ASSOCIATION OF GENETIC AND NONGENETIC VARIABILITIES WITH PHENYTOIN AND CARBAMAZEPINE RESPONSE PHENOTYPES

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Abstract

In spite of the availability of an increasing numbers of antiepileptic drugs (AEDs), drug treatment of epilepsy remains symptomatic with a manifestation of a large variability in drug response to AEDs among patients. At present, it is unclear how drug-resistant epilepsy as well as the variability in response among individuals to AEDs happens. Drug-resistance and variability to drug response are proposed to be a multifactorial and complex genetic trait. The variability is under the influence of several putative factors including patient's genetic and nongenetic variability that affect drug disposition and action.

Therefore, the objective of the two pharmacogenetic studies include in this dissertation was to investigate the combined associations of common genetic variations in genes encoding drug metabolizing enzymes, drug transporters and drug target along with nongenetic variations on the clinical phenotypes of drug response to phenytoin (PHT) and carbamazepine (CBZ) namely maintenance dose and drug exposure.

These two studies were cross-sectional genetic association studies using a candidate gene approach. Retrospective data were used. The study populations were patients with epilepsy who were unrelated Caucasian Americans or African Americans that were enrolled in the P50 studies.

For PHT pharmacogenetic study, a dataset of 54 adult Caucasian patients with epilepsy on PHT maintenance therapy was used. Demographic and clinical variables were retrieved. Genomic DNA samples were used to genotype for 5 candidate single nucleotide polymorphisms (SNPs): *SCN1A* c.IVSN5+5 G>A, *ABCB1*c.3435C>T, *CYP2C9*2* (g.3608C>T), *CYP2C9*3* (g.42614A>C) and *CYP2C19*2* (g.19154G>A). Steady-state $AUC_{0\text{-}12\ hr}$ was determined from PHT plasma concentrations at 0, 0.08, 0.25, 0.5, 1, 2, 4, 6 and 12 hours after an oral dose. Bivariate analysis as well as stepwise multiple linear regression analysis were used to determine the association of genetic and nongenetic variants with PHT maintenance dose and $AUC_{0\text{-}12\ hr}$. This study identified two non-genetic factors (body weight

and age) and three genetic variants (*CYP2C9*2, CYP2C9*3* and *CYP2C19*2)* that were strongly associated with variability in PHT maintenance dose in adult Caucasian patients with epilepsy. These covariates explained about 40% of variability in PHT dose requirement in this sample. Moreover, PHT dose and a genetic factors including *CYP2C9*3* and *ABCB1*c.3435C>T were found to be associated with increase in phenytoin AUC $_{0\text{-}12\ hr}$ in the same group of adult Caucasian patients. These covariates explained about 42% (R^2= 0.422, *P*= 0.007) and 76% (R^2= 0.760, *p*< 0.001) of variability in phenytoin AUC $_{0\text{-}12\ hr}$ in two different multiple linear regression models.

For CBZ pharmacogenetic study, demographic and clinical variables were retrieved from datasets of 55 unrelated adult Caucasian American and 32 African American patients with epilepsy on CBZ maintenance therapy. Genomic DNA samples were used to genotype for 5 candidate SNPs including *CYP3A5*3* (g.6986A>G), *CYP3A5*6* (g.14690G>A), *CYP3A5*7* (g.27131_27132insT), *SCN1A* c.IVSN5+5 G>A and *ABCB1*c.3435C>T. Steady-state AUC$_{0\text{-}24\ hr}$ was determined from CBZ plasma concentrations at 0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 12 and 24 hours after oral doses. Bivariate analysis as well as stepwise multiple linear regression analysis were used to determine the association of genetic and non-genetic variants with CBZ response phenotypes, namely CBZ maintenance dose and AUC$_{0\text{-}24\ hr}$ -to-dose ratio (ADR) in Caucasians or African American patients. By using multiple linear regression analysis, this study found a significant association between CBZ maintenance dose and a non-genetic factor, age, and a genetic variant, *CYP3A5*3.* The two covariates explained about 9% (R^2 = 0.089, *P* = 0.020) of inter-individual variability in CBZ maintenance dose*.* In line with that, carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio was found to be associated with the presence of *CYP3A5*3* alleles. The model explains about 32% of the variability in carbamazepine AUC $_{0\text{-}24\ hr}$-to-dose ratio (R^2= 0.324, *P*< 0.001).

In addition, it was found that carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio was significantly different between African and Caucasian American patients. In bivariate analysis, carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio was found to be associated with race, *CYP3A5*3,*

*CYP3A5*7* or *ABCB1c*.3435C>T allele suggesting that there might be the influence of multiple polymorphisms on CBZ pharmacokinetics which may resulted in the different exposure to CBZ between African and Caucasian patients.

These two pharmacogenetic studies clearly confirm that drug response is a complex multifactorial phenotype influenced by many genetic and nongenetic factors. However, the genes and variants identified so far explain only a small fraction of variability in response to AEDs, and still needed to be replicated by other independent study.

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Chapter One
Introduction

Epilepsy is one of the world's most common noncommunicable diseases. It is estimated that the mean prevalence of active epilepsy is 8.2 per 1,000 of general population[1]. Thus around 50 million people worldwide have epilepsy at any one time[2]. As a result patients with epilepsy, especially with chronic epilepsy, have a higher risk of premature death comparing to general population[3].

In spite of the availability of increasing numbers of antiepileptic drugs (AEDs), the treatment of epilepsy remains symptomatic. Furthermore, there is a large variability in drug response to AEDs among patients. Namely, more than 30% of patients with epilepsy continue to have intractable seizures despite an adequate drug treatment[4-7]. As for the individual patient, the outcome and response to drug treatment may be inherent, and therefore seizure control in some patients may be difficult from the onset [1].This results in substantial individual and social costs[8-11].

To date, the goal of antiepileptic drug therapy is to completely prevent seizures without causing side effects. This goal is still difficult to achieve, since there is a marked variability in clinical response among patients to AEDs. As the result, even with an appropriate drug of choice there is a wide range of effective doses required by each individual[12].

At present, the etiology of the majority of epilepsies remains undetermined. In addition, the mechanisms of action of many AEDs are still poorly understood. Therefore, it is unclear how intractable or drug-resistant epilepsy as well as the variability in response among individuals to AEDs happens.

Drug-resistance and variability to drug response are thought to be a multifactorial and complex genetic trait[13]. The variability is under the influence of several putative factors that

include the patient's genetic background, pathogenesis of epilepsy, autoimmunity, age, gender, comorbidities and comedications that affect drug disposition and action [14, 15]. The major proposed genetic mechanisms are the alteration in protein functions in pharmacokinetic and pharmacodynamic pathways of AEDs. First, alterations of the disposition or delivery of AEDs, by altering expression or functions of drug metabolizing enzymes or drug transporters, may result in reduction of active drug concentration in the blood and therefore at their sites of action in the brain. Second, genetic or functional alterations of drug targets and their down steam pathways, may impair the therapeutic effects of AEDs at their specific targets, even though the drug can reach the brain properly [14, 16-18].

Phenytoin (PHT) and carbamazepine (CBZ) are the effective and inexpensive AEDs that are used as the first choice in the treatment of simple partial, complex partial and generalized tonic-clonic seizures. However, control of epilepsy with PHT or CBZ is difficult because of their narrow therapeutic index and complicated pharmacology. Furthermore, adverse drug reactions (ADRs) are common in both drugs [12].

Given the response to AEDs is un-predictable, the optimal dose is often determined by the process of trial and error which may take months [19, 20]. Therefore, the ability to estimate the dose required by individual patients to improve both efficacy and safety of AED therapy is important.

Therefore, this pharmacogenetic study is conducted to investigate genetic and non-genetic factors that are associated with variability in response to phenytoin and carbamazepine in Caucasian and African American patients with epilepsy. The goal is to quantify the association between the AEDs' phenotypic response and these factors and to develop an algorithm which ultimately may be used for individualized AED therapy.

Statement of problem

In spite of the availability of new drugs, successful treatment outcomes in epilepsy with antiepileptic drugs is approximately 70%, and there is a large inter-individual variability in response to AEDs.

Phenytoin and carbamazepine are cost-effective AEDs that are used widely as first-line drugs in the treatment of epilepsies [21]. However, about 30% of newly treated patients with epilepsy do not respond to treatment with the single AED[22, 23]. It is known that patients who have inadequate response to initial treatment with AEDs are likely to have refractory epilepsy[6].

In addition, control of epilepsy with PHT or CBZ is difficult because of their narrow therapeutic index, complicated pharmacology, and common adverse drug reactions. It is also accepted that some patients may have adverse effects or drug toxicities generated from low doses of PHT and CBZ and limits their clinical utility [24, 25]. It is clear that the currently available strategies and treatments are not universally effective. Given the response to AEDs such as PHT and CBZ is unpredictable, the usefulness of these effective drugs has been limited. Therefore, it is crucial to quickly identify the right drug and the appropriate doses for individual patients to decrease the danger of ineffective treatment. This ability will not only increase efficacy but also safety of the AED therapy.

Purpose

This present pharmacogenetic study seeks:

1. To investigate the association of genetic variants, single nucleotide polymorphisms (SNPs) in candidate genes that encode proteins involving the main pharmacological pathways of PHT and nongenetic variants with variability in response to PHT treatment in Caucasian American patients with epilepsy.

2. To use the identified knowledge to develop a statistic model that combines genetic and nongenetic factors to estimate PHT maintenance dose a priori.

3. To investigate the association of genetic variants, single nucleotide polymorphisms (SNPs) in candidate genes that encode proteins involving the main pharmacological pathways of CBZ and nongenetic variants with variability in response to CBZ treatment in African American and Caucasian American patients with epilepsy.

Significance of the study

The knowledge gains from this pharmacogenetic study would provide evidence that variants in genes encoding drug target, drug transporters and drug metabolizing enzymes combined with nongenetic variants could explain variability in drug response to phenytoin or carbamazepine in patients with epilepsy.

These finding may also prove the concept that drug responsiveness is a complex polygenic phenotype, where variability in drug response is affected by several genetic variants, each contributing only a small effect to the variability in response to AED.

Furthermore, the results of this study would provide insight into phenotypic variation among African and Caucasian American patients with epilepsy on CBZ therapy. If phenotypic variation exists, it may in part be explained by racial or ethnic differences in genetic background.

More importantly, as it is accepted that by using the available strategy to treat epilepsy, there are difficulties associated with selection of appropriate AEDs and dose adjustments. The use of multivariate analysis to search for and quantify the association of genetic and nongenetic variants with variability in responsiveness to AEDs will offer the ways to identify subgroups of patients most likely to respond well, unlikely to respond or likely to develop toxicity. The findings from this study may therefore, lead to a prospective evaluation of how pharmacogenetic tests can guide the selection of the most appropriate AED and/or dosage for

individual patients. This could potentially result in individualized therapy with better seizure control, fewer side effects and ultimately improvements in a patient's quality of life.

Chapter Two
Literature review

The need for a more effective approach for antiepileptic drug therapy

Epilepsy is one of the most common serious neurological disorders. The prevalence rates of active epilepsy have been estimated to be between 4-10 /1,000 people in general population [1]. In resource-poor countries, the prevalence and incidence rates of epilepsy are higher [1, 2]. It is estimated that more than 80% of people with epilepsy live in developing countries where the majority of them do not receive any effective treatment [26]. In addition to limited availability of the much needed antiepileptic treatment in developing countries, antiepileptic drugs (AEDs) themselves also pose a significant drawback in advancing epileptic treatment. Despite new antiepileptic drugs (AEDs) developed, drug treatment in epilepsy is still characterized as unpredictable in clinical efficacy, adverse drug reactions and optimal dosage for individual patients.

Large variability in drug response to AEDs is an important problem in epilepsy therapy. Among patients with clinically identical seizures, some remain easily controlled with a simple medication, while some become seizure-free without medication, and the others are not satisfactorily managed with the present available drugs or become drug resistant. This variability in response to AEDs results in a wide range of maintenance doses required by different patients. The required dose may differ up to four-fold among individuals.[25] Regarding a varied and complicated natural history of epilepsy, studies have shown that more than 30% of epileptic patients do not respond to treatment with a single AED [6, 22, 23]. Furthermore, patients who did not respond to the first AED are likely to have refractory epilepsy [6]. These patients are often disabled by their illness, have an unsatisfactory quality of life, and are at a higher risk of sudden death. [27, 28]

The burden of drug resistant or refractory epilepsy can be conceptualized as the economic, social, and psychological consequences on patients, families and society at large [29]. In the United States, Begley et al. assessed economic burden of intractable epilepsy and showed that the only 35% of intractable epilepsy responsible for 79% ($8.5 billion USD) of all lifetime costs of the epilepsy population ($11.1 billion USD) [10, 30]. In addition, intractable epilepsies have larger indirect costs or time loss due to illness or health care (88%) as compared to those with treatable epilepsy (15%) [10, 30].

Given the response to AEDs is un-predictable, the optimal therapy is often determined by the process of trial and error which may take months.[19, 20] Therefore, the more effective approach to determine the optimal therapy required by individual patients to improve both efficacy and safety of AED therapy is important.

Current understanding of underlying mechanisms of variability in drug response

At present, the mechanisms underlying the development of drug-resistant epilepsy and drug response variability are complex and not fully understood. Variability to drug response is thought to be a multifactorial and complex genetic trait.[31] In other words, they are likely to be caused by genetic variation in several genes, together with non-genetic factors. [15, 17] The interplay of these factors determines the profile of the plasma concentration that will reach its target site of action. Too little exposure leads to an ineffective drug regimen, whereas too much exposure may lead to adverse effects.[14, 32] Furthermore, even though an appropriate drug level may reach its target, variability in drug target sensitivity may leads to variable drug response.[32]

First line antiepileptic drugs: therapeutic management problems remain

Phenytoin (PHT) and carbamazepine (CBZ) are effective and inexpensive drugs of first choice in the treatment of simple partial, complex partial and generalized seizures.[21] However, control of epilepsy with PHT or CBZ is difficult because of large interindividual variability in drug response. PHT has a narrow therapeutic index and nonlinear pharmacokinetics. CBZ exhibits auto-induction of metabolism.[33, 34] Ultimately adverse drug reactions (ADRs) are common in both drugs.

Phenytoin and carbamazepine: Pharmacogenetics

Phenytoin (5, 5-diphenylhydantoin; PHT) is metabolized in humans almost completely by oxidation. The major and rate-limiting metabolic pathway in human is *4*-hydroxylation of phentoin to form 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH) and dihydrodiol pathway. HPPH is an inactive metabolite and accounts for 70-90 % of the total urinary metabolite. There is a large inter-individual variability in the metabolism of PHT.[35]

Studies *in vitro* and *in vivo* clearly showed that the major enzymes responsible for PHHP formation is cytochrome P450 **(**CYP)2C9 [36] [37] [38] with minor contribution by CYP2C19 [36, 39, 40] and CYP3A4 [41]. CYP2C9 catalyzed the formation of both (*R*) and (*S*)-HPPH but is highly stereoselective for (*S*)-HPPH. CYP2C19, on the other hand, is more responsible for the formation of the (R) HPPH. [36]

Genetic variations in *CYP2C9* AND *CYP2C19*

Genes encoding the CYP2C subfamily are located on chromosome 10q24 and comprises four genes arranged in the order of *CYP2C8-CYP2C9-CYP2C19-CYP2C18*. The *CYP2C9* and *CYP2C19* genes are highly polymorphic. At present, more than thirty variant alleles of *CYP2C9* have been identified where the most common are *CYP2C9*2* and *CYP2C9*3*. [42] The wild-type, *CYP2C9*1*, encodes enzyme containing arginine at position 144 and isoleucine at position 359. The variant allele, *CYP2C9*2,* is due to g.3608C>T substitution on exon 3 which

produces enzyme with a single amino acid substitution at position 144, an arginine to cysteine (Arg144Cys). [43] *CYP2C9*3* variant allele is associated with g.42614A>C substitution on exon7. This results in an enzyme with substitution of isoleucine by leucine at position 359 (Ile359Leu). While the metabolic activity of CYP2C9.2 is moderately decreased compared with that of CYP2C9.1[43] [44]; CYP2C9.3 shows a markedly decreased activity.[45]

In contrast to *CYP2C9*, most variant alleles of *CYP2C19* result in nonfunctional or absent enzymes.[46] The wild type forms are *CYP2C19*1A* and *CYP2C19*1B*. The variant *CYP2C19*2* allele is due to g.19154G>A substitution on exon 5, whereas *CYP2C19*3* is associated with g.17948G>A substitution on exon 4, both leading to aberrant splice sites and inactive enzymes.[46, 47]

Poor metabolizers of phenytoin have been shown to have either variant in *CYP2C9* or *CYP2C19*. Although *CYP2C9*3* is the primary determinant of slow phenytoin metabolism, defective *CYP2C19* alleles also contribute, especially at high doses.[40, 48] A 4-fold increase in AUC and decrease in PHT clearance have been shown in individuals with homozygous *CYP2C9*3* allele compared with wide-type subjects [49, 50]. Decreased maximum metabolic rate (Vmax), increased PHT serum concentration and increased Michaelis constant (Km) have been reported in poor metabolisers (PMs) of *CYP2C19*2* which is 54% higher than that of extensive metabolizers (EMs).[51-53]

There is a significant difference in *CYP2C9* allelic variants frequencies among racial groups. The allele frequency of *CYP2C9*2* has been reported as 8-19% in Caucasians, 1-4% in Africans and 0% in East Asians. Slightly different from *CYP2C9* allelic variants, the allele frequency of *CYP2C9*3* has been reported as 3-15%, 0.5-2%, and 1-6% in Caucasians, Africans and East Asians, respectively.[42, 54, 55] Similar to *CYP2C9*, the variant allele frequency of *CYP2C19* varies significantly among ethnic groups. The allele frequency of *CYP2C19*2* has been reported as 11-13% in Caucasians and Africans, but 27-37% in East

Asians. In contrast, *CYP2C19*3* allele is rare or even absent in Caucasians; however, its allele frequency is slightly more prevalent in East Asians (5-11%).[56]

CBZ is extensively metabolized in the body where its biotransformatoin occurs mainly in the liver. Several matabolites are formed by parallel or consecutive reactions. The major pathways of CBZ biotransformation include the epoxide-diol pathway, aromatic hydroxylation, and conjugation. Among its three main pathways, epoxide-diol pathway is the most important CBZ biotransformation. First, CBZ is oxidized at the 10,11 double bond to the chemically stable CBZ epoxide which is pharmacologically active.[57] CBZ epoxide is then extensively hydrolyzed to trans-CBZ-diol. The diol is excreted into the urine as a glucuronide conjugated and unconjugated form. [58]

CBZ epoxide has an anticonvulsant effect in the animal model with epilepsy and might therefore contribute to the clinical effects of CBZ in human.[58] On the other hand, CBZ-diol and other metabolites have no or little anticonvulsant activity.

CBZ epoxidaion is mediated by CYP isozymes, mainly by CYP3A4 and partly by CTP2C8.[57, 59] By using heterologously expressed CYP3A4 and CYP3A5 and phenotyped human liver microsome, Huang et al. has shown that CYP3A5 exhibited comparable metabolic activity as CYP3A4 toward CBZ epoxidation [60]. Large interindividual variabilion in CYP3A expression both in the liver and small intestine may contribute greatly to variability in oral bioavailability and systemic clearance of CYP 3A substrates.

