INFLUENCE OF GENETIC VARIABILITY ON THE CLINICAL PHARMACOLOGY OF CARBAMAZEPINE AND LAMOTRIGINE

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Summary

Epilepsy is a common and serious but treatable neurological disorder. Current treatment is limited by high rates of adverse drug effects and lack of complete seizure control in a significant proportion of patients. Epilepsy is suitable for pharmacogenetic exploration because response to antiepileptic drug (AED) treatment can be quantified and adverse effects can be assessed by several measures. Furthermore, there is substantial knowledge of the pharmacodynamics and pharmacokinetics of AEDs and some candidate genes implicated in the disorder have been identified. However, recent studies examining the association of particular genes and their genetic variants with seizure control and adverse drug effects have not provided definitive conclusions.

Commonly, pharmacogenetic studies focus primarily on single nucleotide polymorphisms, which are single base variations in the sequence of genes. This form of genetic variant has the potential to influence the structure and/or function of the proteins those genes encode. Candidate genes for pharmacogenetic studies in epilepsy are those that encode proteins directly involved in the pharmacokinetics (drug metabolizing enzymes (DMEs), drug transporters (DTPs) and pharmacodynamics (voltage-gated sodium channels, γ-aminobutyric acid receptors (GABA) of AED action. These principals have been applied to this research programme, investigating the influence of genetic variability on the clinical pharmacology of carbamazepine (CBZ) and lamotrigine (LTG).

Although many genes could influence response to these drugs, obvious candidates are known. CBZ and LTG target the α-subunit of the voltage-gated sodium channels. CBZ metabolism involves several DMEs such as CYP3A4, CYP3A5, CYP1A2, microsomal epoxide hydrolase (mEH) and UGT2B7, and LTG is a probable substrate of P-

glycoprotein (P-gp). Common polymorphisms in genes encoding these proteins were examined as initial genetic factors that may influence the response to CBZ and LTG monotherapy. These polymorphisms include CYP3A4 g.-392A>G, CYP3A5 g.6986A>G, CYP1A2 g.5734C>A, EPHX1 c.337T>C, EPHX1 c.416A>G, UGT2B7 c.802C>T, ABCB1 c.1236C>T, ABCB1 c.2677G>T/A, ABCB1 c.3435C>T and SCN2A c.56G>A.

The prevalence of these common polymorphisms was evaluated in a 400-strong study population from a single research unit. The polymorphisms were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), with the exception of EPHX1 c.337T>C, which was identified by direct sequencing assay. Genotype distribution of each polymorphism was examined for Hardy-Weinberg equilibrium (HWE). Minor allele frequency ranged between 3.5% (CYP3A4 -392G) and 48.0% (ABCB1 1236T). Allele and genotype distributions were comparable to published data for other Caucasian populations. All genotype distributions were consistent with HWE. As such, no apparent systematic error in the genotyping assay was identified.

The influence of common polymorphisms in DME and sodium channel genes on the optimal dose of CBZ was assessed in a cohort of 70 patients. Optimal dose in this study was defined as the final dose given to a patient that successfully maintained optimal seizure control without intolerable side effects. Several basic clinical factors such as age and gender were also examined as potential predictors. The effect of predictors on the optimal dose of CBZ was investigated using linear regression analysis. This study revealed that age and common polymorphisms in the EPHX1 gene (c.337T>C and c.416A>G) are potential predictors for optimal dose of CBZ. The significant effects of EPHX1 variants may be explained by their significant contribution to the inactivation of

CBZ. These potential predictors explain approximately 15% of the inter-individual variation in CBZ dose requirements.

The prevalence of common polymorphisms in genes that encode CBZ-related DMEs was examined in an effort to identify a unique adverse effect profile. A total of 14 out of 104 patients receiving CBZ monotherapy experienced at least one intolerable adverse effect which led to withdrawal of treatment. Of these, 9 patients experienced central nervous system (CNS)-related effects and 5 patients experienced idiosyncratic reactions. The variant allele distributions were compared between patients who experienced the adverse effects and those who did not by an appropriate contingency table test. There was no significant difference in allele distributions between these groups, either for CNS-related effects or idiosyncratic reactions. These preliminary findings suggest that common polymorphisms in DME genes do not form a unique profile which increases the risk of developing intolerable CBZ adverse effects.

The prevalence of common polymorphisms in ABCB1 and SCN2A genes was investigated between seizure free and non-seizure free patients who received LTG monotherapy as the first AED treatment. A total of 79 patients were involved in this study cohort. Of these, 39 patients had reached seizure free status within the first year of treatment. Differences in the distribution of gene variants were examined by logistic regression analysis. No difference was observed in the distribution of these polymorphisms between the two groups, whether analysed by allele, genotype, haplotype or diplotype frequency. It is unlikely that common polymorphisms in ABCB1 and SCN2A genes influence the expression and/or activity of their respective proteins to the level at which they can dictate response to LTG therapy.

The influence of common polymorphisms in ABCB1 and SCN2A genes on the optimal dose of LTG was assessed in a cohort of 94 patients. Optimal dose in this study was defined as the final dose given to the patients that successfully maintained seizure freedom for at least 1 year with LTG monotherapy. Several basic clinical factors such as age and gender were also examined as potential predictors. The effect of predictors on the optimal dose of LTG was investigated using linear regression analysis. This study revealed that gender and common polymorphisms in the ABCB1 gene (c.1236C>T and c.3435C>T) are potential predictors for optimal dose of LTG. These predictors explain approximately 17% of the inter-individual variation in LTG dose requirement. These findings further highlight the potential role of P-gp in the management of epilepsy

The final study investigated the influence of ABCB1 c.1236C>T and ABCB1 c.3435C>T polymorphisms on the pharmacokinetics of LTG. A total of 156 blood samples from 50 patients receiving LTG monotherapy were available for analysis. The influence of ABCB1 variants was evaluated by population pharmacokinetics. This approach successfully estimated the oral clearance of LTG monotherapy at steady-state. However, the absorption rate constant (K_a) and volume of distribution (V_d) of LTG were poorly estimated. The inclusion of common polymorphisms in the ABCB1 gene in the pharmacokinetic model did not improve the estimation of oral clearance. This may indicate that common variants of ABCB1 do not influence clearance of LTG.

In conclusion, these results suggest that optimal doses of CBZ and LTG are associated with polymorphisms in genes involved in their respective pharmacokinetics. Common genetic polymorphisms of other DMEs that are responsible for CBZ metabolism did not significantly associate with CBZ dose and none were associated with CBZ adverse effects. Polymorphisms in the ABCB1 gene may not be useful in predicting response to LTG treatment and did not influence the oral clearance of LTG. The present study is

preliminary but provides encouragement for future investigation, particularly, large scale studies of multiple polymorphisms and combinations thereof which attempt to identify a panel of genotypes that can be used as predictors of an individual patient's response to AED treatment.

Table of Contents

SUMMARY.....ii
LIST OF TABLES.....xiii
LIST OF FIGURES.....xvii
LIST OF ABBREVIATIONS.....xxi
ACKNOWLEDGEMENTS.....xxiii
AUTHOR'S DECLARATION.....xxiv
PUBLICATIONS.....xxv
CHAPTER 1 - GENERAL INTRODUCTION.....1
1.1 Epilepsy.....2
1.1.1 History of epilepsy.....2
1.1.2 Epilepsy and its syndromes.....3
1.1.3 Classification of seizures.....4
1.1.4 Diagnosis of epilepsy.....7
1.2 Treatment of Epilepsy.....8
1.2.1 History of antiepileptic drug development.....8
1.2.2 Indications of antiepileptic drugs.....9
1.2.3 Mechanisms of action of antiepileptic drugs.....11
1.2.4 Clinical pharmacokinetics of antiepileptic drugs.....14
1.2.5 Variability in the response to antiepileptic drug treatment.....19
1.3 Pharmacogenetics.....19
1.3.1 Human genetics.....20
1.3.2 Glossary of genetics.....21
1.3.3 Genetic variations among humans.....22
1.3.4 Nomenclature of genetic sequence variations.....23
1.3.5 History of pharmacogenetics.....24
1.4 Pharmacogenetics of antiepileptic drugs.....26
1.4.1 Pharmacogenetics of antiepileptic drugs: The influence of pharmacodynamic genes.....26
1.4.2 Pharmacogenetics of antiepileptic drugs: The influence of pharmacokinetic genes.....28
1.4.3 Antiepileptic drugs as drugs of pharmacogenetic interest.....30
1.5 Carbamazepine.....31
1.5.1 History and indications.....31
1.5.2 Mechanisms of action.....32
1.5.3 Clinical pharmacokinetics.....32
1.5.4 Metabolism of carbamazepine.....33
1.5.5 Effects of autoinduction on carbamazepine pharmacokinetics.....36
1.5.6 Concentration-response relationship.....38

1.5.7 Side effects and adverse reactions 39
1.5.8 Potential factors that influence the variability in clinical pharmacology of carbamazepine 40
1.6 Lamotrigine 42
1.6.1 Indication and efficacy 42
1.6.2 Mechanism of action 42
1.6.3 Clinical pharmacokinetics 43
1.6.4 Concentration – response relationship 45
1.6.5 Adverse effects 46
1.6.6 Potential factors that influence the variability in clinical pharmacology of lamotrigine 47
1.7 Drug metabolising enzyme, drug transporter protein and sodium channel genes and their common polymorphisms 49
1.7.1 Cytochrome P450: History and structure 49
1.7.2 Cytochrome P450 3As 52
1.7.2.1 Functions and tissue distribution of CYP3As 52
1.7.2.2 CYP3A gene locus 53
1.7.2.3 Genetics and common polymorphisms of CYP3A4 54
1.7.2.4 Genetics and common polymorphisms of CYP3A5 54
1.7.2.5 Phenotype profile of common CYP3A4 and CYP3A5 variants 55
1.7.3 Cytochrome P450 1A2 57
1.7.3.1 Functions and tissue distribution of CYP1A2 57
1.7.3.2 Genetics and common polymorphisms of CYP1A2 57
1.7.3.3 Phenotype profile of common CYP1A2 variants 59
1.7.4 Microsomal epoxide hydrolase 59
1.7.4.1 Tissue distribution and structure of microsomal epoxide hydrolase 60
1.7.4.2 Microsomal epoxide hydrolase activity 61
1.7.4.3 Genetics and common polymorphisms of EPHX1 62
1.7.4.4 Phenotype profile of common EPHX1 variants 63
1.7.5 UDP-glucuronosyltransferases 64
1.7.5.1 UDP-glucuronosyltransferase 2B7: Functions and tissue distribution 65
1.7.5.2 Genetics and common polymorphisms of UGT2B7 65
1.7.5.3 Phenotype profile of common UGT2B7 variants 66
1.7.6 Drug Transporter Proteins 67
1.7.6.1 P-glycoprotein 69
1.7.6.2 Functions and tissue distribution of P-gp 70
1.7.6.3 Genetics and common polymorphisms of ABCB1 71
1.7.6.4 Phenotype profile of common ABCB1 variants 72
1.7.7 Voltage-gated Sodium Channels 73
1.7.7.1 Tissue distribution of voltage-gated sodium channels 75
1.7.7.2 Genetics and common polymorphisms of SCN2A 75
1.7.7.3 Phenotype profile of common SCN2A variants 76
1.8 Study Aims 77

CHAPTER 2 - MATERIALS, EXPERIMENTAL PRINCIPLES AND RECURRENT METHODS 78

2.1 Materials 79

2.1.1 Chemicals and reagents 79

2.1.2 Pharmaceuticals 80

2.1.3 Materials for gel electrophoresis 80

2.1.3.1 Electrophoresis buffer 80

2.1.3.2 Agarose gel 80

2.1.3.3 Loading dye 80

2.1.4 Equipment 81

2.2 Experimental Principles 82

2.2.1 Polymerase chain reaction 82

2.2.1.1 Polymerase chain reaction components 82

2.2.1.2 PCR primer design 83

2.2.1.3 Polymerase chain reaction assay 83

2.2.1.4 Examining the PCR product 84

2.2.2 Identification of single nucleotide polymorphisms 85

2.2.2.1 Restriction fragment length polymorphism assay 85

2.2.2.2 Gel electrophoresis 87

2.2.2.3 Direct sequencing 87

2.2.3 Methods of assessing the response to antiepileptic drug treatment 88

2.2.3.1 Assessing the efficacy of antiepileptic drugs 89

2.2.3.2 Assessing the adverse effects of antiepileptic drugs 90

2.3 Recurrent Methods 92

2.3.1 Study subjects 92

2.3.2 Control DNA samples 92

2.3.3 DNA extraction 94

2.3.4 DNA quantification 95

2.3.5 Polymerase chain reaction 95

2.3.5.1 PCR primers 95

2.3.5.2 Optimization of polymerase chain reaction assay 96

2.3.5.3 Quality control of polymerase chain reaction assay 97

2.3.6 Restriction fragment length polymorphism assay 97

2.3.6.1 Choosing suitable restriction enzymes 97

2.3.6.2 Optimisation of restriction fragment length polymorphism assay 98

2.3.6.3 Quality control of restriction fragment length polymorphism assay 98

2.3.7 Statistical analyses *99*

CHAPTER 3 - POLYMORPHISMS OF DRUG METABOLISING ENZYME, DRUG TRANSPORTER PROTEIN AND SODIUM CHANNEL GENES 100

3.1 The Prevalences of Common Polymorphisms in the Drug Metabolizing Enzyme, Drug Transporter Protein and Sodium Channel Genes in a West of Scotland Epilepsy Population 101

3.1.1 Introduction 101

3.1.2 Aims 102

3.1.3 Methods 102

3.1.3.1 DNA samples and extraction 102

3.1.3.2 PCR-RFLP optimization 102

3.1.3.3 Identification of CYP3A4 g.-392A>G polymorphism by PCR-RFLP 102

3.1.3.4 Identification of CYP3A5 g.6986A>G polymorphism by PCR-RFLP 104

3.1.3.5 Identification of CYP1A2 g.5734C>A polymorphism by PCR-RFLP 106

3.1.3.6 Identification of EPHX1 c.337T>C polymorphism by PCR-RFLP 108

3.1.3.7 Identification of EPHX1 c.416A>G polymorphism by PCR-RFLP 110

3.1.3.8 Identification of UGT2B7 c.802C>T polymorphism by PCR-RFLP 111

3.1.3.9 Identification of ABCB1 c.1236C>T polymorphism by PCR-RFLP 113

3.1.3.10 Identification of ABCB1 c.2677G>T/A polymorphism by PCR-RFLP 115

3.1.3.11 Identification of ABCB1 c.3435C>T polymorphism by PCR-RFLP 118

3.1.3.12 Identification of SCN2A c.56G>A polymorphism by PCR-RFLP 119

3.1.3.13 Identification of EPHX1 c.337T>C polymorphism by direct sequencing 121

3.1.3.14 Predicting the net activity of mEH 124

3.1.3.15 Comparison of allele and genotype distributions between ethnic groups 125

3.1.3.16 Hardy-Weinberg Equilibrium test 126

3.1.4 Results 126

3.1.4.1 Allele and genotype distributions of CYP3A4 g.-392A>G and CYP3A5 g.6986A>G polymorphisms 126

3.1.4.2 Allele and genotype distributions of CYP1A2 g.5734C>A polymorphism 128

3.1.4.3 Allele and genotype distributions of EPHX1 c.337T>C and c.416A>G polymorphisms 130

3.1.4.4 Allele and genotype distributions of UGT2B7 c.802C>T polymorphism 130

3.1.4.5 Allele and genotype distributions of ABCB1 c.1236C>T, c.2677G>T/A and c.3435C>T polymorphisms 134

3.1.4.6 Allele and genotype distributions of SCN2A c.56G>A polymorphism 137

3.1.5 Discussion 138

3.1.5.1 The prevalence of common polymorphisms in DME, DTP and sodium channel genes in comparison with other ethnic groups 138

3.1.5.2 Hardy-Weinberg equilibrium test 143

3.1.6 Conclusion 144

CHAPTER 4 - PHARMACOGENETICS OF CARBAMAZEPINE. 145

4.1 Genetic Predictors of Carbamazepine Optimal Dose 146

4.1.1 Introduction 146

4.1.2 Aims 147

4.1.3 Methods 147

4.1.3.1 Definitions 147

4.1.3.2 Study subjects 147

4.1.3.3 Genotype analysis and phenotype assessment 148

4.1.3.4 Statistical analysis 148

4.1.4 Results 150

4.1.4.1 Patient characteristics and carbamazepine dosage regimen 150

4.1.4.2 Genetic predictors of carbamazepine optimal dose 150

4.1.5 Discussion 159

4.1.6 Conclusion 165

4.2 A Preliminary Genetic Analysis of Drug Metabolising Enzymes in Patients with Carbamazepine Adverse Effects 166

4.2.1 Introduction 166

4.2.2 Aims 167

4.2.3 Methods 168

4.2.3.1 Study subjects 168

4.2.3.2 Adverse reaction documentation 168

4.2.3.3 Genotype analysis and phenotype assessment 169

4.2.3.4 Genotype-phenotype analysis 169

4.2.3.5 Statistical analysis 169

4.2.4 Results 170

4.2.4.1 Patient characteristics and adverse effect profile 170

4.2.4.2 The pattern of polymorphisms in drug metabolising genes in patients with carbamazepine adverse effects 171

4.2.5 Discussion 171

4.2.6 Conclusion 177

CHAPTER 5 - PHARMACOGENETICS OF LAMOTRIGINE 178

5.1 Association between ABCB1 and SCN2A gene polymorphisms and the Response to Lamotrigine Therapy in Newly Diagnosed Epilepsy Patients. 179

5.1.1 Introduction 179

5.1.2 Aims 182

5.1.3 Methods 182

5.1.3.1 Study subjects 182

5.1.3.2 Genotype analysis and phenotype assessment 182

5.1.3.3 Haplotype and diplotype inference 183

5.1.3.4 Evaluation of clinical response 183

5.1.3.5 Statistical analysis 184

5.1.4 Results 184

5.1.4.1 Patient demographics and lamotrigine dosage regimen 184
5.1.4.2 Associations between polymorphisms in the ABCB1 and SCN2A genes and the response to lamotrigine monotherapy 186
5.1.5 Discussion 186
5.1.6 Conclusion 194
5.2 Genetic Predictors of Lamotrigine Optimal Dose 195
5.2.1 Introduction 195
5.2.2 Aims 196
5.2.3 Methods 196
5.2.3.1 Definitions 196
5.2.3.2 Study subjects 196
5.2.3.3 Genotype analysis and phenotype assessment 197
5.2.3.4 Statistical analyses 197
5.2.4 Results 199
5.2.4.1 Patient demographics and lamotrigine dosage regimen 199
5.2.4.2 Genetic predictors of lamotrigine optimal dose 203
5.2.5 Discussion 208
5.2.6 Conclusion 211
5.3 The Effects of ABCB1 Gene Polymorphisms on the Pharmacokinetics of Lamotrigine Monotherapy 212
5.3.1 Introduction 212
5.3.2 Aims 214
5.3.3 Methods 214
5.3.3.1 Study subjects 214
5.3.3.2 Blood sampling 215
5.3.3.3 ABCB1 c.1236C>T and c.3435C>T polymorphism analysis and phenotype assessment 215
5.3.3.4 Lamotrigine assay 215
5.3.3.5 Database construction 217
5.3.3.6 Population pharmacokinetic analysis 217
5.3.4 Results 220
5.3.4.1 Patient demographics 220
5.3.4.2 Genotype distribution of ABCB1 c.1236C>T and c.3435C>T polymorphisms 221
5.3.4.3 Lamotrigine population pharmacokinetics 221
5.3.5 Discussion 227
5.3.6 Conclusion 233
CHAPTER 6 - GENERAL DISCUSSION & CONCLUSION 234
6.1 General Discussion 235
6.2 Future Research Directions 247
6.3 Conclusion 250
LIST OF REFERENCES 251

List of Tables

CHAPTER 1

TABLE 1.1	International classification of epileptic seizure.	5
TABLE 1.2	Approved indications for antiepileptic drugs.	10
TABLE 1.3	Proposed mechanisms of antiepileptic drugs.	13
TABLE 1.4	The pharmacokinetics of antiepileptic drugs.	16
TABLE 1.5	Antiepileptic drugs and their related hepatic drug metabolizing enzymes.	18
TABLE 1.6	Single nucleotide polymorphisms of selected drug metabolising enzyme, drug transporter protein and sodium channel genes.	51

CHAPTER 3

TABLE 3.1	Allele and genotype distributions of the CYP3A4 g.-392A>G polymorphism in comparison to previously published data from different populations.	127
TABLE 3.2	Allele and genotype distributions of the CYP3A5 g.6986A>G polymorphism in comparison to previously published data from different populations.	128
TABLE 3.3	Allele and genotype distributions of the CYP1A2 g.5734C>A polymorphism in comparison to previously published data from different populations.	129
TABLE 3.4	Allele and genotype distributions of the EPHX1 c.337T>C polymorphism in comparison to previously published data from different populations.	131

TABLE 3.5	Allele and genotype distributions of the EPHX1 c.416A>G polymorphism in comparison to previously published data from different populations.	132
TABLE 3.6	Allele and genotype distributions of the UGT2B7 c.802C>T polymorphism in comparison to previously published data from different populations.	134
TABLE 3.7	Allele and genotype distributions of the ABCB1 c.1236C>T polymorphism in comparison to previously published data from different populations.	135
TABLE 3.8	Allele and genotype distributions of the ABCB1 c.2677G>T/A polymorphism in comparison to previously published data from different populations.	136
TABLE 3.9	Allele and genotype distributions of the ABCB1 c.3435C>T polymorphism in comparison to previously published data from different populations.	137
TABLE 3.10	Allele and genotype distributions of the SCN2A c.56G>A polymorphism in comparison to previously published data from a different population.	138
CHAPTER 4		
TABLE 4.1	Patient demographics and carbamazepine doses.	151
TABLE 4.2	Genotype distribution comparison between the carbamazepine cohort and the 400-strong study population.	155
TABLE 4.3	Summary of univariate regression analysis of variables predicting the optimal dose of carbamazepine.	157

TABLE 4.4	Summary of hierarchical multivariate regression analysis of variables predicting the optimal dose of carbamazepine.	158
TABLE 4.5	Demographic profile of patients who experienced intolerable carbamazepine adverse effects.	170
TABLE 4.6	The incidence of common polymorphisms in drug metabolising enzyme genes in patients with central nervous system related carbamazepine adverse effects.	173
TABLE 4.7	The incidence of common polymorphisms in drug metabolising enzyme genes in patients with carbamazepine idiosyncratic reactions.	174
CHAPTER 5		
TABLE 5.1	Patient demographics and lamotrigine doses.	185
TABLE 5.2	Genotype distributions of common SCN2A and ABCB1 polymorphisms between the lamotrigine cohort and the overall 400-strong study population.	187
TABLE 5.3	Comparison of the genotype distribution of common polymorphisms in SCN2A and ABCB1 between the seizure free and non-seizure free groups.	188
TABLE 5.4	Logistic regression analysis of response to lamotrigine monotherapy on the basis of genotype and allele of common polymorphisms in SCN2A and ABCB1 genes.	189
TABLE 5.5	The distribution of ABCB1 haplotypes between seizure free (n = 39) and non-seizure free (n = 40) patients.	190
TABLE 5.6	Logistic regression analysis of response to lamotrigine monotherapy on the basis of ABCB1 haplotype.	190

TABLE 5.7	Logistic regression analysis of response to lamotrigine monotherapy on the basis of major diplotype of the ABCB1 gene.	191
TABLE 5.8	Patient demographics and lamotrigine doses.	199
TABLE 5.9	Comparison of distributions of common SCN2A and ABCB1 polymorphisms between the lamotrigine cohort and the 400-strong study population.	204
TABLE 5.10	Summary of univariate regression analysis of variables predicting the optimal dose of lamotrigine.	205
TABLE 5.11	Summary of hierarchical multivariate regression analysis of variables predicting the optimal dose of lamotrigine.	206
TABLE 5.12	Hierarchy of structural and covariate pharmacokinetic models, error models and their minimum value of objective function (OBJF).	223
TABLE 5.13	Lamotrigine pharmacokinetic parameter estimates and their relative standard errors (RSE) using various models.	228

List of Figures

CHAPTER 1

FIGURE 1.1	Year of introduction of antiepileptic drugs to the clinical management of epilepsy in the United Kingdom.	9
FIGURE 1.2	The pathway of carbamazepine metabolism.	35
FIGURE 1.3	Chemical structure of carbamazepine and the sites of CYP450 action.	36
FIGURE 1.4	Chemical structure of lamotrigine.	43
FIGURE 1.5	A schematic view of the secondary structure of the CYP3A4 protein.	52
FIGURE 1.6	The location of the CYP3A gene locus on Chromosome 7 and arrangement of CYP3A genes in the locus.	53
FIGURE 1.7	Distribution of polymorphisms in the CYP3A5 gene.	55
FIGURE 1.8	The location of the CYP1A gene locus on Chromosome 15 and distribution of polymorphisms in the CYP1A2 gene.	58
FIGURE 1.9	A schematic view of the secondary structure of the epoxide hydrolase protein.	61
FIGURE 1.10	The location of the EPHX1 gene locus on Chromosome 1.	63
FIGURE 1.11	The location of the UGT2B gene locus on Chromosome 4 and distribution of polymorphisms in the UGT2B7 gene.	66
FIGURE 1.12	A postulated model representing drug and metabolite movement in the intestinal epithelium and hepatocyte.	68

FIGURE 1.13 A postulated model representing the location of ABCB1 P-glycoprotein in the blood-brain barrier and brain parenchyma. 69

FIGURE 1.14 The putative 2-D structure of ABCB1 P-glycoprotein with the sites of common polymorphisms. 70

FIGURE 1.15 The location of ABCB genes on Chromosome 4. 71

FIGURE 1.16 Schematic diagram of α- and β-subunits of sodium channel $Na_v1.2$. 74

FIGURE 1.17 The location of the SCN2A gene on Chromosome 2. 76

CHAPTER 2

FIGURE 2.1 Distribution of 400 study subjects to each individual project. 93

CHAPTER 3

FIGURE 3.1 Electrophoresis patterns for CYP3A4 g.-392A>G genotypes analysed with PCR-RFLP assay. 104

FIGURE 3.2 Electrophoresis patterns for CYP3A5 g.6986A>G genotypes analysed with PCR-RFLP assay. 106

FIGURE 3.3 Electrophoresis patterns for CYP1A2 g.5734C>A genotypes analysed with PCR-RFLP assay. 107

FIGURE 3.4 Electrophoresis patterns for EPHX1 c.337T>C genotypes analysed by PCR-RFLP assay. 109

FIGURE 3.5 Electrophoresis patterns for EPHX1 c.416A>G genotypes analysed by PCR-RFLP assay. 111

FIGURE 3.6 Electrophoresis patterns for UGT2B7 c.802C>T genotypes analysed with PCR-RFLP assay. 113

FIGURE 3.7 Electrophoresis patterns for ABCB1 c.1236C>T genotypes analysed by PCR-RFLP assay. 114

FIGURE 3.8 Electrophoresis patterns for ABCB1 c.2677G>T/A genotypes analysed by PCR-RFLP assay. 117

FIGURE 3.9 Electrophoresis patterns for ABCB1 c.3435C>T genotypes analysed by PCR-RFLP assay. 119

FIGURE 3.10 Electrophoresis patterns for SCN2A c.56G>A genotypes analysed by PCR-RFLP assay. 121

FIGURE 3.11 DNA sequencing signals of the four nucleotides around the EPHX1 c.337T>C polymorphism for the T/T, T/C, and C/C genotypes. 125

FIGURE 3.12 The distribution of predictive net activity of microsomal epoxide hydrolase in the 400-strong study population. 133

CHAPTER 4

FIGURE 4.1 Correlation between carbamazepine optimal dose and age of the patient at the time optimal dose was attained. 152

FIGURE 4.2 Distribution of carbamazepine optimal dose between genders. 153

FIGURE 4.3 Distribution of carbamazepine optimal dose between epilepsy syndromes. 154

FIGURE 4.4 Correlation between the observed and predicted carbamazepine optimal doses based on the optimal multivariate regression model. 159

CHAPTER 5

FIGURE 5.1 Distribution of lamotrigine optimal dose between genders. 200

FIGURE 5.2 Distribution of lamotrigine optimal dose and age at achieving 12 months of seizure freedom. 201

FIGURE 5.3 Distribution of lamotrigine optimal dose between epilepsy syndromes. 202

FIGURE 5.4 Correlation between observed and predicted lamotrigine optimal dose based on the optimal multivariate regression model. 207

FIGURE 5.5 Relationship between individual estimates of oral clearance and clinical covariates; age (A), gender (B), body weight (C) and duration of lamotrigine therapy (D). 224

FIGURE 5.6 Relationship between individual estimates of oral clearance and genotypes of ABCB1 c.1236C>T (A) and c.3435C>T (B) polymorphisms. 225

FIGURE 5.7 A plot of Akaike Information Criterion value *versus* univariate/multivariate inclusion in Model 2. 226

FIGURE 5.8 Plots of measured lamotrigine concentration *versus* population and individual predicted lamotrigine concentration for base model (A and B) and optimal model (C and D). 229

List of Abbreviations

ABC	ATP-binding cassette
AED	antiepileptic drug
AIC	Akaike Information Criterion
BBB	blood brain barrier
BLAST	Basic Local Alignment Search Tool
CAR	constitutive androstane receptor
CBZ	carbamazepine
CBZ-E	carbamazepine-10,11-epoxide
CL/F	oral clearance
CNS	central nervous system
cv	coeffient of variation
CYP	cytochrome P450
DME	drug metabolising enzyme
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
DTP	drug transporter protein
EEG	electroencephalogram
F	bioavailability
GABA	γ-aminobutyric acid
GAM	Generalised Additive Modelling
GEFS+	generalised epilepsy with febrile seizure plus
GR	glucocorticoid receptor
HNF	hepatic nuclear factor
HWE	Hardy-Weinberg equilibrium
IBE	International Bureau for Epilepsy
ILAE	International League Against Epilepsy
K_a	constant rate of absorption
LD	linkage disequilibrium
LTG	lamotrigine
mEH	microsomal epoxide hydrolase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
OATP	organic anion-transporting polypeptide

OBJF	objective function
OR	odds ratio
PB	phenobarbital
PCR	polymerase chain reaction
P-gp	P-glycoprotein
PHT	phenytoin
PNS	peripheral nervous system
PXR	pregnane X receptor
RFLP	restriction fragment length polymorphism
RMT	resting motor threshold
RNA	ribonucleic acid
SMEI	severe myoclonic epilepsy of infancy
SNP	single nucleotide polymorphism
STR	simple tandem repeat
$t_{1/2}$	half-life
Taq	*Thermus aquaticus*
Tm	melting temperature
tRNA	transfer ribonucleic acid
UGT	UDP-glucuronosyl transferase
V_d	apparent volume of distribution
VPA	valproic acid

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Author's Declaration

I hereby declare that this thesis submitted in fulfilment of the requirement for the degree of Doctorate of Philosophy represents my own work, except where specifically stated in the acknowledgements and in the text of this thesis. This thesis has not been previously submitted to this or any other institution for any degree.

Mohd Makmor Bakry

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Publications

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Review

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Epilepsy

1.1.1 History of epilepsy

The word 'epilepsy' originates from a Greek word, *epilambanein*, which means 'to seize' or 'to attack' (World Health Organization, 2001), and the term 'epilepsia' is used to denote a seizure. In ancient times, epilepsy or an epileptic attack was depicted as an abrupt suspension or seizing of a person's senses (Gastaut & Broughton, 1972). The basic concepts of epilepsy in ancient Indian medicine were identified during the Vedic period in 4500–1500 BC. The complete Ayurvedic medical system, Charaka Samhit, described epilepsy as '*apasmara*' which means 'loss of consciousness'. A description of epilepsy was also found in a Babylonian textbook of medicine dating as far back as 1067 BC, and which emphasized the supernatural nature of epilepsy (Pierce, 2002). A later description of epilepsy appeared in the Hippocratic text entitled "On the Sacred Disease". In describing epilepsy, Hippocrates wrote the following statement:

> "It is thus with regard to the disease called Sacred: it appears to me to be nowise more divine nor more sacred than other diseases, but has a natural cause from the originates like other affections (Hippocrates, 400 B.C.E.)"

Hippocrates has highlighted the fact that epilepsy is just one kind of brain disorder. This notion has countered the superstitious thought that epilepsy is a curse or a prophetic power. Galen (AD 130–200) described three types of fits, and suggested that epilepsy was a brain disorder related to an accumulation of "thick humours" (Pierce, 2002). Later, in the 19th-century, John Hughlings Jackson deduced the basic pathophysiology of epileptic seizures through his clinical observation (Hogan & Kaiboriboon, 2003).

Jackson's contributions to the field of epilepsy continue to be recognised in modern medical sciences.

1.1.2 Epilepsy and its syndromes

Epilepsy is one of the most important clinical syndromes, with a prevalence range of 3 to 7.3 per 1000 population (Wright *et al.*, 2000; Al Rajeh *et al.*, 2001; Oun *et al.*, 2003; Hui & Kwan, 2004). It is a difficult disorder to control (Spear, 2001) and may even cause sudden unexplained death (Pedley & Hauser, 2002). The term epilepsy refers to the recurrence of seizures from all types of pathological states or diseases (Janz, 1985). The International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE) consensus defined an epileptic seizure and epilepsy as follows:

> "An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain (Fisher *et al.*, 2005)"

> "Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure (Fisher *et al.*, 2005)"

Seizures occur due to a sudden, excessive, rapid discharge in the grey matter of some part of the brain; this might spread further all over the brain, to produce generalised syndromes. The brain's electrical discharge may result in an almost instantaneous loss of consciousness, alteration of perception or impairment of psychic function, convulsive

movement, disturbance of sensation, or some combination thereof (Victor & Ropper, 2001).

Epilepsy syndromes are commonly characterised by their clinical presentation such as seizure type, age at onset, brain electrical pattern, causal factors and trend of genetic inheritance (Brodie & French, 2000). Generally, epilepsy syndromes are widely grouped into idiopathic, cryptogenic and symptomatic (Commission, 1989; Brodie & French, 2000). Idiopathic or primary epilepsies are epileptic syndromes with an unknown cause except perhaps a genetic one; cryptogenic epilepsies are epileptic syndromes with uncertain nature of origin and the seizures may be the only sign of brain abnormalities; meanwhile symptomatic or secondary epilepsies are epileptic syndromes with clearly known causes (Victor & Ropper, 2001; Brodie *et al.*, 2005). Epilepsy treatment decisions are based principally on the type of epilepsy syndrome and the kinds of seizures that occur.

1.1.3 Classification of seizures

The present commonly used classification was proposed by the ILAE more than twenty years ago (TABLE 1.1; Commission, 1981) using clinical description and electroencephalogram (EEG) characteristics. The limitations of this classification are becoming increasingly apparent with advances in imaging technology and neurophysiology. Nevertheless, the present system has gained widespread acceptance and provides an effective means of communication among clinicians.

The current classification divides seizures broadly into partial and generalised. Partial seizures, also called focal, are those seizures that begin in a focal region of the cortex in

one hemisphere. Based on the symptoms of the seizures, partial seizures can be subdivided into simple partial seizures and complex partial seizures.

TABLE 1.1. International classification of epileptic seizure. Adapted from Commission (1981).

I	**Partial (focal) seizures (seizures beginning locally)**			
	A	Simple (consciousness not impaired)		
		i	With motor symptoms	
		ii	With somatosensory or special sensory symptoms	
		iii	With autonomic symptoms or signs	
		iv	With psychic symptoms	
	B	Complex (with impaired consciousness)		
		i	Simple partial onset followed by impairment of consciousness	
		ii	Impaired consciousness at onset	
	C	Partial seizures evolving into secondary generalised seizures		
II	**Generalised seizures (convulsive or non-convulsive)**			
	A	Absence seizures		
		i	Typical (petit mal)	
		ii	Atypical	
	B	Myoclonic seizures		
	C	Clonic seizures		
	D	Tonic seizures		
	E	Tonic-clonic seizures		
	F	Atonic seizures		
III	**Unclassified seizures**			

Simple partial seizures have no impact on the level of consciousness. Depending on the area of the brain affected, these seizures could be expressed as shaking of a small part of the body, an unusual tingling or numbness of a localised body part, or even an unusual

smell, visual hallucination, or ill-defined feeling. Complex partial seizures occur when the abnormal electrical activity involves parts of the brain that affect level of consciousness. Thus, the critical feature of a complex partial seizure is that the person has altered consciousness, so that he may be confused or staring unresponsively. There may also be subtle, repetitive and stereotypical movements of the face or extremities. Sometimes a complex partial seizure can start in just one area and spread throughout the entire brain, resulting in a generalised tonic-clonic seizure. This seizure type is known as complex partial with secondary generalisation.

Generalised seizures are caused by abnormal electrical activity that occurs over the entire brain simultaneously. This group of seizures affect the level of awareness and muscle movement of all extremities. Absence seizures are also known as "petit mal". They are described as staring spells. These seizures typically start in childhood and are often outgrown by adolescence, although adults can occasionally have absence seizures. Myoclonic seizures are characterized by sudden brief jerks of a single muscle or muscle group. It may appear as if sufferers have been startled and you may also see the head or body suddenly bend forward or backward. Atonic seizures are also very sudden brief seizures, but they involve loss of all muscle tone. The patient will suddenly go limp and fall to the ground. There is a significant risk of head injury during the fall. Tonic seizures involve stiffening of parts of the body or the entire body. Unlike tonic-clonic seizures, there is no progression to a clonic phase. Tonic-clonic seizures are also known as "grand mal". They generally start with a tonic phase with stiffening of the entire body. The eyes may roll back in the head, the back arches, and arms and legs stiffen. The clonic part is described as rhythmic jerking of the entire body. Once the seizure is over, the patient may feel worn out and may even sleep for a period of time. They may also experience some confusion.

1.1.4 Diagnosis of epilepsy

The diagnosis procedure aims to determine whether the patient has epilepsy, and if so, the syndromic classification and underlying aetiology. Epilepsy may be difficult to diagnose in the early stages especially in the absence of a witnessed account. It is imperative that the physician obtains a complete patient history, including details of birth, childhood, family history, and medication regimen; a thorough medical history, including illnesses of the nervous system; and a thorough history of drug and alcohol use. A detailed description of the seizures is important to distinguish seizure types.

The primary tool in the diagnosis of epilepsy is the EEG (King *et al.*, 1998), which measures electrical activity in the brain. Seizure disorders produce characteristic patterns in an EEG test. In patients with partial epilepsy, the EEG may also help to localise the area of seizure onset. Inpatient video-EEG monitoring is used to localise seizure onset in patients undergoing evaluation for epilepsy surgery. It is also used to confirm epilepsy when the diagnosis is uncertain. Neuroimaging approaches are another means to identify any underlying epileptogenic structural abnormalities and are indicated in patients suspected to have localisation-related epilepsy. Magnetic resonance imaging (MRI) is undoubtedly the investigation of choice (Duncan, 1997). However, computer tomography should be used in preference to MRI in patients with cardiac pace makers, metal aneurysm clips, or severe claustrophobia, or when MRI is not available.

Imaging techniques that show cerebral function play a growing role in the evaluation of epilepsy. These techniques include functional MRI, magnetoencephalography, magnetic resonance spectroscopy, single photon emission computed tomography, and positron emission tomography (Duncan 1997). Making an accurate diagnosis of an epileptic syndrome may allow the clinician to define the likely prognosis, provide appropriate counselling and choose the most appropriate treatment (Brodie & French, 2000).

1.2 Treatment of Epilepsy

Treatment approaches for the epilepsies can include pharmacotherapy using AEDs, neurosurgical procedures, removal of causative and precipitating factors, and regulation of physical and mental activities (Victor & Ropper, 2001). AED treatment is the commonest initial approach to treat epilepsy. AEDs can reduce or stop seizures altogether. Some patients with epilepsy do not respond to several AEDs and in this situation those with partial epilepsy might be considered for surgery. Another option for patient who are resistant to AEDs is vagal nerve stimulation (Brodie *et al.*, 2005).

1.2.1 History of antiepileptic drug development

The first effective agent in the treament of epilepsy was bromide (Pearce, 2002). However, the usage of bromide was limited due to an association with frequent side effects. In 1912, phenobarbital (PB) was introduced as a better-tolerated antiepileptic agent. In subsequent years, Merritt and Putnam introduced a new non-sedating AED, phenytoin (PHT; Merritt & Putnam, 1938). Since its introduction, PHT has been a first-line medication for the prevention of partial and tonic-clonic seizures, and the acute treatment of seizures and status epilepticus. In 1953, CBZ was synthesised at the Geigy laboratory by Schindler and Blattner (Schmutz, 1985). It was originally developed as an antipsychotic and its antiepileptic properties were not observed clinically until 1963 (Brodie & Dichter, 1997). Sodium valproate (VPA) was serendipitously discovered in 1963 by Pierre Eymard, when it was used as a solvent in testing other potential AEDs (Brodie & Dichter, 1997). Since the introduction of VPA, many new AEDs have been developed and marketed (FIGURE 1.1). Generally, AEDs can be divided into two generations, the established AEDs and the modern AEDs. The modern AEDs are

believed to possess better efficacy and tolerability profiles than their established counterparts.

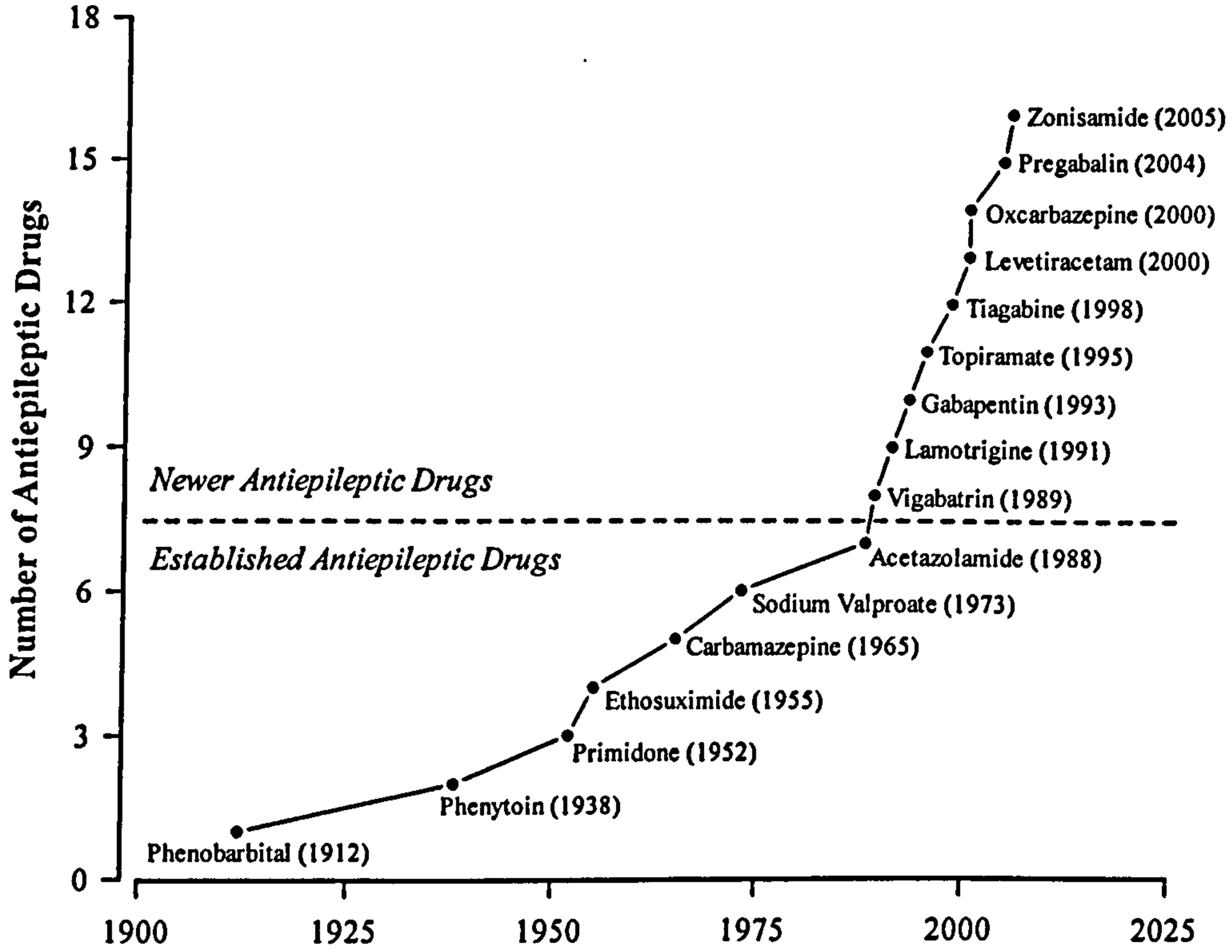

FIGURE 1.1. Year of introduction of antiepileptic drugs to the clinical management of epilepsy in the United Kingdom.

1.2.2 Indications of antiepileptic drugs

Starting treatment with an AED is a major event for the patient and should not be undertaken without careful evaluation. AED therapy is long term, usually for at least three years and, depending on circumstances, sometimes for life. The modern AEDs complement established AEDs that are well known to most clinicians. In addition to

providing more options for the adjunctive treatment of seizures, some of these new compounds have been approved for monotherapy (TABLE 1.2).

TABLE 1.2. Approved indications for antiepileptic drugs.

Drug	Partial seizure[a]	Broad spectrum[b]	Infantile spasms	Absence only
Established AEDs				
Carbamazepine	+			
Ethosuximide				+
Phenobarbitone	+	+		
Phenytoin	+			
Valproic acid	+	+	+	
New AEDs				
Gabapentin	+			
Lamotrigine	+	+		
Levetiracetam	+	+		
Oxcarbazepine	+			
Pregabalin[c]	+			
Tiagabine[c]	+			
Topiramate	+	+		
Vigabatrin[c]	+		+	
Zonisamide[c]	+	+	+	

[a] Including secondarily generalised

[b] Most seizure types, including partial, absence, myoclonic, tonic and both primary and secondarily generalised tonic-clonic

[c] Licensed as adjunctive treatment/add-on therapy only

(Kwan *et al.*, 2001; Perucca, 2001; Bazil & Pedley, 2003)

One of the advantages of using established AEDs is that their spectrum of efficacy, adverse-effect profiles, drug-drug interactions, and idiosyncrasies are relatively well

known. Although some side effects of the established AEDs may be undesirable, they are usually predictable. AEDs are generally chosen according to seizure type. LTG, controlled-release CBZ, topiramate or VPA are first-line monotherapy options for partial seizures. For generalised seizures, LTG, topiramate or VPA are the options and, as these drugs are broad spectrum, they may also be used when there is doubt about the classification (Kwan *et al.*, 2001; Perucca, 2001; Bazil & Pedley, 2003). Broad spectrum AEDs usually cover partial, absence, myoclonic, tonic and both primary and secondarily generalised tonic-clonic seizures. Although as effective as other established AEDs, PHT is no longer considered as a first-line treatment because of its side effect profile when employed on a chronic basis and its saturation kinetics, which require routine monitoring of its plasma concentrations.

1.2.3 Mechanisms of action of antiepileptic drugs

Experimental studies (both *in vitro* and *in vivo*) together with clinical observations have contributed to understanding of the mechanisms of action of AEDs. However, current knowledge on mechanisms of action is of limited application in the initiation of therapy for individual patients. This is because of a poor understanding of seizure pathophysiology. Therefore, most first line drugs are usually chosen on the basis of available clinical evidence (Perucca, 2001). Knowledge of mechanisms of action can have an important role in determining which AEDs to add-on in those patients who fail to respond to the first drug (Deckers *et al.*, 2000). Combining AEDs with different mechanisms of action can improve the percentage of patients with controlled seizures, possibly up to 20 percent (Stephen & Brodie, 2002). The recent influx of newer AEDs into clinical practice may elevate knowledge of mechanisms of action amongst other potentially important drug selection criteria, particularly in the absence of sufficient clinical evidence or experience (Brodie, 1999; Kwan *et al.*, 2001).

The mechanisms of action of AEDs are presented in TABLE 1.3. Three mechanisms have been proposed to explain the effects of AEDs: modulation of voltage-dependent ion channel (sodium, calcium, potassium), potentiation of GABA-mediated inhibitory neurotransmission and inhibition of glutamate-mediated excitatory neurotransmission (Meldrum, 2001). The opening of voltage-gated sodium channels is responsible for the upstroke of the neuronal action potential and these proteins essentially control the intrinsic excitability of the nervous system (Clare *et al.*, 2000). Sodium channels cycle between functional states (open, closed, inactivated) within a matter of milliseconds. This character is important in producing the rapid burst of action potentials during normal neuronal transmission, and particularly in the production of epileptic discharges (Kwan *et al.*, 2001). Many AEDs such as PHT, CBZ and LTG block the neuronal voltage-gated sodium channels as their principal antiseizure action (Tunnicliff, 1996; White, 1999).

The key structure and sequence homology of voltage-dependent calcium channels are shared with sodium channels (Anderson & Greenberg, 2001). Low and high activation thresholds are used to widely categorise calcium channels. L-type calcium channels are expressed in muscle, brain and endocrine cells. N-, P/Q- and R- type calcium channels are found mainly in neurones. All of these channels require a strong depolarization (i.e. high threshold) to be activated. T-type calcium channels are low threshold channels and are found in various cell types (Ertel *et al.*, 2000). The rhythmic 3-Hz spike-and-wave discharge that is present in generalised absence seizures is believed to be a product of activation of these low-threshold T-type calcium channels (Sohal & Huguenard, 2001). Blockade of the voltage-dependent calcium channels in a subtype-specific manner explains the effects of several AEDs. For example, LTG reduces N- and P-type calcium currents, diminishing repetitive neuronal firing, and consequently controlling seizure, without affecting L-type calcium currents (Pisani *et al.*, 2004).

TABLE 1.3. Proposed mechanisms of antiepileptic drugs.

Drug	↓ Na^+ channels	↓ Ca^{2+} channels	↑ K^+ channels	↑ inhibitory transmission	↓ excitatory transmission
Established AEDs					
Carbamazepine	+++				
Ethosuximide		+++			
Phenobarbital		+		+++	+
Phenytoin	+++				
Valproic acid	+	+		++	+
New AEDs					
Felbamate	++	++		++	++
Gabapentin	+	+++		+	
Lamotrigine	+++	+			
Levetiracetam		+	+	+	++
Oxcarbazepine	+++	+	+		
Tiagabine				+++	
Topiramate	++	++		++	++
Vigabatrin				+++	
Zonisamide	++	++			

+++ = primary action, ++ = probable action and + = possible action (Kwan *et al.*, 2001; Madeja *et al.*, 2003).

The tetrameric structure of neuronal voltage-gated potassium channels is derived from the association of large protein complexes. Their monomers are related to sodium and calcium channel subunits (Anderson & Greenberg, 2001). Activation of potassium channels suppresses the generation of action potentials (Spitzer *et al.*, 2002). Studies have reported levetiracetam (Madeja *et al.*, 2003) and retigabine (Rundfeldt, 1999) activate potassium channel conductance as one of their mechanisms of antiseizure action.

GABA is the principal inhibitory neurotransmitter in mammalian brain and is released at up to 40% of all synapses (Olsen & Avoli, 1997). Insufficiency of GABAergic neurones has been proposed as a mechanism of seizure generation (Loscher & Schmidt, 2002). GABA-related mechanisms, such as the promotion of GABA synthesis, increase in GABA release, facilitation of GABA receptors and a decrease in GABA inactivation are associated with AEDs such as VPA, gabapentin, vigabatrin and tiagabine (Errante *et al.*, 2002).

The complementary neurotransmitter to GABA is glutamate, which is the principal excitatory neurotransmitter in the mammalian CNS (Meldrum *et al.*, 1999). Seizures have been associated with glutamatergic system abnormalities, including over-activation of glutamatergic transmission and defective ionotropic and metabotropic glutamate receptor properties (Meldrum *et al.*, 1999). None of the licensed AEDs act solely on the glutamatergic neurones, but glutamate receptor blockade is associated with some AEDs (Meldrum, 2001). For example, felbamate and topiramate are believed to act on the NMDA- and AMPA-subtypes of glutamate receptors, respectively (Harty & Rogawski, 2000; Kaminski *et al.*, 2004).

1.2.4 Clinical pharmacokinetics of antiepileptic drugs

Evidence supporting the relationship between plasma drug concentration and clinical response to established AEDs is available to aid dosage individualization (Perucca, 1999; Affolter *et al.*, 2003). Drug therapeutic ranges were derived from population pharmacokinetic studies using statistical concepts in which most of the patients demonstrated an acceptable clinical response with no intolerable side effects (Patsalos, 2001). Nevertheless, some patients may demonstrate good seizure control below the minimum limit of the therapeutic range, and intoxication may also be present in some

patients with plasma drug concentrations within the therapeutic range (Browne, 1998). Hence, the therapeutic range is regarded as a guide to AED dosage, and an individual patient's clinical conditions should always be considered in cases of poor response or intolerable side effects.

To achieve optimal control of seizures, AEDs must be well absorbed from the gastrointestinal tract, distributed into body tissues, and reach sufficient concentration in the brain. Almost all AEDs have more than 90 percent oral bioavailability, with the exception of gabapentin and vigabatrin (TABLE 1.4). Interestingly, some AEDs such as PHT are not only well absorbed in the small intestine but absorption also extends up to the colonic region (Stevenson *et al.*, 1997). After the absorption phase, AEDs are distributed into body compartments depending on their physicochemical properties. In the blood, AEDs such as PHT, VPA and tiagabine are highly bound to plasma proteins. AEDs with this characteristic have a higher risk for drug interactions through competitive depletion from the site of protein binding. This is clearly observed in polytherapy regimens comprising PHT and VPA. One should aim for a lower plasma concentration of PHT when used concomitantly with VPA, because of plasma protein binding displacement (Perucca *et al.*, 1980b; Patsalos & Perucca, 2003). Reduced binding to plasma proteins may shorten the AED half-life, requiring a more frequent dosing schedule as noted with the newer AED, gabapentin (Perucca, 2001).

The magnitude and duration of the antiepileptic actions of AEDs are predominantly determined by dose and pharmacokinetic profile (Perucca, 1999). The AED dose and pharmacokinetic profile are in turn influenced by enzymatic biotransformation and drug transporters (Browne, 1998; Kim, 2002a; Ramachandran & Shorvon, 2003). Most AEDs undergo enzymatic biotransformation to form a more water-soluble compound before being excreted by the kidney. Nevertheless, some AEDs can be excreted in an

unchanged form or with only minor enzymatic action, for example gabapentin, topiramate and vigabatrin (Patsalos, 1999).

TABLE 1.4. The pharmacokinetics of antiepileptic drugs.

Drug	Bioavailability (%)	Plasma protein binding (%)	t½ (h)	Usual effective plasma concentration (μmol/l)	T_{max} (h)	Route of elimination
Established AEDs						
Carbamazepine	89[a]	75	8-22	Up to 50	4-8	>90% hepatic
Ethosuximide		<5	60	Up to 700	3-7	65% hepatic
Phenobarbital	≥ 90	45	100	Up to 170	2-8	>90% hepatic
Phenytoin	≥ 90	90-95	22	Up to 80	3-8	>90% hepatic
Valproic acid	≥ 90	80-90	15-20	Up to 700	3-8	>95% hepatic
New AEDs						
Felbamate	≥ 80	20-25	14-23	125-250	2-6	60% hepatic (oxidation), renal excretion
Gabapentin	< 60	<5	5-7	70-120	2-3	>95% renal excretion
Lamotrigine	98	55	15-30[b]	10-60	2-5	>90% hepatic (glucuronide conjugation)
Levetiracetam	≥ 90	<10	6-8	35-120	1	>65% renal excretion
Oxcarbazepine	-	40	8-15	50-140	3-13	>90% hepatic (glucuronide conjugation)
Tiagabine	≥ 90	96	4-13	50-250	1	>90% hepatic (oxidation)
Topiramate	≥ 80	13	20-30	15-60	4-10	Renal excretion, 30% hepatic (oxidation)
Vigabatrin	≥ 60	0	5-8	6-278	1	Renal excretion
Zonisamide	≥ 90	50	50-70	45-180	2-6	70% hepatic (glucuronide conjugation, acetylation, oxidation)

[a]extended release formulation; [b]comedication with VPA and enzyme inducers.

(Patsalos, 1999; Perucca, 1999; Perucca, 2001; Ramsay & Wilder, 2002; Bazil & Pedley, 2003)

Biotransformation occurs mainly through an oxidation process and takes place primarily in the liver with the involvement of DMEs such as cytochrome P450 (CYP450) isoenzymes, hydrolases and conjugating enzymes. More than 12 isoenzymes of CYP450 are involved in drug metabolism. Metabolism of AEDs is predominantly driven by a variety of CYP450 subfamilies, including CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A (Ramachandran & Shorvon, 2003), mEH and conjugating enzymes such as UDP-glucuronosyltransferases (UGTs; TABLE 1.5). The reaction catalysed by

CYP450s involves the oxidation of AEDs where there is a heterocyclic ring, an aromatic ring, an alkane, or an alkene substituent. CYP450 metabolism converts AEDs into more water-soluble compounds, which are more easily conjugated and excreted from the body. CYP450 metabolism also converts some AEDs, such as CBZ, PB and PHT, to intermediate epoxide metabolites. Many of these epoxide metabolites are reactive and can covalently bind to cell macromolecules, resulting in cytotoxicity (Madden *et al.*, 1996). Detoxification of these reactive epoxide metabolites is mediated by mEH. mEH converts epoxides to trans-dihydrodiols which can be conjugated and excreted from the body. Conjugation of AEDs or AED metabolites is predominantly mediated by UGT isoenzymes in a process known as glucuronidation. Glucuronidation involves chemical binding of a substance to glucuronic acid via a glycosidic bond. The resulting glucuronide metabolite is typically much more water soluble than the original substance. Examples of AEDs that are mainly metabolised through glucuronidation are VPA and LTG (Staines *et al.*, 2004).

