk large tumor suppressor, and Zonula occludens-1 protein—which were first discovered to share a domain). Five SNPs mapped within intergenic loci on chromosomes 1, 4, 11, and 12 [128].

Krintel et al. studied 196 Danish patients with moderate to severe RA, treated with IFN, ETN, or ADA and analyzed 486,450 SNPs for association with response to anti-TNF therapy. The findings of the earlier GWAS by Lui et al. and Plant et al. could not be confirmed in this study, and no SNPs achieved significance despite the wide array studied [112]. Another multistage, GWAS of 882 patients with RA from the Dutch Rheumatoid Arthritis Monitoring (DREAM) registry evaluated 2,557,253 SNPs for response to anti-TNF therapy. Although no single SNP reached significance, three SNPs (rs1568885, rs1813443, and rs4411591) showed directional consistency and eight genetic loci were suggestive of association with response in this cohort. However, none of the associations found in earlier studies could be confirmed in this study as well [129].

Cui et al. recently reported a GWAS meta-analysis, looking at two million common variants in 2,706 RA patients from 13 different cohorts. The SNP (rs6427528) was associated with higher CD84 gene expression in peripheral blood mononuclear cells and

a better response by DAS scores in patients treated with ETN. CD84 is a cell surface receptor found on immune cells including T cells, B cells, monocytes, and platelets. It is thought to play a role in signaling T cell activation and IFNγ secretion [130, 131]. The variant rs6427528 was not associated with response to therapy with either INF or ADA [132]. Acosta-Colman et al. recently described a SNP, rs3794271 in the fourth intron of the *SLCO1C1* gene in their cohort of 315 patients which was associated with a good EULAR response to anti-TNF therapy. SLCO1C1 is a cell membrane transporter important in drug metabolism. The GWAS study by Krintel et al. described above had reported an intergenic SNP rs11045392, located between the 5′ end of *SLCO1C1* and 3′ end of the *PDE3A* gene (which codes for a phosphodiesterase) as a putative marker of response to anti-TNF therapy. The authors speculated these two SNPs, rs3794271 and rs11045392, may be in linkage disequilibrium, and therefore the PDE3A-SLCO1C1 locus may be an important determinant of response to anti-TNF therapy [133].

2.4.2 Rituximab

Rituximab is an antiCD20 chimeric antibody with proven efficacy in RA. Several candidate gene studies have looked at pharmacogenetic associations affecting response to rituximab therapy. In a small study by Daien et al. [134], 63 patients were analyzed for 13 SNPs in nine genes including IL10, LTA, TGFβ1, TNF-α, TNFRSF1B, TRAF1-C5, STAT4, TNFAIP3, and PTPN22. The following SNPs, PTPN22 rs2476601, STAT4 rs7574865, TRAF1-C5 rs1081848, and TNFAIP3 rs6920220, have been associated with RA susceptibility in prior studies [135, 136].

Two SNPs in TGFβ1, rs1800470 in codon 10 and rs1800471 in codon 25 were associated with a good response to rituximab. At codon 10, the CT genotype was more prevalent in responders as compared to the TT genotype (OR 1.6, 95 % CI 1.2–2.3; $p = 0.002$), the CC genotype was equally present in responders and non-responders. At TGFβ1 codon 25, all patients with the GC genotype were responders, while 63 % of patients with the GG genotype were responders (OR 1.6, 95 % CI 1.3–1.9; $p = 0.025$). Patients with both SNPs had an even better response to rituximab (OR 2.6, $p = 0.008$) [134]. SNPs in codon 10 and 25 have been associated with lower TGFβ1 production which may explain the better response to rituximab therapy [137].

B lymphocyte stimulating factor (BLyS) is a B lymphocyte survival factor. BlyS levels increase after treatment with rituximab. Polymorphisms in the BlyS gene promoter may affect serum BlyS levels and B cell repopulation after rituximab therapy. The polymorphism –871C>T in the BlyS promoter has been studied in this respect. In a study of 115 patients with RA, the 871CC genotype was associated with a better EULAR response to rituximab than the TT genotype (OR 6.9, 95 % CI 1.6–29.6; $p = 0.03$) [138].

This association of the –871C>T SNP with rituximab response could not be confirmed in another study of 152 Italian patients which examined four polymorphisms –2841T>C, –2704T>C, –2701T>A, and –871C>T in the BLySS promoter that are in linkage disequilibrium. The BLyS haplotype TTTT was associated with a good response to rituximab (OR 14.4, 95 % CI 1.77–117.39; $p=0.003$) only in patients who were seropositive and had a prior poor response to anti-TNF agents. This finding was replicated in an additional 115 patients in the same study. However, no association was found with the –871C>T SNP [139].

Some studies looking at the F158V polymorphism in the FCGRIIIA gene (rs396991) found the V variant to be significantly associated with a good response to rituximab therapy. In one study with 111 patients, V allele carriage was associated with a higher response rate (91 % of responders versus 70 % of non-responders) (OR 4.6, 95 % CI 1.5–13.6; $p=0.006$) [140]. Similar results were seen in 212 RA patients where 89.5 % of patients with the VV genotype had a good EULAR response at 6 months versus 66 % with the VF genotype and 66.2 % with the FF genotype ($p=0.01$) [141]. Kastbom et al. however reported that heterozygotes (158 V/F) had a better response than homozygotes (158 VV or 158FF) [142]. Sarsour et al. did not find a difference in response to either rituximab or TNF inhibitors in patients with FCGRIIIA polymorphisms [143].

IL6 is another B cell survival factor. Fabris et al. studied the –174G>C polymorphism in IL-6 and the D358A polymorphism in IL-6-receptor alpha (IL-6Ra) genes; these polymorphisms are associated with expression of IL6 and IL6Ra. The IL-6 –174CC genotype (rs1800795) was associated with a poor response to rituximab by EULAR criteria (39.1 %) as compared to the GC/CC genotypes (18.5 %) (OR 2.83, 95 % CI 1.10–7.27; $p=0.031$). No association was found between the D358A polymorphism in IL-6-Ra and response [144].

2.4.3 Tocilizumab

Tocilizumab is a humanized monoclonal IL6 receptor antagonist. Recently, Wang et al. reported the first GWAS demonstrating genetic variants associated with response to tocilizimab. This study pooled data from six studies with a total of 1,683 patients. Two hundred and fifty-three variants showed an association with tocilizumab therapy, of which four SNPs, rs11886534, rs850246, rs13302591, and rs12110787 reached genome-wide significance. Seven of these two hundred and fifty-three variants (rs11052877, rs4910008, rs9594987, rs10108210, rs703297, rs703505, and rs1560011) achieved significance on conformational analysis. Of these, rs11052877 is located in the 3′-untranslated region of CD69, and rs1560011 is an intronic SNP in CLEC2D which blocks osteoclast function [145].

3 Conclusions and Future Directions

Thus, there is a growing body of literature on the pharmacogenetics of therapies used in RA. Clearly, inherited differences in drug-metabolizing enzymes, drug receptors, and drug targets are important in determining an individual's response to a given drug. Nonetheless, several caveats need to be considered before pharmacogenetics can be brought in to the clinic.

In several of the studies reviewed above, the strength of the association between genotype and phenotype can be called in to question for several different reasons. Whether many of these studies were adequately powered is questionable—most of the studies described had small sample sizes and associations observed in one study were not necessarily reproducible in another. Some of them were retrospective and may have over or under estimated drug effects, particularly adverse effects. Moreover, ethnicity may have strong influences on pharmacogenetic associations and the populations examined in most of the above studies were ethnically homogeneous. Our study examining the frequencies of SNPs in the MTX pathway in different racial groups demonstrated significant differences in the allele frequencies of several SNPs between Caucasians and African-Americans with RA [146]. Hence, genotype–phenotype associations may differ significantly in ethnically diverse populations. For example, in the study by Criswell et al. although certain MHC/TNF haplotypes were predictive of response to ETN in a Caucasian population, whether these results will apply to other populations remains unknown [104].

As many of the drugs in RA (such as MTX, TNF antagonists) work through several different cellular (and genetic) pathways, examination of SNPs in different metabolic pathways rather than a single pathway may be more predictive of response [21]. It is also worth noting that if a variant is only weakly associated with response, this may be due to the fact that this variant may occur in tandem or close proximity to the gene which is the actual marker of response. For reasons described above, haplotype analyses may be more useful than single SNP analyses in predicting response [104, 108]. Genome-wide association studies in pharmacogenetics are also emerging, as described above, with some promising results. Finally, the cost-effectiveness of pharmacogenetic testing is an important issue to consider before pharmacogenetics can be incorporated in to daily clinical practice [147]. Drugs with a narrow therapeutic index, severe side effects, a well-established association between a specific genotype and phenotype (usually toxicity), and for which the frequency of the genetic variant of interest is high are the ideal candidates for pharmacogenetic testing.

Notwithstanding these caveats, as genotyping becomes more readily available and less expensive, and major funding agencies display an increasing commitment to pharmacogenetic research

(International HapMap Consortium (www.hapmap.org) and the Pharmacogenetics Research Network (http://www.nigms.nih.gov/pharmacogenetics/) by the National Institutes of Health), it is quite likely that genotype-guided therapy of patients with RA will happen in the not too distant future.

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Chapter 17

Pharmacogenomics of Osteoporotic Fractures

José A. Riancho and Flor M. Pérez-Campo

Abstract

Osteoporosis is a prevalent disease that typically reduces bone strength and predisposes to fractures. It is a multifactorial disorder resulting from the interaction of genetic and acquired factors. Candidate gene studies and, more recently, genome-wide studies have identified a number of polymorphisms significantly associated with bone mass and fractures. Anti-resorptive drugs, which inhibit the differentiation and activity of osteoclasts, are frequently used to treat patients with osteoporosis.

Several candidate gene studies have explored the association of genetic factors with drug response, including some common polymorphisms of the gene encoding FDPS (Farnesyl diphosphate synthase), an enzyme that is the main target of aminobisphosphonates. Although scarce data are available, interesting opportunities are open for a better understanding of the pharmacogenetics of osteoporosis and osteoporotic fractures. They include the reanalysis of data already available from epidemiological studies and clinical trials, as well as obtaining pharmacogenetic data in new studies. However, based upon the experience with previous genome-wide association studies, large collaborative efforts would be likely needed to obtain meaningful results.

Key words Osteoporosis, Bone mineral density, Bisphosphonates, Wnt, Genome-wide association studies

1 Osteoporosis and Osteoporotic Fractures

Fractures are the consequence of the action of physical forces that exceeds the strength of the bones on which they are applied. They may result from intense high-energy impacts whose intensity goes beyond those the skeleton is designed to resist (i.e., falls from a height), or from relatively small loads (including those applied to the skeleton during daily activities), or from minor trauma, (such as falling from the standing position) that act on a debilitated skeleton.

Bone strength depends on a number of factors [1, 2]:

- *Bone mass.* Bone mass depends on bone volume and bone density, that is, the amount of bone tissue inside the bone volume (most bones have an outer layer of compact tissue and

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a core of "spongy" or trabecular bone). Bone density is usually measured in clinical practice as "bone mineral density" (BMD) by using double energy X-ray densitometry (DXA).

- *Bone geometry.* The ability of a bone with a given mass to stand a physical stress depends on the spatial distribution of the bone mass. This is a universal phenomenon that applies to any material (biological or inert), and it is well recognized in the engineering and construction fields. In general, for a tubular structure (like a long bone), the farther the mass is distributed from the center, the higher its ability to resist mechanical loads [3–5].
- *Bone quality.* The intrinsic characteristics (quality) of a material determine its mechanical properties. Factors determining the mechanical quality of bone tissue are not completely known, but they are likely to include some properties of the bone matrix (such as the cross-linking of collagen fibers, which is the major organic component of the matrix, or the degree and homogeneity of mineralization), as well as the spatial distribution and connectivity of the trabeculae of spongy bone (often referred to as the "microarchitecture") and the porosity and thickness of the cortical bone [1]. Nevertheless, these factors are rarely assessed in clinical practice.

Bone tissue is constantly being renewed by the concerted action of osteoblasts and osteoclasts. Bone remodeling has two main phases: a resorption phase consisting in the removal of old bone by osteoclasts, and a later phase of new bone formation driven by osteoblasts [6]. Thus, the activity of osteoblasts and osteoclasts determines bone mass, bone geometry, bone quality, and, subsequently, bone strength [7, 8]. Osteoporosis is a prevalent disorder consisting in decreased bone mass and/or abnormal bone microarchitecture that impairs bone strength and increases the risk of fracture. Therefore, patients with osteoporosis may suffer fractures as a result of minor trauma, or even in the absence of trauma. The most common osteoporotic fractures are those of the vertebral bodies, the hip, the wrist, the shoulder, and the pelvis.

Osteoporosis is a multifactorial disorder, resulting from a complex interplay of genetic and acquired factors. Although it has been reported that the heritability of BMD is about 60–80 % [9], the predisposition to fracture does not only depend on BMD but also on other skeletal characteristics, as explained above. It also depends on nonskeletal factors, such as the tendency to fall, and the defense response when falling. In turn, the tendency to fall depends on muscle strength, balance and other factors (risk behavior, visual acuity, environmental factors, etc.). Therefore, not unexpectedly, most studies have reported lower heritability for fractures (usually between 24 and 48 %) than for BMD [10, 11].

2 Genome-Wide Studies: Expectations and Realities

After the seminal study by Morrison et al. [12], many investigators explored the possible relationship between polymorphisms in biologically plausible candidate genes and osteoporosis. However, most studies were underpowered, and the results reported by one group were infrequently confirmed by other investigators, thus frustrating prior expectations.

More recently, when microarray technology has become available, several groups managed to screen this association between genetic polymorphisms and osteoporosis with a hypothesis-free, genome-wide approach. Interestingly, as is the case with other complex diseases, researches collaborated to share and combine the data from individual studies to reach higher statistical power and replicate the results. The GEFOS/GENOMOS consortium is a good example of such efforts. The investigators were able to get DNA data from 133,000 individuals and they found 56 single nucleotide polymorphisms (SNPs) significantly associated with BMD and 6 SNPs associated with osteoporotic fractures, with p-values $<5 \times 10^{-8}$. This is the commonly used threshold for statistical significance in genome-wide association studies (GWAS) [13]. The study included a first meta-analysis stage of meta-analysis of 17 GWAS and a second stage of replication of the suggestive SNPs in several independent cohorts. Therefore, the association of those SNPs with these phenotypes is highly reliable. They were found in several genes related to pathways that are known to regulate the differentiation and activity of bone cells, such as Wnt, estrogen, and RANKL pathways, as well as in several novel genes which had not been previously linked to osteoporosis.