Genetic variations in *CYP 3A5*

The CYP3As are the most abundant drug metabolizing enzyme expressed in human liver, intestine and kidney. [61] [62] Among the four members, CYP3A4 is responsible for most CYP3A-mediated metabolism with some contribution of the minor isoforms CYP3A5, CYP3A7 and CYP3A43. There is a large interindividual variability in CYP3A expression and function. [61] CYP3A4 alone cannot fully explain the variability because its genetic variants are

uncommon and have limited effect on enzyme function. On the other hand, CYP3A5 which is highly variable expressed in human, may greatly contribute to the variability.[63]

The human *CYP3A* gene is located on position q21-q22.1 of chromosome 7.[64] The *CYP3A* locus consists of *CYP3A4, CYP3A5, CYP3A7* and *CYP3A43* genes and three pseudogenes *CYP3AP1, CYP3AP2* and *CYP3AP3*.[65, 66] The four genes are located in the order of *CYP3A43-CYP3A4-CYP3A7-CYP3A5*. [56]

In most of the human liver, CYP3A4 is expressed at higher levels than the other three minor isoforms and contribute largely to CYP3A-mediated drug metabolism. However, CYP3A5 may be expressed at higher levels in extrahepatic tissues than CYP3A4.[65]

CYP3A5 is polymorphic and its expression is more variable than CYP3A4 [67]. There is a large variability in hepatic and extrahepatic CYP3A5 expression. Only about 20% of human liver expressed this isoform.[68] The highly polymorphic *CYP3A5* leads to a severe decrease in the synthesis of functional CYP3A5 protein. The most common polymorphism is due to a splice site variants of g.6986G>A of intron 3. While the A allele (wild-type or *CYP3A5*1*) encodes a normal splice CYP3A5, the variant G allele (*CYP3A5*3*) introduces a stop codon which leads to premature termination of translation. In contrast to most CYPs of which the *1 is usually the most common allele, for *CYP3A5*, the *3 allele appears to be the most common allele found in some populations including Caucasian. [66] *CYP3A5*3* is also the most common variant associated with the absence of liver CYP3A5. Studies have shown that *CYP3A5*3* homozygotes have no CYP3A5 protein expression in the liver. Individuals with at least one *CYP3A5*1* allele express metabolically active CYP3A5 protein. [67]

The other common variants with altered activity or expression are *CYP3A5*6* and *CYP3A5*7* which are common among African Americans. *CYP3A5*6* is a variant in exon 7, g.14690G>A, resulting in splicing defect and leading to the deletion of this exon from mRNA. The *CYP3A5*7* with a single insertion or g.27131-32insT resulting in frameshift mutation leading to a premature termination of translation.[69]

*CYP3A5*3* is the most common defective allele with an allele frequency of about 90%, 75% and 20% in Caucasians, Asians and Africans, respectively. On the other hand, *CYP3A5*6* and *CYP3A5*7* are not present in Caucasians or Asians but with a frequency of 17% and 8% respectively in Africans.[66]

Both PHT and CBZ are possible substrates for drug efflux transporters, P-glycoprotein (Pgp) [70-73] located in human tumors and several normal tissues including the intestine, liver, kidney and blood brain barrier (BBB).[74, 75] Based on its distribution in organs of drug absorption, distribution, metabolism and excretion, Pgp can be expected to play an important role in the disposion and distribution of its substrates [76]

Genetic variability of *ABCB1*

Pgp is encoded by ATP-binding cassette subfamily B member 1 (*ABCB1*) or multidrug resistance-1 (*MDR-1*) gene located on chromosome 7q21.1. More than one hundred polymorphisms have been identified in *ABCB1* gene. The human *ABCB1* gene is composed of 28 exons. A common synonymous single nucleotide polymorphism (SNP) in exon 26, *ABCB1* c.3435C>T, was the first variant to be associated with altered protein expression and function in humans, although the SNP does not change the encoded amino acid. The 3435T allele was associated with lower levels of Pgp in the duodenum and resulted in higher plasma concentration of digoxin. [77] Pgp over-expression in focal tissue has been found in patients with intractable epilepsy.[78-80]

In 2003, Siddiqui et al. reported the association of *ABCB1* c.3435C>T with resistance to multiple AEDs.[81] Further studies have shown that the polymorphism alone or in haplotype with other polymorphisms in *ABCB1* gene was associated with resistance to AEDs [82-87]. However, some other studies failed to replicate the association of *ABCB1* 3435C>T with drug-resistant epilepsy.[88-93]

The influence of the synonymous variant, *ABCB1*c.3435C>T, on *ABCB1* mRNA and P-gp expression is still controversial. Based on the available evidences, both C and T alleles showed opposite association with P-gp expression in the intestine [77, 94] and epileptic brain[78] in different populations. In the Japanese population, the T allele(s) is associated with higher expression of *ABCB1* mRNA in duodenal enterocytes. On the other hand, the CC genotype was reported to be associated with higher expression of intestinal P-gp than the TT and C/T genotypes in Caucasians.[77] In addition, the T/T genotype was associated with drug resistance epilepsy in Japanese patients with AED therapy[85] in contrast to the C/C genotype in Caucasians[81].

Recently it was found that the synonymous variant, *ABCB1*c.3435C>T, changes substrate specificity by altering conformations, rather than levels, of mRNA and protein. This study demonstrated for the first time that silent SNPs can lead to the synthesis of protein product with the same amino acid sequence but different structural and functional properties.[95]

Marked differences in the allele frequency of *ABCB1*c.3435C>T were observed between the African and Caucasian-Asian populations. The C allele is more frequently present in African population (73% to 90%) compared with Caucasians (43% to 54%) and Asians (34% to 62%).[96]

Together with variation in genes encoding enzymes involving in the major metabolic pathways of PHT and CBZ, variability in drug efflux transporter might alter drug disposition and bioavailability, and prevent their distribution into site of action in the brain, leading to inter-individual variability in drug exposure and subsequently altered maintenance doses.[97, 98]

The primary mechanism of PHT and CBZ to protect against partial and secondarily generalized tonic-clonic seizures is mediated by inibiting the brain voltage-gated sodium (Na_v) channels [99] [100-102]. Voltage-gated Na_v channels mediate regenerative inward currents that are responsible for the initial depolarization (rising phase) of action potentials in the neurons. Several concomitant effects of PHT and CBZ on Na_v channels include reducing the maximal

amplitude of sodium current, reducing the available number of Na_v channels by shifting the inactivation into a hyperpolarization stage and slowing the recovery from inactivation[103] [17]. Both PHT and CBZ have a common diphenyl moiety and bind to a common recognition site on the α-subunit of sodium channels [104]. Complete loss of sodium channel blockage has been shown in hippocampal tissue from patients with carbamazepine-resistant, chronic epilepsy [105].

Voltage-gated Na_v channels are heteromultimeric membrane proteins consisting of a large pore-forming α-subunit and smaller auxiliary β-subunits, β1 and β2. The α-subunit forms the ion conducting pore of the channel and the channel gate that regulate sodium flux. [106] Nine genes (*SCN1A, SCN2A, SCN1A SCN3A, SCN4A, SCN5A, SCN6A, SCN7A, SCN8A* and *SCN9A*) encode different Na_v channel α-subunits ($Na_v1.1$, $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.4$, $Na_v1.5$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$). These different α-subunits however exhibit a high degree of sequence and structure similarity. [107]

Genetic variations in *SCN1A*

The *SCN1A* gene encodes the brain sodium channel α-subunit. To date, more than 300 variants in *SCN1A* gene have been documented. The associated phenotypes range from benign febrile seizures to serious conditions such as Dravet's syndrome as well as variability in drug response. Sodium channel transcripts undergo alternative mRNA splicing. In a previous pharmacogenetic study, a common intronic polymorphisms in a splice donor consensus sequence in the *SCN1A* gene*, SCN1A*IVS5N+5 G>A or formerly *SCN1A* IVS5-91 G>A (rs3812718), has been reported to be significantly associated with high maximum doses during the clinical use of both PHT and CBZ [108].

Further studies suggest that the A allele of the *SCN1A* IVS5N+5 G>A disrupts the consensus sequence of exon 5N affecting the alternative splicing at exon 5 of *SCN1A* and resulting in a decreased expression of Nav1.1-5N transcripts. [108] By using whole-cell patch clamp technique, Thompson et al. have demonstrated that $Na_v1.1$-5N channels exhibited

enhanced tonic block by PHT and lamotrigine compared to the $Na_v1.1$-5A. The results suggested that $Na_v1.1$ channels containing exon 5N are more sensitive to PHT and lamotrigine.[109]

These results provide evidence that polymorphisms in genes encoding drug metabolizing enzymes, drug transporters and drug targets may explain and predict variability in drug response of patients with epilepsy to AEDs.

Pharmacogenetic study of drug response

At present, little is known about genetic variations underlying variability in drug response and drug-resistant epilepsy which is postulated to be a complex trait. As all complex disease phenotypes, an individual's risk typically depends on functions of many known and unknown genetic and environmental factors, each has a moderate effect on phenotypic variation. It is unlikely that a single factor is sufficient for phenotypic variability. In addition to functional studies, pharmacogenetic studies attempt to identify genetic variants that are associated with the functional phenotypes of drug responsiveness or drug resistance. To date, very few susceptible variants have been unambiguously identified to be associated with drug-resistant epilepsy and variability in drug response phenotype. Genetic association studies examine links between genotypes and phenotypes to detect the association between one or more genetic polymorphisms (genotype) and one or more traits (phenotypes) either quantitative or discrete ones. Genetic association studies are therefore used as a statistical tool to identify such genotype-phenotype association that might exist. It is hoped that this will increase our understanding about factors underlying the variability in drug response or drug resistance and lead to the development of improved therapeutic strategies.

Chapter Three
Conceptual framework and Hypotheses

Several genetic and biological theories as well as empirical evidence from experimental and clinical studies guided the conceptual framework of this study. The first section of this chapter lays down the concept of the genetics of complex diseases describing the nature of variability in drug responsiveness. The following section explains genetic and non-genetic factors that influence response to AEDs and the proposed genetic mechanisms. The third section describes the application of the common disease common variant hypothesis. The final section delineates the conceptual models and hypotheses of this pharmacogenetic study formulated from the knowledge in the former three sections.

Complex disease: multifactorial and polygenic trait

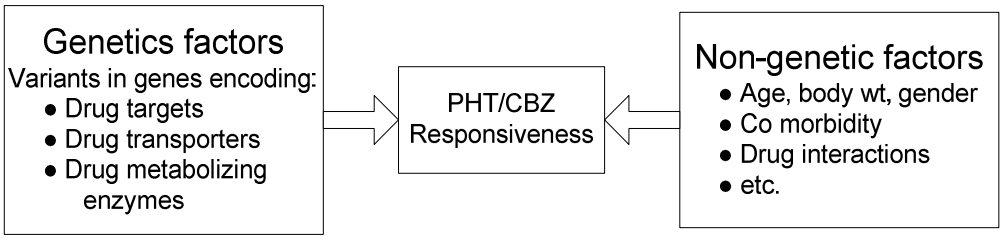
The first theory, the concept of complex disease [110] [111] [112] [113], is applied to describe the nature of variability in drug response phenotypes in this study. The genetic basis of complex disease has been used to describe the etiology of nonmendelian epilepsy [114]. This concept implies that responsiveness to antiepileptic drugs (AEDs), as well as other complex diseases, is a 'multifactorial' and 'polygenic' trait. In other words, many predisposing genetic and non-genetic factors, with their small effects, independently contribute to the patients' different responses to AEDs.

Predisposing factors: genetic and nongenetic factors

Various predisposing factors influence the patient response to AEDs including genetic and non-genetic factors. Once the drug is administered, it is absorbed and distributed to its sites of action where it interacts with targets (such as receptors and enzymes), undergoes metabolism and then is excreted. Each of these processes could be affected by genetic and non-genetic variations that could potentially result in variation in drug response. Genetic factors are responsible for genetic variations on drug response through drug targets, drug transporters,

and drug metabolizing enzymes. Non-genetic factors, or environmental factors, for instances, age, body weight, gender, co-morbidity, and drug interaction, could also alter drug response from person to person. Figure 3.1 shows the overview of the theoretical framework of this study.

Figure 3.1 Theoretical framework of antiepileptic drug pharmacogenetic study

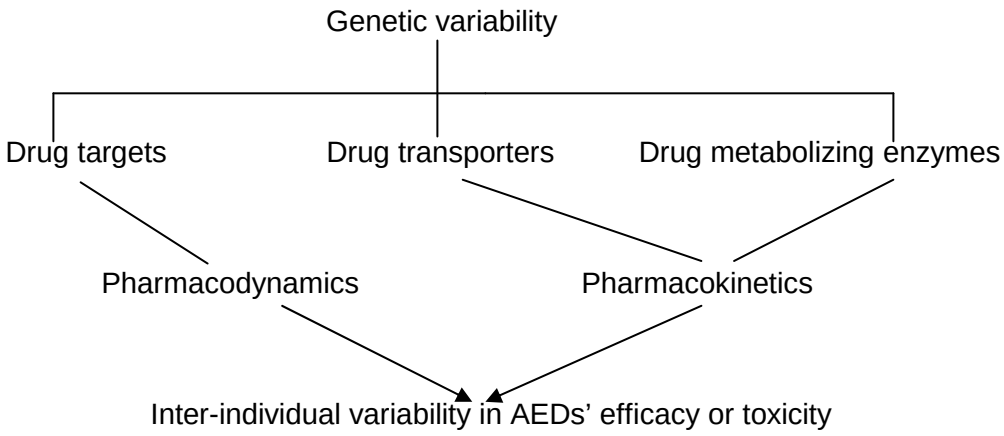


Genetic predisposing factors

Genetic factors are now believed not only to predispose some patients to epilepsy but may also influence patients' responsiveness to AEDs. Recently, several genetic mechanisms that determine drug resistance in epilepsy have been proposed. Three major genetic mechanisms focused on genetic variations that affect pharmacokinetic and pharmacodynamic pathways of AEDs are applied to this study. Firstly, the target hypothesis proposed that genetic variations may alter drug-target sensitivity in epileptogenic brain tissue.[16, 17] Secondly, the multi-drug transporter hypothesis proposed that over expression of multi-drug transporters may not only resulted in the increased removal of AEDs from the epileptogenic tissue but also decreased drug absorption or increase drug excretion.[17, 78, 97] Thirdly, genetic variants that resulted in the loss or gain of functions of drug metabolizing enzymes may alter drug clearance or bioavailability, leading to variability in AEDs blood levels and possibly target tissue levels. [32] The former two putative genetic mechanisms of drug resistance in epilepsy and the latter variation in functions of drug metabolizing enzymes may partly explain inter-individual variability

in drug response to AEDs which are seen in most patients as the differences in dose requirement or adverse effects [35, 54, 97, 115]

Figure 3.2 Genetic variability that affects inter-individual variability in response to AEDs.



Nonspecific genetic mechanisms of non-responsiveness to AEDs

Evidence has demonstrated that patients nonresponsive to the first AEDs have less than a 10% chance of being controlled by other AEDs, in spite of their different mechanisms of actions [6]. This has led to the proposed nonspecific mechanisms of drug resistance. The nonspecific mechanisms include the alteration of drug absorption, distribution, metabolism, excretion and uptake into the brain. These mechanisms are mediated by multidrug transporters and drug metabolizing enzymes.

Gain of function of multidrug transporter hypothesis: Decreased uptake of AEDs into the brain and decreased absorption or increased excretion

In terms of multidrug transporters, the gain of function of multidrug transporters has been hypothesized as a result of decreased uptake of AEDs into the brain and decreased absorption or increased excretion of the drug. [17, 78, 97] Multidrug transporters belong to the adenosine triphosphate (ATP)-binding cassette (ABC) super family and include P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP). In humans, P-gp is a transmembrane

glycoprotein expressed in normal organs involved in absorption and excretion as well as in tumors [74]. These organs include bile canaliculi in the liver, brush-border membrane of intestine, kidney, adrenal grand, uterus and testis [74] [116] [117] [76], as well as in the blood brain barrier (BBB) [75].

P-gp, an efflux transporter located on the apical side of the cells, plays an important role in drug absorption, distribution and excretion.[97, 118] It actively transports substrates from the inside to the outside of cell. Expression of P-gp in the capillary endothelium of the blood-brain barrier is believed to prevent penetration of substrate drug into the central nervous system. Over production of P-gp was first found to be associated with multidrug resistant phenotypes including refractory brain tissue [119] [120] [121]. The proposed mechanism of multidrug resistance is the reduction of cellular accumulation of many structurally diverse AEDs in the brain due to the efflux transporter's function. Its important determinant for drug disposition stems from the fact that it decreases drug absorption from the gut lumen and increases drug excretion into the bile and urine. In addition, P-gp and CYP3A4 are frequently co-expressed in the same cells and share a large number of substrates and modulators. The disposition of such drugs is thus affected by both transporters and metabolism [122].

Therefore, genetic variants resulting in the gain of function of P-gp has been proposed as a possible genetic factor responsible for refractory epilepsy and possibly contributing to variability in drug response to AEDs.

Loss or gain of functions of dug metabolizing enzymes: regulation of AEDs level in the blood and/or target tissues

Drug metabolizing enzymes have an important role in regulating AEDs blood levels, and potentially their availability to the target tissues. Genetic variants that resulted in loss or gain of functions of drug metabolizing enzymes and altered drug clearance or bioavailability have been well documented. In addition, variability in expression of drug metabolizing enzymes among

different ethnic or age groups make them important candidates to explain inter-individual variability in drug responsiveness [123].

Because of a narrow therapeutic index of PHT and CBZ, the ability to identify variant alleles that influence their metabolisms and have significant clinical effect may not only improve drug efficacy but also prevent serious adverse effects.

Specific genetic mechanism of non-responsiveness to AEDs

There is an increasing evidence to support the belief that nonspecific genetic mechanisms that alter pharmacokinetic processes account partly for the observed inter-individual variability in drug response. In addition, genetic variations that alter pharmacodynamic process, such as sensitivity of drug targets are likely to be the determination of variability in drug response.

Drug target hypothesis: Altered sensitivity of ion channels to AEDs

AEDs act by different mechanisms to modulate the excitability of drug responsive epilepsy. Therefore, ion channel mutation may explain resistance to only AEDs that act by modulating the ion channel. Phenytoin and carbamazepine modulate the sodium channel in a use-dependent manner, resulting in normalization of the hyperexcitable neurons.[103]

Mutations to ion channels may not only result in the genesis of epilepsy but also contribute to drug responsiveness /non-responsiveness due to alteration of the structure and/or function of the channel. By using surgical specimens of drug resistant temporal lobe epilepsy, Remy et al showed that the mechanism of action of carbamazepine, a use-dependent block of voltage-dependent sodium channels, is completely lost in carbamazepine-resistant patients [16]. Furthermore, a study by Tate et al suggested a polymorphism in *SCN1A* to be associated with drug responsiveness to PHT and CBZ [108].

Non-genetic or environmental predisposing factors

Several non-genetic or environmental predisposing factors may contribute to inter-individual variability in drug response. These factors include types of seizures, physiological differences, co-morbidity, drug-drug interactions, drug-food interactions and poor drug compliance. However, these factors could not be exclusively controlled in this study. Therefore, some obvious demographic variables including race, age, bodyweight and gender are used as surrogate variables in this study as described below.

Demographic factors: Race, age, bodyweight and gender

Race

Race is an important demographic variable that has been shown to contribute to inter-individual variability in response to several drugs [122]. The effects of race on variability in drug metabolism and response reflect both genetic and environmental differences. Several genetic variations may provide a molecular basis for racial differences in drug metabolizing enzymes, drug transporters and drug targets [122]. Therefore, race can be used as an initial useful surrogate marker for unstudied genetic and environmental factors that result in variability in drug response [124].

Age

Many nonpathological alterations in structure and/or functions of several organs accompanying aging may affect normal physiological processes in the elderly, such as drug disposition. Because of aging, the combination of declines in liver volume, liver blood flow and possibly the amounts or activities of some drug metabolizing enzymes, account for diminished clearance of drugs undergoing hepatic oxidative metabolisms [125-127].

Body weight

Body weight and body fat affect variability in the volume of distribution of drugs. In general, a larger body size results in larger volume of distribution. In addition, greater body fat increases the distribution volume for lipophilic drugs including most AEDs. Therefore, variability in bodyweight would partly contribute to variability in pharmacokinetics and dose requirement of AEDs.

Gender

Biological differences between men and women may lead to differences in both pharmacokinetics and pharmadynamics and ultimately in responses to drug. In addition to variability in volume of distribution between genders, gender based differences can be explained partly by differences in body composition and weight among men and women. Much evidence has shown that differences in hepatic drug metabolism and renal clearance are related to gender differences. These differences may associate in part with differences in clinical dosage required and probably drug response. [128, 129] [130] [131]

Common disease common variants hypothesis

At present, little is known about genetic variations underlying drug-resistant epilepsy and variability in drug response to AEDs. Very few susceptible variants have been unambiguously identified. With all complex disease phenotypes, it is believed that inter-individual variability in drug response depends on many known and unknown functions of genetic and environmental factors. Each of these factors has small or moderate effect on phenotypic variation. It is unlikely that single factor is sufficient for phenotypic variation. Therefore, to succeed in finding true causal genetic variants, a study must be able to detect a relatively weak statistic signal [132].

The third theory, the common disease common variants hypothesis (CDCV) was applied for operational selection of candidate genes and variants [110, 133, 134]. The common disease common variant hypothesis proposed that the genetic factor underlying common complex

disease is often due to a few susceptible variant alleles that are common in general population. Therefore, this pharmacogenetic study focused on susceptible variants that are present at relatively high frequencies (at least 1%) in the study populations [133, 135].

In line with the theoretical framework stated above, the criteria for selection of candidate genes and variants in this study were guided by empirical research findings that are well documented as well as those in controversy. These theories and evidence were applied to identify candidate genes and variants that may explain and predict the variability of responsiveness to PHT and CBZ.

Candidate variant in drug target gene: *SCN1A*

The primary mechanism of PHT and CBZ to protect against partial and secondarily generalized tonic-clonic seizures is believed to be mediated by modulating the brain voltage-gated sodium channels [101, 102]. SCN1A is a gene encoding the brain sodium channels. Recently, one of an intronic polymorphism in the SCN1A gene, rs3812718 (*SCN1A* IVS5N+5 G>A), has been reported to be significantly associated with high maximum doses during the clinical use of both PHT and CBZ [108]. A functional study done by the same research group suggested that the SCN1A polymorphism affects the alternative splicing of exon 5 [108]. Therefore, the variant, rs3812718 or *SCN1A* IVS5N+5 G>A, was selected as a candidate variant related to both PHT and CBZ responsiveness in this study.

Candidate variant in multidrug transporter gene: *ABCB1*

Both PHT and CBZ are possible substrates for P-gp[17]. P-gp is expressed at the blood brain barrier (BBB), blood-cerebrospinal fluid barrier (BCSB) as well as other excretory cells including enterocytes, hepatocytes and proximal tubular cells in the kidney.

In patients with intractable epilepsy, over-expression of P-gp in focal tissue has been found.[78-80] Interestingly, a silent polymorphism (rs1045642 or *ABCB1* c.3435C>T) in *ABCB1* gene was found to be associated with resistance to AEDs in several studies [81, 82].

However, it is unclear whether the silent variant, 3435C>T is a marker or causal variant, since some later studies failed to replicate the association of ABCB1 3435C>T with drug-resistance epilepsy [88, 89].