In order to understand drug disposition, DMEs cannot be studied in isolation, particularly where the roles of DTPs have been reported to be significant (Kim, 2002a; Kim, 2002b). Drug movement across tissue compartments habitually occurs in two directions; influx and efflux. Specific DTPs are responsible for carrying out both of these functions in a concentration-dependent manner (Roden, 2001). The most commonly recognised efflux transporter is P-gp. Several AEDs, such as PHT and LTG, have been shown to be transported by P-gp (Potschka & Loscher, 2001; Potschka *et al.*, 2002). P-gp can be found in various body tissues such as the intestinal lining, liver and brain. Variation in P-gp activities may be present and can potentially affect drug pharmacokinetics. It has been demonstrated that variation in the expression of duodenal P-gp correlates with the variation in plasma digoxin concentrations (Hoffmeyer *et al.*, 2000). Interestingly, DMEs and DTPs that are present at the level of the blood-brain

barrier (BBB), blood-cerebrospinal fluid barrier and various parts of the brain could also alter the elimination process of AEDs (Mcfadyen *et al.*, 1998; de Lange & Danhof, 2002). Therefore, variation in the expression and function of DMEs and DTPs may consequently influence the response to AED treatment.

TABLE 1.5. Antiepileptic drugs and their related hepatic drug metabolizing enzymes.

Drug Metabolizing Enzyme	Antiepileptic Drug								
	CBZ	ESM	PB	PHT	VPA	TGB	TPM	LTG	ZNS
CYP1A2	+			+					
CYP2C9			++	++	+				
CYP2C19			++	+	+		+		+
CYP2D6	+			+					
CYP3A4	++	++		+		++			++
CYP3A5	+	+?		+					+
mEH	+		+?	+?					
UGT1A4								++	
UGT1A6 & 1A9					++				
UGT2B7	+				++				

CYP, cytochrome P450; mEH, microsomal epoxide hydrolase; UGT, UDP-glucuronosyltransferase; CBZ, carbamazepine; ESM, ethosuximide; PB, phenobarbital; PHT, phenytoin; VPA, valproic acid; TGB, tiagabine; TPM, topiramate; LTG, lamotrigine; ZNS, zonisamide; ++, major involvement; +, significant involvement; +?, potential involvement. (Adapted from Ramachandran & Shorvon, 2003).

1.2.5 Variability in the response to antiepileptic drug treatment

The majority of epilepsy patients receive AEDs as the main treatment modality. However, response to AED therapy remains poor, despite the ever-expanding list of modern AEDs, introduced in the last decade and designed to possess improved efficacy and fewer side effects. Poor responders to AED treatment account for about 30 percent of epilepsy patients (Kwan & Brodie, 2000), and only small numbers of them have shown clinically significant improvement with the introduction of additional AEDs (Ramachandran & Shorvon, 2003). Patients with uncontrolled epilepsy not only suffer from health implications but also psychosocial and socioeconomic consequences.

Despite the relative importance of uncontrolled seizures in a large fraction of epilepsy patients, the cellular basis of pharmacoresistance has so far remained elusive. Two major hypotheses have been proposed to explain poor responsiveness to AED therapy; (1) alteration in AED targets such as decreased sensitivity of ion channels and neurotransmitter receptors, and (2) modification in AED-related DMEs and DTPs resulting in decreased absorption, increased metabolism or decreased uptake into the brain (Bazil & Pedley, 2003; Ramachandran & Shorvon, 2003; Remy & Beck, 2006). Any one of these alterations may be the result of variation in genes that express the corresponding proteins (Roden, 2001; Spear, 2001; Ramachandran & Shorvon, 2003; Remy & Beck, 2006).

1.3 Pharmacogenetics

Interindividual variation in the response to drugs is an important clinical issue. Such variation ranges from resistance to treatment to adverse drug reactions and drug-drug interactions. It is now clear that much of the individuality in drug response is inherited

(Hartl & Orel, 1992). This genetically determined variability in drug response defines the research area known as pharmacogenetics (Vogel, 1959). Pharmacogenetic studies create an opportunity to explore individualisation in drug therapy, a concept of tailored drug type and dosage to individuals based not only on traditional clinical factors but also on their genetic profiles (Kruglyak, 1999).

1.3.1 Human genetics

The genome of an organism is its whole hereditary information. The human genome is stored in 23 pairs of chromosomes. A chromosome is a single large macromolecule of deoxyribonucleic acid (DNA), and is the basic 'unit' of DNA in a cell. DNA consists of two associated polynucleotide strands that wind together in a helical fashion. It is often described as a double helix. The DNA is a long chain of nucleotides which consist of deoxyribose (a pentose sugar), phosphoric acid and 4 organic nitrogenous bases (purines - Adenine (A) and Guanine (G), or pyrimidines - Cytosine (C) and Thymidine (T)). The human genome contains approximately 3.2 billion base pairs that are specifically arranged according to the function of a gene and genetic ancestry. A gene is a relatively small segment of DNA that codes for the synthesis of a specific protein. Genes consist of a long strand of DNA that contains a promoter, which controls the activity of a gene, and exons, coding sequences which determine what the gene produces. Genes also contain introns, non-coding regions that do not code for the gene products, but sometimes regulate gene expression. Exons and introns are arranged alternate to each other.

Protein synthesis is a 2-part process that involves a second type of nucleic acid known as ribonucleic acid (RNA). The first process in protein synthesis is called transcription. In this step, the double DNA strands unzip from each other and a single strand of

messenger RNA (mRNA) is then made by pairing up mRNA bases with the exposed DNA nucleotide bases. Splicing is a modification of genetic information after transcription, in which introns are removed and exons are joined. Splicing prepares precursor mRNA in eukaryotes to produce mature mRNA. The second process in protein synthesis is translation. After the mature mRNA is manufactured, it leaves the cell nucleus and travels to a cellular organelle called the ribosome. In the ribosome, the mRNA code is translated into a transfer RNA (tRNA) code which, in turn, is transfered into a protein sequence. In this process, each set of 3 mRNA bases, known as a codon, will pair with a complimentary tRNA base triplet called an anticodon. Each tRNA is specific to an amino acid and as tRNA's are added to the sequence, amino acids are linked together by peptide bonds, eventually forming a protein that is later released by the ribosome.

1.3.2 Glossary of genetics

The commonly used terms in pharmacogenetics include allele, genotype, haplotype, diplotype and phenotype (Guttmacher & Collins, 2002). Alleles are alternative forms of a gene at a particular locus, or location, on a chromosome. Different alleles produce variation in inherited characteristics such as hair colour or blood type. In an individual, one form of the allele (dominant) may be expressed more than another form (recessive). A single allele for each gene locus is inherited from each parent. A pair of alleles forms the genotype. Genotype is the genetic constitution of an organism or cell at a particular locus. Usually, in a pair of alleles, each individual can be assigned to one of 3 possible genotypes.

Haplotype may refer to only one locus or to an entire genome. Haplotype represents a set of closely linked genetic markers present on one chromosome which tend to be

inherited together. A diploid is a full set of genetic material consisting of paired chromosomes. The diploid human genome has 46 chromosomes. Phenotype is an observable characteristic of an organism produced by the organism's genotype interacting with the environment. This contrasts with genotype, which is an organism's genetic composition.

1.3.3 Genetic variations among humans

Even though the DNA sequence between humans is 99.9% identical, millions of base pair differences are still present (Kruglyak & Nickerson, 2001). Genetic variations occur as a result of insertions, deletions, simple tandem repeats (STRs) and single nucleotide polymorphisms (SNPs) of the DNA sequence. In insertion variations, a few base pairs are inserted into a genetic sequence. This can happen due to slipping of DNA polymerase. Insertions in the coding region of a gene may alter splicing of the mRNA or cause a frameshift, both of which may significantly alter the gene product. A sequence deletion is a variation in which a part of a chromosome or a sequence of DNA is missing. Any number of nucleotides can be deleted, from a single base to an entire piece of chromosome. Deletion of a number of base pairs that is not evenly divisible by three will lead to a frameshift mutation, causing all of the codons occurring after the deletion to be read incorrectly during translation, producing a severely altered and potentially non-functional protein. A STR in DNA is a class of polymorphism that occurs when a pattern of two or more nucleotides are repeated and the repeated sequences are directly adjacent to each other.

The most common type of genetic variation is the SNP, of which there are an estimated 1.4 million candidates in the human genome (Kruglyak & Nickerson, 2001). A SNP is a DNA sequence variation which occurs when a single nucleotide in the sequence differs

between members of a species. For a variation to be considered a SNP, it must occur in at least 1% of the population. The estimated frequency of SNPs in the human genome is 1 per 1000 bases (Wang & Moult, 2001). Commonly, each SNP is represented by two alleles. However, SNPs with more than 2 alleles are also present. SNPs may fall within promoter regions, coding sequences, noncoding regions, or in the intergenic regions between genes. SNPs within a coding sequence may change the amino acid sequence of the protein that is produced. A SNP that does not change the polypeptide sequence is termed a synonymous SNP. If a different polypeptide sequence is produced they are termed as non-synonymous SNPs (Guttmacher & Collins, 2002). SNPs in coding and regulatory regions are the most likely to affect gene function (Chakravarti, 1998). Approximately 50% of coding SNPs are non-synonymous, and about 80% of these non-synonymous SNPs have been associated with disease through alteration in protein stability (Wang & Moult, 2001). Nevertheless, SNPs that are not in coding regions may still have consequences for gene splicing and transcription factor binding. Interestingly, some SNPs may simply serve as markers for the unidentified susceptible polymorphisms rather than ascribing a causative role (Chakravarti, 1998; Carlson *et al.*, 2003). In many pharmacogenetic studies, SNPs are commonly used as phenotype markers (Davidson, 2000). SNPs can be identified using several analytical assays such as polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) and direct sequencing.

1.3.4 Nomenclature of genetic sequence variations

A nomenclature system has been suggested to standardise the description of polymorphisms in DNA and protein sequences (den Dunnen & Antonarakis, 2001). Variations are described at the most basic level using either a genomic or a complemetary DNA reference sequence. A genomic reference sequence is preferred

because it includes multiple transcription initiation sites (promoters), alternative splicing, and multiple translation initiation sites (ATG-codons). A complementary DNA reference sequence is used when the entire genomic sequence is not known.

To avoid confusion in the description of a sequence change, a letter indicating the type of reference sequence is used, for example; 'g.' for a genomic sequence (e.g. g.54A>T), 'c.' for a complemetary DNA sequence (e.g. c.54A>T) and 'p.' for a protein sequence (e.g. p.R54K). At the DNA level, nucleotides are designated by the bases; A (adenine), C (cytosine), G (guanine) and T (thymidine). Nucleotide changes are indicated by a number which denotes the position of the substitution in the DNA sequence counted from the first coding nucleotide. Substitutions are designated by a '>' character. For example, 65A>C denotes that at nucleotide 65 an A residue changed to C. Two polymorphisms in one variant are described as "first change + second change", for example, 69A>C+102G>C denotes an A to C transition at nucleotide 69 and a G to C transition at nucleotide 102 in the same variant. For the purposes of standardisation, alleles of a SNP are represented by the position of the SNP and base of the allele, for example, alleles of the c.89A>G substitution are denoted as 89A and 89G. Genotypes of a SNP are represented by the position of the SNP and the pair of allele bases, for example, genotypes of c.89A>G substitution are denoted as 89A/A, 89A/G and 89G/G.

1.3.5 History of pharmacogenetics

Inherited characteristics that were first observed by Mendel (Hartl & Orel, 1992), have now evolved to a more complex and applied discipline known as pharmacogenetics. The earliest documented evidence of individual differences in drug metabolism can be traced back to the ancient Greeks (Pirmohamed, 2001). Sir Archibald Garrod, an English physician brought forward the knowledge of biochemical genetics in his

description of "an inborn error of metabolism" (Garrod, 1902). An incident that occurred during World War II further highlighted the diversity of pharmacogenetic disorders. Service personnel deployed in regions with high malarial risk were given a prophylactic antimalarial drug, primaquine. After the drug was taken, approximately 10% of the African-American servicemen were diagnosed with acute anemia. The affected individuals were found to harbour some mutations in the gene expressing glucose 6-phosphate dehydrogenase (Carson *et al.*, 1956). At the end of World War II, medicine entered the antibiotic era, and isoniazid was introduced to treat tuberculosis. It was noted that some individuals who took isoniazid rapidly developed a peripheral neuropathy. This was later proven to be associated with genetically polymorphic *N*-acetyltransferase, an enzyme responsible for the metabolism of isoniazid (Kalow & Staron, 1957). In the post-war years, Motulsky and Kalow, who were interested in genetically determined differences in drug metabolism, wrote extensive reviews on heredity and response to drugs (Motulsky, 1957; Kalow, 1962). This laid the foundations for 'pharmacogenetics', a term coined by Friedrich Vogel of Heidelberg in 1959 (Vogel, 1959).

The discovery of polymorphic hydroxylation of debrisoquine in man has strengthened the promising future of pharmacogenetics for medicine and science (Mahgoub *et al.*, 1977). Further polymorphisms relating to other cytochrome enzymes were discovered in subsequent decades. Now, the Human Genome Project and other research endeavors are providing information that allows a better understanding of the underlying causes of pharmacogenetic anomalies.

1.4 Pharmacogenetics of antiepileptic drugs

Epilepsy itself has been linked to at least 33 chromosome regions (Prasad *et al.*, 1999). On the basis of prior knowledge of AED pharmacodynamics and pharmacokinetics, candidate genes and SNPs for AED pharmacogenetic study can be identified. Accordingly, genes that have a potential pharmacogenetic impact in epilepsy are divided into four groups; (i) genes that characterize a subclass of the epilepsy, (ii) genes for ion channel and AED receptors, (iii) genes that encode pharmacokinetic-related proteins associated with AED efficacy, and (iv) genes which are associated with AED toxicity (Spear, 2001). Interindividual variations in genes located on or related to regions that express drug receptors, ion channels, DMEs and DTPs may influence the response to AED treatment (Spear, 2001; Holmes, 2002; Clancy & Kass, 2003; Ramachandran & Shorvon, 2003; Ma *et al.*, 2004).

1.4.1 Pharmacogenetics of antiepileptic drugs: The influence of pharmacodynamic genes

Most forms of human idiopathic epilepsy have been associated with genetic variation in ion channels. These include generalised epilepsy with febrile seizure plus (GEFS+) and severe myoclonic epilepsy of infancy (SMEI) that were linked to sodium channel subunit (SCN1A and SCN2A, respectively) variation (Ceulemans *et al.*, 2004; Kamiya *et al.*, 2004) and some idiopathic generalised epilepsy syndromes that have been associated with voltage-gated potassium channel (Chioza *et al.*, 2002) and calcium channel gene polymorphisms (Chioza *et al.*, 2001). Most AEDs are believed to act on ion channels to modulate the excitability of neurones. Therefore, it is possible that there are associations between channel abnormalities and drug responsiveness in epilepsy. AED targets may be altered by the changes in the genetic transcription of ion channel

subunits. These transcriptional changes most probably affect both the density of ion channels in the neuronal membrane, as well as the shape of multi-subunit channel complexes. Other post-transcriptional modifications of ion channel proteins that are induced by seizures may also affect AED sensitivity (Remy & Beck, 2006).

Reversal of excessive neuronal hyperexcitability can be achieved by AED binding to its recognition site in the domain IV, segment 6 region of the voltage-gated sodium channel (Ramachandran & Shorvon, 2003). Alteration of this important binding site could potentially result in drug non-responsiveness. This postulated mechanism is supported by the phenotypic difference between SMEI and GEFS+. Most patients with SMEI carry a truncating mutation which leads to a short $Na_v1.1$ sodium channel α-subunit protein and most likely causes a loss of function (Ceulemans *et al.*, 2004). In these patients, protein truncation might result in destruction of the drug binding site and thereby explain resistance to AEDs acting on the sodium channel. On the other hand, GEFS+ is a more benign phenotype which results from a few SNPs in sodium channel genes, but a nonetheless fully expressed protein (Ceulemans *et al.*, 2004). Compared to SMEI, GEFS+ is associated with a better response to AEDs.

Other than treatment response, variability in the function of ion channels may also influence the amount of AED required to achieve optimal seizure control. It has been demonstrated that an intronic polymorphism IVS5 -91G>A in the SCN1A gene is associated with maximum doses of CBZ and PHT (Tate *et al.*, 2005). Maximum doses of CBZ averaged 1313, 1225, and 1083 mg for individuals with IVS5 -91A/A, A/G, and G/G genotype, respectively; maximum doses of PHT averaged 373, 340, and 326 mg, for individuals with IVS5 -91A/A, A/G, and G/G genotype, respectively, suggesting a trend towards reduction in maximum dose according to genotype. This polymorphism affects alternative splicing of exon 5 of the SCN1A gene. The major IVS5 -91A allele

disrupts the consensus sequence of fetal exon 5N, possibly reducing the expression of this exon relative to the adult exon 5A. Analysis of SCN1A c.DNA from adult human brain tissue of individuals with epilepsy showed some degree of association between the IVS5 -91G>A genotypes and the SCN1A expression (Tate *et al.*, 2005). Therefore, genetic variability in ion channels not only explains epileptogenesis but may also influence drug responsiveness and dosing requirements due to subtle alteration of the channel structure.

1.4.2 Pharmacogenetics of antiepileptic drugs: The influence of pharmacokinetic genes

Investigation of AED pharmacogenetic-pharmacokinetic association can be approached from two directions; (1) genes encoding DTPs and (2) genes encoding DMEs. Although pharmacogenetic studies examining the influence of DTP polymorphisms directly on AED pharmacokinetics are limited, some studies have explored the association between DTP gene polymorphisms and the response to AED treatment (Siddiqui *et al.*, 2003; Tan *et al.*, 2004a; Hung *et al.*, 2005; Sills *et al.*, 2005; Kim *et al.*, 2006; Seo *et al.*, 2006a). The most studied DTP gene is ABCB1, a gene responsible for encoding the efflux transporter, P-gp. P-gp is believed to be a contributor to pharmacoresistance in epilepsy, as several AEDs have been demonstrated to be transported by P-gp (Potschka *et al.*, 2002) and over-expression of P-gp has been identified in medically intractable epileptic brain tissue (Marchi *et al.*, 2004). Variability in P-gp expression may be the result of polymorphisms in the ABCB1 gene and has been postulated to result in interindividual variation in the response to AED treatment. One study has demonstrated that patients with drug-resistant epilepsy are more likely to carry the ABCB1 3435C/C genotype than the 3435T/T genotype (Siddiqui *et al.*, 2003). Further studies examining multiple polymorphic sites have concluded that haplotypes of common polymorphisms

in the ABCB1 gene may be more useful in predicting response to AED treatment (Hung *et al.*, 2005; Seo *et al.*, 2006a). However, other investigations have failed to confirm the original association between the ABCB1 c.3435C>T polymorphisms and drug-resistant epilepsy (Tan *et al.*, 2004a; Sills *et al.*, 2005; Kim *et al.*, 2006). As such, the functional significance of ABCB1 gene polymorphisms in the response to AED treatment remains elusive.

The second dimension of AED pharmacogenetic-pharmacokinetic association studies is that related to DMEs. Among the DMEs that mediate metabolism of AEDs, only isoforms of CYP2C have been studied in depth. (Prasad *et al.*, 1999; Clancy & Kass, 2003; Ramachandran & Shorvon, 2003). CYP2C9 has two functionally important polymorphisms, the c.430C>T and c.1075A>C substitutions (Scordo *et al.* 2004). Lower CYP2C9 activity is associated with the 430T+1075C variant. In a single dose study using 300 mg PHT, there was a significant difference in trough PHT concentrations between the variants of CYP2C9 (Aynacioglu *et al.*, 1999). Compared with the CYP2C9 430C/C+1075A/A variant, the greatest difference in trough PHT concentrations was demonstrated in individuals carrying the CYP2C9 430T/T+1075A/C variant (95% CI: 0.78 – 2.20; Aynacioglu *et al.*, 1999). In a different study, the PHT 0 – 96 hour AUC after a single dose was also significantly higher in patients carrying the 430T+1075C variant than in individuals homozygous for the 430C and 1075A alleles. Overall, carriers of the 430T+1075C variant have 1.8 to 2.7-fold higher PHT AUC than other genetic variants (Caraco *et al.*, 2001).

In addition, the maximal elimination rate of PHT was shown to be lower in individuals with the 1075C allele compared to those with the 1075A allele (Odani *et al.*, 1997), and this may explain the association between higher plasma PHT concentrations and the CYP2C9 1075C allele (Brandolese *et al.*, 2001; Soga *et al.*, 2004). Carriers of the

430T+1075C variant have also been shown to have a higher plasma PHT concentration (Caraco *et al.*, 2001), which corresponded with a mean PHT dose that was 30% lower in a group of patients with the 430T+1075C variant compared to those with the 430C+1075A variant (199 ±38.6 mg *versus* 287 ±80.7 mg, respectively). This difference in PHT dose between variant groups was most evident in patients with PHT steady-state plasma concentrations within the therapeutic range (10 – 20 mg/l; van der Weide *et al.*, 2001). A trend confirming that the PHT dose is associated with CYP2C9 variants was also demonstrated in the prevalence profile of the variants; CYP2C9 430T+1075C alleles were present in 62.5% of the low-dose group, 32.4% of the standard-dose group and 0% of the high-dose group (van der Weide *et al.*, 2001). A study on maximum dose of AEDs has also demonstrated that carriers of CYP2C9 430T+1075C variant received significantly lower PHT and CBZ doses (Tate *et al.*, 2005).

1.4.3 Antiepileptic drugs as drugs of pharmacogenetic interest

A drug with pharmacogenetic interest is one that is likely to demonstrate significant inter-individual variability in mortality, morbidity, quality of life impact and the risk of severe adverse effects. In addition to these criteria, the frequency of drug prescription and the number of exposed patients should also be considered (Kim *et al.*, 2007). Drugs that are frequently prescribed may also be drugs of pharmacogenetic interest because a larger population of patients is at risk of suffering therapeutic failure or severe adverse effects.

The exciting pharmacogenetic findings in relation to PHT metabolism have a limited clinical application since this drug is no longer employed as a first line agent in most European countries, especially for the chronic treatment of epilepsy (National Institute

for Health and Clinical Excellence, 2004). However, a number of other AEDs is available for further exploration. VPA, CBZ and LTG are the three most commonly prescribed AEDs by clinicians in the UK (Morgan *et al.*, 2004). The Standard and New Antiepileptic Drug (SANAD) study has demonstrated that the best AED for newly diagnosed idiopathic generalised epilepsy is VPA, whereas CBZ and LTG are the drugs of choice for patients with partial epilepsy (Marson *et al.*, 2007a; Marson *et al.*, 2007b). The Epilepsy Unit at the Western Infirmary is a tertiary referral centre for adults with new-onset and pre-existing seizure disorders and, as such, has a population which is predominated with partial or localisation-related epilepsy. Given that these patients are most commonly treated with CBZ and LTG, that these drugs have a common pharmacodynamic target, and that their pharmacokinetic and metabolic profiles are well established, it is reasonable to focus on these two important AEDS.

1.5 Carbamazepine

1.5.1 History and indications

CBZ was first synthesised in the laboratories of J.R. Geigy A.G. by Schindler and Blattner in 1957 (Schmutz, 1985). Theoblad and Kunz reported its initial spectrum of anticonvulsant activity six years after CBZ had been discovered (Schmutz, 1985). CBZ or 5*H*-dibenz-[b,f]azepine-5-carboxamide (Himes *et al.*, 1981) has been used as an AED for many decades. It is effective in the treatment of simple partial, complex partial and generalised tonic-clonic seizures. It is ineffective and may even be deleterious in generalised absence seizures (Liporace *et al.*, 1994; Kochen *et al.*, 2002). Other indications for CBZ include the treatment of neuropathic pain, such as trigeminal neuralgia, and psychiatric disorders, such as manic-depressive illness and aggression due to dementia.

1.5.2 Mechanisms of action

Several mechanisms have been proposed to explain the anticonvulsant activity of CBZ. Its principal mechanism is on neuronal ion channels to prevent the generation of high-frequency repetitive firing. CBZ blocks voltage-gated sodium channels and L-type calcium channels (Ambrosio *et al.*, 1999), and potentiates potassium channel conductance (Schmidt & Elger, 2004), which consequently suppresses the generation of high frequency action potentials. CBZ binds to the inactivated state of the voltage-gated sodium channel and changes the gating conformation in a voltage-, frequency- and time-dependent manner (Courtney, 1975; Kuo, 1998; Kwan *et al.*, 2001). It binds to a common receptor site located on the extracellular portion of the sodium channel. The common binding site contains two phenyl groups, which are probably part of the side chain groups of aromatic amino acids constituting the channel. The two phenyl rings in CBZ bind to corresponding phenyl groups in the receptor. Other mechanisms of action reported for CBZ include modulation of synaptic transmission and neurotransmitter receptors such as the purine, monoamine, acetylcholine and N-methyl-D-aspartate (NMDA) receptors (Brodie & Dichter, 1997; Kwan *et al.*, 2001; Bazil & Pedley, 2003; Schmidt & Elger, 2004). However, these mechanisms are not as clear as the first mechanism.

1.5.3 Clinical pharmacokinetics

CBZ is absorbed relatively slowly from the gastrointestinal tract; however, its oral bioavailability is high (>70%). The irregular and delayed absorption is due to its poor dissolution in gastrointestinal fluid, and also alteration of gastrointestinal motility by its weak anticholinergic properties. After a single oral dose, peak plasma CBZ concentrations are achieved within 4 to 8 hours (Spina, 2002). In the blood, CBZ is 75%

bound to albumin, and to a lesser degree to α_1-acid glycoprotein. In terms of physicochemistry, CBZ is neutral and highly lipophilic, making it easy to cross cell membranes. CBZ rapidly distributes to various organs and body tissues. In single-dose studies employing healthy volunteers and patients, the V_d for CBZ has been estimated at between 0.79 and 1.86 l/kg (Spina, 2002). The biotransformation of CBZ occurs mainly in the liver, involving multiple DMEs such as the CYP450s, hydrolases and conjugating enzymes. CBZ does not undergo significant enterohepatic metabolism (Spina, 2002). In humans, less than 2% of CBZ is eliminated unchanged in the urine and approximately 70% of its oral dose is renally excreted (Bertilsson *et al.*, 1997).

1.5.4 Metabolism of carbamazepine

Multiple isoforms of CYP450 enzymes may be involved in the metabolism of a single AED. CYP450-related metabolism has a significant influence on the plasma concentrations of CBZ. CBZ metabolism is mediated by CYP1A2, CYP2B6, CYP2C8, CYP2C9 and CYP3A4, with the latter being identified as the primary isoenzyme (Pearce *et al.*, 2002; Tredger & Stoll, 2002). The phase 1 metabolism of CBZ produces four major metabolites; CBZ-10,11-epoxide (CBZ-E); 2-hydroxy-CBZ; 3-hydroxy-CBZ; and CBZ-acridan (Bernus *et al.*, 1996). Thereafter, mEH converts CBZ-E to CBZ-10,11-diol, prior to conjugation and excretion from the body. A significant amount of CBZ (15%) is subject to direct *N*-glucuronidation, with UGT2B7 identified as the key enzyme (Staines *et al.*, 2004). Most other products of phase 1 metabolism are also conjugated with glucuronides (Maggs *et al.*, 1997; FIGURE 1.2).

The intermediate metabolite, CBZ-E is known to be pharmacologically active. It is often associated with rash and other side effects of CBZ (Ramsay & Wilder, 2002). The rate of CBZ epoxidation is believed to correlate with the content of microsomal CYP3A4 in

human liver (Kerr *et al.*, 1994), while inhibition of CYP3A4 by drug such as ketoconazole can suppress CBZ-E formation by up to 94% (Pelkonen *et al.*, 2001). These findings suggest that CYP3A4 is the principal catalyst of CBZ-E formation in human liver. A minor role of CYP2C8 in CBZ epoxidation has also been demonstrated (Kerr *et al.*, 1994; Pelkonen *et al.*, 2001). A study using cultures of COS-7 cells has shown that the activity of CYP3A5 is approximately one third that of CYP3A4 with regard to the formation of CBZ-E (Ohmori *et al.*, 1998). Another investigation using baculovirus-infected insect cells and human liver microsomes has shown similar levels of activity, with the CYP3A5/CYP3A4 ratio of intrinsic clearance in the convertion of CBZ to CBZ-E approaching 1.0 (Huang *et al.*, 2004). These investigators also reported that the CYP3A4 and CYP3A5 Michaelis-Menten constant values were 248 and 338 μM, and the maximum rate of metabolism values were 4.87 and 5.98 nmol/min/nmol, respectively. These observations indicate a potentially important role of CYP3A5 enzyme in the *in vivo* metabolism of CBZ.

The conversion of CBZ to hydroxy-CBZ metabolites is the second most important metabolic pathway after epoxidation. The hydroxylation process is mediated by multiple CYP450s, including CYP3A4/5 and CYP1A2 (Pelkonen *et al.*, 2001; Pearce *et al.*, 2002; Spina, 2002). Each of these CYP450s has a specific site of action on the CBZ molecule, corresponding to the 1-, 2-, 3-, or 4- positions of the aromatic ring (FIGURE 1.3; Spina, 2002), however, the most common metabolites are 2-hydroxy-CBZ and 3-hydroxy-CBZ (Pearce *et al.*, 2002).

CBZ induces the CYP450 isoenzymes that are involved in its own metabolism. CBZ may increase the amount of CYP3A4 protein and its corresponding activity up to 3- to 4-fold (Wolbold *et al.*, 2003). CBZ induces CYP3A4 activity to a greater extent than other CYP450 isoenzymes such as CYP2C19 (Bertilsson *et al.*, 1997). As CBZ is

primarily metabolised by CYP3A4, this effect will automatically accelerate its own metabolism in a process known as autoinduction (Tredger & Stoll, 2002), and give a unique time-dependent character to the pharmacokinetics of CBZ (Bialer *et al.*, 1998).

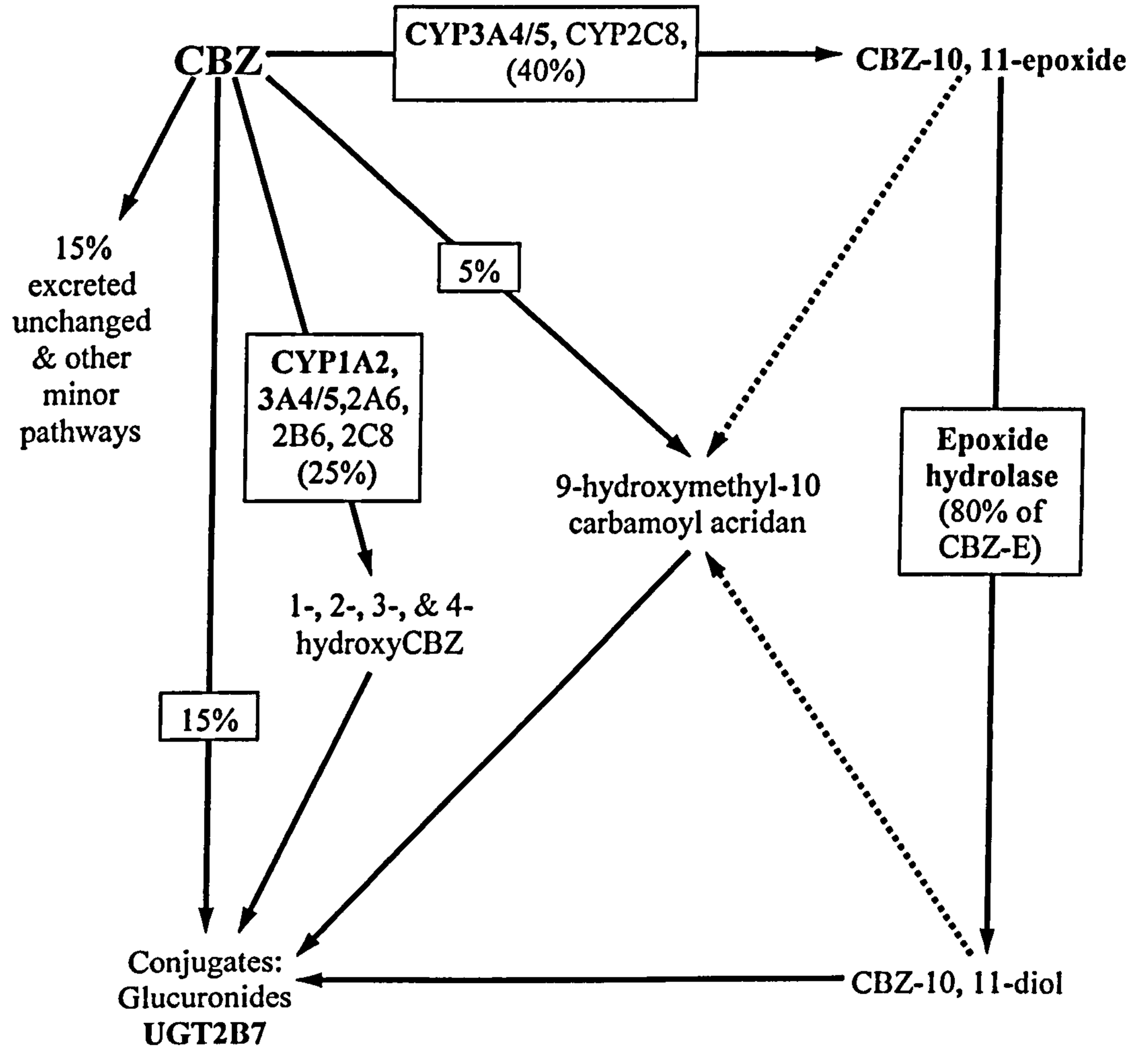

FIGURE 1.2. The pathway of carbamazepine metabolism. Adapted from Pelkonen *et al.* (2001).

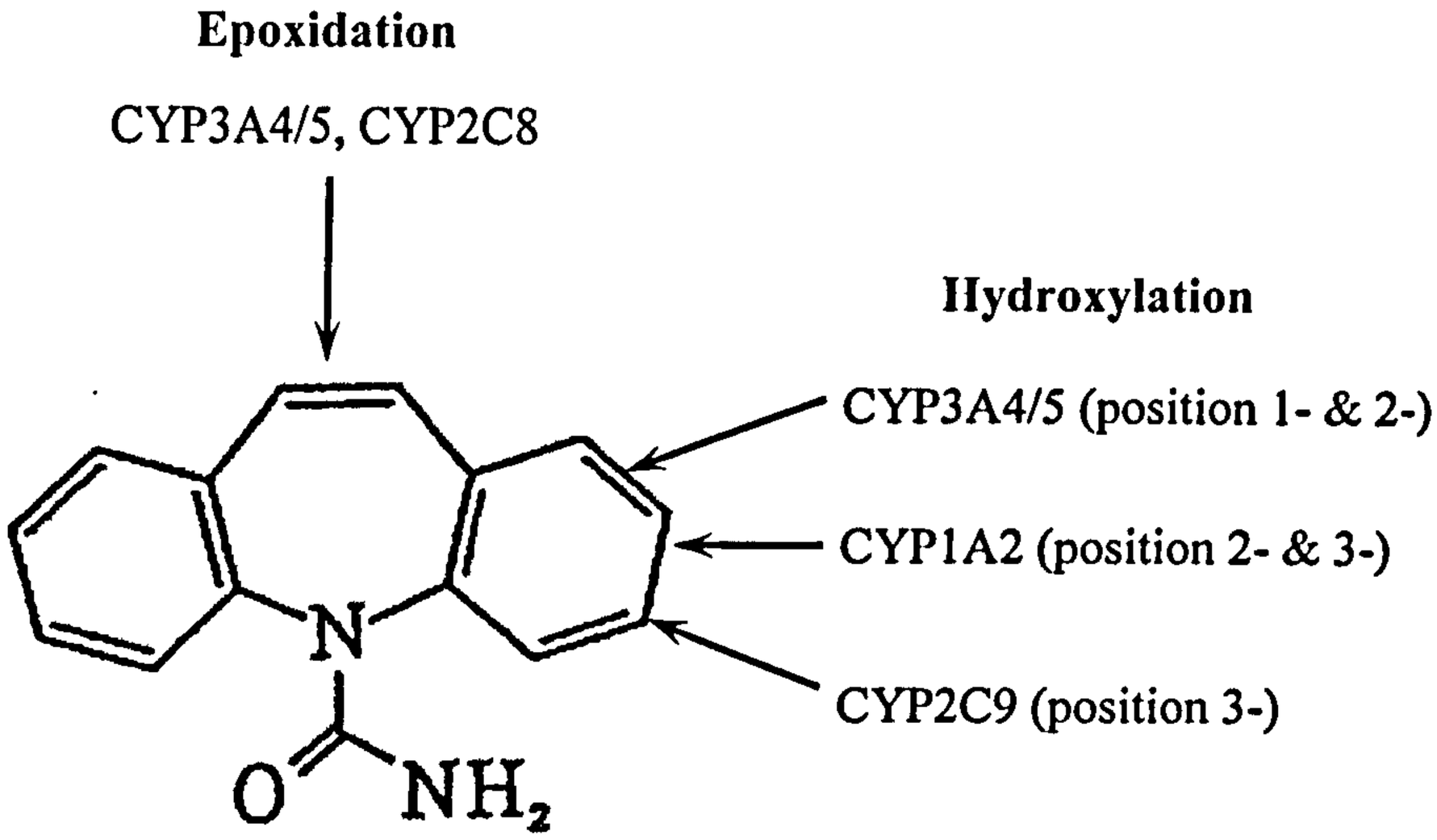

FIGURE 1.3. Chemical structure of carbamazepine and the sites of CYP450 action. Modified from Himes *et al.* (1981), Pelkonen *et al.* (2001) and Spina (2002).

1.5.5 Effects of autoinduction on carbamazepine pharmacokinetics

Autoinduction of CBZ metabolism involves steroid and nuclear receptors such as the constitutive androstane receptor (CAR) and pregnane X receptor (PXR; Fuhr, 2000). The involvement of PXR is crucial at the transcription level of CYP3A4 mRNA (Wolbold *et al.*, 2003). In addition to PXR and CAR, the human glucocorticoid receptor (GR) has also been described as a contributor to CYP3A4 induction by its involvement in the GR-PXR/CAR-cytochrome P450 cascade (Dvorak *et al.*, 2003; Pascussi *et al.*, 2003). Both GR and PXR have been reported to contribute to the transcriptional regulation of the CYP3A4 gene mediated by a large number of xenobiotics (El-Sankary *et al.*, 2001). Although it is recognised that PXR plays a significant role in the transcriptional phase of CYP3A4, genetic variability in the PXR gene does not explain the variation in CYP3A4 expression (Schuetz, 2003) or activity (Zhang *et al.*, 2001).

Autoinduction of CBZ metabolism is a dose dependent process (Liu & Delgado, 1994). Steady-state clearance of CBZ varies between 31 to 110 ml/min depending on the maintenance dose and increases linearly with dose increment (Kudriakova *et al.*, 1992). The ratio of CBZ-E to CBZ ranges between 0.09 ±0.02 at 100 mg/day and 0.21 ±0.06 at 1200 mg/day (Kudriakova *et al.*, 1992). Dose effects on these ratios are more predominant at a lower dosage range. Surprisingly, age does not influence the degree of CBZ autoinduction, despite clear differences in metabolic capacity between young and elderly subjects (Battino *et al.*, 2003).

The effects of autoinduction on CBZ concentrations are critical. Observed concentrations of CBZ are 50% lower than expected concentrations at day-4 of therapy, with observed concentrations falling further during extended follow-up (Eichelbaum *et al.*, 1975). This drop in CBZ plasma concentrations can be detrimental to seizure control (Macphee & Brodie, 1985).

CBZ has been employed as a reference drug in many comparative clinical trials of novel AEDs for the treatment of partial seizures and generalised tonic-clonic seizures in either adult or pediatric subjects (Brodie *et al.*, 1995; de Silva *et al.*, 1996; Reunanen *et al.*, 1996; Tanganelli & Regesta, 1996; Chadwick, 1999; Vasudev *et al.*, 2000; Kwan & Brodie, 2001; Marson *et al.*, 2002; Fakhoury *et al.*, 2004). Due to the effects of autoinduction, the dose of CBZ has to be adjusted (Tanganelli & Regesta, 1996; Arroyo & Sander, 1999; Fakhoury *et al.*, 2004). Dose titration often results in a 2- or more-fold higher dose at the end of study than the initial target dose. In a study of childhood epilepsy, the median doses of CBZ at the start of therapy and after 3 years on therapy were 200 mg/day (50 – 400 mg/day) and 400 mg/day (150 – 1000 mg/day), respectively (de Silva *et al.*, 1996). The range of maximum dose was reported to be wider in CBZ-treated subjects (200 – 1400 mg/day) compared with other AEDs (Chadwick, 1999).

This range is similar to that reported as the final dose required (200 – 1600 mg/day) to achieve full seizure control (Kwan & Brodie, 2001). In clinical studies, some subjects receive a significantly higher dose of CBZ by the end of the study period, however, mean CBZ and CBZ-E plasma concentrations do not differ when measured routinely over a four week period (Schmidt & Elger, 2004) or even up to 48 weeks (Brodie *et al.*, 1995; Hogan *et al.*, 2003). This is a demonstration of how the effect of autoinduction can influence plasma concentrations of CBZ over an extended period of time. These studies have also shown that the degree of CYP3A4 metabolic capacity among patients is varied and may partly associate with interindividual genetic variability.

1.5.6 Concentration-response relationship

The association between plasma CBZ concentrations and clinical response has been investigated comprehensively in seizure disorders. The therapeutic range for CBZ plasma concentrations has been suggested at 4 to 12 mg/l (Bialer *et al.*, 1998), although minor variations on this range have been proposed in numerous other studies (Hvidberg, 1985; Brodie *et al.*, 1995; de Silva *et al.*, 1996; Arroyo & Sander, 1999; Dickinson *et al.*, 1999).

Plasma concentrations of CBZ in excess of 10 to 12 mg/l are likely to produce adverse effects. However, minor signs of toxicity may also be apparent at lower concentrations. Interestingly, some patients are able to tolerate higher levels of CBZ without obvious deleterious consequences. A relationship between fluctuations in plasma CBZ concentration and the emergence of intermittent side effects has also been described (Bialer *et al.*, 1998). Optimal CBZ concentrations vary widely among patients, with lowest concentrations of approximately 1 to 6 mg/l required for minimal effectiveness

(Bialer *et al.*, 1998). However, CBZ concentrations lower than a threshold of 4 to 6 mg/l may not be able to control seizures in difficult to treat epilepsy (Bialer *et al.*, 1998).

1.5.7 Side effects and adverse reactions

Most AEDs have the potential to cause side effects or adverse events. CNS related events represent more than 60% of overall reported side effects or adverse reactions of AEDs (Chadwick, 1999; Carpay *et al.*, 2005). Up to 80% of patients on AEDs experience at least one adverse event (Chadwick, 1999). Adverse events are the most common reason for AED withdrawal in many clinical trials (Reunanen *et al.*, 1996; Fakhoury *et al.*, 2004). The most common side effects of CBZ are CNS related, for example drowsiness, dizziness, asthenia, headache, weakness and somnolence (Brodie *et al.*, 1995; Reunanen *et al.*, 1996; Tanganelli & Regesta, 1996; Vasudev *et al.*, 2000; Hogan *et al.*, 2003).

CBZ is also associated with severe cutaneous reactions such as Stevens-Johnson syndrome, toxic epidermal necrolysis and generalised rash (Rzany *et al.*, 1999). This phenomenon has been associated with the production of reactive iminoquinone, which is an intermediate metabolite in CBZ metabolism (Ju & Uetrecht, 1999). The initial cutaneous reactions may indicate a potentially life-threatening reaction known as anticonvulsant hypersensitivity syndrome (Svensson *et al.*, 2000.). Rash is experienced by 3.4 to 10 percent of patients on CBZ (Reunanen *et al.*, 1996; Chadwick, 1999; Hogan *et al.*, 2003) and is one of the most significant adverse events leading to CBZ withdrawal (Brodie *et al.*, 1995).

1.5.8 Potential factors that influence the variability in clinical pharmacology of carbamazepine

Various factors contribute to variability in pharmacokinetics and pharmacodynamics, which may consequently affect the efficacy and toxicity of CBZ. These factors include patient age, organ function, drug formulation, concomitant medications and genetic status. Age reflects body composition and function. The weight-related V_d of lipophilic drugs is usually higher in adults than in children because of the greater proportion of fat per kg of body weight (Thomson, 2004). Indeed, it has been reported that the V_d of CBZ is strongly associated with body weight and demonstrates an increase when comparing children to adults (Reith *et al.*, 2001). Clearance of CBZ also increases linearly with body weight and nonlinearly with age. With respect to body weight, infants have a higher clearance than older children (Delgado Iribarnegaray *et al.*, 1997; Reith *et al.*, 2000) and the $t_{1/2}$ of CBZ is very short in young children. CBZ is more rapidly metabolized to CBZ-E in children than in adults. This is in accordance with conventional wisdom which suggest that, biotransformation occurs at a faster rate in children than in adults (Gilman *et al.*, 2003; Perucca, 2006). Thus, children may require a higher dose of CBZ per kg of body weight than adults.

The effect of hepatic impairment on the pharmacokinetics of CBZ is not known. Given that CBZ is primarily metabolized in the liver, it is important to exercise caution when initiating CBZ in patients with hepatic dysfunction. The effect of renal impairment on the pharmacokinetics of CBZ is similarly unknown.

Plasma CBZ concentrations can be influenced by drug interactions. These are often pharmacokinetic rather than pharmacodynamic in origin, and result from induction or inhibition of DMEs (Patsalos, 1999; Perucca, 2001; Bazil & Pedley, 2003). The CBZ-E

to CBZ ratio may be significantly increased by the concomitant administration of enzyme-inducing AEDs (PHT and PB) or decreased by enzyme inhibitors (VPA). The metabolism of CBZ can also be inhibited by a variety of therapeutic agents, including macrolide antibiotics, anti-fungals, anti-virals, and anti-hypertensive, all leading to an increased CBZ plasma concentration. Such drug-induced changes in CBZ kinetics are particularly pronounced in children (Battino *et al.*, 1995).

Genetic polymorphisms which result in the production of proteins with altered activity may also have major implications for the variability in CBZ pharmacokinetics and pharmacodynamics. These proteins include DMEs and ion channels. CBZ metabolism involves many DMEs (FIGURE 1.2; Pelkonen *et al.*, 2001). Common polymorphisms in genes encoding these enzymes have been associated with several functional variants. Any such change in the activity of DMEs may consequently influence the plasma and brain concentrations of CBZ. This may in turn increase the risk of treatment failure or the precipitation of adverse effects with CBZ treatment. Blockade of voltage-gated sodium channels is the principal mechanism of CBZ action (Courtney, 1975; Kuo, 1998; Kwan *et al.*, 2001) and polymorphisms in the genes which encode these proteins have been associated with defective channel structure and several forms of epilepsy in children (Meisler & Kearney, 2005). These genetic variants may influence the binding site for CBZ and the sensitivity of the channel to blockade. As such, they have the potential to affect the clinical efficacy and toxicity of the drug.

1.6 Lamotrigine

1.6.1 Indication and efficacy

LTG (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine) is a modified derivative of pyrimethamine, an antifolate compound (FIGURE 1.4). It was approved for the clinical management of seizures in the United Kingdom in 1991. LTG was initially introduced as an adjunctive agent, but was subsequently proven to be effective as monotherapy in the treatment of both partial seizures and primary generalised seizures in newly diagnosed and refractory epilepsy patients (Beghi, 2004). Patients with juvenile myoclonic epilepsy have also benefited from LTG monotherapy (Schimschock *et al.*, 2005). Outwith epilepsy, LTG is employed as a mood stabiliser in depression (Herman, 2004). The effectiveness of LTG monotherapy is comparable to that of CBZ monotherapy (Nieto-Barrera *et al.*, 2001; Kaminow *et al.*, 2003). However, LTG has better tolerability and a better health related quality of life measures than CBZ (Gillham *et al.*, 2000; Nieto-Barrera *et al.*, 2001). Up to 40% of patients who are refractory to other AEDs may respond to LTG (Knoester *et al.*, 2005). LTG is also effective as an alternative AED for patients who experience intolerable adverse effects (Knoester *et al.*, 2005).

1.6.2 Mechanism of action

LTG was identified during the screening of antifolates as putative antiepileptic agents (Ragsdale & Avoli, 1998). However, its anticonvulsant activity does not correlate with its weak inhibition of dihydrofolate reductase, an enzyme involved in folate bio-synthesis (Bazil & Pedley, 2003). LTG acts mainly by prolonging the inactivation of voltage-gated sodium channels. It inhibits sustained repetitive firing of action potentials

in a voltage- and frequency-dependent manner (Cheung *et al.*, 1992; Ragsdale & Avoli, 1998; Kwan *et al.*, 2001). These actions resemble those of the established AEDs, PHT and CBZ. LTG also acts on L-, N-, and P- subtypes of calcium channels to reduce post-synaptic depolarisation (Wegerer *et al.*, 1997; Perucca, 2001). Blockade of voltage-gated sodium and calcium channels by LTG is also believed to prevent the release of glutamate, the principle excitatory neurotransmitter in mammalian brain (Patsalos, 1999; Bazil & Pedley, 2003). LTG has been demonstrated to decrease spontaneous glutamate release and to increase GABA release in rat entorhinal cortex (Cunningham & Jones, 2000). This wide range of pharmacological effects may explain the broad spectrum of clinical activity observed with LTG

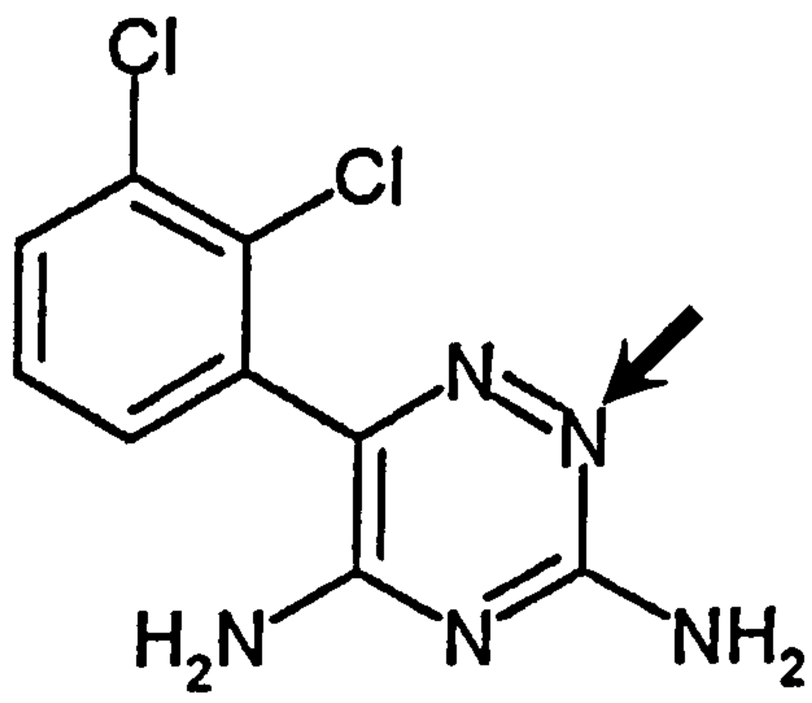

FIGURE 1.4. Chemical structure of lamotrigine. Arrow shows the main position of glucuronidation.

1.6.3 Clinical pharmacokinetics

LTG is rapidly and completely absorbed following oral administration and reaches peak plasma concentrations at 1 to 3 hours after administration (T_{max}; Patsalos, 1999; Bazil & Pedley, 2003; Doose *et al.*, 2003). The oral bioavailability approaches 98% (Patsalos, 1999; Bazil & Pedley, 2003; Doose *et al.*, 2003). LTG absorption is not significantly

influenced by the presence of food in the gastrointestinal tract (Patsalos, 1999). The constant rate of absorption has been estimated at 3.18 l/h. The V_d and $t_{1/2}$ are not dose-dependent, and V_d has been estimated at approximately 1.14 to 1.36 l/kg (Cohen *et al.*, 1987; Ramsay *et al.*, 1991; Hussein & Posner, 1997; Wooton *et al.*, 1997). LTG is approximately 55% bound to human plasma proteins. Thus, any interaction with other therapeutic agents through displacement from protein binding sites is regarded as insignificant.

In healthy volunteers, the total apparent and renal clearances of LTG have been estimated at approximately 0.5 and 0.043 ml/min/kg, respectively (Cohen *et al.*, 1987; Wooton *et al.*, 1997). The $t_{1/2}$ of LTG ranges from 23 to 36 hours (Cohen *et al.*, 1987; Jawad *et al.*, 1987; Rambeck & Wolf, 1993; Wooton *et al.*, 1997; Patsalos, 1999). In patients with epilepsy, concomitant administration of LTG with enzyme-inducing AEDs (PHT, CBZ, primidone or PB) decreases the mean LTG $t_{1/2}$ to 14 hours (Jawad *et al.*, 1987; Ramsay *et al.*, 1991). In contrast, concomitant administration of LTG with enzyme-inhibiting AEDs (VPA) significantly increases the $t_{1/2}$ of LTG (30 – 90 hours; Jawad *et al.*, 1987; Patsalos, 1999; Perucca, 2001; Bazil & Pedley, 2003). Doses may be adjusted accordingly but the dosing interval is unchanged as the relationship between LTG dose and concentration remains linear in both monotherapy and polytherapy (Armijo *et al.*, 1999). Other than concomitant AEDs, several other factors may also influence LTG concentrations. Oral contraceptives can reduce LTG concentration through induction of the glucuronidation process (Sabers *et al.*, 2001) and pregnancy is associated with a decrease in LTG concentrations, especially in the second and third trimesters (Tran *et al.*, 2002; Petrenaite *et al.*, 2005). Severe liver disease, such as cirrhosis, may reduce the elimination of LTG and potentially increase plasma concentrations (Marcellin *et al.*, 2001). A modest autoinduction with LTG has been demonstrated and is complete within 2 weeks of LTG initiation. As a result, the

systemic clearance is increased by an average of 17% and the $t_{1/2}$ decreased from 27.6 to 23.5 hours (Hussein & Posner, 1997).

LTG undergoes 2-N-glucuronidation mainly by UGT1A4 (FIGURE 1.4; Magdalou *et al.*, 1992; Rambeck & Wolf, 1993; Vashishtha *et al.*, 2001). Glucuronide conjugates are the primary (90%) excreted form of LTG. Approximately 70% of oral LTG doses are recovered in the urine (Cohen *et al.*, 1987). The pharmacokinetic profiles of LTG did not differ between healthy volunteers and patients with chronic renal failure, suggesting that renal impairment has little or no effect on LTG plasma concentrations (Wooton *et al.*, 1997).

1.6.4 Concentration – response relationship

Patients with focal and generalised epilepsies are known to possess a lower resting motor threshold (RMT; Tergau *et al.*, 2003). RMT is an indicator of the level of motor cortex excitability. A study performed in healthy volunteers demonstrated a positive linear correlation between LTG plasma concentrations and RMT (Tergau *et al.*, 2003), suggesting that LTG significantly influences the excitability of the motor cortex in a concentration-dependent manner and that higher LTG concentrations may be associated with better seizure control. However, there are no clinical studies which have successfully reproduced this relationship in the epilepsy population. There is no known association between LTG plasma concentration and the percentage reduction in seizure frequency (Mahmood *et al.*, 1998) or total seizure control (Kilpatrick *et al.*, 1996). The literature is, however, less clear with regard to the relationship between LTG concentration and toxicity (Kilpatrick *et al.*, 1996; Hirsch *et al.*, 2004). Concentrations of LTG higher than the recommended therapeutic range (1.5 – 10 mg/l) are often well tolerated and may provide better efficacy (Hirsch *et al.*, 2004), but those greater than 20

mg/l have been reported to produce adverse effects in more than 50% of patients (Hirsch *et al.*, 2004).

There is no evidence to support the appropriateness of monitoring plasma LTG concentration in association with seizure control. However, it may be useful in patients treated with polytherapy, especially when adding or removing concomitant AEDs as LTG concentrations are influenced by enzyme inducers such as PHT, CBZ and PB and enzyme inhibitors like VPA (Perucca, 2001; Bazil & Pedley, 2003). Significant changes in plasma LTG concentrations as a result of the withdrawal of concomitant agents may be detrimental to seizure control or increase the risk of adverse effects.

1.6.5 Adverse effects

The typical adverse effects experienced with LTG are similar to those of CBZ (Brodie *et al.*, 1999; Kaminow *et al.*, 2003). However, the number of patients who experience adverse effects with LTG is relatively low compared to established drugs, such as CBZ and VPA (Nieto-Barrera *et al.*, 2001; Kaminow *et al.*, 2003). Although the incidence of rash is higher with LTG than with CBZ, the percentage of patients with intolerable rashes that lead to drug withdrawal is lower (Brodie *et al.*, 1999; Kaminow *et al.*, 2003; Faught *et al.*, 2004). This was paralleled with a lower number of drop-outs for the LTG group compared to the CBZ group in a head to head monotherapy trial (Brodie *et al.*, 1999).

1.6.6 Potential factors that influence the variability in clinical pharmacology of lamotrigine

Numerous factors can lead to variability in pharmacokinetics and pharmacodynamics, and consequently affect the efficacy and toxicity of LTG. These include patient age, organ function, underlying diseases, drug formulation, concomitant medications and genetic status. Body composition and function are reflected by age. The weight-related V_d of lipophilic drugs is usually higher in adults than in children because of the greater proportion of fat per kg of body weight (Thomson, 2004). However, the weight-normalised V_d for LTG in children aged between 2 to 12 years is higher than that reported in adults (Chen *et al.*, 1999). There is no clear explanation for this observation. LTG metabolism is mainly mediated by glucuronidation in the liver. The rate of glucuronidation is known to be lower in infants aged between 7 and 24 months than in older children or adults (Strassburg *et al.*, 2002). Hence, children under 2 years may exhibit a lesser degree of LTG metabolism. Interestingly, the weight-normalised clearance of LTG is higher in children aged between 2 and 12 years than in adults (Chen *et al.*, 1999). This is in accordance with the observation that, in older children, DMEs mature rapidly and biotransformation occurs at a faster rate than in adults (Gilman *et al.*, 2003; Perucca, 2006). Thus, children may require a higher dose of LTG per kg of body weight than adults. LTG metabolism can also be affected by severe liver disease, such as cirrhosis (Marcellin *et al.*, 2001), whereas renal impairment does not directly affect plasma LTG concentrations but may influence the excretion of glucuronide metabolites of LTG (Wooton *et al.*, 1997). Plasma LTG concentrations can also be influenced by drug interactions. These are often a result from induction or inhibition of DMEs (Jawad *et al.*, 1987; Ramsay *et al.*, 1991; Armijo *et al.*, 1999; Patsalos, 1999; Perucca, 2001; Bazil & Pedley, 2003). It is well known that the concomitant use of LTG and enzyme-

inducing AEDs such as PB, PHT and CBZ may reduce plasma LTG concentrations (Jawad *et al.*, 1987; Ramsay *et al.*, 1991), whereas the interaction between LTG and VPA may result in increase of plasma LTG concentrations (Armijo *et al.*, 1999). Dosage adjustment of LTG may be required when introducing or withdrawing these concomitant AEDs.