The genome-wide coverage and the hypothesis-free approach enable GWAS to identify new genes associated to a specific disease, thus improving the understanding of the pathophysiological mechanisms involved and eventually helping to identify new therapeutic targets.

The research community also had high expectations about how the use of GWAS will help building genetic risk scores useful for predicting the risk of disease. However, those expectations were not fulfilled. Most SNPs identified in GWAS have a very small influence on disease (typical risk ratios of 1.05–1.10), and even the scores resulting from their combination usually explain less than 10 % of the genetic risk of disease. A number of reasons have been suggested to explain these somewhat disappointing results [14, 15].

- GWAS are based upon the "common variation—common disease" hypothesis and can only pinpoint allelic variations which are relatively common in the population (i.e., those with minor allelic frequencies >5 %). However, there may be rare alleles having a larger effect on the phenotype.

- Due to the statistical and computational limitations of the analysis of interactions, most studies have explored the relationship between single SNPs and a given phenotype. However, the interactions between several SNPs and between the polymorphisms and environmental factors are likely important in determining the risk for disease.
- Large consortia allow the combination of data from different studies into a large database. These studies may sometimes include individuals with different genetic backgrounds or disease characteristics in which the role of genetic polymorphisms may be heterogeneous. This fact could clearly impair the power to detect significant associations between genotype and phenotype [16].

3 Pharmacogenetics of Osteoporosis

The influence of genetic polymorphisms on the response to anti-osteoporosis therapy has been explored in a number of candidate gene studies (see recent reviews [17–20]), but the results have rarely been replicated.

Drugs currently used to treat osteoporosis are classified into those that inhibit osteoclastic bone resorption (including bisphosphonates, denosumab, and selective estrogen receptor modulators) and those that stimulate bone formation by osteoblasts (parathyroid hormone and derivatives, such as teriparatide). Strontium ranelate may have a dual effect. Other drugs being tested in clinical trials include inhibitors of cathepsin K (an osteoclastic enzyme critical for bone resorption) and of sclerostin (a negative modulator of the Wnt pathway) [21].

Farnesyl diphosphate synthase (FDPS), an enzyme in the mevalonate pathway, is the main target of the widely used amino-bisphosphonates (alendronate, risedronate, zoledronic acid) [22]. Interestingly, several research groups have explored the role of SNPs of the FDPS gene in determining the response to these drugs. They indeed found that a common polymorphism of FDPS is associated with changes in BMD and bone turnover markers following therapy with these drugs in European postmenopausal women [23, 24], but not in Korean women [25] (Table 1). The sample size was relatively small and the results need to be confirmed in larger studies, but the genetic influence on the therapeutic response suggested by those studies might be relevant from a clinical point of view.

Although anti-osteoporotic drugs have a good safety profile, some patients can suffer cumbersome adverse effects, such as osteonecrosis of the jaw (ONJ). The pathogenesis of ONJ is unclear, but ischemia and genetic predisposition have been suggested to play a role in the development of this condition. Some SNPs of genes

Table 1
Pharmacogenetic studies of bisphosphonates

Study	Drug	Gene	Population	Result
Marc [43]	Etidronate	VDR	Postmenopausal women, Caucasian	Association with BMD increase
Qureshi [44]	Etidronate	COL1A1	Perimenopausal women, Caucasian	Association with BMD increase
Palomba [45]	Alendronate alone or with hormone replacement therapy	VDR	Postmenopausal women, Caucasian	Association with BMD increase
Wang [46]	Aminobisphosphonates	OPG	Postmenopausal women, Asian	Association with BMD increase
Arko [47]	Aminobisphosphonates	ER2	Postmenopausal women, Caucasian	NO association with BMD change
Otrock [48]	Aminobisphosphonates	VDR	Men with thalasemia, Caucasian	NO association with BMD change
Kruk [49]	Aminobisphosphonates	LRP5	Men, Caucasian	NO association with changes in BMD or bone turnover markers
Marini [24]	Aminobisphosphonates	FDPS	Postmenopausal women, Caucasian	Association with BMD increase
Olmos [23]	Aminobisphosphonates	FDPS	Postmenopausal women, Caucasian	Association with BMD increase
Choi [25]	Aminobisphosphonates	FDPS GGPS1	Postmenopausal women, Asian	Association of GGPS1 (but not FDPS) with BMD change

VDR vitamin D receptor, *COL1A1* collagen 1 alpha chain, *OPG* osteoprotegerin, *ER2* estrogen receptor β, *LRP5* lipoprotein receptor-related protein 5, *FDPs* farnesyl diphosphate synthase, *GGPS1* geranylgeranyl diphosphate synthase 1

encoding vascular endothelial growth factor [26], peroxisome proliferator-activated gamma (involved in the differentiation of the common pluripotential precursors towards adipocytes or osteoblasts), or several osteoblast-derived structural or regulatory proteins, such as collagen, osteopontin, metalloproteinases, osteoprotegerin, and RANKL [27, 28], have been associated with ONJ in some studies, but these results have not been replicated yet. A SNP in the CYP2C gene was significantly associated with ONJ in a small GWAS, with an odds ratio of 12.7 [29], but the results were not replicated in another independent study [30]. On the other hand, Marini et al. studied a group of 68 patients treated with zoledronic acid and found an association between ONJ and the

rs2297480 polymorphism of FDPS [31]. The risk of ONJ was higher in individuals with the AA genotype, the genotype also associated with a larger increase in BMD in postmenopausal women treated with oral aminobisphosphonates [23]. This could be consistent with the notion that the genotype is associated with higher sensitivity to bisphosphonates.

4 Pharmacogenetic and Pharmacoepigenetic Studies as Tools for Personalized Medicine and Finding New Drug Targets

The hypothesis-free GWAS approach has rarely been used with a pharmacogenomic objective. However, in theory, it offers promising possibilities for advancing both the pathophysiological and therapeutical knowledge. From a pharmacogenetic point of view, GWAS may help to identify individuals with different degrees of response to therapy or those who are more likely to develop adverse drug-related effects. Moreover, the drug response may be used as a "probe" to identify subsets of cases with peculiar pathophysiological mechanisms despite having common clinical characteristics and diagnosis [32].

4.1 Using Available Data to Get New Information

Given the cost and logistic difficulties inherent in pharmacogenetic GWAS, it could be worthwhile to extract useful information from previous studies with genotypic data. For instance, large cohort studies with GWAS data include individuals with long-term follow-up. Some of these individuals would have likely received drug therapy for osteoporosis. This would make feasible to match the DNA data with the densitometric response or the occurrence of fractures after therapy. On the other hand, genetic material may be available from samples of some of the large industry-sponsored pivotal trials of several currently available drugs. After addressing ethical issues, they could serve as a highly valuable source of pharmacogenetic information.

4.2 Searching for Rare Variants

Similar to the other GWAS, pharmacogenetics-oriented GWAS will not be able to explore the association of rare variants with drug response. This will certainly require new approaches. One includes the use of "exon arrays," which explore many rare allelic variants mainly located on coding regions of the genome. This technology is quite efficient and has a relatively low cost (about 50–100 $ per sample). Therefore, it could be applied to large groups of patients. On the other hand, the exhaustive identification of rare variants and their potential association with drug response will require the resequencing of the entire genome. Although ultrasequencing technology is becoming more widely available, the high cost (roughly 7,000 $ per sample) does not allow its implementation in pharmacogenetic studies of large sample size. An alternative option is genome-wide exome sequencing (including the exome and splice sites). The downside of this approach, however, is that in

order to get sequence data at a lower cost (about 1,000 $ per sample), it omits obtaining data about regulatory regions, which may be key in some cases.

4.3 Alternative Designs

Besides the technologies used, attention must be paid to the design of the studies.

- *Extreme phenotypes.* Up to now, most studies have related the whole spectrum of drug responses to the underlying genetic characteristics. It has been speculated that a more cost-efficient design could consist in restricting the analysis to those individuals with extremely good or bad responses. This so-called "extreme phenotypes" approach has been used in some epidemiological analyses and also in some pharmacogenetic studies [33–35]. However, the true value of this approach is still unclear. There are concerns about the possibility that those individuals with extreme (good or bad) responses might present incorrect diagnoses or disease subtypes with unusual pathogenetic mechanisms. This could cause flaws in the study and limit the applicability of its results
- *Endophenotypes.* Fractures represent the relevant consequence of osteoporosis from a clinical perspective. Thus, they would be the most clinically interesting endpoint in genetic and pharmacogenetic association studies. However, in general, using continuous variables as a measure of effect is more statistically efficient than using a dichotomous variable. Hence, studies using BMD may be more effective in identifying important genetic variants than those using fractures as endpoint. Nevertheless, doing so also has some disadvantages, because a low BMD is not the only risk factor for fractures. Indeed, in most cases, the antifracture efficacy of a drug is poorly correlated with its effect on BMD [36]. Therefore, it could be useful to explore other endophenotypes as endpoint variables to analyze drug-induced changes and their relationship with the genotype. Some of them may include already known parameters, such as the biochemical markers of bone turnover, or some skeletal structural parameters. Others may be discovered in the future, including those that reflect the integrity of the muscle–bone axis [37].

4.4 Pharmacoepigenomics

Epigenetic mechanisms are emerging as critical elements regulating gene expression and cell function. In fact, the methylation and demethylation of the promoter regions of several genes have been recently suggested as a key event in the differentiation of the osteoblastic cells responsible for bone formation [38, 39]. Likewise, we have shown that the methylation of a CpG island in the 5′ region of the SOST gene plays a major role in regulating gene transcription [40]. This gene encodes sclerostin, a protein mainly expressed

by osteocytes and chondrocytes, wich, by binding to the LRP5/6 co-receptor, blocks the stimulatory effect of Wnt ligands on bone formation [41]. In fact, sclerostin-neutralizing antibodies have a potent stimulatory effect on bone formation both in animal models and human subjects.

Despite the important role of DNA methylation and other epigenetic mechanisms in bone biology, little is known about its involvement in the pathogenesis of osteoporosis. Nevertheless, several differentially methylated regions were found in a recent genome-wide study that compared CpG methylation in bone samples from patients with osteoporotic fractures and with osteoarthritis [42]. Thus, studies exploring the relationship between DNA methylation in target tissues and drug responses are certainly interesting from a pharmacogenomic perspective. However, in order to be useful as clinical tools, it must be confirmed that the epigenetic biomarkers can be analyzed and provide informative results in readily available samples (such as blood or urine).

4.5 Collaborative Efforts

The experience accumulated during the past decade has clearly shown that very large sample sizes are needed in order to obtain meaningful data from GWAS. This is also likely the case for pharmacogenomic studies. Therefore, researches in this field should engage in collaborations to enable the building of the extensive datasets needed to elucidate at least some of the complex relationships underlying the interactions between drugs and bone cells.

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Chapter 18

Pharmacogenomics and Pharmacoepigenomics in Pediatric Medicine

Barkur S. Shastry

Abstract

In the past several years, human genetics studies have progressed from monogenic to complex and common diseases because of the advancement in technologies. There is increased knowledge of the pharmacokinetics and pharmacogenomics of the drugs in adults as well as in children. These technological developments provided new diagnostic, prognostic, and therapeutic opportunities. We are now in a position to address many additional ambitious questions. For instance, in clinical medicine, interindividual variation in drug response is a major problem. Some of the heterogeneity of drug safety and efficacy among individuals can be explained by pharmacogenomics. It has also the potential to improve the treatment in both adults and children. In pediatrics however, there is ontogeny and metabolic capacity in children is different compared to adults. Several specific developmental changes may underlie some of the variability in drug response seen in children. They may also be responsible for adverse drug reactions (ADRs). Therefore, much of the diversity in drug effects cannot be explained by studying the genomic diversity alone. It is necessary to include the effect of growth (involves variations in gene expression) along with genetic differences when explaining the variability in treatment response. In this respect epigenomics may expand the scope of pharmacogenomics towards optimization of drug therapy. Future studies must focus on periods of maturation of the drug-metabolizing enzymes and polymorphisms in their genes by using candidate gene approach, gene expression analysis, genome-wide haplotype mapping, and proteomics. The integration of genetic data and clinical phenotypes along with the role of other factors is necessary to evaluate both efficacy and ADRs of any drug. It may require extensive genetic epidemiological studies spanning over many years.

Key words Epigenomics, Gene, Medicine, Pediatrics, Pharmacogenetics, Polymorphism

1 Introduction

In the past several years, the extraordinary progress in genome science has provided unique opportunities to address the questions of genetic basis of diversity among individuals and complex and common disease [1]. Recent advances in genetic technology also identified a new form of genetic variation known as copy number variation (CNV). They are known to be associated with population diversity and several complex and common diseases [2]. In addition, whole genome association studies suggest that the variation

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in nucleotides alone does not account for all the heritable phenotypic variations [3]. There may be another layer of information that may depend upon epigenetics [4–6]. The term epigenetics refers to heritable changes in the pattern of gene expression without changes in the DNA sequence. Epigenetic control mechanisms may differ among tissues and individuals. They may also change in time during aging. Many genes of medical interest are under epigenetic control. However, little is known regarding the role of epigenetics in the pathogenesis of diseases. Therefore, in this age of genomics and personalized medicine we must consider the combination of genetics, pharmacogenetics, and epigenetics to better understand the disease pathogenesis and drug response [7, 8].

Medicinal drugs are mostly prescribed by trial and error approach and any medical treatment initiation will therefore raise questions about its safety and efficacy. It is also widely recognized in clinical practice that not all patients respond in a same way to a given therapy. Some individuals develop a severe adverse drug reaction (ADR) in both adults and children even in a normal recommended dose. In some individuals however, ADRs may cause lethality or permanently disabled conditions. In fact, approximately 7 % of all hospitalization in the United States and United Kingdom are due to ADRs and it is the fifth leading cause of death in the United States [9–13]. A similar incidence rate was reported in France [14]. The incidence of serious and fetal ADRs is found to be more common in children and is a significant cause of mortality [15–17]. This is because most drugs have not been studied in pediatric population [18]. However, in modern medicine pharmacogenomics can be used to individualize drug therapy in newborns to minimize ADRs and to optimize efficiency [19].

2 Ontogeny Plays an Important Role in Pediatric Drug Development and Therapy

Several factors such as age, sex, diet, organ function, and drug interaction or co-medication influence drug metabolism [20]. An altered biotransformation may result significantly in therapeutic failure [21] and ADRs. Therefore, it is essential to characterize drug biotransformation pathways to maximize efficacy and toxicity ratio. In this respect the two related fields namely pharmacogenetics and pharmacogenomics will have considerable potential to improve patient-specific therapy [22–24].