Because of the putative important roles of P-gp in intractable epilepsy and inconclusive findings of its polymorphism, c.3435C>T, it is of interest to select the c.3435C>T variant as an additional candidate variant related to both PHT and CBZ responsiveness in this study.

Candidate variants in genes encoding drug metabolizing enzymes: *CYP2C9, CYP2C19* and *CYP3A5*

Variations in genes encoding enzymes involving in major metabolic pathways of PHT and CBZ may alter drug clearance and bioavailability and lead to variability in required maintenance doses and ultimately drug responsiveness.

PHT is metabolized mainly by hepatic CYP2C9 and with a lesser extent by CYP2C19. Two known common functional polymorphisms of *CYP2C9* (*CYP2C9*3* or rs1057910, g.42614A>C and *CYP2C9*2* or rs1799853, g.3608C>T) which are non-synonymous polymorphisms produce enzymes with a decrease in activities both in vivo and in vitro. *CYP2C19*2* (rs4244285, g.19154G>A), a common variant with a splicing defect that produces an enzyme with no activity, might also affect PHT response. Therefore, *CYP2C9*2*, *CYP2C9*3* and *CYP2C19*2* were selected as candidate variants related to PHT responsiveness.

CBZ is metabolized partly by hepatic and intestinal CYP3A4 and CYP3A5 to an active metabolite, carbamazepine-10,11-epoxide, which further undergoes hepatic metabolism to inactive product. [60] Large inter-individual variability in CYP3A expression both in the liver and small intestine may contribute greatly to variability in oral bioavailability and systemic clearance of CYP 3A substrates including CBZ. CYP3A5 is polymorphic and its expression is more variable than CYP3A4. Studies in Caucasian and African Americans have shown that only people who are heterozygous or homozygous for *CYP3A5*1* express a large proportion of total

CYP3A content in the liver and small intestine and may contribute greatly to overall catalytic activity of CYP3A. *CYP3A5*3* (rs776746, g.6986A>G) and *CYP3A5*6* (rs10264272, g.14690G>A) cause alternative splicing and protein truncation and *CYP3A5*7* (rs41303343, g.27131 27132insT) causes the frameshift mutation. This results in the absence of CYP3A5. Interestingly, different distributions of these variant alleles have been found among Caucasian and African populations [136]. Therefore, functional variants in CYP3A5 including *CYP3A5*3*, *CYP3A5*6* and *CYP3A5*7* may contribute to inter-individual and interracial variations in CBZ bioavailability or systemic clearance that lead to variability in response to CBZ.

Phenotypes: ultimate drug responsive phenotypes and intermediate phenotypes

According to the proposed theoretical framework, genetic variants in genes encoding drug metabolizing enzymes, drug transporter and drug target that alter PHT and CBZ disposition and action may influence patients' responsiveness which ultimately are presented as drug responders or drug non-responders.

Among drug responders, variability in responsiveness can be seen as large inter-individual variability in dose requirement. This phenomenon may also result from variability in drug disposition and sensitivity of drug target. Therefore, responsiveness to several drugs relating to traits can be assessed as drug exposure or dose requirement and were used as surrogate phenotypes to explore genetic basis of responsiveness to PHT and CBZ in this study. These intermediate phenotypes include maintenance doses and area under the drug plasma concentration-time curve (AUC).

Conceptual model and hypotheses

Based on the theoretical framework including the basic concept of a complex disease, the postulated genetic mechanisms of intractable epilepsy, common disease common variants hypotheses, and supported by evidence from previous research findings, the proposed conceptual models that may explain variability in responsiveness to PHT and CBZ therapy are presented in Figures 3.3 and 3.4. In these models, variability in responsiveness to PHT and CBZ were explained by multiple genetic variations in drug metabolizing enzymes, multidrug transporter and drug target in concert with potential non-genetic factors. These combined genetic and non-genetic factors may explain and predict the variability of phenotypic outcomes better than each individual factor.

Therefore, the primary objective of this pharmacogenetic study was to determine genetic variants in a sodium channel gene (*SCN1A* IVS5N+5 G>A), a multidrug-transporter gene (*ABCB1*c.3435C>T) and genes encoding drug metabolizing enzymes (*CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP3A5*3, CYP3A5*6* and *CYP3A5*7*) and non-genetic factors that are associated with variability of the clinically phenotypic response to PHT and CBZ therapy, in Caucasian American and African American patients with epilepsy. The secondary objective was to use these genetic and non-genetic factors to develop an algorithm to explain and estimate the phenytoin dose *a priori.*

Figure 3.3. Proposed conceptual model of the influence of genetic and non-genetic factors on variability in response to phenytoin (PHT) treatment.

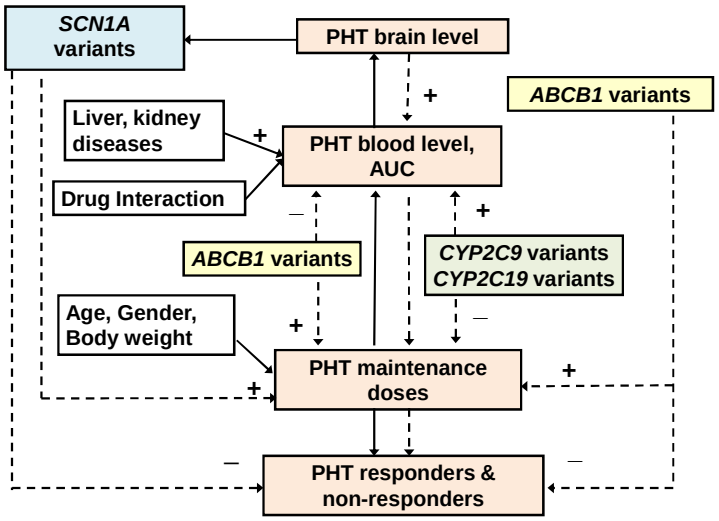
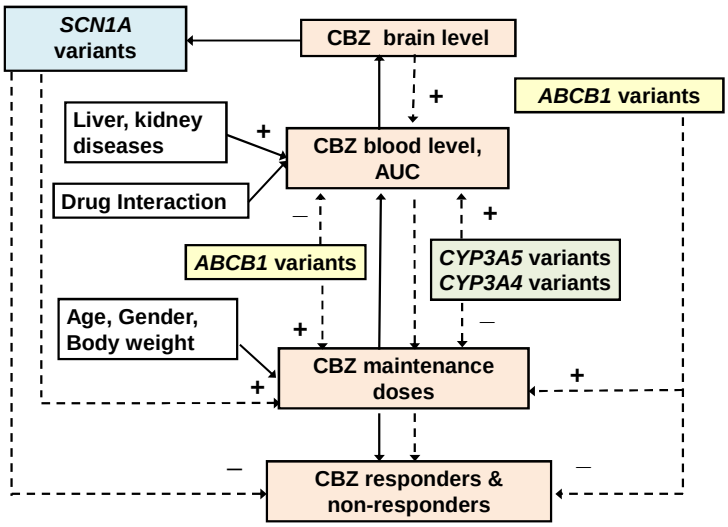


Figure 3.4. Proposed conceptual model of the influence of genetic and non-genetic factors on variability in response to carbamazepine (CBZ) treatment.



Hypotheses

In accordance with the study purpose, the key hypothesis of this study was that common SNPs in the sodium channel α subunit gene (*SCN1A*), a multidrug-efflux transporter gene (*ABCB1*) and the cytochrome P-450 genes encoding the major drug metabolizing enzymes involved in PHT or CBZ metabolism (*CYP2C9*, *CYP2C19* and *CYP3A5*) carried by the patients along with demographic factors would affect the variability in PHT and CBZ response phenotypes. The drug response phenotypes were measured by clinical maintenance doses and AUC in unrelated patients with epilepsy on PHT or CBZ maintenance therapy.

Chapter Four
Methodology

This study is a cross-sectional genetic association study using a candidate gene approach. Retrospective data were used. The datasets were retrieved from two studies: 1) the steady-state pharmacokinetic study of the stable labeled-phenytoin (P50, 1997-2002) and 2) the steady-state pharmacokinetic study of the stable labeled-carbamazepine (P50, 2002-2007).

Population and Setting

Study population was drawn from the study populations of the P50 projects. The P50 studies are the steady-state pharmacokinetic studies of the stable labeled-phenytoin or stable labeled-carbamazepine in the elderly (age > 65 years) comparing to younger adults (age 18-65 years). In this present study, the study populations were patients with epilepsy who were unrelated Caucasian Americans (Americans of European descent) or African Americans (Americans of African descent) that were enrolled in the P50 studies. Patients were ascertained through three epilepsy centers including 1) University of Minnesota (Minnesota) 2) University of Miami (Florida) and 3) Emory University (Georgia). The studies were approved by each local Institutional Review Boards (IRB).

Participant inclusion and exclusion criteria

Patients were eligible if they were 18 years or older, diagnosed with epilepsy, and taking PHT or CBZ as monotherapy or with other non-interacting AEDs at the time of the study. Exclusion criteria were patients taking medications known to affect PHT or CBZ disposition, patients with races or ethnicity other than African American or Caucasian, and women who were pregnant at the time of the study.

Clinical data collection

After completing the required consent procedure, patients were invited to the clinical research centers for a 24-hour study of single dose steady-state pharmacokinetic study of the

stable labeled-phenytoin or carbamazepine. Briefly, upon admission, the site neurologist performed a brief medical and neurological examination including assessment of CNS toxicity. The patients were asked about recent AED medication use and seizure frequency over the last six months. Demographic and additional clinical data were collected by reviewing patient medical records.

An indwelling catheter was placed in each forearm: one for drug administration, the other for blood sample collection. Blood samples were obtained for laboratory tests and genotyping. One hundred mg of the PHT or CBZ stable-labeled isotope was then infused over 20 minutes. At the end of the infusion, the patients took their usual oral morning dose with 100 mg less corresponding to the amount of the 100 mg infused drug. Subsequent to the infusion, blood and urine were collected for the following 24 hours. Venous blood samples were collected at: 0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 10, 12 and 24 hours after venous drug administration. Blood samples (10 ml) were collected in EDTA tube and then centrifuged. Plasma samples were frozen at -20 ºC until assayed for drug concentrations. Another blood sample was collected at time zero and placed in tube containing acid-citrate-dextrose (ACD) solution for DNA extraction.

Selection of candidate genes and genotyping methods

Selection of candidate genes and variants

The principle criteria for selection of candidate genes were genes that encode proteins involving pharmacokinetic and pharmacodynamic pathways of PHT and CBZ. Three groups of candidate genes that were selected for the study included *SCN1A, ABCB1 and CYP* genes. The candidate variants selected were common single nucleotide polymorphisms (SNPs) that were associated with altered functions of the interested proteins (functional variants) or those with prior evidence of association with phenytoin or carbamazepine response. Table 4.1 shows the candidate genes and SNPs that are selected for phenytoin and carbamazepine studies described in chapters five and six respectively.

Table 4.1 Candidate genes and SNPs and their effect on protein products that were seleced for phenytoin and carbamazepine studies

Gene name	Chromosome location	reference SNP ID (variant allele)	Physical location (bp)	Effect	Enzyme activity	ancestral allele	mutant allele
CYP2C9	10q24	rs1799853 (CYP2C9*2)	g.3608C>T	R144C	Decrease	C	T
CYP2C9	10q24	rs1057910 (CYP2C9*3)	g.42614A>C	I359L	Decrease	A	C
CYP2C19	10q24.1	rs4244285 (CYP2C19*2)	g.19154G>A	Splicing defect	Decrease	G	A
CYP3A5	7q21.1	rs776746 (CYP3A5*3)	g.6986A>G	Splicing defect	Severely decrease	A	G
CYP3A5	7q21.1	(CYP3A5*6)	g.14690G>A	Splicing defect	None or Severely decrease	G	A
CYP3A5	7q21.1	rs41303343 (CYP3A5*7)	g.27131 27132insT	346 Frameshift	None or Severely decrease		T
SCN1A	2q24.3	rs3812718	IVS5N+5 G>A	Splicing defect		C (G)	T(A) *
ABCB1	7q21.1	rs1045642 (ABCB1 3434C>T)	c.3435 C>T	None		C	T
Note: *A-G means A and G are polymorphic bases on one strand which are equivalent to T and C on the other strand							

Genotyping methods

Venous blood samples collected into tubes containing acid-citrate-dextrose (ACD) solution were used for DNA extraction. From the whole blood collected, genomic DNA was extracted from white blood cells according to the standard method. The dissolved DNA was stored at 4 ^{o}C. DNA concentration and purity were determined by using standard ultraviolet (UV) spectroscopy method. DNA yields were typically 200 ng/µl or more with 260:280 ratios of 1.8.

All SNP genotyping was performed using *Taq*Man assays (Applied Biosystems, USA), according to the manufacturer instruction. The PCR primers and probes were obtained from Applied Biosystems (ABI) assays-on-demand service. The PCR reactions were performed in a 96-well optical template. In a 25 µl-final volume, each well contained 1 µl of 20 ng/ µl DNA and 24 µl of PCR master mix containing *Tag*man Universal PCR Mix, primers and probes. The PCR amplification cycling protocol was 10 minutes at 95 ºC followed by 40 cycles of 15 seconds at 60 ºC and 2 minutes at 60˚ C. Allelic discrimination were then performed using ABI 7500 Real Time PCR with ABI software.

Data Analysis

The demographic data, clinical data, drug concentrations and genotyping data were entered into a computer system using Excel® for Windows software. WinNonlin version 5.2 (Pharsight, Inc., Mountain View, CA) was used to perform pharmacokinetic analyses. Descriptive data analyses and genetic association analyses were conducted using Statistic Package for Social Sciences (SPSS) version 15.0 (SPSS Inc. IL, USA). In case of missing value of variables for analysis, the case-wise deletion was used for that particular analysis.

Pharmacokinetic data analyses

Pharmacokintic parameters were calculated from PHT and CBZ total plasma concentration-time profiles for each individual by fitting a non-compartmental model with first order elimination. The area under plasma concentration-time curve ($AUC_{0\text{-}12\ hr}$) and $AUC_{0\text{-}24hr}$ were estimated from plasma PHT or CBZ concentrations collected at 0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 10,12 and 24 hours after oral morning dose by using the linear trapezoidal method.

Descriptive statistics

Descriptive statistics were used to summarize the demographic and clinical characteristics of the participants. To compare the distributions or means of demographic or clinical characteristics between or among groups, Student's *t*-test or analysis of variance (ANOVA)

were used for parametric continuous data. Otherwise, Wilcoxon rank sum test (Mann Whitney U test) or Kruskal-Wallis test were used for nonparametric numerical data. Boxplots or Scatter plots were used to demonstrate the distributions of observations.

Genetic association analyses

To determine the stability of the allele and genotype frequencies of each SNP in the study population and to assess the quality of the genotyping data, we tested for deviation from Hardy-Weinberg equilibrium (HWE). HWE was calculated by Chi-square test using Microsoft Office Excel 2007. A *p* value of < 0.05 was considered as departure from HWE.

The association analyses included bivariate analyses, multiple linear regression, analysis of covariance (ANCOVA) and model validation analyses.

Bivariate analysis

To test bivariate linear association, Pearson's correlation was first used to determine the association between response phenotypes or dependent variables versus continuous independent variables (body weight and age). Spearman rank correlation procedure was also used to assess correlation between dependent variable and nominal independent variables (gender and genetic variants). The strength of the association between the two variables was assessed by correlation coefficient (r). A *p* value of < 0.05 was determined as significant correlation. Scatter plots were used to demonstrate the relationship between the two variables.

Multiple linear regression analysis

The assumptions for multivariate analyses including linearity, bivariate normal distribution and homogeneity of variance were tested. Characteristics of the distribution of the variables were assessed using boxplot and Kolmogorov-Siminov test for normal distribution. A *p* value of <0.10 is determined as non-normal distribution. Levene's test was used to determine equality of variances of two or more groups. A *p* value of < 0.05 is determined as uneqal variance.

Stepwise multiple regression analysis [137-139] was used to test the combined association of genetic variants and non-genetic factors with PHT or CBZ daily maintenance dose or pharmacokinetic parameters.

Genetic variants and non-genetic factors were used as independent variables, and PHT and CBZ doses or AUC were used as dependent variables. Dependent variables were assessed whether the data fit the assumptions of multiple linear regression. If not, the dependent variables were transformed: PHT dose was transformed by deviding by body weight (kg) to be body weight normalized doses (mg/day/kg); AUC was transformed by deviding by doses. For independent variables: age and body weight were used as continuous variables, whereas, genetic variants, gender were transformed to multiple categorical variables (dummy variables).

The relative strength of the association of each covariated was determined from standardized regression coefficiants (β), whereas, the unstandardized regression coefficient (b) was used in the regression model to predict the dependent variables. Multiple testings were not taken into account, therefore a p value of < 0.05 was considered to be significant [140].

Correlation matrix was used to examine collinearity. Independent variables that showed evidences of collinearity or multicolinearilty (correlated at > 0.8) were subject to dropping from further analyses.

Histograms, normal probability plots and standardized residual plots were used to assess normality of residual distribution to detect violation of model assumption and identify influential observations.

Validation of model

A statistical model for predicting phenytoin dose requirement was constructed using multiple linear regression model. Nonparametric or distribution-free computational intensive statistical techniques including a bootstrap was used for validation of model based on limited sample data.

The bootstrap procedure was used to estimate the confidence intervals of the regression coefficients. One thousand bootstrap resamples of the same size of the original samples were simulated by performing 1,000 random resamplings with replacement from the original observed samples. The regression coefficients were computed and the bootstrap distribution of the statistic was obtained. The 2.5 percentile and 97.5 percentile were used to construct 95% bootstrap confidence intervals (CIs) of the regression coefficients. [141]

Analysis of Covariance (ANCOVA)

ANCOVA [138] was used to determine the effect of each genetic variant on the mean values of PHT response phenotypes by controlling the influence of other genetic covariates and potential confounding variables (age, body weight and gender) were controlled or adjusted. Post-hoc comparisons (Student's *t*-test or ANOVA) were conducted to compare the adjusted means across subgroups.

Statistical significance was defined as $p<0.05$ and was not adjusted for multiple comparisons. [140]

Chapter Five

Association of genetic variants in *CYP, ABCB1* and *SCN1A* and non-genetic variants with phenytoin response phenotypes

Introduction

Phenytoin (PHT) is the most cost-effective first-line antiepileptic drug used worldwide.[21] However, in addition to the fact that about 30% of newly treated patients with epilepsy do not respond to treatment with the first AED, control of epilepsy with PHT is difficult because of its narrow therapeutic index, complicate pharmacology and common adverse drug reactions.[6] Large variability in PHT response phenotypes namely dosage and pharmacokinetic parameters are proposed to be simultaneously affected by genetic and non-genetic factors. PHT is a substrate of P-glycoprotein efflux transporter (ATP-binding cassette [ABC] B1) and initially metabolized by cytochrome P450 (CYP) 2C9 and CYP2C19 and exerts its antiepileptic activity by modulating the brain voltage-gated sodium channels. It has been widely known that specific genes encode proteins that involve pharmacodynamic and pharmacokinetic properties of PHT. Variations of such genes result in varying phenotypes. Therefore, genetic factors including common single nucleotide polymorphisms (SNPs) in such genes, together with non-genetic factors were investigated for their combined association with PHT response phenotypes, maintenance doses and the area under the plasma concentration- time curve (AUC). By using a candidate gene approach, relating genes including sodium channel gene (*SCN1A)*, drug efflux transporter gene (*ABCB1)* and cytochrome P450 genes (*CYP2C9, CYP2C19)* with their common functional SNPs were studied.

Furthermore, given that PHT has a narrow therapeutic index, the ability to use pharmacogenetic knowledge to explain the variability in PHT dose required among individuals and to estimate therapeutic dose for each individual at the time of treatment initiation will reduce the time to achieve therapeutic effect and the risk of overdosing and hence adverse effect.

Purpose

This study aimed to investigate the associations of the combined genetic variants in *SCN1A, ABCB1* and *CYP* genes and non-genetic factors with phenytoin maintenance dose as well as the AUC at phenytoin maintenance dose, and to use combined genetic and non-genetic factors to develop a statistical model to estimate PHT dose.

Study design

This study was a cross-sectional genetic association study using the candidate gene approach.

Procedure

A dataset of 56 adult Caucasian patients with epilepsy on PHT maintenance therapy was used. Demographic and clinical variables were retrieved. Genomic DNA samples were used to genotype for 5 candidate SNPs: *SCN1A* c.IVSN5+5 G>A, *ABCB1*c.3435C>T, *CYP2C9*2* (c.430C>T), *CYP2C9*3* (c.1075A>C) and *CYP2C19*2* (c.861G>A). All participants gave written informed consent. Steady-state $AUC_{0\text{-}12\ hr}$ was determined from phenytoin plasma concentrations at 0, 0.08, 0.25, 0.5, 1, 2, 4, 6 and 12 hours after an oral dose by using the WinNonlin software program. Stepwise multiple linear regression analysis was used to determine the association of genetic and non-genetic variants with phenytoin phenotypes.

Bivariate analyses were first used to select genetic and non-genetic factors that were associated with PHT doses or AUC using significance criteria of $p < 0.1$. Stepwise multiple linear regression analyses were then used to quantify the association of all factors with PHT phenotypes, namely maintenance doses and AUC. Only variables that were independently associated with PHT doses or AUC ($p < 0.05$) were retained in the final model. The bootstrap resampling procedure was used to validate the model by constructing the 95% confidence interval (CI).