Genetic polymorphisms which result in the production of proteins with altered activity may have major implications for the variability in LTG pharmacokinetics and pharmacodynamics. These proteins include DMEs, DTPs and specific subunits of neuronal ion channels. Polymorphisms in UGT genes can influence the activity of glucuronidation enzymes (Sawyer *et al.*, 2003; Thibaudeau *et al.*, 2006), and any such change in the activity of UGTs involved in LTG metabolism may consequently affect plasma and brain drug concentrations. This may increase the risk of treatment failure or the precipitation of adverse effects with LTG treatment. LTG is believed to be a substrate for P-gp (Potschka *et al.*, 2002). Polymorphisms in the ABCB1 gene which encodes P-gp can alter the expression and function of P-gp (Hoffmeyer *et al.*, 2000). This protein is expressed in various body tissues, including the intestine, liver, kidney and brain (Kim, 2002a; Marchi *et al.*, 2004). Variability in the ABCB1 gene has the potential to influence the absorption, distribution, and elimination of LTG. Polymorphisms in the pharmacodynamic targets of LTG may also have an impact on its efficacy. Blockade of voltage-gated sodium channels is the principal mechanism of LTG action (Cheung *et al.*, 1992; Ragsdale & Avoli, 1998; Kwan *et al.*, 2001) and polymorphisms in the genes which encode the principal protein subunits of these channels have been associated with defective channel structure and several forms of epilepsy in children (Meisler & Kearney, 2005). These genetic variants have the potential to influence the binding site for LTG and the sensitivity of the channel to

pharmacological blockade. As such, they might contribute to interindividual variability in the clinical efficacy and toxicity of the drug.

1.7 Drug metabolising enzyme, drug transporter protein and sodium channel genes and their common polymorphisms

In accordance to the aims of this project, special attention is given to DMEs that are involved in CBZ metabolism, P-gp that has been reported to transport LTG, and voltage-gated sodium channels which are blocked by CBZ and LTG in exerting their antiepileptic effects. Common polymorphisms in these DME, P-gp and voltage-gated sodium channel genes are described in this section. Published studies examining these gene variants in association with AED responsiveness are limited. As such, studies investigating other pharmacological agents are presented to illustrate the functional relevance of these gene variants. The common polymorphisms examined throughout this research programme are listed in TABLE 1.6. These are the most widely recognised genetic polymorphisms of the respective proteins at the time this project was planned.

1.7.1 Cytochrome P450: History and structure

In 1940s, cytochrome P450 monooxygenase was first systematically documented in studies exploring the *in vivo* metabolic pathway of compounds in animals (Schenkman, 1993). Extended work through the 1960s by many investigators revealed the presence of mixed function oxidase in the process of drug and xenobiotic oxidation. In 1958, Garfinkel and Klingenberg identified and reported CYP450 as a new pigment in mammalian liver microsomes (Lipscomb & Gunsalus, 1973; Sato *et al.*, 1973). When

treated with carbon monoxide, the reduced form of the pigment demonstrated an unusually strong absorption band at 450 nm. For that reason, the pigment was known as Pigment-450 (P450; Sato *et al.*, 1973). Most of these pigments are present in a membrane-bound state of several tissues, for example, in the liver, kidney, adrenal cortex, intestinal mucosa and several endocrine glands (Sato *et al.*, 1973). CYP450s are named with the root CYP derived from **CY**tochrome **P**450 followed by an Arabic numeral denoting the gene family, a letter for the subfamily and another numeral designating the gene number (Gonzalez, 1993). Human CYP450 has more than 30 isoforms.

CYP450 has a molecular weight of about 57 000 Daltons (~500 residues) and contains one equivalent of β-type haem per polypeptide. The overall hydrophobicity of CYP450 is about 36%, and this gives a significant non-polar property to the molecule. Computational modelling of CYP450 suggests the existence of an α-helix and a β-strand in the molecule. The structure undergoes 'open' and 'closed' transitions to facilitate substrate binding and product/metabolite release (FIGURE 1.5; Black, 1993). Further investigations in 3-dimensional models have demonstrated that CYP450 structure is specific for certain substrates (Guengerich, 1987).

TABLE 1.6. Single nucleotide polymorphisms in selected drug metabolising enzyme, drug transporter protein and sodium channel genes.

Protein	Gene	Single nucleotide polymorphism	Amino acid substitution	dbSNP reference number
CYP3A4	CYP3A4	g.-392A>G	-	rs2740574
CYP3A5	CYP3A5	g.6986A>G	Splicing defect	rs776746
CYP1A2	CYP1A2	g.5734C>A	-	rs762551
mEH	EPHX1	c.337T>C	Y113H	rs1051740
		c.416A>G	H139R	rs2234922
UGT2B7	UGT2B7	c.802C>T	H268Y	rs7439366
P-gp	ABCB1	c.1236C>T	G412G	rs1128503
		c.2677G>T/A	A893S/T	rs2032582
		c.3435C>T	I1145I	rs1045642
$Na_v1.2$	SCN2A	c.56G>A	R19K	rs17183814

mEH = microsomal epoxide hydrolase; P-gp = P-glycoprotein; $Na_v1.2$ = voltage-gated sodium channel alpha-2 subunit; amino acid code, A = Alanine, G = Glycine, H = histidine, I = Isoleucine, K= lysine, R = arginine, S = Serine, T = Threonine, Y = tyrosine; dbSNP = SNP database from National Center for Biotechnology Information available at http://www.ncbi.nlm.nih.gov/.

FIGURE 1.5. A schematic view of the secondary structure of the CYP3A4 protein. The thick arrow shows the substrate pocket. Adapted from Williams *et al.* (2004).

1.7.2 Cytochrome P450 3As

1.7.2.1 Functions and tissue distribution of CYP3As

Almost 50% of all clinically used drugs are metabolised by CYP3A isoforms, mainly CYP3A4 and CYP3A5 (Kuehl *et al.*, 2001; Tredger & Stoll, 2002; Schuetz *et al.*, 2004). Both CYP3As are also involved in the metabolism of more than 50% of AEDs. Other than xenobiotics, CYP3A also catalyses the metabolism of endogenous substances such as steroids, fatty acids and prostagladins (Ohmori *et al.*, 1998). CYP3A proteins are present in many tissues throughout the body. CYP3A expression is greatest in the liver, which is the main organ of drug elimination. On average, CYP3A comprises 30 to 40% of total hepatic CYP450 (Shimada *et al.*, 1994). In the small intestine, CYP3A can be found in the villus epithelium (Paine *et al.*, 1997). CYP3A hepatic expression shows a variation as much as 40-fold among surgically excised and organ donated livers (Lamba

et al., 2002a; Ozawa *et al.*, 2004). A large interindividual variability has also been reported for the CYP3A content in the small intestine (Paine *et al.*, 1997; Lown *et al.*, 1994).

The CYP3A4 isoform is the dominant CYP3A enzyme in the adult liver. In contrast, CYP3A5 is expressed mainly in the gastrointestinal tract. Nevertheless, the CYP3A5 isoform can also be detected in other tissues throughout the body, such as adrenal gland, lung, prostate and kidney (Kolars *et al.*, 1994; Lown *et al.*, 1994; Gervot *et al.*, 1996; Haehner *et al.*, 1996; Hukkanen *et al.*, 2001; Koch *et al.*, 2002).

1.7.2.2 CYP3A gene locus

The CYP3A gene locus is located on position q22.1 of chromosome 7 (Ozawa *et al.*, 2004). Duplication of an ancestral CYP3A gene cassette of 40-55 kb forms the CYP3A locus. Each of the CYP3A genes contains 13 exons, which encode a 503-amino acid CYP3A protein (Wojnowski, 2004). The CYP3A family consists of four members; CYP3A4, CYP3A5, CYP3A7 and CYP3A43 (FIGURE 1.6; Kuehl *et al.*, 2001).

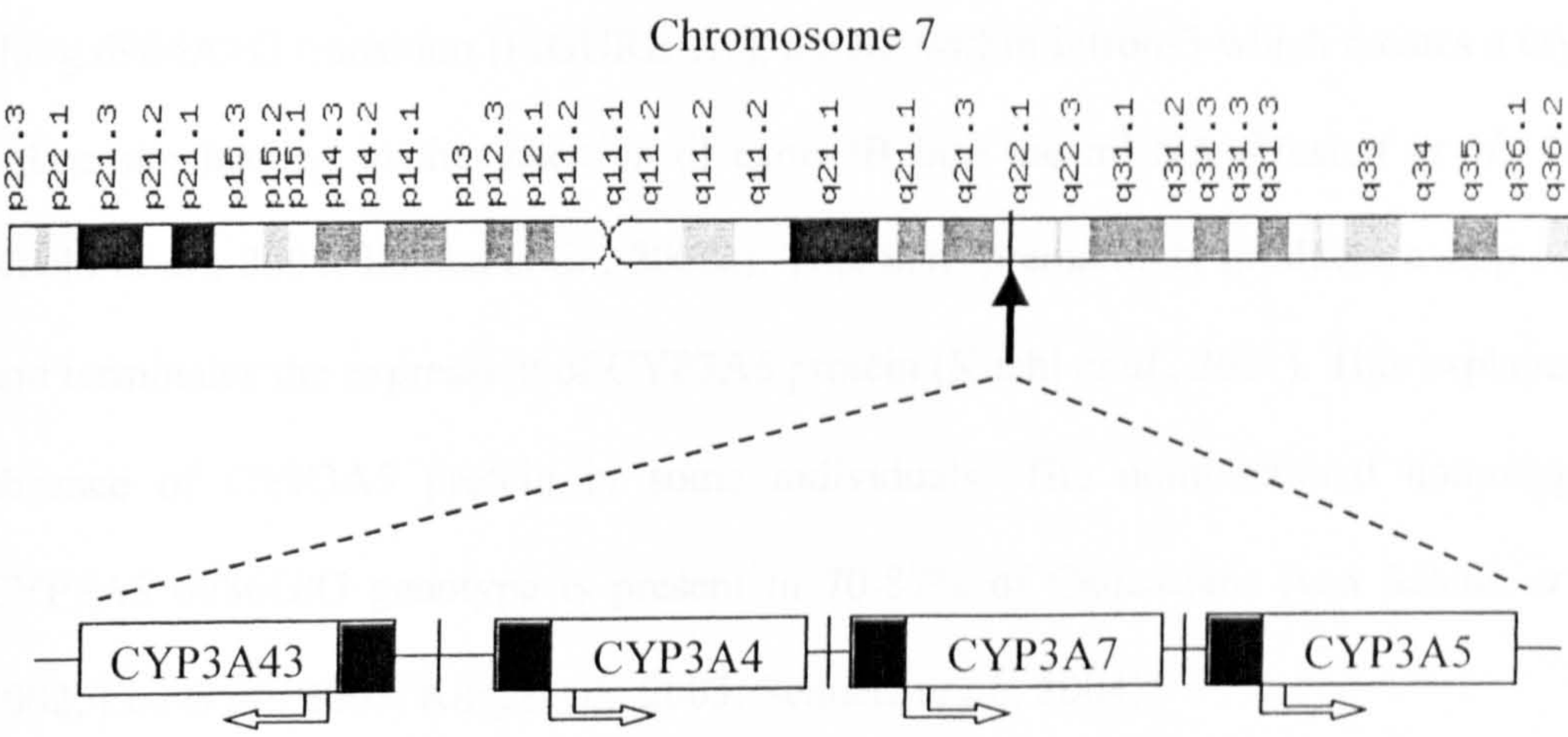

FIGURE 1.6. The location of the CYP3A gene locus on Chromosome 7 and arrangement of CYP3A genes in the locus. Adapted from Wojnowski (2004).

1.7.2.3 Genetics and common polymorphisms of CYP3A4

Genetic polymorphisms in the flanking, intronic and exonic regions of the CYP3A4 gene may influence its level of expression and function. In the 5'-flanking region of CYP3A4 gene alone, at least six different SNPs have been identified (Lamba *et al.*, 2002b). The polymorphic alleles of the CYP3A4 present at a lower frequency in Caucasians than Oriental populations, except for CYP3A4 g.747C>G (Hamzeiy *et al.*, 2002) and CYP3A4 g.-392A>G (Sata *et al.*, 2000; Kuehl *et al.*, 2001; Gracia-Martin *et al.*, 2002; Hamzeiy *et al.*, 2002). The CYP3A4 g.-392A>G polymorphism, previously known as CYP3A4-V, is a SNP in the promoter region of the CYP3A4 gene and was first described in association with the risk of developing prostate carcinoma (Rebbeck *et al.*, 1998).

1.7.2.4 Genetics and common polymorphisms of CYP3A5

Clear polymorphic CYP3A5 expression is found in the adult liver, small intestine and other organs, with individuals presenting a low level of protein (Haehner *et al.*, 1996; Kuehl *et al.*, 2001; Lin *et al.*, 2002). The most recognised polymorphism of CYP3A5 is the g.6986A>G transition (FIGURE 1.7), a SNP within intron-3 which creates a cryptic splice site leading to the insertion of exon-3B into the mRNA (Hustert *et al.*, 2001; Kuehl *et al.*, 2001; Lamba *et al.*, 2002a). This SNP prematurely produces a stop codon and terminates the expression of CYP3A5 protein (Kuehl *et al.*, 2001). This explains the absence of CYP3A5 protein in some individuals. The nonfunctional homozygous CYP3A5 6986G/G genotype is present in 70-87% of Caucasians (van Schaik *et al.*, 2002; Lee *et al.*, 2003; King *et al.*, 2003; Schuetz *et al.*, 2004).

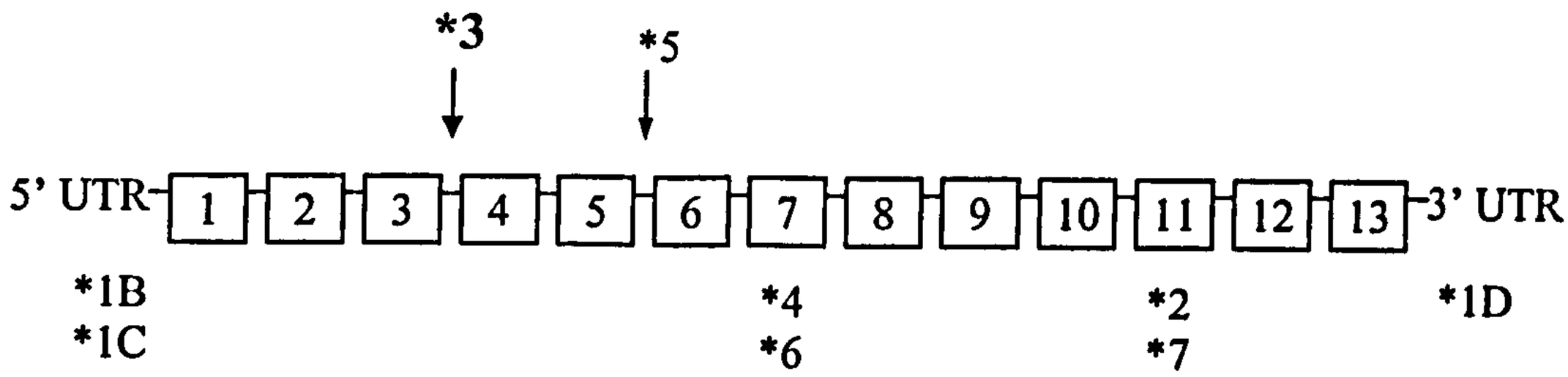

FIGURE 1.7. Distribution of polymorphisms in the CYP3A5 gene. Boxes 1 to 13 represent the exons. The *3 symbolizes the g.6986A>G polymorphism.

1.7.2.5 Phenotype profile of common CYP3A4 and CYP3A5 variants

Hepatic CYP3A4 protein expression varies as much as 90-fold among individuals (Hustert *et al.*, 2001). The high interindividual variation observed in CYP3A4 levels is believed to be genetically related (Chelule *et al.*, 2003; Ingelman-Sundberg, 2004). The functional significance of CYP3A4 gene variants for AED metabolism is unknown. For substrates such as nifedipine and alphatoxin B_1, the significance of genetic variability in CYP3A4 activity has been demonstrated among Japanese and Caucasian populations (Shimada *et al.*, 1994). This is further supported by a meta-analysis of 16 studies employing 10 different CYP3A4 substrates, which revealed strong genetic control on the variability of CYP3A4-dependent metabolism of adriamycin, ethylestradiol, erythromycin, midazolam, nifedipine and nitrendepine (Ozdemir *et al.*, 2000). Some additional evidence to support the importance of CYP3A4 gene variants in drug metabolism has also been reported. For example, a significant difference in the systemic clearance of midazolam, statins, tacrolimus and docetaxel has been demonstrated between the carriers of the CYP3A4 -392A and -392G alleles (Wandel *et al.*, 2000; Hesselink *et al.*, 2003; Goto *et al.*, 2004; Kajinami *et al.*, 2004; Tran *et al.*, 2006).

The second most highly expressed CYP3A protein, CYP3A5, provides another potential avenue for exploration. Some studies suggest that CYP3A5 might not directly contribute to CYP3A variability due to its low protein expression in the liver (Westlind-Johnsson *et al.*, 2003). Nevertheless, some investigators have demonstrated positive findings. It has been reported that some Caucasian subjects with the CYP3A5 6986A allele are able to express CYP3A5 protein at a level which corresponds to 50% of the total hepatic CYP3A content (Kuehl *et al.*, 2001). Therefore, polymorphisms of CYP3A5 may influence the total CYP3A activity (Kuehl *et al.*, 2001) and there is evidence to support this hypothesis. A significant influence of CYP3A5 polymorphisms has been demonstrated on the efficacy of drugs which undergo CYP3A5-dependent metabolism, such as lovastatin, simvastatin, artovastatin (Kivisto *et al.*, 2004) and saquinavir (Frohlich *et al.*, 2004). Carriers of the CYP3A5 6986G allele were found to possess a lower midazolam clearance (Wong *et al.*, 2004). However, a similarly designed study in a Korean population failed to reproduce this finding (Yu *et al.*, 2004). These conflicting results might be explained by ethnic differences between studies, which are not uncommon, and further investigation is required for clarification.

The degree of linkage disquilibrium between common polymorphisms in CYP3A4 and CYP3A5 appears to be high (>90%) when calculated from all concordant homozygous and heterozygous genotypes (Dally *et al.*, 2004). A 60% homology between the 5'-flanking region of CYP3A5 and the corresponding region of CYP3A4, from nucleotide -1 to -1432 (Jounaidi *et al.*, 1994), appears to contribute to this association. Linkage may play an important role in the overall activities of the CYP3A protein (Lamba *et al.*, 2002b; Wilkinson, 2004). For substrates that are metabolised mainly by one of the CYP3A enzymes, effects of the CYP3A5 g.6986A>G polymorphism are usually opposed to the effects of the CYP3A4 g.-392A>G (Saito *et al.*, 2004).

The importance of inducer effects on CYP3A4 and CYP3A5 gene polymorphisms has been demonstrated in a handful of studies. Subjects who were treated with rifampicin and who carry the CYP3A4 –392A and CYP3A5 6986G polymorphisms appear to demonstrate higher midazolam clearance (Floyd *et al.*, 2003). The extent of induction was also found to be approximately 50% greater in subjects with the CYP3A5 6986G/G genotype (Floyd *et al.*, 2003). This evidence suggests that genetic polymorphisms can significantly influence the pharmacological induction of drug metabolism.

1.7.3 Cytochrome P450 1A2

1.7.3.1 Functions and tissue distribution of CYP1A2

CYP1A2 is a DME that catalyzes the hydroxylation of xenobiotics such as caffeine, theophylline, clozapine and CBZ (Pelkonen *et al.*, 2001). Some of the metabolites produced via the CYP1A2 pathway can be mutagenic and carcinogenic to human cells. In mammals, CYP1A2 is constitutively expressed in the liver and the olfactory mucosa (Zhang *et al.*, 2000). CYP1A2 represents approximately 13% of human liver CYP450 (Shimada *et al.*, 1994). Interestingly, CYP1A2 was found to be more active in men than in women (Rasmussen *et al.*, 2002). CYP1A2 activity can be induced by drugs such as CBZ (Parker *et al.*, 1998).

1.7.3.2 Genetics and common polymorphisms of CYP1A2

The CYP1A gene locus is located on Chromosome 15q24.1 of the human genome (FIGURE 1.8). The CYP1A locus contains two genes, CYP1A1 and CYP1A2. The CYP1A2 gene comprises 7 exons that encode 515 amino acids. It also contains an upstream region of about 3 kb (Quattrochi *et al.*, 1986). The CYP1A2 gene is separated by 23 kb from the CYP1A1 gene in the CYP1A locus. These two genes are orientated in

opposite directions with respect to one another, and share a common 5'flanking region, which contains the xenobiotic response elements and hepatic transcription factors, including hepatic nuclear factors (HNFs; Corchero *et al.*, 2001).

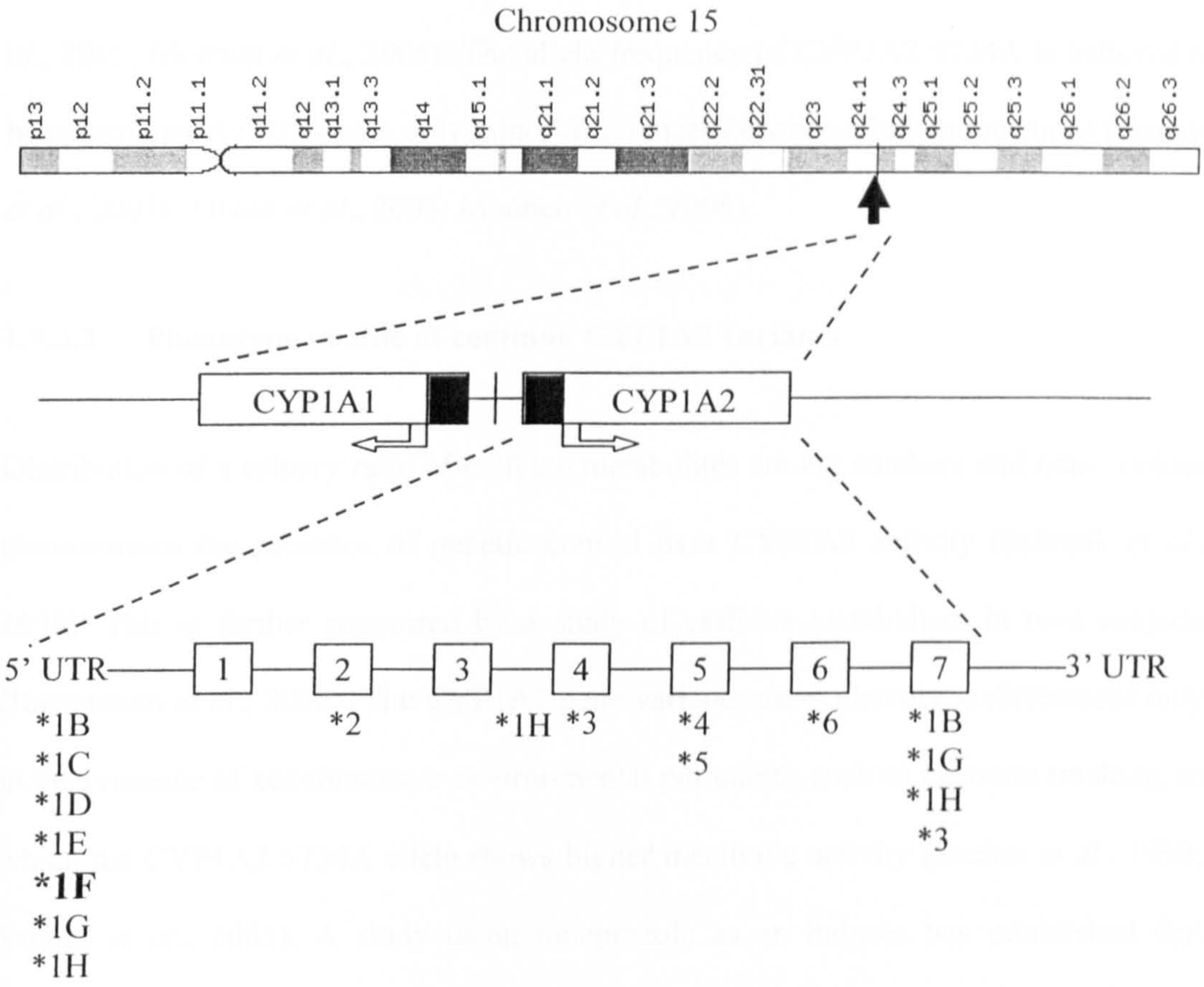

FIGURE 1.8. The location of the CYP1A gene locus on Chromosome 15 and distribution of polymorphisms in the CYP1A2 gene. Boxes 1 to 7 represent the exons. The *1F symbolizes the g.5734C>A polymorphism.

Currently, more than 13 polymorphisms of CYP1A2 have been identified (Human Cytochrome P450 (CYP) Allele Nomenclature Committee, 2001). Among these polymorphisms only the CYP1A2 g.-2467delT and CYP1A2 g.5734C>A are commonly found in Caucasian populations (Sachse *et al.*, 2003; Pavanello *et al.*, 2005). CYP1A2 g.-2467delT is believed to be under strong linkage with CYP1A2 g.5734C>A in

Caucasians (Sachse *et al.*, 2003). Little is known about the influence of CYP1A2 variants on its metabolic activity, except in the case of the CYP1A2 g.5734C>A substitution, where the CYP1A2 5734A allele has been reported to be highly inducible (Sachse *et al.*, 1999) and capable of adjusting the risk of developing disease (Cornelis *et al.*, 2005; Moonen *et al.*, 2005). The allele frequency of CYP1A2 5734A is believed to be approximately 70%, with only minor differences between ethnic populations (Hamdy *et al.*, 2003a; Obase *et al.*, 2003; Moonen *et al.*, 2005).

1.7.3.3 Phenotype profile of common CYP1A2 variants

Distribution of a urinary ratio of caffeine metabolites among smokers and non-smokers demonstrates the presence of genetic control over CYP1A2 activity (Schrenk *et al.*, 1998). This is further supported by a study of caffeine metabolism in twin subjects (Rasmussen *et al.*, 2002). The CYP1A2 gene variants show phenotype differences only in the presence of xenobiotics or environmental pollutants, such as cigarette smoking, in which the CYP1A2 5734A allele shows higher metabolic activity (Sachse *et al.*, 1999; Sachse *et al.*, 2003). A study using omeprazole as an inducer has established that variation in CYP1A2 induction is also associated with the presence of the CYP1A2 5734C>A polymorphism (Han *et al.*, 2002). This phenomenon may be applicable to the study of CBZ because it has the potential to significantly induce CYP1A2 activity, as demonstrated in the drug-drug interaction involving concomitant use of CBZ with clozapine and olanzapine (Jerling *et al.* 1994; Lucas *et al.* 1998).

1.7.4 Microsomal epoxide hydrolase

The epoxide hydrolases are categorised under a broad group of hydrolytic enzymes, which include esterases, proteases, dehalogenases and lipases (de Vries & Janssen,

2003). Epoxide hydrolases play an important function in the activation and detoxification of exogenous chemicals, such as polycyclic aromatic hydrocarbons. At least five forms of epoxide hydrolases are present in mammalian species. These are microsomal cholesterol 5,6-oxide hydrolase, hepoxilin A_3 hydrolase, leukotriene A_4 hydrolase, and soluble and microsomal epoxide hydrolases (Fretland & Omiecinski, 2000). The first four hydrolases participate in the metabolism of endogenous compounds. In contrast to other epoxide hydrolases, mEH activity is geared more towards the metabolism of xenobiotic agents (Fretland & Omiecinski, 2000).

1.7.4.1 Tissue distribution and structure of microsomal epoxide hydrolase

mEH has been found in all tissues studied to date. The highest expression of mEH protein is in the liver followed by prostate, lung and kidney (Rebhan *et al.*, 1997). mEH is also present in brain tissue and may have an important role in the metabolism of drugs that penetrate the BBB, such as AEDs.

The first description of epoxide hydrolase structure was based on protein derived from *Agrobacterium radiobacter* AD1 (FIGURE 1.9; Nardini *et al.*, 1999). It consists of 2 domains: the first or the core domain shows a typical feature of the α/β hydrolase-fold topology and the second or the cap domain is primarily comprised of α-helices. Substrates enter the active site through a long tunnel that is filled with water molecules. The tunnel is located between the core domain and the cap domain. The back of the tunnel or the active site of epoxide hydrolase replenishes the water molecules after the metabolic reaction (Nardini *et al.*, 1999; de Vries & Janssen, 2003).

FIGURE 1.9. A schematic view of the secondary structure of the epoxide hydrolase protein. Epoxide hydrolase is built from α-helices, β-strands and coils, and has a narrow hydrophobic active site (black arrow). Source from Nardini *et al.* (1999).

1.7.4.2 Microsomal epoxide hydrolase activity

mEH catalyzes the trans-addition of water to a broad range of epoxide substrates such as xenobiotic alkene and arene oxides. mEH substrates are quite selective, with little or no metabolism by other epoxide hydrolases (Fretland & Omiecinski, 2000). The narrow hydrophobic tunnel of the active site limits the ability of mEH to catalyze the hydrolysis of large epoxide molecules (Fretland & Omiecinski, 2000). Typical mEH substrates include toxic and procarcinogenic compounds, as well as epoxide metabolites of AEDs. In an animal study, mEH activity was significantly increased by PB treatment (Slawson *et al.*, 1996). However, the level of mEH induction is moderate compared to CYP450s (Pirmohamed *et al.*, 1994; Hassett *et al.*, 1998). A study employing human lymphocytes also demonstrated that mEH activities varied by 3.3-fold and 4.7-fold in control subjects and patients receiving enzyme inducing drugs, respectively (Pirmohamed *et al.*, 1994).

Indirect pharmacokinetic evidence in human and animal studies suggests that hepatic mEH is also inducible by CBZ (Eichelbaum *et al.*, 1985; Regnaud *et al.*, 1988; Kudriakova *et al.*, 1992).

In a study employing CBZ-E as a substrate for mEH, it was demonstrated that the median log metabolic ratio of transdihydrodiol to epoxide was slightly greater in chronic PB and PHT treated subjects, compared to subjects not treated with inducing agents (Kroetz *et al.*, 1993). However, the distribution of the log metabolic ratios did not differ significantly from normality (Kroetz *et al.*, 1993). These findings indicate that the rate of CBZ-E metabolism is not significantly influenced by the induction of mEH. As such, although CBZ may induce mEH activity, it is unlikely to enhance the elimination of CBZ-E.

1.7.4.3 Genetics and common polymorphisms of EPHX1

A single functional gene, EPHX1, located at the q42.1 of chromosome 1, encodes the human mEH protein (FIGURE 1.10; Skoda *et al.*, 1988; Hassett *et al.*, 1994a). The EPHX1 gene contains nine exons, although the first exon is noncoding. Protein coding begins with the sixth nucleotide of the second exon (Hassett *et al.*, 1994a). As many as 33 polymorphisms in EPHX1 have been reported (Saito *et al.*, 2001). Two polymorphisms that have been studied most extensively are the substitution of c.337T>C on exon-3 and c.416A>G on exon-4, and particularly, in relation to the risk of developing cancer (Jourenkova-Mironova *et al.*, 2000; Zhou *et al.*, 2001; Lebailly *et al.*, 2002; Sonzogni *et al.*, 2002; Cajas-Salazar *et al.*, 2003), emphysema (Smith & Harrison, 1997; Budhi *et al.*, 2003) and liver disease (Sonzogni *et al.*, 2002). The frequency distribution reported for the 337C allele is much higher in Oriental populations (45-56%; Takeyabu *et al.*, 2000; Budhi *et al.*, 2003; Zhang *et al.*, 2003)

compared to African and Caucasian populations (21-41%; Jourenkova-Mironova *et al.*, 2000; London *et al.*, 2000; Wong *et al.*, 2000), while the 416G allele is observed at a frequency of around 15-24% in most populations (Jourenkova-Mironova *et al.*, 2000; Wong *et al.*, 2000; Lebailly *et al.*, 2002).

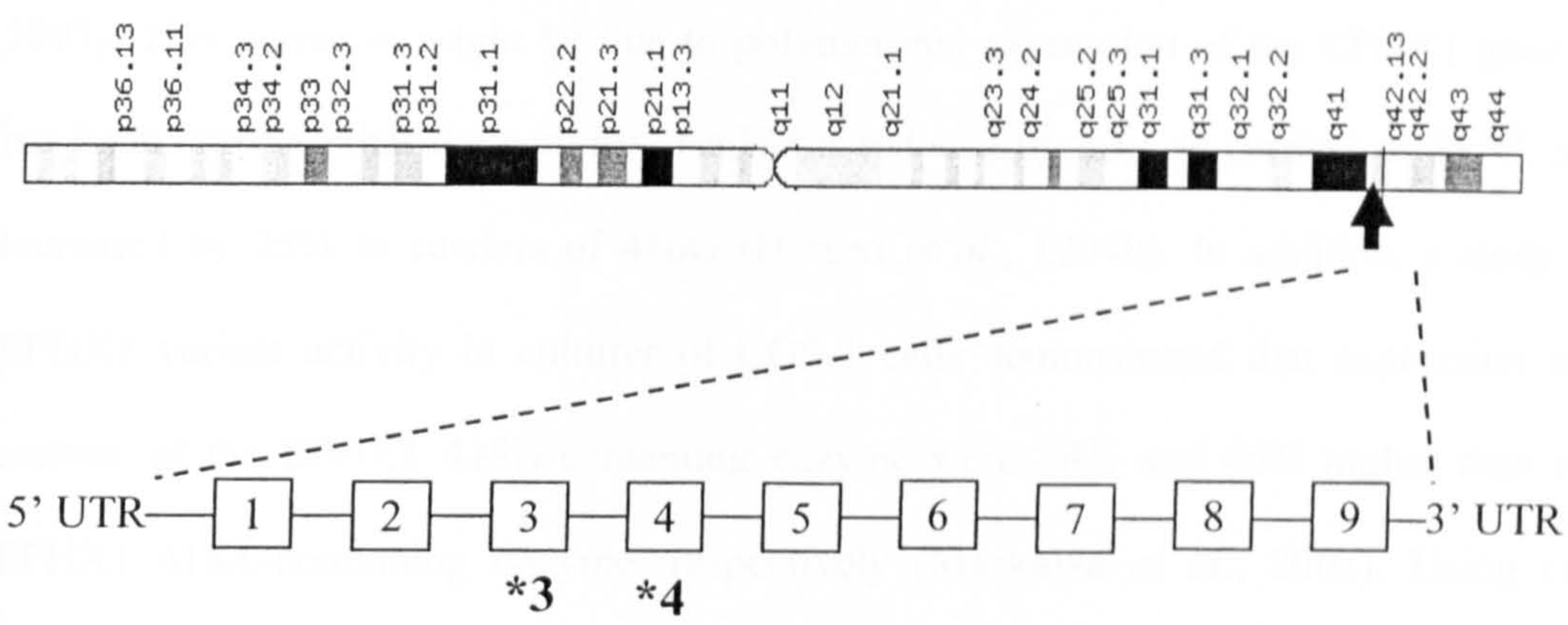

FIGURE 1.10. The location of the EPHX1 gene locus on Chromosome 1. Boxes 1 to 9 represent the exons. The *3 and *4 symbolize the c.337T>C and c.416A>G polymorphisms, respectively.

1.7.4.4 Phenotype profile of common EPHX1 variants

In vivo effects of EPHX1 variants have been demonstrated in some cases of cancer, and in lung and liver diseases, especially in the presence of xenobiotic or environmental factors (London *et al.*, 2000; Wong *et al.*, 2000; Ulrich *et al.*, 2001; Zhou *et al.*, 2001; Cajas-Salazar *et al.*, 2003; Lin *et al.*, 2006). The gene-disease association is believed to be related to the ability of EPHX1 variants to convert a less active compound to a more active compound in individuals with 416G (fast metaboliser) or a more active compound to a less active compound in individuals with 337C (slow metaboliser). It has

been proposed that polymorphic expression of EPHX1 may also contribute to the deficiency in mEH, which could be partly responsible for AED-induced idiosyncratic reactions such as those observed with CBZ-E (Pirmohamed *et al.*, 1992).

mEH-mediated hydrolysis of benzo[a]pyrene-4,5-epoxide varied 8.5- and 7-fold between liver samples at low and high substrate levels, respectively (Hassett *et al.*, 1997). This variation might be due to polymorphic expression of the EPHX1 gene. It has been reported that the activity of mEH is reduced by 50% in carriers of 337C, and increased by 25% in carriers of 416G (Hassett *et al.*, 1994b). In addition, a study of EPHX1 variant activity in cultures of COS-1 cells demonstrated that expression and activity of the EPHX1 416G-containing enzyme were 28% and 40% higher than the EPHX1 416A-containing enzyme, respectively (Maekawa *et al.*, 2003). Using cis-stilbene oxide and benzo[a]pyrene-4,5-oxide as substrates, the hydrolysis reaction rates of mEH were significantly lower for the 337C/416G allele than for other EPHX1 variants (Hosagrahara *et al.*, 2004). In many studies, diplotypes of 337T>C and 416A>G polymorphisms are commonly used to predict the levels of net mEH activity (Smith & Harrison, 1997; Sarmanova *et al.*, 2000; Takeyabu *et al.*, 2000; Zhou *et al.*, 2001; Sonzogni *et al.*, 2002; Lebailly *et al.*, 2002; Cajas-Salazar *et al.*, 2003). Using these diplotypes, the net mEH activity can be categorised as low, medium or high.

1.7.5 UDP-glucuronosyltransferases

Conjugation reactions may involve glucuronidation, sulphation, glutathionation, acetylation and methylation. The glucuronidation process is mediated by UGTs. To date, 15 functionally active human UGTs have been identified. UGTs catalyze the biotransformation of a vast array of structurally diverse endogenous compounds and xenobiotics. Substrates of UGT are conjugated with glucuronic acid from a sugar donor.

Glucuronidation can affect both parent compounds and metabolites. Other than a deactivation property, UGTs may also generate bioactive and even toxic compounds (Radominska-Pandya *et al.*, 2001).

1.7.5.1 UDP-glucuronosyltransferase 2B7: Functions and tissue distribution

UGT2B7 is an important isoform that participates in glucuronidation of physiologically important endogenous compounds and variety of clinically used drugs (Turgeon *et al.*, 2001; Court *et al.*, 2003; Staines *et al.*, 2004). Examples of UGT2B7 substrates include estrogens, catecholestrogens, bile acids, 3'-azido-3'-deoxythymidine, opioids, and CBZ. The UGT2B7 protein is highly expressed in liver and kidney; however, it is also detected in mammary gland, small intestine, lungs and brain tissue (King *et al.*, 1999; Turgeon *et al.*, 2001).

1.7.5.2 Genetics and common polymorphisms of UGT2B7

At present, the human UGT2B subfamily includes 7 genes; UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17 and UGT2B28 (Riedy *et al.*, 2000; Levesque *et al.*, 2001). Most of the UGT2B genes are believed to be located in one gene locus on Chromosome 4q13.2 (FIGURE 1.11; Riedy *et al.*, 2000). The UGT2B7 gene comprises 6 exons that encode 529 amino acids (Carrier *et al.*, 2000; Riedy *et al.*, 2000).

More than 80 SNPs have been reported for the UGT2B7 gene (National Center for Biotechnology Information, National Library of Medicine, Bethesda, USA; http://www.ncbi.nlm. nih.gov/). Of these SNPs, the UGT2B7 c.802C>T polymorphism has been reported to be present at a significant level in many populations. This polymorphism occurs in exon-2 of the UGT2B7 gene, and leads to replacement of histidine by tyrosine at codon 268. The presence of the UGT2B7 802T allele is higher in

Caucasian populations than other ethnic groups, ranging between 49% – 54% (Bhasker *et al.*, 2000; Lampe *et al.*, 2000). Although the UGT2B7 802C>T substitution causes an amino acid change, its functional significance in terms of endogenous substrate and xenobiotic metabolisms is unclear.

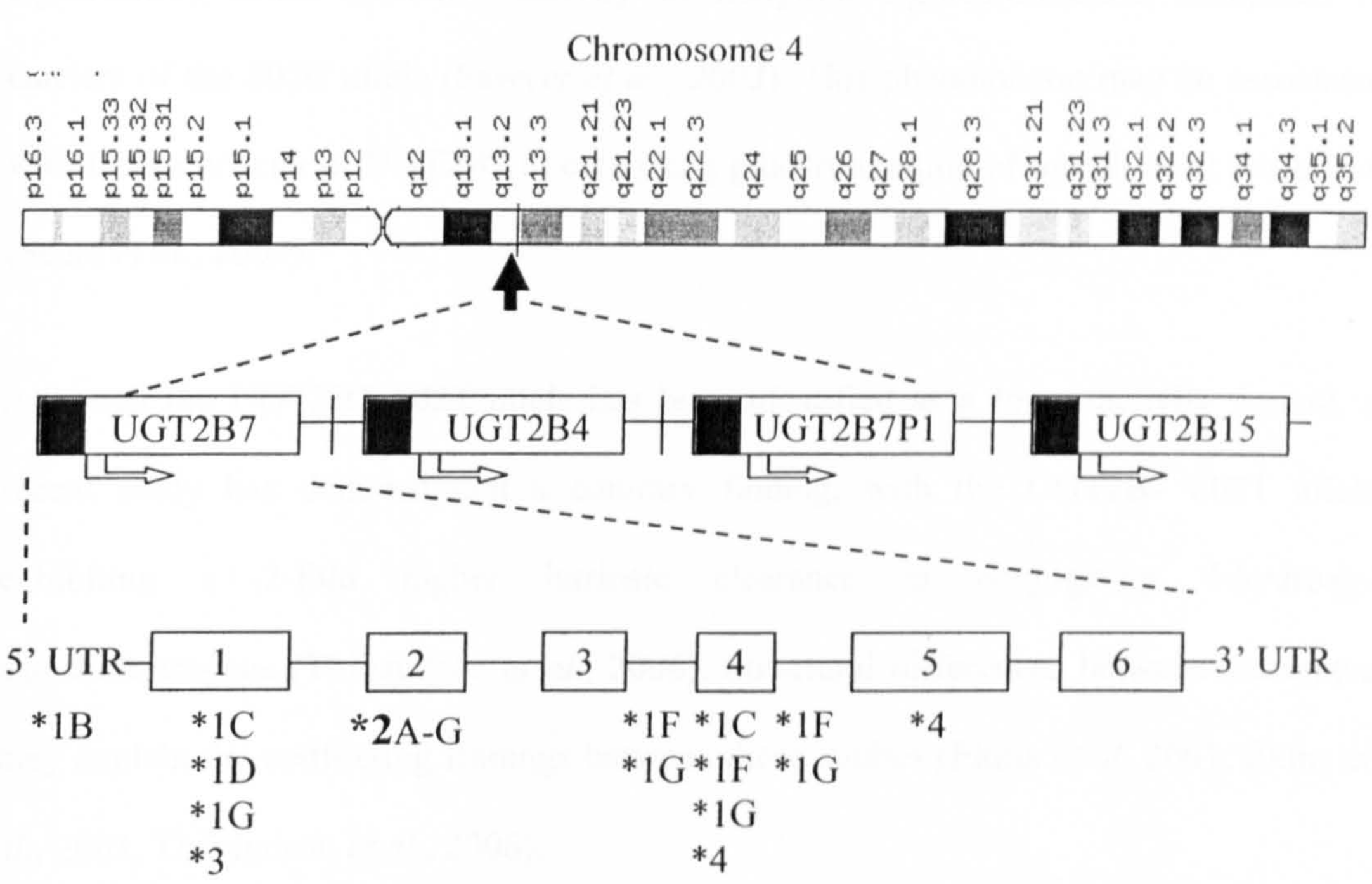

FIGURE 1.11. The location of the UGT2B gene locus on Chromosome 4 and distribution of polymorphisms in the UGT2B7 gene. Boxes 1 to 6 represent the exons. The *2 symbolizes the c.802C>T polymorphism.

1.7.5.3 Phenotype profile of common UGT2B7 variants

Several studies have been performed to determine the effects of the UGT2B7 802C>T polymorphism on xenobiotic metabolism, however, none of these studies directly examined AED metabolism. An *in vitro* investigation using two types of cell culture has concluded that the c.802C>T substitution may not be responsible for the variability in

plasma and urine concentrations of opioid compounds, menthol, oxazepam, propranolol or androgens (Coffman *et al.*, 1998). The UGT2B7 802T allele also appears to lack a functional effect on morphine 3-glucuronidation (Bhasker *et al.*, 2000; Holthe *et al.*, 2003). Nevertheless, the carriers of the 802T allele have been shown to possess a significantly lower UGT2B7 activity in morphine-6-glucuronidation compared to carriers of the 802C allele (Sawyer *et al.*, 2003). This phenomenon may be associated with the specificity of UGT2B7 in catalysing glucuronidation of morphine at position-6 (Soars *et al.*, 2004).

Although the UGT2B7 802T allele has been identified as a lower activity variant, a recent study has demonstrated a contrary finding, with the UGT2B7 802T allele exhibiting a 2-fold higher intrinsic clearance in conjugating 4-hydroxy-catecholestrogens (Thibaudeau *et al.*, 2006). Structural differences between substrates may explain the conflicting findings between these studies (Ekins *et al.* 2001; Ekins *et al.*, 2003; Thibaudeau *et al.*, 2006).

1.7.6 Drug Transporter Proteins

DTPs can be important determinants of drug absorption, distribution and excretion. The functional significance of DTPs is dependent upon their expression, and this can be modulated by genes and many substances (Ishikawa *et al.*, 2004). DTPs are known to be involved in drug-drug interactions, for example between CBZ and talinolol (Lown *et al.*, 1997; Kim, 2002a; Giessmann *et al.*, 2004).

DTPs have also been associated with non-responsiveness to AEDs, as they significantly influence intestinal drug absorption and BBB drug permeability (Huai-Yun *et al.*, 1998, Potschka *et al.*, 2001; Potschka *et al.*, 2002). Some hepatic DTPs such as P-gp may

affect the influx of AEDs into hepatocytes, and the efflux of AED metabolites into the biliary system, which subsequently influences the rate and extent of AED metabolism (FIGURE 1.12). The expression of intestinal P-gp can also be induced by drugs such as CBZ (Giessmann *et al.*, 2004). This phenomenon may further reduce drug absorption and enhance clearance.

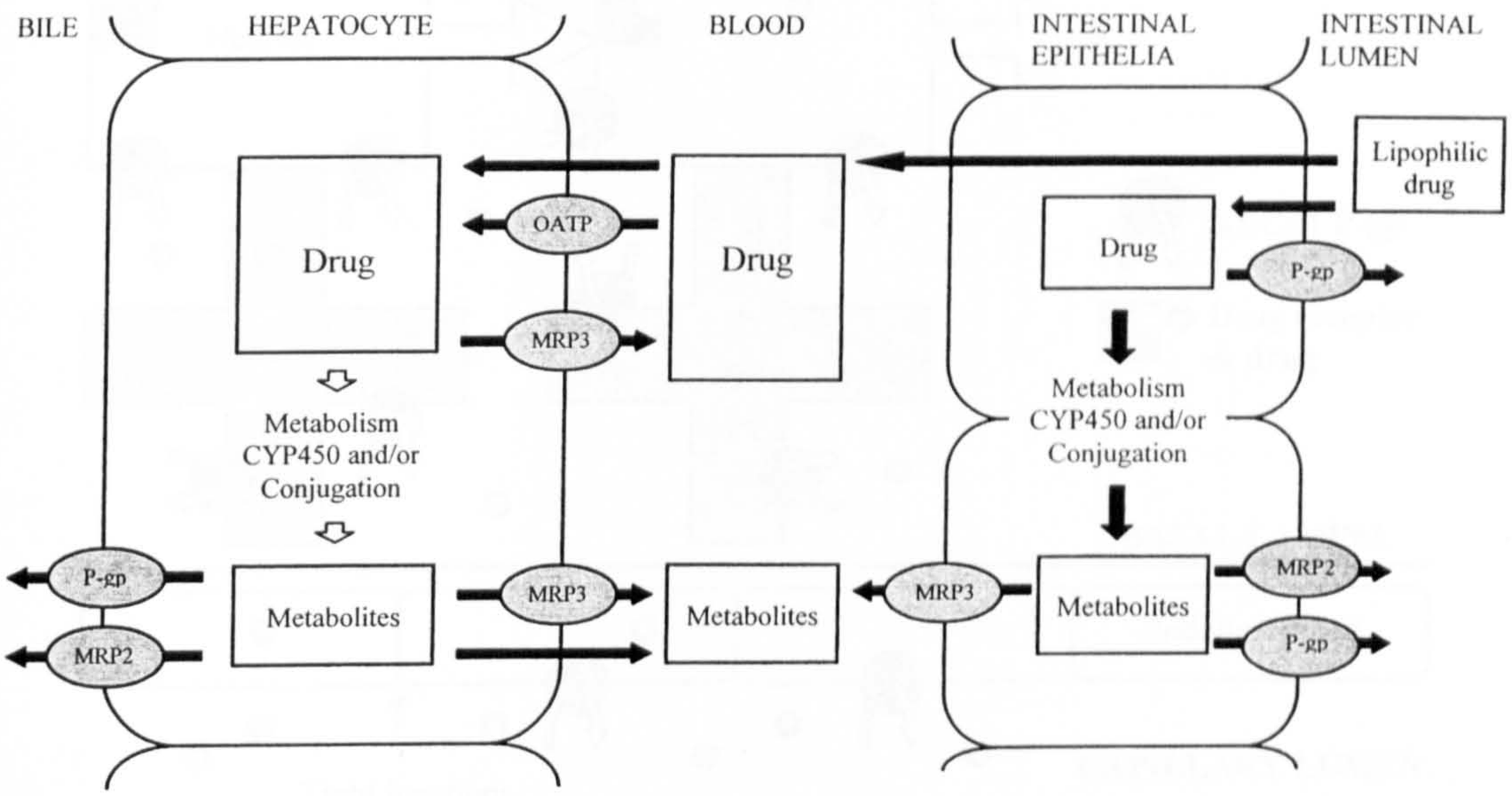

FIGURE 1.12. A postulated model representing drug and metabolite movement in the intestinal epithelium and hepatocyte. OATP = Organic Anion Transporter Protein, P-gp = P-glycoprotein, MRP = Multidrug Resistance Associated Protein. Adapted from Kim (2002b).

Drug distribution into brain tissues is determined by a host of factors including barrier systems such as the BBB, physicochemical properties of the drugs themselves, cerebral blood flow, drug metabolism, and pathological conditions (de Lange & Danhof, 2002). Expression of P-gp at the luminal membrane of cerebrovascular endothelial cells and within the brain parenchyma has been suggested to restrict drug accumulation at

neuronal target sites (FIGURE 1.13; Tsuji & Tamai, 1997; Marchi *et al.*, 2004). P-gp has also been reported to reduce the penetration of several AEDs into the brain (Potschka *et al.*, 2002). These findings support the functional relevance of P-gp in influencing the distribution of AEDs into brain tissue.

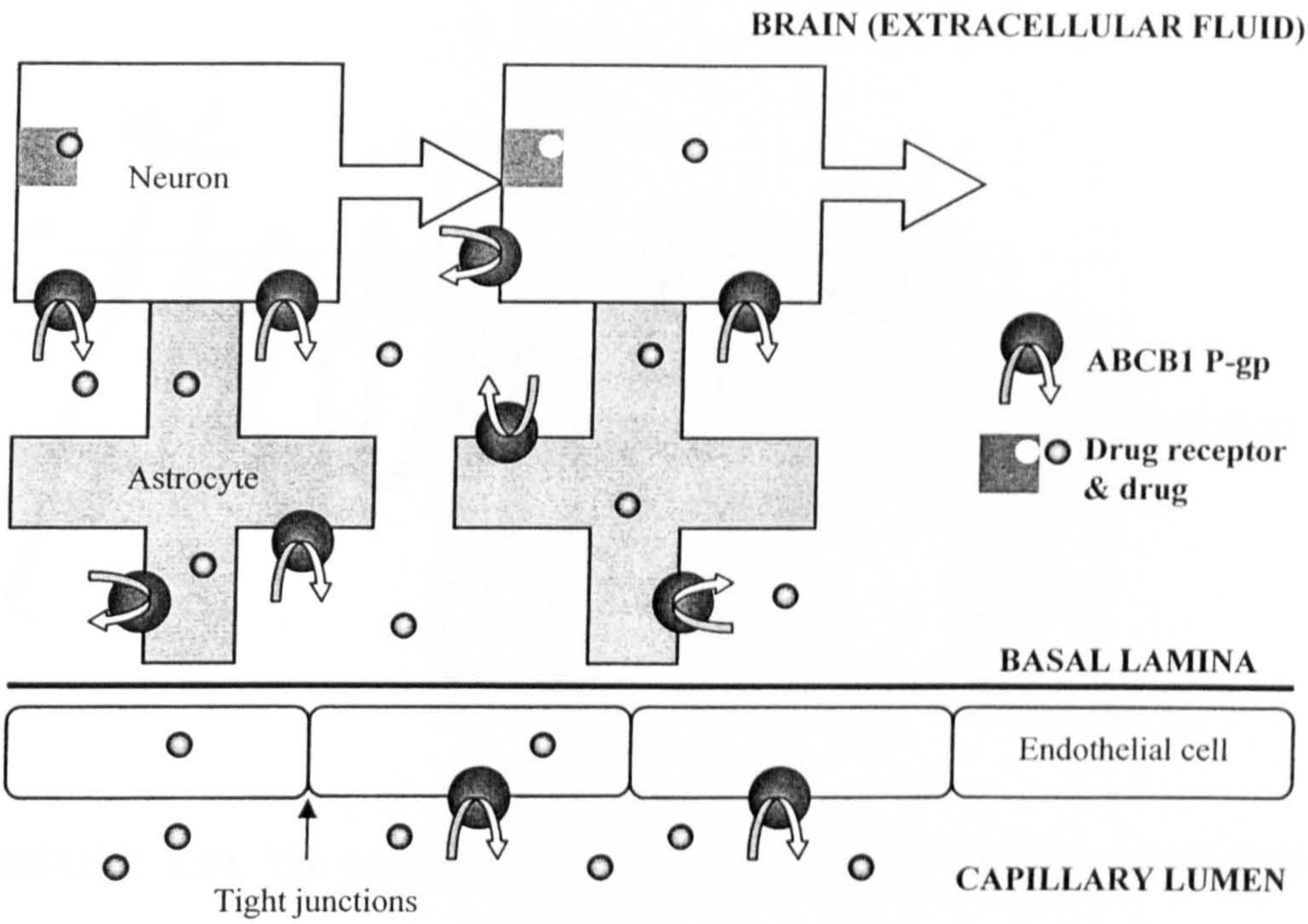

FIGURE 1.13. A postulated model representing the location of ABCB1 P-glycoprotein in the blood-brain barrier and brain parenchyma. Adapted from Marchi *et al.*, 2004).

1.7.6.1 P-glycoprotein

In the 1970s, a surface glycoprotein was first identified on Chinese hamster ovary cells with altered permeability to colchicine (Juliano & Ling, 1976). The ~170 kD glycoprotein was then designated as P-gp. The N-terminus of the P-gp molecule

contains 6 transmembrane domains, followed by a large cytoplasmic domain with an ATP binding site, and then a second section with 6 transmembrane domains and a further ATP binding site which shows over 65% amino acid homology with the first half of the polypeptide (FIGURE 1.14). P-gp has been studied extensively with regard to its role in chemotherapy disposition and resistance (Chan *et al.*, 2004).