Pharmacogenomics explores the relationship between genetic variability and responses to pharmacological intervention. However in pediatrics, this area of research is in its infancy and only a small number of investigations linked to therapeutic response in children. This is mainly due to limited patient number, use of only a candidate gene approach, paucity of meta-analysis, use of different research methods across studies, lack of replication of studies, and

exclusion of nongenetic factors [25, 26]. In children, diseases tend to differ from adults because of developmental growth. Their body size and organ functions are different than adults. There may be age-specific changes in drug pharmacokinetic and pharmacodynamic parameters and that may have an effect on pediatric drug response [27–30]. For instance, it is well known that differences in gene expression occur as the embryo develops [31]. Some developmental genes are switched off in adulthood and this could be associated with either efficacy or toxicity of drugs. Therefore, the pharmacogenomic information obtained in adults may not be the same in children. Physiological and environmental factors in addition to genetic variation may affect the ontogeny of drug biotransformation [32–36].

Additionally, many genes encoding drug-metabolizing enzymes, receptors, transporters, and signal transduction molecules are involved in drug response [37]. It is well known that genetic difference in these genes contributes to the variation in drug response in adults but little is known about their genetically abnormal responses in infancy and childhood [38, 39]. In this respect studies addressing changes in genotype–phenotype relationship during growth and developments are valuable [34]. For instance, the variability in warfarin dose (40 %) in adults depends on variations in genes encoding cytochrome P450 (CYP2C9) and vitamin K epoxide reductase complex 1(VKORC1) whereas in children 50 % of dose depend upon the age rather than genotype of CYP2C9 and VKORC1 [40, 41]. This simple example illustrates that adult experience of pharmacogenomics is not useful in pediatrics and it may mislead the pediatricians to prescribe the medication.

3 Investigations of Pediatric Biomarkers

Biomarkers play an important role in medicine because they can be used to diagnose the disease, to monitor the treatment response of a disease, and in drug development [42, 43]. However, such biomarkers are limited in pediatric population [44] and biologic surrogates are used in pediatric medicine. In order to develop biomarkers specific to children, again ontogeny of children and pathophysiology of pediatric diseases must be considered. One example to illustrate this is the postnatal development of renal function, glomerular filtration, tubular function, and renal drug deposition [45–47]. Additionally, gamma-glutamyltransferase has been used as a marker in disease of hepatobiliary dysfunction in adults. The levels of this enzyme in neonates and young children are several times that of adult level [48]. Similarly, hemoglobin levels in younger children are lower as compared with adult value [49]. Therefore, innovative and new approaches are needed to develop

biomarkers for pediatric medical care [50]. Adult biomarkers are not applicable to pediatric diseases and an understanding of age-related changes is critical for pediatric disease processes.

It is also relevant to add that it is not only the age but also sex and weight must be taken into consideration in pediatric population. Many diseases in children have relatively lower prevalence suggesting that different pathogenic mechanisms must be operative relative to adults. However, some diseases such as pancolitis and uveitis are more prevalent in children compared with adults. Additionally, bronchopulmonary dysplasia, patent ductus arteriosus, and several cancers are encountered almost exclusively in children. Thus, it is difficult to rely on adult experience when applying pharmacogenomics to children. A comprehensive approach such as multicenter collaborative programs targeting pediatric diseases may be necessary to identify biomarkers appropriate for children. This may help uncover novel target for future drug development that may ultimately result in personalized medicine avoiding patient to patient variability in drug response.

4 Pharmacogenomic Studies of Pediatric Disorders

The ultimate goal of pediatric pharmacogenetics and pharmacogenomics is the optimization of disease treatment. This can be achieved by identifying genetic and developmental factors that influence interindividual variability in drug deposition and response in children of the entire age spectrum. Several diseases such as acute lymphoblastic leukemia (ALL), neuroblastoma, asthma, autism, attention-deficit hyperactivity disorder, and type I diabetes occur during childhood. They are associated with age-related differences in drug delivery, dosing, and therapeutic response [51]. As mentioned previously, many factors such as genetic variations in drug-metabolizing enzymes, drug transporters, drug interactions, absorption, distribution, and age play a role in efficacy and toxicity of medication. They also play an important role in immunosuppressive therapy in organ transplantation and anesthetic practice as well as in rheumatology [52–61]. It is quite possible to achieve the goal of pharmacogenomics because the human genome sequence as well as the international HapMap [62] project is available for studies. In addition, inherited genetic changes remain stable throughout the lifetime of a person. Therefore, pharmacogenomics can be applied to identify the risk factors for drug toxicity. One example is the identification of polymorphisms in human leukocyte antigen (HLA) as risk factors for several ADRs [63, 64].

In children with cancer therapy ADRs are a very serious problem because often they are more frequent and more severe. It is estimated that of all hospital admissions 22 % are caused by ADRs of pediatric cancer treatment [65] and 40 % of cancer survivors are

left with long-term problem [66]. For instance, thiopurines, mercaptopurine (6-MP), and thioguanine (6-TG) are frequently used to treat children with the most common type of childhood cancer ALL. However, some patients suffer with hematopoietic toxicity to thiopurines and some patients (25 %) fail therapy. This toxicity could be due to reduced activity of thiopurine S-methyltransferase (TPMT) gene because of genetic variation [67–71]. Therefore, TPMT genotype can be used to choose the appropriate dose of thiopurine for an individual child [72]. However, this approach is reported to be accounting for only a portion of myelotoxicity suggesting that other genes involved in the metabolism of thiopurine drug also could contribute to thiopurine toxicity [73, 74]. Further studies will help to evaluate the cumulative effect of thiopurine toxicity and develop a new diagnostic test [75–77]. Similarly, tacrolimus has been widely used as an immunosuppressive agent in solid organ transplantation in children but it produces nephrotoxicity [78]. It is possible that tissue matching, cytokine polymorphisms, gene expression, function, and polymorphisms of genes encoding multiple enzymes may influence immunosuppressant deposition and final treatment outcome [79–82].

Many pediatric patients were also treated with warfarin for thrombotic events but some patients develop excessive bleeding and blood clots that can lead to serious risk [83]. Recent genetic studies revealed that vitamin K epoxide reductase complex 1 (VKORC1) and cytochrome P450 (CYP 2C9) genes account for nearly all of the genetic variations of warfarin dose in adult patients [84–86]. However in children, the coagulation system is not the same as in adults because it is continuously changing and developing over time [87]. As mentioned earlier, it is not clear whether the same genetic polymorphisms in CYP2C9 and VKORC1 have the same effect in children. According to one study it appears that age was the most important factor determining the dose [41, 88]. Similarly, treatment with vincristine [89], cisplatin [90–92], anthracyclines [93, 94], methotrexate [95, 96], and glucocorticoid [97] produces undesirable adverse events (Table 1). Recent studies suggest that variants in corresponding genes are associated with drug toxicity [98–108]. In children cisplatin-induced cytotoxicity is severe and frequent. Therefore, pharmacogenetic test will help to identify these higher risk patients and improve treatment.

Asthma is a complex disorder in children with a highly variable response to pharmacological therapy. Genetic and environmental factors are the important determinants of the risk of development of asthma. It is estimated that 70–80 % of variability in individual responses to therapy may have a genetic basis and several candidate genes of asthma and atopy have been reported [109–114]. However, genetic interactions, host factors, and environmental factors can also have an influence on drug response [115].

Table 1
A partial list of relationship between variant genes and drug toxicity

Drug	Disease treated	Toxicity	Variant genes	Reference
Thiopurines	ALL	Hematopoietic	TPMT	[67–71]
Vincristine	Malignancies	Peripheral neuropathy	CYP3A5	[97]
Cisplatin	Cancer	Nephrotoxicity, peripheral neurotoxicity, and hearing loss	TPMT GST COMT	[99, 101]
Anthracyclines	Cancer	Cardiotoxicity	?	–
Warfarin	Thrombotic events	Excessive bleeding and blood clot	VKORC1 CYP2C9	[84–86]
Methotrexate	ALL	Leukoencephalopathy, mucositis	MTHFR	[104, 105]

ALL acute lymphoblastic leukemia, *TPMT* thiopurine S-methyltransferase, *GST* glutathione S-transferase, *CYP* cytochrome P450, *MTHFR* methylene tetrahydrofolate reductase, *VKORC1* vitamin K epoxide reductase complex 1, *COMT* catechol O-methyltransferase

Although genetic variations can improve the response to asthma therapy, at present, data do not support routine genotyping of all patients prior to treatment. It appears that asthma pharmacogenetic studies in different populations with a large number of subjects are required before tailoring the asthma therapy based on the genetic makeup of an individual patient [116].

5 Pharmacoepigenomics in Pediatric Medicine

It has become clear in recent years that mammalian genome alone cannot explain many phenomena such as discordance of monozygotic twins, disease susceptibility of an individual, variability in phenotype of a single genotype, and interindividual variability in drug response. Therefore, there must be another layer of information and this additional layer of information could be due to epigenetic modifications. These modifications can mediate gene regulation and vary with age as well as from tissue source [117]. It is always assumed that genotype and gene expression do not change over time. However, abnormalities in epigenetic programming can lead to pathological conditions. Studies on monozygotic twins suggest that non-Mendelian and complex diseases are likely to be caused by the combination of genetic and epigenetic factor [118]. The most common epigenetic modifications involve DNA methylation, various modifications of histones, microRNA, and small noncoding RNA expression. All these factors can modulate gene expression. For instance, DNA methylation may disrupt the transcriptional activity of genes and genomic hypomethylation can

lead to genomic instability [119]. Similarly, histone modifications may affect chromatin structure and microRNAs may regulate the translation of mRNAs.

It is not only the polymorphisms of genes encoding drug-metabolizing enzymes and drug transporters but epigenetic factors are also involved in interindividual variations in drug response. This is because epigenetic factors may control the expression of drug-related genes [5, 7, 120–125]. Therefore, epigenetic factors could provide additional information on molecular markers to predict the responsiveness of disease to therapy. It is also necessary to address the influence of environmental factors including nutrition and hormones on chromatin modifiers [126, 127]. Epigenetic profile may not be maintained indefinitely in a differentiated cell and cellular stress as well as microenvironment may contribute to significant changes throughout the genome and throughout the life of an individual [128]. In this regard two emerging fields namely pharmacoepigenetics and pharmacoepigenomics play an important role in developing new drugs and individualized therapy. Further knowledge of epigenomic changes and identification of factors that mediate alterations in the epigenome may lead to new drug targets and therapies.

6 Ethical Considerations in Pediatric Care

The human genome project has provided multiple opportunities to make significant contributions to clinical pediatrics. Pharmacogenetic testing in children holds a great promise to improve drug safety and efficacy by identifying genetic determinant that influences drug deposition (absorption, metabolism, distribution, and excretion). For instance, children with functional bowel disorder may be benefited by studying polymorphism in serotonin transporter gene and different therapeutic responses to serotonergic agents [129, 130]. A similar example about TPMT was discussed previously [72]. However, before the genetic test is implemented there are several ethical, legal, and social issues such as threat of discrimination, insurance, cost of testing, and use and storage of genetic information that need to be addressed [131–133]. These issues must be reevaluated from time to time as new issues and technologies develop. Similar ethical issues of pharmacogenomic testing in adult population have been addressed previously [134]. In case of children, this issue becomes more complex because children will not participate in decision-making.

Recommendations to guide pediatricians should be developed and genetic information should be used to improve drug therapy only. It is necessary to obtain appropriate informed parental consent, pay attention to protect confidentiality and identity of subjects, and minimize potential risk. Additionally, the test

should be scientifically sound and significant. Childhood diseases with relatively low incidence rate are very challenging because of lack of sufficient number of affected children to apply genomic marker [135]. On the other hand childhood diseases with relatively high prevalence may provide a valuable information on the mechanisms of pediatric diseases but phenotypic expression of diseases must be considered in evaluating potential genomic markers. Additionally, appropriate control samples for genomic studies are difficult to obtain until the development of large repositories. Despite these limitations, efforts are made to identify genes for Kawasaki disease and asthma [136, 137]. These studies may provide valuable information to develop effective therapeutic strategies for these disorders in children.

ADRs are complex and are caused by many factors. It is important that scientists, clinicians, health-care providers, government, and industry people work together to understand the pediatric ADRs. Details of phenotypes and relevant clinical data such as medication dose, concurrent medications, and ancestry data are necessary to understand the role of genes and other factors that influence drug toxicity. Physicians and parents must be educated in terms of complex terminology and limitations of genetic testing. All potential risks and benefits need to be discussed. It is also important that pediatric nurse practitioners understand the pharmacological effects of treatment of children with behavioral issues [138]. In addition, pharmacists must be involved in educating parents and families about pharmacogenomic testing and the development of the genetically influenced drug discovery. This will provide safe and effective medications to patients of all ages [139]. After all, the safety and efficiency of medications are most important in health care and it is an international concern.

7 Concluding Remarks

Interindividual variations in drug response are due to multiple factors including genetic polymorphisms in genes encoding drug-metabolizing enzymes, drug transporters, and receptors [140–144]. Although substantial studies that link genetic variants to interindividual difference in drug response in adults have been reported such studies are rare in pediatrics. Neonates and older infants belong to a different group of individuals. They may experience a different range of drug reactions that may have long-term implications for their development [145]. Thousands of deaths every year are caused by fetal drug reactions and ADRs have been shown to be three times more frequent among pediatric patients than in adults. These ADRs are not necessarily predictable from

the adult experience [146]. Therefore, a systematic research and education in safety issues are needed to understand and prevent adverse events [147].

In Neuropsychiatric disorders, a marked variability in clinical response has been observed. For instance, autism is a lifelong developmental disorder but little has been reported on the factors that underlie the variability in individual response to the treatment. Recently, several polymorphisms in several genes have been reported [148–150] to be responsible for the efficacy and safety of risperidone and escitalopram treatment. Similarly, several studies have found an association of genotype and methylphenidate treatment response in attention-deficit hyperactivity disorder [151] but clinically relevant genetic marker remains a challenge. In addition, in some populations there is no association between polymorphisms in the genes and response to drugs [152]. It is less likely that genetic variation alone could explain variations in drug response. Investigations on gene–gene interactions, environmental factors, and whole genome approach are needed to identify validated biomarkers for children. An understanding of the gene networks that are involved in growth and development of an individual could provide insight into the susceptibility of a child to pediatric drug response [31, 153]. Thus, laboratory tests to diagnose ADRs and pharmacogenomics approach to prevent ADRs are important in the development and utilization of new therapeutic methods in children [154–156].