Results

Study participants and participants' characteristic

Demographic data, clinical data and DNA samples were gathered from patients with epilepsy on PHT maintenance therapy who were enrolled in the P-50 study. Of the total 56 patients, most patients were Caucasian American (54 patients) while the rest were African American (2 patients). Therefore, further analysis on racial subpopulation could not be done. The analysis of this study was based on the 54 unrelated Caucasian patients.

Demography

The demographic characteristics of the 54 study patients are summarized in Table 5.1. In this sample, most of the patients were Caucasian and non-Hispanic or Latino (96.3%). Given the small number of Hispanic or Latino patients, ethnicity was not taken into account in further analyses. More than half of the patients were male (57.4%). About 60% of the patients were elderly with age over 65 years old. There was a very wide range of the patients' body weight (46.6 - 127.4 kg).

Table 5.1 Demographic characteristics of the patients

Demographic characteristics	Total N	n (%)	mean± S.D.	range
Ethnicity	54			
Caucasian		52 (96.3%)		
Hispanic or Latino		2 (3.7%)		
Gender	54			
Male		31 (57.4%)		
Female		23 (42.6%)		
Age(years)	54		62.6 ± 19.4	21.0-93.0
20-65		22 (40.7%)	42.7 ± 13.0	21.0-65.0
>65		32 (59.3%)	76.3 ± 7.4	67.0-93.0
Body weight (kg)	54		74.8 ± 18.2	46.6-127.4

Antiepileptic drug (AEDs) therapy

Table 5.2 shows clinical characteristics of antiepileptic drug therapy of the 54 study patients. The majority of the patients were on PHT monotherapy (57.4%). Of the 23 patients who were on PHT combined therapy, almost 90% of them used newer non-interacting AEDs including levetiracetam, lamotrigine, gabapentin, clonazepam and zonisamide. Only three patients were given older AEDs that may alter PHT pharmacokinetics, including phenobarbital, carbamazepine and valproic acid. Given the small number of patients that were co-administered these older AEDs, further subgroup analysis on these drugs was not conducted.

Phenytoin maintenance doses

A large variability in required PHT maintenance doses was found among these patients. The daily maintenance PHT doses ranged from 150.0 to 700.0 mg/day or 1.8 to 8.7 mg/day/kg. The required doses of PHT differ about 4-fold among individuals. The mean of PHT doses was 355.7 mg/day and the mean of body weight normalized dose was 4.9 mg/day/kg. PHT doses

required for patients with PHT combined therapy were seemingly higher than those in patients with PHT monotherapy. However, neither mean of PHT dose ($p = 0.537$, Wilcoxon rank sum test, two tailed) nor body weight normalized PHT dose ($p = 0.126$, Wilcoxon rank sum test, two tailed) was significantly different between PHT monotherapy and combined therapy groups.

Phenytoin plasma concentrations

Of the 54 patients, only 49 PHT plasma samples were available. The trough plasma PHT steady state total concentrations between patients who received PHT monotherapy (11.2 mg/L) and PHT combined therapy (14.3 mg/L) were significantly different ($p = 0.024$, Wilcoxon rank sum test, two tailed).

Table 5.2 Clinical characteristics of phenytoin (PHT) administration of the adult Caucasian American patients with epilepsy on phenytoin maintenance therapy (N=54) on the day of study.

Clinical characteristics	Total N	n (%)	Mean ± S.D.	range
Patients with PHT on the last visit day	54			
PHT monotherapy		31 (57.4%)		
PHT combined therapy:				
Two drugs		14 (25.9%)		
Three drugs		9 (16.7%)		
Combined drugs:				
Levetiracetam		6 (26.1%)		
Lamotrigine		6 (26.1%)		
Gabapentin		6 (26.1%)		
Clonazepam		1 (4.3%)		
Zonisamide		1 (4.3%)		
Carbamazepine		1 (4.3%)		
Phenobarbital		1 (4.3%)		
Valproic acid		1 (4.3%)		
PHT maintenance dose (mg/day)	54		355.7 ± 105.0	150.0-700.0
PHT monotherapy			341.6 ± 90.9	150.0-500.0
PHT combined therapy			374.8 ± 121.0[a]	200.0-700.0
PHT maintenance dose (mg/day/kg)	54		4.9 ± 1.4	1.8-8.7
PHT monotherapy			4.6 ± 1.4	1.8-8.7
PHT combined therapy			5.3 ± 1.4 [a]	3.4-8.7
Trough PHT steady state total concentration, Css (mg/L)	49		12.6 ± 6.8	3.3-24.5
PHT monotherapy		27 (55.1%)	11.2 ± 7.1	4.12-14.0
PHT combined therapy		22 (44.9%)	14.3 ± 6.0[b]	3.3-24.5

Note: [a] Compared with PHT monotherapy ($p > 0.05$, Wilcoxon rank sum test, two tailed)
[b] Compared with PHT monotherapy ($p < 0.05$, Wilcoxon rank sum test, two tailed)

Genotype distributions and allele frequencies

Table 5.3 summarizes the genotype distributions of the five candidate SNPs in the study patients. All observed allele frequencies of *CYP2C9*2, CYP2C9*3, CYP2C19*2* and *ABCB1 (rs1045642*) were consistent with those of the American population with European descent in public databases. No homozygous mutant genotypes, *CYP2C9*2/*2, CYP2C9*3/*3* and *CYP2C19*2/*2*, were observed in this study sample. In the present sample of Caucasian Americans, while the minor allele frequency (MAF), A allele, of *SCN1A*c.IVS5-91G>A was 0.40, the T allele frequency of the *ABCB1c.*3435C>T was 0.61.

All observed genotype frequencies of the *CYP2C9*2, CYP2C9*3, CYP2C19*2, SCN1A*c.IVS5-91G>A and *ABCB1c.*3435 C>T of the study patients were in Hardy-Weinberg (HW) equilibrium (HWE $P > 0.05$).

Table 5.3 Genotype distribution and allele frequencies of *CYP2C9*2, CYP2C9*3, CYP2C19*2, SCN1A*c.IVS5-91G>A and *ABCB1*c.3435 C>T of the study patients (n=54).

Genetic variables	Genotype frequencies (n)			HWE *P*- value	MAF (Nucleotide)
*CYP2C9*2*	*1/*1	*1/*2	*2/*2	0.80	0.10 (T)
(rs1799853, g.3608C>T)	0.80(43)	0.20(11)	0(0)		
*CYP2C9*3*	*1/*1	*1/*3	*3/*3	0.90	0.05 (C)
(rs1057910, g.42614A>C)	0.91(49)	0.09(5)	0(0)		
*CYP2C19*2*	*1/*1	*1/*2	*2/*2	0.84	0.13 (A)
(rs4244285, g.19154G>A)	0.74(41)	0.26(13)	0(0)		
SCN1A	G/G	G/A	A/A	0.92	0.40 (A)
(rs3812718, c.IVS5N+5 G>A)	0.37(20)	0.46(25)	0.17(9)		
ABCB1	C/C	C/T	T/T	0.59	0.39 (C)
(rs1045642, c.3435 C>T)	0.18(10)	0.41(22)	0.41(22)		

Association between demographic factors and genetic variants with phenytoin doses

Bivariate analysis

Correlation coefficient

Correlation analyses revealed significant associations of PHT maintenance dose with demographic factors including body weight, age and gender (P = 0.007, 0.001 and < 0.001, respectively), as well as genetic variants which included *CYP2C9*2 and CYP2C9*3* (P = 0.014,

0.010, respectively). The weight normalized maintenance dose (mg/day/kg) was found significantly associated with only age (*P* = 0.002) and the genetic variants of *CYP2C9*3* and *CYP2C19*2* (*P* = 0.003 and 0.021, respectively). Table 5.4 summarizes the correlations of each variable with PHT doses.

Table 5.4 Bivariate analysis: Associations of genetic variants and demographic factors with phenytoin (PHT) daily maintenance dose (mg/day) and weight normalized dose (mg/day/kg).

Dependent Variables	**PHT dose (mg/day)**		**Normalized PHT dose (mg/day/kg)**	
Independent variables	correlation coefficients (r)	*p*-value (2-tailed)	correlation coefficients (r)	*p*-value (2-tailed)
Bodyweight (kg)	0.365[a]	0.007	-	-
Age (years)	-0.428[a]	0.001	-0.412[a]	0.002
Gender	-0.508[b]	<0.001	-0.086[b]	0.550
CYP2C9*2	-0.333[b]	0.014	-0.234[b]	0.089
CYP2C9*3	-0.319[b]	0.010	-0.397[b]	0.003
CYP2C19*2	-0.045[b]	0.748	-0.314[b]	0.021
SCN1Ac.IVS5N+5G>A	0.126[b]	0.365	0.098[b]	0.481
ABCB1c.3435 C>T	0.075[b]	0.589	0.093[b]	0.506

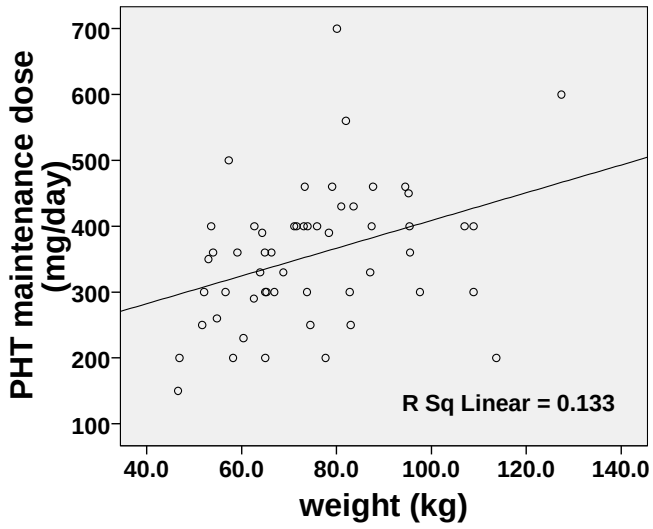
[a] Pearson's correlation
[b] Spearman correlation

The scatter plots

Figure 5.1-5.6 shows scatter plots, or boxplots where appropriate, of phenytoin dose data, maintenance dose (mg/day) and body weight normalized maintenance dose (mg/kg/day) with demographic variables and genetic variants. Each plot shows the marginal relationships between each pair of phenotype dose and independent variable ignoring the effect of all other independent variables as described below.

Body weight: (Figure 5.1) As expected, increased bodyweight was associated with increased daily maintenance doses of PHT (Pearson's correlation coefficient (r) = 0.365, *p* = 0.007). Therefore, bodyweight was used to normalize the daily maintenance dose of PHT. The body weight normalized PHT dose (mg/day/kg) had a more normal distribution and was then used in the subsequent analyses.

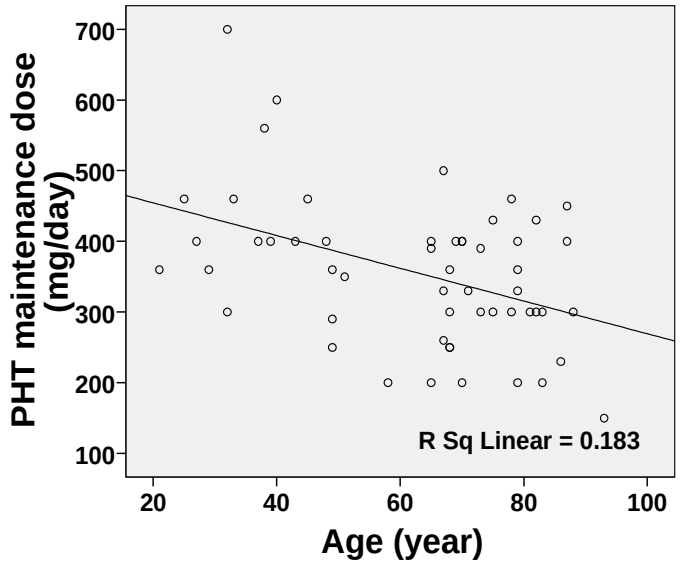
Figure 5.1 Correlation of bodyweight with phenytoin dose (mg/day).



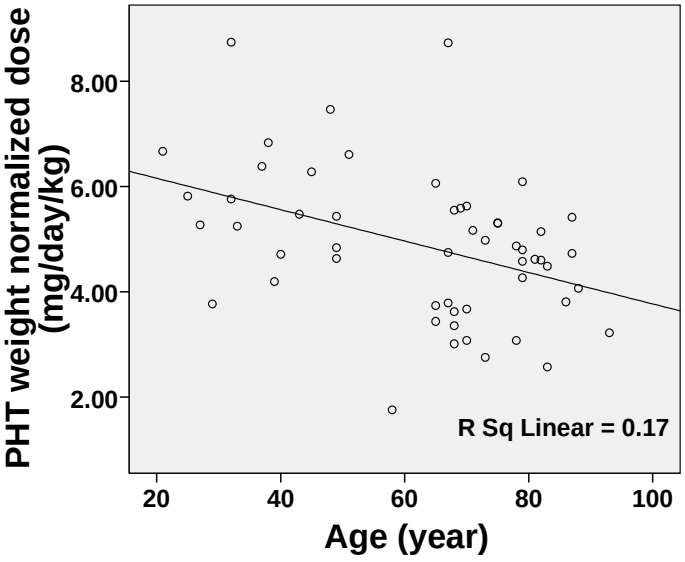
Age: (Figure 5.2) Increased age, on the other hand, was significantly associated with decreased PHT doses, both in absolute doses (r = - 0.428, *p* = 0.001) and body weight normalized doses (r = - 0.412, *p* = 0.002).

Figure 5.2 Correlations of age with (A) phenytoin doses (mg/day) and (B) body weight normalized phenytoin dose (mg/day/kg).

A

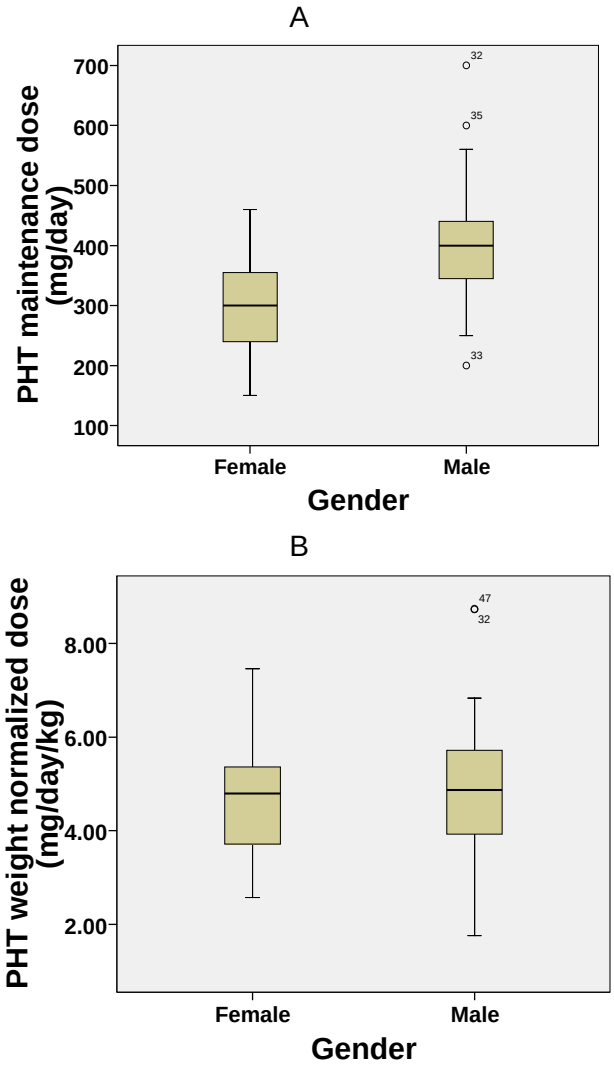


B



Gender: (Figure 5.3) Female gender was found to be strongly associated with decreased phenytoin absolute doses (r = -0.508, $p < 0.001$). However, after dose was normalized by bodyweight, such association was not significant (r = -0.086, $p = 0.539$).

Figure 5.3 Boxplots of (A) phenytoin doses (mg/day) and (B) body weight normalized phenytoin dose (mg/day/kg) across genders.



CYP2C9 and *CYP2C19* variants: (Figure 5.4 – 5.6) The presence of *CYP2C9*3* allele was significantly associated with lower absolute PHT doses (r= -0.319, $p = 0.010$) and body weight normalized PHT doses (r = - 0.397, $p = 0.003$). On the other hand,

*CYP2C9*2* allele was only significantly associated with absolute PHT dose (r = -0.333, *p* = 0.014) and *CYP2C19*2* allele was only significantly associated with body weight normalized PHT dose (r = -0.314, *p* = 0.021).

Figure 5.4 Boxplots of (A) phenytoin(PHT) dose (mg/day) and (B) body weight normalized phenytoin dose (mg/day/kg) across *CYP2C9*3* genotypes.

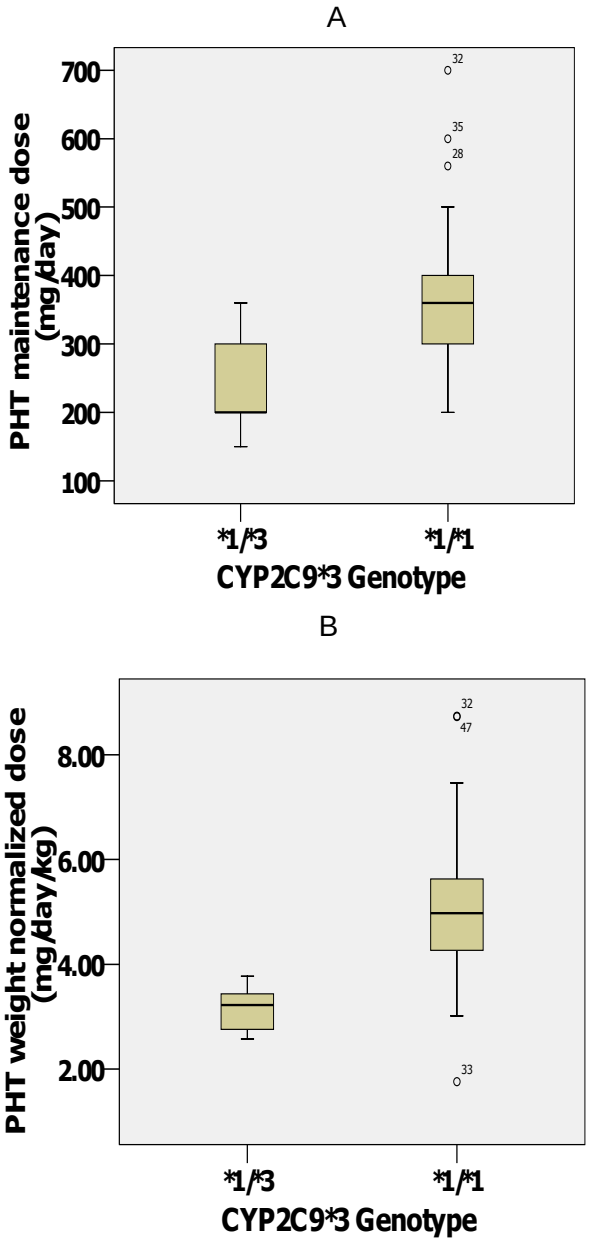
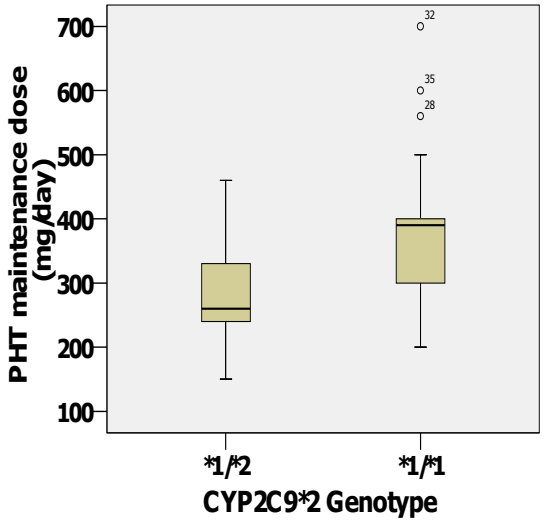


Figure 5.5 Boxplots of (A) phenytoin (PHT) dose (mg/day) and (B) body weight normalized phenytoin dose (mg/day/kg) across *CYP2C9*2* genotypes.