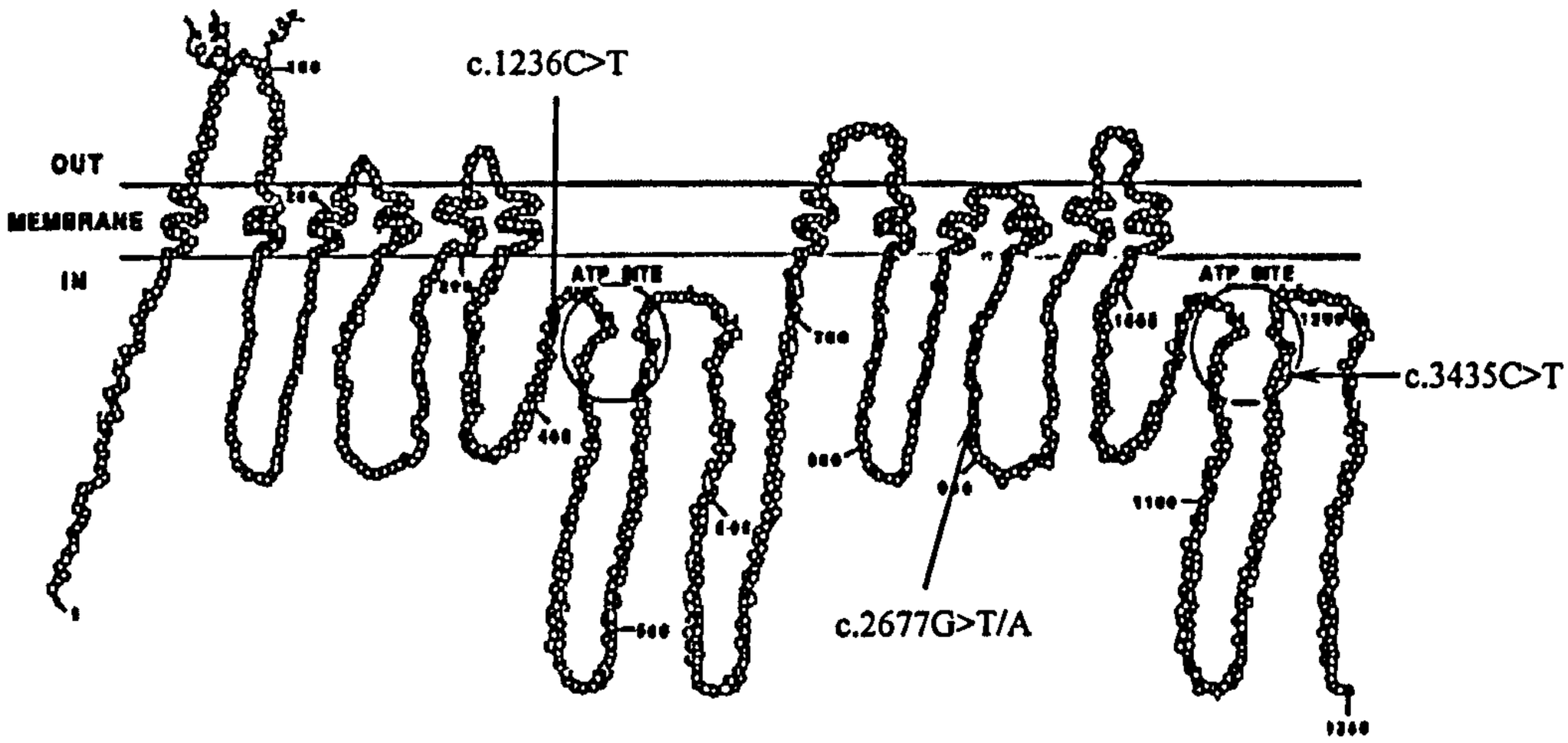

FIGURE 1.14. The putative 2-D structure of ABCB1 P-glycoprotein with the sites of common polymorphisms. Adapted from Pastan *et al.* (1991).

1.7.6.2 Functions and tissue distribution of P-gp

P-gp is an ATP-dependent transmembrane efflux pump with a wide range of amphipathic hydrophobic substrates (Kim, 2002a). P-gp transports substances with diverse chemical structures, such as anticancer agents, cardiac drugs, HIV protease inhibitors, immunosuppressants, β-adrenoceptor antagonists, AEDs and also drug metabolites (Potschka *et al.*, 2002; Schwab *et al.*, 2003). Usually, CYP3A generated metabolites undergo subsequent sulfation and glucuronidation. In humans, P-gp is commonly co-localised and co-regulated with CYP3A in many tissues. Thus, P-gp

substrates were originally speculated to include most drug sulfates and glucuronides (Zhu, 1999). P-gp may serve as a functional barrier against drug entry. P-gp knock-out mice demonstrate significant elevation in the disposition of P-gp substrates (Schinkel *et al.*, 1996). P-gp can be detected in many tissues such as the intestine, liver, kidney, adrenal gland and brain (Kim, 2002a; Marchi *et al.*, 2004). It can also be found in the apical membrane of many barrier tissues such as the BBB and blood-placenta barrier (Cascorbi, 2006).

1.7.6.3 Genetics and common polymorphisms of ABCB1

P-gp belongs to the transporter superfamily of the ATP-binding cassette (ABC). In humans, the ABCB1 gene encodes P-gp (Borst, 1997; Kusuhara & Sugiyama, 2001). The total length of the ABCB1 gene is approximately 209 kb, which consists of a core promoter region and 29 exons (FIGURE 1.15; Bodor *et al.*, 2005). The gene encodes a 1280-amino acid transporter (Kim, 2002a; Schwab *et al.*, 2003).

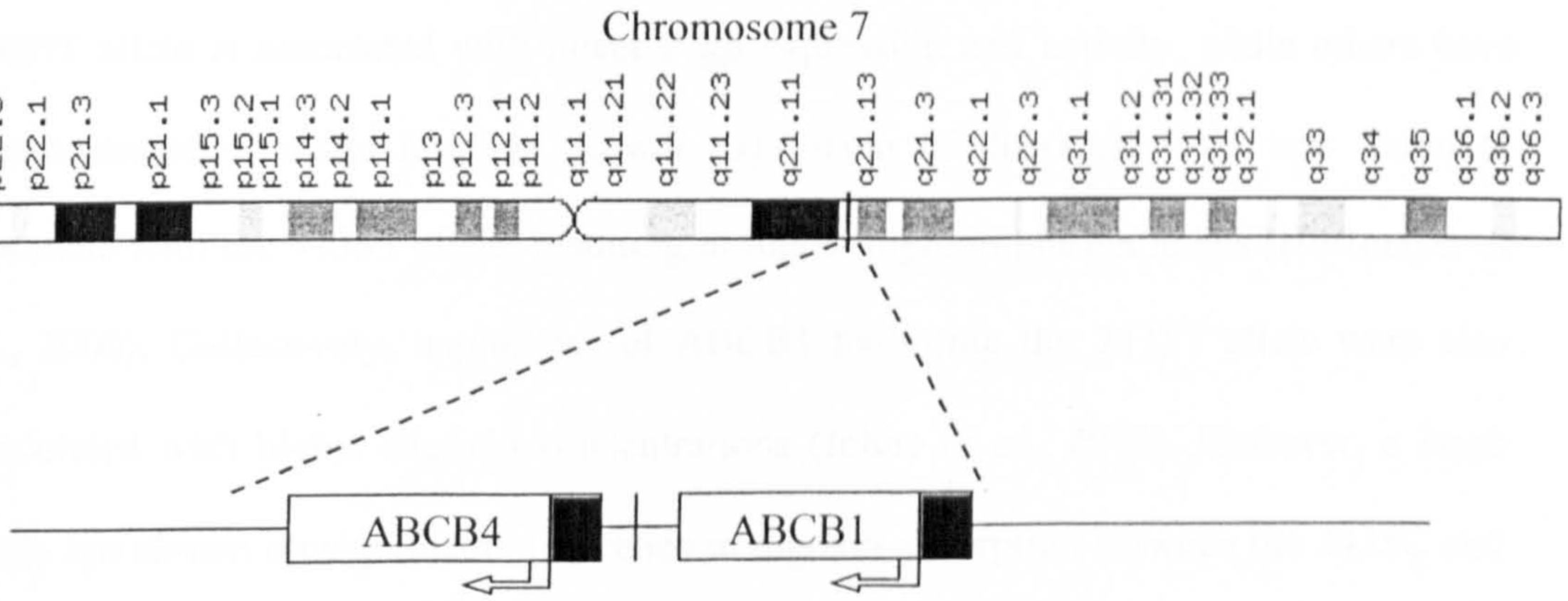

FIGURE 1.15. The location of ABCB genes on Chromosome 4.

More than 35 SNPs have been identified in the ABCB1 gene, of which eight SNPs cause non-synonymous amino acid changes (Cascorbi, 2006). In many populations, the highest allele frequencies have been documented for the c.1236C>T (21 – 41%), c.2677G>T/A (10 – 44%) and c.3435C>T (10 – 54%) transitions (Pauli-Magnus & Kroetz, 2004; Cascorbi, 2006). Significant ethnic differences exist in the frequency of allele and genotype distributions of the 3435C>T polymorphism of ABCB1 (Schwab *et al.*, 2003). In a Japanese study, strong association was demonstrated between c.2677G>T/A and c.3435C>T alleles (Tanabe *et al.*, 2001). Carriers of the ABCB1*2 variant, which signified the haplotype of 1236T-2677T-3435T were more commonly found among European Caucasians (62%) and South East Asians (40%) than African-Americans (13%; Kim *et al.*, 2001; Tang *et al.*, 2002; Tang *et al.*, 2004).

1.7.6.4 Phenotype profile of common ABCB1 variants

Among the SNPs that have been documented for the ABCB1 gene, polymorphisms of c.1236C>T, c.2677G>T/A and c.3435C>T have been studied extensively (Kim, 2002a). However, study findings are inconsistent. Some investigations have shown that the 3435T allele is associated with lower P-gp expression and activity, while others have demonstrated opposite findings. Lower expression of duodenal P-gp was found to correlate with the 3435T allele, resulting in higher digoxin plasma levels (Hoffmeyer *et al.*, 2000). Collectively, haplotypes of ABCB1 harboring the 3435T allele were also associated with higher digoxin concentrations (Johne *et al.*, 2002). However, a large study has shown no significant difference in digoxin absorption between the 3435C and 3435T alleles and other variants (Gerloff *et al.*, 2002). A similar conflict was also observed with studies related to epilepsy. A higher prevalence of the ABCB1 3435C/C genotype was found among poor-responders to AED treatment (Siddiqui *et al.*, 2003), suggesting that the 3435C/C genotype was associated with drug resistant epilepsy.

However, three subsequent studies examining this polymorphism failed to confirm the original finding (Tan *et al.*, 2004a; Sills *et al.*, 2005; Kim *et al.*, 2006). Some further investigations have suggested that haplotypes of these three loci might be more useful in predicting drug resistance in epilepsy (Hung *et al.*, 2005) In this regard, a study focusing on CBZ has shown that the T-T-T haplotype at the 1236, 2677 and 3435 positions of the ABCB1 gene was associated with a better response to CBZ therapy (Seo *et al.*, 2006a).

1.7.7 Voltage-gated Sodium Channels

Voltage-gated sodium channels play a crucial role in the initiation and propagation of action potentials in neurones and other electically excitable cells (Goldin, 2003). The channel protein consists of a complex of a 260 kDa α-subunit in association with one or more auxiliary β-subunits (Goldin, 2003; Yu & Catterall, 2003). The sodium channel α-subunit folds into 4 domains (I-IV), which are similar to one another and each domain contains 6 α-helical transmembrane segments (S1-S6; FIGURE 1.16). The S4 region acts as the channel's voltage sensor. When stimulated by a change in transmembrane voltage, this region moves towards the extracellular side of the cell membrane allowing the channel to become permeable to sodium ions. A re-entrant loop between S5 and S6 is embedded into the transmembrane region of the channel to form a narrow, ion-selective filter at the extracellular end of the channel. The cytoplasmic loop linking domains III and IV is important for channel function. This loop plugs the channel after prolonged activation and contributes to its inactivation. A total of nine α-subunits ($Na_v1.1$-$Na_v1.9$) have been functionally characterized (Yu & Catterall, 2003).

The essential properties that enable voltage-gated sodium channels to carry out their physiological roles include rapid voltage-dependent activation, which opens the

channel, and inactivation, which closes the channel until recovery (Goldin, 2003). The inactivation process determines the frequency of neuronal action potential firing. In general, the inactivation process can be divided to two phases, fast and slow. Fast inactivation occurs when the cytoplasmic loop occludes the channel. On the other hand, slow inactivation does not involve the cytoplasmic loop. The slow inactivation process is believed to involve a conformational change of the channel. Many drugs including AEDs affect sodium channel inactivation.

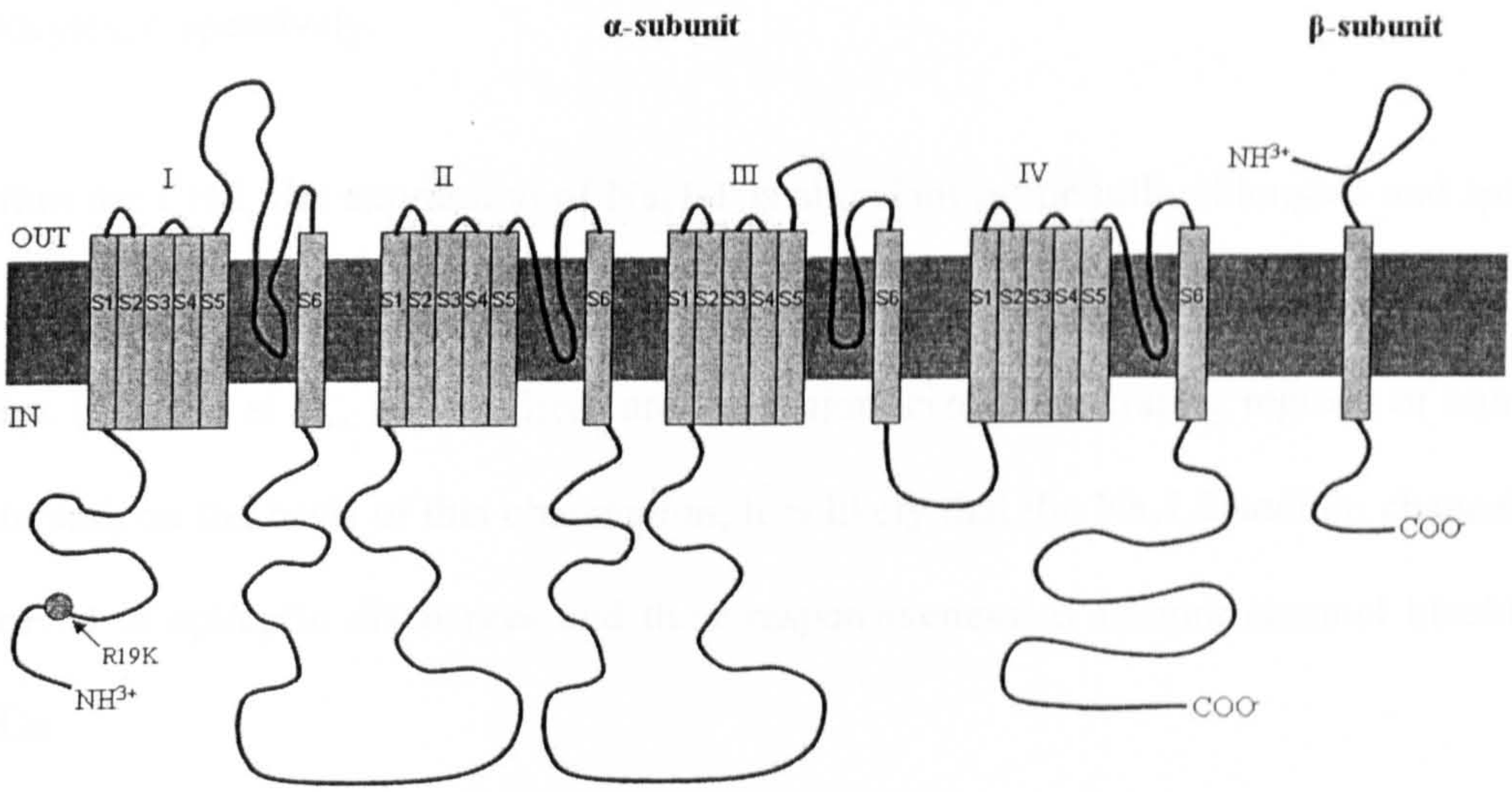

FIGURE 1.16. Schematic diagram of α- and β-subunits of sodium channel $Na_v1.2$. The α-subunit gene has four repeats (domains I - IV). Each of the repeats has six membrane regions (S1-S6). The S4 region is the voltage sensor. The grey circle represents the position of the amino acid substitution (R19K) which results from the c.56G>A polymorphism. Adapted from Goldin (2003).

1.7.7.1 Tissue distribution of voltage-gated sodium channels

Consistent with a distinct role for each channel in human physiology, sodium channels have differential expression profiles during development and different sub-cellular localization in adulthood (Mandel, 1992). $Na_v1.1$ and $Na_v1.3$ are localised to the soma of the neurone, where they control neuronal excitability. $Na_v1.2$ is expressed in unmyelinated axons where action potential conduction takes place. $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ are the most abundantly expressed in the peripheral nervous system (PNS). $Na_v1.1$ and $Na_v1.6$ are also significantly expressed in the PNS. Finally, $Na_v1.4$ and $Na_v1.5$ are muscle sodium channels that control the excitability of skeletal and cardiac myocytes, respectively.

Within the CNS, the expression of $Na_v1.1$ is abundant in medulla oblongata and spinal cord. In contrast, the expression of $Na_v1.2$ is highest in the hippocampus and cerebral cortex (Gordon *et al.*, 1987). These are the major seizure generating regions of human brain and, on the basis of this observation, it is likely that the $Na_v1.2$ sodium channel is involved in epileptic discharges and their responsiveness to sodium channel blocking AEDs

1.7.7.2 Genetics and common polymorphisms of SCN2A

The α-subunit is the principal subunit of neuronal sodium channels and is expressed by at least 5 genes in human brain, namely SCN1A, SCN2A, SCN3A, SCN8A and SCN9A (Kohling, 2002). In general, the clusters of genes encoding sodium channels are located on chromosomes 2 and 3. The SCN2A gene that encodes the $Na_v1.2$ protein is located on Chromosome 2q24.3 (FIGURE 1.17). This gene covers approximately 120 kb of g.DNA, contains 29 exons, and encodes a 2005 amino acid protein (Kasai *et al.*, 2001).

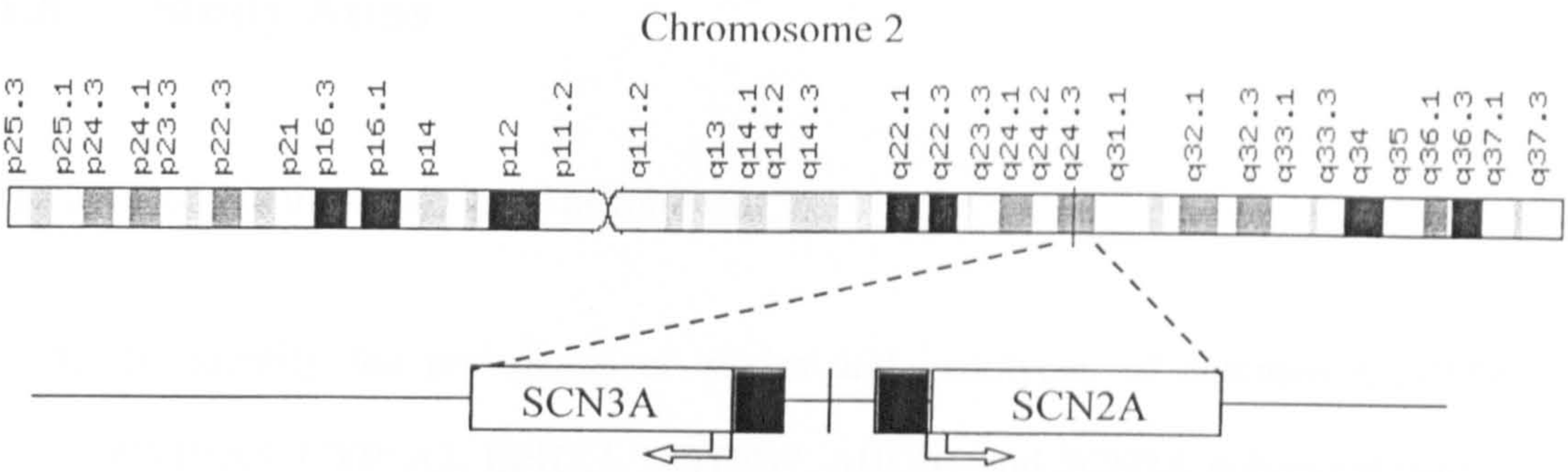

FIGURE 1.17. The location of the SCN2A gene on Chromosome 2.

Several SCN2A SNPs have been identified (Kaplan & Lacey, 1983; Kasai *et al.*, 2001; Heron *et al.*, 2002; Berkovic *et al.*, 2004). However, data regarding the prevalence of these polymorphisms is limited. The c.56G>A substitution in SCN2A is a well known non-synonymous polymorphism (Ito *et al.*, 2004). The c.56G>A polymorphism which results in an amino acid substitution at codon-19 of the $Na_v1.2$ protein has been reported to have a minor allele (56A) frequency at 6% in a Japanese population (Nakayama *et al.*, 2002).

1.7.7.3 Phenotype profile of common SCN2A variants

Several non-synonymous SNPs in the SCN2A gene, including the c.56G>A substitution, have been associated with paediatric seizure disorders such as febrile seizure and benign familial neonatal infantile seizures (Sugawara *et al.*, 2001; Heron *et al.*, 2002; Berkovic *et al.*, 2004). The 56A allele was found to be more frequent in children with febrile seizure than healthy controls (Sugawara *et al.*, 2001). The mechanism by which this polymorphism alters the predispotion of seizures is unknown. However, any significant modification of sodium channel structure might reasonably explain seizure generation and could, in theory, influence the response to AED treatment (Ramachandran & Shorvon, 2003).

1.8 Study Aims

The aims of this thesis are listed as follows:

1. To identify the prevalence of alleles and genotypes of common CYP3A4, CYP3A5, CYP1A2, EPHX1, UGT2B7, ABCB1 and SCN2A polymorphisms in a West of Scotland epilepsy population.

2. To establish an association between common polymorphisms in SCN2A, CYP3A4, CYP3A5, CYP1A2, EPHX1, UGT2B7 genes and the optimal dose of CBZ.

3. To establish an association between common polymorphisms in CYP3A4, CYP3A5, CYP1A2, EPHX1 and UGT2B7 genes and CBZ adverse effects.

4. To establish an association between common polymorphisms in ABCB1 and SCN2A genes and the response to LTG monotherapy.

5. To establish an association between common polymorphisms in ABCB1 and SCN2A genes and the optimal dose of LTG.

6. To establish the influence of common ABCB1 gene polymorphisms on the pharmacokinetics of LTG in newly diagnosed epilepsy patients.

Studies exploring aim (1) are presented in Chapter 3, studies exploring aims (2) and (3) are described in Chapter 4, and studies exploring aims (4), (5) and (6) are presented in Chapter 5.

CHAPTER 2

Materials, Experimental Principles and Recurrent Methods

2.1 Materials

2.1.1 Chemicals and reagents

Promega® wizard DNA purification kit was purchased from Promega (Southampton, UK). Bromophenol blue, ethylene diamine tetraacetic acid (EDTA), ethanol, ethidium bromide, glacial acetic acid, isopropanol, tris base (Trizma®), and xylene cyanole FF were obtained from Sigma-Aldrich Co. Ltd. (Poole, UK). Chlorine tablets were purchased from Prime Source (Birmingham, UK). Deoxyribonucleotide triphosphates (dNTP mix), magnesium chloride ($MgCl_2$), 10x PCR buffer (200 mM Tris HCl & 500 mM KCl), and Taq polymerase were purchased from Invitrogen Ltd. (Paisley, UK). Molecular biology grade water was obtained from Eppendorf AG (Hamburg, Germany). PCR primers were purchased from MWG-Biotech (Ebersberg, Germany).

Agarose powder was obtained from BioGene Ltd. (Kimbolton, UK). Bovine serum albumin (100x), 10x NE buffer, restriction enzymes (*Dde*I, *Dpn*II, *EcoO*1091, *EcoR*V, *Fok*I, *PspOM*I, *Pst*I and *Rsa*I), and DNA ladders (25 and 50 bp) were bought from New England Biolabs (Hitchin, UK). AMPure® system, DYEnamic ET Terminator Cycle Sequencing Kit and DYEnamic® ET Terminator dilution buffer were purchased from Beckman Coulter Ltd. (High Wycombe, UK). Ethyl acetate and methanol were bought from Rathburn Chemicals Ltd. (Walkerburn, UK). Sodium hydroxide was purchased from Fisher Scientific UK Ltd. (Loughborough, UK).

2.1.2 Pharmaceuticals

LTG and the internal standard (BWA725C) for high performance liquid chromatography (HPLC) were obtained from Glaxo Smith Kline (Stevenage, UK).

2.1.3 Materials for gel electrophoresis

2.1.3.1 Electrophoresis buffer

A total of 3.7224 g of EDTA was dissolved in 1 litre of distilled water. The pH of the EDTA solution was adjusted to 7.0 by slow addition of 5N sodium hydroxide. In a separate container, 48.4 g of tris base (Trizma®) was dissolved in 5 litres of distilled water. The Tris Base solution was then added to the EDTA solution. The pH of the final solution was slowly adjusted to 8.0 with glacial acetic acid. Finally, distilled water was added to a total volume of 10 litres.

2.1.3.2 Agarose gel

Appropriate quantities of agarose powder (2.0, 2.5 and 3.0 g) were dissolved in 100 ml of electrophoresis buffer to make 2.0, 2.5 and 3.0% gels, respectively. The mixture was boiled in a microwave oven. After the agarose powder was completely dissolved, 0.5 µl of ethidium bromide (10 mg/ml) was added and mixed thoroughly. The mixture was poured into a gel tray and allowed to set for approximately 1 hour.

2.1.3.3 Loading dye

Small pinches (approximately 1 mg) of both bromophenol blue and xylene cyanole FF were mixed with 20 ml of 50% glycerol/water solution.

2.1.4 Equipment

Centrifugation: Blood samples were centrifuged in a ALC® PK130 centrifuge (DJB Labcare Ltd., Newport Pagnell, UK).

DNA quantification: genomic DNA samples were quantified using a WPA Biotech photometer model UV1101 (Biochrom Ltd., Cambridge, UK)

DNA storage: The genomic DNA samples were stored in a NapCOIL UF400 –80°C freezer (Jencons-PLS, Forrest Row, UK).

Direct sequencing assay: The sequencing equipment consisted of MegaBACE 1000™ DNA Analysis System and SPRIPlate 96R® plate (Beckman Coulter Ltd., High Wycombe, UK)

LTG assay: The experimental equipment consisted of Shimadzu SIL-9A autosampler, Shimadzu SPDF-6A UV detector, Shimadzu LC-10AT pump (Shimadzu UK Ltd., Milton Keynes, UK) and Heto Maxi Dry Plus vacuum centrifuge (Heto-Holten A/S, Allerød, Denmark). The HPLC column was a Zorbax sil column (250 x 4.6 mm; DuPont UK Ltd., Stevenage, UK)

Polymerase chain reaction: PCR was performed in a PxE 2.0 Thermal Cycler® (Thermo Electron Co., Basingstoke, UK)

Restriction fragment length polymorphism assay: Separation of DNA fragments was performed in a Sub-cell® GT tank. A PowerPac Basic was used as a power system for the electrophoresis process. Separation of DNA fragments was visualised under ultraviolet light using Gel Doc 1000 system (all Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

2.2 Experimental Principles

2.2.1 Polymerase chain reaction

PCR provides a sensitive means of amplifying small quantities of DNA. PCR was invented by Kary B. Mullis in 1983 (Rabinow, 1996). The discovery of polymerase derived from the bacterium *Thermus aquaticus* (Taq) which is found in the region of hot springs makes this method feasible. Taq polymerase is stable at the high temperatures required to perform the amplification, whereas other polymerases would denature. The concept of PCR is based on the fundamentals of natural DNA polymerization reaction.

2.2.1.1 Polymerase chain reaction components

PCR requires several basic components which include a DNA template that contains the region of the DNA to be amplified, two primers which are complementary to the DNA regions at the 5' and 3' ends of the DNA sequence to be amplified, a DNA polymerase (Taq polymerase) used to synthesize a DNA copy, dNTPs from which the DNA polymerase builds new DNA, buffer solution which provides a suitable chemical environment for optimum activity and stability of the DNA polymerase, and the divalent cation magnesium which is a necessary cofactor for Taq polymerase activity. Magnesium concentration is a crucial factor that can affect the success of the amplification. Template DNA concentration, dNTP concentration and the presence of proteins can affect the amount of free magnesium in the reaction. In the absence of adequate free magnesium, Taq polymerase is inactive. In contrast, excess free magnesium reduces enzyme fidelity and may increase the level of nonspecific amplification

2.2.1.2 PCR primer design

Good primer design is essential for successful reactions. A number of criteria have been proposed for optimal primer design. A primer length of 18-24 bases is appropriate for most PCR applications. Shorter primers lead to amplification of nonspecific PCR products. The specificity of PCR depends strongly on the primer melting temperature (Tm), a temperature at which half of the primer has annealed to the DNA template. Usually, optimal amplification is achieved when the Tm for both primers is between 52-58°C. The G and C nucleotide content of a primer should be between 40 and 60%. To assist in fulfilling these criteria, several online resources and software programmes have been created, such as Primer3 (Rozen & Skaletsky, 2000) and GeneFisher (Giegerich *et al.*, 1996). The template DNA sequence that is required by these programmes can be obtained from established DNA databases, such as GenBank (National Center for Biotechnology Information, National Library of Medicine, Bethesda, USA; http://www.ncbi.nlm.nih.gov/Genbank/). The specificity of a primer produced by these programmes can be examined using Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, National Library of Medicine, Bethesda, USA; http://www.ncbi.nlm.nih.gov/BLAST/). This evaluation program compares the primer sequence to DNA sequences available in a comprehensive genomic database and calculates the statistical significance of matches between primer and targeted DNA sequence.

2.2.1.3 Polymerase chain reaction assay

There are three major steps in a PCR, which are repeated for 30 to 40 cycles. This is done on an automated thermocycler, which can heat and cool PCR tubes within a very short period of time. It is common that additional denaturation and extension steps are

included before and after the PCR cycles, respectively. In each cycle, the DNA template is denatured at 94°C, the primers are annealed to the target sequence at 50-60 °C and the two new strands are extended by Taq polymerase at 72 °C, doubling the amount of DNA present in a single cycle. The PCR is usually terminated with soaking at 4 °C to prevent any further reaction before the PCR product undergoes further sequence analyses.

During the denaturation, the double strand melts open to single stranded DNA, and all enzymatic reactions stop. During the annealing stage, ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little longer (especially where primers that fit exactly) and on that piece of double stranded DNA (template and primer), the polymerase can attach and start to copy the template. Once 3 to 4 dNTPs are incorporated, the ionic bond between template and primer is sufficiently strong to prevent further breakage. During the extension phase, primers have a stronger ionic attraction to the template than the any forces attempting to break these attractions. Primers in positions with no exact match are loosely bound and do not support extension. The dNTPs are successively coupled to the primer in the 5' to 3' direction and in direct complementary to the DNA template. Approximately 1000 dNTPs can be linked to form a complementary DNA sequence in 1 minute at 72°C.

2.2.1.4 Examining the PCR product

The PCR product has to be examined before it is used in further applications. Not every PCR is successful. There is a possibility that the quality of amplified DNA is poor because one of the primers was poorly designed, there were too many annealing site for the primers or that the annealing temperature or magnesium concentration was not

optimised. It is also possible that there is a PCR product but of incorrect size. In this case, one of the primers likely anneals to a part of the gene closer (or further) than expected. It is also possible that both primers might anneal to a totally different gene. In some cases, it is possible that the primers anneal firmly to the desired locations, but also to other locations in the genome. In this case, multiple DNA fragments will be formed. Multiple DNA fragments can also produced if a lower than optimal annealing temperature is employed. In such cases, the primers anneal in a non-specific manner to the DNA template. Examination of PCR product is carried out by gel electrophoresis. The size of the PCR product should be known in advance and can be confirmed by a DNA ladder which is included in the electrophoresis process. For successful DNA sequence analysis, a single PCR product of uniform size should be produced.

2.2.2 Identification of single nucleotide polymorphisms

SNPs can be detected using many methods, including hybridization, primer extension, oligonucleotide ligation, allele-specific PCR and restriction enzyme cleavage. However, each of these methods has its advantages and disadvantages (Syvanene, 2001; Zhang *et al.*, 2005).

2.2.2.1 Restriction fragment length polymorphism assay

RFLP assays are based on the principle of restriction enzyme cleavage to identify the individual alleles of a genetic polymorphism. This method is classic, relatively inexpensive and reliable (Zhang *et al.*, 2005). However, it is not recognised as a high-throughput technique.

RFLP is a technique in which genotypes are differentiated by analysis of fragments derived from cleavage of a larger DNA fragment by restriction enzyme. The restriction

enzyme makes two incisions, one through each of the sugar-phosphate backbones of the double helix without damaging the nitrogenous bases. Any SNP that alters the recognition sequence of a restriction enzyme can be genotyped by RFLP.

When the SNP does not alter the enzyme recognition sequence, mismatched PCR-RFLP can be employed to introduce an artificial restriction enzyme site (Haliassos *et al.*, 1989). This method requires the careful selection of restriction enzymes and the design of appropriate mismatched PCR primers. Mismatched PCR–RFLP uses a primer containing an additional mismatched nucleotide or nucleotides adjacent to the SNP site. This allows restriction recognition sequences to cover the mismatch base and SNP site. The artificial restriction enzyme recognition site is introduced in just one of the two allelic sequences.

The presence of genetic polymorphisms is responsible for the variation in the length of DNA fragments created by the restriction enzyme digest of PCR products. The restriction enzyme is selected such that its recognition sequence contains the nucleotide of interest and that the enzyme cuts the target DNA at only one of the two possible alleles. Suitable restriction enzymes for a SNP site can be identified using online resources, such as RestrictionMapper (http://www.restrictionmapper.org) and NEB cutter (http://tools.neb.com/NEBcutter2/index.php; Vincze *et al.*, 2003). The restriction enzyme recognises specific sequences and cuts the amplified DNA strand into series of fragments of different lengths. The length of the fragments can be analysed by gel electrophoresis. The pattern of DNA fragments is used to differentiate one allele from another. This is a key tool in DNA fingerprinting, reflecting the existence of different alleles in the individual.

2.2.2.2 Gel electrophoresis

Gel electrophoresis is a method that separates macromolecules, either nucleic acids or proteins, on the basis of size and electric charge. The term electrophoresis describes the migration of charged particles under the influence of an electric field. DNA has a negative charge in solution, so it will migrate to the anode in an electric field. Agarose and polyacrylamide are the most common separation media used in gel electrophoresis. In gel electrophoresis, the DNA is forced to move through a sieve of molecular proportions. The end result is that large pieces of DNA move more slowly than small pieces. The DNA fragments in the gel are visualised by the inclusion of ethidium bromide. This compound binds to DNA and the complex DNA and ethidium bromide fluoresces under ultraviolet light. A digital image can be obtained as a permanent record of the gel electrophoresis.

2.2.2.3 Direct sequencing

The direct sequencing method is a valuable complement to other methods of polymorphism identification such as PCR-RFLP (Yandell & Dryja, 1989). One of the advantages of direct sequencing is that it detects all polymorphic sites within amplified sequence (Engelke *et al.*, 1988). For a small-scale study with a limited number of targeted polymorphism sites, direct sequencing can be expensive. In recent years, direct sequencing of DNA has undergone vast development (Yandell & Dryja, 1989). The latest sequencing procedures involve hybridisation of an oligonucleotide primer to a clean DNA template fragment, extension of the primer with DNA polymerase and energy transfer dye-terminator reagent, resolution of the DNA fragment using capillary electrophoresis, and nucleotide identification using laser scanning. Most sequencing

processes are now undertaken by a fully automated system (Innis *et al.*, 1988). These are high-throughput and increasingly economical.

2.2.3 Methods of assessing the response to antiepileptic drug treatment

The most common difficulty in pharmacogenetic studies, particularly in epilepsy, is the ability to identify an unequivocal drug response phenotype. Phenotypes related to AED treatment can be pharmacokinetic or pharmacodynamic in nature and can include drug dose, plasma drug concentration, response to treatment (efficacy) and occurrence of adverse effects (toxicity). Individual drug response can be affected by both environmental and genetic factors (Terwilliger & Weiss, 1998). In population-based studies, AED response phenotypes can also be influenced by many clinical and experimental factors that are directly and indirectly associated with AED treatment, such as circadian variability in pharmacokinetics, ethnicity, population size, heterogeneity of epilepsy, gradations of response, drug interactions, life-style and methods of assessing outcome (Sills *et al.*, 2005; French, 2006). Non-genetic factors such as renal and hepatic impairments may directly influence variability in drug pharmacokinetics, especially for drugs that are mainly eliminated through both of these organs. Ethnicity is usually associated with genetic heterogeneity but also variability in diet and life-style, and thus, populations with mixed-ethnicity may also have a broad heterogeneity in terms of environmental interaction. Population size can influence selection and randomisation biases and epilepsy type may demonstrate different levels of response to a given AED, such that differences in the distribution of epilepsy syndromes between studies may contribute to conflicting findings. Finally, drug-drug interactions can influence the effective dose and concentration of AEDs, and thus, influence the response to treatment.

Assessing the response to AED treatment is complicated, however various methods have been proposed. AED treatment response phenotypes are commonly evaluated by two sets of measures related to efficacy and to safety. The primary outcome measures related to efficacy are seizure frequency and seizure severity, whereas the primary outcome measures related to safety are the incidence of adverse drug effects (Perucca, 1997; Gilliam, 2005).

2.2.3.1 Assessing the efficacy of antiepileptic drugs

In clinical practice, the response of an individual patient to a specific AED cannot be known in advance; there is no treatment that is universally effective or universally ineffective. As such, it is difficult to establish whether a particular treatment is likely to be effective for a particular patient. The most attractive indicator of treatment success is complete seizure control (Perucca, 1997; Gilliam, 2005; French, 2006). However, the probability of achieving seizure-freedom varies greatly depending on the severity of the seizure disorder, pre-treatment seizure frequency and duration of the assessment period (Kwan & Brodie, 2000; Gilliam, 2005). The hidden difficulty in using such a definition is the time factor. How long the patient must be treated with the medication before complete seizure control can be established or discounted? It is important that the duration of follow-up is sufficiently long to ensure optimal individualisation of dosage and meaningful assessment of response at that dosage. Individuals with low frequency pre-treatment seizure frequencies may have to be observed for considerably longer than those with higher frequencies.

Other useful endpoints include the percentage change in seizure frequency for the group as a whole, as well as the proportion of patients achieving at least 50% reduction in seizure frequency compared with pre-treatment baseline (Perucca, 1997; Gilliam, 2005).

However, this approach, while statistically sound, has less practical applicability as it is arguable that a 50% reduction in seizure frequency may not be adequate to produce a significant improvement in quality of life. Counting of seizures can be done directly by the patient or less common by an external observer. Self-reported seizure frequency may be inaccurate as some patients are not aware of their seizures. Patients with the lowest self-reported seizure frequency often have the highest proportion of unrecognised seizures (Gilliam, 2005). In addition, those with newly diagnosed epilepsy may not have had a sufficient number of seizures to permit a reliable definition of seizure frequency; in such circumstances, the measurement of reduction in seizure frequency can be meaningless. Another potential measurement of treatment efficacy is the reduction in seizure severity (French, 2006). Seizure severity requires a definition that takes several factors into account and physiological consequences of the seizure must be quantified. The most obvious factors include fall, loss of consciousness, and intensity or duration of postictal effect. It is clear for the above that variability in the techniques of assessing the efficacy of AED treatment can contribute to the variation in phenotype characterization.

2.2.3.2 Assessing the adverse effects of antiepileptic drugs

Evaluation of adverse effects is fraught with difficulties. One of these is the lack of current standardisation in the methodology for the detection and categorisation of adverse events. Recording of adverse effects is often based on spontaneous reporting by the patient, physical examination, laboratory tests or a combination thereof. Spontaneous reporting has clear advantages in highlighting effects which are clinically relevant, but it is associated with distinct variability in the accuracy of detection and with significant under-reporting (Perucca, 1997; Gilliam, 2005). Patients may not be able to report signs and symptoms of adverse effects accurately as they emerge

(Gilliam, 2005). An alternative to the reliance on spontaneous reporting is the use of standardised procedures with respect to clinical questioning and physical examination. Of course, a further problem is the ability to quantitatively assess the severity of adverse symptoms, especially for CNS-related adverse effects (Perucca, 1997; Gilliam, 2005). Another important issue is the need to obtain information about the time course of a given adverse effect, as there is a difference in the relative importance between acute, transient and chronic effects which persist throughout the treatment period.

In clinical practice, the assessment of adverse effects may be considered as "biological", and "cognitive" or "behavioral". Biological adverse effects are those that are objective and evident by examination or measured by clinical tests. This category of adverse effects includes rash, hair loss, liver failure, or nephritis. Supplemental tests may be required when the clinical history indicates a particular possibility. Biological adverse effects can be divided into acute and chronic events. Acute effects are generally idiopathic and by definition cannot be predicted or anticipated for an individual patient, for example aplastic anaemia, hepatitis, nephritis, or Stevens-Johnson syndrome. Chronic effects include disorders such as folate deficient anemia, AED-induced rickets, neuropathy, gingival hyperplasia, and cerebellar degeneration. Chronic adverse effects appear to be related more to cumulative toxicity.

Individual sensitivities to the cognitive and behavioral side effects of AEDs are commonly reported by patients and their families. Behavior and cognition are altered by many aspects of the epileptic disorder and these may be confused with medication effects (Gilliam, 2005). In clinical practice, measuring a change in the patient's personality or behavior is almost never objective. There is almost no area of clinical medicine more in need of objective measures than the area of adverse drug effects. As

with efficacy, variability in the techniques of assessing adverse effects of AEDs may contribute significantly to the variation in phenotype characterization.

2.3 Recurrent Methods

2.3.1 Study subjects

Approvals to obtain a peripheral blood sample for the extraction and pharmacogenetic analysis of DNA from all appropriate patients with epilepsy was granted by the West Research Ethics Committee (North Glasgow University Hospitals NHS Trust; Ref 02/119(2)) in September 2002. All patients attending the Epilepsy Unit at the Western Infirmary, Glasgow, Scotland were invited to participate. The first 400 patients (201 male; median age 40 years, range 14 to 85 years) who provided informed consent contribute to the experiments described in this thesis. Among these patients, 398 self-identified as being of European ancestry and the other two subjects were of Asian origin. The 400 DNA samples were collected continuously from September 2002 until December 2005. Throughout this thesis, this group of 400 patients is denoted as the "400-strong study population". A flow chart showing the distribution of these 400 patients to each individual project is presented in FIGURE 2.1.

2.3.2 Control DNA samples

A total of 10 control DNA samples were obtained from members of staff of the Epilepsy Unit. All of these subjects self-identified as being of European ancestry. These samples were employed for the purposes of assay optimisation and validation and thereafter as control samples of known genotype in individual analyses.

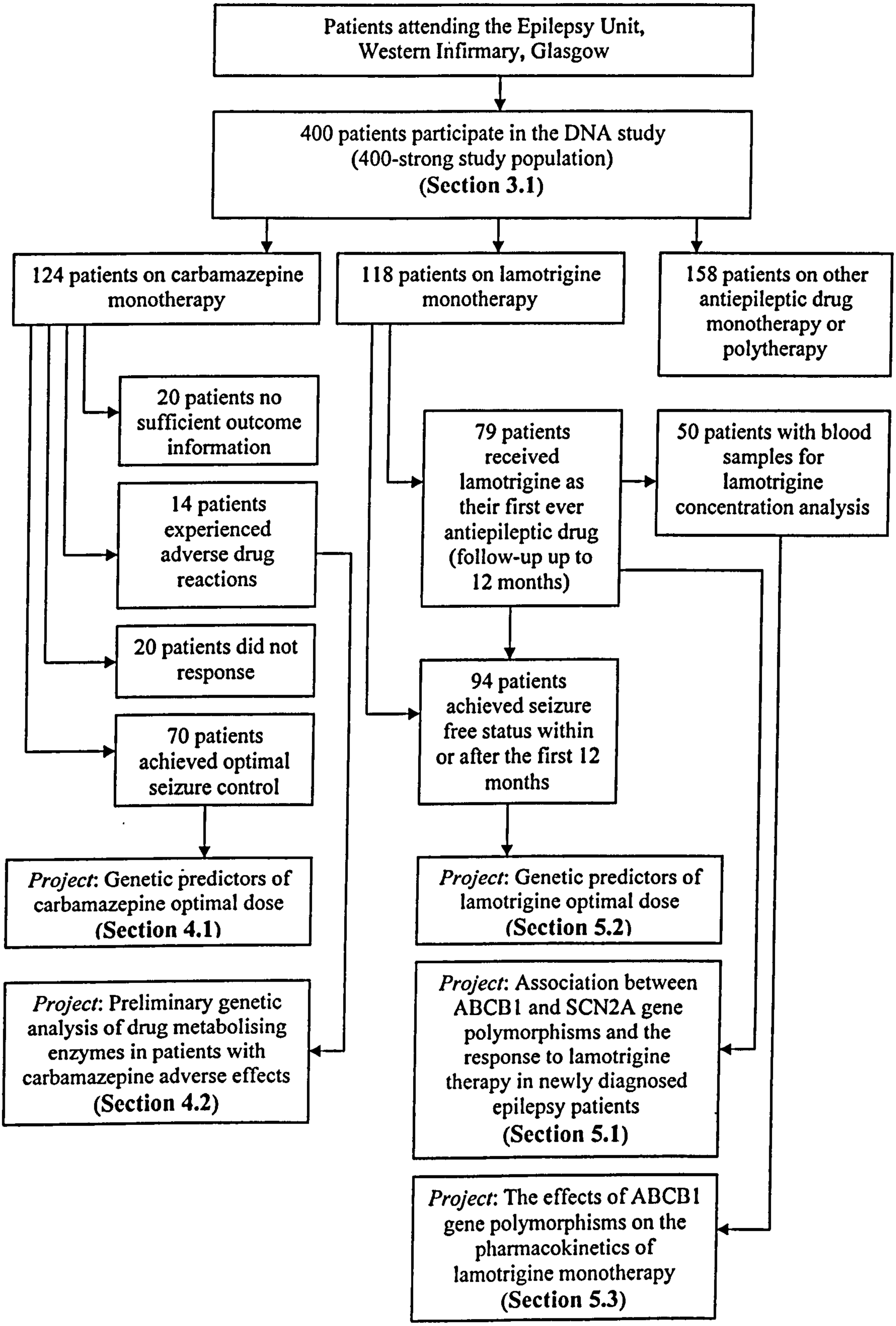

FIGURE 2.1. Distribution of 400 study subjects to each individual project.

2.3.3 DNA extraction

A total of 16 ml (4 ml x 4 tubes) of venous blood was collected in EDTA-coated tubes by qualified clinical personnel and stored at 4 °C. Genomic DNA was extracted from leukocytes using the Promega® Wizard DNA purification kit as describe below.

The DNA extraction was started with lysis of the red blood cells and separation of the white blood cells. A total of 30 ml of cell lysis solution was placed in a 50 ml centrifuge tube and 10 ml of whole blood added. The solution was mixed by inversion and then incubated at room temperature (20°C) for 10 minutes. The mixture was then centrifuged at 2000 x g for 10 minutes at room temperature (20°C). The supernatant was removed and discarded into a waste container containing a chlorine tablet, leaving approximately 1.4 ml of residual liquid and a white pellet. These steps were repeated if the pellet appeared red in color.

The next step was to release the DNA from the nucleus of the white blood cells. The centrifuge tube containing the white pellet was vortexed to resuspend the white blood cells. A 10 ml aliquot of nuclei lysis solution was added to the tube and mixed using a pasteur pipette. The resulting solution was then incubated at 37 °C for approximately 2 hours. The next step was to remove the cellular proteins by a salt precipitation technique. A 3.3 ml aliquot of protein precipitation solution was added to the nuclear lysate and vortexed for 10 – 20 seconds. The solution was then centrifuged at 2000 x g for 10 minutes at room temperature (20°C) to separate the cellular protein (dark brown pellet).

The final step was to concentrate and desalt the DNA. Without disturbing the cellular protein pellet, the supernatant was decanted to a universal tube containing 10 ml of

isopropanol. This solution was then mixed by inversion until the white "thread-like" DNA strands formed a visible mass. Following that, the solution was centrifuged at 2000 x g for 3 minutes at room temperature (20°C) and the DNA emerged as a small white pellet. The supernatant was discarded and 10 ml of 70% ethanol was added to the DNA pellet. The pellet was resuspended and the tube washed by several inversions. The solution was again centrifuged at 2000 x g for 3 minutes at room temperature (20°C). The ethanol was decanted, the tube was inverted on clean absorbent paper, and the pellet allowed to air-dry for 10 – 15 minutes. A 200 µl volume of molecular biology grade water was added to rehydrate the DNA and left overnight prior to quantification. Thereafter, the dissolved DNA was transferred to a 250 µl cryovial using a sterile pipette and stored frozen at –80 °C.

2.3.4 DNA quantification

The concentration of DNA was quantified using ultraviolet spectroscopy. A 1 µl aliquot of DNA solution was diluted with 70 µl of molecular biology grade water in a polystyrene cuvette. The optical density (O.D.) of the DNA solution was determined at a wavelength of 260 nm. The genomic DNA concentration was calculated using the following equation:

$$\text{genomic DNA } (\mu g/\mu l) = 0.05 * \text{O.D.} * \text{dilution factor}$$

2.3.5 Polymerase chain reaction

2.3.5.1 PCR primers

Primer sequences were usually obtained from existing publications, however, on occasion, an online software package, Primer3 (Rozen & Skaletsky, 2000), was used to

design the PCR primers. Some characteristics were standardised for all primer design, such as length of primers (18 – 24 bp), GC content (50%) and primer Tm (55°C). The genetic sequence of interest was obtained from GenBank. The specificity of the primers was evaluated using BLAST.

2.3.5.2 Optimization of polymerase chain reaction assay

All PCR assays were optimised using a standard protocol. A total of 10 control DNA samples were employed for this purpose. The initial PCR assay was performed in a 30 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each PCR primer, 1.5 mM $MgCl_2$, 3 µl of 10x PCR buffer and 0.25 mM dNTP mix, and the final reaction volume adjusted with molecular biology grade water. The initial PCR conditions consisted of denaturation at 94°C for 5 minutes; followed by 35 cycles at 94°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (extension), and thereafter a final extension at 72°C for 5 minutes and soaking at 4°C. The initial annealing temperature was close to the mean Tm of both PCR primers. The amplified DNA fragment was separated using 2.0% agarose gel electrophoresis at 80V for 60 minutes and then visualised under ultraviolet light. The size of amplified DNA fragment was estimated relative to a DNA ladder of known size (25 bp or 50 bp) that was incorporated in each electrophoresis. The size of observed DNA fragment was compared with the size of expected fragment obtained from the primer design tool. The quantity of amplified PCR product was assessed based on the brightness of the band under ultraviolate light. PCR assays with poor DNA amplification were systematically modified and re-evaluated. The annealing temperature was adjusted in a stepwise manner without changing any other initial PCR conditions. In the case of low quantities of PCR amplification, the annealing temperature was reduced by 1 degree each time. In the case of multiple bands, the

annealing temperature was increased by 1 degree each time. The annealing temperature was typically evaluated between 50 and 60°C. The magnesium concentration was adjusted as a second step of optimisation once the optimal annealing temperature has been identified. In the case of low quantities of PCR product, the magnesium concentration was increased in a stepwise manner by 0.5 mM. In the case of multiple bands, the magnesium concentration was reduced in a stepwise manner by 0.5 mM. The magnesium concentration was typically evaluated between 0.5 and 2.5 mM. The PCR conditions were adjusted until a bright and reproducible PCR product band was identified on gel electrophoresis.

2.3.5.3 Quality control of polymerase chain reaction assay

To assess risk of contamination, two blank samples were included in each analysis, in which the DNA template was replaced by 1 µl of molecular biology grade water. Each batch of PCR analyses consisted of 18 reaction tubes (16 patient samples and 2 blanks). Blanks were randomly located across the thermal cycler block and also randomly assigned to lanes on gel electrophoresis. The blank samples were not expected to produce any DNA amplification.

2.3.6 Restriction fragment length polymorphism assay

2.3.6.1 Choosing suitable restriction enzymes

An online restriction enzyme mapping software, NEB cutter (http://tools.neb.com/NEBcutter2/index.php), was utilised to identify suitable restriction enzymes for a given SNP site. On occasion, restriction enzymes were identified from existing publications.

2.3.6.2 Optimisation of restriction fragment length polymorphism assay

Each of the RFLP assays developed in this project was optimised using a standard procedure. PCR products from 10 control DNA samples were employed for this purpose. Each restriction digest was performed in a 10 µl volume containing 5 µl of PCR product, 1 µl x 10x NE buffer, restriction enzyme (2.5, 5 to 7.5 U) and the final reaction volume adjusted with molecular biology grade water. These reaction mixtures were incubated at 37°C for up to 4 hours. At the end of 2 and 4 hours incubation time, an aliquot of digest product (5 µl) was removed from reaction tube and each mixed with a small quantity of loading dye (1 µl), and immediately loaded onto a 2.0% agarose gel. A DNA ladder of known size (25 bp or 50 bp) and one sample of undigested PCR product (5 µl) were also included in each electrophoresis run. The electrophoresis conditions were set at 80V for 60 to 120 minutes. At the end of electrophoresis, the agarose gel was visualised under ultraviolet light. The expected size of digested fragments was estimated relative to the DNA ladder. Incomplete digestion was evaluated by digesting the PCR product in a high concentration of restriction enzyme (10 U) and a longer incubation period (24 hours). In the case of poor resolution between fragments, the electrophoresis was repeated using a higher agarose concentration (increased in a stepwise manner by 0.5%) to permit clearer separation of digest fragments.

2.3.6.3 Quality control of restriction fragment length polymorphism assay

Each batch of RFLP analysis comprised one DNA ladder (25 or 50 bp), 2 water blanks, 16 patient samples, one undigested control sample (PCR product), and three control DNA samples of known genotype, corresponding to each of the expected genotypes in

the population. Once all 400 patients had been genotyped, 5% were selected randomly and re-genotyped for confirmatory purposes.

2.3.7 Statistical analyses

MINITAB™ Statistical Software Release 13.32 (Minitab Ltd., Coventry, UK) was used to perform all statistical analyses. Logistic regression analysis was used to assess the association between genetic predictors and the response to AED therapy. The odds ratio (OR) together with its 95% confidence interval (95% CI) and *p*-value were used to evaluate the strength of association. Genetic predictors for optimal dose of AEDs were identified and characterised using univariate and multivariate linear regression analyses. To explore interaction effects, cross-product terms were added as independent variables. A goodness-of-fit (r^2) value more than 0.8 indicates a strong predictive model. Pearson's correlation (r) value more than 0.8 indicates a strong association between variables. For this analysis, *p*-values less than 0.05 indicate a significant association or significant predictor effect. No formal power calculation and multiple testing corrections were undertaken for the statistical analysis; therefore, the research projects presented in this thesis are considered to be exploratory in an effort to generate research hypothesis.

CHAPTER 3

POLYMORPHISMS OF DRUG METABOLISING ENZYME, DRUG TRANSPORTER PROTEIN AND SODIUM CHANNEL GENES

3.1 The Prevalences of Common Polymorphisms in the Drug Metabolizing Enzyme, Drug Transporter Protein and Sodium Channel Genes in a West of Scotland Epilepsy Population

3.1.1 Introduction

In keeping with the aims of this research project, genes of interest were derived from current knowledge regarding the pharmacokinetics and pharmacodynamics of CBZ and LTG. Potentially functional variants of these genes were then selected from previously published information (as presented in section 1.7). The common genetic polymorphisms evaluated in this project are listed in TABLE 1.6. In a population based pharmacogenetic study, the prevalence of variant genotypes is expected to adhere to Hardy-Weinberg Equilibrium (HWE) conditions (Xu *et al.*, 2002). HWE is based on the assumption that, under constant conditions after a generation of random mating, genotype frequencies throughout a population at a specified gene locus become fixed at a specific equilibrium value (Hosking *et al.*, 2004). The HWE test is carried out by comparing the genotype distribution of each SNP against the genotype distribution that is predicted by HWE at that locus. Deviation from HWE can be the result of one or more of five violations. The first violation is inbreeding, which causes an increase in homozygosity for all genes. The second violation is assortative mating, which causes an increase in homozygosity only for those genes involved in the trait that is assortatively mated. The third violation is the occurrence of new mutations. The fourth violation is gene flow through migration in or out of the population. The fifth violation is related to population size, which causes a random change in genotypic frequencies, particularly if the population is very small. This is due to a sampling effect, and is known as genetic drift (Xu *et al.*, 2002). Testing for HWE is also commonly used for the quality control

of large-scale genotyping and is one of the few methods which can be useful to identify systemic genotyping errors in unrelated individuals (Xu *et al.*, 2002; Hosking *et al.*, 2004; Salanti *et al.*, 2005; Wittke-Thompson *et al.*, 2005).

3.1.2 Aims

The aim of this project was to evaluate the prevalences of common gene polymorphisms (TABLE 1.6) in a West of Scotland epilepsy population and to compare the allele and genotype distributions with previously published data from different ethnic groups. The genotype distribution was further examined using the HWE test as an early quality control measure for the genotyping methodology.

3.1.3 Methods

3.1.3.1 DNA samples and extraction

A total of 400 DNA samples from the study population described in section 2.3.1 were utilised in this project. DNA extraction was performed following the method described in section 2.3.3.

3.1.3.2 PCR-RFLP optimization

Each PCR-RFLP methods presented below have been optimised using the method described in Chapter 2 (sections 2.3.5.2 and 2.3.6.2).

3.1.3.3 Identification of CYP3A4 g.-392A>G polymorphism by PCR-RFLP

The method described by van Schaik *et al.* (2000) was modified for the purposes of this study. Modification was undertaken to optimise the yield of PCR product which

included alteration in the total reaction volume, the quantity of reagents and the annealing temperature (as described in section 2.3.5.2). The 5'-flanking promoter region of the CYP3A4 gene containing the g.-392A>G polymorphism was amplified using forward, 5'-GGA CAG CCA TAG AGA CAA CTG CA-3' and reverse, 5'-CTT TCC TGC CCT GCA CAG-3' primers. A PCR product containing the CYP3A4 -392G allele with restriction site for the *Pst*I enzyme was produced by incorporating the underlined mismatched nucleotides into the forward primer. The PCR assay was performed in a 30 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 2 mM $MgCl_2$, 3 µl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 94°C for 7 minutes; 35 cycles of 94°C for 1 minute (denaturation), 56°C for 1 minute (annealing), 72°C for 1 minute (extension); and followed by a final extension at 72°C for 7 minutes and soaking at 4°C. A PCR product with a size of 334 bp was expected from the amplification.

The CYP3A4 -392A allele was discriminated from the -392G allele by digesting the PCR product with *Pst*I endonuclease. The PCR product was digested for at least 2 hours at 37°C in a 20 µl reaction volume containing 15 µl of PCR product, 5 U of *Pst*I restriction enzyme, 2 µl of 10x NE buffer, 0.2 µl of 100x BSA (10 mg/ml) and an appropriate volume of molecular biology grade water. *Pst*I cut the PCR product at position 5'-CTGCA/G-3', and produced 220-bp, 81-bp and 33-bp fragments for the CYP3A4 -392A allele; and 199-bp, 81-bp, 33-bp and 21-bp fragments for the CYP3A4 -392G allele.