In order to achieve this goal, we need to have epidemiological and gene expression studies along with a statistical model for the accurate evaluation of the drug gene association [135, 157, 158] or genetic association of drug response. Polymorphism in a gene may vary from population to population or in different age groups and therefore validation of markers across different groups is important. This approach requires population screening early in the clinical trial to identify subpopulations that is most or least likely benefited or experiences ADRs with the treatment [159, 160]. There are several pharmacogenetics and pharmacogenomics networks that are established in several countries. Their goal is to improve drug safety by identifying genetic markers that predict the therapeutic response [161, 162]. For instance, the Canadian pharmacogenomic network utilizes genetic information of patients to identify genetic variants that have potential to provide relevant predictions of efficacy and toxicity of drug [159]. In addition, they also created a database of clinical ADRs and biobanks containing tissue samples to help conduct genome-wide association studies [163]. It is hoped that further efforts will be made to utilize the pharmacogenetic and pharmacogenomic findings to develop individualized medicine for all pediatric patients group [164].

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Chapter 19

Pharmacogenomics in Children

Michael Rieder

Abstract

Historically genetics has not been considered when prescribing drugs for children. However, it is clear that genetics are not only an important determinant of disease in children but also of drug response for many important drugs that are core agents used in the therapy of common problems in children. Advances in therapy and in the ethical construct of children's research have made pharmacogenomic assessment for children much easier to pursue. It is likely that pharmacogenomics will become part of the therapeutic decision making process for children, notably in areas such as childhood cancer where the benefits and risks of therapy are considerable.

Key words Children, Pharmacogenomics, Pharmacology, Drug safety, Genetics, Drug ontogeny, Childhood cancer

1 Children and Genetics

The expanding interest in pharmacogenomics and personalized medicine over the past decade suggests that this is a recent phenomenon. In fact, there has been interest in how genetically determined variations may impact on therapy for children for many years, dating back to comments made by Sir Archibald Garrod, the father of inborn errors of metabolism, who at the dawn of the last century observed that, in addition to controlling key metabolic pathways, genetics was also likely to control some of the variations observed in terms of response to drugs [1]. This should not be surprising. The role of genetics in human disease has been recognized by pediatricians for many years. Given the impact of disorders with a genetic basis such as cystic fibrosis and Down syndrome, child-health-care clinicians and researchers have been sensitized to the importance of genetics to a greater extent than their colleagues with a primary focus on adults.

Indeed, children were among the patients studied by David Price Evans and colleagues in their landmark paper describing genetic control of isoniazid metabolism [2]. However, despite

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the clear and compelling reasons that genetic variation in drug action and clearance should be important as outlined above, pharmacogenomics has been a relative later comer to the forefront in pediatric research. There are several likely reasons for this. While it was appreciated that genetics may play an important role in understanding sources of variability in human drug response in children, it was also appreciated that ontogeny—changes in drug disposition related to maturation of key elements in human drug disposition and clearance—was also a major issue, certainly with respect to drug safety [3]. Indeed, much of the fundamental research conducted in pediatric clinical pharmacology over the past three decades has focused on understanding the impact of ontogeny on drug handling in children and the subsequent effects on efficacy and safety [4]. There have been additional pragmatic issues that have limited the extent to which 245 nM/Ls could be studied in children. These included the amount of blood initially required to conduct studies and the practical problems in obtaining biological samples from children, especially very young children, as well as ethical issues relative to the fact that the person consenting for genetic studies was not the person on whom the study was being conducted [5, 6]. Additionally, there have several myths with respect to drug utilization in children—that drugs were used relatively infrequently in the care of most children and then that the vast majority of drugs used were antibiotics—that have reduced the enthusiasm of investigators to pursue these studies in children [7].

Over the past decade many of these challenges have been addressed and there has been a substantial increase in the amount and quality of pharmacogenomic research being conducted in children. The amount of sample needed and the cost of doing analysis have dramatically decreased, while the use of alternate sample sources—such as saliva—has made the conduct of studies much more feasible. There has been research and discourse on the issues of the ethical conduct of genetic studies in children. It has also been appreciated that drug in children is indeed both common and complex, with studies showing, for example, that on average a Canadian child has four prescriptions per year and that these are from a range of 2,400 therapeutic entities [7]!

2 Sources of Variation in Drug Response in Children

The paradox of drug development is that drugs are developed and evaluated for safety and efficacy in populations, but the clinician treats individual patients [8]. This paradox is especially germane for children, in that many drugs used for the routine care of children have been approved based on studies in adult patients. In

this case, drugs are used off-label—that is to say, use of the drug is not guided by dosage, safety, or efficacy data from the product monograph. Off-label drug use is not uncommon—indeed off-label drug use in adults frequently happens for off-indication prescribing—but in pediatrics this is also frequently off-evidence. While in many cases off-label drug use in adult medicine is in the context of robust data supporting the indication, dose and safety profile of the drug in question, frequently this data is lacking for children. In children, off-label drug use has been associated with an increased risk of adverse drug reactions [9].

A key issue is variability in drug response, which is to say that there are some children in whom the drug works and is safe, some in which it does not produce the desired therapeutic effect and some in which the drug produces adverse events. There are a number of sources of variation in drug response in children [10]. These include the usual sources of variability in adults such as host-dependent variability in drug disposition and clearance, the impact of the disease being treated or other concurrent disease and the effect(s) of other drugs or environmental influences (Fig. 1). In addition to this, the impact of ontogeny on drug disposition, effect and clearance is extremely important, notably for children under a year of age [4]. A final factor that to date has been rarely considered for children is how genetically determined variations in drug disposition and clearance—or in drug response—may impact on variability [11].

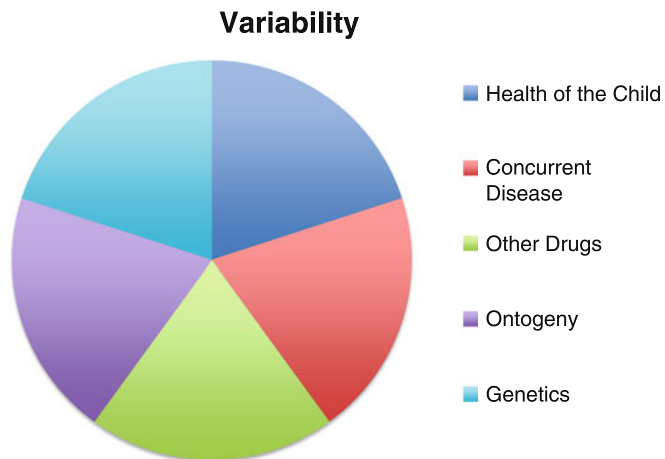

Fig. 1 Sources of variability in drug response in children. These include factors inherent to the child, the effect(s) of the disease being treated or other diseases, the effects of other therapies, ontogeny, and genetically controlled variation in drug disposition, action, and clearance

As noted above, the fact that genetics may be important the wellbeing of children is not a mystery to child health care providers. However, many of the genetic disorders that were historically most relevant to child health were chromosomal polysomies such as Down Syndrome or disorders with classical Mendelian or X-linked inheritance such as Cystic Fibrosis or Duchenne Muscular Dystrophy in which mechanism or effects can be traced to a single event (polysomy) or a clear mechanistic pathway (for example reduced function of the cystic fibrosis transmembrane conductance regulator or alterations in dystrophin) while many pharmacogenetically determined variations are due to more complex mechanisms or the interactions of several genes. The degree to which these variations are clinically relevant in children has been debated, and the on-going controversy as to how useful pharmacogenetic testing is likely to be in patient care has been part of the reason that pharmacogenomic approaches have been relatively slow to come to the clinic in children compared to adults. This is perhaps best illustrated by comparing two drugs for whom pharmacogenomic determinants have been identified that determine toxicity—6-mercaptorpurine and codeine.

3 6-Mercaptopurine and Codeine: A Tale of Two Drugs

An enzyme for which pharmacogenetic variability was identified for some time is Thiopurine Methyltransferase (TPMT). This enzyme catalyzes the S-methylation of thiopurines such as the chemotherapeutic agent 6-mercaptopurine (Fig. 2) [12]. It was appreciated in the 1970s that there was considerable variability in toxicity when this drug was administered to patients with cancer. A landmark study conducted by Drs. Richard Weinshilbaum and Susan Sladek at the Mayo Clinic established that there was a pharmacogenetic basis for this variability, an activity pattern consistent with autosomal codominant inheritance for alleles for low and high TPMT activity; 88.6 % of subjects had high enzyme activity, 11.1 % had intermediate activity, and 0.3 % had no detectable activity [13]. Relevant to considerations of pharmacogenomics in children, 115 of the subjects in this study were children, on average age of 13 years. Translating this to clinical relevance, it has been demonstrated that patients who are homogenous for low TPTM activity—the 0.3 % with no detectable activity—were at significantly greater risk for toxicity—such as neutropenia—when being treated with 6-mercaptopurine [14].

Given the potential mortality and known morbidity associated with febrile neutropenia, it may seem surprising that the routine use of TPMT genotyping in defining dose regimens for children with cancer did not gain early wide spread acceptance in the broader community of pediatric oncologists [15]. There are several

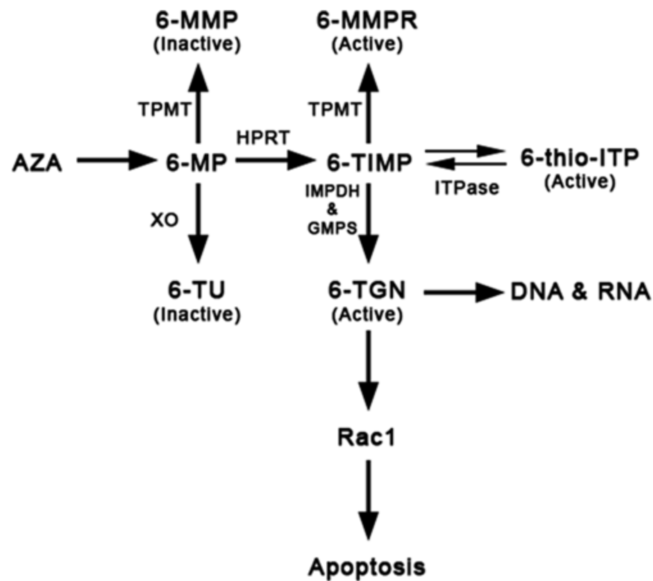

Fig. 2 Metabolism of 6-mercaptopurine (6-MP) via xanthine oxidase (XO) to the inactive metabolite 6-thiouric acid (6-TU), thiopurine *S*-methyltransferase (TPMT) to the inactive metabolite 6-methylmercaptopurine (6-MMP), and hypoxanthine guanine phosphoribosyl transferase (HPRT) to 6-thioinosine monophosphate (6-TIMP) which is then further metabolized to thioguanine nucleotides (6-TGN), 6-methylmercaptopurine ribonucleotides (6-MMPR) or 6-thio-inosine triphosphate (6-thio-ITP), these all being active metabolites

likely reasons for this. A pragmatic reason is the volume of blood required at the time to conduct genotyping studies. This was at the time significantly larger than the amount of blood needed to monitor for toxicity—this being by a complete blood count. The cost of genotyping has historically been a consideration, previously being severalfold larger than the cost of most routine laboratory tests. The outcome of interest—neutropenia—had been known for some times and protocols were in place to evaluate this using simple assessments—such as routine use of complete blood counts. The value of genotyping above and beyond conventional monitoring was initially well defined. Finally there is the consideration of frequency. There was a debate as to just how valuable routine genotyping was for the broader population, given that the frequency of patients homozygous for low activity genes was in the range of 0.3–0.5 %. A key element of the debate was the extent to which heterozygotes were likely to need dosage alteration [16]. While this was not clear initially, there has been emerging data that suggests that dose alteration may need to occur in other groups than the homozygous low activity patients—for example, patients with high activity may need increased dosage, while risk of secondary malignancies may be related in part to variability in TPMT

activity [17, 18]. It has been increasingly appreciated that gene–gene interactions may play an important role in determining toxicity and that analysis to determine risk should factor in multiple variables including age and concurrent therapy as well as genotypic variation [19, 20].

In this case, a pharmacogenetic determinant of variability was described more than 30 years ago, and the precise role(s) of how this variant will be used to alter therapy are still being investigated and defined. However, it is safe to say that there is increasing acceptance that routine genotyping of TPMT in the context of the therapy for childhood cancer. As well, there is increasing interest in the use of TPMT genotyping to guide dosing for other drugs and in other disorders, for example in treating patients with inflammatory bowel disease with azathioprine [21].

Let us now consider codeine. Codeine is an opiate alkaloid that is the second most abundant alkaloid in opium. Although present naturally codeine for therapeutic use is most commonly synthesized. Codeine has been recognized for more than a century as having analgesic properties and has enjoyed wide spread use for this indication, including being listed as part of the WHO "Pain Ladder" [22–24]. Codeine is considered a weak opiate and has been recommended as part of a stepwise approach to treating pain. There are many advantages to using codeine; it is inexpensive, available as an oral formulation in both liquid and tablet form, and is extremely stable. However, there are important—and until recently largely unrecognized—pharmacogenomic variables in drug disposition which can significantly alter the benefit–risk profile of codeine.

To appreciate these differences it is important to first recognize that codeine is, as an analgesic, a prodrug. Chemically codeine is 3-methylmorphine, which pharmacologically is relatively inactive compound. After ingestion of codeine, the drug enters the liver via the portal circulation where it undergoes metabolism via both Phase I and Phase II pathways (Fig. 3) [25]. Codeine is demethylated by CYP2D6 to produce morphine, which historically has been viewed to be the major mechanism by which codeine exerts its analgesic effects [26]. Codeine also is conjugated by Glucuronyltransferase (more specifically, UDP-Glucuronosyltransferase-2B7) to codeine-3-glucuronide and codeine-6-glucuronide, with the 6-glucuronide being active as an analgesic [27]. A minority of codeine is metabolized by CYP2D6, typically accounting for approximately 5 % of the dose. Once metabolized to morphine, further metabolism occurs via glucuronidation to morphine-3-glucuronide and morphine 6-glucuronide, with the 6-glucuronide also being pharmacologically active, having roughly half the potency of morphine. The major route of conjugation is typically via 3-glucuronidation, typically eight to tenfold greater than the production of the 6-glucuronide [26, 27].