A



B

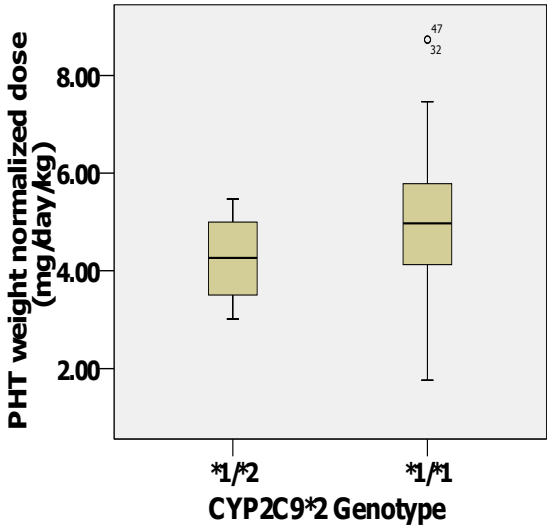
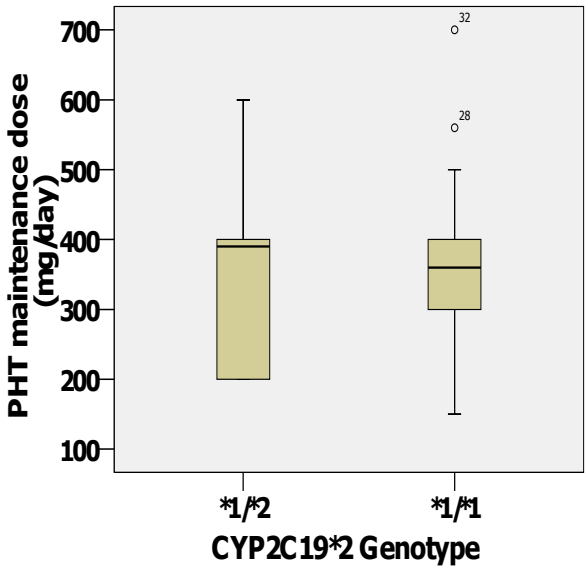
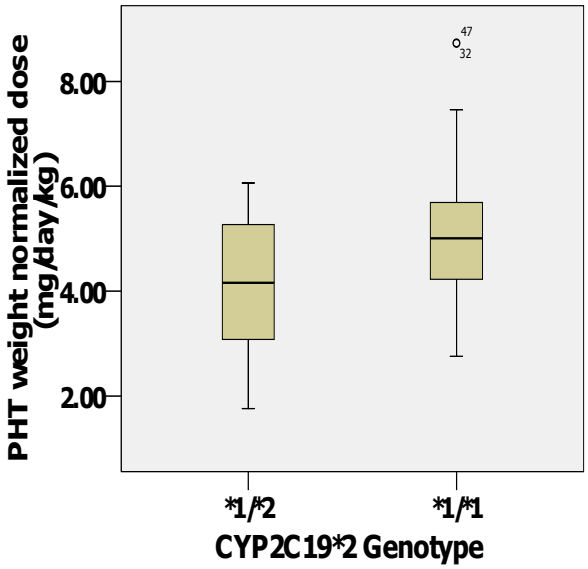


Figure 5.6 Boxplots of (A) phenytoin (PHT) dose (mg/day) and (B) weight normalized phenytoin dose (mg/day/kg) across *CYP2C19*2* genotypes.

A



B



<u>*SCN1A* variant and *ABCB1* variant</u>: (Figure 5.7 – 5.8) No significant association of variants in *SCN1A* c.IVS5N+5 G>A and *ABCB1c.*3435 C>T with phenytoin doses was seen in the present study.

Figure 5.7 Boxplots of (A) phenytoin (PHT) dose (mg/day) and (B) weight normalized phenytoin dose (mg/day/kg) across *SCN1A* c.IVS5N+5 G>A genotypes.

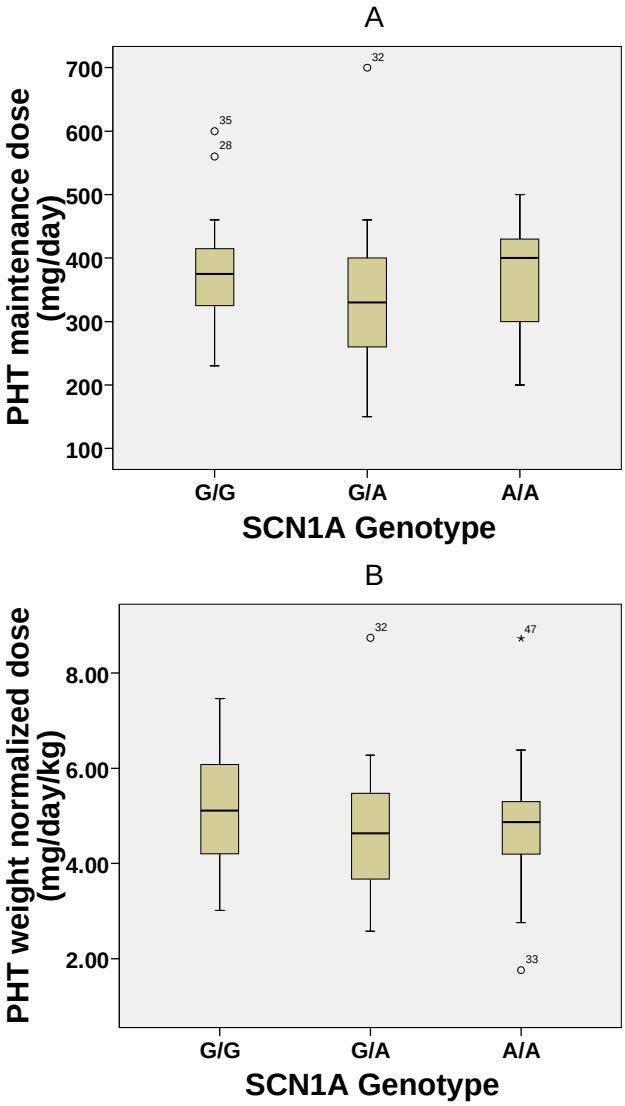
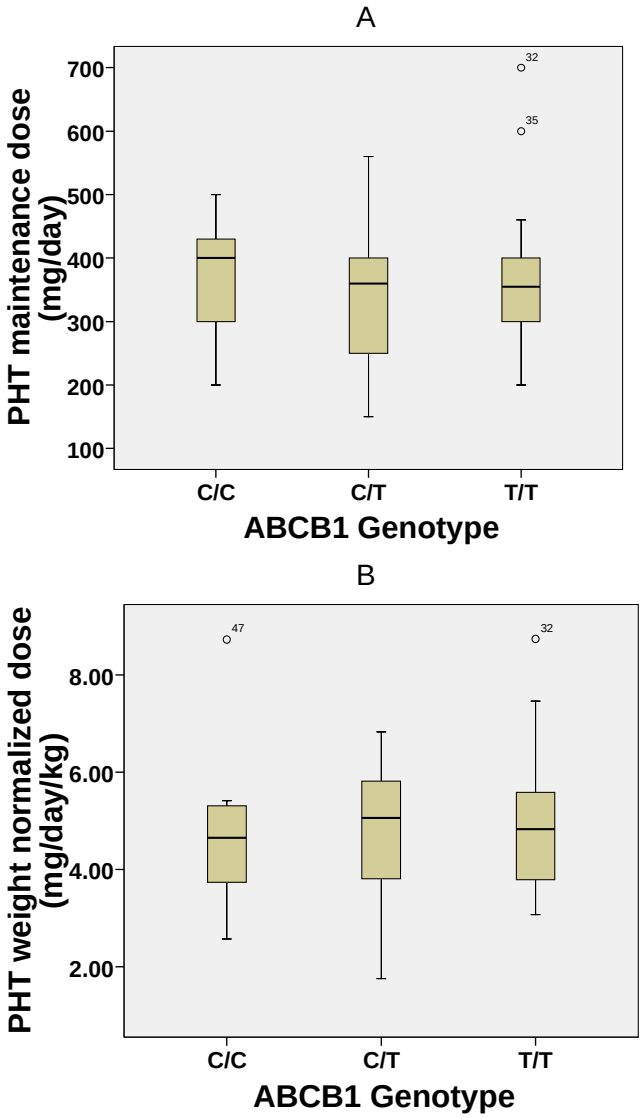


Figure 5.8 Boxplots (A) phenytoin (PHT) doses (mg/day) and (B) weight normalized phenytoin dose (mg/day/kg) across *ABCB1c*.3435 C>T genotypes.



Combined association of genetic variants and demographic factors with phenytoin maintenance doses

Multiple linear regression model

Stepwise multiple linear regression analysis showed the combined association of demographic factors and multiple genetic variants with PHT maintenance dose (mg/day/kg).

Table 5.5 summarizes the main findings of this study. In a multiple linear regression model, increasing age and the presence of *CYP2C19*2*, *CYP2C9*3* and *CYP2C9*2* alleles were independently associated with decreased PHT dose (mg/kg/day). The two candidate SNPs, *SCN1A* c.IVSN5+5 G>A and *ABCB1c.*3435 C>T, as well as gender were not significantly associated with the weight normalized PHT maintenance dose in this study.

Age was the first variable that was entered into the model. The negative and largest standardized regression coefficients of age (β = - 0.386, *P* = 0.002) indicated that older age has the largest influence on lower PHT dose. In accordance with that, the presence of either one allele of *CYP2C19*2* (β = - 0.359, *P*=0.003) or *CYP2C9*3* (β = -0.334, *P* = 0.005) were the second and third covariates entered into the model and had a similar magnitude of effect on lower PHT dose. Finally, *CYP2C9*2* (β = -0.251, *P* = 0.032), the last covariate entering the model, had the least influence on lower PHT dose in this study. The model that include these factors explained 44% (R^2 =0.443, *P* < 0.001) of the variance in the PHT maintenance dose.

Table 5.5 Multiple regression analysis: Association of phenytoin daily maintenance dose (mg/day/kg) with genetic variants and demographic factors.

Independent Variables	Unstandardized Regression coefficients		Standardized Regression coefficients	*p*-value
	b	Standard error	β	
Constant	7.677	0.709	-	<0.001
Age (year)	-0.028	0.008	-0.386	0.002
Gender	0.002	0.326	0.001	0.995
CYP2C9*2	-0.868	0.393	-0.251	0.032
CYP2C9*3	-1.609	0.542	-0.334	0.005
CYP2C19*2	-1.143	0.358	-0.359	0.003
*SCN1A*c.IVSN5+5 G>A	-0.187	0.220	-0.094	0.400
*ABCB1c.*3435 C>T	-0.213	0.226	-0.113	0.351
R^2 = 0443, P < 0.001				

Main effects of genetic variants and demographic factors on phenytoin dose

The results from multiple regression analysis showed the simultaneous association of multiple genetic variants and demographic factors with PHT dose requirement without significant interaction effect. Therefore, the main effects of each covariate on PHT dose were estimated as shown in Table 5.6. When the adjusted means of PHT dose associated with each covariate were estimated and compared, there were significant differences between the adjusted means between age groups and genotype groups.

To investigate the main effect of age on phenytoin dose requirement, we compared the adjusted mean doses between two age groups, adult (age 18 to 65 years) and the elderly (age > 65 years), after controlled for the effects of *CYP2C9*2, CYP2C9*3* and *CYP2C19*2*. The corrected mean ± S.E. of PHT doses (mg/day/kg) in the elderly group (4.52 ± 0.20 mg/day/kg,

n=32) was significantly lower than that in the adult group (5.42 ± 0.26 mg/kg/day, n = 24) (p = 0.006).

To investigate the main effect of each SNP on PHT dose, the corrected mean PHT doses of each heterozygous variant group were compared with the corresponding homozygous wild-type (*1/*1) group with an adjustment for effects of age and other SNPs. It was found that corrected means of PHT doses of the heterozygous *CYP2C9*2* or *CYP2C9*3* groups after controlled for the effect of age, *CYP2C19*2*, *CYP2C9*3* or *CYP2C9*2* were significantly different from its corresponding *1/*1 group.

There was one patient who had compound heterozygote of *CYP2C9*2/*3*. The patient's PHT dose was 3.22 mg/day/kg which was apparently lower than the corrected mean dose of the homozygous wild-type of *CYP2C9*2* group (5.07 mg/day/kg) and *CYP2C9*3* group (5.03 mg/day/kg).

Similarly, the corrected mean of PHT doses of the heterozygous *CYP2C19*2* group after adjusted for the effects of age, *CYP2C9*2* and *CYP2C9*3* was significantly different from the PHT dose of its homozygous wild-type group. On the other hand, no significant main effect of *SCN1A* c.IVSN5+5 G>A and *ABCB1c.*3435 C>T as well as gender on PHT dose was found in this small study sample.

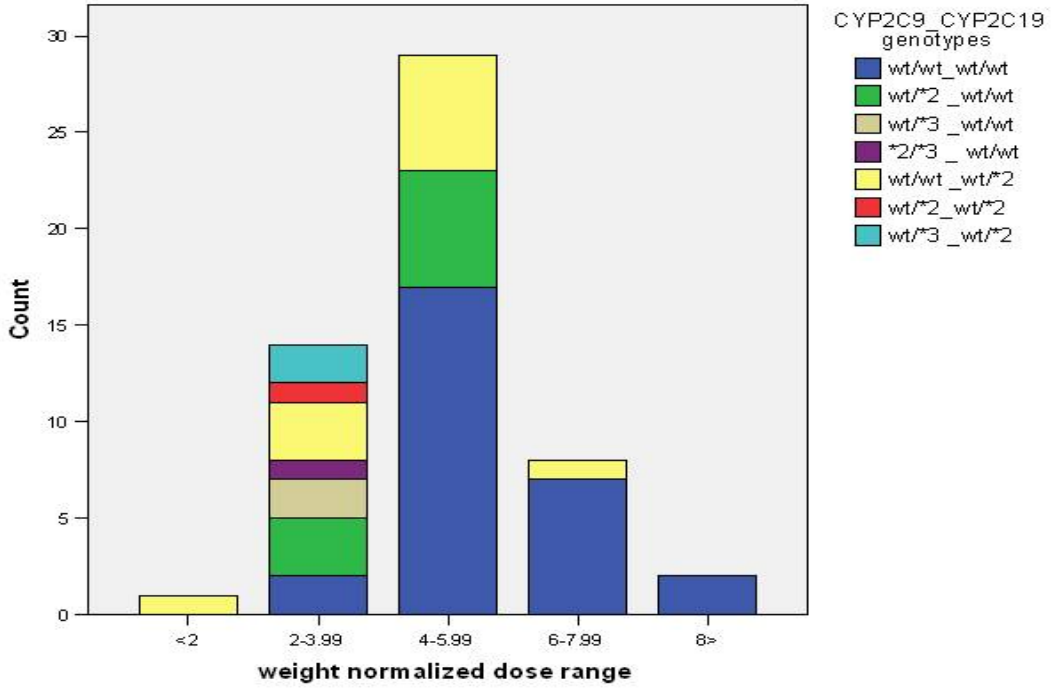
Table 5.6 Corrected means of phenytoin doses across genetic variants and demographic factors.

Independent variables	N total	n (%)	PHT Dose (mg/day/kg), adjusted mean ± S.E.	p-value
Age (year)	54			
18-65		22 (40.4%)	5.42±0.24	
>65		32 (59.3%)	4.52±0.20*	0.006
CYPC19*2	54			
*1/*1		40 (74.1%)	5.16±0.17	
*1/*2		14 (25.9%)	4.09±0.30*	0.003
CYPC9*3	54			
*1/*1		49 (90.7%)	5.03±0.16	
*1/*3		5 (9.3%)	3.45±0.49*	0.004
CYPC9*2	54			
*1/*1		43 (79.6%)	5.07±0.16	
*1/*2		11 (20.4%)	4.16±0.34*	0.021

Note: * ANCOVA adjusted for other covariates, significant $p < 0.05$

Figure 5.9 shows the distribution of weight normalized PHT dose (mg/day/kg) with genotype combinations of *CYP2C9* and *CYP2C19*. The results clearly showed that patients who required low PHT daily doses carried at least one variant allele of *CYP 2C9* or *CYP2C19.* On the other hand, most of the patients with high PHT daily doses carry wild-type alleles of both *CYP2C9* and *CYP2C19*.

Figure 5.9 Distribution of weight normalized PHT dose (mg/day/kg) with CYP 2C9 and CYP2C19 genotypes combination.



Pharmacogenetic model: use of genetic and non-genetic factors to estimate phenytoin dose

The regression models were constructed with the aim to predict PHT dose. Using stepwise multiple linear regression, the final model that estimated the PHT maintenance dose (mg/kg/day) was as follows:

PHT maintenance dose (mg/day/kg) = 7.105 - 0.026Age (year) - 1.577*CYP2C9*3* - 1.069*CYP2C19*2* - 0.906*CYP2C9*2*

The four covariates explained about 40% (adjusted R^2 = 0.398) of inter-individual variability in PHT maintenance dose according to the regression model ($p < 0.021$) in this sample.

Validation of model

Bootstraping

The bootstrap procedure was used to validate the model by taking random samples of the subjects from the dataset with replacement and averaging the results obtained from multiple samples. The results were used to construct the 95% confidence interval (CI). By using 1,000 bootstrap samples with a random sampling of 54 patients each time, the 95% CIs of all regression coefficients were obtained as shown in Table 5.7. The bootstrapping procedure results support the significant association of the four predictors. The average R^2 was 0.376, supporting that the model could explain about 40% of the variation in PHT dose requirement.

Table 5.7 Stepwise multiple regression analysis and bootstrapping: independent predictors of PHT doses (mg/day/kg).

Independent Variables	Regression coefficients			*Observed P*-value	95% Confidence interval
	Unstandardized		Standardized β		
	b	S.E.			
Constant	7.105	0.517	-	<0.001	(6.165, 8.007)
Age	-0,026	0.008	-0.356	0.002	(-0.034, -0.013)
CYP2C9*3	-1.577	0.518	-0.328	0.004	(-2.557, -0.454)
CYP2C19*2	-1.069	0.348	-0.336	0.003	(-2.041, -0.483)
CYP2C9*2	-0.906	0.378	-0.262	0.021	(-1.414, -0,302)
R^2 = 0.376					

Association of genetic variants with PHT plasma concentration-time curve at maintenance doses

Multiple linear regression analysis

Multiple linear regression analysis showed the association of phenytoin $AUC_{0\text{-}12\ hr}$ and genetic variants and PHT dosage. Table 5.8 and 5.9 summarize findings of this study. In a

multiple linear regression model fit with intercept (N=48), PHT oral daily dose (mg) (standardized coefficient, β = 0.731, $p < 0.001$) and *CYP2C9*3* (β = 0.366, $p = 0.013$) were independent predictors associated with increased mean of phenytoin $AUC_{0\text{-}12\ hr}$. A trend of association was also seen with *CYP2C19*2* (β = 0.263, $p = 0.073$). The model explains 42% of variability in $AUC_{0\text{-}12\ hr}$ (R^2= 0.422, *P*= 0.007).

Table 5.8 Multivariate regression analysis: Association of phenytoin $AUC_{0\text{-}12hr}$ with daily dose and genetic variants (N=48).

Independent Variables	Unstandardized Regression coefficients		Standardized Regression coefficients	*P*-value
	b	Standard error	β	
Constant	2.383	84.298	-	0.978
PHT oral daily dose (mg)	0.521	0.136	0.731	<0.001
Age (year)	-0.192	0.577	-0.048	0.741
Weight (kg)	-0.616	0.686	-0.149	0.375
Gender	11.488	27.048	0.074	0.673
CYP2C9*2	28.358	27.015	0.145	0.300
CYP2C9*3	91.664	35.161	0.366	0.013
CYP2C19*2	44.192	23.923	0.263	0.073
SCN1A c.IVS5-91 G>A	-0.522	14.392	-0.005	0.971
*ABCB1c.*3435 C>T	22.455	14.019	0.216	0.117
R^2 = 0.422, *P* = 0.007				

In a stepwise multiple linear regression model fit without intercept (N=48), Dose (mg) (standardized coefficient, β = 0.356, $p < 0.001$), *ABCB1*c.3435T/T (β = 0.469, $p < 0.001$), *ABCB1*c.3435C/T (β = 0.344, $p < 0.001$) and *CYP2C9*3* (β = 0.186, $p = 0.015$) were independent predictors associated with increased mean of phenytoin $AUC_{0\text{-}12\ hr}$. The model explains 76% of variability in $AUC_{0\text{-}12\ hr}$ (R^2= 0.760, $P < 0.001$).

Table 5.9 Stepwise Multivariate regression analysis: Association of phenytoin AUC $_{0\text{-}12hr}$ with daily dose and genetic variants (N=48).

Independent Variables	Unstandardized regression coefficients		Standardized regression coefficients β	*P*-value
	b	Standard error		
PHT oral daily dose (mg)	0.522	0.127	0.356	<0.001
ABCB1 T/T	118.097	19.879	0.469	<0.001
ABCB1 C/T	84.403	20.005	0.344	<0.001
CYP2C9*3	91.147	35.841	0.186	0.015
R^2 = 0.760, *P* < 0.001				

Key findings

This study aimed to investigate the associations of the combined genetic variants in *CYP2C9, CYP2C19, ABCB* and *SCN1A* genes and non-genetic factors with PHT maintenance dose as well as PHT exposure, and to use the identified factors to develop a statistical model to explain or estimate PHT dose.

Of the three demographic factors and five genetic variants studied, this study identified two non-genetic factors (body weight and age) and three genetic variants (*CYP2C9*2, CYP2C9*3* and *CYP2C19*2)* that were strongly associated with variability in phenytoin maintenance dose in adult Caucasian patients with epilepsy. These covariates explained about 40% of variability in PHT dose requirement in this sample. However, gender, *SCN1A* c.IVSN5+5 G>A and *ABCB1c.*3435 C>T were not significantly associated with PHT dose in this small study.

Moreover, PHT dose and a genetic factors including *CYP2C9*3* and *ABCB1*c.3435C>T were found to be associated with increase in phenytoin AUC $_{0\text{-}12\ hr}$ in the same group of adult Caucasian patients with epilepsy that are on PHT maintenance therapy. These covariates explained about 42-76% of variability in phenytoin AUC $_{0\text{-}12\ hr}$ in this study.