A total of 5 µl of digest product was mixed with 1 µl of loading dye and loaded on a 2.5% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis

conditions were set at 100 V for 100 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination (FIGURE 3.1).

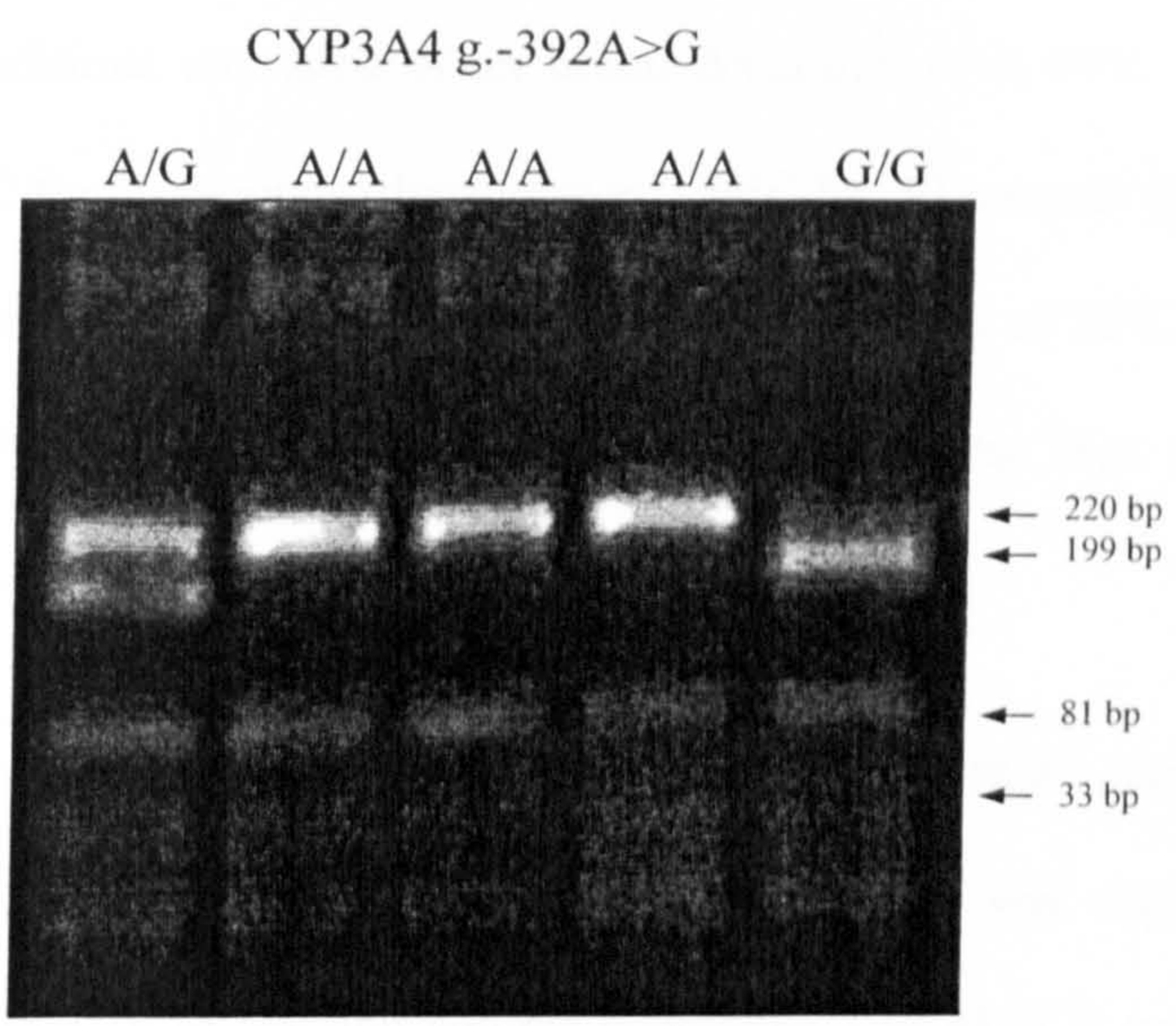

FIGURE 3.1. Electrophoresis patterns for CYP3A4 g.-392A>G genotypes analysed with PCR-RFLP assay.

3.1.3.4 Identification of CYP3A5 g.6986A>G polymorphism by PCR-RFLP

The method described by Fukuen *et al.* (2002) was modified for the purposes of this study. Modification was undertaken to optimise the yield of PCR product which included alteration in the total reaction volume, the quantity of reagents and the annealing temperature (as described in section 2.3.5.2). The intronic region of the CYP3A5 gene containing the g.6986A>G polymorphism was amplified using forward, 5'-CTT TAA AGA GCT CTT TTG TCT CTC A-3' and reverse, 5'-CCA GGA AGC CAG ACT TTG AT-3' primers. A PCR product containing the CYP3A5 6986G allele with restriction site for the *Dde*I enzyme was produced by incorporating the undelined mismatched nucleotide into the forward primer. The PCR assay was performed in a 30

μl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 1.5 mM $MgCl_2$, 3 μl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 95°C for 10 minutes; 35 cycles of 94°C for 30 seconds (denaturation), 56°C for 30 seconds (annealing), 72°C for 30 seconds (extension); and followed by a final extension at 72°C for 5 minutes and soaking at 4°C. A PCR product with a size of 200 bp was expected from the amplification.

The CYP3A5 6986A allele was discriminated from the 6986G allele by digesting the PCR product with *Dde*I endonuclease. The PCR product was digested for at least 2 hours at 37°C in a 10 μl reaction volume containing 5 μl of PCR product, 5 U of *Dde*I restriction enzyme, 1 μl of 10x NE buffer and an appropriate volume of molecular biology grade water. *Dde*I cut the PCR product at position 5'-C/TNAG-3', and produced 129-bp and 71-bp fragments for the CYP3A5 6986A allele; and 107-bp, 71-bp and 22-bp fragments for the CYP3A5 6986G allele.

A total of 5 μl of digest product was mixed with 1 μl of loading dye and loaded on a 3.0% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis conditions were set at 100 V for 80 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination (FIGURE 3.2).

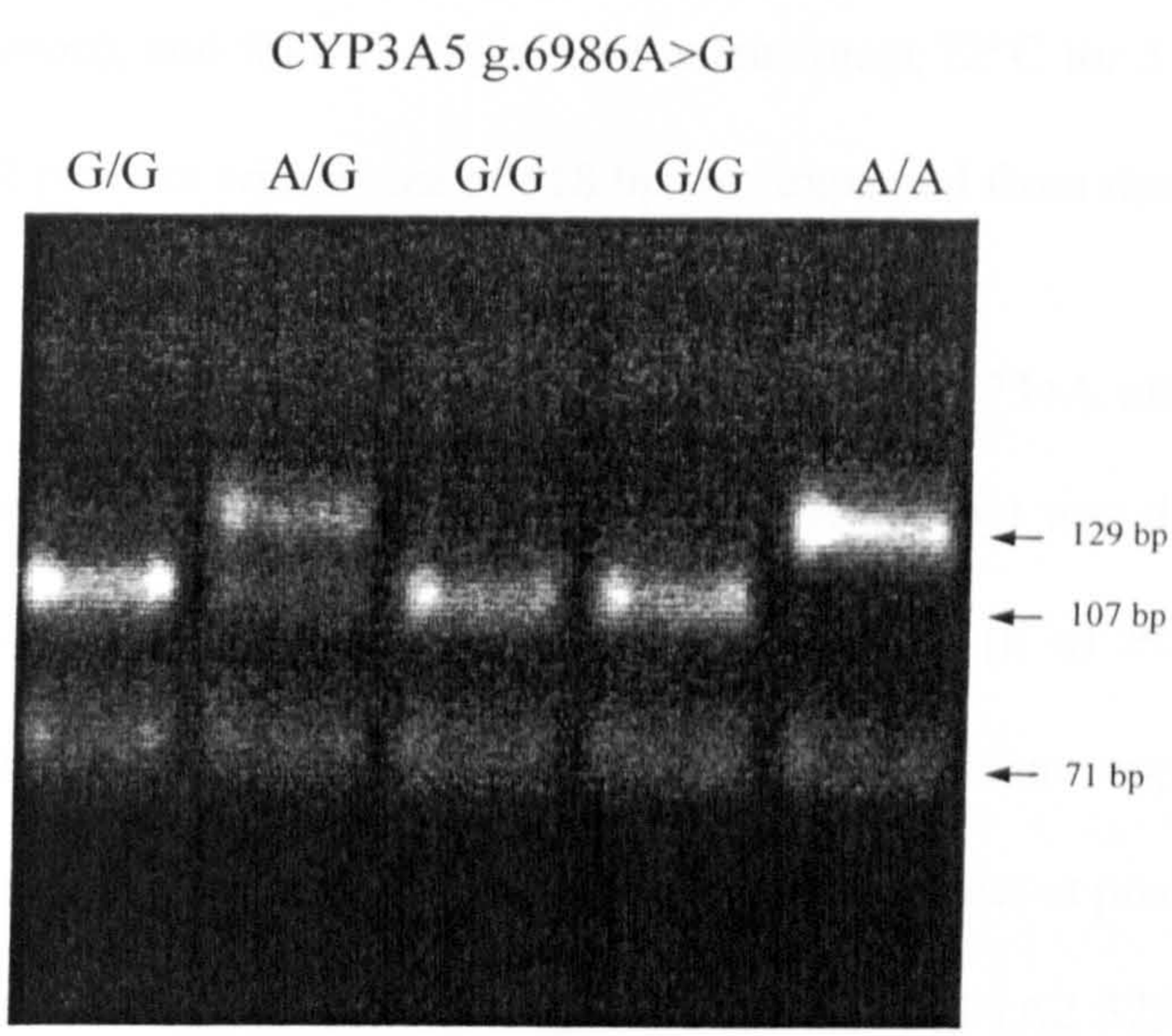

FIGURE 3.2. Electrophoresis patterns for CYP3A5 g.6986A>G genotypes analysed with PCR-RFLP assay.

3.1.3.5 Identification of CYP1A2 g.5734C>A polymorphism by PCR-RFLP

A new, self-designed PCR-RFLP method for the identification of CYP1A2 g.5734C>A was utilised in this study. The intron-1 region of the CYP1A2 gene containing the g.5734C>A polymorphism was amplified using forward, 5'-CCC TTG GGT ATA TGG AAG GTA-3' and reverse, 5'-CTT GAG CAC CCA GAA TAC CA-3' primers. The PCR assay was performed in a 30 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 2 mM $MgCl_2$, 3 µl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds (denaturation), 56°C for 30 seconds (annealing), 72°C for 1

minute (extension); and followed by a final extension at 72°C for 5 minutes and soaking at 4°C. A PCR product with a size of 518 bp was expected from the amplification.

The CYP1A2 5734C allele was discriminated from the 5734A allele by digesting the PCR product with *PspOM* I endonuclease. The PCR product was digested for at least 2 hours at 37°C in a 10 µl reaction volume containing 5 µl of PCR product, 5 U of *PspOM* I restriction enzyme, 1 µl of 10x NE buffer and an appropriate volume of molecular biology grade water. *PspOM* I cut the PCR product at position 5'-G/GGCCC-3' and produced 268-bp and 250-bp fragments for the CYP1A2 5734C allele. The 518-bp PCR product remained uncut for the CYP1A2 5734A allele (FIGURE 3.3).

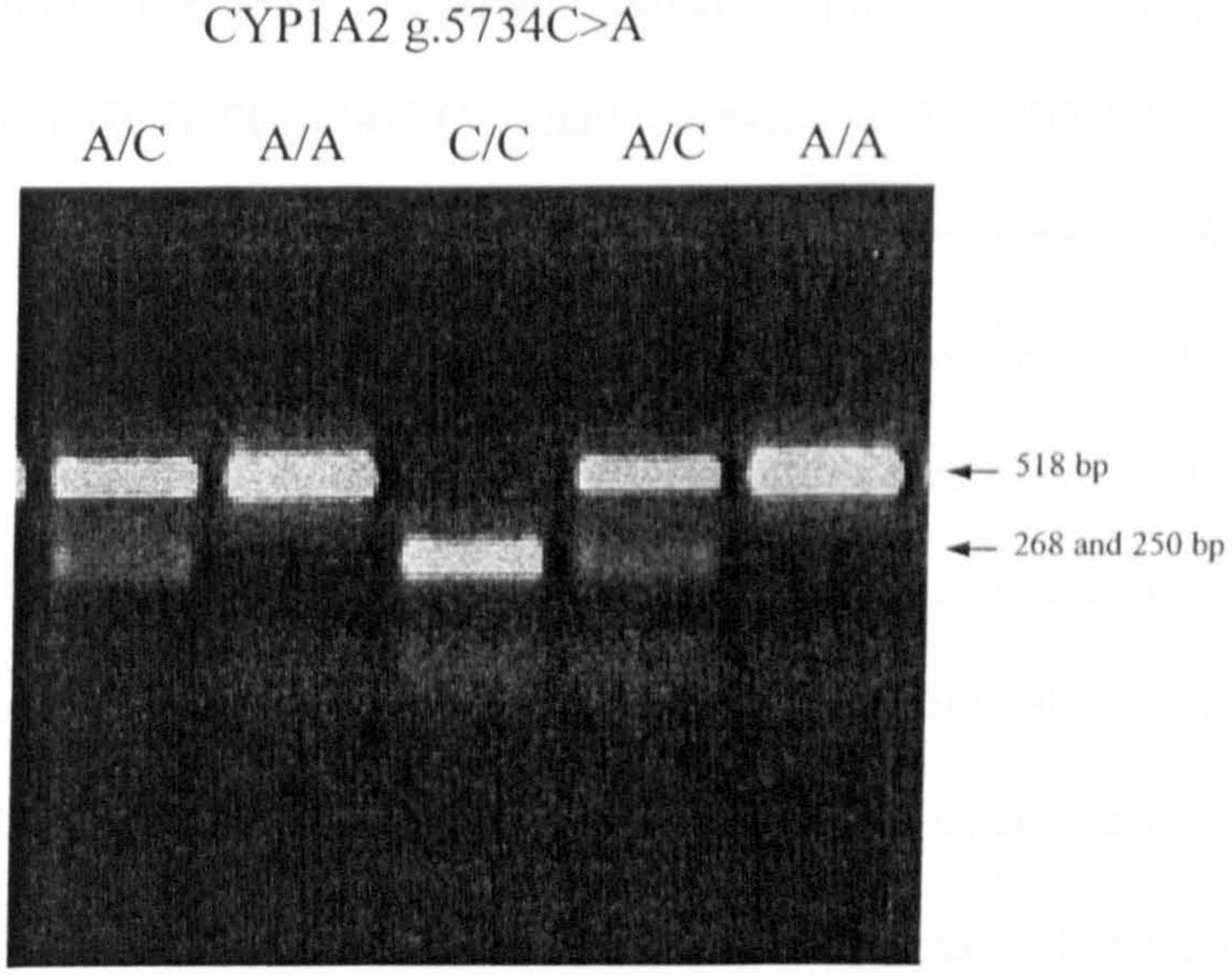

FIGURE 3.3. Electrophoresis patterns for CYP1A2 g.5734C>A genotypes analysed with PCR-RFLP assay. The 268-bp and 250-bp fragments did not resolve clearly after 45 minutes electrophoresis on a 2.0% agarose gel.

A total of 5 µl of digest product was mixed with 1 µl of loading dye and loaded on a 2.0% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis

conditions were set at 90 V for 45 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination. The 268-bp and 250-bp fragments did not resolve clearly after 45 minutes electrophoresis, however, this did not affect the genotype identification.

3.1.3.6 Identification of EPHX1 c.337T>C polymorphism by PCR-RFLP

The method described by Budhi *et al.* (2003) was modified for the purposes of this study. Modification was undertaken to optimise the yield of PCR product which included alteration in the total reaction volume, the quantity of reagents and the annealing temperature (as described in section 2.3.5.2). The exon-3 region of the EPHX1 gene containing the c.337T>C polymorphism was amplified using forward, 5'-GAT CGA TAA GTT CCG TTT CAC C-3' and reverse, 5'-CAA TGT TAG TCT TGA AGT GAG GAT-3' primers. The reverse primer contains mismatched nucleotides for codon 113 (shown as bold and underlined) and codon 119 (shown as underlined only). The mismatched nucleotide at codon 113 was introduced to create a restriction site for the *EcoR*V enzyme and recognition of the 337T allele (Smith & Harrison, 1997). The mismatched nucleotide at codon 119 was introduced to eliminate the effect of the adjacent c.357G>A polymorphism, which is reported to influence the estimation of genotypes at codon 113 (Budhi *et al.*, 2003). The PCR assay was performed in a 30 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 2 mM $MgCl_2$, 3 µl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 94°C for 7 minutes; 35 cycles of 94°C for 1 minute (denaturation), 56°C for 1 minute (annealing), 72°C for 1 minute

(extension); and followed by a final extension at 72°C for 7 minutes and soaking at 4°C. A PCR product with a size of 164 bp was expected from the amplification.

The EPHX1 337T allele was discriminated from the 337C allele by digesting the PCR product with *EcoR*V endonuclease. The PCR product was digested for at least 2 hours at 37°C in a 10 µl reaction volume containing 5 µl of PCR product, 5 U of *EcoR*V restriction enzyme, 1 µl of 10x NE buffer, 0.1 µl of 100x BSA (10 mg/ml) and an appropriate volume of molecular biology grade water. The *EcoR*V cut at position 5'-GAT/ATC-3' and produced 140-bp and 24-bp fragments for the EPHX1 337T allele. The 164-bp PCR product remained uncut for the EPHX1 337C allele (FIGURE 3.4).

A total of 5 µl of digest product was mixed with 1 µl of loading dye and loaded on a 3.0% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis conditions were set at 80 V for 100 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination.

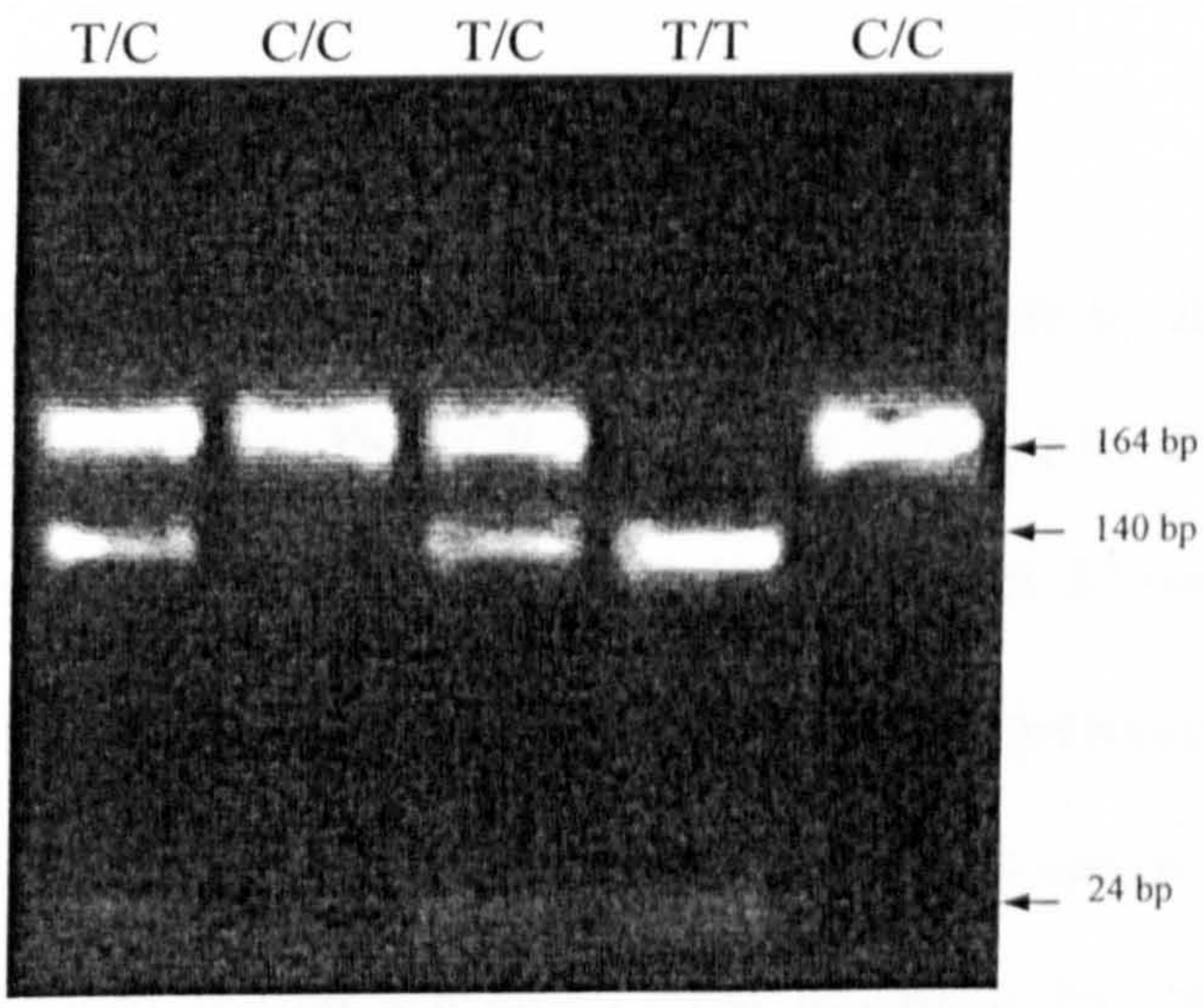

FIGURE 3.4. Electrophoresis patterns for EPHX1 c.337T>C genotypes analysed by PCR-RFLP assay.

3.1.3.7 Identification of EPHX1 c.416A>G polymorphism by PCR-RFLP

The method described by Smith & Harrison (1997) was modified for the purposes of this study. Modification was undertaken to optimise the yield of PCR product which included alteration in the total reaction volume, the quantity of reagents and the annealing temperature (as described in section 2.3.5.2). The exon-4 region of the EPHX1 gene containing the c.416A>G polymorphism was amplified using forward, 5'-ACA TCC ACT TCA TCC ACG T-3 and reverse, 5'-ATG CCT CTG AGA AGC CAT-3' primers. The PCR assay was performed in a 30 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 1.5 mM $MgCl_2$, 3 µl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 94°C for 5 minutes; 37 cycles of 94°C for 30 seconds (denaturation), 56°C for 30 seconds (annealing), 72°C for 30 seconds (extension); and followed by a final extension at 72°C for 5 minutes and soaking at 4°C. A PCR product with a size of 210 bp was expected from the amplification.

The EPHX1 416A allele was discriminated from the 416G allele by digesting the PCR product with *Rsa*I endonuclease. The PCR product was digested for at least 2 hours at 37°C in a 10 µl reaction volume containing 5 µl of PCR product, 5 U of *Rsa*I restriction enzyme, 1 µl of 10x NE buffer and an appropriate volume of molecular biology grade water. *Rsa*I cut the PCR product at position 5'-GT/AC-3' and containing the EPHX1 416G allele and produced 164-bp and 46-bp sized fragments for the EPHX1 416G allele. The 210-bp PCR product remained uncut for the EPHX1 416A allele.

A total of 5 µl of digest product was mixed with 1 µl of loading dye and loaded on a 2.5% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis conditions were set at 80 V for 80 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination (FIGURE 3.5).

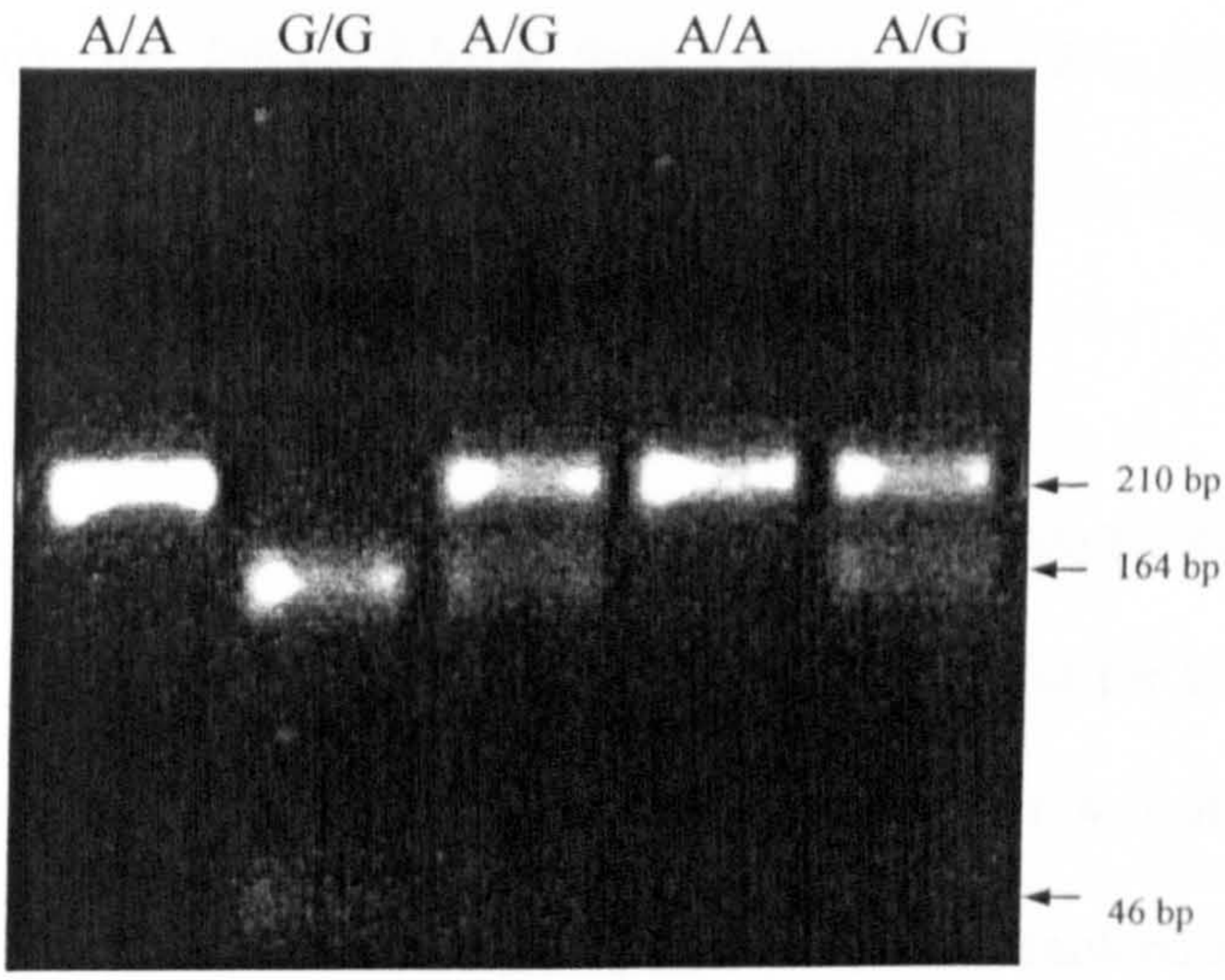

FIGURE 3.5. Electrophoresis patterns for EPHX1 c.416A>G genotypes analysed by PCR-RFLP assay.

3.1.3.8 Identification of UGT2B7 c.802C>T polymorphism by PCR-RFLP

The method described by Bhasker *et al.* (2000) was modified for the purposes of this study. Modification was undertaken to optimise the yield of PCR product which included alteration in the total reaction volume, the quantity of reagents and the annealing temperature (as described in section 2.3.5.2). The exon-2 region of the UGT2B7 gene containing the c.802C>T polymorphism was amplified using forward, 5'-TGC CTA CAT TAT TCT AAC C-3' and reverse, 5'-TCT CTG AAA ATT CTG

CAC T-3' primers. The PCR assay was performed in a 30 µl reaction volume containing 150 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 2 mM $MgCl_2$, 3 µl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds (denaturation), 59°C for 30 seconds (annealing), 72°C for 30 seconds (extension); and followed by a final extension at 72°C for 5 minutes and soaking at 4°C. A PCR product with a size of 579 bp was expected from the amplification.

The UGT2B7 802C allele was discriminated from the 802T allele by digesting the PCR product with *Fok*I endonuclease. The PCR product was digested for at least 2 hours at 37°C in a 10 µl reaction volume containing 6 µl of PCR product, 4 U of *Fok*I restriction enzyme, 1 µl of 10x NE buffer and an appropriate volume of molecular biology grade water. *Fok*I cut the PCR product at position 5'-GGATG$(N)_9$/-3' and produced 457-bp, 79-bp and 42-bp fragments for the UGT2B7 802T allele; and 346-bp, 111-bp, 79-bp and 42-bp fragments for the UGT2B7 802C allele.

A total of 10 µl of digest product was mixed with 1 µl of loading dye and loaded on a 2.5% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis conditions were set at 80 V for 45 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination (FIGURE 3.6).

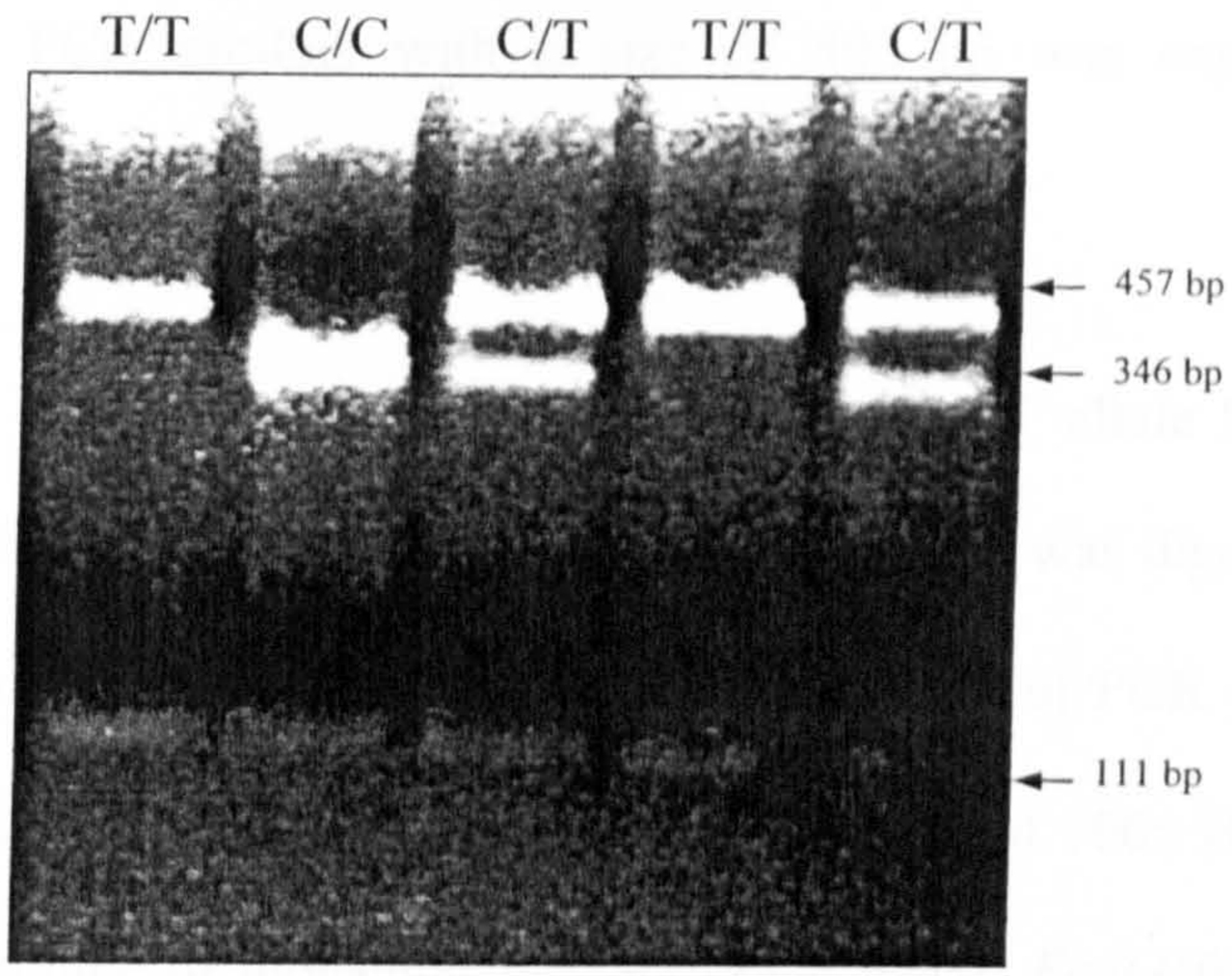

FIGURE 3.6. Electrophoresis patterns for UGT2B7 c.802C>T genotypes analysed with PCR-RFLP assay

3.1.3.9 Identification of ABCB1 c.1236C>T polymorphism by PCR-RFLP

The method described by Tang *et al.* (2002) was modified for the purposes of this study. Modification was undertaken to optimise the yield of PCR product which included alteration in the total reaction volume, the quantity of reagents and the annealing temperature (as described in section 2.3.5.2). The exon-12 region of the ABCB1 gene containing the c.1236C>T polymorphism was amplified using forward, 5'-TCT TTG TCA CTT TAT CCA GC-3' and reverse, 5'-TCT CAC CAT CCC CTC TGT-3' primers. The PCR assay was performed in a 20 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 1.5 mM $MgCl_2$, 2 µl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 94°C for 10 minutes; 35 cycles of 94°C for 30 seconds (denaturation), 60°C for 30 seconds (annealing), 72°C for

30 seconds (extension); and followed by a final extension at 72°C for 5 minutes and soaking at 4°C. A PCR product with a size of 502 bp was expected from the amplification.

The ABCB1 1236C allele was discriminated from the 1236T allele by digesting the PCR product with *EcoO*1091 endonuclease. The PCR product was digested for at least 2 hours at 37°C in a 20 µl reaction volume containing 10 µl of PCR product, 5 U of *EcoO*1091 restriction enzyme, 2 µl of 10x NE buffer, 0.2 µl of 100x BSA (10 mg/ml) and an appropriate volume of molecular biology grade water. *EcoO*1091 cut the PCR product at position 5'-RG/GNCCY-3' and produced 380-bp and 122-bp fragments for the ABCB1 1236C allele. The 502-bp PCR product remained uncut for the ABCB1 1236T allele (FIGURE 3.7).

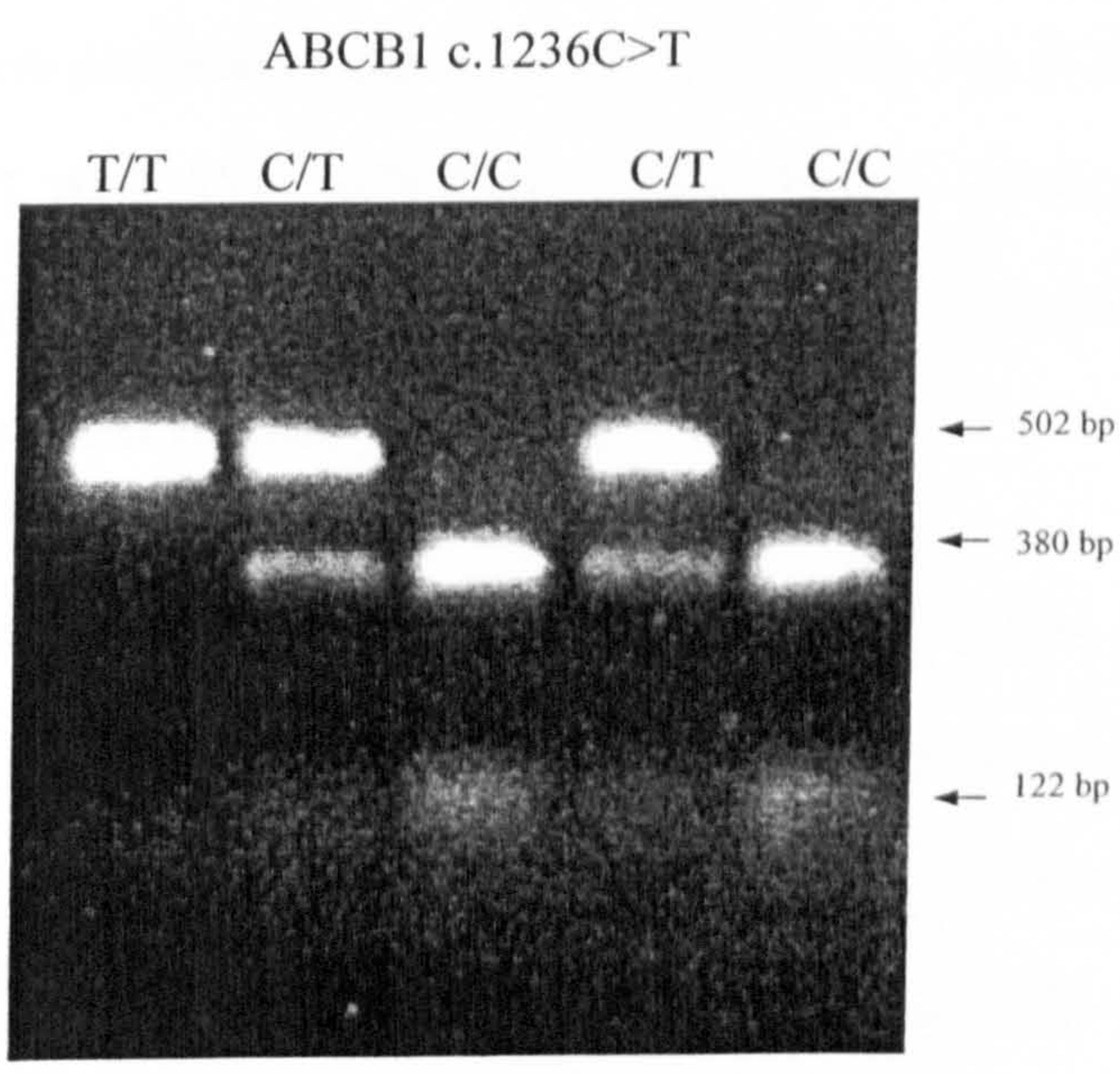

FIGURE 3.7. Electrophoresis patterns for ABCB1 c.1236C>T genotypes analysed by PCR-RFLP assay.

A total of 5 µl of digest product was mixed with 1 µl of loading dye and loaded on a 2.5% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis

conditions were set at 80 V for 90 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination.

3.1.3.10 Identification of ABCB1 c.2677G>T/A polymorphism by PCR-RFLP

The method described by Cascorbi *et al.*, (2001) was modified for the purposes of this study. Modification was undertaken to optimise the yield of PCR product which included alteration in the total reaction volume, the quantity of reagents and the annealing temperature (as described in section 2.3.5.2). The exon-21 region of the ABCB1 gene containing the c.2677G>T/A polymorphism was amplified using two PCR assays, one for the identification of the 2677A allele (PCR1) and the other for the identification of the 2677T allele (PCR2). PCR1 was performed using forward, 5'-TGC AGG CTA TAG GTT CCA GG-3' and reverse, 5'-GTT TGA CTC ACC TTC CCA G-3' primers. PCR2 was performed using the same forward primer as PCR1, together with the reverse, 5'-TTT AGT TTG ACT CAC CTT CCC G-3' primer. A mismatched nucleotide (underlined) in the reverse primer of PCR2 was employed to introduce a restriction site for the *Ban*I enzyme.

PCR1 and PCR2 reaction components and conditions were identical. The PCR assay was performed in a 20 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 2 mM $MgCl_2$, 2 µl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds (denaturation), 62°C for 30 seconds (annealing), 72°C for 30 seconds (extension); and followed by a final extension at 72°C for 7 minutes and soaking at 4°C. A PCR product with a size of 220 bp was expected from the PCR1

amplification. A PCR product with a size of 224 bp was expected from the PCR2 amplification.

The ABCB1 2677non-A allele was discriminated from the 2677A allele by digesting the product of PCR1 with *Rsa*I endonuclease. The PCR1 product was digested for at least 2 hours at 37°C in a 10 µl reaction volume containing 5 µl of PCR1 product, 5 U of *Rsa*I restriction enzyme, 1 µl of 10x NE buffer and an appropriate volume of molecular biology grade water. *Rsa*I cut the PCR1 product at position 5'-GT/AC-3', and produced 118-bp and 102-bp fragments for the ABCB1 2677non-A allele; and 118-bp, 82-bp and 20-bp fragments for the ABCB1 2677A allele.

The ABCB1 2677non-T allele was discriminated from the 2677T allele by digesting the product of PCR2 with *Ban*I endonuclease. The PCR2 product was digested for at least 2 hours at 37°C in a 10 µl reaction volume containing 5 µl of PCR2 product, 5 U of *Ban*I restriction enzyme, 1 µl of 10x NE buffer and an appropriate volume of molecular biology grade water. *Ban*I cut the PCR2 product at position 5'-G/GYRCC-3', and produced 198-bp and 26-bp fragments for the ABCB1 2677non-T allele. The 224-bp product of PCR2 remained uncut for the ABCB1 2677T allele.

The electrophoresis conditions for both PCR1 and PCR2 assays were identical. A total of 5 µl of digest product was mixed with 1 µl of loading dye and loaded on a 3.0% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis conditions were set at 80 V for 120 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination. Images from both PCR1 and PCR2 were analysed simultaneously to determine one of the 6 possible genotypes (G/G, G/T, T/T, G/A, A/A, T/A; FIGURE 3.8).

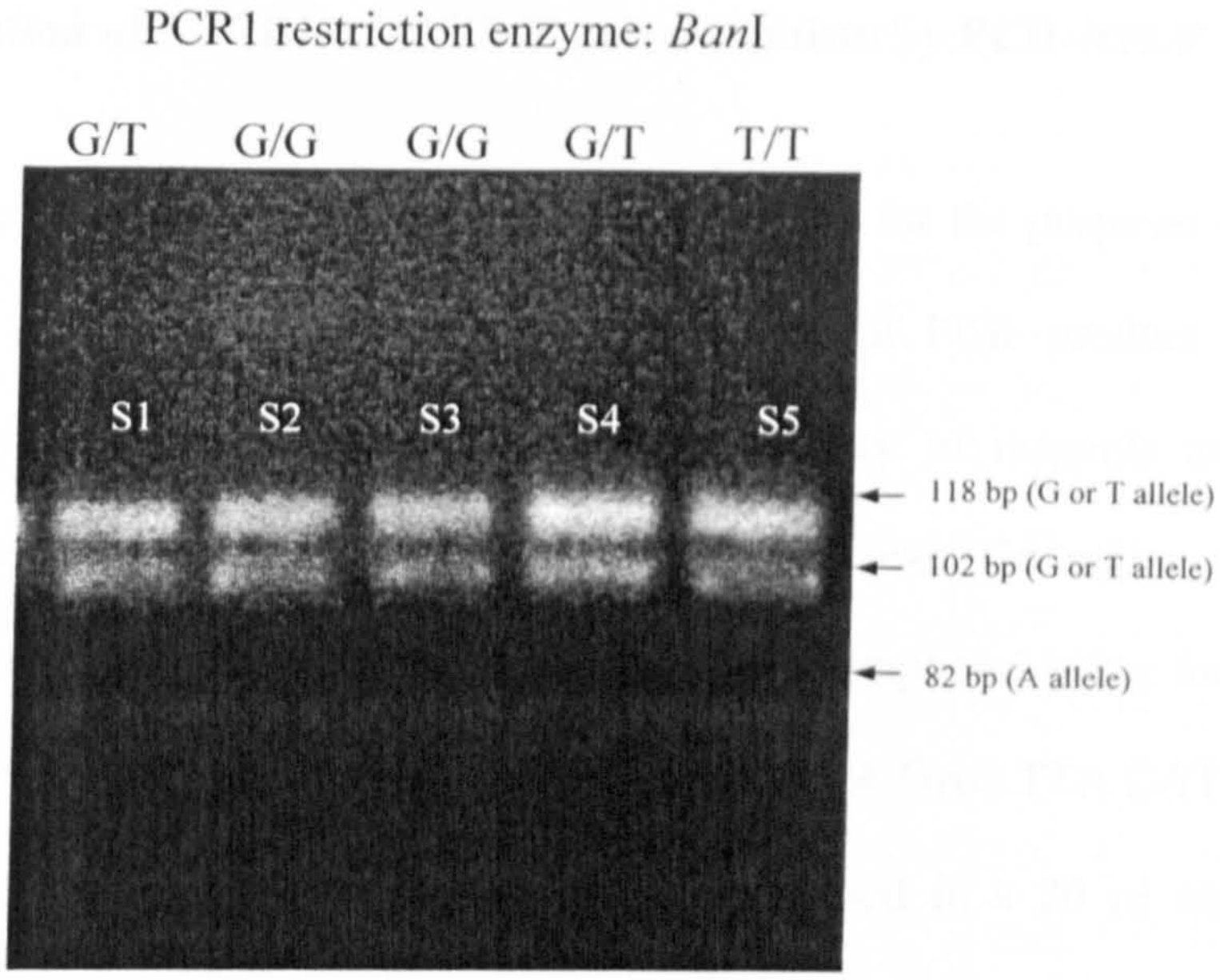

FIGURE 3.8. Electrophoresis patterns for ABCB1 c.2677G>T/A genotypes analysed by PCR-RFLP assay. PCR1 and PCR2 were carried-out in two separate reactions. The product of PCR1 was digested by *Ban*I and the product of PCR2 was digested by *Rsa*I. Digital images of the gels from both PCR-RFLP assays were examined simultaneously to determine the genotype. No fragment for the 2677A allele was observed in either gel. S1 to S5 represent individual subjects, their respective electrophoresis patterns, and ABCB1 c.2677G>T/A genotypes.

3.1.3.11 Identification of ABCB1 c.3435C>T polymorphism by PCR-RFLP

The method described by Hamdy *et al.* (2003b) was modified for the purposes of this study. Modification was undertaken to optimise the yield of PCR product which included alteration in the total reaction volume, the quantity of reagents and the annealing temperature (as described in section 2.3.5.2). The exon-26 region of the ABCB1 gene containing the c.3435C>T polymorphism was amplified using forward, 5'-ACT CTT GTT TTC AGC TGC TTG-3' and reverse, 5'-AGA GAC TTA CAT TAG GCA GTG ACT C-3' primers. The PCR assay was performed in a 20 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 1.5 mM $MgCl_2$, 2 µl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 94°C for 5 minutes; 33 cycles of 94°C for 30 seconds (denaturation), 56°C for 30 seconds (annealing), 72°C for 30 seconds (extension); and followed by a final extension at 72°C for 5 minutes and soaking at 4°C. A PCR product with a size of 231 bp was expected from the amplification.

The ABCB1 3435C allele was discriminated from the 3435T allele by digesting the PCR product with *Dpn*II endonuclease. The PCR product was digested for at least 2 hours at 37°C in a 20 µl reaction volume containing 10 µl of PCR product, 5 U of *Dpn*II restriction enzyme, 2 µl of 10x NE buffer and an appropriate volume of molecular biology grade water. *Dpn*II cut the PCR product at position 5'-/GATC-3' and produced 163-bp and 68-bp fragments for the ABCB1 3435T allele. The 231-bp PCR product remained uncut for the ABCB1 3435C allele.

A total of 5 µl of digest product was mixed with 1 µl of loading dye and loaded on a 2.5% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis conditions were set at 80 V for 120 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination (FIGURE 3.9).

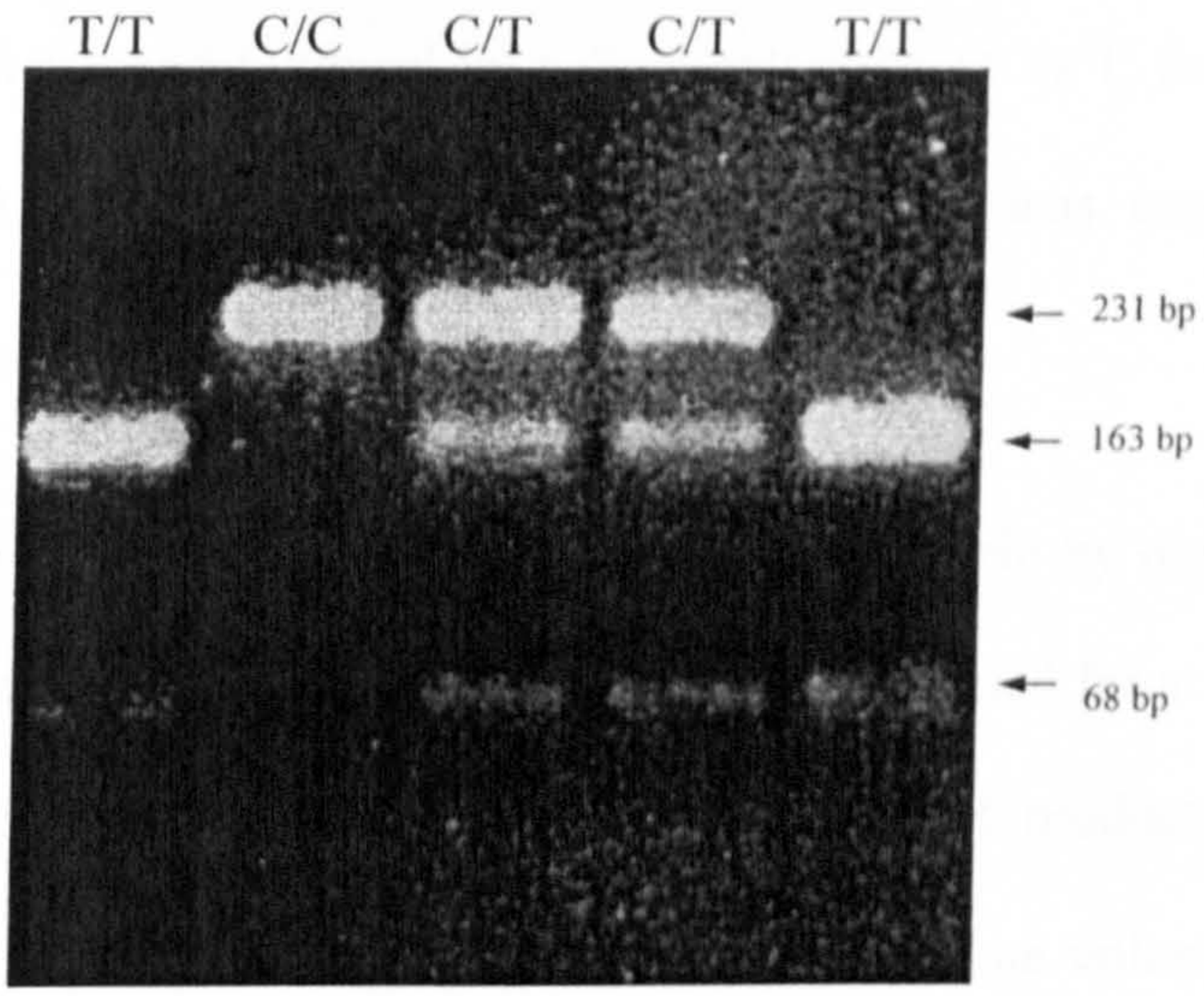

FIGURE 3.9. Electrophoresis patterns for ABCB1 c.3435C>T genotypes analysed by PCR-RFLP assay.

3.1.3.12 Identification of SCN2A c.56G>A polymorphism by PCR-RFLP

The method described by Nakayama *et al*. (2002) was modified for the purposes of this study. Modification was undertaken to optimise the yield of PCR product which included alteration in the total reaction volume, the quantity of reagents and the annealing temperature (as described in section 2.3.5.2). The exon-2 region of the SCN2A gene containing the c.56G>A polymorphism was amplified using forward, 5'-AAT CAC CTT TTA TTC TAA TGG TC-3' and reverse, 5'-CAG TGA AGG CAA

CTT GAC TAA GA-3' primers. The PCR assay was performed in a 20 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq polymerase, 20 pmol of each primer, 1.5 mM $MgCl_2$, 2 µl of 10x PCR buffer, 0.25 mM dNTP mix, and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 95°C for 10 minutes; 35 cycles of 94°C for 30 seconds (denaturation), 60°C for 30 seconds (annealing), 72°C for 30 seconds (extension); and followed by a final extension at 72°C for 7 minutes and soaking at 4°C. A PCR product with a size of 400 bp was expected from the amplification.

The SCN2A 56G allele was discriminated from the 56A allele by digesting the PCR product with *Scr*FI endonuclease. The PCR product was digested for at least 2 hours at 37°C in a 20 µl reaction volume containing 10 µl of PCR product, 5 U of *Scr*FI restriction enzyme, 2 µl of 10x NE buffer and an appropriate volume of molecular biology grade water. *Scr*FI cut the PCR product at position 5'-CC/NGG-3', and produced 178-bp, 130-bp, 64-bp and 28-bp fragments for the SCN2A 56G allele; and 206-bp, 130-bp and 64-bp fragments for the SCN2A 56A allele.

A total of 5 µl of digest product was mixed with 1 µl of loading dye and loaded on a 3.0% agarose gel containing 300 ng/ml ethidium bromide. The gel electrophoresis conditions were set at 80 V for 120 minutes. A digital image of the agarose gel was captured at the end of the electrophoresis process under ultraviolet transillumination (FIGURE 3.10).

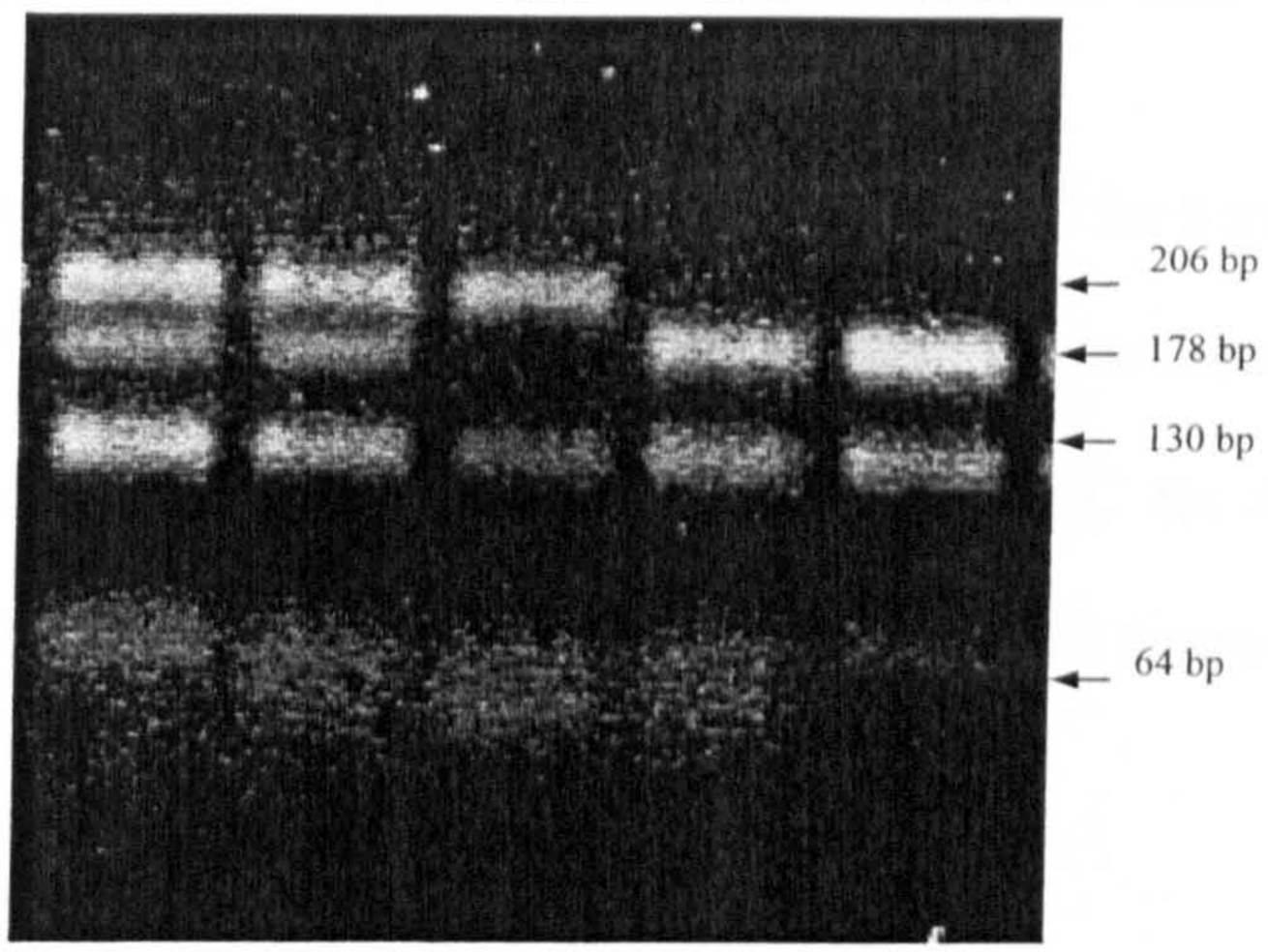

FIGURE 3.10. Electrophoresis patterns for SCN2A c.56G>A genotypes analysed by PCR-RFLP assay.

3.1.3.13 Identification of EPHX1 c.337T>C polymorphism by direct sequencing

The genotype distribution of EPHX1 c.337T>C identified by PCR-RFLP assay (section 3.1.3.6) was not consistent with HWE ($p < 0.05$). This was believed to be the result of the close proximity of a polymorphism at codon 119, and its consequent influence on the accuracy of genotyping by the RFLP technique. For this reason, a newly designed direct sequencing assay was employed to re-analyse all 400 DNA samples. Four steps of sample preparation are required before the DNA fragment can be analysed by the automated MegaBACE 1000^{TM} DNA Analysis System

PCR amplification of the target DNA fragment: Forward (5'-GTA GCC AGT GAT GTG G-3') and reverse (5'-CAT GTA TGT GTT CCT GCC TA-3') primers were used to amplify a DNA fragment of exon-3 of the EPHX1 gene. The PCR assay was performed in a 30 µl reaction volume containing 100 ng of genomic DNA, 1 U of Taq

polymerase, 20 pmol of each primer, 2 mM $MgCl_2$, 3 µl of 10x PCR buffer, 0.25 mM dNTP mix and an appropriate volume of molecular biology grade water.