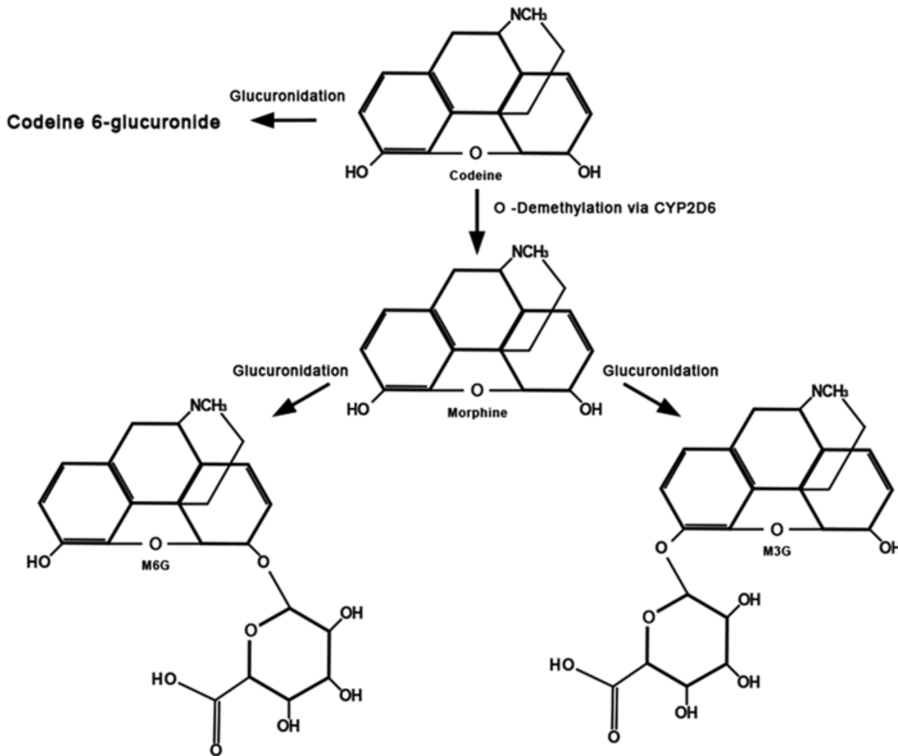

Fig. 3 Metabolism codeine to the active metabolites morphine and codeine-6-glucuronide, the demethylation to morphine being catalyzed by CYP2D6; morphine is further metabolized to morphine-3-glucuronide and morphine-6-glucuronide

Codeine toxicity—with the classical hallmarks of coma, miosis, and bradypnea—has long been recognized as an adverse event associated with codeine overdose [28]. However, in 2006 Koren and colleagues described the case of a breast-fed infant who died of opiate toxicity related to maternal use of codeine for analgesia post episiotomy [29]. In this case, a detailed analysis of blood and breast milk coupled with genetic studies demonstrated that, despite the mother taking prescribed and conventional doses of a codeine–acetaminophen combination product, that the infant did indeed have very high concentrations of morphine in the blood, the post-mortem blood morphine concentration being 245 nM/L [29]. To put this in context, patients on chronic high-dose opiate therapy have been found to have blood concentrations of 190 nM/L [30]. The reason for this very high morphine concentration became clear when genetic studies were undertaken. The mother was an ultrarapid metabolizer for CYP2D6 and therefore converted much more codeine to morphine than expected, a fact validated in studies of the morphine concentration of her breast milk [29].

Table 1
Ethnic distribution of CYP2D6 phenotypes

Population	PM phenotype (%)	UM phenotype (%)
Northern European	7.7–8.9	1
Mediterranean Littorial	2	8
Horn of Africa	2	29
South African	19	
Chinese	1	1
Saudi Arabian	1	21

Derived from *The Oncologist*, Vol. 11, No. 2, 126–135, 2006

In this case the particular genetic variability had been known for some time. CYP2D6 is a polymorphic enzyme, in that it can be demonstrated in three distinct phenotypes—extensive metabolizers (EMs), poor metabolizer (PMs) and ultrarapid metabolizers (UMs) [31]. These phenotypes are the product of the extensive polymorphisms known with respect to the gene encoding CYP2D6. Currently there are more than 80 alleles for *CYP2D6*, with many of these producing the PM phenotype [32]. An additional factor complicating this polymorphism is the variable expression of these phenotypes in different populations (Table 1) [33, 34]. Codeine was originally isolated in France in 1832, and the original use of codeine was among northern European populations, among whom the UM polymorphism is uncommon. As the use of codeine has expanded—and codeine has been among the most popular opiates used worldwide—codeine therapy has become more common in populations with much higher rates of the UM genotype. It is not surprise that, in these populations, problems with codeine toxicity have emerged—not only among newborns, but also among children following surgery [35–37].

The recognition of these toxicities led to a brisk response by regulators and hospitals, including a formal Contraindication being issued by the US FDA for the use of codeine in children with obstructive sleep apnea after tonsillectomy or adenoidectomy [38]. Many hospitals and practice groups have removed codeine from their formularies—some with careful deliberation and some with changes that appear be less than fully thought out (for example, replacing codeine with oxycodone—which is O-demethylated by CYP2D6). A common change has been to replace codeine with morphine, which is sensible given that much of the analgesic activity of codeine is probably related to metabolism to morphine [39]. It should be noted that many of these changes occurred within 5 years of the publication of the index case that triggered concerns as to genetically determined toxicity in children [29].

Here we have two drugs both of which have genetically determined toxicities yet in one case there has been a profound change in use and regulation within half a decade of the description of the issues of concern yet in the case of the other drug there have been no widespread major changes in use based on genetic testing although more than three decades have passed since potential issues were identified. This probably reflects in part the fact that although of codeine is in fact rare the toxicity is often extreme—with a high incidence of fatal outcomes [40]. In contrast, while febrile neutropenia during chemotherapy is common fatal outcomes are relatively uncommon and there are well defined, evidence-based protocols to guide management [41]. The success of pediatric oncology has largely been based on the use of carefully developed evidence-based protocols, and the requirements needed to change protocols are substantial. In addition, pediatric oncologists are prepared to take risks considerably larger than many other physicians given the severity of the disorders being treated and the fact that outcomes in pediatric cancer, despite the many adverse effects of the treatments prescribed, are in fact the best outcomes for almost any cancers, with approximately 75 % of children with cancer being "cured" of their disease [42]. The very fact that genetically determined variations in TPMT activity influence the concentration of chemotherapeutic agents has been recognized for more than three decades means that these great improvements in outcome have occurred without assessment of TPMT activity beyond the crude but doubtless relevant phenotype of febrile neutropenia. The fact that many of the technical and economic issues that made routine genotyping of children problematic three decades ago have been resolved can then to be seen to be less of a factor in deciding when to routinely use TPMT in management.

4 Pharmacogenomics and Childhood Cancer

As noted above, drug use in children is in fact more common than is usually appreciated, and tends to be concentrated in approximately 25 % of children, who account for 70 % of drug use [7, 43]. This would suggest that the most appropriate groups of children for whom pharmacogenomic testing may be of utility are among this 25 %, and prominent among these children are children with cancer. Chemotherapy is the mainstay of the treatment of children with cancer and for this indication has been strikingly effective [42, 44, 45]. However, while survival rates for children with cancer have improved dramatically, this has not been without cost. Part of this has been economic, in that cancer treatment involves considerable time in hospital and clinic and substantial use of drugs and laboratory resources [46]. Part of this has been in terms of health-related quality of life [47]. Health-related quality of life among

children with cancer is impacted most significantly during therapy and when children develop terminal illness [47]. As well, adverse drug reactions produce a significant burden, including being a very common cause of hospital admission for children with cancer [48]. Among the increasing number of survivors of childhood cancer, health-related quality of life is relatively high—except among children who have sustained comorbidities, which are frequently long-term adverse events of therapy [47]. This may be as common as two-third of all survivors, with a large follow-up study demonstrating that a quarter of the adult survivors of childhood cancer had a serious chronic health condition related to their therapy with one-quarter having three or more chronic health problems [49]. Thus, adverse drug reactions are a problem not only during therapy but also for many years thereafter. Given these troubling numbers, clearly strategies to reduce the risk of adverse drug reactions—and long-term health risk—are important go-forward elements in research in childhood cancer.

Genetics has already been incorporated into the care of children with cancer; an example is the Philadelphia Chromosome, a chromosomal anomaly produced as a result of reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)] that is most commonly seen as a marker of chronic myeloid leukemia [50]. The presence of this chromosomal anomaly is associated with altered prognosis—and the need for different therapy—when seen in the context of acute lymphoblastic leukemia in childhood [51]. An area of oncology care where genetics has not been widely used has been in the evaluation—and possibly prevention—of adverse drug reactions [52].

As noted above, adverse drug reactions are very common among children with cancer, and current strategies for prevention and monitoring are largely based on evaluation of clinical phenotypes [53]. This can be effective—an excellent example is the use of Mesna during ifosfamide therapy, which has been quite clearly shown to sharply reduce the risk of bladder toxicity [54]. However, these strategies can be difficult to develop and often require much more mechanistic insights that are currently available.

Pharmacogenomic testing offers the potential of being able to identify children at altered risk for adverse events—notably serious adverse events—so that monitoring can be performed on a more regular or consistent manner or novel therapies can be used to reduce adverse drug reaction risk. Given how oncology treatment is undertaken in children, ideally this could be done at the time of diagnosis and initial work-up, at which time considerable effort is put into informing the family and in the implementation of evidence-based treatment protocols. Additionally, the protocols used in oncology are very clear both as to the drugs used and their dose and timing. Thus, pediatric oncology seems to be an area in which pharmacogenomics could make a relatively early entry into routine care.

Three drugs commonly used in oncology illustrate the potential. Cisplatin is a platinum-based chemotherapeutic agent that forms complexes which in vivo bind to DNA producing cross-linkages which trigger apoptosis [55]. This drug is a highly effective chemotherapeutic agent used for the therapy of a variety of solid tumors. Although a very useful drug, cisplatin is associated with many adverse events, one of the most serious being ototoxicity [56, 57]. Cisplatin-induced ototoxicity is a feared adverse event associated with therapy, with known risk factors including concurrent therapy with other ototoxic drugs, male gender, and age; children under the age of 5 have 20-fold greater risk than do adults [58]. Our group has described a genetic association with cisplatin-induced ototoxicity; in a cross-Canada study which included a network of 16 pediatric academic health science centers from which cases and matched controls were recruited [61]. We described the association of tag single nucleotide polymorphisms (SNPs) in the *thiopurine S-methyltransferase* (TPMT) gene (rs12201199 and rs12201199) and in the *catechol-O-methyltransferase* (COMT) gene (rs9332377) with cisplatin-induced ototoxicity [59, 60]. This has been validated by other investigators [61]. Modeling the predictive value of these alleles, the presence of three or more risk alleles predicts a very high 5-year risk of not having normal hearing compared to children who have no risk alleles, who have a 60 % chance of normal hearing (Fig. 4). While the place of genetic testing for cisplatin-induced ototoxicity is being discussed, economic

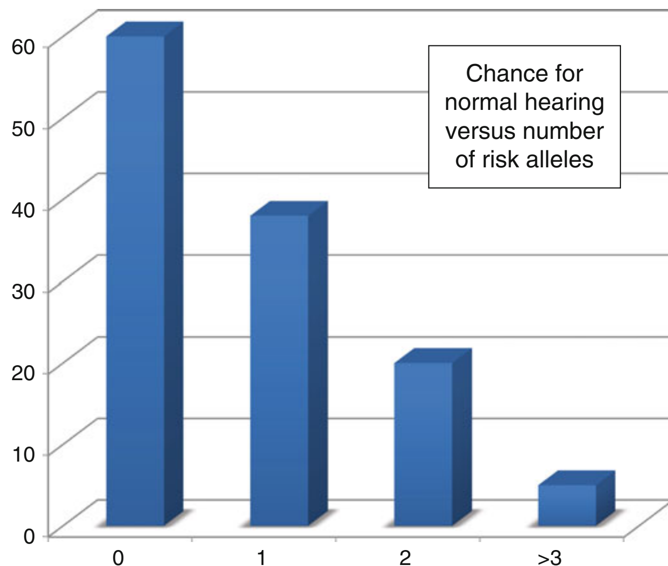

Fig. 4 5-year chance of having normal hearing following cisplatin therapy related to having 0, 1, 2, or 3 or more risk alleles for cisplatin-induced ototoxicity; percentages estimated based on data derived by our group and published in *Nat Genet* 2009;41:1345–9

analysis suggest that there may be a significant health care saving over the longer term associated with genetic evaluation of hearing risk at the onset of chemotherapy [62].

It is worth commenting on how these SNPs were identified. One might consider that a logical approach would be to develop a study strategy investigating genes regulating pathways known to be key mechanistic elements in the pathogenesis of the disorder of interest. However, in the case of cisplatin-induced ototoxicity the pathophysiological mechanism(s) remain controversial, and consequently our group elected to genotype patient and control samples for 1,949 SNPs which captured genetic variation among 220 genes involved in drug metabolism and distribution (including Phase I and II enzymes, drug transporters and drug receptors) and disease-specific genes related to physiological pathways impacted by cisplatin. This broad approach was an important aspect of the success of our study in identifying unique SNPs associated with the toxicity of interest as a more selective search may well have missed them. In addition to informing clinicians and patients as to risk, our findings have also provided insights into potential mechanism(s) which are now being evaluated to better define the pathophysiology of cisplatin-induced hearing loss.

The anthracyclines are antitumour antibiotics whose mechanisms of action include intercalation between base pairs on the DNA/RNA strand, inhibition of topoisomerase II, generation of iron-mediated free oxygen radicals and inducing histone eviction from chromatin [63]. The anthracyclines are the cornerstone of therapy for many of the more common cancers in children, including most of the hematoreticular malignancies [64]. Although very effective, the anthracyclines are associated with serious adverse effects, the most feared of which is cardiotoxicity [65–67]. Risk factors for anthracycline-induced cardiotoxicity include cumulative dose as well as age, with children under the age of 4 years having a significantly higher risk for cardiotoxicity [65, 66]. Additional risk factors include female gender, higher dose rates, and cranial irradiation; preventive strategies to date have not produced consistently robust results in terms of efficacy [68]. Anthracycline-induced cardiotoxicity is associated with both significant morbidity as well as mortality and consequently strategies to identify patients at risk would be of considerable utility.

Using a similar approach to our studies of cisplatin-induced ototoxicity, we have identified a series of SNPs associated with anthracyline-induced cardiotoxicity in children with cancer in Canada, a finding that we have verified in a replication cohort from the Netherlands [69, 70]. We identified a series of risk and protective alleles that can be related at least in good part to the known pharmacology of the anthracyclines; these variants include protective variants characterized by loss-of-function for influx transporters for anthracyclines as well as risk variants characterized by loss-of-function for efflux transporters for anthracyclines (Table 2, Fig. 5) [69].