Discussion and conclusions

In this present study, it was found that the *CYP2C19*2* null allele along with the loss of function variants, *CYP2C9*3* and *CYP2C9*2*, were associated with decreased PHT dose requirement. This finding has further documented the important role of variants in *CYP2C19* in addition to *CYP2C9* in the inter-individual variability in PHT metabolism and dose requirement. Phenytoin is almost completely cleared by hepatic metabolism. The major and rate-limiting pathways in humans is p-hydroxylation of PHT in the CYP system to form (S)- and (R)- 5-(4-hydroxyphenyl)-5-phentlhydrantoin (HPPH). The main enzyme involved in PHT clearance is CYP2C9. A previous study has shown that the metabolic ratio of PHT (HPPH/PHT) in volunteers receiving a single oral dose of 300 mg PHT was lower in all individuals with CYP2C9 variant genotypes compared with those with homozygous wild-type genotypes (*CYP2C9*1/*1*). [50] A four-fold increase in AUC and decrease in PHT clearance have been shown in individuals homozygous for the *CYP2C9*3* allele compared with wide-type subjects [49] [50]. Lower levels of the maximum dose of PHT has been reported to be associated with patients who carry the *CYP2C9*3* allele [108]. Mean PHT doses of patients with 1 and 2 copies of *CYP2C9*3* were found to be lower than those with *CYP2C9*1/*1*. The variant, *CYP2C9*3*, could explain only 6.5% of the total variability in PHT dose. In contrast, *CYP2C9*2* did not show a significant association with dose in that study. [108]

Although CYP2C9 is the primary determinant, a number of studies have confirmed that the activity of CYP2C19 is clinically significant to overall phenytoin metabolism. Studies *in vitro* indicate that CYP2C19 is also involved in PHT metabolism and it appears that its contribution increases as the concentration of PHT increases. This suggests that CYP2C19 might be more important when CYP2C9 is saturated. Odani et al studied the effect of genetic polymorphisms of CYP2C9 and CYP2C19 on the pharmacokinetics of PHT and noted that there was a decrease of maximum metabolic rate (Vmax) in Japanese individuals with CYP2C19 variants compared to those from homozygous wild-type or extensive metabolizers (EMs).[51] Mamiya et al also reported that the mean Km value in poor metabolizers (PMs) of *CYP2C19*2* was 54%

higher than that of EMs.[52] In accordance with the results based on a population pharmacokinetic analysis, Hung et al (2004) found that the pharmacokinetic parameters (V_{max}, $CL_{intrinsic}$) decreased in Taiwanese patients who were carriers of both variant alleles of *CYP2C9* and *CYP2C19.* However, studies have found a strong association between *CYP2C9* variants, *CYP2C9*2* and **3*, and PHT dose requirement, but could not find the association with *CYP2C19*2*.[142] [108]

There is a significant difference in *CYP2C9* allelic variants frequencies among racial groups. The allele frequency of *CYP2C9*2* is estimated as 8% to 19% in Caucasians but zero in Asians. The allele frequency of *CYP2C9*3* has been reported as 3% to 15% in Caucasians and 1% to 6% in Asians.[42] Similar to *CYP2C9*, the variant allele frequency of *CYP2C19* varies significantly among ethnic groups. In contrast, the allele frequency of *CYP2C19*2* has been estimated as 11% to 13% in Caucasians, but 27% to 37% in Asians. In addition, *CYP2C19*3* allele is rare or absent in Whites, whereas its allele frequency is slightly higher in Asians (5% to 11%).[56] Since the incidence of variants in *CYP2C19* is more frequent in the Asian population, further study on the influence of CYP2C19 variants on PHT dose requirement in Asians should be conducted.

Furthermore, the finding that increased PHT exposure as measured by its $AUC_{0\text{-}12hr}$ was associated with *ABCB1*c.3435C>T allele, suggesting the role of Pgp on PHT pharmacokinetics. Hoffmeyer et al has first shown that the 3435T allele was associated with lower levels of Pgp in the duodenum and resulted in higher plasma concentration of digoxin.[77] Basic et al studied the effect of ABCB1 c.3435C>T on phenobarbital concentration in the cerebrospinal fluid (CSF) and serum (S) to assess the relationship of the polymorphism to phenobarbital penetration across blood brain barrier in epileptic patients receiving phenobarbital monotherapy. The result showed that compared to the CT heterozygotes and TT homozygotes, the CC homozygotes had a significantly lower CSF phenobarbital concentration and CSF/S concentration ratio with increased seizure frequency.[143] However, the association of *ABCB1*c.3435T allele with PHT

AUC $_{0\text{-}12hr}$ but lack of association with PHT dose in this study might be interpreted as the modest effect of *ABCB1* variant that could be masked by the effects of *CYPs* variants.

No significant association was found between the variant *SCN1A* c.IVSN5+5 G>A and PHT dose requirement or exposure in this study. The lack of association of *SCN1Ac.*IVS5N+5 G>A with PHT dose was inconsistent with the recent finding that this variant was associated with maximum dose of both PHT and CBZ in an epileptic patient cohort. In that study, CBZ dose was found to be lowest in patients with the GG genotype, highest in those with the AA genotype and intermediate in those with the heterozygous GA genotype.[108] Although another study by the same group of researchers failed to provide a similar association with PHT maximum and maintenance doses there was a significant association with PHT serum concentrations at maintenance dose. Further study has showed that the ancestral G allele of the *SCN1A* c.IVS5N+5 G>A expressed $Na_v1.1$ transcripts containing both exon 5A and 5N of the Nav1.1 transcripts. The variant A allele disrupts the consensus sequence of exon 5N and affects the alternative splicing at exon 5 of *SCN1A* resulting in a decreased expression of Nav1.1-5N transcripts.[108] More interestingly, the recent functional study conducted by Thompson et al has demonstrated that $Na_v1.1$-5N channels exhibited enhanced tonic block by PHT and lamotrigine compared to the $Na_v1.1$-5A. The results suggested that $Na_v1.1$ channels containing exon 5N are more sensitive to PHT and lamotrigine.[109]

For non-genetic factors, the negative and largest standardized regression coefficients of age indicated that older age has the largest influence on lower PHT dose (mg/day/kg).Previous study by Battino et al.on the influence of aging on the pharmacokinetics of PHT at steady state has shown that the apparent oral clearance values (CL/F) were negatively correlated with age ($r = -0.28$, $P < 0.05$). On average, CL/F values decreased about one third between 65 and 85 years of age. Evidently age explain 7.8% of the variation in CL/F in the elderly group.[144] Based on their findings, the authors suggested the use of smaller initial dosage in old patients. This may be helpful for preventing overdose in the elderly. In addition to age, increased body weight was clearly associated with increased PHT maintenance dose, suggesting the

contribution of body weight on PHT pharmacokinetics. Regarding gender, a significant association of female gender with lower PHT dose but not with weight-normalized dose might reflect the effect of lower body weight of female gender than that of male individuals.

By using multiple linear regression to quantify the association of PHT dose with genetic and non-genetic variants, the proposed regression model explain or predict the influence of advanced age and the presence of genetic variants *CYP2C9*3* or **2* and *CYP2C19*2* on lower PHT dose requirement. The influence is larger if individual carries variants of both *CYP2C9* and *CYP2C19*. An algorithm that included these factors could explain 40% of the variance in the PHT maintenance dose. In line with that, a retrospective analysis of 281 epileptic patients found that the maximum dosage of PHT was associated with the CYP2C9*3 allele. However, the polymorphism could explain only 6.5% of the total variation in doses in that study. [108]

To overcome the small sample size of the present study, the model was validated using a bootstrap re-sampling technique. The results strongly confirm the association of age and the presence of *CYP2C9*3, CYP2C9*2* and *CYP2C19*2* alleles on PHT dose requirement. The results support the hypothesis that the dosing algorithm could explain more than 30% of the variation in PHT dose.

In conclusion, the present study clearly demonstrates that in addition to non-genetic factors, age and body weight, multiple genetic factors including the presence of variants in gene encoding drug metabolizing enzymes, *CYP2C19* 2* and *CYP2C9*3 and CYP2C9*2*, contribute to the variability in PHT dose requirement and exposure. The findings suggest a dosage reduction for patients who are carriers of the loss of function alleles of *CYP2C9* and *CYP2C19*. Identifying subgroups of patients who carry these variant alleles would be necessary in order to decrease the incidence of concentration drug dependent intoxication. The findings also suggest the role of a variant in *ABCB1,* the *3435C>T,* on drug absorption,

distribution or elimination of PHT. Further studies should be conducted to identify other genetic and environmental factors.

Furthermore, since the application of pharmacogenetics on PHT dosing is based on the extent of non-genetic and pharmacogenetic factors that affect the PHT dose, such association should be quantified in future research. With sufficient information, maintenance PHT dose could possibly be estimated from these demographic and pharmacogenetic factors before treatment initiation to improve safety and effectiveness of PHT therapy.

Chapter Six

Association of *CYP3A5, ABCB1* and *SCN1A variants* and non-genetic variants with carbamazepine response phenotypes

Introduction

Carbamazepine (CBZ) is one of the most widely used first-line antiepileptic drug for the treatment of partial and generalized tonic-clonic epilepsies. However, when used as an antiepileptic drug, carbamazepine response or dose requirement varies widely from patient to patient. Several difficulties associated with carbamazepine dose selection include its narrow therapeutic index, auto-induction of its metabolic enzymes, as well as the formation of active metabolite.[33, 34, 145] In addition to nongenetic factors, it is increasingly recognized that genetic variability in the genes encoding proteins that involve in the biotransformation, distribution, excretion and action of many therapeutic drugs may complicate their dosing requirements.

Carbamazepine is a sodium channel blocker and a possible p-glycoprotein substrate. It is metabolized mainly by CYP3A4 and CYP3A5 to an active metabolite. Therefore common genetic variants in genes encoding these proteins may have a significant influence on CBZ dosing requirements and exposure.

Carbamazepine is mainly metabolized by CYP3A4 to an active metabolite, carbamazepine-10,11-epoxide, which further undergoes hepatic metabolism to inactive products [57] [60]. CYP3A5 has been shown to exhibit comparable metabolic activity as CYP3A4 toward CBZ epoxidation [60]. CYP3A5 is polymorphic and its expression is more variable than CYP3A4 [67]. Therefore, three variants in the *CYP3A5* gene (which associate with loss of enzyme function) include *CYP3A5*3, CYP3A5*6* and *CYP3A5*7* and may contribute to inter-individual variation in CBZ disposition and lead to variability in drug exposure and dosing requirement.

Furthermore, as a possible P-gp substrate, it is hypothesized that genetic variation in *ABCB1* gene which encodes a drug efflux transporter, P-gp, would influence CBZ absorption from the intestine or distribution to the brain. A synonymous polymorphism of the *ABCB1* gene, 3435 C>T, which has been reported to be associated with antiepileptic drug resistance, was selected as a candidate polymorphism for this study.[81, 88]

With respect to the drug target, an intronic polymorphism in the sodium channel gene, *SCN1A* c.IVSN5+5 G>A, has been reported to be associated with maximum doses of CBZ [108] and was also selected as a candidate polymorphism. In addition, inter-individual variability can be further affected by non-genetic factors. Therefore the hypothesis of this study was that polymorphisms in gene encoding *CYP 3A5, ABCB1, SCN1A* and nongenetic factors may influence CBZ dose requirement and exposure.

Purpose

This study aimed to investigate the associations of the combined genetic variants in *CYP 3A5, ABCB1 and SCN1A* genes and non-genetic factors including age, body weight and gender with carbamazepine maintenance dose and the area under the plasma concentration-time curve (AUC) at carbamazepine maintenance doses in Caucasian American and African American patients with epilepsy.

Study design

This study was a cross-sectional genetic association study using the candidate gene approach.

Procedure

Demographic and clinical variables were retrieved from a dataset of 55 unrelated adult Caucasian American and 32 African American patients with epilepsy on CBZ maintenance therapy. Genomic DNA samples were used to genotype 5 candidate SNPs including *CYP3A5*3* (g.6986A>G), *CYP3A5*6* (g.14690G>A), *CYP3A5*7* (g.27131_27132insT), *SCN1A*

c.IVSN5+5 G>A and *ABCB1*c.3435C>T by using the TaqMan assay for allele discrimination according to the manufacturer's instruction. Steady-state $AUC_{0\text{-}24\ hr}$ was determined from carbamazepine plasma concentrations at 0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 12 and 24 hours after an oral dose by using WinNonlin software program. Carbamazepine $AUC_{0\text{-}24\ hr}$ -to-dose ratio (ADR) was calculated by dividing the steady-state $AUC_{0\text{-}24\ hr}$ in mg-hr/L by CBZ oral morning dose in milligram. Bivariate analysis including correlation and simple linear regression analysis as well as stepwise multiple linear regression analysis were used to determine the association of genetic and non-genetic variants with carbamazepine response phenotypes, namely CBZ maintenance dose (mg/day) and ADR in Caucasian or African American patients. Genotype frequencies in each group were tested for Hardy-Weinberg equilibrium. A *P* value of < 0.05 was considered statistical significance.

Results

Study participants and participants' characteristic

Demographic and clinical characteristics

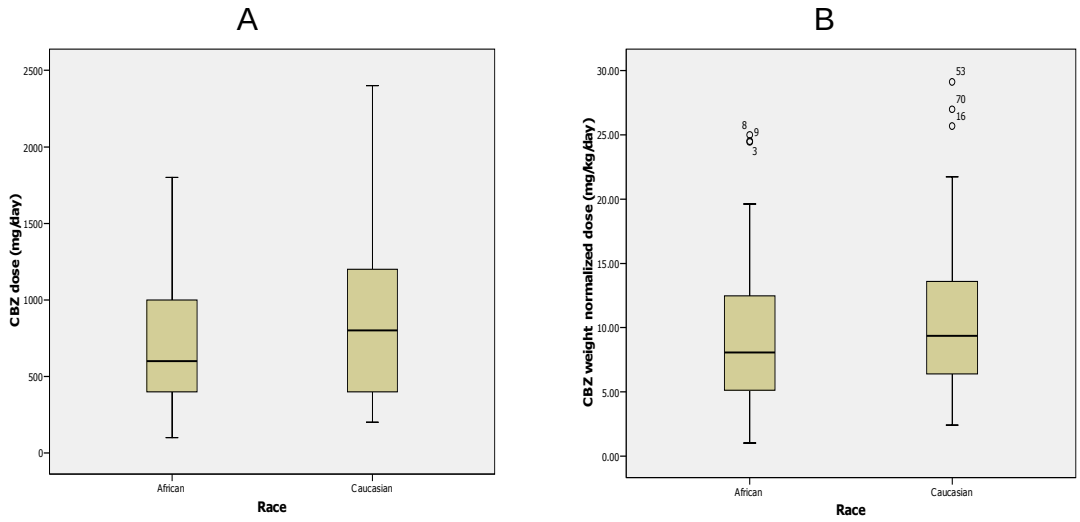
The demographic characteristics, means of CBZ daily dosage, $AUC_{0\text{-}24\ hr}$ and $AUC_{0\text{-}24\ hr}$ -to-dose ratio of the 32 African American and 55 Caucasian patients are summarized in Table 6.1. Boxplots comparing carbamazepine maintenance daily doses and weight normalized daily dose are shown in Figure 6.1. Carbamazepine daily maintenance doses seemed to be lower in the African American patients than those in the Caucasian patients with no statistical significance. While no significant differences in the $AUC_{0\text{-}24\ hr}$ between the two racial groups ($P>0.05$), there was a significant difference of carbamazepine $AUC_{0\text{-}24\ hr}$-to-dose ratio (ADR) between the two population samples. Carbamazepine ADR in the African American patients was significantly higher than that in the Caucasian patients ($P=0.018$).

Table 6.1 Demographic and clinical characteristics of the patients.

	African Americans (N=32)	Caucasian Americans (N=55)
Demographic variables		
Age, years, mean ± SD (range)	41.8 ±11.9 (19-68)	49.6 ± 16.1(20-87)
Bodyweight, Kg, mean ± SD (range)	84.3 ± 20.9 (48.0-152.9)	81.4 ± 18.1 (50.4-125.1)
Gender: Men / Women, n (%)	14 (44%) / 18 (56%)	32 (58%) / 23(42%)
Clinical variables		
Carbamazepine maintenance dose (mg/day), mean ± SD (range)	771.9 ± 420.6 (100-1,800)	858.2 ± 505.8 (200-2,400)
Carbamazepine maintenance dose (mg/kg/day), mean ± SD (range)	9.6 ± 6.2 (1.0-25.0)	10.7 ± 5.9 (2.4-29.1)
Carbamazepine AUC $_{0\text{-}24\ hr}$ (mg.hr/ml), mean ± SD (range)	169.7± 59.2 (76.07-253.96)	166.8± 53.1 (52.64-271.67)
Carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio, mean ± SD (range)	1.26 ± 0.76 (0.31-2.32)	0.75 ± 0.40 [a] (0.20-1.62)

[a] *P*= 0.018, independent Student's t test

Figure 6.1 Boxplots comparing carbamazepine maintenance daily doses (mg/day) (A) and weight normalized daily dose(mg/kg/day) (B) in African Americans (N=32) and Caucasian Americans (N=55).



Genotype distributions and allele frequencies

Tables 6.2 and 6.3 summarize the genotype distributions of the five candidate SNPs in the study patients separated by race. In each race group, all of the observed genotype frequencies of the *CYP3A5*3* (g.6986A>G), CYP3A5*6 *(g.14690G>A)*, *CYP3A5*7* (g.27131_27132insT), *SCN1A* c.IVSN5+5 G>A and *ABCB1*c.3435C>T of the study patients were in Hardy-Weinberg (HW) equilibrium (HWE $P > 0.05$). The allele frequencies of the tested variants in Caucasians and African Americans are shown in table 6.4. The frequency of variant alleles shows interracial differences. Most of the Caucasian individuals were detected with *CYP3A5*3* allele (91%); whereas only 45% of the African American individuals were detected with this allele. Furthermore, only patients in the African group were detected with *CYP3A5*6* and *CYP3A5*7,* while no patient in the Caucasian group was detected. Therefore, the allele frequencies of *CYP3A5*6* and *CYP3A5*7* were zero in the Caucasian group. In addition, there was a difference in the frequencies of *ABCB1*c.3435C>T allele between the African and Caucasian groups. Only 14% of the *ABCB1*c.3435 T allele was detected in the African group comparing to 46% in the Caucasian group.

Table 6.2 Genotype distribution and frequencies of *CYP3A5*3, CYP3A5*6, CYP3A5*7, SCN1A c.IVSN5+5 G>A* and *ABCB1c.3435 C>T* of the African Americans.

Genetic variables	**Genotype frequencies (n)**			**HWE P value**
*CYP3A5*3*	*1/*1	*1/*3	*3/*3	
(g.6986A>G)	36.36(12)	36.36(12)	27.27(9)	0.7231
*CYP3A5*6*	*1/*1	*1/*6	*6/*6	
(g.14690G>A)	81.82(27)	18.18(6)	0(0)	0.4520
*CYP3A5*7*	*1/*1	*1/*7	*7/*7	
(g.27131_27132insT)	63.64(21)	21.21(7)	15.15(5)	0.9183
SCN1A	G/G	G/A	A/A	
*(*c.IVSN5+5 G>A)	40.00(14)	45.71(16)	14.29(5)	0.3425
ABCB1	C/C	C/T	T/T	
(c.3435 C>T)	71.43(25)	28.56(10)	0(0)	0.5691

Table 6.3 Genotype distribution and frequencies of *CYP3A5*3, CYP3A5*6, CYP3A5*7, SCN1A c.IVSN5+5 G>A* and *ABCB1c.3435 C>T* of Caucasian Americans.

Genetic variables	**Genotype frequencies (n)**			**HWE P value**
*CYP3A5*3*	*1/*1	*1/*3	*3/*3	
(g.6986A>G)	0(0)	18.18(10)	81.82(45)	0.4583
*CYP3A5*6*	*1/*1	*1/*6	*6/*6	
(g.14690G>A)	100(55)	0(0)	0(0)	-
*CYP3A5*7*	*1/*1	*1/*7	*7/*7	
(g.27131_27132insT)	100(55)	0(0)	0(0)	-
SCN1A	G/G	G/A	A/A	
(c.IVSN5+5 G>A)	17.74(11)	51.61(32)	30.65(19)	0.6953
ABCB1	C/C	C/T	T/T	
(c.3435 C>T)	24.19(15)	59.68(37)	16.13(10)	0.1128

Table 6.4 Allele frequencies of the tested variants in African Americans (N=32) and Caucasian Americans (N=55).

Genetic variables	Effect	Allele frequencies	
		African Americans	Caucasian Americans
SCN1A c.IVSN5+5 A allele	Reduced fetal exon 5N expression	0.37	0.56
ABCB1 c.3435 T allele	Altered ABCB1 efflux transporter expression	0.14	0.46
*CYP3A5*3* allele	Reduced CYP3A5 expression	0.45	0.91
*CYP3A5*6* allele	Reduced CYP3A5 expression	0.09	0
*CYP3A5*7* allele	Reduced CYP3A5 expression	0.26	0

Association between demographic factors and genetic variants with carbamazepine doses

Bivariate analysis

Correlation coefficient

Correlation analyses revealed significant associations of CBZ maintenance dose with a demographic factor, age, as well as a genetic variant, *CYP3A5*3*. Table 6.5 summarizes the correlation coefficients between each variable and CBZ daily maintenance doses. Increasing age was significantly associated with decreased CBZ doses (r = -0.227, *P* = 0.009). Body weight and gender, on the other hand, were not significantly correlated with CBZ doses. Furthermore the presence of the *CYP3A5*3* allele was found to be significantly associated with increased CBZ doses (r = 0.181, *P* = 0.046). No significant correlation of variants in *CYP3A5*6, CYP3A5*7, SCN1A* c.IVS5-91 G>A and *ABCB1c.*3435 C>T with carbamazepine doses were seen in this study.