The PCR conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds (denaturation), 60°C for 30 seconds (annealing), 72°C for 30 seconds (extension) and finally followed by 72°C for 5 minutes (final extension) and soaking at 4°C. A PCR product with a size of 542 bp was expected from the amplification.

Purification of PCR product: The purification step was based on protocol 000601v024 published by Agencourt Bioscience Corporation, Beverly, MA, USA (http://www.agencourt.com). The purification reaction was performed in a 96-well plate. In each well, a total of 15 µl of PCR product was mixed with 27 µl of a magnetic bead solution (AMPure®). The reaction was mixed thoroughly by vortexing for 30 seconds. The reaction mixtures were then left to incubate for 3-5 minutes at room temperature (20°C). The magnetic beads were separated from the reaction mixture by placing the plate onto a magnetic plate (SPRIPlate 96R®) for 5-10 minutes. The supernatant was aspirated from the reaction plate and discarded. With the reaction plate remaining on the SPRIPlate 96R®, 200 µl of 70% ethanol was dispensed into each well and the plate incubated for a further 30 seconds at room temperature (20°C). A total of two washes were performed using 70% ethanol. The reaction plate was then placed on the bench to air-dry, before 40 µl sterile water was added to each well for elution of the purified PCR product. The plate was vortexed for 30 seconds to promote maximum elution. The eluant was then used in the sequencing PCR step.

Sequencing PCR: The sequencing PCR process was based on DYEnamic ET Terminator Cycle Sequencing Kit (product instruction US81050PL 2002) by Amersham

Biosciences (http://www4.gelifesciences.com/aptrix/upp01077.nsf/content/). A 96-well plate was used to run the sequencing PCR. The PCR was performed in a 10 µl reaction volume containing 4 µl of purified DNA template, 5 pmol of the reverse primer (5'-CAT GTA TGT GTT CCT GCC TA-3'), 1 µl of DYEnamic® ET Terminator reagent, 3 µl of DYEnamic® ET Terminator dilution buffer and 1 µl of sterile water. The PCR conditions consisted of 25 cycles of 95°C for 20 seconds (denaturation), 50°C for 15 seconds (annealing) and 60°C for 60 seconds (extension). The sequencing PCR product was then cleaned using CleanSEQ® system (Agencourt Bioscience Corporation, Beverly, MA, USA).

Removal of dye-terminator: The dye-terminator removal process was based on protocol 000600v031 published by Agencourt Bioscience Corporation, Beverly, MA, USA (http://www.agencourt.com). The cleanup reaction was performed in a 96-well plate. In each well, 5 µl of the sequencing PCR product was mixed with 10 µl of magnetic bead solution (CleanSEQ®) and 31 µl of 85% ethanol. The magnetic beads were separated from the reaction mixture by placing the plate onto a SPRIPlate 96R® for 3 minutes. The clear supernatant was aspirated from the reaction plate and discarded. With the reaction plate remaining on the SPRIPlate 96R®, 100 µl of 85% ethanol was dispensed into each well and the plate incubated for 30 seconds at room temperature (20°C). The ethanol was then aspirated from the reaction plate and discarded. A total of two washes with 85% ethanol were performed. After the washes, the reaction plate was placed on the bench to air-dry, before 20 µl of sterile water was added to each well for elution. The eluant was then used in the MegaBACE 1000™ sequencing analysis.

MegaBACE 1000™ sequencing analysis: MegaBACE 1000™ DNA Analysis System utilised parallele capillary electrophoresis. High-pressure nitrogen gas was used to inject

the capillaries with linear polyacrylamide, a separation matrix. Eluant was loaded into the analyser in a 96-well plate and a electrical current was established at the tip of each capillary, where it came into contact with the sample. Samples were electro-kinetically injected into the linear polyacrylamide and travelled the length of the capillary during the course of the run. All sequencing samples were automatically base-called and presented as DNA sequencing chromatograms. The DNA sequencing signals for each genotype of the EPHX1 c.337T>C polymorphism are presented in FIGURE 3.11.

3.1.3.14 Predicting the net activity of mEH

The net activity of mEH was predicted on the basis of an association between EPHX1 gene variants and *in vitro* activity of mEH described by Hassett *et al.* (1994b). Individuals who carry the 337 + 416 diplotypes of C/C + A/A, C/C + A/G, T/C + A/A and C/C + G/G were predicted as having the low activity mEH, those with 337+416 diplotypes of T/T + A/A, T/C + A/G and T/C + G/G were predicted to have medium activity mEH, and those with 337+416 diplotypes of T/T + G/G and T/T + A/G were predicted to have high activity mEH. This categorical method was also described by Sarmanova *et al.* (2000).

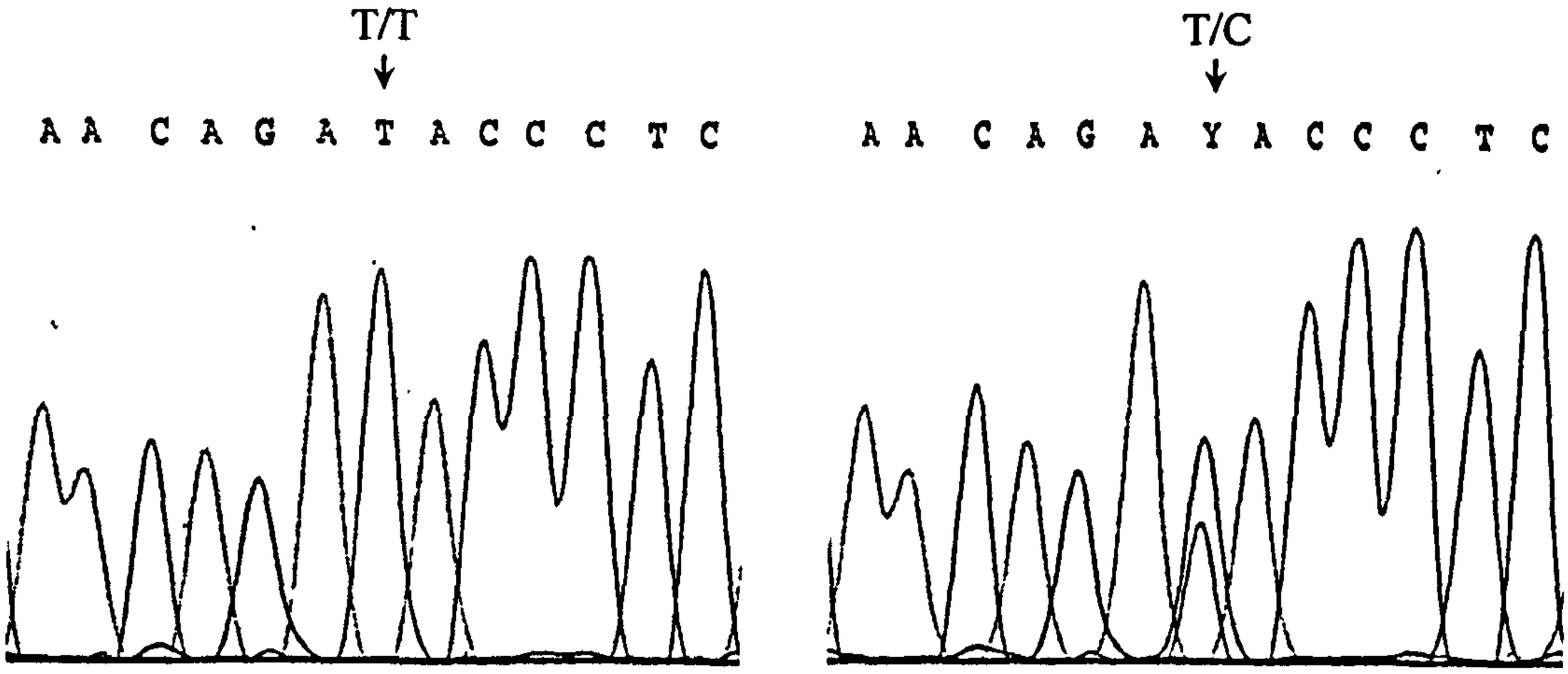

FIGURE 3.11. DNA sequencing signals of the four nucleotides around the EPHX1 c.337T>C polymorphism for the T/T, T/C, and C/C genotypes.

3.1.3.15 Comparison of allele and genotype distributions between ethnic groups

Allele and genotype distributions identified in the current study population were compared with data from other populations that are available in the literature. Fisher's Exact test or X^2 test was employed for this purpose. A *p*-value of greater than 0.05

indicated that no significant differences in the allele and genotype distributions between study populations.

3.1.3.16 Hardy-Weinberg Equilibrium test

The HWE test is one of the quality control measures for genotyping method as suggested by several authors (Xu *et al.*, 2002; Hosking *et al.*, 2004; Salanti *et al.*, 2005; Wittke-Thompson *et al.*, 2005). Adherence to HWE was tested using an online calculator, freely available on an established website (The Online Encyclopedia for Genetic Epidemiology, available from <http://www.genes.org.uk/software/hardy-weinberg.shtml>). The online calculator compared the genotype distribution identified in the current study with a distribution predicted by HWE using X^2 test. A *p*-value of greater than 0.05 indicated that the observed genotype distribution was consistent with HWE assumptions.

3.1.4 Results

3.1.4.1 Allele and genotype distributions of CYP3A4 g.-392A>G and CYP3A5 g.6986A>G polymorphisms

Allele and genotype distributions of the CYP3A4 g.-392A>G and CYP3A5 g.6986A>G polymorphisms in comparison with other populations are presented in TABLE 3.1 and TABLE 3.2, respectively. The CYP3A4 -392A and CYP3A5 6986G alleles were predominant in the 400-strong study population, with allele frequencies of 96.5% and 92.6%, respectively. Only one individual was homozygous for the CYP3A4 -392G/G genotype and four individuals were homozygous for the CYP3A5 6986A/A genotype. The allele and genotype distributions of CYP3A4 g.-392A>G and CYP3A5 g.6986A>G polymorphisms observed in the current study were consistent with HWE ($p > 0.05$) and

comparable ($p > 0.05$) with other Caucasian populations but different ($p < 0.05$) from previously published Oriental and African-American populations.

TABLE 3.1. Allele and genotype distributions of the CYP3A4 g-392A>G polymorphism in comparison to previously published data from different populations.

Study	Frequency					
	Allele		Genotype			
	A	G	A/A	A/G	G/G	Ref.
Current study†, n = 400	**0.965**	**0.035**	**0.932**	**0.065**	**0.003**	
Chinese & Japanese‡, n = 80	1.000	0.000	1.000	0.000	0.000	1
Hispanic‡, n = 121	0.890	0.110	0.800	0.180	0.020	1
African-American‡, n = 116	0.455	0.545	0.190	0.530	0.280	1
Caucasian (German)□, n = 432	0.971	0.029	0.944	0.054	0.002	2
Caucasian (American)□, n = 230	0.935	0.065	0.891	0.087	0.022	3
African-American‡, n = 64	0.477	0.523	0.281	0.391	0.328	3

†A *vs* G, 0.965 *vs* 0.035 (95% CI, 0.024 – 0.050); HWE: $X^2 = 0.57$, $p = 0.752$; Allele and genotype distributions in comparison with current study findings, ‡$p < 0.05$ and □$p > 0.05$; References: 1 = Paris *et al.* (1999), 2 = Dally *et al.* (2003), 3 = Zeigler-Johnson *et al.* (2004).

TABLE 3.2. Allele and genotype distributions of the CYP3A5 g.6986A>G polymorphism in comparison to previously published data from different populations.

Study	Frequency					
	Allele		Genotype			
	A	G	A/A	A/G	G/G	Ref.
Current study†, n = 400	**0.074**	**0.926**	**0.010**	**0.128**	**0.862**	
Japanese‡, n = 400	0.232	0.768	0.070	0.325	0.605	1
Caucasian (German)□, n = 432	0.063	0.937	0.002	0.121	0.877	2
Caucasian (American)□, n = 230	0.048	0.952	0.011	0.073	0.915	3
African-American‡, n = 64	0.638	0.362	0.446	0.385	0.169	3

†A *vs* G, 0.074 *vs* 0.926 (95% CI, 0.906 – 0.942); HWE: $X^2 = 1.78$, $p = 0.410$; Allele and genotype distributions in comparison with current study findings, ‡$p < 0.05$ and □$p > 0.05$; References: 1 = Sata *et al.* (2000), 2 = Dally *et al.* (2003), 3 = Zeigler-Johnson *et al.* (2004).

3.1.4.2 Allele and genotype distributions of CYP1A2 g.5734C>A polymorphism

Allele and genotype distributions of the CYP1A2 g.5734C>A polymorphism in comparison with other populations are presented in TABLE 3.3. Most (93.2%) of the 400-strong study population carried at least one 5734A allele. Only small numbers were homozygous for the 5734C allele (6.8%). The allele and genotype distributions of the CYP1A2 g.5734C>A polymorphism observed in the 400-strong study population were

consistent with HWE ($p > 0.05$), and comparable ($p > 0.005$) with other Caucasian populations.

TABLE 3.3. Allele and genotype distributions of the CYP1A2 g.5734C>A polymorphism in comparison to previously published data from different populations.

Study	Frequency					
	Allele		Genotype			
	C	A	C/C	A/C	A/A	Ref.
Current study†, n = 400	**0.275**	**0.725**	**0.068**	**0.415**	**0.517**	
Caucasian (German)□, n = 236	0.322	0.678	0.102	0.441	0.457	1
Caucasian (Italian)□, n = 95	0.332	0.668	0.095	0.474	0.431	2
Caucasian (Dutch)□, n = 94	0.280	0.720	0.080	0.400	0.520	3
Egyptian□, n = 212	0.320	0.680	0.108	0.420	0.472	4
Hispanic & Mestizo□, n = 932	0.300	0.700	0.090	0.420	0.490	5
Japanese‡, n = 159	0.386	0.614	0.163	0.447	0.390	6

†C vs A, 0.27 vs 0.73 (95% CI, 0.69 – 0.77); HWE: $X^2 = 0.66$, p = 0.717; Allele and genotype distributions in comparison with current study findings, ‡$p < 0.05$ and □$p > 0.05$; References: 1 = Sachse *et al.* (1999), 2 = Pavanello *et al.* (2005), 3 = Moonen *et al.* (2005), 4 = Hamdy *et al.* (2003a), 5 = Cornelis *et al.* (2005), 6 = Obase *et al.* (2003).

3.1.4.3 Allele and genotype distributions of EPHX1 c.337T>C and c.416A>G polymorphisms

When analysed with PCR-RFLP, the genotype distribution of EPHX1 c.337T>C was inconsistent with HWE ($p < 0.05$). This was believed to be the result of the close proximity of a polymorphism at codon 119, and its consequent influence on the accuracy of genotyping by the RFLP technique. For this reason, the 400-strong study population was re-analysed by direct sequencing. The genotype distribution of EPHX1 c.337T>C identified this method was consistent with HWE ($p > 0.05$), was deemed to be accurate, and has subsequently been reported and employed in the remainder of this thesis.

Allele and genotype distributions of the EPHX1 c.337T>C and EPHX1 c.416A>G polymorphisms in comparison with other populations are presented in TABLE 3.4 and TABLE 3.5, respectively. The allele and genotype frequencies of EPHX1 337T>C and EPHX1 416A>G polymorphisms were consistent with HWE ($p > 0.05$) and comparable ($p > 0.05$) to those of other Caucasian populations. Most of study subjects were predicted to carry low and medium net activities of mEH, accounting for 39.5% and 40.5% of the 400-strong study population, respectively (FIGURE 3.12). Patients who carried a diplotype of 337T/T and 416A/A dominated the medium mEH activity group (69.8%).

3.1.4.4 Allele and genotype distributions of UGT2B7 c.802C>T polymorphism

Allele and genotype distributions of the UGT2B7 c.802C>T polymorphism in comparison with other populations are presented in TABLE 3.6. The genotype distribution of the UGT2B7 c.802C>T polymorphism in the 400-strong study population was consistent with HWE ($p > 0.05$) and comparable ($p > 0.05$) with other

Caucasian populations. Allele frequency of the UGT2B7 c.802C>T polymorphism approached equivalence (50%) for the C and T substitutions. The 802C/T genotype (54.7%) was the most common genotype observed in the study population.

TABLE 3.4. Allele and genotype distributions of the EPHX1 c.337T>C polymorphism in comparison to previously published data from different populations.

Study	Frequency					
	Allele		Genotype			
	T	C	T/T	T/C	C/C	Ref.
Current study[§], n = 400 (PCR-RFLP)	0.640	0.360	0.468	0.345	0.187	
Current study[†], n = 400 (Direct Sequencing)	**0.689**	**0.311**	**0.482**	**0.413**	**0.105**	
Caucasian (American)[□], n = 458	0.718	0.282	0.517	0.402	0.081	1
African-American[‡], n = 242	0.791	0.209	0.632	0.318	0.050	1
Caucasian (Australian)[□], n = 496	0.672	0.328	0.452	0.439	0.109	2
Chinese[‡], n = 252	0.443	0.557	0.302	0.282	0.416	3
Japanese[‡], n = 172	0.558	0.442	0.326	0.465	0.209	4

[§]The genotype distribution identified by PCR-RFLP was inconsistent with HWE ($X^2 = 25.26$, $p < 0.001$) and was reanalysed using direct sequencing; [†]T *vs* C, 0.69 *vs* 0.31 (95% CI, 0.28 – 0.34); HWE: $X^2 = 0.57$, $p = 0.750$; Allele and genotype distributions in comparison with current study findings, [‡]$p < 0.05$ and [□]$p > 0.05$; References: 1 = London *et al.* (2000), 2 = Gsur *et al.* (2003), 3 = Zhang *et al.* (2003), 4 = Budhi *et al.* (2003).

TABLE 3.5. Allele and genotype distributions of the EPHX1 c.416A>G polymorphism in comparison to previously published data from different populations.

	Frequency					
	Allele		Genotype			
Study	A	G	A/A	A/G	G/G	Ref.
Current study†, n = 400	**0.806**	**0.194**	**0.650**	**0.312**	**0.038**	
Caucasian (American)□, n = 458	0.806	0.192	0.659	0.297	0.044	1
African-American‡, n = 242	0.709	0.291	0.492	0.434	0.074	1
Caucasian (Australian)□, n = 496	0.822	0.178	0.677	0.290	0.032	2
Japanese□, n = 172	0.848	0.152	0.744	0.209	0.067	3

†A *vs* G, 0.81 *vs* 0.19 (95% CI, 0.17 – 0.22); HWE: $X^2 < 0.01$, $p = 0.999$; Allele and genotype distributions in comparison with current study findings, ‡$p < 0.05$ and □$p > 0.05$; References: 1 = London *et al.* (2000), 2 = Gsur *et al.* (2003), 3 = Budhi *et al.* (2003).

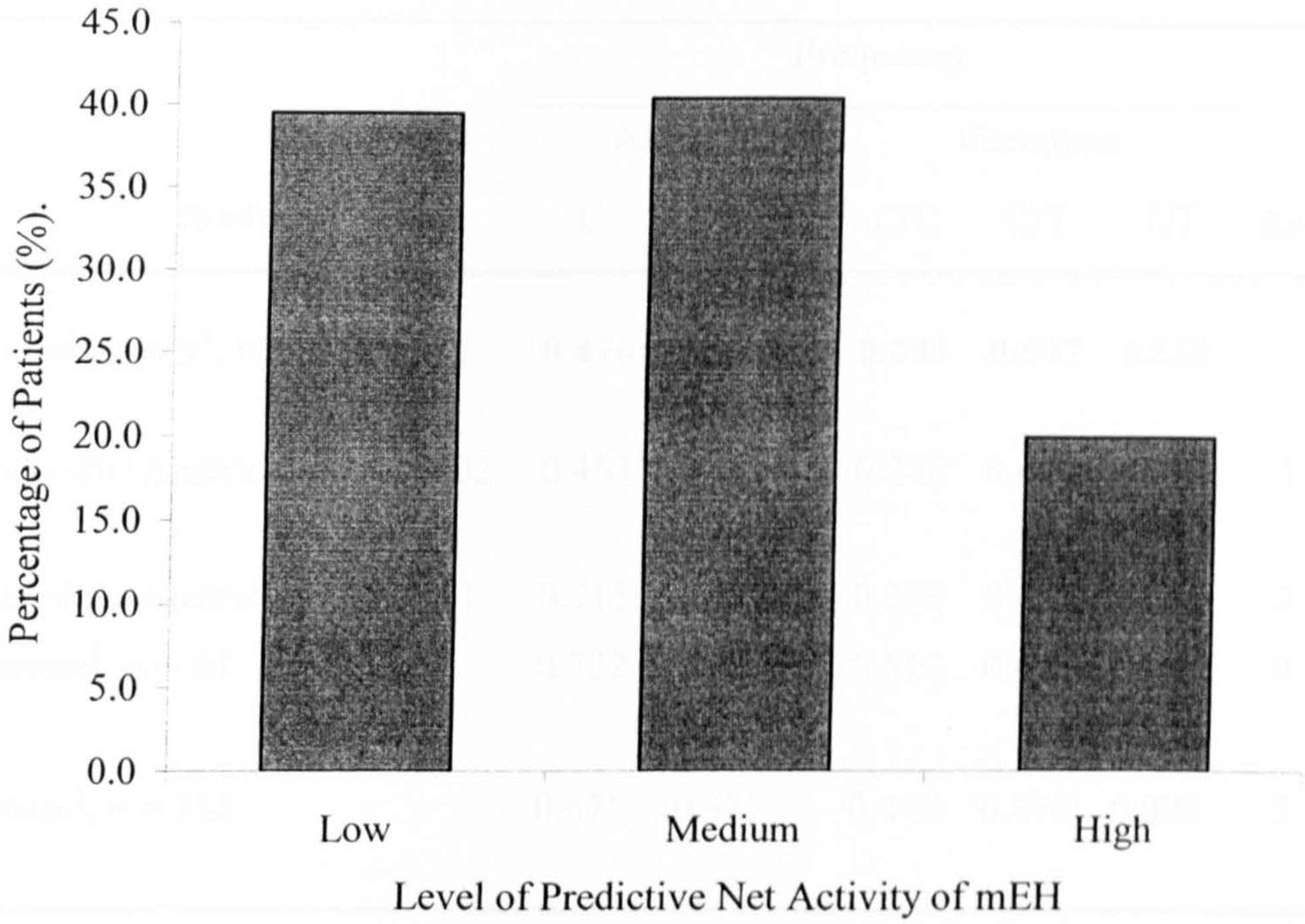

FIGURE 3.12. The distribution of predictive net activity of mEH in the 400-strong study population. The net activity of mEH was predicted using a series of diplotypes derived from the EPHX1 c.337T>C and EPHX1 c.416A>G polymorphisms. Low activity mEH = C/C+A/A, C/C+A/G, T/C+A/A and C/C+G/G, medium activity mEH = T/C+A/G, T/T+A/A and T/C+G/G, and high activity mEH = T/T+A/G and T/T+G/G. References: Hassett *et al.* (1994b); Sarmanova *et al.* (2000).

TABLE 3.6. Allele and genotype distributions of the UGT2B7 c.802C>T polymorphism in comparison to previously published data from different populations.

Study	Frequency					
	Allele		Genotype			
	C	T	C/C	C/T	T/T	Ref.
Current study†, n = 400	**0.476**	**0.524**	**0.203**	**0.547**	**0.250**	
Caucasian (American)□, n = 202	0.463	0.537	0.218	0.490	0.292	1
Caucasian (Australian)□, n = 91	0.511	0.489	0.275	0.472	0.253	2
Japanese‡, n = 84	0.732	0.268	0.512	0.440	0.048	2
Chinese‡, n = 218	0.675	0.325	0.440	0.470	0.090	3

†C *vs* T, 0.48 *vs* 0.52 (95% CI, 0.47 – 0.57); HWE: $X^2 = 3.80$, $p = 0.149$; Allele and genotype distributions in comparison with current study findings, ‡$p < 0.05$ and □$p > 0.05$; References: 1 = Lampe *et al.* (2000), 2 = Bhasker *et al.* (2000), 3 = Lin *et al.* (2005).

3.1.4.5 Allele and genotype distributions of ABCB1 c.1236C>T, c.2677G>T/A and c.3435C>T polymorphisms

Allele and genotype distributions of the ABCB1 c.1236C>T, ABCB1 c.2677G>T/A and ABCB1 c.3435C>T polymorphisms in comparison with other populations are presented in TABLE 3.7, TABLE 3.8 and TABLE 3.9, respectively. The allele and genotype distributions of the ABCB1 c.1236C>T, ABCB1 c.2677G>T/A and ABCB1 c.3435C>T polymorphisms in the 400-strong study population were consistent with HWE ($p > 0.05$) and comparable ($p > 0.05$) to those of other Caucasian populations. The majority

of patients were heterozygous for these polymorphisms (47.0% for 1236C/T, 44.3% for 2677G/T and 48.5% for 3435C/T).

TABLE 3.7. Allele and genotype distributions of the ABCB1 c.1236C>T polymorphism in comparison to previously published data from different populations.

Study	Frequency					
	Allele		Genotype			
	C	T	C/C	C/T	T/T	Ref.
Current study†, n = 400	**0.520**	**0.480**	**0.285**	**0.470**	**0.245**	
Caucasian (American)□, n = 100	0.540	0.460	-	-	-	1
African-American‡, n = 100	0.805	0.195	-	-	-	1
Chinese‡, n = 92	0.380	0.620	-	-	-	1
Malays‡, n = 92	0.375	0.625	-	-	-	1
Indians‡, n = 95	0.347	0.653	-	-	-	1
Japanese‡, n = 69	0.348	0.652	0.101	0.493	0.406	2

†C vs T, 0.52 vs 0.48 (95% CI, 0.43 – 0.53); HWE: $X^2 = 1.37$, $p = 0.504$; Allele and genotype distributions in comparison with current study findings, ‡$p < 0.05$ and □$p > 0.05$; '-' mark indicates unavailable data; References: 1 = Tang *et al.* (2004), 2 = Goto *et al.* (2002).

TABLE 3.8. Allele and genotype distributions of the ABCB1 c.2677G>T/A polymorphism in comparison to previously published data from different populations.

Study	Frequency									Ref.
	Allele			Genotype						
	G	T	A	G/G	G/T	T/T	G/A	T/A	A/A	
Current study†, n = 400	**0.545**	**0.449**	**0.006**	**0.320**	**0.443**	**0.225**	**0.007**	**0.005**	**0.000**	
Caucasian (Polish)□, n = 141	0.574	0.408	0.018	0.333	0.461	0.170	0.021	0.015	0.000	1
Caucasian (German)□, n = 51	0.520	0.450	0.030	0.236	0.529	0.176	0.039	0.020	0.000	2
Caucasian (Swedish)□, n = 200	0.560	0.420	0.020	-	-	-	-	-	-	3
Korean‡, n = 126	0.642	0.377	0.171	0.190	0.381	0.135	0.143	0.103	0.048	4
African-American‡, n = 200	0.895	0.100	0.005	-	-	-	-	-	-	5

†HWE: $X^2 = 3.668$, $p = 0.300$; Allele and genotype distributions in comparison with current study findings, ‡$p < 0.05$ and □$p > 0.05$;

References: 1 = Tan *et al.* (2004), 2 = Meissner *et al.* (2004), 3 = Green *et al.* (2006), 4 = Park *et al.* (2007), 5 = Kroetz *et al.* (2003).

TABLE 3.9. Allele and genotype distributions of the ABCB1 c.3435C>T polymorphism in comparison to previously published data from different populations.

Study	Frequency					
	Allele		Genotype			
	C	T	C/C	C/T	T/T	Ref.
Current study†, n = 400	**0.425**	**0.575**	**0.183**	**0.485**	**0.332**	
Caucasian (Scottish)□, n = 190	0.480	0.520	0.240	0.480	0.280	1
Japanese‡, n = 69	0.557	0.443	0.304	0.507	0.188	2
Chinese‡, n = 98	0.460	0.540	0.240	0.440	0.320	3
Malays‡, n = 99	0.480	0.520	0.250	0.460	0.280	3
Indians‡, n = 93	0.380	0.620	0.180	0.390	0.430	3

†C vs T, 0.42 vs 0.58 (95% CI, 0.53 – 0.63); HWE: $X^2 = 0.02$, $p = 0.988$; Allele and genotype distributions in comparison with current study findings, ‡$p < 0.05$ and □$p > 0.05$; References: 1 = Ameyaw *et al.* (2001), 2 = Goto *et al.* (2002), 3 = Balram *et al.* (2003).

3.1.4.6 Allele and genotype distributions of SCN2A c.56G>A polymorphism

Allele and genotype distributions of the SCN2A c.56G>A polymorphism in comparison with a Japanese population are presented in TABLE 3.10. The genotype distribution of the SCN2A c.56G>A polymorphism in the 400-strong study population was consistent with HWE ($p > 0.05$) and comparable ($p > 0.05$) with the reported Japanese population.

The 56G/G genotype (87.5%) was the most common genotype observed in the study population.

TABLE 3.10. Allele and genotype distributions of the SCN2A c.56G>A polymorphism in comparison to previously published data from a different population.

Study	Frequency					
	Allele		Genotype			
	G	A	G/G	G/A	A/A	Ref.
Current study†, n = 400	**0.931**	**0.069**	**0.875**	**0.112**	**0.013**	
Japanese□, n = 100	0.940	0.060	0.880	0.120	0.000	1

†G *vs* A, 0.93 *vs* 0.07 (95% CI, 0.05 – 0.10); HWE: $p = 0.052$; Allele and genotype distributions in comparison with current study findings, □p > 0.05; References: 1 = Nakayama *et al.* (2002).

3.1.5 Discussion

3.1.5.1 The prevalence of common polymorphisms in DME, DTP and sodium channel genes in comparison with other ethnic groups

The frequencies of minor alleles ranged between 3.5% (CYP3A4 -392G) and 48.0% (ABCB1 1236T). A relatively low frequency of the CYP3A4 g.-392G allele has also been observed in many other studies (Paris *et al.*, 1999; Sata *et al.*, 2000; Kuehl *et al.*, 2001; Gracia-Martin *et al.*, 2002; Hamzeiy *et al.*, 2002; Dally *et al.*, 2003; Zeigler-

Johnson *et al.*, 2004). This low frequency may indicate that, in Caucasian populations, a large sample size is required to identify a statistically meaningful association between the CYP3A4 g.-392A>G variant and any respective phenotype. The g.-392A>G allele and genotype frequencies for the 400-strong study population were distinct from previously published data in Asian, Hispanic and African-American populations (Paris *et al.*, 1999; Chelule *et al.*, 2003; Zeigler-Johnson *et al.*, 2004). In contrast, the non-functional CYP3A5 genotype (6986G/G) has been identified at a high frequency (30 – 87%) in many populations (Fukuen *et al.*, 2002; van Schaik *et al.*, 2002; King *et al.*, 2003; Lee *et al.*, 2003; Schuetz *et al.*, 2004), including the current study population. These CYP3A5 variant allele and genotype frequencies are similar to those of other studies performed in Caucasian populations (van Schaik *et al.*, 2002; King *et al.*, 2003; Lee *et al.*, 2003; Schuetz *et al.*, 2004), but different to those of Japanese and African-American populations (Fukuen *et al.*, 2002; Zeigler-Johnson *et al.*, 2004). These findings may signify that, in most Caucasian individuals, including the current 400-strong study population, CYP3A activity is mainly dependent on CYP3A4. However, a contribution of the CYP3A5 protein to overall CYP3A activity may be important in around 14% of individuals who carry at least one CYP3A5 6986A allele.

The g.5734C>A polymorphism is the most studied CYP1A2 gene variant (Sachse *et al.*, 1999; Moonen *et al.*, 2005; Pavanello *et al.*, 2005). In the current study population, the genotype distribution of the CYP1A2 g.5734C>A polymorphism is almost identical to that of other populations. The high frequency of the 5734A allele observed in our study was similarly reported for other Caucasian, Egyptian and Hispanic populations (Sachse *et al.*, 1999; Hamdy *et al.*, 2003a; Cornelis *et al.*, 2005; Pavanello *et al.*, 2005). In contrast, a study performed in Japanese subjects has reported a much higher 5734C/C frequency than in other ethnic populations (Obase *et al.*, 2003).

mEH plays a vital role in metabolising drugs and detoxifying environmental pollutants (de Vries & Janssen, 2003). The common EPHX1 gene variants, c.337T>C and c.416A>G, that express polymorphic mEH have been associated with the risk of developing several types of cancers and other diseases (London *et al.*, 2000; Wong *et al.*, 2000; Ulrich *et al.*, 2001; Zhou *et al.*, 2001; Cajas-Salazar *et al.*, 2003). The EPHX1 337T and EPHX1 416A alleles predominate in the majority of patients in the current study population. EPHX1 c.337T>C and c.416A>G allele and genotype distributions observed in the 400-strong study population were similar to those of other Caucasian populations (Smith & Harrison, 1997; London *et al.*, 2000; Zhou *et al.*, 2001; Cajas-Salazar *et al.*, 2003; Gsur *et al.*, 2003). However, the 337C allele frequency was different from that of previously published Chinese, Japanese and African-American populations (Takeyabu *et al.*, 2000; Budhi *et al.*, 2003; Zhang *et al.*, 2003), whereas the 416G allele frequency was different only to that of an African-American population (London *et al.*, 2000).

It has been shown that the expression and activity of mEH is influenced by the c.337T>C and c.416A>G polymorphisms (Hassett *et al.*, 1994b; Maekawa *et al.*, 2003). Therefore, diplotypes of these polymorphisms have been used to predict the net activity of mEH (Smith & Harrison, 1997; Sarmanova *et al.*, 2000; Takeyabu *et al.*, 2000; Zhou *et al.*, 2001; Lebailly *et al.*, 2002; Sonzogni *et al.*, 2002; Cajas-Salazar *et al.*, 2003). Most of the 400-strong study population were predicted to have low and medium activity mEH, which associates mainly with the T/C-A/A and T/T-A/A diplotypes, respectively. However, the frequency of patients with high net mEH activity may be important as they accounted for up to 20% of the study population. The implications of this are on the clinical pharmacology of drugs which are metabolised by this pathway.

The most studied UGT2B7 variant is the c.802C>T polymorphism (Sawyer *et al.*, 2003; Wiener *et al.*, 2004; Lin *et al.*, 2005; Thibaudeau *et al.*, 2006). Allele and genotype distributions of the UGT2B7 c.802C>T polymorphisms in the current study population were comparable with other Caucasian populations from two different continents, North America and Australia (Bhasker *et al.*, 2000; Lampe *et al.*, 2000). However, the prevalence of the 802C/C genotype was 2-fold higher in the present study than that reported in Oriental populations (Bhasker *et al.*, 2000; Saeki *et al.*, 2004; Lin *et al.*, 2005).

The most widely investigated ABCB1 gene variants are the c.1236C>T, c.2677G>T/A and c.3435C>T polymorphisms. In the 400-strong study population, the 1236T, 2677T/A and 3435C were identified as the minor alleles. The variant allele and genotype distributions were similar to those reported in other Caucasian populations (Hoffmeyer *et al.*, 2000; Tan *et al.*, 2004b; Tang *et al.*, 2004), but different from those in Asian populations (Goto *et al.*, 2002; Balram *et al.*, 2003; Park *et al.*, 2007). A previous study employing a non-epileptic Scottish population reported distributions of c.3435C>T alleles and genotypes that were comparable to the current study (Ameyaw *et al.*, 2001). A significant difference in allele and genotype distributions of the ABCB1 c.1236C>T polymorphism is observed between Caucasians and African-Americans (Tang *et al.*, 2004). Although pharmacogenetic studies in epilepsy have focused on these specific polymorphisms, the functional significance of ABCB1 variants remains controversial (Siddiqui *et al.*, 2003; Tan *et al.*, 2004a; Sills *et al.*, 2005; Kim *et al.*, 2006). Nevertheless, several studies have suggested that haplotypes based on these three SNPs may be useful in predicting drug resistance in epilepsy (Hung *et al.*, 2005) An investigation focusing specifically on CBZ has shown that the T-T-T haplotype at the 1236, 2677 and 3435 positions of the ABCB1 gene was associated with a better response to CBZ treatment (Seo *et al.*, 2006a).

The SCN2A c.56G>A polymorphism has previously been associated with febrile seizures in children (Nakayama *et al.*, 2002). Information regarding the prevalence of SCN2A c.56G>A variants is limited, especially in Caucasian populations. In the current study, the 56A allele was identified in just 1.3% of the population. Unlike most other SNPs reported here, the SCN2A c.56G>A allele and genotype distributions in the 400-strong study population were comparable with a Japanese population (Nakayama *et al*, 2002).

Differences in allele and genotype distributions between ethnicities are not uncommon (Tishkoff & Kidd, 2004). In general, most of the variant allele and genotype distributions identified in the 400-strong study population were different from those reported for non-Caucasian populations. Greater differences were observed between populations of Caucasian and African ancestry than between Caucasian and Asian populations. This might be explained by the length of genetic divergence, which is approximately twice as long between Caucasoid and Negroid than between Caucasoid and Mongoloid (Ameyaw *et al.*, 2001). Genetic divergence is the process of one species diverging over time into more than one species: Passing small random characteristic changes over time from one generation to the next generations. Genetic divergence operates on a genetic level favoring 2 or more alleles or 2 or more mix of alleles over the original mixture of alleles in a population. There is increasing evidence that different ethnic groups demonstrate significant differences in SNP distribution and linkage disequilibrium profiles (Tishkoff & Kidd, 2004). Furthermore, the frequency of minor alleles observed for CYP3A4 g.-392A>G, CYP3A5 g.6896A>G and SCN2A c.56G>A is low, which suggests that a large sample size is required to obtain sufficient statistical power in any study comparing the effects of the major and minor alleles at these loci.

3.1.5.2 Hardy-Weinberg equilibrium test

With the exception of the PCR-RFLP derived data for EPHX1 c.337T>C, the genotype distributions of all polymorphisms reported in this project were consistent with HWE. This would indicate that the distribution of variant genotypes in the 400-strong study population did not violate HWE assumptions and that there was no apparent systematic error in the genotyping assays (Xu *et al.*, 2002; Hosking *et al.*, 2004; Salanti *et al.*, 2005). As reported in section 3.1.4.3, the genotype distribution of EPHX1 c.337T>C polymorphism identified by PCR-RFLP assay was inconsistent with HWE. Re-analysis using a direct sequencing technique redressed this inconsistency. This would suggest that the PCR-RFLP assay is not the optimal method for identification of EPHX1 c.337T>C polymorphisms. Other investigators have reported similar difficulties in detecting this polymorphism by PCR-RFLP (Takeyabu *et al.*, 2000; Gsur *et al.*, 2003; Godderis *et al.*, 2004). It is believed that the recognition site for the *EcoR*V restriction enzyme may be altered when the mismatched reverse primer anneals to another SNP (c.357G>A) adjacent to the c.337T>C polymorphism (Takeyabu *et al.*, 2000; Gsur *et al.*, 2003). As a result, the DNA fragment amplified loses the recognition site for *EcoR*V and the prevalence of the 337C/C genotype is overestimated. A modified reverse primer containing a second mismatched nucleotide for the c.357G>A site has been suggested to improve the performance of PCR-RFLP-based analysis (Budhi *et al.*, 2003). However, the effectiveness of this second mismatched nucleotide in improving the accuracy of the assay remains questionable. This was the approach employed in the current study without significant advantage. Any conclusions from previous studies which utilised a PCR-RFLP assay to identify the EPHX1 c.337T>C polymorphism may be undermined and should be interpreted with caution.

3.1.6 Conclusion

The prevalences of common polymorphisms in genes encoding DMEs, DTPs and a voltage-gated sodium channel in a West of Scotland epilepsy population are comparable with data previously reported for other Caucasian populations. The heredity of common polymorphisms in DME, P-gp and sodium channel genes are consistent with HWE assumptions. Based on HWE, no apparent systematic errors in the genotyping assays were identified, except in the PCR-RFLP assay for EPHX1 c.337T>C.

CHAPTER 4

PHARMACOGENETICS OF CARBAMAZEPINE

4.1 Genetic Predictors of Carbamazepine Optimal Dose

4.1.1 Introduction

The response to CBZ therapy varies from patient to patient; some will have full seizure control and others will fail to show any response. Finding the optimal dose of CBZ that maintains complete seizure control with minimal side effects is a challenge. The CBZ optimal dose is commonly reached by dosage adjustment based on the level of clinical response, the occurrence of side effects, and the effects of autoinduction. Patients that show modest but progressive seizure reductions might undergo continuous dosage adjustment for more than 1 year before the optimal dose is finally found. Within this period, quality of life might be compromised by ongoing seizures. Most patients are controlled on 600 mg/day but some patients may require up to 1600 mg/day of CBZ to attain complete seizure control (Kwan & Brodie, 2001). Patients with a high dose requirement are not easily distinguished from complete non-responders. Therefore, it may be useful to determine the individual CBZ dosage requirement in order to limit unnecessary or unreasonable titration to high doses of CBZ.

Some studies have reported that optimum treatment response and target therapeutic concentrations of CBZ are achieved with a broad range of doses (200 - 1600 mg/day; Brodie *et al.*, 1995; Chadwick, 1999; Kwan & Brodie, 2001). The variability in dosage requirement is believed to be associated with interindividual differences in CBZ pharmacokinetics and pharmacodynamics. CBZ pharmacokinetics are predominantly influenced by the DMEs (Browne, 1998; Kim, 2002b; Ramachandran & Shorvon, 2003), while on the other hand, CBZ pharmacodynamics are primarily affected by the function of ion channels.

Polymorphisms in genes that encode DMEs and $Na_v1.2$ sodium channel could alter the expression and function of their respective proteins (as described in Chapter 3) and, in turn, affect CBZ metabolism and the conformation of the sodium channel. These may consequently influence CBZ concentrations in plasma and in brain and sodium channel sensitivity to CBZ. As such, common polymorphisms in the DME and SCN2A genes may be able to predict the optimal dose of CBZ.

4.1.2 Aims

This study was designed to evaluate the association between common polymorphisms in CYP3A4, CYP3A5, CYP1A2, EPHX1, UGT2B7 and SCN2A genes and the optimal dose of CBZ. Clinical factors such as age and gender were also evaluated as additional candidate predictors.

4.1.3 Methods

4.1.3.1 Definitions

The optimal dose was defined as the final dose given to a patient that successfully maintained optimal seizure control without intolerable side effects. Optimal seizure control was defined as complete seizure freedom or a significant (≥ 50%) reduction in pre-treatment seizure frequency which was maintained over a period of at least 12 months.

4.1.3.2 Study subjects

From the 400-strong study population, 124 patients were identified as being treated with CBZ monotherapy. Through retrospective evaluation of individual medical records,

fifteen patients were excluded due to insufficient clinical and CBZ dosage information. Five patients that had been referred from other centres or from primary care did not have sufficient information on the outcome of therapy. Thirty-four of the remaining 104 patients with complete outcome information did not respond to CBZ (n = 20) or experienced intolerable side effects (n =14) which resulted in the premature withdrawal of medication. These patients were excluded from the study because an optimal dose could not be established. As a result, 70 patients with optimal seizure control on CBZ monotherapy and with complete information on dosage regimen and relevant clinical data were available for analysis. Information on the clinical response of these patients was confirmed by an experienced clinician prior to data analysis. None of these patients were concomitantly treated with other AEDs or non-AEDs that may have influenced CBZ metabolism. Most of the patients (91.4%) were treated with a sustained release formulation and only a minority (8.6%) received standard release tablets.

4.1.3.3 Genotype analysis and phenotype assessment

Genotypes of the common CBZ-related DME and SCN2A polymorphisms were identified using the PCR-RFLP and direct sequencing assays described in Chapter 3. Genotype determination for the DME and SCN2A polymorphisms was completed on February 2006. Phenotype assessment was started on March 2006 and completed on April 2006.

4.1.3.4 Statistical analysis

Clinical and demographic differences between the CBZ cohort and the 400-strong study population were investigated using the X^2 test. The distribution of age was compared using Student's t-test. The possibility of genotype selection bias was explored by comparing the distribution of individual polymorphisms between the CBZ cohort and

the overall 400-strong study population using the X^2 test. Differences in the mean optimal dose of CBZ in relation to predictors such as gender and genotype of individual DME polymorphisms were initially compared by Student's t-test and/or analysis of variance (ANOVA).

To fit the genetic information into the regression database, a specific score was assigned to the genotype of each polymorphisms according to the number of substituting alleles; no substituting allele was given score 1, one substituting allele was given score 2 and two substituting alleles was given score 3. Interaction between polymorphisms in a single gene or genomic region was identified by correlation test, represented by the multiplicative value of polymorphism scores, and denoted "SNP interaction".

Significant predictors of CBZ optimal dose were then identified using univariate and multivariate linear regression analysis. An additive model was used in the analysis with an assumption that the combined effects of genetic alleles at two or more gene loci are equal to the sum of their individual effects. SNP interactions were added to the multivariate analysis and acted as a non-linear component. Predictors were added into or taken out from the predictive model using a stepwise approach, depending on the degree of impact indicated by the *p*-value. Data transformation was employed to optimise the regression modelling, specifically transformation from linear to natural logarithmic form. Regression coefficients of predictors that significantly influenced the predictive model were used to construct the CBZ optimal dose predictive equation. Pearson's correlation (*r*-value) was used to evaluate the linearity of the predictive model by correlating the observed CBZ optimal dose and the predicted CBZ optimal dose. A *p*-value less than 0.05 indicated the presence of a strong predictor effect. Both $P_{residual}$, which represented the *p*-value of residual analysis, and r^2, that represented the goodness-of-fit, indicated how well the data fitted the predictive model.

4.1.4 Results

4.1.4.1 Patient characteristics and carbamazepine dosage regimen

Demographic information for all 70 patients included in the CBZ cohort is presented in TABLE 4.1. Distributions of gender ($p > 0.05$) and epilepsy syndromes ($p > 0.05$) were comparable with the 400-strong study population. The median age of patients in the CBZ cohort was 34 years (range 14 to 72). In 16% of the CBZ cohort the optimal dose of CBZ was less than the maximum dose to which those patients had been exposed. Thus, the optimal dose represents a composite measure of efficacy and tolerability. FIGURE 4.1 shows that increasing age was associated with a modest reduction in CBZ optimal dose, however this effect was not statistically significant ($r = -0.171$, $p = 0.156$). Gender and epilepsy syndrome were not associated with mean CBZ optimal dose ($p > 0.05$; FIGURE 4.2 and 4.3, respectively).

4.1.4.2 Genetic predictors of carbamazepine optimal dose

Genotype distributions for all of the common DME gene polymorphisms were similar between the CBZ cohort and the 400-strong study population ($p > 0.05$, TABLE 4.2). A total of 2 SNP interactions were identified, between CYP3A4 c.-392A>G and CYP3A5 c.6986A>G ($r = -0.285$, $p = 0.017$) and between EPHX1 c.337T>C and EPHX1 c.416A>G ($r = -0.261$, $p = 0.029$).

TABLE 4.1. Patient demographics and carbamazepine doses.

		CBZ cohort *(n = 70)*	400-strong study population *(n = 400)*	*p*-value
Gender	Male	48.6%	50.3%	0.796
	Female	51.4%	49.7%	
Age (years)	Median	34	40	0.343
	Min	14	14	
	Max	72	85	
Epilepsy syndrome	IGE	27.1%	29.5%	0.689
	LRE	71.5%	67.5%	
	UNC	1.4%	3%	
Optimal Dose (mg/day)	Mean	874.3		
	Median	800		
	Min	300		
	Max	2000		

Min = minimum; Max = maximum; IGE = idiopathic generalised epilepsy; LRE = localisation related epilepsy; UNC = unclassified epilepsy. Statistical significance was determined by X^2 test. The *p*-value represents the comparison between the WSEP and CBZ cohort.

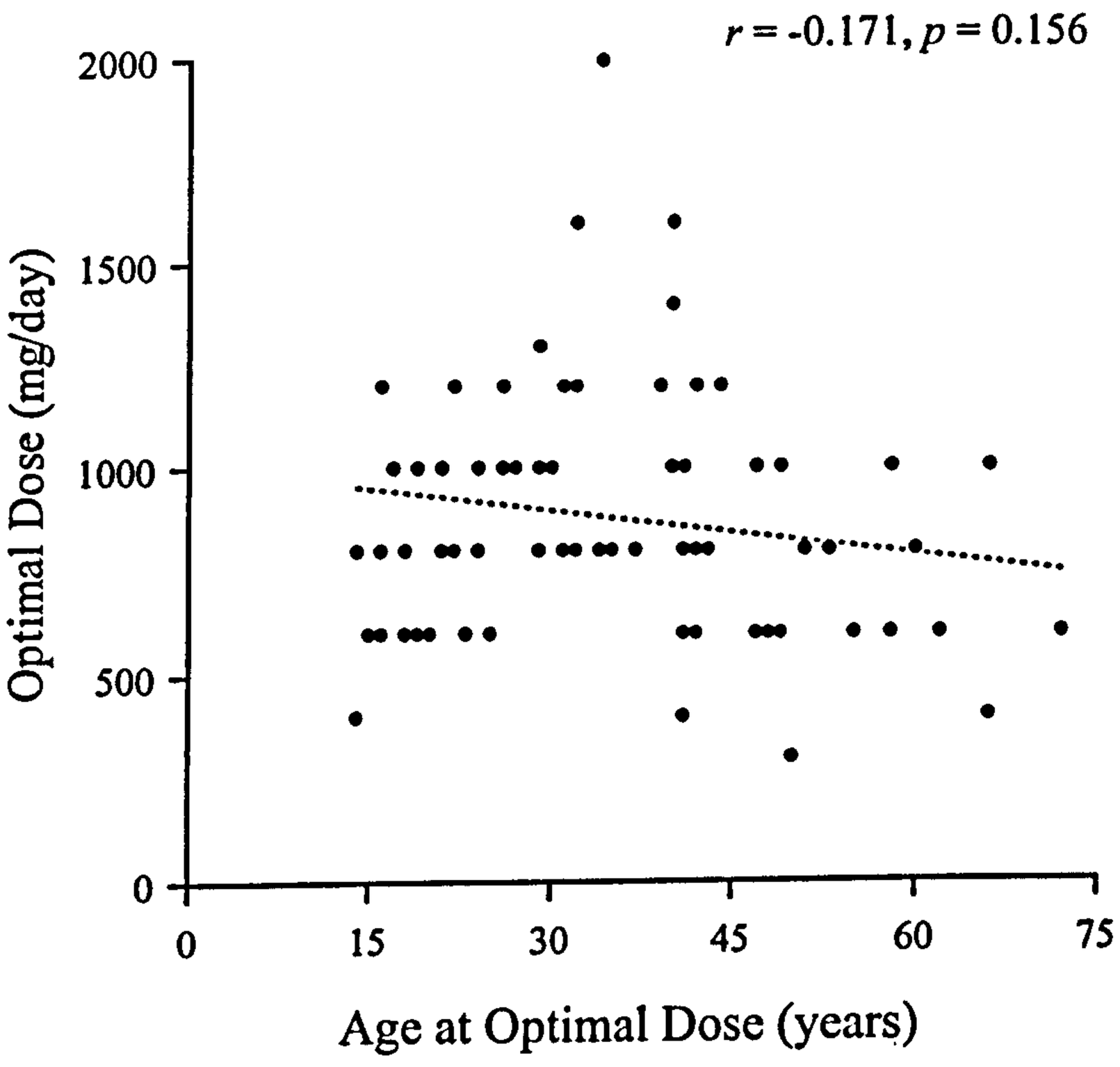

FIGURE 4.1. Correlation between carbamazepine optimal dose and age of the patient at the time optimal dose was attained. Correlation was determined by Pearson's correlation test. Dotted line represents the linear correlation between variables.

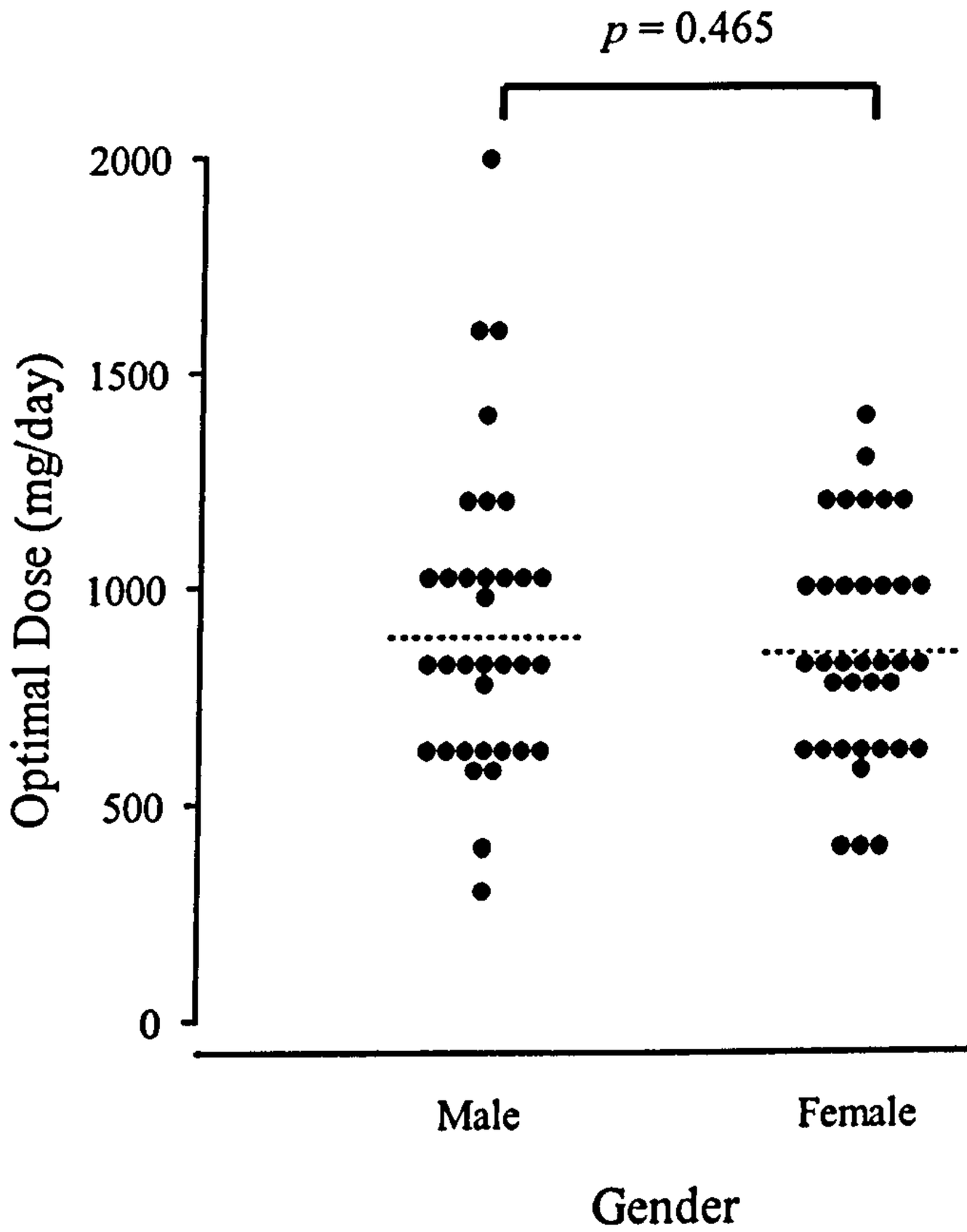

FIGURE 4.2. Distribution of carbamazepine optimal dose between genders. Statistical significance was determined by Student's t-test. Dotted line represents the mean CBZ optimal dose.

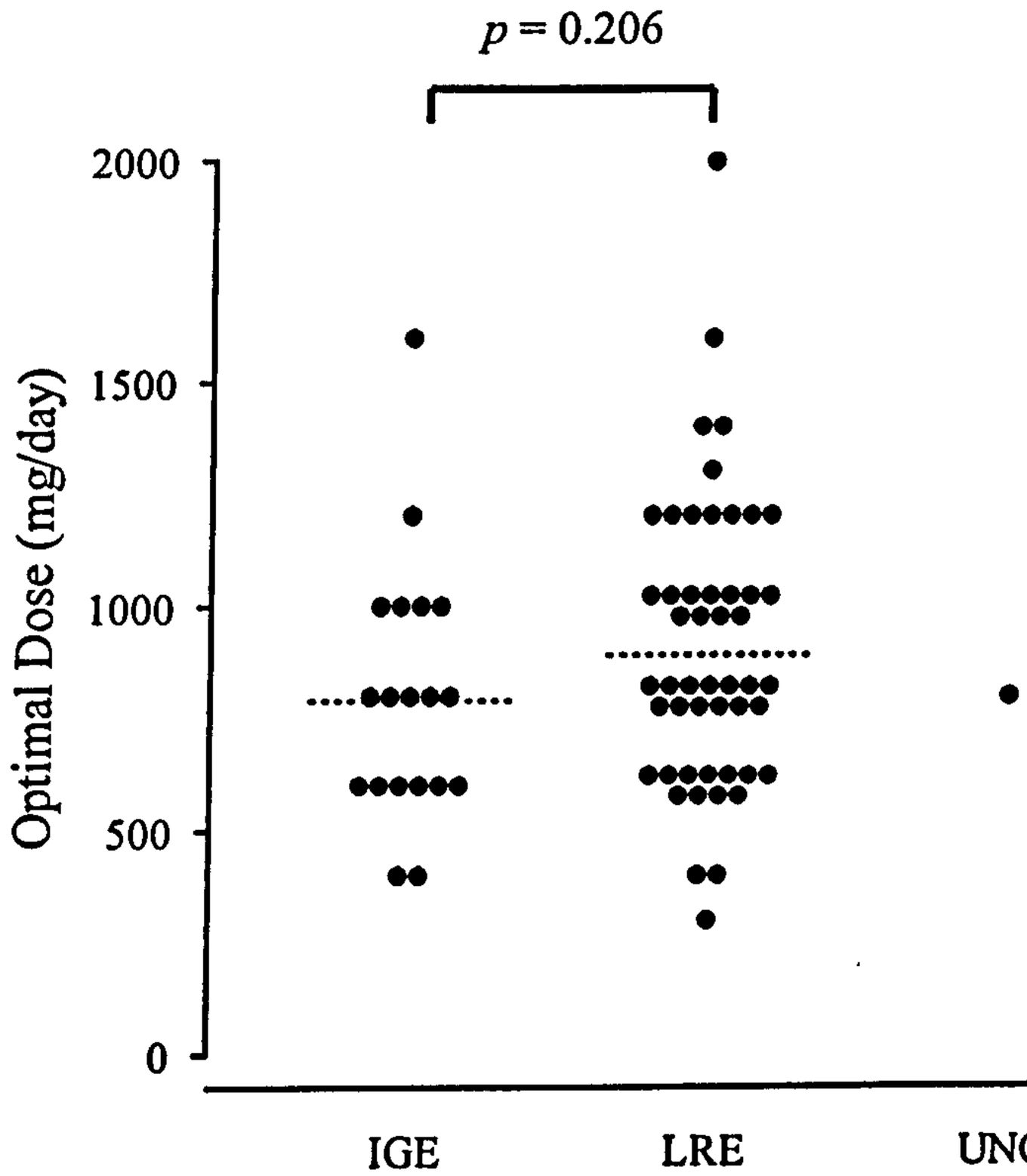

FIGURE 4.3. Distribution of carbamazepine optimal dose between epilepsy syndromes. Statistical significance was determined by Student's t-test. IGE = idiopathic generalised epilepsy, LRE = localisation related epilepsy and UNC = unclassified epilepsy.