Table 2
Risk and protective variants predicting anthracycline cardiotoxicity

Gene	Predictive value	SNP rs-ID
UGT1A6	Risk	rs6759892
ABCB4	Risk	rs1149222
ABCC1	Risk	rs4148350
HNMT	Risk	rs17583889
SCL28A3	Protective	rs78583889
FMO2	Protective	rs2020870
SPG7	Protective	rs2019604
SLC10A2	Protective	rs9514091
SLC28A3	Protective	rs4877847

Derived from *J Clin Oncol* 2012;30:1422–8

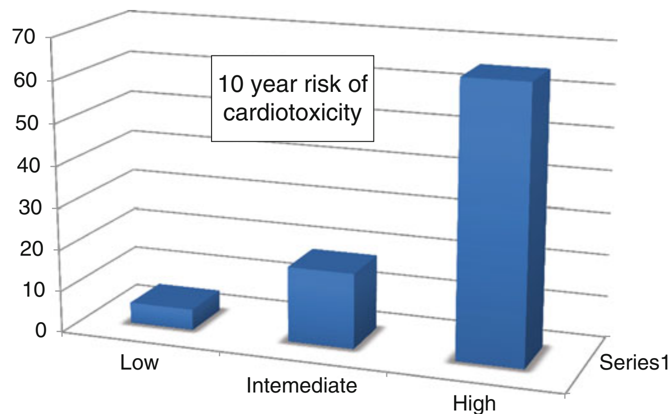

Fig. 5 10-year chance of developed cardiotoxicity related to being low, intermediate or high risk; risk category based on number of protective versus risk alleles and developed estimated based on data derived by our group and published in *J Clin Oncol* 2012;30:1422–8

A third drug to consider is ifosfamide. Ifosfamide is a positional isomer of cyclophosphamide that is used as an alkylating agent in the treatment of solid tumors [70, 71]. Ifosfamide itself is a prodrug that must be activated to ifosfamide mustard to produce tumoricidal effects, in the case of ifosfamide mustard by DNA alkylation at the N-7 position of guanine which leads to interstrand and intrastrand cross-links causing cell death [71]. While very useful for the management of solid tumors, ifosfamide has been associated with a high risk of nephrotoxicity, with known long-term

complications in terms of morbidity and mortality [72–74]. While the risk factors have been debated, it does appear that age under 3 years is a significant risk factor for the development of ifosfamide-induced nephrotoxicity [74, 75].

In the case of ifosfamide, the mechanism of ifosfamide-induced nephrotoxicity has been the topic of intense study which has yielded important clues as pathophysiology [54, 76–82]. As noted above, ifosfamide is a prodrug that must undergo activation to ifosfamide mustard to exert its anticancer effects (Fig. 6). Ifosfamide metabolism can produce either ifosfamide mustard by ring hydroxylation or chloracetaldehyde by side chain oxidation [71, 77, 79]. It has been demonstrated that chloracetaldehyde produced by intrarenal metabolism can act as a potent renal toxin, both in vitro and in vivo [76, 77, 80]. It has also been clearly demonstrated that the use of concurrent antioxidant therapy—in the case of ifosfamide with *N*-acetylcysteine—can prevent ifosfamide-induced renal injury, again both in vitro and in vivo [79–81]. This approach has been shown in a small number of case reports to be effective in children with cancer [82].

In this case the metabolism and putative mechanistic basis of the toxicity of interest are known in some depth. How does pharmacogenomics factor in? The proposed course of action—concurrent therapy with *N*-acetylcysteine—is associated with some risk of adverse effects from *N*-acetylcysteine and also introduces additional complexity into therapy. Given that the risk for nephrotoxicity in the highest risk group is approximately 30 %, this suggests that identifying factors which predict risk—such as genetically determined variation in drug activation—may be of considerable utility in better defining which patients should—and should not—receive concurrent antioxidant therapy. There are several steps along the metabolic pathway regulated by enzymes known to be polymorphic, suggesting that pharmacogenomic studies of these pathways could be "low hanging fruit" in defining the contribution of genetics to ifosfamide-induced nephrotoxicity and in helping to better define care that is optimally safe and effective (Fig. 6) [81–84].

These three examples serve to illustrate the considerable potential that pharmacogenomics offers in improving care in children, even in an area such as pediatric oncology where great strides in improving child health have already taken place.

5 Personalized Medicine for Children

The examples cited above with respect to pediatric oncology should not be taken to mean that this is the only care area in which pharmacogenomics for children should be investigated, and indeed there are a number of other areas—notably for children with

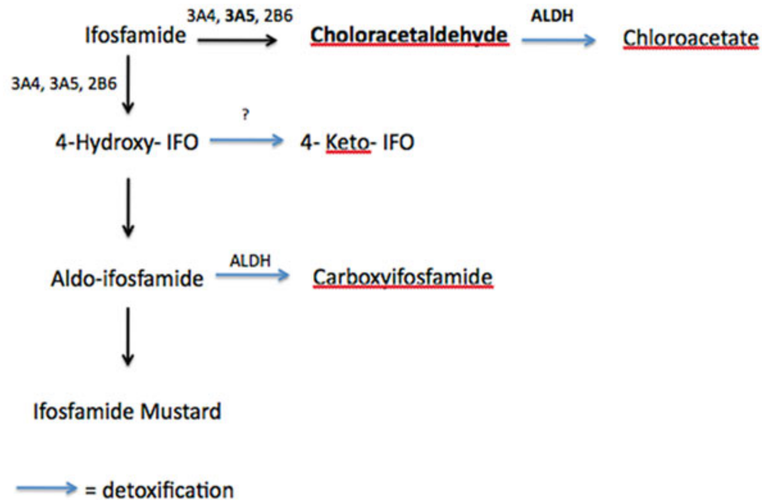

Fig. 6 Metabolism of Ifosfamide. Metabolism of the prodrug ifosfamide produces either the desired chemotherapeutic agent, ifosfamide mustard, or the nephrotoxin chloroacetaldehyde. Potential polymorphisms of interest include *CYP 3A5* polymorphisms (low and high expressors) such as CYP 3A5*3 and *6 = absence of or low expression, CYP 3A5 *1 = high expression and *ALDH* polymorphisms (low and high expressors) such as ALDH2*1 = normal or ALDH2*2 = low function

complex chronic disorders—where pharmacogenomics offers great promise. There are a number of drugs—ranging from morphine to warfarin to proton pump inhibitors—where there are promising findings suggesting that pharmacogenomics offers the link to develop truly personalized medicine for children [11, 85–88]. As well, studies need to be conducted to correlate the results of findings in adults with children—for example, in the area of biomarkers for serious adverse events [11, 89]. One area in which pharmacogenomics and personalized medicine offers tremendous promise in bringing clear direction is the area of drugs for central nervous system disorders [90–95].

Over the past two decades there has been a large increase in the use of drugs impacting on the central nervous system in children. This has resulted in a number of questions as to efficacy and safety. Given that in many schools in North America as many as 5 % of students are taking some type of psychoactive drug, this strongly suggests that research into the variability of drug response—particularly genetically governed areas of variability—is of considerable importance in developing evidence-based optimal therapy that is both effective and safe. As well, understanding these sources of variability permits the development of guidelines and guides the creation of tools which can be used to direct safer therapy [96].

There are serious questions that need to be asked when considering such studies. One obvious one is which drugs to study? Given that more than 2,400 drugs are routinely used in the care of children, which ones should be priority targets for study? Fortunately, this issue has been given some thought, and there are algorithms available which can be used to generate robust solutions when prioritizing as to which drugs would be the most suitable targets for further study [97]. There are practical issues in the conduct of studies, such as ensuring adequate numbers of patients; the creation of large national and even international networks greatly facilitates such studies [98]. While there have been technical and economic issues historically, samples now can be collected relatively noninvasively by using saliva and the costs of genomic testing have fallen dramatically over the past decade [11, 99].

6 Ethical Issues

No consideration of pharmacogenomic testing in children would be complete without a consideration of the ethical issues involved. The ethics of any type of genetic testing in children are complex. When obtaining informed consent, a complication is that the person giving the consent—typically a parent or guardian—is not the person from whom the genetic information is being obtained. This is particularly problematic when this information can be used for risk analysis for adverse events or disease that may occur at some time in the distant future [5, 100].

There have been a number of studies exploring the attitudes of patients and health care practitioners towards pharmacogenomics and personalized medicine [6, 100–103]. Patients appear to be reasonably optimistic with respect to the potential for improving outcomes; interestingly, a major reservation has been that patients have been concerned that their own health care practitioners may not have the right knowledge set to adequately address the new information presented! The literature on attitudes of parents towards genetic testing of their children is much sparser. We are in the process of studying this area and have found that overall parents—and, interestingly, non-parents—are reasonably comfortable with genetic testing to guide drug dosing children with the proviso that this should be directed and purposeful. That is to say, genetic testing done to direct the results of a therapy planned for now appears to be much more acceptable than genetic testing for less well defined purposes, although interestingly even if the results of testing predicted outcome—without ability to influence the outcome—parents still wanted to know them. Germane to this is the relative lack of difficulty our group has had in recruiting large numbers of children—patients and controls—for our pharmacogenomic studies [59, 69, 70, 97]. Clearly much work needs to be done, but in

general it would be reasonable to conclude that parental attitudes would not be a barrier to well planned, well communicated pharmacogenomic research in children.

7 Pharmacogenomics and Drug Development for Children

It is clear that pharmacogenomics is an increasingly important part of the drug development and drug regulation process, not only for adults but for children as well. When developing new therapeutic agents the potential for genetic variation impacting on efficacy and/or safety should be part of the research considerations. There is a potential risk in that drug development guided by pharmacogenomic testing could potentially restrict entry into clinical trails to those patient sub-groups most likely to benefit and least likely to experience harm. This situation should be avoided, notably as the therapeutic world is truly global and drugs need to be tested among the populations who are likely to use them. Clinical trials can and should take advantage of genetic studies to better define which patient populations are likely to benefit—or to be at special risk—from the agent under study. Historically children's therapy has lagged behind therapy for adults—this should not be the case in the era of personalized medicine.

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Index

A

ABCB1. *See* ATP-binding cassette, subfamily B (MDR/TAP), member 1 (ABCB1)
Absorption, distribution, metabolism and elimination (ADME) 70, 75, 111, 577
Accessory proteins 121–144, 160, 172
ACE. *See* Angiotensin-converting enzyme (ACE)
Activator of G protein signaling (AGS) 130, 143
Acute lymphoblastic leukemia (ALL) 674–676, 696
Adaptation 4–6, 247
Addiction 195, 205, 206, 214–217, 589–613
ADHD. *See* Attention-deficit hyperactivity disorder (ADHD)
Admixture 79, 81–82, 249
Adverse drug reactions (ADRs) 24, 68–71, 75, 76, 111–112, 191, 224, 324, 376, 471, 518–520, 558, 561, 570, 639, 672, 674, 678, 679, 689, 696
Agonist 124–126, 135–141, 143, 154, 159, 161, 167, 168, 172, 174, 177, 178, 194, 196, 208, 209, 211–213, 216, 217, 222, 247, 249–252, 261, 274, 283–286, 334, 376, 377, 379–382, 391–395, 399, 400, 403, 408, 421, 426, 435–439, 442, 444–445, 447, 450, 454–456, 458, 460, 469, 470, 472, 473, 489–494, 496, 497, 499, 500, 504, 510, 511, 559, 564, 570, 572, 593–595, 597–602, 604–606, 609, 625, 641–652
AGS. *See* Activator of G protein signaling (AGS)
Alcohol 73, 195, 197, 203, 204, 215, 317, 339, 349, 446, 472, 474, 477, 493, 499, 589–606, 610–613
ALL. *See* Acute lymphoblastic leukemia (ALL)
Alzheimer's disease (AD) 5, 13, 14, 127, 131, 197, 205, 323–520
Ancestry informative markers (AIMs) 81, 518, 572
Angiotensin-converting enzyme (ACE) 198, 201, 245, 246, 248, 253–255, 262, 263, 281, 354, 379, 449, 450, 471
Antagonist 172, 177, 179, 206, 209, 214, 216, 222, 274, 334, 376, 377, 393, 399, 400, 403, 408, 421, 426, 436, 438, 439, 447, 450, 456, 458, 460, 469, 470, 472, 473, 490, 491, 493, 497, 499, 500, 510, 511, 559, 564, 570, 572, 593–595, 598–600, 602, 605, 606, 625, 641–643, 645, 648, 651, 652
Antihypertensive agents 449–450
Antipsychotics 102, 203–205, 325, 363, 489, 511, 557–580, 595
Anxiety 326, 416, 456, 558, 598, 604
Apolipoprotein 261, 265, 329, 330, 335, 347, 348, 356, 374, 489, 500, 502, 504, 519
Arginine vasopressin (AVP) 157, 173, 174, 176, 177
Arthritis 6, 324, 332, 334, 338, 339, 346, 347, 353, 357, 358, 625–653, 668
Artificial neural networks (ANN)
Asthma 12, 155, 157, 159, 179–180, 192–194, 197–199, 206–218, 337, 338, 674–676, 678
Atherogenesis 264–266, 268, 270, 275, 287
Atheroprotection 262, 272, 283
Atherosclerosis 73, 259–270, 272–276, 281, 283, 285–287, 339, 343, 346, 349, 353, 367, 436, 460, 505, 507
ATP-binding cassette proteins 77, 92, 314
ATP-binding cassette, subfamily B (MDR/TAP), member 1 (ABCB1) 77, 92, 93, 98–100, 102–108, 110, 304, 305, 307, 310, 312, 314, 330, 340, 363, 375, 415, 473, 487–504, 508, 509, 511, 561–563, 595, 607–608, 626–628, 633
Attention-deficit hyperactivity disorder (ADHD) 195–197, 203–205, 215, 674, 679
Autism 202, 344, 674, 679
AVP. *See* Arginine vasopressin (AVP)
Azathioprine (AZA) 490, 625, 636–640, 692

B

BBB. *See* Blood–brain barrier (BBB)
BDNF. *See* Brain-derived neurotrophic factor (BDNF)
BHR. *See* Bronchial hyperreactivity (BHR)
Bioactivation 314, 560
Bioavailability 91, 92, 105, 107, 109, 412, 509, 510, 559–561, 641
Bioinformatics 4, 19–32, 192
Biomarkers 7, 12–14, 20, 22, 24, 25, 27, 31, 32, 35–41, 191, 270, 324, 326, 363, 369–376, 425, 438, 443, 463, 502, 515, 517, 519, 567, 668, 673–674, 679, 701
Bisphosphonates 664–666
Blood–brain barrier (BBB) 107, 108, 361, 362, 427, 434, 445, 449, 452, 465, 488, 501–505, 561, 607
BMD. *See* Bone mineral density (BMD)
Bone mass 661–662
Bone mineral density (BMD) 166, 201, 662–667