Figure 6.2-6.9 shows scatter plots or boxplots where appropriate, of CBZ maintenance dosage with demographic variables and genetic variants.

Table 6.5 Bivariate analysis: Associations of genetic variants, demographic factors and ethnicity with carbamazepine (CBZ) daily maintenance dose (mg/day) in combined African (N=32) and Caucasian (N=55) American patients.

Dependent Variables	**CBZ dose (mg/day)**	
Independent variables	correlation coefficients (r)	*P*-value (1-tailed)
Race	0.086[b]	0.188
Bodyweight (kg)	0.150[a]	0.060
Age (years)	-0.227[a]	0.009
Gender	-0.063[b]	0.258
*CYP3A5*3*	0.181[b]	0.046
*CYP3A5*6*	-0.038[b]	0.356
*CYP3A5*7*	-0.047[b]	0.330
SCN1A c.IVSN5+5 G>A	0.079[b]	0.221
ABCB1c.3435 C>T	0.004[b]	0.484

[a] Pearson's correlation
[b] Spearman's correlation

Figure 6.2 Correlation of bodyweight with carbamazepine (CBZ) doses (mg/day) in combined African (N=32) and Caucasian (N=55) American patients.

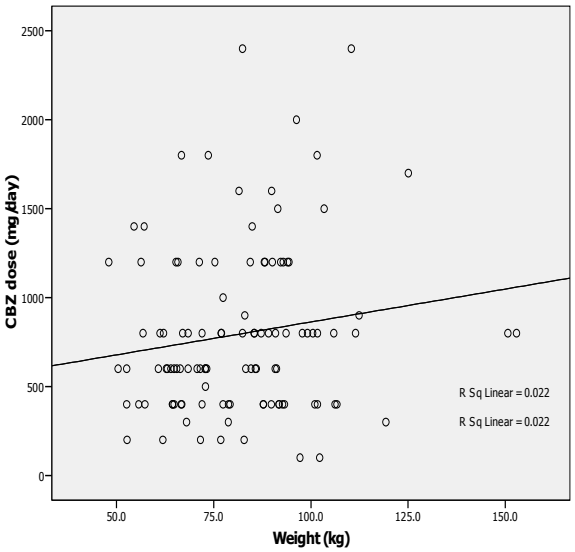


Figure 6.3 Correlations of age with carbamazepine (CBZ) doses (mg/day)

in combined African(N=32) and Caucasian (55) American patients.

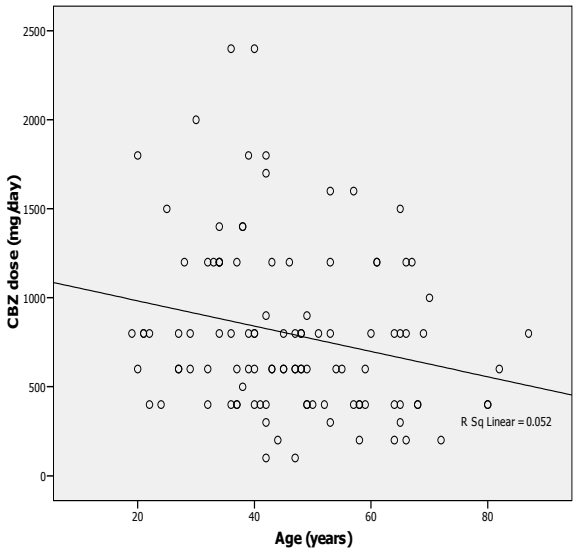


Figure 6.4 Boxplots of carbamazepine (CBZ) doses (mg/day) across genders in combined African (N=32) and Caucasian (N=55) American patients.

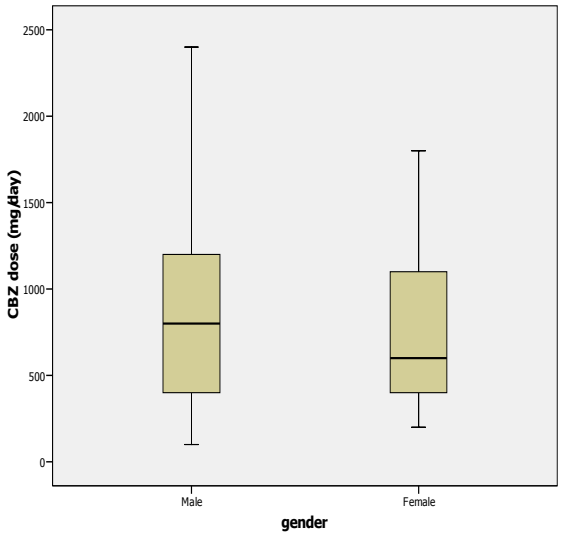


Figure 6.5 Boxplots of carbamazepine (CBZ) dose (mg/day) across *CYP3A5*3* genotypes in combined African (N=32) and Caucasian (N=55) American patients.

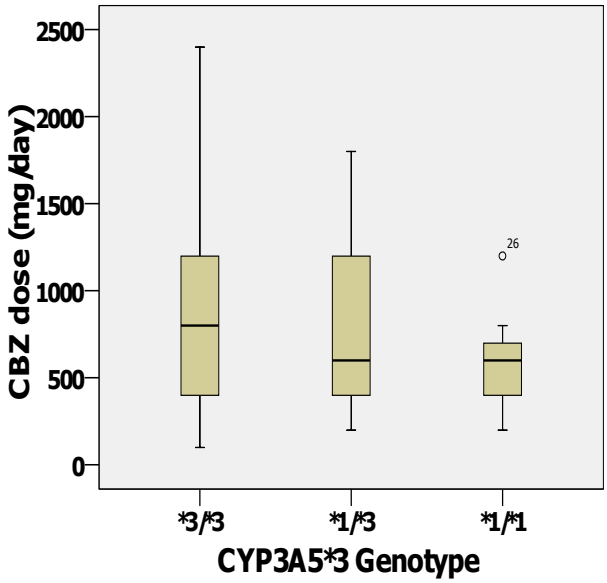


Figure 6.6 Boxplots of carbamazepine (CBZ) dose (mg/day) across *CYP3A5*6* genotypes in combined African (N=32) and Caucasian (N=55) American patients.

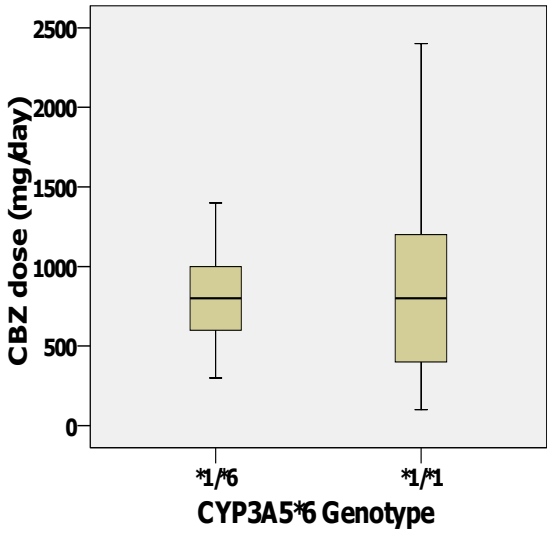


Figure 6.7 Boxplots of carbamazepine (CBZ) dose (mg/day) across *CYP3A5*7* genotypes in combined African (N=32) and Caucasian (N=55) American patients.

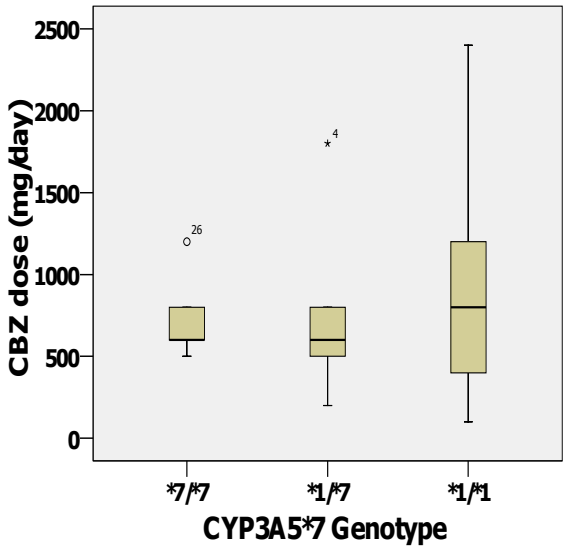


Figure 6.8 Boxplots of carbamazepine (CBZ) dose (mg/day) across *SCN1A* c.IVSN5+5 G>A genotypes in combined African (N=32) and Caucasian (N=55) American patients.

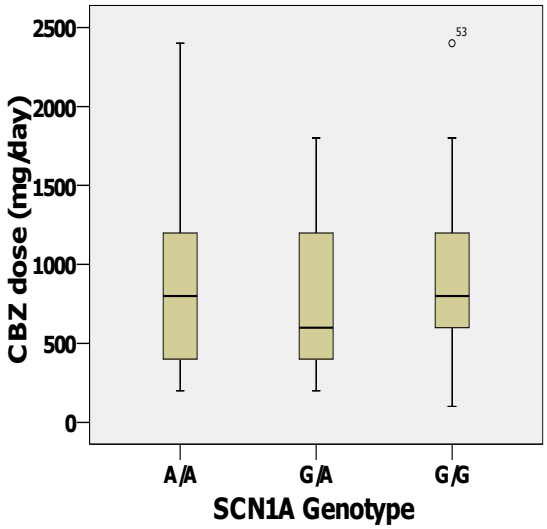
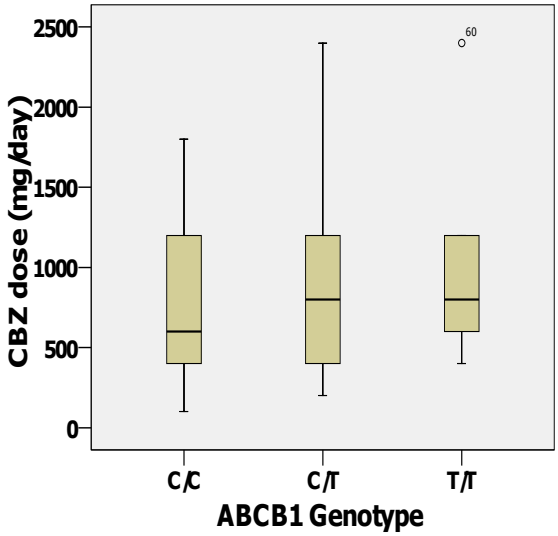


Figure 6.9 Boxplots of carbamazepine (CBZ) doses (mg/day) across *ABCB1c.*3435 C>T genotypes in combined African (N=32) and Caucasian (N=55) American patients.



Simple linear regression analysis

In simple linear regression analysis, as suggested by results from correlation analyses, there was a significant association between increasing age with decreased CBZ maintenance doses (regression coefficient, b = -7.112, P = 0.018). There was also a trend of association between the presence of CYP3A5*3 allele with increased CBZ doses (b = 132.789, P = 0.064). Table 6.6 summarizes the associations between CBZ doses with each of demographic and genetic variable as assessed by simple linear regression analysis.

Table 6.6 Simple regression analysis: Association of carbamazepine daily maintenance dose (mg/day) with genetic variants and demographic factors.

Independent Variables	Unstandardized Regression coefficients		Standardized Regression coefficients	*P*-value	R^2
	b	Standard error	β		
Race	101.515	93.693	.105	0.281	0.011
Age (year)	-7.112	2.948	-0.227	0.018	0.052
Gender	-50.338	91.063	-0.053	0.582	0.003
Body Weight (kg)	3.700	2.365	0.150	0.121	0.022
CYP3A5*3	132.789	70.745	0.198	0.064	0.039
CYP3A5*6	-12.875	192.760	-0.007	0.947	0.000
CYP3A5*7	-60.901	100.260	-0.065	0.545	0.004
SCN1A c.IVSN5+5 G>A	-32.323	69.904	-0.047	0.645	0.002
*ABCB1c.*3435 C>T	12.152	76.749	0.016	0.875	0.000

Multiple linear regression analysis

When the combined influence of genetic and non-genetic variants were taken into account by multivariate analysis, a stepwise multiple linear regression analysis showed the combined association of age and genetic variant with CBZ maintenance dose (mg/day). Table 6.7 summarizes the main findings of this study. In a multiple linear regression model, increasing age was independently associated with decreased CBZ dose (b = -6.842, *P* = 0.037). Whereas

the presence of *CYP3A5*3* alleles was independently associated with increased CBZ dose (b = 140.214, *P* = 0.048). The other candidate SNPs including *CYP3A5*6, CYP3A5*7, SCN1A* c.IVSN5+5 G>A and *ABCB1c.*3435 C>T, as well as body weight and gender were not significantly associated with the CBZ maintenance dose in this study. The two covariates explained about 9% (R^2 = 0.089, *P* = 0.020) of inter-individual variability in CBZ maintenance dose according to the regression model in this study.

Table 6.7 Multiple regression analysis: Association of carbamazepine daily maintenance dose (mg/day) with genetic variants and demographic factors.

Independent Variables	Unstandardized Regression coefficients		Standardized Regression coefficients	*P*-value
	b	Standard error	β	
Constant	940.844	186.191	-	< 0.001
Age (year)	-6.842	3.233	-0.221	0.037
CYP3A5*3	140.214	69.993	0.209	0.048
R^2 = 0.089, *P* = 0.020				

Association of genetic and non-genetic variants with CBZ plasma-concentration time curve at maintenance doses

Bivariate analysis

Correlation coefficients

Table 6.8 summarizes the correlation coefficients between each variable and carbamazepine $AUC_{0\text{-}24\ hr}$ -to- dose ratio (ADR). Correlation analyses revealed significant associations of carbamazepine ADR with race and multiple genetic variants including *CYP3A5*3*, *CYP3A5*7* and *ABCB1c.*3435 C>T. As shown in table 6.8, Caucasian race was correlated with lower carbamazepine ADR compared to African Americans (r = -0.464, *P* = 0.001). Decreased carbamazepine ADR was also significantly correlated with the presence of

the CYP3A5*3 allele (r = -0.569, *P* < 0.001) and *ABCB1c.*3435 T alleles (r = -0.405, *P* = 0.003). On the other hand, the presence of CYP3A5*7 allele was significantly correlated with increased carbamazepine ADR (r = 0.371, *P* = 0.007). Age, body weight, gender, *CYP3A5*6 and SCN1A* c.IVSN5+5 G>A were not significantly correlated with CBZ ADR in this study.

Figure 6.10- 6.18 shows scatter plots or boxplots where appropriate, of CBZ ADR with demographic variables and genetic variants.

Table 6.8 Bivariate analysis: Correlations of race, nongenetic and genetic variants with carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio in combined African Americans (N=17) and Caucasian Americans (N=37).

Dependent Variables	AUC $_{0\text{-}24\ hr}$ -to- dose ratio	
Independent variables	correlation coefficients (r)	*P*-value (1-tailed)
Race	-0.464[b]	0.001
Bodyweight (kg)	-0.136[a]	0.189
Age (years)	0.006[a]	0.485
Gender	-0.084[b]	0.294
*CYP3A5*3*	-0.569[b]	<0.001
*CYP3A5*6*	-0.093[b]	0.274
*CYP3A5*7*	0.371[b]	0.007
SCN1A c.IVSN5+5 G>A	-0.209[b]	0.087
ABCB1c.3435C>T	-0.405[b]	0.003

[a] Pearson's correlation
[b] Spearman's correlation

Figure 6.10 Boxplots comparing carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio in African Americans (N=17) and Caucasian Americans (N=37).

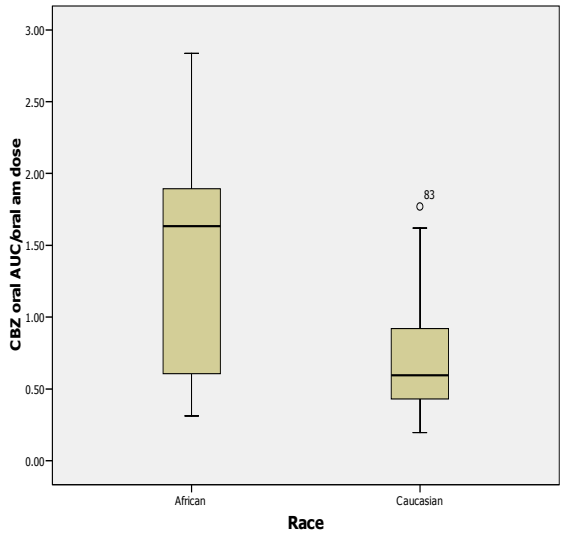


Figure 6.11 Scatter plot of body weight with carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio in combined African Americans (N=17) and Caucasian Americans (N=37).

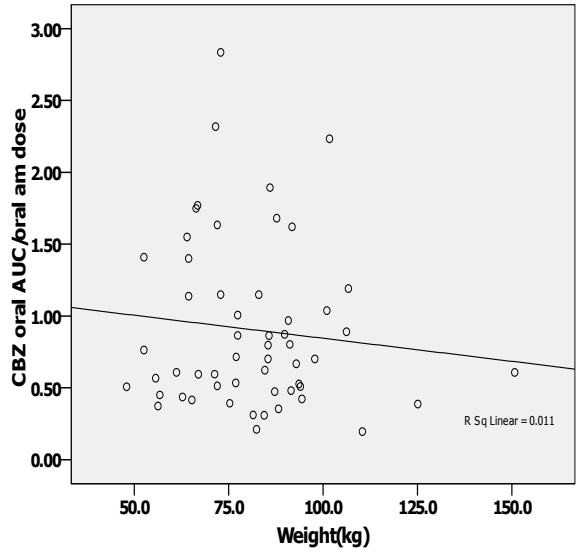


Figure 6.12 Scatter plot of age with carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio in combined African Americans (N=17) and Caucasian Americans (N=37).

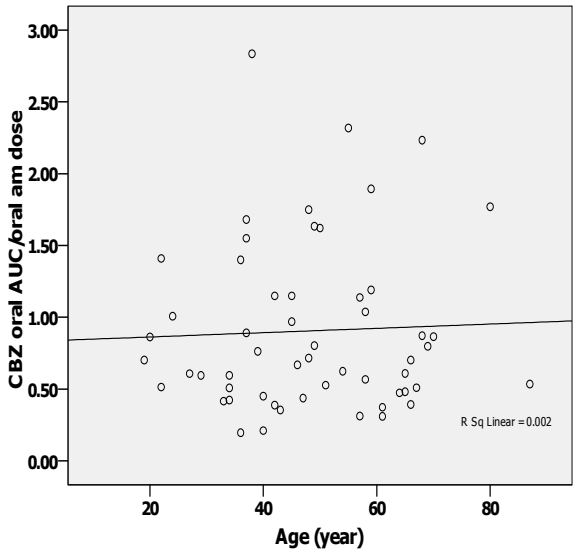


Figure 6.13 Boxplots of carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio across genders in combined African Americans (N=17) and Caucasian Americans (N=37).

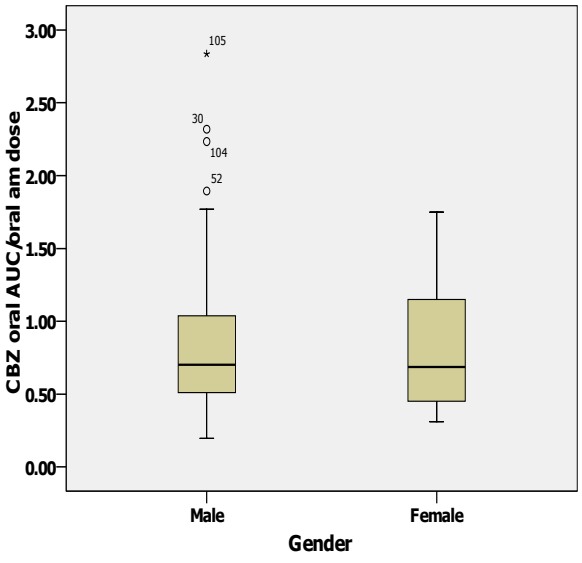


Figure 6.14 Boxplots of carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio across *CYP3A5*3* genotypes in combined African Americans (N=17) and Caucasian Americans (N=37).

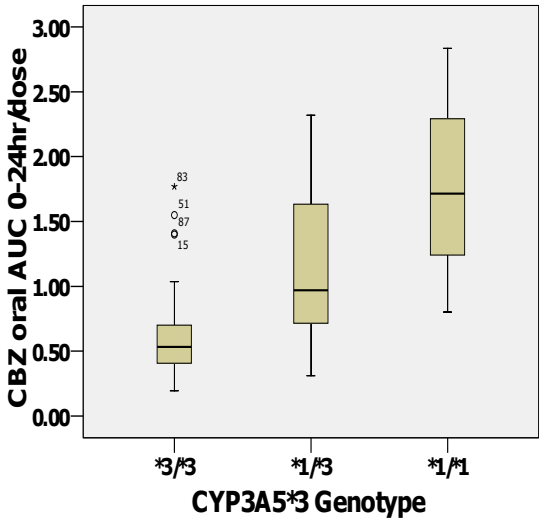


Figure 6.15 Boxplots of carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio across *CYP3A5*6* genotypes in combined African Americans (N=17) and Caucasian Americans (N=37).