TABLE 4.2. Genotype distribution comparison between the carbamazepine cohort and the 400-strong study population.

Variant	Sample	Genotype frequency			*p*-value
		Minor	*Hetero*	*Major*	
SCN2A c.56G>A	SSP	0.013	0.112	0.875	0.606
	CBZ	0.000	0.100	0.900	
CYP3A4 g.-392A>G	SSP	0.003	0.065	0.932	0.333
	CBZ	0.000	0.100	0.900	
CYP3A5 g.6986A>G	SSP	0.010	0.128	0.862	0.290
	CBZ	0.014	0.171	0.815	
CYP1A2 g.5734C>A	SSP	0.068	0.415	0.517	0.792
	CBZ	0.057	0.457	0.486	
EPHX1 c.337T>C	SSP	0.105	0.412	0.483	0.748
	CBZ	0.086	0.386	0.528	
EPHX1 c.416A>G	SSP	0.038	0.312	0.650	0.417
	CBZ	0.057	0.243	0.700	
UGT2B7 c.802C>T	SSP	0.202	0.548	0.250	0.061
	CBZ	0.157	0.457	0.386	

Statistical significance was determined by X^2 test or Fisher's Exact test. SSP = 400-strong study population; CBZ = CBZ cohort with 70 patients; Minor = homozygous for minor allele; Hetero = heterozygous for major and minor alleles; Major = homozygous for major allele. The *p*-value represents the comparison between CBZ cohort and WSEP.

Univariate linear regression analysis showed that none of the candidate predictors was significantly associated with CBZ optimal dose (TABLE 4.3). A multivariate predictive model (Model 1) incorporating all candidate predictors (gender, age, genotypes of 7 DME and SCN polymorphisms and 2 SNP interactions) identified age as the only predictor of CBZ optimal dose (p = 0.026). However, the overall model fit was not statistically significant (r^2 = 19.3%, $p_{residual}$ = 0.270, TABLE 4.4). Remodelling of Model 1 using stepwise regression analysis produced Model 2 which identified age, EPHX1 c.337T>C and EPHX1 c.416A>G as potential predictors (p = 0.023 to 0.059) with a significant overall model fit (r^2 = 13.3%, $p_{residual}$ = 0.023). Enhancement of Model 2 using simple data transformation (from linear form to natural logarithmic form) further improved the overall model fit (r^2 = 15.50%, $p_{residual}$ = 0.011) and revealed 3 significant predictors (age, p = 0.023; EHPX1 c.337T>C, p = 0.019; EPHX1 c.416A>G, p = 0.027; TABLE 4.4).

A predictive equation for optimal dose of CBZ was constructed from the constant value and regression coefficients given by Model 3. The predictive equation is presented below:

$$\ln OD_{CBZ} = 6.477 - 0.006*Age + 0.156*Poly1 + 0.164*Poly2$$

Where $\ln OD_{CBZ}$ is the natural logarithm of the optimal dose (mg/day) of CBZ, Poly1 is the genotype score for EPHX1 c.337T>C (T/T = 1, T/C = 2, C/C = 3), and Poly2 is the genotype score for EPHX1 c.416A>G (A/A = 1, A/G = 2, G/G = 3).

The correlation between the observed CBZ optimal doses and the predicted CBZ optimal doses generated by the predictive equation was statistically significant (p = 0.002) but moderate in strength (r = 0.362, 95%CI 0.14 to 0.55; FIGURE 4.4).

TABLE 4.3. Summary of univariate regression analysis of variables predicting the optimal dose of carbamazepine.

Predictor	**Coef.**	**r^2 (%)**	***p*-value**
Gender	-55.72	0.8	0.460
Age	-3.581	2.9	0.156
SCN2A c.56G>A	-82.5	0.0	0.512
CYP3A4 g.-392A>G	76.2	0.5	0.545
CYP3A5 g.6986A>G	-57.58	0.6	0.508
CYP1A2 g.5734C>A	-5.11	0.0	0.935
EPHX1 c.337T>C	95.75	4.0	0.098
EPHX1 c.416A>G	80.71	2.3	0.208
UGT2B7 c.802C>T	55.66	1.6	0.301

The relative impact of each predictor is denoted by the coefficient value (*Coef.*). The goodness-of-fit value (r^2) indicates how well the individual predictors fit the model. The *p*-value indicates the probability that the predictor influences CBZ optimal dose.

TABLE 4.4. Summary of hierarchical multivariate regression analysis of variables predicting the optimal dose of carbamazepine. Models were developed in a stepwise manner from Model 1 to Model 3.

Predictor	Model 1		Model 2		Model 3 (natural log)	
	Coef.	*p*-value	Coef.	*p*-value	Coef.	*p*-value
Constant	173.10	0.856	649.50	<0.001	6.477	<0.001
Gender	-93.63	0.249	-	-	-	-
Age	-6.36	0.026	-4.68	0.059	-0.006	0.023
SCN2A c.56G>A	-148.90	0.263	-	-	-	-
CYP3A4 g.-392A>G	591.10	0.402	-	-	-	-
CYP3A5 g.6986A>G	174.60	0.603	-	-	-	-
CYP3A SNP interaction	-189.30	0.497	-	-	-	-
CYP1A2 g.5734C>A	25.57	0.695	-	-	-	-
EPHX1 c.337T>C	255.40	0.172	132.79	0.023	0.156	0.019
EPHX1 c.416A>G	283.80	0.181	127.77	0.035	0.164	0.027
EPHX1 SNP interaction	-115.8	0.435	-	-	-	-
UGT2B7 c.802C>T	27.72	0.637	-	-	-	-
r^2 (%)	19.3		13.3		15.5	
$P_{residual}$	0.270		0.023		0.011	

The relative impact of each predictor is denoted by the coefficient value (*Coef.*). The *p*-value indicates the probability that the predictor influences CBZ optimal dose. The goodness-of-fit value (r^2) indicates how well the individual predictors fit the predictive model. The significance of model fitting is represented by the *p*-value of residual analysis ($p_{residual}$). '-' mark represents a predictor that was eliminated by stepwise regression analysis.

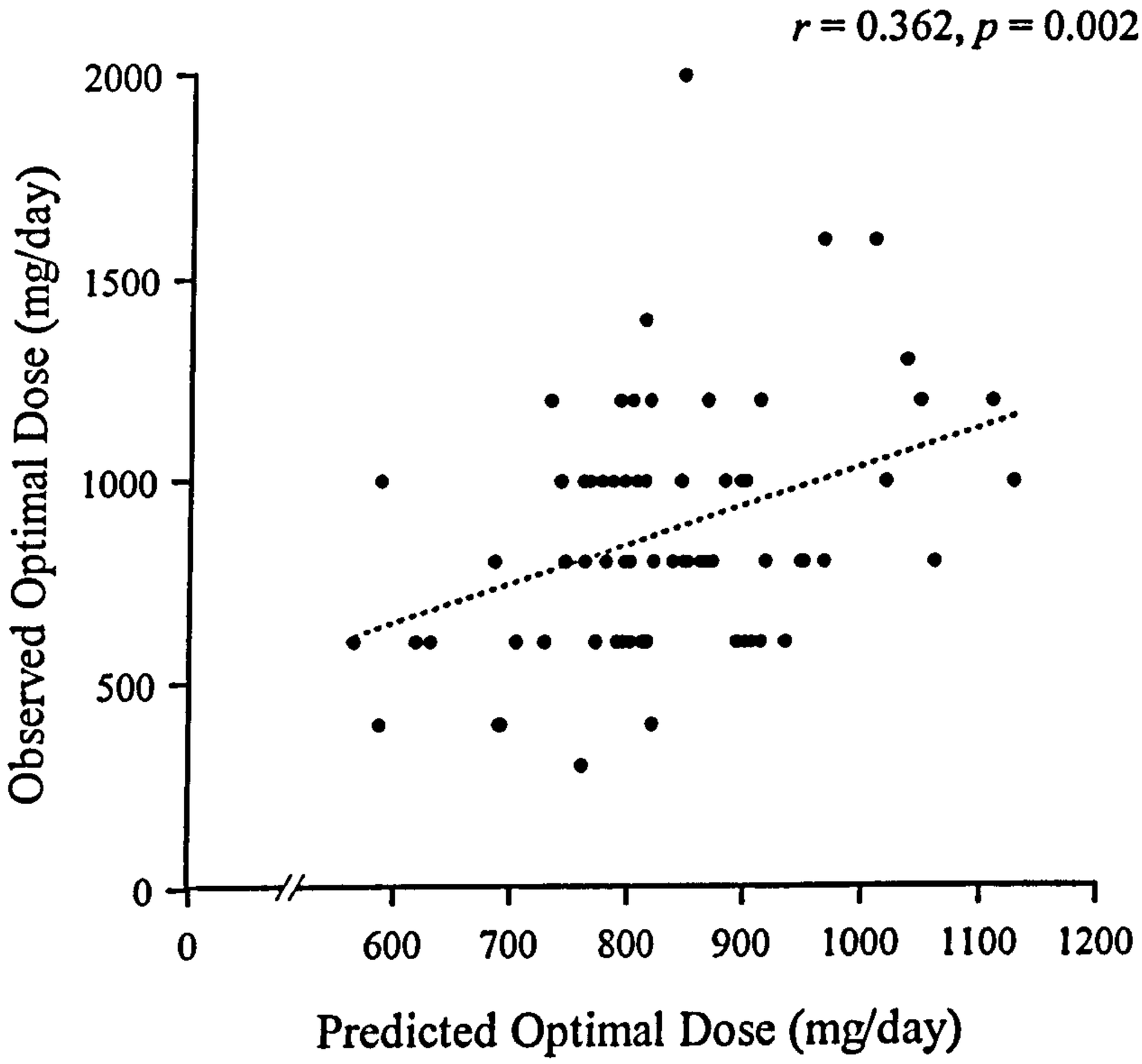

FIGURE 4.4. Correlation between the observed and predicted CBZ optimal doses based on the optimal multivariate regression model. Correlation was determined by Pearson's correlation test. Dotted line is the linear correlation between observed and predicted doses.

4.1.5 Discussion

The main objective of epilepsy pharmacotherapy is to achieve complete seizure control without apparent adverse effects. The ability to predict the required CBZ dose for an individual patient may improve the CBZ dosage decision in patients who are responders, and limit the inevitable titration of CBZ in patients who may be refractory.

Therefore, early prediction of the CBZ optimal dose may shorten the time to achieve optimal seizure control and reduce unnecessary exposure to a high CBZ doses.

The optimal dose of CBZ is determined by individual pharmacokinetic and pharmacodynamic characteristics. Both characteristics are regulated by many factors including physiological and genetic factors. Candidate genetic predictors of optimal dose of CBZ were identified from current knowledge regarding the pharmacokinetics and pharmacodynamics of the drug. The ability of each candidate to predict optimal dose of CBZ was examined through regression modelling. This analysis was not only able to identify potential predictors, but also to describe how much each predictor contributed to interindividual variation in the optimal dose of CBZ.

This study revealed that increasing age was associated with a reduction in the required CBZ dose. A similar observation has been reported in a study examining the relationship between CBZ dose and plasma concentrations (Battino *et al.*, 2003). These investigators found that elderly patients required less CBZ to achieve the recommended therapeutic concentration. Increasing age is known to influence multiple physiological functions, including the drug elimination process, most likely as a result of decreased function of excretory organs such as liver and kidney (Bourdet *et. al.*, 2001). Advancing age may also increase individual sensitivity to drugs and increase the risk of developing side effects (Bourdet *et. al.*, 2001). Therefore, elderly patients are likely to require a lower amount of drug to achieve a similar response to that of younger patients.

Genetic factors influencing both pharmacokinetics and pharmacodynamics contribute to the overall CBZ pharmacogenetic profile. Interindividual differences in pharmacokinetics are commonly associated with DME and DTP gene polymorphisms. However, the role of DTPs, such as P-gp, in CBZ disposition is controversial (Owen *et*

al., 2001) and potentially minimal as the CBZ molecule is highly non-polar, easily able to penetrate cell membranes and distributed well in many body tissues (Spina, 2002). A previous study failed to demonstrate any correlation between a common polymorphism in the ABCB1 gene encoding P-gp and the maximum dose of CBZ (Tate *et al.*, 2005). For this reason, the present study focused solely on genetic polymorphisms of DMEs as potential contributors to interindividual variability in CBZ pharmacokinetics.

In this study, only two common polymorphisms in the EPHX1 gene were identified as significant predictors of CBZ optimal dose when included in a predictive model incorporating patient age. It is possible that polymorphisms in other DMEs do not significantly influence the expression and activity of their respective proteins or that these proteins do not play a principal and/or irredeemable role in the metabolism of CBZ.

The g.-392A>G transition is the most widely studied polymorphism in the CYP3A4 gene. The CYP3A4 -392G allele has also been associated with a reduced capacity to metabolise drugs such as nifedipine and tacrolimus (Shimada *et al.*, 1994; Hesselink *et al.*, 2003). The g.6986A>G substitution is the most commonly recognised polymorphism in the CYP3A5 gene. Variation in CYP3A5 metabolic capacity associated with the g.6986A>G polymorphism has been observed for drugs such as midazolam, statins, alprazolam and tacrolimus (Hesselink *et al.*, 2003; Goto *et al.*, 2004; Kivisto *et al.*, 2004; Wong *et al.*, 2004; Park *et al.*, 2006). The present study, did not identify any significant difference in the mean optimal dose of CBZ between individual genotypes of CYP3A4 g.-392A>G and CYP3A5 g.6986A>G. Even in the multivariate regression analysis, both polymorphisms failed to predict CBZ optimal dose. The reason why these polymorphisms do not appear to influence CBZ dosing is unclear, however, one possible explanation may be related to the strong linkage between these SNPs. In

Caucasian populations, the CYP3A4 -392G allele is usually co-localised with the CYP3A5 6986A allele and *vice versa* (Dally *et al.*, 2003). Low activity of CYP3A4 associated with the -392G allele is therefore balanced out by possession of a functional CYP3A5 enzyme in carriers of the 6986A allele (Ohmori *et al.*, 1998; Huang *et al.*, 2004). Thus, there is some degree of redundancy in the genetic influences on CYP3A-mediated metabolism, at least in the Caucasian population. This redundancy is not observed in Japanese subjects, who do not express the CYP3A4 -392G allele (Paris *et al.*, 1999; Seo *et al.*, 2006b). In the resulting absence of compensatory linkage between CYP3A polymorphisms, the CYP3A5 c.6986A>G variant is associated with interindividual variability in CBZ pharmacokinetics in this population (Seo *et al.*, 2006b).

The CYP1A2 5734A allele has been reported to be highly inducible especially among smokers (Sachse *et al.*, 1999), and is capable of affecting the risk of developing heart disease and cancer (Cornelis *et al.*, 2005; Moonen *et al.*, 2005; Pavanello *et al.*, 2005). Although hydroxylation of CBZ involves the CYP1A2 enzyme, the present study did not find any association between the CYP1A2 g.5734C>A polymorphism and the CBZ optimal dose. Up to 25% of the parent CBZ compound undergoes hydroxylation, but at least 4 DMEs other than CYP1A2 have been reported to catalyze the hydroxylation pathway (FIGURE 1.2; Pelkonen *et al.*, 2001). Thus, the contribution of CYP1A2 to CBZ hydroxylation is potentially very minor, reducing the likelihood of detecting any association between CYP1A2 variants and CBZ dose.

The importance of mEH in the metabolism of CBZ can be predicted from the CBZ metabolic pathway (FIGURE 1.2; Pelkonen *et al.*, 2001). mEH may be responsible for more than 30% of overall CBZ metabolism. The EPHX1 c.337T>C transition causes substitution of tyrosine with histidine at codon-113 and the EPHX1 c.416A>G

transition causes substitution of histidine to arginine at codon-139. In an *in vitro* study, these amino acid changes were shown to influence the stability of mEH (Hassett *et al.*, 1994b). As a potential consequence of this alteration in mEH stability, and its recognised ability to metabolise several carcinogens, both polymorphisms have been associated with an adjusted risk of developing cancers and other diseases (Smith & Harrison, 1997; Jourenkova-Mironova *et al.*, 2000; Sonzogni *et al.*, 2002; Budhi *et al.*, 2003). Studies employing benzo(a)pyrene-4,5-epoxide and cis-stilbene oxide as substrates have shown that EPHX1 337C and EPHX1 416G alleles are associated with lower and higher mEH activities, respectively (Hassett *et al.*, 1994b; Hassett *et al.*, 1997). Interestingly, the opposite was observed in a study employing CBZ-E as a substrate (Nakajima et al., 2005). This would indicate that the influence of EPHX1 variants on enzyme activity is substrate-specific. This is supported by an investigation which examined the hydrolytic activity of mEH on three substrates: cis-stilbene, CBZ-E, and naphthalene oxide, in a panel of microsome samples obtained from human livers (Kitteringham *et al.*, 1996). Thus, the functional significance of EPHX1 variants and the percentage of mEH involvement in CBZ metabolism would support the notion that polymorphisms of EPHX1 gene are potential predictors of CBZ optimal dose. The predictive model that was constructed in the current study further strengthens this postulation. A significant alteration in the capacity for CBZ-E metabolism has been demonstrated in patients with haplotypes of EPHX1 harbouring both 337C and 416G alleles (Nakajima *et al.*, 2005). As CBZ-E is commonly associated with the occurrence of adverse effects arising from CBZ therapy (Ramsay & Wilder, 2002) and mEH is the major determinant of CBZ-E metabolism, these EPHX1 polymorphisms may also be useful in predicting the maximum tolerable dose which should not be exceeded when adjusting CBZ treatment regimens.

Approximately 15% of parent CBZ compound undergoes glucuronidation which is predominantly mediated by UGT2B7. The c.802C>T substitution is a recognised polymorphism in the UGT2B7 gene, but its functional significance is controvesial. The 802T allele has been associated with an altered capacity for the metabolism of morphine and 4-hydroxy-catecholestrogens (Sawyer *et al.*, 2003; Thibaudeau *et al.*, 2006), but other substrates such as oxazepam, propranolol and androgens are unaffected (Coffman *et al.*, 1998). In the present study, the c.802C>T polymorphism, did not significantly predict the optimal dose of CBZ, suggesting that this variant does not significantly influence the role of UGT2B7 in the glucuronidation of CBZ.

CBZ acts predominantly on voltage-gated sodium channels to exert its antiepileptic effects. Changes in the structure of sodium channels may influence their sensitivity to CBZ. In the current study, there was no association between the SCN2A c.56G>A polymorphism and the optimal dose of CBZ. This might suggest that the SCN2A c.56G>A polymorphism does not significantly affect the function of the $Na_v1.2$ protein or its sensitivity to blockade by CBZ. Of course, CBZ can also act on other voltage-gated sodium channels, potentially compensating for any functional or structural defect in the $Na_v1.2$ protein. A previous study has reported that the maximum dose of CBZ is significantly associated with a polymorphism located within intron-5 of SCN1A gene (Tate *et al.* 2005). However, there was a clear difference in the definition of dose between the Tate *et al.* (2005) study and the current investigation. The measure of optimal dose considers both efficacy and tolerability to CBZ treatment, whereas maximum dose reflects tolerability alone. Nevertheless, this SCN1A polymorphism is a potential candidate for future pharmacogenetic investigations of CBZ optimal dose and may be able to explain additional interindividual variability in the CBZ dose requirement.

The predictive model of CBZ optimal dose proved statistically relevant only when a physiological factor (age) was combined with genetic predictors. When each candidate predictor was analysed in isolation, none showed significant ability to predict the optimal dose, indicating that individual dosing requirement are determined by multiple factors, many of which remain to be discovered. The present study showed that potentially important predictors for CBZ optimal dose include patient age and polymorphisms in the EPHX1 gene. Although the model revealed a significant correlation between predicted and observed optimal doses, the strength of the prediction was not sufficient for application in clinical practice. The small size of the CBZ cohort and the retrospective design of this study have influenced its findings and their applicability. This study has revealed an intriguing and potentially significant role of EPHX1 SNPs in individual CBZ dose requirements, however it should be repeated independently and prospectively. The predictive model that was established in this study may serve as a springboard for future studies of SNP-based prediction of optimal drug doses.

4.1.6 Conclusion

None of the input factors, gender, age, and polymorphisms in DME and SCN2A genes can be used as a single predictor for CBZ optimal dose. However, the EPHX1 c.337T>C & c.416A>G polymorphisms appear to be potential predictors for optimal dose in a predictive model incorporating age. More candidate predictors in a larger population are required to strengthen the model. The additional candidate predictors may include factors that influence CBZ pharmacodynamics such as polymorphisms in genes encoding other voltage-gated ion channels that represent the pharmacological targets of CBZ.

4.2 A Preliminary Genetic Analysis of Drug Metabolising Enzymes in Patients with Carbamazepine Adverse Effects

4.2.1 Introduction

Major adverse drug reactions associated with CBZ treatment can be divided into two categories; CNS toxicity and idiosyncratic reactions. Several mechanisms have been proposed to explain the occurrence of CBZ adverse effects. One hypothesis is that CNS toxicity is dependent on CBZ dose and concentration (Delcker *et al.*, 1997). The severity of adverse effects has been reported to be directly associated with increasing plasma CBZ concentrations (Weaver *et al.*, 1988; Semah *et al.*, 1994). In addition, this has also been demonstrated by the relationship between intermittent CNS effects and the dosing-interval fluctuation in CBZ concentrations at steady-state (Tomson, 1984). In some patients, CNS related adverse effects are experienced at modest CBZ doses (less than 600 mg/day), suggesting that these individuals possess low activity DMEs, which result in higher plasma CBZ concentrations. Polymorphisms in genes that encode DMEs may be responsible for this phenomenon. Another hypothesis is that CNS related adverse effects are associated with the rate of rise in plasma CBZ concentrations (Lesser *et al.*, 1984; Wildin *et al.*, 1993; Delcker *et al.*, 1997). An adaptation effect of the brain in response to changes in CBZ concentration is believed to be responsible for the occurrence of these adverse effects (Lesser *et al.*, 1984).

Idiosyncratic reactions are independent of CBZ dose and/or concentration. Rashes are the most common cutaneous manifestation of CBZ idiosyncratic reactions, and a cardinal sign of CBZ hypersensitivity. Idiosyncratic reactions to CBZ can arise as a

result of cross-sensitivity to other AEDs (Shear & Spielberg, 1988; Moss *et al.*, 1999). Although a relatively small proportion of patients experience idiosyncratic reactions, unattended cases can result in severe morbidity and even fatality (Shear & Spielberg, 1988; Moss *et al.*, 1999; Rzany *et al.*, 1999; Maldonado *et al.*, 2002; Pirmohamed & Park, 2003). Idiosyncratic reactions are believed to be associated with an increased activation and/or decreased deactivation of reactive intermediate metabolites (Pirmohamed & Park, 2003). In an animal model, the production of reactive metabolites of CBZ such as arene oxides and quinones is mediated by CYP450 isoenzymes such as CYP3As and CYP1A2 (Madden *et al.*, 1996; Pearce *et al.*, 2002). In contrast, the deactivation process is largely mediated by mEH and UGTs (Green *et al.*, 1995; Maggs *et al.*, 2000).

Evidence suggests that polymorphisms in DME genes are able to alter enzyme activity (as described in Chapter 3). Polymorphisms in genes that encode activation and deactivation enzymes for CBZ may be causal in the precipitation of adverse drug effects. Together with other physiological and immunological factors, genetically compromised DMEs may contribute to the generation of both categories of CBZ-related adverse effects. Collectively, these polymorphisms may produce a unique adverse effect phenotype. As a result, common genetic polymorphisms of DMEs that are involved in CBZ metabolism were examined as potential genetic markers of CBZ adverse effects.

4.2.2 Aims

In an effort to identify a potentially unique phenotype of CBZ adverse effects, the incidence of common DME polymorphisms in patients experiencing intolerable CBZ adverse effects was investigated.

4.2.3 Methods

4.2.3.1 Study subjects

From the 400-strong study population, 104 patients treated with CBZ monotherapy and possessing sufficient outcome data were identified and retrospectively screened for any incidence of adverse effects. A total of 14 patients had experienced at least one intolerable adverse effect that led to CBZ withdrawal (9 CNS-related, 5 idiosyncratic). The remaining 90 patients did not experience any adverse effect of sufficient severity to necessitate drug withdrawal, despite at least 6 months exposure to CBZ. These patients were employed for control purposes and randomely assigned to one of two distinct control groups using an online random number generator (http://www.randomizer.org/form.htm). A total of five individuals were selected at random from control group 1 and employed for comparison with those patients experiencing idiosyncratic reactions with CBZ. Control group 2 was first pared to include only those patients who had been exposed to CBZ at doses above 800 mg/day and then nine individuals were selected at random for comparison with those patients experiencing intolerable CNS-related adverse effects. Applying a minimum dose exposure criterion ensured that control group 2 was relatively free from dose-related adverse events and sufficiently distinct from those patients who discontinued CBZ due to CNS-related adverse effects and who had done so at a median maximum dose of just 400 mg/day.

4.2.3.2 Adverse reaction documentation

Major adverse effects were divided into two categories, CNS-related effects and idiosyncratic reactions. Information regarding CBZ adverse effects was obtained retrospectively from patient medical records. None of the patients was concomitantly treated with other medications (AEDs or non-AEDs) at the time of CBZ adverse effect.

4.2.3.3 Genotype analysis and phenotype assessment

The genotypes of recognised polymorphisms in DMEs associated with CBZ metabolism were identified using the PCR-RFLP and direct sequencing assays described in section 3.1.3. Genotype determination for the DME polymorphisms was completed on February 2006. Phenotype assessment was started on March 2006 and completed on April 2006.

4.2.3.4 Genotype-phenotype analysis

Minor alleles were chosen as potential genetic markers for CBZ adverse effects. The minor alleles were represented by the CYP3A4 -392G, CYP3A5 6986A, CYP1A2 5734C, EPHX1 337C, EPHX1 416G and UGT2B7 802C variants. CYP3A4, CYP3A5 and CYP1A2 were regarded as potential activation enzymes in the precipitation of CBZ-related idiosyncratic reactions, whereas UGT2B7 and EPHX1 were regarded as potential deactivation enzymes. The incidence of the minor alleles of the recognised polymorphisms in DME genes was compared between the adverse effects groups and their respective control groups by the method proposed by Green *et al.* (1995).

4.2.3.5 Statistical analysis

The distributions of gender and epilepsy syndromes were initially compared by X^2 test. The pattern of genetic polymorphisms was evaluated by allelic term. The distribution of minor and major alleles between adverse effect and respective control groups was compared using an appropriate contingency table test (Fisher's Exact test or X^2 test). *P*-values less than 0.05 represented a significant difference in the distribution of minor and major allele between the two groups.

4.2.4 Results

4.2.4.1 Patient characteristics and adverse effect profile

There was no significant difference in the distribution of gender ($p = 0.388$) or epilepsy syndrome ($p = 0.503$) between adverse effect and control groups (TABLE 4.5). In the majority of cases, adverse effects were observed at lower median CBZ doses (400 mg/day) than was employed in the population as a whole (800 mg/day; $p = 0.001$; TABLE 4.5).

TABLE 4.5. Demographic profile of patients who experienced intolerable carbamazepine adverse effects.

		Intolerable Adverse Events		
		Yes, n = 14	*No, n = 90*	*p*-value
Gender	Male	60%	48%	0.388
	Female	40%	52%	
Epilepsy Syndrome	IGE	13%	28%	0.503
	LRE	87%	70%	
	UNC	0%	2%	
CBZ maximum dose (mg/day)	Median	400	800	0.001
	Min	400	300	
	Max	1200	2000	

IGE, idiopathic generalised epilepsy; LRE, localisation related epilepsy; UNC, unclassified epilepsy. Statistical significance was determined by X^2 test for gender and epilepsy syndrome, and Student's t-test for CBZ maximum dose. The *p*-value represents the comparison between patients who experienced intolerable CBZ and the population from which they are drawn.

Drowsiness and dizziness (6 out of 14 cases) and rash (5 out of 14 cases) were the most common intolerable adverse effects leading to CBZ withdrawal. Some patients experienced more than one adverse event simultaneously. In all patients, the adverse effects resolved with discontinuation of CBZ.

4.2.4.2 The pattern of polymorphisms in drug metabolising genes in patients with carbamazepine adverse effects

The incidence of common polymorphisms in DME genes are presented in TABLE 4.6 and TABLE 4.7 for the CNS-related adverse effects and idiosyncratic reaction groups, respectively. For CNS related adverse effects, there was a marginally lower incidence of minor alleles in the adverse effect group compared to the control group (total = 20 *vs* 25 alleles, $p = 0.402$). For idiosyncratic reactions, the presence of minor alleles in DME genes was similar between adverse effect and control groups (total = 10 *vs* 11 alleles, $p = 0.882$). The presence of minor alleles in activation DMEs was comparable between the idiosyncratic reaction group and controls (3 *vs* 2 alleles, $p = 0.999$) and there was similarly no difference in the distribution of minor alleles in the deactivation DME genes between the idiosyncratic reaction group and respective control group (7 *vs* 9 alleles, $p = 0.559$). Overall, there was no single polymorphism that was evidently related to the occurrence of CBZ adverse effects.

4.2.5 Discussion

The risk of developing adverse drug effects has been associated with several polymorphisms in DME genes, such as CYP2C9 and CYP2D6 (Royer, 1997). The expression and activity of DMEs may be decreased or increased by the effects of genetic polymorphisms. A decrease in DME activity may result in higher plasma drug concentration at routine dose, increasing the risk of developing dose or concentration

dependent adverse effects. This low activity phenotype may also be associated with accumulation of reactive metabolites that could in turn be responsible for idiosyncratic reactions. In contrast, the high activity phenotype may also be partly responsible for the development of idiosyncratic reactions through increased production of reactive metabolites (Shear & Spielberg, 1988; Green *et al.*, 1995; Royer, 1997; Pirmohamed & Park, 2003). Accordingly, polymorphisms in genes encoding DMEs that mediate CBZ metabolism were hypothesised to be causal factors in the development of CBZ adverse effects. However, there was no clear association between the incidence of common polymorphisms in DME genes and CBZ adverse effects that might be considered as the sole determinant of treatment withdrawal. Common polymorphisms in DME genes such as CYP3A4, CYP3A5, CYP1A2, EPHX1 and UGT2B7 are unlikely to be a predominant factor in the development of CBZ adverse reactions. It is more likely that other factors acting concurrently with DMEs may be responsible in predisposing an individual to CBZ adverse effects. The overall balance between physiological factors, bioactivation, detoxification and immune responsiveness probably determines whether an adverse effect will occur.

In many studies, the occurrence of CNS-related adverse effects is associated with the rate of rise in plasma CBZ concentration (Lesser *et al.*, 1984; Wildin *et al.*, 1993; Olling *et al.*, 1999). The CNS symptoms are manifested when the brain attempts to adapt to the increasing CBZ concentrations (Lesser *et al.*, 1984; Wildin *et al.*, 1993). The rising phase of plasma CBZ concentration is controlled by three principle factors, absorption, distribution and elimination. The influence of absorption is most pronounced in the immediate aftermath of drug intake, early in the dosing interval and when the rate of rise in concentration is at its greatest. Therefore, factors that affect the rate of CBZ absorption may be more responsible for the generation of CNS-related adverse effects than those which influence distribution and elimination.

TABLE 4.6. The incidence of common polymorphisms in drug metabolising enzyme genes in patients with central nervous system related carbamazepine adverse effects.

Patient ID	Gender	Seizure Type	CYP3A4 g.-392A>G	CYP3A5 g.6986A>G	CYP1A2 g.5734C>A	EPHX1 c.337T>C	EPHX1 c.416A>G	UGT2B7 c.802C>T	Adverse Effects
Central nervous system related effect group									
N1	M	IGE	A/A	G/G	**C**/A	T/T	A/A	T/T	Nausea
N2	F	LRE	A/A	G/G	A/A	T/T	A/A	**C**/T	Drowsiness
N3	M	LRE	A/A	G/G	**C**/A	T/T	**G**/**G**	T/T	Drowsiness
N4	F	LRE	A/A	G/G	A/A	**C**/**C**	A/A	T/T	Dizziness, numbness
N5	F	LRE	A/A	G/G	A/A	T/T	A/A	**C**/T	Dizziness, nausea
N6	M	LRE	A/A	G/G	A/A	T/**C**	A/A	**C**/**C**	Headache
N7	F	LRE	A/A	A/G	**C**/**C**	T/T	A/A	**C**/T	Drowsiness
N8	M	LRE	A/A	G/G	**C**/A	T/**C**	A/A	**C**/T	Drowsiness
N9	M	LRE	A/A	G/G	A/A	T/**C**	A/A	**C**/T	Confusion
Control Group									
C1	F	LRE	A/A	G/G	A/A	T/**C**	A/A	**C**/T	
C2	M	IGE	A/A	G/G	**C**/A	T/T	A/**G**	**C**/T	
C3	F	LRE	A/A	G/G	**C**/A	T/**C**	A/A	**C**/**C**	
C4	M	LRE	A/A	A/G	A/A	T/T	A/A	**C**/T	
C5	M	LRE	A/A	G/G	**C**/A	T/**C**	A/**G**	T/T	
C6	M	LRE	A/A	G/G	A/A	T/**C**	A/A	**C**/T	
C7	M	LRE	A/A	G/G	**C**/A	T/**C**	A/A	T/T	
C8	F	LRE	A/**G**	A/G	**C**/A	T/**C**	A/A	**C**/**C**	
C9	F	UNC	A/A	G/G	A/A	T/T	A/A	**C**/T	

M = male; F = female, IGE = idiopathic generalized epilepsy; LRE = localisation related epilepsy; UNC = unclassified epilepsy; Polymorphisms are presented as genotype. Bold nucleotide represents the minor allele.

TABLE 4.7. The incidence of common polymorphisms in drug metabolising enzyme genes in patients with carbamazepine idiosyncratic reactions. The activation of reactive carbamazepine metabolites is potentially mediated by CYP3A4, CYP3A5 and CYP1A2. The deactivation of reactive carbamazepine metabolites is potentially mediated by microsomal epoxide hydrolase (EPHX1) and UGT2B7.

			Activation Enzyme			**Deactivation Enzyme**			
Patient ID	**Gender**	**Seizure Type**	**CYP3A4 g.-392A>G**	**CYP3A5 g.6986A>G**	**CYP1A2 g.5734C>A**	**EPHX1 c.337T>C**	**EPHX1 c.416A>G**	**UGT2B7 c.802C>T**	**Adverse Effects**
Idiosyncratic reaction group									
R1	M	LRE	A/A	G/G	**C**/A	T/**C**	A/A	**C**/T	Rash
R2	F	IGE	A/A	G/G	A/A	T/**C**	A/**G**	T/T	Rash
R3	F	LRE	A/A	G/G	A/A	T/T	A/**G**	T/T	Rash
R4	M	LRE	A/A	G/G	**C**/A	T/T	A/**G**	T/T	Rash, hair loss
R5	M	LRE	A/A	G/G	**C**/A	T/T	A/A	**C**/T	Rash
Control group									
C10	M	IGE	A/A	G/G	A/A	T/T	A/A	**C**/**C**	
C11	M	IGE	A/A	G/G	**C**/A	T/T	A/**G**	**C**/**C**	
C12	F	LRE	A/A	G/G	A/A	T/**C**	A/A	T/T	
C13	F	IGE	A/A	G/G	A/A	T/**C**	A/A	**C**/T	
C14	M	LRE	A/A	G/G	**C**/A	T/T	A/A	**C**/T	

M = male; F = female; IGE = idiopathic generalized epilepsy; LRE = localization related epilepsy; Polymorphisms are presented as genotype. Bold nucleotide represents the minor allele.

Factors that affect the rate of absorption include gastrointestinal function and drug formulation (Wildin *et al.*, 1993; Olling *et al.*, 1999). Any given CBZ formulation is subject to high interindividual variability in the rate of absorption (Cotter *et al.*, 1977; Perucca *et al.*, 1980a; Olling *et al.*, 1999), highlighting important differences in gastrointestinal function between individuals. Gastrointestinal disease may also alter intestinal motility and the potential for drug absorption. The pattern of dosing-interval fluctuation in plasma CBZ concentrations can also be affected by formulation (May & Rambeck, 1989; Stevens *et al.*, 1998) and is closely associated with the intensity of adverse effects (Tomson, 1984). The dosing-interval fluctuation in concentrations observed with extended-release formulations is lower than that observed with standard release tablets and consequently fewer adverse effects are noted (Miller *et al.*, 2004).

Idiosyncratic reactions may be associated with the imbalance between activation and deactivation of reactive metabolites of CBZ, and also immune responsiveness (Shear & Spielberg, 1988; Green *et al.*, 1995; Royer, 1997; Pirmohamed & Park, 2003). The reactive metabolites, arene oxides, can precipitate cellular damage if not sufficiently deactivated (Madden *et al.*, 1996). Low molecular weight drugs and reactive metabolites bind covalently to cellular proteins and form haptens that trigger an immunological response (Svensson *et al.*, 2000). The isoforms of CYP450 involved in CBZ metabolism are present in human skin (Wolkenstein *et al.*, 1998) and stable binding between reactive metabolites of CBZ and skin CYP450s has been demonstrated (Wolkenstein *et al.*, 1998). The hapten produced can precipitate a cutaneous reaction. As a result, the production of hapten has been postulated as one possible explanation for the occurrence of CBZ cutaneous reactions. The presence of anti-CYP450 antibodies in patients who suffer hypersentivity reactions further supports this hypothesis (Leeder *et al.*, 1992). The findings of the current study suggest that common DME polymorphisms

do not significantly influence the pathway of activation/deactivation or the binding between DMEs and reactive metabolites.

Previous studies have also failed to demonstrate any association between polymorphisms in the EPHX1 gene and CBZ hypersensitivity (Green *et al.*, 1995; Pirmohamed & Park, 2003). Polymorphisms in genes that express DMEs may be less important than those that express immunological factors. The human major histocompatibility complex (MHC) which determines immune system recognition and which responds to CBZ-related antigen production is a possible causal immunological factor. Expression of genetically polymorphic MHCs has been identified (Leeder *et al.*, 1992). Furthermore, haplotypes of MHC and other immunological factors such as tumour necrosis factor have been associated with the severity of drug hypersensitivity reactions (Pirmohamed *et al.*, 2001).

The present study should be considered as preliminary because of the limited number of patients who experienced intolerable CBZ-related adverse effects. In addition, the study findings may also be influenced by the method of assessing adverse effects as described in section 2.2.3.2. Most of the adverse effects documented in this study arose from spontaneous reporting. Spontaneous reporting has advantages in highlighting effects which are clinically relevant, but is associated with extreme variability in the accuracy of detection and with significant under-reporting (Perucca, 1997; Gilliam, 2005). The retrospective nature of this study may have further compromised the accurate identification and recording of relevant adverse effects. Accordingly, this study focused only on those adverse effects which were sufficiently severe to necessitate CBZ withdrawal.

4.2.6 Conclusion

This preliminary study failed to reveal any association between CBZ adverse effects and common polymorphisms in DME genes. A large prospectively designed study is required to establish whether there is any functional relevance of DME gene polymorphisms in the development of CBZ adverse effects. Genetic variability in drug absorption and in proteins which contribute to the immune response should be targeted as potentially causal precipitants of CBZ adverse effects in future explorations.

CHAPTER 5

PHARMACOGENETICS OF LAMOTRIGINE

5.1 Association between ABCB1 and SCN2A gene polymorphisms and the Response to Lamotrigine Therapy in Newly Diagnosed Epilepsy Patients

5.1.1 Introduction

Partially responsive and non-responsive epilepsy patients may try a number of AEDs over a long period of time before they are definitely identified as being refractory. This may result in an increased exposure to adverse drug effects, seizure progression and an elevation in the cost of treatment (Elger, 2003). Resistance to AED treatment remains as a major therapeutic challenge, despite the increasing numbers of AEDs in routine clinical use. Two major theories have been proposed to explain the phenomenon of therapeutic failure in epilepsy (Elger, 2003; Loscher, 2005), both arising from a basic knowledge of clinical pharmacology. Firstly, the pharmacokinetic hypothesis suggests that AEDs do not reach the epileptic focus in sufficient concentration (Elger, 2003; Loscher, 2005; Szoeke *et al.*, 2006), and secondly, the pharmacodynamic hypothesis suggests that alteration in the subunit composition of ion channels and neurotransmitter receptors reduces their sensitivity to AEDs (Elger, 2003; Loscher, 2005).

The pharmacokinetic profile of AEDs is predominantly determined by the activities of DMEs and DTPs. Brain tissue obtained from patients with medically intractable seizures has been shown to express a higher level of the efflux transporter protein, P-gp (Tishler *et al.*, 1995; Marchi *et al.*, 2004) and several AEDs are believed to be substrates for P-gp-mediated transport (Potschka *et al.*, 2002). Overexpression of P-gp may result in an insufficient penetration of AEDs into brain tissue (Ramachandran & Shorvon, 2003) and has been proposed to be responsible for the phenomenon of pharmacoresistance in epilepsy. Seizure activity itself can increase the expression of P-

gp (Rizzi *et al.*, 2002; van Vliet *et al.*, 2004), suggesting that any delay in achieving optimal seizure control might increase the likelihood of long-term intractability to AED treatment.

A significant percentage of newly diagnosed epilepsy patients have been shown to respond poorly to LTG monotherapy (Kwan & Brodie, 2000) and one could argue that overexpression of P-gp may contribute to this poor response. It is widely recognised that the expression of P-gp can be influenced by polymorphisms in the ABCB1 gene (Hoffmeyer *et al.*, 2000; Tanabe *et al.*, 2001; Nakamura *et al.*, 2002) and that these genetic variants may influence the ability of P-gp to transport substrates across biological membranes. Given that LTG is believed to be a substrate for P-gp (Potschka *et al.*, 2002) and that this protein is up-regulated in epileptic brain tissue, it is not unreasonable to suggest that common polymorphisms in the ABCB1 gene could impact on the efficacy and/or toxicity of LTG monotherapy in newly diagnosed epilepsy.

The most commonly recognised SNPs in the human ABCB1 gene are the c.1236C>T, c.2677G>T/A and c.3435C>T substitutions. Several studies have explored the functional relevance of the ABCB1 c.2677G>T/A and c.3435C>T variants but inconsistent findings have been reported when employing drug pharmacokinetics as a phenotype (Hoffmeyer *et al.*, 2000; Kim *et al.*, 2001; Nakamura *et al.*, 2002; Oselin *et al.*, 2003). Other studies have evaluated the association between the ABCB1 c.3435C>T polymorphism and response to AED treatment and again have produced conflicting results (Siddiqui *et al.*, 2003; Tan *et al.*, 2004a; Sills *et al.*, 2005; Kim *et al.*, 2006). The 3435C allele was initial associated with poor response to AED treatment (Siddiqui *et al.*, 2003), but at least three further studies have failed to corroborate this original finding (Tan *et al.*, 2004a; Sills *et al.*, 2005; Kim *et al.*, 2006). Rather than investigate single genotypes, some investigators have suggested that haplotypes of the three

common polymorphisms in ABCB1 could be more useful in predicting drug responsiveness in epilepsy (Hung *et al.*, 2005; Seo *et al.*, 2006a).

Discrepancies between the results of individual studies may be attributed to sample size, or genetic background of the respective cohorts, or they may simply have arisen by chance (Sills *et al.*, 2005). Differences in AED regimens between invidual studies may have also significantly influenced the findings. Not all AEDs are believed to be transported by P-gp (Owen *et al.*, 2001; Potschka *et al.*, 2004). Therefore, examining the association between ABCB1 polymorphisms and the response to monotherapy treatment with an AED that has been shown to be a substrate of P-gp may be more appropriate.

In contrast to the transporter hypothesis discussed above, the pharmacodynamic theory of refractory epilepsy suggests that variations in the structure and subunit composition of ion channels may be associated with poor response to AED treatment (Ramachandran & Shorvon, 2003). This phenomenon may also contribute to poor seizure control with LTG therapy. LTG acts mainly on voltage-gated sodium channels to exert its antiepileptic effect (Zona & Avoli, 1997; Kohling, 2002), and any changes in sodium channel structure may affect the activity of LTG. As described in Chapter 3, the SCN2A c.56G>A substitution, results in a nonsynonymous amino acid change from arginine to lysine at codon 19 of the $Na_v1.2$ α-subunit protein (Ito *et al.*, 2004). This polymorphism has the potential to affect the conformation of the sodium channel and consequently its sensitivity to LTG. As such, this SCN2A variant may also be associated with interindividual variability in the responsiveness to LTG treatment.

5.1.2 Aims

In light of the pharmacokinetic and pharmacodynamic hypotheses of pharmacoresistant epilepsy, the prevalences of the ABCB1 c.1236C>T, ABCB1 c.2677G>T/A, ABCB1 c.3435C>T and SCN2A c.56G>A polymorphisms were investigated in a series of newly diagnosed epilepsy patients in an attempt to predict the response to LTG monotherapy.

5.1.3 Methods

5.1.3.1 Study subjects

From the overall 400-strong study population, 118 patients were identified as being treated with LTG monotherapy. A total of 79 patients from the 118 patients received LTG as their first ever AED, irrespective of seizure type. All of these patients were involved in a head to head monotherapy trial comparing LTG with VPA (Stephen *et al.*, 2007). Each patient was treated for at least 1 year with LTG. Patients who withdrew from LTG therapy for any reason before the end of the 12-month study period were excluded. None of the participants was concomitantly treated with any other medication (AEDs or non-AEDs) that might have influenced LTG metabolism.

5.1.3.2 Genotype analysis and phenotype assessment

Genotypes of the common SCN2A and ABCB1 gene polymorphisms were identified using the PCR-RFLP assays described in Chapter 3. Genotype determination completed for the SCN2A polymorphism on February 2006 and for ABCB1 polymorphisms on May 2006. Phenotype assessment was started on June 2006 and completed on July 2006.

5.1.3.3 Haplotype and diplotype inference

The haplotypes of ABCB1 polymorphisms were inferred using THESIAS software (Testing Haplotype Effects In Association Studies; Tregouet & Garelle, 2007). The haplotypes were presented in the arrangement of c.1236C>T-c.2677G>T/A-c.3435C>T. This study was not powered for this haplotype analysis due to limited sample size, therefore, the result was considered observational. ABCB1 diplotypes were inferred using combination of genotypes of the c.1236C>T, c.2677G>T/A and c.3435C>T polymorphisms, as described by Coulbault *et al.* (2006).

5.1.3.4 Evaluation of clinical response

Information regarding the outcome of LTG therapy was obtained retrospectively from the clinical study notes. Response to LTG was assessed by an experienced clinician and recorded in the study folders and electronic database. Complete medical history, seizure type and basic clinical factors such as height, weight, age and gender were documented at the first study visit. Progress was evaluated at study visits, scheduled every 4 to 6 weeks, from the day of treatment initiation and for at least 12 months thereafter. However, patients are allowed to contact or visit the clinic at anytime in cases of worsening of seizure control and adverse drug reaction. Records of each study visit included details of seizure frequency, adverse effects, and complete LTG treatment regimen. Patients were categorised as seizure free if no seizure was experienced within the last 6 months of the study period. In contrast, patients with uncontrolled seizures or those who had experienced seizure break-through within the last 6 months were categorised as non-seizure free.

5.1.3.5 Statistical analysis

Clinical and demographic differences between the seizure free and non-seizure free groups were investigated using the X^2 test. The distributions of age and maximum LTG dose were compared using Student's t-test. The possibility of genotype selection bias was explored by comparing the distribution of individual polymorphisms between the LTG cohort and the overall 400-strong patient population using the X^2 test.

The genotype distribution of each polymorphism was initially compared between seizure free and non-seizure free groups by contingency table tests. Logistic regression analysis was then used to evaluate differences in the prevalences of ABCB1 c.1236C>T, c.2677G>T/A, c.3435C>T and SCN2A c.56G>A polymorphisms. The analyses were conducted using allele, genotype, diplotype and haplotype frequencies. Specifically for haplotype analysis, the haplotype with the highest frequency was selected as the default reference. The odds ratio (OR) together with its 95% confidence interval (95% CI) was used as a measure of association between the gene polymorphisms and response to LTG treatment. All reported *p*-values are two-tailed.

5.1.4 Results

5.1.4.1 Patient demographics and lamotrigine dosage regimen

A total of 39 patients became seizure-free within the first 12 months of LTG treatment, while the remaining 40 patients continued to experience seizures despite attempts to optimise LTG therapy. The median age of patients was 35 years (range 13 to 80) and there were no significant differences in the distribution of gender ($p = 0.216$), age at evaluation ($p = 0.165$) or epilepsy syndrome ($p = 0.332$) between the seizure free and the non-seizure free groups (TABLE 5.1). At the end of one year, the non-seizure free

patients had reached a significantly higher daily LTG dose than the seizure free patients ($p < 0.001$; TABLE 5.1).

TABLE 5.1. Patient demographics and lamotrigine doses.

		Response to LTG Therapy		
		Seizure free *n = 39*	*Non-seizure free* *n = 40*	*p*-value
Gender	Male	56.4%	43.6%	0.216
	Female	42.5%	57.5%	
Age at evaluation	Mean	39.4	34.5	0.165
	Median	36.0	32.5	
	Min	13	14	
	Max	80	78	
Epilepsy syndrome	IGE	20.5%	30.0%	0.332
	LRE	79.5%	70.0%	
Maximum dose (mg/day)	Mean	180	294	< 0.001
	Median	150	300	
	Min	100	150	
	Max	400	600	

IGE = idiopathic generalised epilepsy; LRE = localisation related epilepsy. Statistical significance was determined by X^2 test for gender, age and epilepsy syndrome, and Student's t-test for LTG maximum dose. The *p*-value represents the comparison between seizure free and non-seizure free groups.

5.1.4.2 Associations between polymorphisms in the ABCB1 and SCN2A genes and the response to lamotrigine monotherapy

There was no significant difference in the genotype distributions of ABCB1 c.1236C>T, c.2677G>T/A, c.3435C>T and SCN2A c.56G>A polymorphisms between the LTG cohort and the overall 400-strong study population ($p > 0.05$; TABLE 5.2). The distributions of ABCB1 c.1236C>T, c.2677G>T/A, c.3435C>T and SCN2A c.56G>A genotypes were similar between the seizure free and the non-seizure free patients ($p > 0.05$; TABLE 5.3) and there was no significant association between the alleles or genotypes of common ABCB1 and SCN2A polymorphisms and the response to LTG monotherapy ($p > 0.05$; TABLE 5.4). The distribution of ABCB1 haplotypes is presented in TABLE 5.5. The major haplotypes of ABCB1 in the LTG cohort were the T-T-T (38.8%) and C-G-C (36.9%; TABLE 5.5). There was no significant difference in the response to LTG treatment when common ABCB1 haplotypes were compared with the T-T-T default reference haplotype ($p > 0.05$; TABLE 5.6) There was similarly no association between the major diplotypes in ABCB1 and the response to LTG treatment ($p > 0.05$; TABLE 5.7).

5.1.5 Discussion

Overexpression of P-gp has been postulated to be one explanation for the poor response to AED treatment. Brain tissue from patients with medically intractable seizures has a high level of P-gp expression and several AEDs are believed to be transported by this protein (Tishler *et al.*, 1995; Potschka *et al.*, 2001; Marchi *et al.*, 2004). LTG has been shown to be a substrate for P-gp (Potschka *et al.*, 2001) and thus, it is possible that a P-gp-related mechanism might be responsible for any poor clinical response to LTG treatment.

TABLE 5.2. Genotype distributions of common SCN2A and ABCB1 polymorphisms between the lamotrigine cohort and the overall 400-strong study population

		Genotype frequency			
Polymorphism	**Cohort**	***Minor***	***Hetero***	***Major***	***p*-value**
SCN2A c.56G>A	SSP	0.013	0.112	0.875	0.532
	LTG	0.025	0.140	0.835	
ABCB1 c.1236C>T	SSP	0.245	0.470	0.285	0.657
	LTG	0.203	0.599	0.278	
ABCB1 c.2677G>T/A	SSP	0.23	0.45	0.32	0.457
	LTG	0.10	0.52	0.30	
ABCB1 c.3435C>T	SSP	0.182	0.485	0.333	0.723
	LTG	0.177	0.532	0.291	

SSP = 400-strong study population (n = 400); LTG = LTG cohort (n = 79); Minor = homozygous of minor allele; Hetero = heterozygous of minor and major alleles; Major = homozygous of major allele. Statistical significance was determined by X^2 test. The *p*-value represents the comparison between LTG cohort and SSP.

TABLE 5.3. Comparison of the genotype distribution of common polymorphisms in SCN2A and ABCB1 between seizure free and non-seizure free groups.

Gene Polymorphism	Genotype	Seizure free n = 39	Non-seizure free n = 40	*p*-value
SCN2A c.56G>A	GG	34	32	0.755
	GA	4	7	
	AA	1	1	
ABCB1 c.1236C>T	CC	12	10	0.605
	CT	21	20	
	TT	6	10	
ABCB1 c.2677G>T	GG	13	11	0.908
	GT	19	22	
	TT	6	7	
	TA	1	0	
ABCB1 c.3435C>T	CC	7	7	0.985
	CT	21	21	
	TT	11	12	

Statistical significance was determined by X^2 test or Fisher's Exact test. The *p*-value represents the comparison of the genotype distribution between seizure free and non-seizure free groups.

TABLE 5.4. Logistic regression analysis of response to lamotrigine monotherapy on the basis of genotype and allele of common polymorphisms in SCN2A and ABCB1 genes.

Gene Polymorphism	Genotype	Allele	Odds Ratio	95% CI	p-value
SCN2A c.56G>A	GG vs not GG		1.70	0.50 – 5.74	0.393
	GA vs not GA		0.54	0.14 – 2.01	0.357
	AA vs not AA		1.03	0.06 – 17.01	0.986
	GG vs AA		1.06	0.06 – 17.71	0.966
		G vs A	0.66	0.22 – 1.94	0.448
ABCB1 c.1236C>T	CC vs not CC		1.33	0.50 – 3.58	0.568
	CT vs not CT		1.17	0.48 – 2.82	0.732
	TT vs not TT		0.55	0.18 – 1.68	0.292
	CC vs TT		2.00	0.54 – 7.45	0.301
		C vs T	0.73	0.39 – 1.37	0.333
ABCB1 c.2677G>T	GG vs not GG		1.32	0.50 – 3.45	0.573
	GT vs not GT		0.78	0.32 – 1.88	0.577
	TT vs not TT		0.86	0.26 – 2.82	0.800
	GG vs TT		1.38	0.36 – 5.34	0.642
		G vs T	0.97	0.43 – 2.19	0.941
ABCB1 c.3435C>T	CC vs not CC		1.03	0.32 – 3.27	0.958
	CT vs not CT		1.06	0.44 – 2.56	0.905
	TT vs not TT		0.92	0.35 – 2.42	0.861
	CC vs TT		1.09	0.29 – 4.12	0.898
		C vs T	0.96	0.51 – 1.79	0.887

TABLE 5.5. The distribution of ABCB1 haplotypes between seizure free (n = 39) and non-seizure free (n = 40) patients.

	Haplotype frequency	
ABCB1 haplotype	Seizure free	Non-seizure free
T-T-T	0.368	0.408
C-G-C	0.393	0.346
C-G-T	0.142	0.026
T-G-C	0.054	0.078
C-T-T	0.041	0.028
T-T-C	0.001	0.014

TABLE 5.6. Logistic regression analysis of response to lamotrigine monotherapy on the basis of ABCB1 haplotype. Only haplotypes with a frequency of more than 1% in both seizure free and non-seizure free patients were examined.

ABCB1 haplotype	Odds ratio	95% confidence interval	*p*-value
T-T-T	1.00	reference	
C-G-C	0.82	0.43 – 1.57	0.559
C-G-T	0.87	0.36 – 2.14	0.769
T-G-C	1.42	0.46 – 4.44	0.545
C-T-T	0.71	0.12 – 4.33	0.702

TABLE 5.7. Logistic regression analysis of response to lamotrigine monotherapy on the basis of major diplotype of the ABCB1 gene.

Diplotype of ABCB1*	Odds Ratio	95% CI	p-value
CC-GG-CC vs not CC-GG-CC	1.03	0.30 – 3.52	0.393
CT-GT-CT vs not CT-GT-CT	1.30	0.52 – 3.27	0.580
TT-TT-TT vs not TT-TT-TT	0.83	0.23 – 2.99	0.780
CC-GG-CC vs TT-TT-TT	1.20	0.23 – 6.19	0.827

*Diplotype arrangement: ABCB1 c.1236C>T-c.2677G>T/A-c.3435C>T.