Qing Yan (ed.), *Pharmacogenomics in Drug Discovery and Development*, Methods in Molecular Biology, vol. 1175, DOI 10.1007/978-1-4939-0956-8, © Springer Science+Business Media New York 2014

Brain-derived neurotrophic factor
(BDNF)........346, 390, 400, 402, 406, 415, 433, 446, 447, 456, 457, 463–465, 469, 562, 567, 571, 595, 608–609
Bronchial hyperreactivity (BHR)........208, 209, 212–214

C

CAA. *See* Cerebral amyloid angiopathy (CAA)
CAD. *See* Coronary artery disease (CAD)
Calcium channel........343, 379, 444, 449, 490, 497, 612
Calcium-sensing receptor (CASR)........122, 155, 158, 161–167, 182, 192, 193, 200, 201, 223, 224, 335
CAMs. *See* Constitutively active mutants (CAMs)
Cancer........5, 6, 12–14, 23, 26, 27, 30, 31, 46, 56, 60, 99, 110, 125, 217, 220–222, 302, 311, 313–317, 319, 323, 324, 330, 331, 334, 336, 338–344, 346, 347, 349, 351–353, 355, 359, 365, 368, 401, 403, 431, 433, 436, 440, 487, 488, 502, 504, 518, 634, 674–676, 690, 692, 695, 696, 698, 700
Candidate gene association studies
(CGAS)........75–77
Cannabinoid........140, 190, 214–216, 379, 384, 400, 470
Cardiovascular disease (CVD)........5, 67, 201, 259–263, 265, 266, 268–270, 285, 339, 342, 570
CASR. *See* Calcium-sensing receptor (CASR)
CASs. *See* Complex adaptive systems (CASs)
Catechol-O-methyltransferase (COMT)........358, 474, 476, 481, 562, 564, 573, 577, 595, 676, 697
Central nervous system (CNS)........10, 25, 28, 202, 323, 325, 326, 361, 365, 366, 373, 399, 400, 405, 436, 451, 457, 459, 461, 463, 467, 468, 472, 473, 476, 487, 502, 504, 505, 508, 510, 518, 611, 701
Cerebral amyloid angiopathy (CAA)........357, 362, 427, 435
Cerebrospinal fluid (CSF)........92, 365, 367, 370–374, 376, 424, 425, 428, 431, 438, 442, 449, 462, 502, 507, 517, 564, 599
Chemoattractant........212, 217, 268, 270
Chemokines........158, 199, 200, 217–220, 268, 274, 284, 334, 353, 380, 443
Chemotaxis........219, 260, 266
Chemotherapy........7, 301–303, 313, 315, 316, 400, 595, 695, 698
  chemotherapy induced peripheral neurotoxicity
  (CIPN)........301–319
Childhood cancer........675, 692, 695–700
Chromatin remodeling........28, 361, 611
Chromosome........39, 128, 164, 173, 175, 179, 205, 208, 212–214, 216, 218, 253, 327, 343, 374, 402, 568, 596, 600, 604, 605, 607–611, 627–629, 631, 632, 635, 636, 639, 642, 644–646, 649, 696
Chronobiology........29, 31
Chronomics........21
Clinimetrics........318, 319
CNS. *See* Central nervous system (CNS)
Coenzyme Q10 (CoQ10)........78, 380, 394, 444, 446
Cognitive function........400, 416, 419, 425–427, 435, 438, 443, 450, 452, 453, 458, 469, 504, 513, 515, 564, 604
Combination therapy........465–466, 472, 484, 510
Combined behavioral intervention (CBI)........603
Complex adaptive systems (CASs)........3–8, 14
Complexity........3, 4, 8–12, 14, 19, 28, 31, 130, 191, 201, 202, 208, 209, 252, 271, 325, 326, 507, 700
COMT. *See* Catechol-O-methyltransferase (COMT)
Constitutively active mutants
(CAMs)........142, 154–156, 159, 180, 195, 207
CoQ10. *See* Coenzyme Q10 (CoQ10)
Coronary artery disease (CAD)........6, 36, 262, 270, 271, 274, 340, 342, 348, 352, 356
CSF. *See* Cerebrospinal fluid (CSF)
CVD. *See* Cardiovascular disease (CVD)
Cystic fibrosis........687, 690
Cytochrome (CYP)........74, 76, 111, 314, 345, 351, 352, 364, 400, 405, 416, 434, 465, 472, 474–478, 482, 483, 486–487, 510, 511, 559, 560, 563, 577, 580, 595, 596, 609–610, 673, 675, 676, 701
  CYP2C9........76, 472–474, 476, 477, 482, 484–486, 510, 511, 519, 577, 673, 675, 676
  CYP2C19........412, 472–474, 476, 478, 482, 484–486, 511, 519, 561, 576, 577
  CYP2D6........76, 472–474, 476, 478, 482–484, 486, 508–511, 519, 559, 561, 562, 568, 572, 575–577, 579, 580, 595, 609–610, 692–694
Cytokines........5, 207, 217, 218, 220, 260, 267, 268, 270, 273, 274, 278, 279, 285, 287, 365, 404, 417, 457, 464, 469, 490, 495, 648, 675
Cytomegalovirus (CMV)........219, 220

D

Databases........21–25, 27, 28, 30, 38, 59–69, 71, 222–224, 246, 327, 483, 664, 679
Data integration........20, 26, 29–32
Data mining........20, 29–32
dbSNP. *See* Single nucleotide polymorphism database (dbSNP)
Decision support........20, 29–32, 80
Deconvolution........40–41
Dementia........323–327, 329, 336, 338, 341, 350, 353, 360, 361, 363, 365, 369–372, 374–376, 402, 410, 412, 443, 449, 471, 472, 476, 484, 485, 502, 505–507, 512, 514–519
De-orphanized receptors........155, 179
Depression........11, 12, 14, 27, 196, 197, 215, 216, 324, 326, 353, 376, 434, 455, 558, 600, 604

DHA. *See* Docosahexaenoic acid (DHA)
Diabetes....5, 27, 73, 157, 173–178, 197, 199, 215, 221, 260, 262, 317, 333, 334, 337–340, 342, 345, 346, 348, 351, 354, 355, 358, 403, 421, 436, 460, 505, 518, 570, 674
Diagnosis....12–14, 20, 24, 29, 32, 69, 175, 209, 302, 324, 325, 370, 371, 373, 374, 516, 517, 519, 666, 696
Diagnostic technique....46
Disease modifying anti-rheumatic drugs (DMARDs).... 625, 626, 639, 641
DNA
  methylation....23, 26, 27, 361, 468, 506, 592, 668, 676
  repair.... 28, 282, 302, 365
Docosahexaenoic acid (DHA).... 364, 380, 445–446
Drug metabolism....314, 326, 471, 472, 477–484, 487, 519, 563, 572, 577, 580, 636, 650, 672
Drug ontogeny.... 673, 688, 689
Drug resistance....6, 21, 487
Drug safety.... 28, 677, 679, 688
Dynamical diseases....11–12
Dynamical medicine....3–14, 19–32

E

ECM. *See* Extracellular matrix (ECM)
Efficacy.... 75, 77, 91, 108, 109, 138, 154, 155, 159, 179, 190–192, 195, 201, 203, 206, 207, 211, 213, 216, 245, 246, 301, 399, 409, 416, 423, 425, 427–429, 443, 453, 459, 487, 505, 508, 509, 512, 518–520, 557, 561, 564–567, 576–578, 580, 594, 602, 603, 606, 608, 613, 626, 630–636, 642, 648, 650, 667, 672–674, 677, 679, 688, 689, 698, 701, 703
Eicosapentaenoic acid (EPA)....445, 446
Electroencephalography (EEG).... 369, 370, 372
Electronic medical records (EMRs).... 26, 32, 71, 81
Electropherograms....59, 60, 64
Emergence....4, 5
EMRs. *See* Electronic medical records (EMRs)
Endophenotypes....83–84, 215, 216, 667
Endothelial cells (ECs).... 92, 102, 154, 211, 261, 264, 269, 271–273, 276, 277, 281, 284, 286, 340, 361, 450, 465, 487, 488, 501, 503
Endothelin....157, 178, 199, 212, 269, 273, 330, 491, 500
Epigenetic.... 13, 23, 26, 27, 190, 325, 327, 361, 380, 468, 563, 592, 611, 667, 668, 672, 676, 677
Epigenomics.... 21, 23, 26–27, 677
Epilepsy.... 11, 165, 195, 326, 331, 335, 336, 344, 351, 357, 358, 420, 471
Epithelial cells....92
Epstein–Barr virus (EBV)....220
Estrogen....278, 339, 350, 380, 439–440, 476, 482, 573, 663–665
Extracellular matrix (ECM)....260, 267, 268, 276, 368
Ezetimibe....96, 260–262, 280–281, 491, 495

F

Familial glucocorticoid deficiency (FGD)....172, 173
Familial hypocalciuric hypercalcemia (FHH).... 158, 162–164, 193
Fatigue....263
Feedback....6–7, 12, 124, 220, 266, 272
Feedback loops.... 6–7, 12, 220, 266
Follicle-stimulating hormone (FSHR)....153, 156, 168–170

G

Gain of function....124, 125, 141, 155, 164–166, 174, 177–178, 198
Gene clustering....217, 325, 326, 518, 519
Gene expression....4, 10, 13, 22–25, 27–31, 35–41, 83, 134, 190, 201, 223, 264–267, 269–275, 278–282, 284, 285, 305, 325, 360, 361, 415, 419, 437, 468, 487, 563, 564, 572, 601, 611, 627, 635, 649, 667, 672, 673, 675, 676, 679
Gene selection strategy....317
Gene set enrichment analysis (GSEA)....39
Gene silencing....376, 381, 394, 430, 432–433, 467–468
Gene therapy....381, 466, 517
Genome-wide association studies (GWAS).... 24, 32, 79–83, 112, 162, 190, 192, 202, 204–206, 212, 213, 215, 216, 218, 219, 225, 303, 309, 313, 316, 317, 319, 364, 374, 559, 567–568, 570, 571, 573, 575, 576, 580, 642, 649–652, 663, 665, 666, 668
Genotype.... 27, 29, 77, 78, 80, 81, 98, 105, 106, 109, 110, 191, 209, 223, 247, 249, 251, 252, 254, 255, 303, 309–311, 315, 316, 318, 360, 372, 374, 375, 446, 449, 472, 486, 501, 502, 504, 506–509, 511–513, 515, 519, 560–564, 568, 571–573, 576, 598–600, 603–612, 628–632, 634, 635, 637–639, 641, 642, 645–653, 664, 666, 667, 673, 675, 676, 679, 694, 698
  Genotyping....71, 75, 79, 81, 98–101, 111–112, 224, 255, 576, 577, 580, 609, 632, 638, 639, 652, 676, 690–692, 695
Gonadotropin-releasing hormone (GnRHR)....157, 170–172
G protein-coupled receptor (GPCR)....121–143, 153–180, 189–225, 248, 252, 269, 465, 609
G protein-coupled receptor kinases (GRK)....122, 126, 127, 132, 135–139, 141–143, 159, 248, 252
GWAS. *See* Genome-wide association studies (GWAS)

H

Haplotypes ... 78, 99–101, 108, 112, 132, 134, 135, 162, 194–196, 199, 205, 208, 212, 224, 225, 249, 310, 486, 488, 502, 509, 560, 563, 573, 603–605, 608, 628, 632, 643, 646–648, 651, 652
HDL. *See* High density lipoprotein (HDL)
Health informatics ... 20, 26, 29–32
Heart failure (HF) ... 5, 194, 199, 206, 207, 245–255, 346
Heat maps ... 39–40
Heat shock proteins ... 367, 375, 380, 394, 440–441, 644
High density lipoprotein (HDL) ... 260–266, 271, 278, 279, 283, 286, 287, 412, 504
High-throughput (HTP) ... 9, 13, 19, 24, 26, 190, 225, 271, 471, 508
Hippocampus ... 165, 363, 368–370, 375, 400, 404, 407, 408, 411–413, 425, 426, 428, 433, 437, 443, 444, 447, 452, 454, 457, 458, 460, 461, 467, 567
Histone modifications ... 361, 592, 677
HIV. *See* Human immunodeficiency virus (HIV)
HLA. *See* Human leukocyte antigen (HLA)
Homeostasis ... 79, 162, 165, 167, 168, 174, 201, 269, 272, 280, 345, 365–367, 402, 404, 413, 417, 436, 440, 449, 462, 487, 504
Homozygous ... 110, 142, 158, 162, 166, 168, 171, 200, 208, 248–254, 315, 457, 472, 509, 571, 598, 599, 601–610, 612, 627, 629–632, 635, 649, 691
HTP. *See* High-throughput (HTP)
5HTT. *See* Serotonin transporter gene (*5HTT*)
Human immunodeficiency virus (HIV) ... 102, 108, 158, 200, 217–219, 353, 491, 500, 589
Human leukocyte antigen (HLA) ... 574, 576, 642–644, 646, 647, 674
Hypercalciuria ... 164–166, 193
Hypertension ... 122, 125, 127, 131, 132, 134–136, 142–143, 194, 198, 201, 207, 263, 330, 333, 340, 348, 353, 367, 369, 471, 505, 507, 511, 512, 518
Hyperthyroidism ... 160, 161
Hypocalcemia ... 158, 161–167, 200, 335
Hypoparathyroidism ... 164–166

I

Idiopathic hypogonadotropic hypogonadism (IHH) ... 157, 170–172
Immunofluorescence ... 176
Immunotherapy ... 376, 378, 382, 419–422, 427, 502
Inflammation ... 5, 212, 213, 219, 222, 264–268, 273–275, 278, 284–285, 365, 407, 424, 432, 436, 446, 448, 449, 452, 455, 506
Infradian ... 10

K

Knowledge discovery (KD) ... 20, 29–31

L

Leptin ... 167, 270, 367, 432, 571
Lipidomics ... 21, 22, 25, 83, 84
Loss of function (LOF) ... 124, 125, 129, 154, 155, 157, 158, 162, 164, 166, 167, 169, 171, 175, 176, 178, 180, 198, 200, 207, 267, 591, 698
Low density lipoprotein (LDL) ... 77, 175, 260–262, 264–268, 271, 280, 286, 287, 330, 334, 335, 348, 349, 355, 412, 501
Luteinizing hormone (LH) ... 155, 156, 169–170, 333