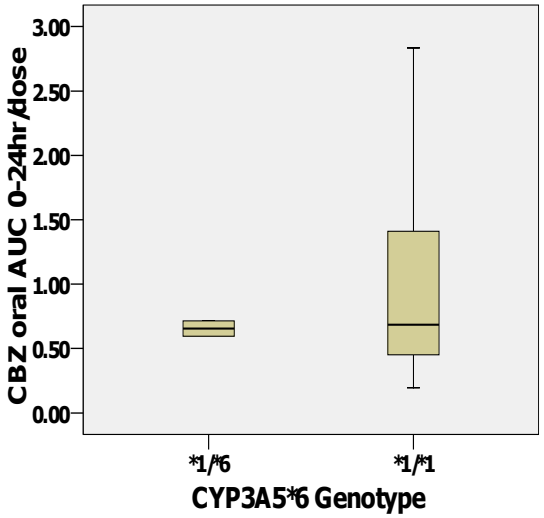


Figure 6.16 Boxplots of carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio across *CYP3A5*7* genotypes in combined African Americans (N=17) and Caucasian Americans (N=37).

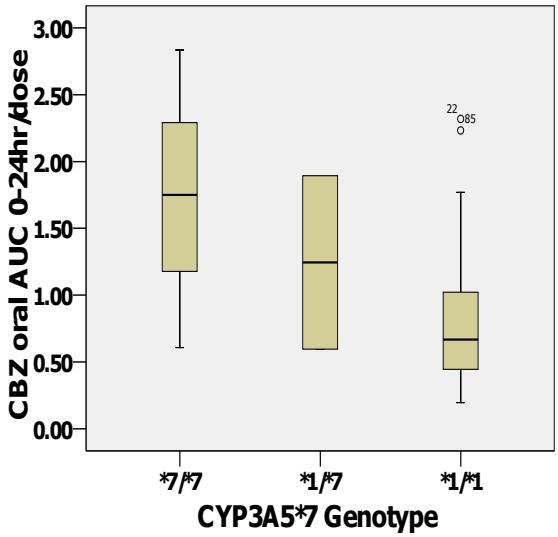


Figure 6.17 Boxplots of carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio across *SCN1A* c.IVSN5+5 G>A genotypes in combined African Americans (N=17) and Caucasian Americans (N=37).

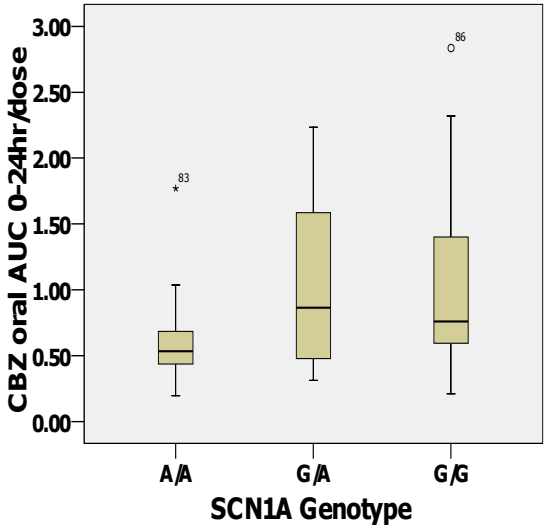
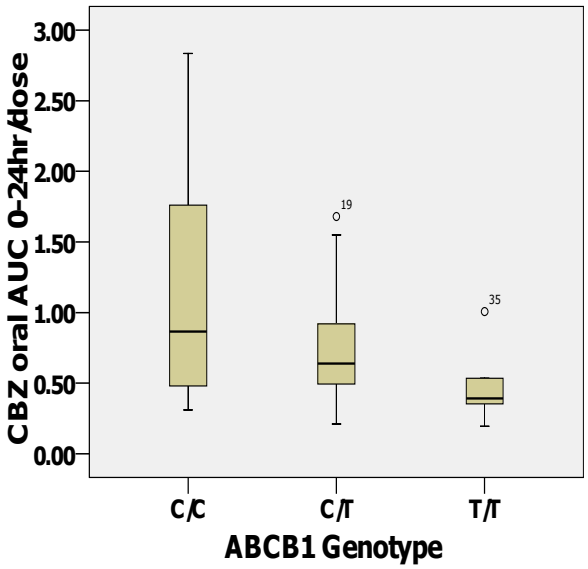


Figure 6.18 Boxplots of carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio across *ABCB1c.*3435 C>T genotypes in combined African Americans (N=17) and Caucasian Americans (N=37).



Simple linear regression analysis

In simple linear regression analysis, as suggested by correlation analyses, there were significant associations between carbamazepine ADR with race, as well as genetic variants including *CYP3A5*3*, *CYP3A5*7* and *ABCB1c.*3435 C>T. Caucasian race was associated with decreased carbamazepine ADR compared with African Americans (regression coefficient, b = -0.505, *P* = 0.002). Decreased carbamazepine ADR was also associated with the presence of CYP3A5*3 allele (b= -0.546, *P* < 0.001) and *ABCB1c.*3435 T alleles (b= -0.344, *P* = 0.007). On the other hand, the presence of a CYP3A5*7 allele was significantly associated with increased carbamazepine ADR (b = 0.437, *P* = 0.013). No significant association was found with age, body weight, gender, *CYP3A5*6* and *SCN1A* c.IVSN5+5 G>A. Table 6.8 summarizes the associations between carbamazepine AUC $_{0\text{-}24hr}$ –to-dose ratio (ADR) with each demographic and genetic variables assessed by simple linear regression analysis.

Table 6.8 Simple linear regression analysis: Association of carbamazepine AUC $_{0\text{-}24hr}$ –to-dose ratio (ADR) with genetic and non-genetic variants.

Independent Variables	Unstandardized Regression coefficients		Standardized Regression coefficients	*P*-value	R^2
	b	Standard error	β		
Race (Caucasian)	-0.505	0.159	-0.404	0.002	0.163
Age (year)	0.002	0.005	0.041	0.769	0.002
Gender	-0.086	0.162	-0.073	0.598	0.005
Body weight (kg)	-0.003	0.004	-0.103	0.453	0.011
CYP3A5*3	-0.546	0.122	-0.569	<0.001	0.324
CYP3A5*6	-0.281	0.465	-0.093	0.549	0.009
CYP3A5*7	0.437	0.169	0.371	0.013	0.137
SCN1A c.IVSN5+5 G>A	-0.177	0.119	-0.214	0.144	0.046
*ABCB1c.*3435 C>T	-0.344	0.122	-0.384	0.007	0.148

Multiple linear regression analysis

Stepwise multiple linear regression analysis showed the association of carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio and a genetic variant.

Table 6.9 summarizes the findings of this study. In a multiple linear regression model, *CYP3A5*3* (b = -0.546, *P*=< 0.001) was an independent predictor associated with decreased mean of carbamazepine AUC $_{0\text{-}24\ hr}$-to-dose ratio. The model explains about 32% of the variability in carbamazepine AUC $_{0\text{-}24\ hr}$-to-dose ratio (R^2 = 0.324, *P* < 0.001).

Table 6.9 Multivariate regression analysis: Association of carbamazepine AUC $_{0\text{-}24hr}$ –to-dose ratio with genetic variant.

Independent Variables	Unstandardized regression coefficients (b)	Standard error	*P*-value	Standardized regression coefficients (β)
Constant	1.754	0.202	<0.001	-
CYP3A5*3	-0.546	9.122	<0.001	-0.569
R2 = 0.324, P < 0.001				

Key findings

This study aimed to investigate the associations of the combined genetic variants in *CYP 3A5, ABCB1 and SCN1A* genes and non-genetic factors with CBZ maintenance dose and exposure.

By using multivariate analysis, a significant association was found between CBZ maintenance dose and a non-genetic factor, age, and a genetic variant, *CYP3A5*3.* Carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio was also found to be associated with the presence of *CYP3A5*3* alleles.

In addition, it was found that carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio was significantly different between African and Caucasian American patients. In bivariate analysis, carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio was found to be associated with race, *CYP3A5*3, CYP3A5*7* and *ABCB1c*.3435C>T allele suggesting that there might be the influence of multiple polymorphisms on CBZ pharmacokinetics which may result in the different exposures to CBZ between African and Caucasian American patients.

Discussion and conclusions

The findi that the presence of *CYP3A5*3* was associated with higher CBZ maintenance doses and with lower carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio seems to be a paradox since

*CYP3A5*3* encodes a shorter, less active CYP3A5 enzyme. By using heterologously expressed CYP3A4 and CYP3A5 and phenotyped human liver microsome, Huang et al showed that CYP3A5 exhibited comparative metabolic activity of CYP3A4 toward CBZ epoxidation. [60] The authors suggested that, under conditions where CYP3A5 was expressed to a significant fraction of the total CYP3A content, the contribution of CYP3A5 to the disposition of some drugs may be an important source of inter-individual variability in drug disposition.[60] In human, the *CYP3A* locus contains *CYP3A4*, *CYP3A7* and *CYP3A5* genes lying in order [64]. Linkage disequilibrium analysis has shown that *CYP3A5*3* is closely linked to *CYP3A4*1* [146]. In Caucasians and Asians the most common *CYP3A* haplotype includes *CYP3A4*1A, CYP3A7*1, CYP3A7*_3925 6T and *CYP3A5*3*. In the adult liver, this haplotype is associated with the phenotype that only CYP3A4 is expressed. [66] Furthermore, studies have shown that CYP3A5*3 homozygotes have no CYP3A5 protein expression in the liver. Only individuals with at least one CYP3A5*1 allele expressed metabolically active CYP3A5 protein.[67] Therefore the apparent association of low AUC $_{0\text{-}24\ hr}$ -to- dose ratio of CBZ with the defective allele *CYP3A5*3* may reflect the association with *CYP3A4*1A* which encodes CYP3A4 with normal enzyme activity.

The observation that there was a marginal association between increased carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio and the CYP3A5*7 allele, is in agreement with the fact that the frame shift variant *CYP3A5*7* was associated with no expression and loss of function of CYP3A5 in some individuals. The loss of function variants in *CYP3A5* therefore contribute to the increased AUC or exposure to CBZ.

The association of Caucasian race with decreased carbamazepine ADR would be explained by the different allele frequency of genetic variants between Caucasian and African patients. The present study shows that *CYP3A5*3* is the most common defective allele found with an allele frequency of about 90% in Caucasian Americans but only 45% in African Americans. While *CYP3A5*6* and *CYP3A5*7* are not present in Caucasian, they are found with 9% and 26% frequency respectively in African Americans. Therefore, very high frequency of

*CYP3A5*3* homozygotes (82%) and heterozygotes(18%) in Caucasian Americans found in this study, or in other words, a very low frequency of *CYP3A5*1* found, implies that the impact of CYP3A5 in Caucasian patients would be of less significance compared to African patients. Therefore, the lower carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio in Caucasian group would reflect the association with *CYP3A4*1A* as suggested before.

Furthermore, the marginal association of *ABCB1*c.3435T allele with decreased carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio should be interpreted cautiously. The opposite finding has been reported in previous studies. Hoffmeyer et al. first reported that the 3435TT genotype was associated with decreased expression of intestinal Pgp and increased digoxin plasma levels compared to those with CC genotype in Caucasian volunteers.[77] Basic et al. also reported that the *ABCB1c*.3435CC genotype was associated with lower phenobarbital levels in the cerebrospinal fluid (CFS) and lower CSF/plasma ratio.[143] However, at present the influences of the synonymous variant, *ABCB1*c.3435C>T, on *ABCB1* mRNA and P-gp expression are still uncertain. It was recently found that the silent variant, *ABCB1*c.3435C>T, changes substrate specificity rather than alters mRNA and protein levels. [95]

Further more, while the association of *CYP3A5*3* with carbamazepine AUC $_{0\text{-}24\ hr}$ -to- dose ratio explained about 32 % of variability in ADR, the association of *CYP3A5*3* with carbamazepine dose explained only 9% of the variability in dose. This suggests the modest influence of *CYP* variants on dose requirement. Other undetermined genetic factors in the complicated metabolic pathways of carbamazepine and clinical factors would influence inter-individual variability in CBZ dose requirement.

As shown in this study, the frequency of variant alleles shows interracial differences. In Caucasian descendants, the loss of function allele *CYP3A5*3* background was found in most individuals. In African descendants, on the other hand, *CYP3A5*1* allele is more common along with the presence of the loss of function allele CYP3A5*6 and the null allele *CYP3A5*7*. Large interracial differences of functional variant allele frequencies of *CYP3A5* and others may

contribute to inter-individual and interracial differences in CBZ exposure, and potentially, response among Caucasian and African descendants.

No significant association was found between the variant in *SCN1A*IVS5N+5 G>A and CBZ dose requirement or exposure. In a previous pharmacogenetic study, the variant was found to be associated with maximum dose of both PHT and CBZ in a cohort of patients with epilepsy. In that study, CBZ dose was found to be the lowest in patients with GG genotype, highest in those with the AA genotype and intermediate in those with the GA genotype.[108] Study by Abe et al. found that the frequency of the AA genotype was significantly higher in carbamazepine-resistant patients and AED-resistant patients.[147]

For non-genetic factors, age was found to be negatively correlated with CBZ dose ($r = -0.227$, $P = 0.009$) in bivariate analysis and was confirmed as an independent predictor in multiple linear regression analysis ($b = -6.842$, $P = 0.037$). In agreement with our finding, age has been reported to be negatively correlated with apparent oral clearance (CL/F) in a retrospective study of steady state serum CBZ concentration in a group of 157 elderly patients with epilepsy compared with younger subjects.[148] The presumed reduction in the rate of CYP mediated drug metabolism was suggested.[148]

In conclusion, the results from this study suggest the influences of *CYP* and *ABCB1* variants and a non-genetic factor, age, on CBZ exposure and dose requirement. Further studies are needed to characterize the effect of other undetermined genetic variants and clinical factors on CBZ response. Racial differences in allele frequencies of multiple functional variants and the different combinations of variants may influence inter-individual differences in CBZ response. Further studies in racial genetic differences may explore racial differences in CBZ pharmacokinetics or pharmacodynamics and therefore improve the existing therapeutic efficacy and safety.

Chapter Seven
Conclusions and Future Directions

The objective of the two studies included in this dissertation was to investigate the combined associations of common genetic variations in genes encoding drug metabolizing enzymes, drug transporters and drug target along with non-genetic variations on the clinical phenotypes of drug response, namely maintenance dose and exposure, of the two drugs, phenytoin and carbamazepine.

For genetic factors, the results from these pharmacogenetic studies shows that common functional genetic variants in gene encoding drug metabolizing enzymes have strong influence on drug response phenotypes both in terms of maintenance dose and AUC. For PHT, it was clearly demonstrated that the loss of function variant alleles including *CYP2C9*3* and *CYP2C9*2* along with *CYP2C19*2* were strongly associated with decreased PHT maintenance dose and increased AUC. It was shown that patients who carried the combination of these three variant alleles were clearly associated with the lower PHT dose requirement compared to those who carried the wild-type allele.

Unlike the PHT pharmacogenetic study, the association of CBZ maintenance dose and exposure with the loss of function allele *CYP3A5*3* which might be a surrogate of *CYP3A4*1A* was found to be paradoxical. This was because it was found that the loss of function allele *CYP3A5*3* was associated with increased CBZ maintenance dose and decreased ADR. In contrast, *CYP3A5*7* which encoded a truncated protein was found to be associated with increased carbamazepine ADR. The association of *CYP* variant alleles with CBZ response phenotype was partly unclear which may be attributable to undetermined factors. The most possible cause is that the true effect might be masked by the influence of many other undetermined genetic variations in complex CBZ metabolic pathways. Based on a general principle, a genetic variant would exert its effect on metabolism of a drug that has only one

major pathway for elimination. The findings on genetic variants on CBZ metabolism are thus inconclusive.

The association of drug response phenotypes with the most studied genetic variation in drug transporter gene, *ABCB1*c.3435C>T, is still inconclusive. The association of the *ABCB1*c.3435T allele along with *CYP2C9*3* with increased mean of phenytoin $AUC_{0\text{-}12\ hr}$ has a biological plausibility. The silent allele, *ABCB1*c.3435T, was postulated to be associated with decreased Pgp expression and function or altered substrate specificity.[77] [95] The finding is in line with the proposed hypothesis that drug efflux transporter, Pgp, works in concert with drug metabolizing enzyme to play an important role in drug disposition. However, the observation that the *ABCB1*c.3435T allele was associated with decreased carbamazepine $AUC_{0\text{-}24\ hr}$ -to- dose ratio was not consistent with the proposed hypothesis. The possible explanation for this opposite observation has not been found except for the finding by Seo et al that the *ABCB1*c.3435TT genotype was associated with drug-resistance to carbamazepine in Japanese patients.[149] The finding was opposed to the first finding by Siddiqui et al that multidrug-resistant epilepsy was significantly associated with the *ABCB1*c.3435CC allele. [81] Furthermore, in contrast to PHT which has been demonstrated to be a substrate of Pgp, such findings on CBZ are not clear. [72, 73, 150]

In the present studies, we did not find the association of a candidate genetic variant in sodium channel gene, *SCN1A*IVS5N+5 G>A with either PHT or CBZ response phenotypes. A small sample size which could potentially lead to insufficient power of the study to detect the association might partly contribute to this limitation.

For non-genetic factors, age was the only factor that was consistently associated with PHT and CBZ dose requirements. These results confirm the previous finding that patients with old age are associated with lower apparent oral clearance compared with young adult matched control and therefore require lower PHT and CBZ dosages.[144, 148]

Increased body weight was associated with increased PHT dose requirement. This association could be explained by the effect of increased drug volume of distribution. In contrast, no such association was found between body weight and CBZ dose. Other undetermined factors, such as CBZ autoinduction after chronic use, may mask the influence of body weight.

Biological differences between men and women may lead to differences in both pharmacokinetics and pharmacodynamics and ultimately result in differences in drug responses. In the present study, gender was found to be confounded with body weight. Female gender was associated with lower body weight. As a result, gender was not a significant covariate after adjusting for body weight. To interpret the influence of gender, one should be aware of this possible association.

Taken together, the findings of the present studies demonstrate that the combined association of genetic and non-genetic variants with PHT and CBZ response phenotypes strongly confirms that multiple genetic and non-genetic factors work in concert to influence inter-individual variation in drug response.

This multi-factorial architecture precludes the usefulness of conventional bivariate genetic analysis and requires application of alternative methods. Therefore, genetic analysis of complex phenotypes such as multiple linear regression analysis, is a powerful tool to identify multiple genetic variants associated with continuous traits. The multivariable analysis is a tool to identify different associated factors and their relative independent contributions to variability of a phenotypic outcome. Multiple variable analysis allows us to simultaneously assess the impact of multiple independent variables on outcome. Furthermore the results are able to be adjusted for factors that normally determine response of many drugs including age, weight as well as other associated variables.

Genetic differences among racial groups usually reflect differences in the distribution of polymorphic traits, which occur in different frequencies in different populations. For instances,

polymorphisms of *CYP2C9, CYP2C19* and *CYP3A5* the enzymes responsible for drug metabolisms of PHT and CBZ are distributed differently between Caucasian and African Americans, so the proportion of people with impaired metabolism differs between these two populations. In the CBZ pharmacogenetic study, race was used as a surrogate variable for unstudied genetic and environmental factors that may result in variability in drug response and a significant difference in carbamazepine $AUC_{0\text{-}24\ hr}$-to-dose ratio was found between the two racial groups. Therefore, different allele frequency of pharmacogenetic variant alleles in different racial groups would be an important predisposing factor that may give rise to variability in drug response among different racial descendants. Multiethnic studies enable us to look into a boarder picture of complex disease etiology by putting together several pieces of evidences from various views. Therefore, multiethnic studies are not only necessary but also advantageous for dissecting the etiology of complex disease [151]. What we need are clearly defined phenotypes, carefully designed recruitment procedures and rigorous analytic approaches. Together, these components will advance our knowledge about complex diseases in all humans [151].

There are several limitations in this study that should be acknowledged including the limited sample size and the limited number of clinical factors that may contribute to variation in drug response to antiepileptic drugs. Some of these clinical factors, such as the types of epilepsy, the age of onset of epilepsy and the number of seizures before starting treatment have been reported to be associated with refractory epilepsy. [6] Furthermore, potential drug-drug interactions should also be considered. Including these factors as independent covariates in future analyses might further explain variability in antiepileptic drug response.

Finally, it should be kept in mind that association is not causal. Statistics alone can not prove that a relationship between an associated factor and outcome is causal. Causality is established on the basis of biological plausibility and well designed study which minimize sources of potential bias. Therefore, any findings from pharmacogenetic studies should be replicated in a well-designed larger independent study to minimize the false positive findings.

Other undetermined genetic variants, non-genetic and clinical factors such as types of seizures, age of seizure onset, drug-drug or food-drug interactions which are possible responsible for variable responsiveness among patients need further study. Functional study is also crucially needed to verify biological plausibility.

Concluding remarks

This dissertation clearly confirms that drug response is a complex multifactorial phenotype influenced by many genetic and non-genetic factors. However, the genes and variants identified so far explain only a small fraction of variability in response to AEDs, and still need to be replicated by other independent studies. Pharmacogenetic or pharmacogenomic studies of complex disease and drug response is a major challenge for contemporary pharmacogeneticists. There will be a need to improve the association studies strategy.

As differences in drug response in terms of clinical efficacy or dosage requirement among patients are very common, pharmacogenetic research will enhance our understanding of underlying mechanisms and finding genetic tests for predicting drug efficacy in patients with epilepsy. The development and implication of a pharmacogenetic model to quantify the association and to predict response phenotypes at the treatment initiation would be valuable.

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