M

Macrophage ... 213, 217, 218, 260, 264–266, 268, 270, 271, 274–276, 278, 279, 283–287, 330, 358, 369, 382, 442, 443, 448, 464
MCI. *See* Mild cognitive impairment (MCI)
Melanocortin ... 156, 167–168, 191, 224, 354
Melatonin ... 382, 454–455
Membrane transporters ... 91–112, 628, 650
Meta-analyses ... 645
Metabolism ... 11, 27, 70, 75, 76, 97, 111, 162, 166, 172, 216, 220, 278– 280, 282–284, 286, 302, 314, 326, 364, 369, 375, 418, 419, 436, 471–474, 477, 483, 484, 486, 487, 505–507, 510, 511, 515, 519, 559–563, 568–570, 572, 576, 577, 580, 592, 607, 609, 632, 634, 636, 638, 650, 672, 675, 677, 687, 691–694, 698, 700, 701
Metabolites ... 22, 25, 70, 76, 96, 98, 108, 365, 375, 402, 406, 410, 411, 438, 440, 444, 447, 487, 494, 500, 509, 560, 572, 574, 594, 597, 609, 611, 627, 636, 691, 693
Metabolomics ... 4, 21, 25, 370, 374–375, 517
Methotrexate (MTX) ... 94–97, 492, 496, 500, 625–636, 646, 652, 676
Methylenetetrahydrofolate reductase (MTHFR) ... 331, 471, 474, 510, 577, 596, 610–611, 626, 627, 629–634, 676
MicroRNAs (miRNAs) ... 11, 27, 35, 41, 83, 360, 361, 366, 368, 369, 376, 383, 394, 433, 466–468, 517, 677
Mild cognitive impairment (MCI) ... 361, 362, 365, 371–376, 449, 453, 463, 502, 506–508
Monogenic disease ... 154, 155, 169, 180, 191
MTHFR. *See* Methylenetetrahydrofolate reductase (MTHFR)
MTX. *See* Methotrexate (MTX)
Multifactorial therapy ... 512–516
Mutations ... 45, 59, 61, 64, 99, 124, 125, 129, 135, 139, 141, 142, 153–180, 192, 209, 220–224, 267, 317, 327, 450, 472, 487, 571

Myalgias 69, 70, 78
Myasthenia gravis (MG) 194, 207–208
Myocardial infarction 122, 201, 260, 262, 270, 330, 339, 349, 352, 354, 356
Myopathy 67–84, 109, 112, 260–262, 334, 341, 355
Myotoxicity 69, 70

N

Nephrogenic diabetes insipidus (NDI) 157, 173–178
Neurodegeneration 329, 360, 363–367, 369, 422, 441, 449, 461, 462, 467, 468, 471, 501, 517
Neuropathology 370, 400, 402, 424, 438, 450, 507
Neuropathy 108, 301–319, 330, 352, 676
Neurotransmitter 201–206, 336, 353, 363, 395, 399, 409, 437, 438, 442, 459, 472, 505, 558, 559, 563, 570, 572, 592, 594, 595, 599, 1990
Next generation sequencing (NGS) 23, 27, 101, 190, 192, 223–225
Nonlinearity 4, 5, 7–8, 10–12, 31
  nonlinear interactions 5, 11

O

Obesity 5, 11, 27, 122, 127, 128, 131, 156, 167–168, 194, 195, 197, 204, 207, 215–217, 260, 333, 334, 337, 341, 342, 348, 571
Oguchi disease 132, 135, 136, 141, 142, 160
Omega-3 445
Opioid 205–206, 223, 492, 496, 589–605, 608, 609, 611–613
Oscillations 10, 12, 31
Osteoporosis 11, 216, 217, 339, 349, 661–664, 666–668

P

Paraffin tumoral tissue 52
Parathyroid hormone receptors 129, 131, 157, 166
Parkinson's disease 11, 326, 335, 336, 339, 342, 349, 352, 353, 367, 376, 448, 451
Pathogenesis 31, 78, 134, 166, 179, 218, 264, 272, 273, 282, 286, 324–326, 360, 362, 364, 365, 367, 368, 415, 438, 443, 447, 449, 450, 463, 465, 471, 484, 487, 488, 501–503, 519, 648, 664, 668, 672, 698
Pathophysiology 7–8, 141, 203, 209, 213, 263, 326, 361, 365, 443, 462, 511, 563, 673, 698, 700
Pathways 5–7, 12, 13, 22, 24, 25, 30, 31, 35, 37–39, 41, 45, 75, 78, 83, 84, 107, 124–126, 130, 137, 139–143, 154, 155, 160, 161, 164, 167, 168, 171, 174, 178, 179, 192, 201, 203, 209, 213, 218, 220, 221, 260, 262, 263, 267, 268, 270–275, 278, 279, 281, 282, 284–288, 302, 314, 343, 364–366, 373, 395, 403, 406, 407, 416, 417, 419, 421, 425, 426, 428, 432–437, 439, 440, 443–445, 451, 455–458, 460, 462, 468, 501, 506, 509, 519, 563, 590, 592, 594, 597, 600, 602, 613, 626, 627, 632–634, 636, 638, 642, 646–648, 652, 663, 664, 672, 687, 690, 692, 698, 700
PCR. *See* Polymerase chain reaction (PCR)
Pediatrics 571, 671–679, 688–690, 695–697, 700
Personalized medicine 3–8, 13, 14, 19, 21–23, 27–32, 71, 190, 191, 206, 209, 301–319, 325–326, 558, 580, 613, 666–668, 672, 674, 687, 700–703
Personalized therapy 45, 46
PET imaging 370
P-glycoprotein (P-gp) 92, 97, 314, 362, 363, 366, 391, 412, 415, 416, 434, 436, 445, 470, 487, 488, 493, 501–504, 509, 561, 563, 607, 628
Pharmacodynamics (PD) 75, 191, 246, 463, 471, 510
Pharmacoepigenomics 667–668, 671–679
Pharmacogenetics 46, 59, 61, 80, 91–112, 127, 154–156, 169, 179, 189–225, 246, 247, 249–255, 470, 476, 482, 484, 486, 511, 518, 557–580, 589–613, 625–653, 664–668, 672, 674–677, 679, 690, 692
Pharmacogenomics 3–14, 19, 45–64, 79, 111, 121–144, 172, 190, 245–255, 259–288, 301, 323–520, 578, 591, 661–668, 671–679, 687–701
Pharmacokinetics (PK) 75, 77, 92, 98, 104, 107–110, 191, 246, 314, 317, 437, 438, 463, 471, 509, 510, 561, 563
Pharmacology 4, 131, 132, 154–156, 158, 179, 190, 191, 194, 196, 198, 200, 202–206, 209, 211–213, 216, 217, 219, 221, 223, 688, 698
Pharmacotherapy 245, 246, 254, 255, 590–600, 603, 606, 607, 612, 613
Phenotype 5–7, 12, 24, 27–29, 32, 35, 38, 40, 41, 81, 83, 100, 124, 127–129, 131, 132, 136, 141–143, 154–156, 158–161, 166–171, 173, 175–178, 180, 190–194, 196, 198, 200–202, 204–206, 208, 209, 213–216, 219, 221, 223, 225, 246, 253, 254, 274, 363, 374, 400, 421, 426, 440, 466, 483–486, 507, 509, 560, 573, 575, 576, 639, 652, 663, 664, 667, 676, 678, 694–696
  phenotyping 69–71, 83, 84, 219
Polymerase chain reaction (PCR) 46–51, 54–59, 61, 63, 101
Polymorphisms 6, 23, 76, 77, 79, 83, 98–101, 104–112, 125, 127, 132, 134–135, 142–143, 154, 155, 157, 158, 162, 163, 166, 167, 179, 190, 193, 195, 200–207, 209, 212–215, 217–219, 223, 224, 246–248, 265, 270, 301, 310, 313–318, 327, 361, 486, 501, 502, 506, 508, 510, 511, 518, 559, 561–567, 570–575, 577, 591, 592, 599, 600, 603, 605, 627–635, 637–648, 650, 651, 663, 664, 666, 674, 675, 677–679, 694, 697, 701
Polyunsaturated fatty acids (PUFAs) 364, 391, 445–446
Posttraumatic stress disorder (PTSD) 7

Potency ... 124, 138, 154, 155, 159, 179, 194, 206, 207, 211, 212, 287, 395, 396, 405, 412, 432, 437, 456, 692
Prognosis ... 12–14, 20, 29, 32, 361, 367, 376
Proteomics ... 4, 9, 13, 21, 25, 28, 83, 364, 370, 374–375, 515, 517, 519
Pyrosequencing ... 101

Q

Quality of Life (QoL), ... 259, 288, 302, 319, 695, 696

R

Regulator of G protein signaling (RGS) ... 122, 126, 128, 130, 132–135, 143, 269, 512
Renal dysfunction ... 263
Retinoic acid (RA) ... 159, 384, 392, 401, 404, 444, 445, 493, 499
Rhabdomyolysis ... 69–72, 76, 81, 83, 261, 262, 281, 282
Rheumatoid arthritis (RA) ... 6, 332, 334, 338, 339, 346, 353, 357, 358, 625–653
Rhodopsin ... 121, 123, 132, 136, 138, 141–143, 155, 156, 159–160, 191, 193, 211, 224
Rhythms ... 7, 10–12, 31, 36, 171, 372
  circadian ... 10, 171, 507
Rituximab ... 313, 625, 650–651

S

Schizophrenia ... 11, 27, 191, 195–198, 202–204, 215, 326, 331, 349–351, 358, 376, 511, 557–580, 597, 600, 608
Selective serotonin reuptake inhibitors (SSRIs) ... 605
Self-organization ... 4, 6–7
Serotonin reuptake transporter (SERT) ... 363, 505
Serotonin transporter gene (*5HTT*) ... 562, 564, 577, 605, 606, 677
Signaling ... 11, 30, 84, 121–143, 154, 155, 159–161, 164, 167, 168, 170, 173, 174, 178–180, 192, 197, 202, 204, 206–208, 210, 212–215, 220, 221, 264, 267, 269, 270, 273–275, 278, 279, 284, 286, 343, 365, 366, 376, 395, 399, 402–404, 406–408, 411, 417, 421, 431–434, 437, 439, 441, 442, 445, 447, 451, 452, 455, 511, 517, 592, 593, 597, 608, 611, 642, 644, 647, 648, 650
Single molecule real-time (SMRT) sequencing ... 101
Single nucleotide polymorphism database (dbSNP) ... 21, 23, 30, 193, 224
Single nucleotide polymorphisms (SNPs) ... 21, 23, 76–82, 98–101, 104, 105, 108–110, 112, 131, 132, 134, 143, 155–157, 162, 168, 190, 191, 193, 195–205, 207, 208, 212, 214, 218, 224, 265, 301, 305, 306, 308, 313–318, 327, 360, 361, 374, 484–488, 501, 502, 508, 510, 512, 518, 559, 563, 574, 583, 591, 597, 599, 601, 606, 608, 610–612, 627–632, 634, 635, 642–652, 663–665, 697, 698
Spatial ... 7–11, 13, 14, 28, 29, 271, 368, 395, 415, 416, 437, 438, 440, 441, 447, 448, 451, 454, 457, 460, 466, 469, 662
  dimension ... 8–9, 13
Statins ... 67–71, 74–81, 84, 95–97, 101, 109, 110, 112, 260–262, 271–282, 393, 432, 493
Stem cells ... 92, 368, 464, 468, 469, 517
Stress ... 5, 7, 9, 130, 250, 260, 270, 281, 282, 314, 315, 361, 364, 366, 367, 369, 373, 395, 403–405, 409, 415, 418, 432, 435, 443, 445, 446, 452, 453, 460, 466, 519, 572, 595, 605, 612, 662, 677
Stroke ... 122, 260–262, 265, 324, 340, 349, 354, 363, 400, 414, 435, 460, 461, 505
Sulfasalazine ... 96, 109, 494, 496, 625, 639, 641
Symptom clusters ... 557, 558
Systems biology ... 3–14, 19, 25, 29, 31
Systems medicine ... 3, 21–26

T

Tardive dyskinesia (TD) ... 195, 196, 203, 204, 331, 352, 557–559, 570, 572–575, 580
Telomere ... 366, 368
Temporal ... 7–14, 28, 29, 271, 361, 363, 369, 371, 375
Temporal dimension ... 8–11, 14, 28
Tensor-based morphometry (TBM) ... 371
Testotoxicosis ... 128, 169–170
Therapeutic mechanisms ... 263, 269–271
Therapeutic strategies ... 4, 20, 213, 263, 323–520, 678
Therapeutic targeting ... 265, 267
Thiopurine methyltransferase (TPMT) ... 472, 476, 481, 636–640, 675–677, 690–692, 695, 697
Thrombosis ... 260, 268, 276–277, 358
Thyrotropin ... 129, 155
Time-series ... 10, 12, 13, 31
TNF. *See* Tumor necrosis factor (TNF)
Tocilizumab ... 625, 651
TPMT. *See* Thiopurine methyltransferase (TPMT)
Transcription factor ... 83, 220, 272, 280, 332, 343, 349, 352, 354, 358, 369, 394, 444, 448, 452, 453, 460, 563, 570, 610, 611, 627, 648, 649
Transcriptome ... 13, 25, 28, 29, 31, 35, 41, 278, 325, 326, 375, 502
Transcriptomics ... 13, 21, 23, 27, 35–41, 83, 281, 517
Tumor necrosis factor (TNF) ... 285, 338, 343, 394, 404, 410, 448, 450, 469, 494, 499, 500, 625, 641–650
Type 2 diabetes ... 5, 197, 221, 260, 342, 345, 421

U

Ultradian ... 10

V

Variable number tandem repeat (VNTR)
Variants ... 45, 46, 59–61, 64, 76–83, 92, 96, 98–101, 105, 106, 108–110, 112, 122–125, 127–129,

131–136, 142, 143, 154–156, 158–162, 165–170, 172, 173, 178, 180, 190–198, 201–219, 221–225, 246, 247, 250–255, 269, 307, 327, 329, 353, 360, 364, 365, 370, 441, 472, 482, 483, 486–488, 502, 505, 506, 508–512, 519, 559–564, 566–571, 573–580, 590–593, 595–597, 599–601, 603–605, 607–612, 628, 629, 631, 635–637, 639, 641, 642, 646–652, 666–667, 675, 676, 678, 679, 692, 698, 699
Vasodilation....273, 435
Vasopressin receptor....157, 173–175
Vitamin C....443
Vitamin D....349, 443–444, 494, 500, 503, 665
Vitamin E....444, 492, 494, 496, 498–500

W

Whole genome sequencing....80–81
Wnt....343, 412, 437, 663, 664, 668

X

Xenobiotics....107, 314, 475, 479, 487, 494