Variability in the expression and activity of P-gp can be influenced by polymorphisms in the encoding gene (Hoffmeyer *et al.*, 2000). Of the three most widely studied polymorphisms in ABCB1, only the c.2677G>T/A transition is known to produce an amino acid change which might in turn be associated with a functionally relevant phenotype (Cascorbi, 2006). The common polymorphisms of the ABCB1 gene are strongly linked to each other (Kim *et al.*, 2001; Tanabe *et al.*, 2001; Tang *et al.*, 2002; Tang *et al.*, 2004), most likely because they exist within a single block of linkage disequilibrium. Accordingly, the association between common ABCB1 polymorphisms and the response to LTG treatment was investigated both individually and in combination analyses using allele, genotype, haplotype, and diplotype of the ABCB1 gene.

This study took advantage of an existing monotherapy comparison between VPA and LTG in order to isolate a cohort of newly diagnosed epilepsy patients treated with a known substrate of P-gp as monotherapy. This allowed the elimination of a number of

confounding factors which may have contributed to the conflicting results of previous investigations (Siddiqui *et al.*, 2003; Tan *et al.*, 2004a; Sills *et al.*, 2005; Basic *et al.*, 2006; Kim *et al.*, 2006). Evaluating the response to AED treatment without considering the spectrum of individual drugs to which the patients were exposed may have undermined the conclusions of previous studies.

There is currently no firm evident to suggest that all clinically used AEDs are substrates for P-gp. This is clearly emphasised in recent studies of both CBZ (Owen *et al.*, 2001; Potschka *et al.*, 2001) and levetiracetam (Potschka *et al.*, 2004). It is therefore unlikely that any changes in the expression or activity of P-gp resulting from ABCB1 gene polymorphisms would influence the penetration of these AEDs into brain tissue. Hence, their inclusion in efficacy studies would weaken or possibly mask any evidence of association between genetic variants and response to AED treatment, particularly where significant numbers of CBZ and levetiracetam exposed patients are involved. In addition, AED polytherapy is commonly used in the management of patients with partially responsive epilepsy and this fact could also have affected study findings. The concomitant use of multiple AEDs may result in unpredictable interactions at the level of both DMEs and DTPs. Furthermore, some AEDs have been shown to stimulate the expression of P-gp (Weiss *et al.*, 2003). Thus, drug-drug and drug-Pgp interactions could independently influence the response to AED treatment. This underlines the importance of performing such studies in patients with newly diagnosed epilepsy treated with AED monotherapy.

There were no significant differences in the distribution of clinical factors such as age, gender and epilepsy syndromes between seizure free and non-seizure free groups. This would indicate that any variability arising from clinical factors was equally proportioned. The significantly higher daily dose of LTG in the non-seizure free group

is symptomatic of ongoing titration and excludes any suggestion of undertreatment in these poor-reponders. Almost 50% of the patients in the LTG cohort failed to achieve seizure free status within the first 12 months of LTG monotherapy. This percentage is higher than the value previously reported in newly diagnosed epilepsy patients (Kwan & Brodie, 2000). Differences in the duration of follow-up may explain this discrepancy.

No significant association was observed between ABCB1 c.1236C>T, c.2677G>T/A and c.3435C>T polymorphisms and the response to LTG treatment, irrespective of whether analyses were based on allele, genotype, haplotype or diplotype frequencies. These findings would support the observations reported previously by Tan *et al.* (2004a), Sills *et al.* (2005) and Kim *et al.* (2006). If P-gp expression and activity are genuinely influenced by common polymorphisms in the ABCB1 gene, the current study findings would question the role of P-gp in mediating resistance to treatment with LTG. Either the distribution of LTG in human brain is not significantly affected by P-gp or the function of P-gp at the BBB is not influenced by common ABCB1 polymorphisms. However, there has been a suggestion that pathological overexpression of P-gp in the epileptic focus may overcome any genetically determined variability (Sills *et al.*, 2005). In addition, it is reasonable to speculate that LTG may also be transported by other, as-yet-unidentified DTPs (Cascorbi, 2006). Other DTPs could potentially compensate for any deficiency in the function of P-gp related to genetic variability. As such, changes in P-gp activity secondary to polymorphic expression of the ABCB1 gene might not be demonstrable in a clinical study of drug efficacy. One must also appreciate that the response to LTG treatment can be influenced by a multitude of additional pharmacokinetic and pharmacodynamic factors, such as the conformation of voltage-gated sodium channels and the activity of glucuronidation enzymes.

For AEDs such as LTG, which act predominantly on voltage-gated sodium channels, changes in the structure or function of the sodium channel may significantly influence the response to treatment. In this study, the SCN2A c.56G>A polymorphism did not appear to be associated with the response to LTG treatment. This might suggest that the SCN2A c.56G>A polymorphism does not significantly affect the expression or function of the $Na_v1.2$ protein in the neuronal tissue or its sensitivity to blockade by LTG. However, it is also recognised that LTG can act on other voltage-gated sodium channels, potentially compensating for any functional or structural defect in the $Na_v1.2$ protein.

This investigation failed to demonstrate any association between the ABCB1 c.1236C>T, c.2677G>T/A, c.3435C>T and SCN2A c.56G>A polymorphisms and the response to initial LTG monotherapy. Some common issues to pharmacogenetic studies that may influence their findings should be considered, especially the consensus of phenotype criteria. At the very least, it is important to recognise that results are strongly influenced by the duration of follow-up which was, in this case, 12 months of LTG therapy. The duration of follow-up can influence the number of patients who are categorised as seizure free and non-seizure free. Hence, the conclusions of any such study should be considered alongside limitations of study design.

5.1.6 Conclusion

There was no evidence of any association between the prevalences of ABCB1 c.1236C>T, c. 2677G>T/A, c.3435C>T and SCN2A c.56G>A polymorphisms and the response to LTG monotherapy within the first 12 months of treatment. This study included only a small number of patients which could have had a significant bearing on the results. The influence of ABCB1 and SCN2A polymorphisms should be investigated

prospectively in a considerably larger cohort of patients with newly diagnosed epilepsy and treated with AED monotherapy in order that a more definitive conclusion can be reached. In the meantime, any functional significance of ABCB1 and SCN2A gene variants on pharmacoresistant epilepsy remains unclear.

5.2 Genetic Predictors of Lamotrigine Optimal Dose

5.2.1 Introduction

AED dosing is typically based on a trial and error approach allied to the individual clinical experience. In the absence of a rational approach to identify optimal dose, the duration of uncontrolled seizures may be prolonged and some patients may be exposed to unnecessarily high AED doses. A wide interindividual variability in dosage requirement has been documented for most AEDs. For example, the effective doses leading to seizure freedom with CBZ range between 200 and 1600 mg/day, and for VPA between 200 and 2500 mg/day (Kwan & Brodie, 2001). Differences in the required dose between individuals are believed to be linked to genetic variability in genes associated with AED pharmacodynamics and pharmacokinetics. Genetic variants that influence dosing regimen have been reported for both PHT and CBZ, with maximum doses of these drugs associated with polymorphisms in the SCN1A and CYP2C9 genes (van der Weide *et al.*, 2001; Tate *et al.*, 2005).

A study by Kwan and Brodie (2001) reported that a modest dose of LTG (100 to 200 mg/day) can provide complete seizure control in a large number of patients. However, some patients may require doses up to 600 mg/day to achieve a similar degree of efficacy. Interindividual variability in LTG dosage requirement is likely to be influenced by both pharmacodynamic and pharmacokinetic factors. Although the

previous study (section 5.1) failed to demonstrate any association between the response to LTG therapy and common SCN2A and ABCB1 polymorphisms, it did not directly investigate any association between these genetic variants and LTG dose. It is important to note that the response to LTG treatment is not correlated with plasma concentration or dose (Kilpatrick *et al.*, 1996; Mahmood *et al.*, 1998).

Thus, it is reasonable to speculate that the SNPs which were studied in the previous section may be able to influence the LTG dose requirement. In this section, these polymorphisms were investigated in relation to the dose of LTG, rather than its efficacy.

5.2.2 Aims

This study was designed to evaluate the association between common polymorphisms in the SCN2A and ABCB1 genes and the optimal dose of LTG. Basic clinical factors such as age and gender were also included as potential predictors of optimal dose.

5.2.3 Methods

5.2.3.1 Definitions

Optimal dose was defined as the final dose given to a patient that successfully maintained optimal seizure control without intolerable side effects and which was maintained throughout the period of follow-up without the need for adjustment. Optimal seizure control was defined as seizure freedom for at least 1-year on LTG monotherapy.

5.2.3.2 Study subjects

From the overall 400-strong study population, a total of 118 patients were identified as being treated with LTG monotherapy. Through retrospective evaluation of individual

patient records, 94 of these patients were identified as having achieved seizure free status for a period of at least 1 year. These patients included those who achieved seizure free within and after the first 12 months of LTG initiation and those received LTG as a second line monotherapy AED. Complete information on LTG dosing regimen and other basic clinical data was available for all of these individuals. None was concomitantly treated with any other medication (AEDs and non-AEDs) that might have influenced LTG metabolism.

5.2.3.3 Genotype analysis and phenotype assessment

Genotypes of the common SCN2A and ABCB1 gene polymorphisms were identified using the PCR-RFLP assays described in Chapter 3. Genotype determination completed for the SCN2A polymorphism on February 2006 and for ABCB1 polymorphisms on May 2006. Phenotype assessment was started on June 2006 and completed on October 2006.

5.2.3.4 Statistical analyses

Clinical and demographic differences between the LTG cohort and the 400-strong study population were investigated using X^2 test. The distribution of age was compared using Student's t-test. Differences in the mean LTG optimal dose between categorical variables (gender and epilepsy syndromes) were compared by t-test or analysis of variance (ANOVA), as appropriate. The association between age and optimal dose of LTG was evaluated using Pearson's correlation test. Evidence of genotype selection bias was investigated by comparing the distribution of each polymorphism between the LTG cohort and the overall 400-strong study population.

To fit the genetic information into the regression database, a score was assigned to the genotype of each polymorphism according to the number of substituting alleles; no substituting alleles was given score 1, one substituting allele was given score 2 and two substituting alleles was given score 3. Interaction between polymorphisms was investigated by Pearson's correlation test and represented by the multiplicative value of polymorphism scores.

Candidate predictors included age, gender and the genotypes of each polymorphism. The influence of each predictor on LTG optimal dose was analysed individually using univariate linear regression analysis and, in combination, using multivariate linear regression analysis. An additive model was used in the analysis with an assumption that the combined effects of genetic alleles at two or more gene loci are equal to the sum of their individual effects. SNP interactions were added to the multivariate model and acted as a non-linear component. Predictors were added or removed using a stepwise approach, depending on the degree of impact indicated by the respective *p*-value. Data transformation from linear to natural logarithmic form was employed to optimise regression modelling. Coefficients of regression of the predictors which significantly influenced the model were used to construct a predictive equation for LTG optimal dose. Pearson's correlation test was used to evaluate the relationship between the observed optimal doses and the optimal doses predicted by the equation. Pearson's correlation value (r) of more than 0.8 indicated a strong correlation between variables. A *p*-value of less than 0.05 indicated a significant predictor effect. Both $P_{residual}$, which represented the *p*-value of residual analysis, and r^2, which represented the goodness-of-fit, indicated how well the model fitted the data.

5.2.4 Results

5.2.4.1 Patient demographics and lamotrigine dosage regimen

Demographic information for patients in the LTG cohort is presented in TABLE 5.8. Distribution of gender ($p = 0.887$) and epilepsy syndromes ($p = 0.429$) were comparable with the 400-strong study population. The median age of patients in the LTG cohort was 38.5 years (range 16.5 to 85).

TABLE 5.8. Patient demographics and lamotrigine doses.

		LTG Cohort (n = 94)	*400-strong study population (n = 400)*	*p-value*
Gender	Male	51.0%	50.3%	0.887
	Female	49.0%	49.7%	
Age (year)	Median	38.5	40	0.460
	Min	16.5	14	
	Max	85	85	
Epilepsy syndrome	IGE	35.1%	29.5%	0.429
	LRE	60.6%	67.5%	
	UNC	4.3%	3.0%	
Optimal dose (mg/day)	Median	200		
	Min	50		
	Max	600		

Min = minimum, Max = maximum; IGE = idiopathic generalised epilepsy; LRE = localisation related epilepsy; UNC = unclassified epilepsy. Statistical significant was determined by Student's t-test or X^2 test, where appropriate. The *p*-value represents the comparison between the LTG cohort and 400-strong study population.

All of the patients received LTG on a twice-daily basis with total daily dose ranging from 50 to 600 mg. Female patients received higher LTG doses in order to achieve optimal seizure control (male *vs* female, 201 *vs* 279 mg/day respectively, $p = 0.002$; FIGURE 5.1). There was no significant association between LTG dose and age of the patients ($r = -0.096$, $p = 0.356$; FIGURE 5.2) or epilepsy syndromes ($p = 0.674$; FIGURE 5.4).

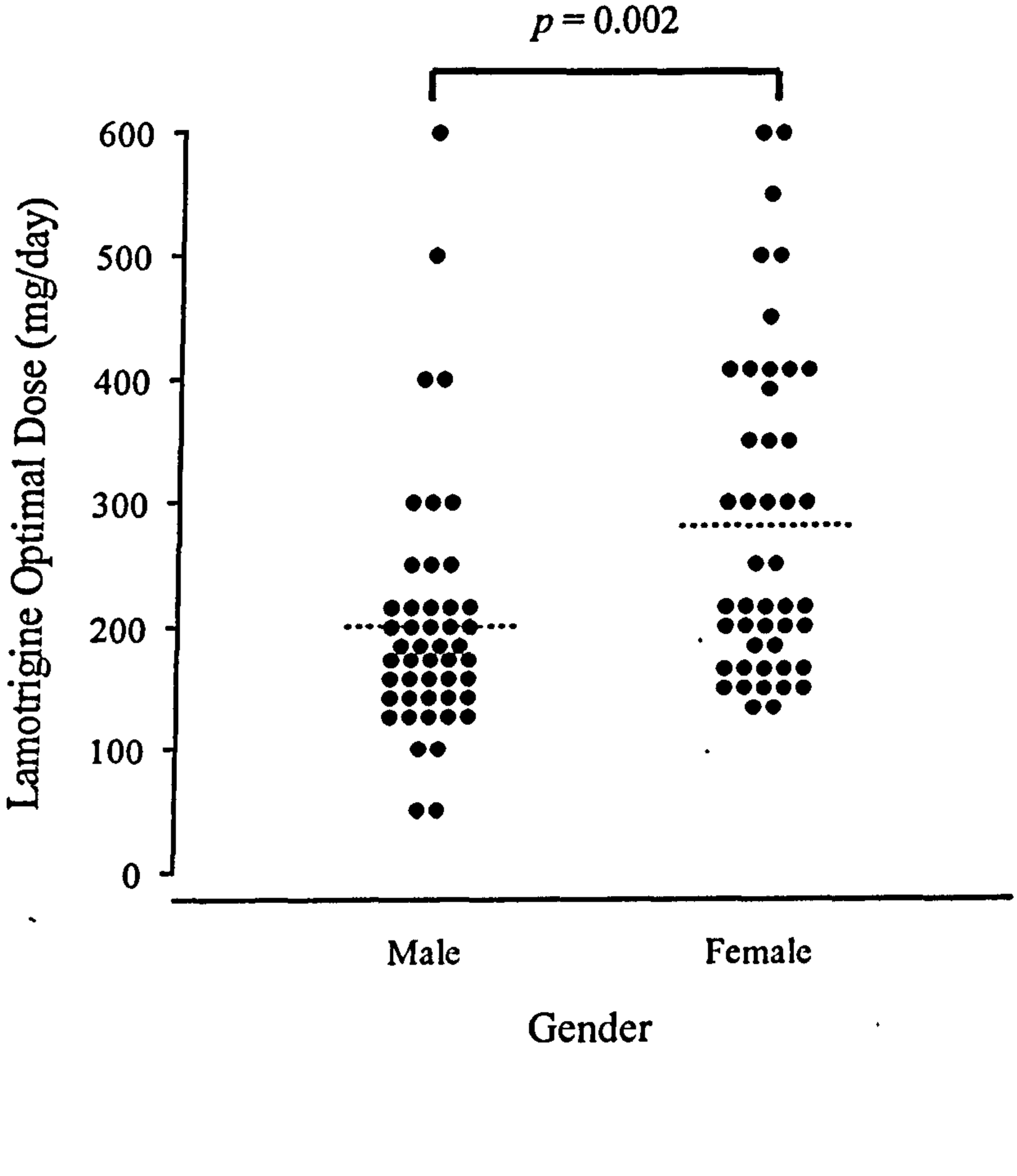

FIGURE 5.1. Distribution of lamotrigine optimal dose between genders. Statistical significance was determined by Student's t-test. Dotted lines represent the average optimal dose for respective gender.

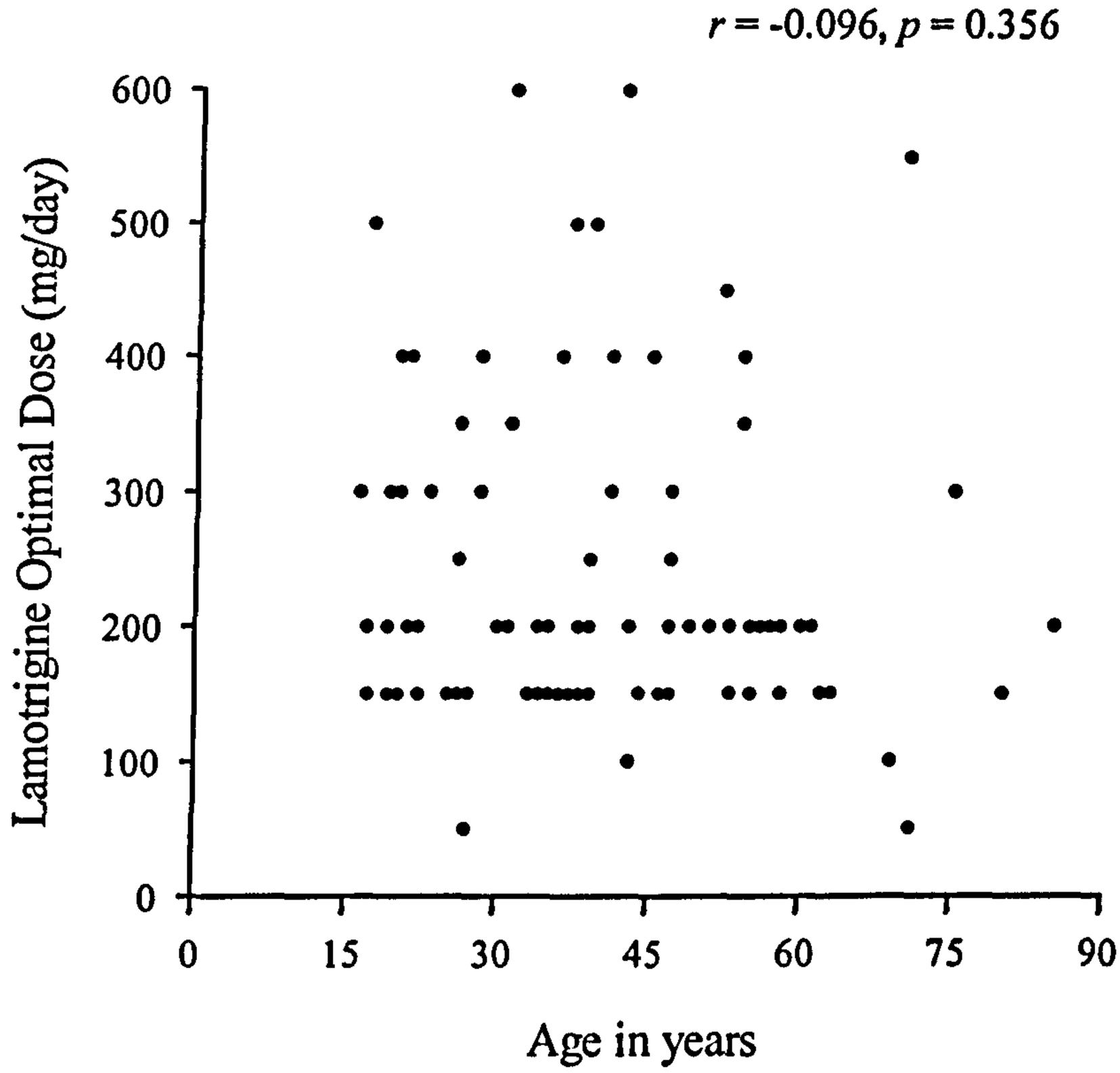

FIGURE 5.2. Distribution of lamotrigine optimal dose and age at achieving 12 months of seizure freedom. Correlation was examined by Pearson's correlation test.

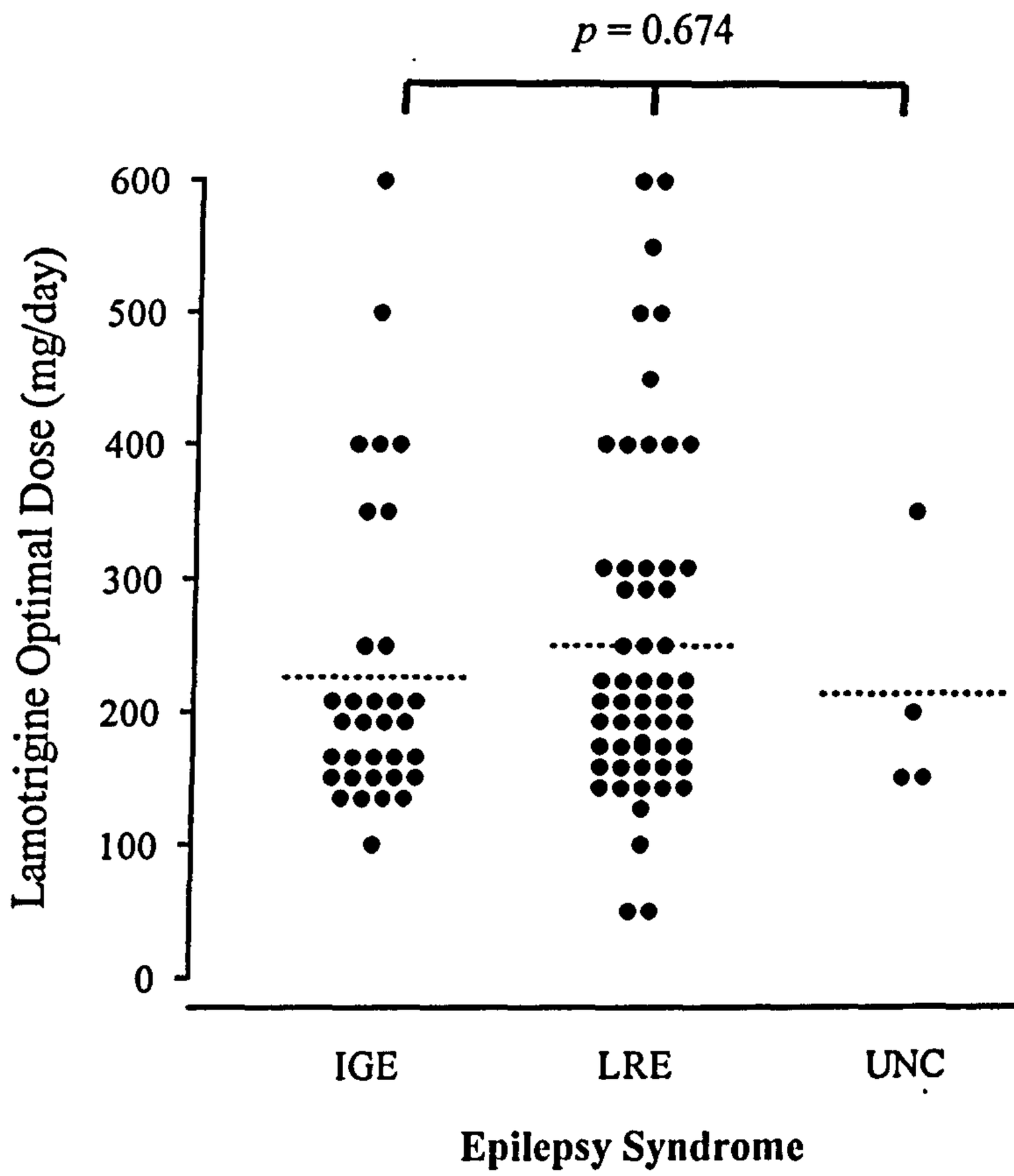

FIGURE 5.3. Distribution of lamotrigine optimal dose between epilepsy syndromes. Statistical significance was determined by ANOVA. Dotted lines represent the average optimal dose for respective epilepsy syndrome. IGE = idiopathic generalised epilepsy; LRE = localisation related epilepsy; UNC = unclassified epilepsy.

5.2.4.2 Genetic predictors of lamotrigine optimal dose

There were no significant differences in the distribution of common polymorphisms in the ABCB1 and SCN2A genes between the LTG cohort and the overall 400-strong study population (TABLE 5.9). Three potential SNP interactions were identified between ABCB1 c.1236C>T and c.2677G>T/A ($r = 0.751$, $p < 0.001$), c.1236C>T and c.3435C>T ($r = 0.549$, $p < 0.001$), c.2677G>T/A and c.3435C>T ($r = 0.618$, $p < 0.001$). Univariate linear regression analysis demonstrated that single predictors for LTG optimal dose included gender ($p = 0.002$) and genotype of ABCB1 c.1236C>T ($p = 0.010$). However, the strength of prediction was weak in both cases ($r^2 < 10\%$; TABLE 5.10). In an effort to improve the strength of prediction, the candidate predictors were evaluated using a multivariate linear regression analysis (TABLE 5.11). The basic regression model (Model 1) represents the combination effects of all candidate predictors and showed a better overall model fit ($r^2 = 11.2\%$, $p = 0.023$) than any individual univariate analysis. Stepwise multivariate regression analysis identified gender and the SNP interaction between ABCB1 c.1236C>T and c.3435C>T as the optimal combination of predictors ($r^2 = 14.1\%$, $p < 0.001$; Model 2). Transformation of optimal dose data from linear to natural logarithmic form further improved the overall model fit ($r^2 = 16.6\%$, $p < 0.001$; Model 3). A predictive equation for LTG optimal dose was constructed from the regression constant and coefficients of Model 3 and is presented below:

$$\ln OD_{LTG} = 4.68 + 0.32*\text{Gender} + 0.05*(\text{ABCB1 c.1236C>T}*\text{c.3435C>T})$$

where; $\ln OD_{LTG}$ = natural log of LTG optimal dose (mg/day); scores for gender, 1 = male, 2 = female; scores for ABCB1 c.1236C>T and c.3435C>T genotypes, 1 = C/C, 2 = C/T, 3 = T/T.

A statistically significant correlation was observed between predicted and observed LTG optimal doses ($p < 0.001$), however, the strength of the association was modest ($r = 0.400$, 95%CI 0.22 to 0.56; FIGURE 5.4).

TABLE 5.9. Comparison of distributions of common SCN2A and ABCB1 polymorphisms between the lamotrigine cohort and the 400-strong study population.

		Genotype frequency			
Polymorphism	Cohort	*Minor*	*Hetero*	*Major*	*p*-value
SCN2A c.56G>A	SSP	0.013	0.112	0.875	0.974
	LTG	0.010	0.106	0.884	
ABCB1 c.1236C>T	SSP	0.245	0.470	0.285	0.300
	LTG	0.170	0.521	0.309	
ABCB1 c.2677G>T/A	SSP	0.230	0.450	0.320	0.147
	LTG	0.140	0.500	0.360	
ABCB1 c.3435C>T	SSP	0.182	0.485	0.333	0.305
	LTG	0.181	0.564	0.255	

SSP = 400-strong study population; LTG = LTG cohort (94 patients); Minor = homozygous for minor allele; Hetero = heterozygous for minor and major alleles; Major = homozygous for major allele. Statistical significance was determined by X^2 test. The *p*-value represents comparison of LTG and SSP data.

TABLE 5.10. Summary of univariate regression analysis of variables predicting the optimal dose of lamotrigine.

Predictor	***Coef.***	***r^2 (%)***	***p-value***
Gender	78.31	9.2	0.002
Age	-0.72	0.9	0.358
SCN2A c.56G>A	-35.07	0.0	0.612
ABCB1 c.1236C>T	47.72	5.9	0.010
ABCB1 c.2677G>T/A	27.81	1.3	0.143
ABCB1 c.3435C>T	29.01	1.3	0.135

The relative impact of each predictor is presented by the coefficient value (*Coef.*). The goodness-of-fit value (r^2) indicates how well the individual predictors fit the model. The *p*-value indicates the probability of the predictor influencing LTG optimal dose.

TABLE 5.11. Summary of hierarchical multivariate regression analysis of variables predicting the optimal dose of lamotrigine.

Predictor	Model 1		Model 2		Model 3 (natural log)	
	Coef.	***p*-value**	**Coef.**	***p*-value**	**Coef.**	***p*-value**
Constant	176.30	0.121	74.79	0.077	4.68	<0.001
Gender	83.11	0.001	76.13	0.002	0.32	0.001
Age	-0.14	0.853	-	-	-	-
SCN2A c.56G>A	-45.15	0.207	-	-	-	-
ABCB1 c.1236C>T	-18.12	0.791	-	-	-	-
ABCB1 c.2677G>T/A	57.51	0.499	-	-	-	-
ABCB1 c.3435C>T	-100.0	0.382	-	-	-	-
ABCB1 1236C>T*2677G>T/A†	8.87	0.813	-	-	-	-
ABCB1 1236C>T*3435C>T†	-4.13	0.923	12.46	0.015	0.05	0.009
ABCB1 2677G>T/A*3435C>T†	22.48	0.591	-	-	-	-
r^2 (%)	11.2		14.1		16.6	
$p_{residual}$	0.023		<0.001		<0.001	

The relative impact of each predictor is presented by the coefficient value (*Coef.*). The *p*-value indicates the probability of the predictor affecting LTG optimal dose. The goodness-of-fit value (r^2) indicates how well the predictors fit the model. The significance of model fitting is represented by the *p*-value of residual analysis ($p_{residual}$). '-' mark represents predictors that were eliminated in the stepwise regression analysis. †SNP interaction between predictors.

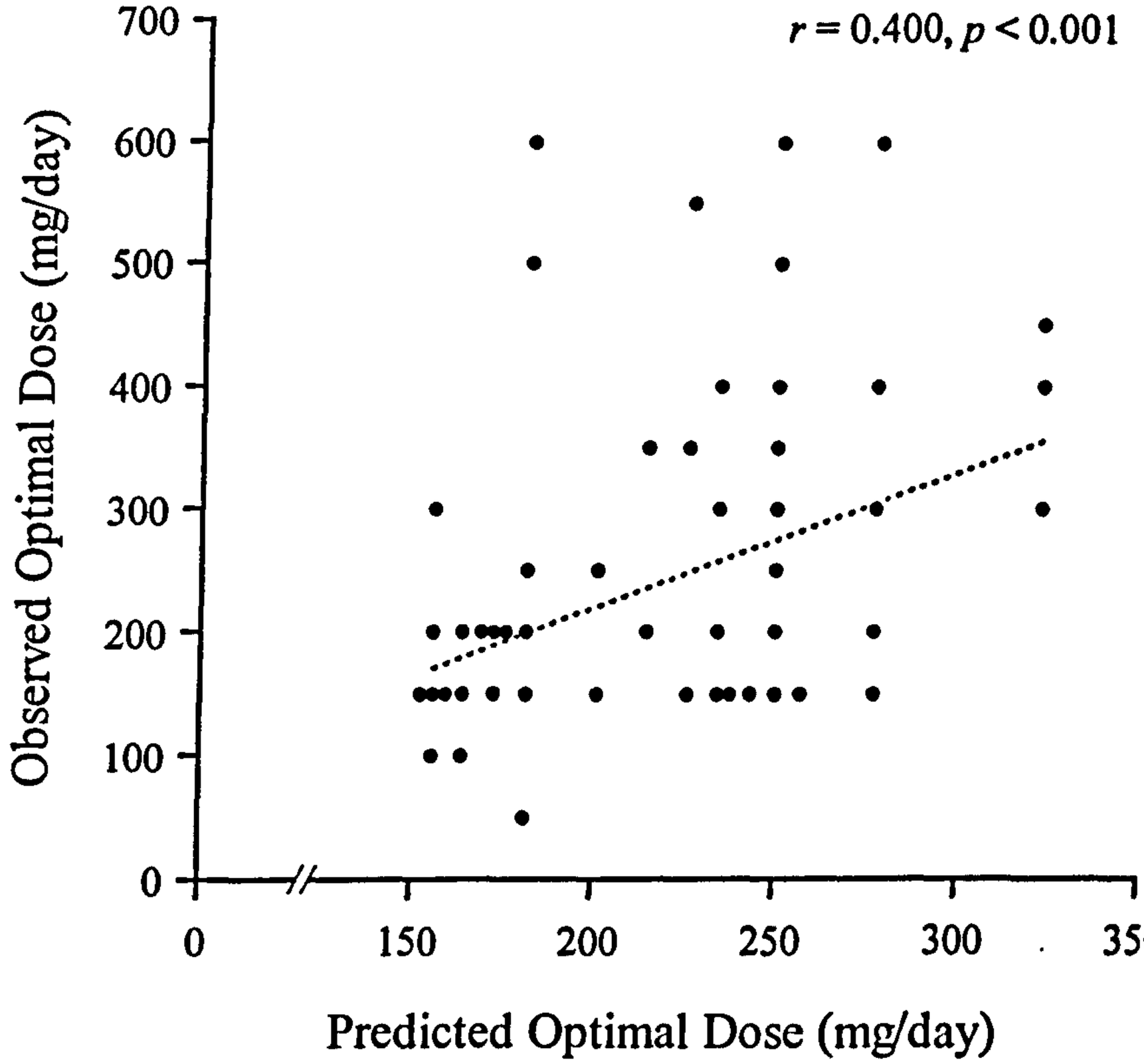

FIGURE 5.4. Correlation between observed and predicted lamotrigine optimal dose based on the optimal multivariate regression model. Statistical significance was determined by Pearson's correlation test. A single data point (●) may represent more than one value. Dotted line is the correlation between observed and predicted doses.

5.2.5 Discussion

One of the biggest challenges in the treatment of epilepsy is the identification of the best dose of any given AED for a particular patient. Conventionally, optimal doses are established through a trial and error method. During the period of dosing adjustment, quality of life may be impacted by either under-treatment or over-treatment. This concern applies to all epilepsy patients and all AEDs, including LTG. Although complete control of seizures can be achieved with doses of 100 to 200 mg/day of LTG in a large number of patients, higher doses of up to 600 mg/day may be required by some individuals (Kwan & Brodie, 2001). Continuous titration of LTG may be undertaken in an attempt to achieve complete seizure control and this may expose non-responsive patients to unnecessarily high doses of LTG, increasing the risk of adverse effects and the cost of treatment. Prediction of LTG optimal dose may be useful in guiding dosing strategies and could improve the usage of LTG by employing slow titration and low target doses in potentially sensitive individuals and a quicker titration and higher target doses in less sensitive patients.

In addition to conventional therapeutic drug monitoring, a genetic-based approach to the optimisation of AED dose may be useful (Tate *et al.*, 2005; Ferraro & Buono, 2005). Potential genetic predictors of optimum dose are genes which encode proteins involved in the pharmacokinetics and pharmacodynamics of LTG such as DMEs, DTPs and voltage-gated ion channels. LTG has been suggested to be a substrate for P-gp (Potschka *et al.*, 2002) and its principle mechanism of action is believed to be blockade of sodium channels (Zona & Avoli, 1997; Kohling, 2002). Therefore, polymorphisms in the ABCB1 gene that encodes P-gp and the SCN2A gene which encodes the $Na_v1.2$ sodium channel were evaluated as potential predictors of LTG optimal dose. Basic

clinical factors such as age and gender were included in the evaluation as additional candidate predictors.

This study failed to demonstrate any association between age and LTG optimal dose. This is not surprising given that a previous report suggested that LTG pharmacokinetics were not significantly influenced by age in an adult population (Hussein & Posner, 1997). The SCN2A c.56G>A polymorphism was similarly unrelated to LTG optimal dose when assessed either individually or in combination with other candidate predictors. If the function of the $Na_v1.2$ sodium channel is genuinely influenced by the SCN2A c.56G>A polymorphism, then it is possible any effect of this polymorphism on LTG dosage requirement was masked by the interaction of LTG with other sodium channel subtypes (Zona & Avoli, 1997; Kohling, 2002). A polymorphism in the SCN1A gene has been associated with maximum doses of both PHT and CBZ (Tate *et al.*, 2005) and plasma concentrations of PHT at maintenance dose (Tate *et al.*, 2006). It is reasonable to suggest that this particular polymorphism in the SCN1A gene should be included in any future search for candidate predictors of LTG optimal dose.

Gender and the interaction between ABCB1 c.1236C>T and c.3435C>T polymorphisms were shown to possess the greatest predictive capacity for the optimal dose of LTG when employed together in a multivariate regression model. How gender might influence the LTG optimal dose is unclear, as the pharmacokinetics of LTG are not believed to differ between males and females (Hussein & Posner, 1997). It is possible that this observation is based on differences in body weight, fat composition, or in sex hormone activity between the genders (Gallagher *et al.*, 1996; Tanaka, 1999). Females have a higher fat composition than males (Lemieux *et al.*, 1993; Gallagher *et al.*, 1996), and this may increase the V_d of lipophilic drugs (Mangoni & Jackson, 2003; Thomson, 2004). LTG is highly lipophilic in nature (Mashru *et al.*, 2005) and, therefore, a higher

dose may be required by females to achieve a similar target concentration. Unfortunately, the current study did not include measures of body weight, or further investigate the effect of fat composition, which may have been enlightening with regard to the relationship between dose and gender.

The association between the ABCB1 c.1236C>T polymorphism and LTG dose was significant, with or without the presence of gender information. Although the c.1236C>T polymorphism does not result in an amino acid substitution in the encoded protein, this polymorphism may be linked to an as yet unidentified causal variant which could in theory influence the activity of P-gp or indeed any other LTG-related pharmacokinetic and pharmacodynamic factor. Previous studies exploring the functional significance of the ABCB1 gene polymorphisms on drug pharmacokinetics have been largely inconclusive (Tanabe *et al.*, 2001; Goto *et al.*, 2002; Nakamura *et al.*, 2002), with differences in substrate and study design believed to be the major contributing factor to the conflicting findings. The c.3435C>T substitution is the most studied polymorphism in the ABCB1 gene. There is no general consensus with regard to the functional significance of ABCB1 variants associated with the c.3435C>T polymorphism. The 3435T allele has been associated with both higher and lower expression of P-gp depending on substrate studied (Hoffmeyer *et al.*, 2000; Nakamura *et al.*, 2002). Elevation in P-gp expression is believed to decrease absorption and increase elimination of many drugs. These effects may explain the association between the 3435T allele and reduced concentrations of fexofenadine (Kim *et al.*, 2001) and also the poor response to chemotherapy (Sohn *et al.*, 2006). As the 3435C>T and 1236C>T polymorphisms are strongly under linkage disequilibrium (Kim *et al.*, 2001; Tang *et al.*, 2002; Tang *et al.*, 2004), a similar mechanism may also explain the association between LTG dose and the 1236T allele. This would, in turn, explain why the association

between the c.1236C>T polymorphism and optimal dose of LTG was much stronger when the c.3435C>T polymorphism was included in the multivariate analysis.

The small number of participants and the retrospective nature of this study may limit the potential significance of the findings. A larger prospective study in newly diagnosed epilepsy must be undertaken before the clinical implications of these results can be realistically assessed. With respect to LTG therapy, this study provides some information which may guide future dosing decisions. The combination of ABCB1 c.1236C>T-c.3435C>T SNP interaction and gender explains approximately 17% of the interindividual variability in LTG optimal dose and may be an important clinical indicator of whether a patient will require low or high doses of LTG to achieve complete seizure control. This could potentially prevent the under-treatment of responsive individuals or reduce the risk of unnecessary exposure to high doses of LTG in patients who are non-responders. Despite this encouraging finding, more candidate predictors are required to strengthen the model. These should include polymorphisms of genes that encode other sodium channel subunits and, given that LTG metabolism is primarily mediated by UGT1A4, any known variants in the UGT1A4 gene (Magdalou *et al.*, 1992; Rambeck & Wolf, 1993; Vashishtha *et al.*, 2001).

5.2.6 Conclusion

This study suggests that patient gender and the genotypes of ABCB1 c.1236C>T and c.3435C>T polymorphisms are useful in predicting the LTG optimal dose. In contrast, there was no significant association between a common polymorphism in the SCN2A gene and doses of LTG. The use of genetic markers in predicting LTG optimal dose may be advantageous in any future dose selection strategies. This study has also added

further support to the proposed involvement of the ABCB1 gene and P-gp in epilepsy management.

5.3 The Effects of ABCB1 Gene Polymorphisms on the Pharmacokinetics of Lamotrigine Monotherapy

5.3.1 Introduction

The single and multiple dose pharmacokinetics of LTG, when used as both monotherapy and polypharmacy have been studied in healthy volunteers and patients (Jawad *et al.*, 1987; Ramsay *et al.*, 1991; Hussein & Posner, 1997). In general, drug pharmacokinetics are affected by both extrinsic factors, such as concomitant medications, mealtime and composition, chronopharmacology, and lifestyle, and intrinsic factors, such as gender, age, body size, renal and hepatic function, phenotype and disease conditions. The influence of some of these factors on the interindividual variability in LTG pharmacokinetics has been explored previously, for example, comedication with other AEDs and renal impairment (Jawad *et al.*, 1987; Ramsay *et al.*, 1991; Wooton *et al.*, 1997).

Common and clinically significant pharmacokinetic parameters include clearance, V_d and K_a. In healthy volunteers, the total apparent clearance and renal clearance of LTG have been estimated at 0.5 and 0.043 ml/min/kg, respectively (Cohen *et al.*, 1987; Wooton *et al.*, 1997). Renal impairment does not influence the clearance of LTG (Wooton *et al.*, 1997). LTG has clinically important pharmacokinetic interactions with other AEDs. Concomitant administration of LTG with enzyme-inducing AEDs (PHT, CBZ, primidone or PB) increases the clearance of LTG through induction of UGT enzymes (Jawad *et al.*, 1987; Ramsay *et al.*, 1991), whereas co-medication with VPA

decreases the clearance of LTG through inhibition of glucuronidation (Jawad *et al.*, 1987; Patsalos, 1999; Perucca, 2001; Bazil & Pedley, 2003). Consequently, the LTG concentration/dose ratio can be altered by interactions with other AEDs. LTG concentrations can also be affected by oral contraceptives (Sabers *et al.*, 2001), which are believed to induce LTG glucuronidation. The apparent V_d has been reported in the range of 1.14 to 1.36 l/kg (Cohen *et al.*, 1987; Ramsay *et al.*, 1991; Hussein & Posner, 1997; Wooton *et al.*, 1997). No significant relationship has been identified between the pharmacokinetic parameters of LTG and either age or gender in adult population (Hussein & Posner, 1997). However, in special populations such as pregnancy, the clearance of LTG is higher in the second and third trimesters when compared with the preconception and postpartum periods (Tran *et al.*, 2002; Petrenaite *et al.*, 2005).

To further understand interindividual variability in LTG pharmacokinetics, additional factors should be considered. These should perhaps include genetic variants of P-gp since LTG has been suggested to be a substrate for P-gp-mediated transport (Potschka *et al.*, 2002). As P-gp is significantly expressed in many organs related to drug absorption and elimination (Kim, 2002a), alteration in the activity of P-gp may consequently influence the pharmacokinetics of LTG. The expression and function of P-gp has been associated with polymorphisms in the encoding gene, ABCB1 (Hoffmeyer *et al.*, 2000). The ABCB1 gene has several common polymorphisms including the c.1236C>T, c.2677G>T/A and c.3435C>T transitions. However, conflicting results have been reported regarding the association between these polymorphisms and their respective phenotypes (Hoffmeyer *et al.*, 2000; Kim *et al.*, 2001; Gerloff *et al.*, 2002; Johne *et al.*, 2002; Yates *et al.*, 2003).

In the current study, the effects of ABCB1 c.1236C>T and c.3435C>T polymorphisms on the pharmacokinetics of LTG monotherapy were examined by utilizing plasma LTG

concentration data obtained during a head-to-head monotherapy trial of LTG and VPA in newly diagnosed epilepsy patients (Stephen *et al.*, 2007). LTG pharmacokinetic parameters were estimated using a population pharmacokinetic approach. A similar approach has been used to examine the pharmacokinetics of other AEDs (Ingwersen *et al.*, 2000; Jiao *et al.*, 2003).

5.3.2 Aims

The aim of this study was to perform an exploratory, retrospective evaluation of: (1) the population pharmacokinetics of LTG monotherapy in newly diagnosed epilepsy patients; and (2) the influence of ABCB1 c.1236C>T and c.3435C>T polymorphisms on LTG pharmacokinetic parameters such as the oral clearance (CL/F; where F = bioavailability), volume of distribution (V_d/F) and K_a.

5.3.3 Methods

5.3.3.1 Study subjects

Subjects were identified from the 400-strong study population. A total of 118 patients were identified as treated with LTG monotherapy. All of these patients were drawn from on going head to head monotherapy trial comparing LTG with VPA in newly diagnosed epilepsy (Stephen *et al.*, 2007). Study visits were scheduled every 4 to 6 weeks, at which demographic data such as age, gender and body weight were documented, efficacy and tolerability was recorded, and venous blood samples were obtained. A total of 50 patients had sufficient demographic, pharmacokinetic and genotypic data to be included in the pharmacokinetic analysis. Routine biochemical data representing the level of organ function were not included because blood samples were not obtained for this purpose.

5.3.3.2 Blood sampling

The mean number of blood samples obtained from each patient was 3 (range 1 to 4). Each of these samples was taken at a different study visit. Blood samples obtained in this study were collected at a steady-state concentrations between 0.9 to 13.5 hours post dose (mean 3.8; median 3.0). Plasma was separated from whole blood by centrifugation at 800 x g for 10 minutes and was stored below -10°C until required for analysis. In total, 156 blood samples were available for drug concentration measurement.

5.3.3.3 ABCB1 c.1236C>T and c.3435C>T polymorphism analysis and phenotype assessment

Genotypes of common ABCB1 gene polymorphisms were identified using the PCR-RFLP assays described in Chapter 3. Genotype determination completed for ABCB1 polymorphisms on May 2006. Phenotype assessment was started on November 2006 and completed on March 2007.

5.3.3.4 Lamotrigine assay

The LTG concentration was determined by a modified version of the method described by Kilpatrick *et al.* (1996).

Standards: Blank plasma obtained from drug-free healthy volunteers was used as a diluent for the stock and standard solutions. LTG stock solution was prepared by dissolving 20 mg of LTG in 20 ml of methanol. The stock solution was then diluted to 10 mg/l LTG with blank plasma. Further dilution of the 10 mg/l LTG solution with blank plasma was required to achieve the full range of calibration standard solutions (1, 2.5, 5, 7.5 and 10 mg/l). The 1 mg/l calibration standard solution was also used as a

quality control standard. Internal standard solution (2 mg/l) was prepared by dissolving 1 mg of BWA725C in 1 ml of methanol and diluting to 500 ml with ethyl acetate. The calibration and internal standard solutions were stored at -20°C for up to seven days.

Sample preparation: A 10 µl aliquot of the internal standard solution and 50 µl of 2 M sodium hydroxide were added to 100 µl of calibration standard solutions (1, 2.5, 5, 7.5 and 10 mg/l LTG), quality control solution (1 mg/l) and patient plasma samples alike. The calibration standards were used to construct a linear calibration plot that was then employed to estimate LTG concentrations in the patient samples. Three of quality control standards were included in each batch analysis to determine and monitor intra- and inter-assay reproducibility.

LTG extraction: LTG was extracted from standards and patient samples into ethyl acetate under alkalinised conditions. A total of 1 ml of ethyl acetate was added to both standards and samples before vortex-mixing for 10 seconds. The organic layer was separated from the aqueous layer by centrifugation at 12 100 x g for 30 seconds. The organic layer was removed into a conical centrifuge tube, and the ethyl acetate evaporated using vacuum centrifuge (Heto Maxi Dry Plus). The residue was reconstituted with 300 µl of flow solvent. The flow solvent consisted of hexane, ethanol and 35% ammonia solution in a ratio of 79.75/20/0.25 by volume. Reconstituted standards and samples were transferred to autosampler microvials before chromatography.

High-performance liquid chromatography: Chromatography was carried out at room temperature (20°C) on a Zorbax sil normal-phase column (250 x 4.6 mm). The chromatographic system consisted of a Shimadzu LC-10AT pump, a Shimadzu SIL-9A autoinjector and a Shimadzu SPDF-6A ultraviolet spectrophotometer. The injection

volume was set at 100 µl. Flow rate was 1.75 ml/min throughout. The detector was set at wavelength 306 nm with a 1 second rise time. The lower limit of detection was 0.25 mg/l LTG. The intra-assay and inter-assay coefficients of variation were <4% and <6% at 1 mg/l LTG, respectively.

Calculations: Chromatograms were recorded and integrated on a Jones Chromatography JCL6000 chromatography data system (Crawford Scientific, Strathaven, UK). LTG concentrations were determined by comparison of peak height ratios of analyte to internal standard, quantified in relation to volume, and expressed in mg/l.

5.3.3.5 Database construction

Details of the LTG dosage regimen, the time of last dose and the time of blood sampling were recorded in a specific database that could be read by NONMEM® (Beal & Sheiner, 1992). All patients received a twice-daily LTG regimen, which is believed to be associated with optimal compliance. Where the time of the previous evening dose was not available, a standardised dosing interval of 12 hours was assumed. The time at which the morning and evening doses were taken is required because daily LTG doses may not be divided equivalently. A scoring system was employed to incorporate categorical data into the database, such as, gender (1 = female; 0 = male), and genotypes of the ABCB1 c.1236C>T and c.3435C>T polymorphisms (0 = C/C; 1 = C/T; 2 = T/T).

5.3.3.6 Population pharmacokinetic analysis

The population pharmacokinetic analysis of LTG was performed by the NONMEM® software package, version V, level 1.1 (GloboMax L.L.C., Hanover, USA). NONMEM subroutines ADVAN2 and TRANS2 were specified to fit a model with first-order

absorption and first-order elimination to the LTG concentration data. The first-order conditional estimation (FOCE) algorithm was used throughout the modelling procedure. FOCE uses conditional estimates of the random interindividual variability while estimating the population parameters. The choice of a structural pharmacokinetic model was based on prior knowledge of LTG disposition and preliminary data from the present study.

The base model (without any covariates) was initially used to describe LTG plasma concentration-time data and to obtain initial estimates of CL/F, V_d/F and K_a. Covariate modelling in NONMEM® was undertaken using the following general model structures:

Base model:	$TVCL = \theta_{CL}$
Linear model:	$TVCL = \theta_{CL} * WT$
Linear model with intercept:	$TVCL = \theta_{CL} * (1 + \theta_{WT} * (WT - \text{median WT}))$
Nonlinear model:	$TVCL = \theta_{CL} * (WT/\text{median WT})^{\theta_{nonlinear}}$

where: TVCL is the typical population estimate of CL/F; 0 is the population parameter estimate; WT is the body weight.

Typical values (TVCL, TVV_d and TVK_a) represent the population estimates of CL/F, V_d/F and K_a, respectively. For each patient, the magnitude of interindividual variability in parameters, such as CL/F, was modelled as follows:

$$CL_j = TVCL * \exp^{\eta_{j,CL}}$$

where: CLj is the value of CL/F for the j^{th} patient; TVCL is the typical population estimate of CL/F; and $\eta_{j,CL}$ was assumed to be a random Gaussian variable with zero mean and variance ω_{CL}^2 that distinguished the j^{th} patient's parameter from the population

estimate as predicted by the model. A similar model was used to estimate V_d/F. Individual estimates of CL/F and V_d/F were obtained from individual η_i estimates which were obtained as part of the FOCE analysis.

The magnitude of the residual variability in plasma concentrations was modelled using 3 error models:

Additive error model: $C_{obs,ij} = C_{pred,\,ij} + error_1$

Proportional error model: $C_{obs,ij} = C_{pred,\,ij}(1 + error_2)$

Combined error model: $C_{obs,ij} = C_{pred,\,ij}(1 + error_2) + error_1$

where: $C_{obs,ij}$ is the i^{th} observed LTG concentration in the j^{th} patient; $C_{pred,\,ij}$ is the predicted LTG concentration corresponding to the i^{th} observed LTG concentration for the j^{th} patient; $error_1$ is the additive error; $error_2$ is the proportional error and the magnitude of error was assumed to be a random Gaussian variable with mean of zero and variance σ^2. Additive, proportional and combined error models were tested in this study.

The potential influence of clinical factors on the population parameter estimates was first examined subjectively using scatterplots of individual estimates of CL/F against the available clinical factors. Generalised Additive Modelling (GAM) was then used as an additional screening tool to objectively evaluate the effect of univariate and multivariate factors on the base model. The Akaike Information Criterion (AIC) value produced by the GAM analysis was compared between the model that included clinical factors and the base model. A lower AIC value indicates a better goodness-of-fit of the statistical model to the data.

Various structural and covariate models were analysed using NONMEM®. Covariate models included body weight and ABCB1 c.3435C>T and ABCB1 c.1236C>T polymorphisms. Hierarchical models were compared by a likelihood ratio test. A decrease in the value of objective function (OBJF) as a result of structural pharmacokinetic remodeling or the inclusion of a parameter indicates a better fit. A change in OBJF of more than 6.63 and 9.21 are required to reach statistical significance ($p = 0.01$) for the addition of 1-fixed or 2-fixed effects, respectively. In addition to the above statistical condition, the following two criteria were also required to be met: good fit in the diagnostic plots (predicted *versus* measured concentration and weighted residuals *versus* predicted concentration), and low relative standard errors of the parameter estimates. The coefficient of variation (cv) which represents a measure of dispersion of a probability distribution was calculated for each parameter estimated.

5.3.4 Results

5.3.4.1 Patient demographics

The study population consisted of 50 newly diagnosed epilepsy patients of whom 52% were male. The mean age and weight of the participants was 39 years (range 13.5 to 80.5 years) and 75.4 kg (range 48.3 to 129 kg), respectively. There was no association between weight and age ($r = 0.139$, $p = 0.334$), or weight and genotypes of ABCB1 c.1236C>T ($p = 0.279$) and c.3435C>T ($p = 0.340$). Female patients had a slightly lower body weight than male patients (70.8 kg *vs* 79.0 kg; $p = 0.057$). The duration of treatment from initiation of LTG to time of final blood sample, was between 1 and 131 weeks (mean 62 weeks). The daily doses of LTG ranged between 50 mg and 550 mg (median 150 mg).

5.3.4.2 Genotype distribution of ABCB1 c.1236C>T and c.3435C>T polymorphisms

The genotype frequencies of the ABCB1 c.1236C>T polymorphism was 14% C/C, 52% C/T, and 34% T/T. The genotype frequencies of the c.3435C>T polymorphism was 28% C/C, 46% C/T, and 26% T/T. The genotype distributions of ABCB1 c.1236C>T and c.3435C>T polymorphisms were consistent with HWE ($p = 0.853$ and $p = 0.841$, respectively).

5.3.4.3 Lamotrigine population pharmacokinetics

The OBJF values for selected pharmacokinetic models fitted using NONMEM® are presented in TABLE 5.12. The first pharmacokinetic model incorporating a combined error model had an OBJF value of 257.7 with an aborted covariance step. The OBJF value did not significantly change when the interindividual variability in K_a was fixed to zero, however, the covariance step managed to achieve completion. This simpler model was chosen as the base model (Model 2) and provided the following initial estimates of population values (mean value with relative standard error expressed as a percentage):

$\theta_{CL} = 2.46$ l/h (7.5%) $cv_{CL} = 45.3\%$ (22.4%)

$\theta_V = 51.5$ l (41.0%) $cv_V = 132\%$ (62%)

$\theta_{Ka} = 1.57$ /h (73.0%)

Plots of individual estimates of CL/F *versus* clinical factors are presented in FIGURE 5.5. There was no significant correlation between the individual estimates of CL/F and the clinical factors, except for body weight ($r = 0.490$, $p < 0.001$). Plots of individual estimates of CL/F *versus* genotypes of the ABCB1 c.1236C>T and c.3435C>T polymorphisms are presented in FIGURE 5.6. There was no significant difference in

individual estimates of CL/F between genotypes of either ABCB1 c.1236C>T (p = 0.690) or c.3435C>T (p = 0.384).

The GAM analysis produced similar results to those obtained with the scatterplots (FIGURE 5.7). None of the factors tested improved the AIC value of the base model (Model 2), except body weight. Addition of other factors into the model did not improve the AIC value in the presence of weight. These analyses indicated that weight was the only factor that was likely to improve the population model fit.

A linear effect of weight on the population value of CL/F improved the pharmacokinetic model fit (Model 3) and reduced the interindividual coefficient of variation in clearance from 45% to 37%. There was no advantage in using a non-linear model to describe the influence of weight. Although no influence of weight could be detected on V_d/F, this was deemed to be physiologically appropriate and therefore included (Model 4; TABLE 5.12). The residual error was adequately described using a proportional error model. Model 4 was therefore used to evaluate the effects of ABCB1 gene polymorphisms on LTG pharmacokinetics. Addition of ABCB1 c.1236C>T and c.3435C>T genotype scores into model 4, either individually or in combination, did not significantly improve the OBJF value (Models 5 & 6; TABLE 5.12). The model fitting was consistent with the results of GAM analysis and visual examination of the scatterplots.

TABLE 5.12. Hierarchy of structural and covariate pharmacokinetic models, error models and their minimum value of objective function (OBJF).

Model	CL/F	V/F	K_a	Error model	OBJF
1	$\theta_{CL}*e^{\eta_{CL}}$	$\theta_V*e^{\eta_V}$	$\theta_{Ka}*e^{\eta_{Ka}}$	Combined	257.7
2 (Base model)	$\theta_{CL}*e^{\eta_{CL}}$	$\theta_V*e^{\eta_V}$	θ_{Ka}	Combined	257.7
3	$\theta_{CL}*WT*e^{\eta_{CL}}$	$\theta_V*e^{\eta_V}$	θ_{Ka}	Combined	236.4
4 (Optimal model)	$\theta_{CL}*WT*e^{\eta_{CL}}$	$\theta_V*WT*e^{\eta_V}$	θ_{Ka}	**Proportional**	**236.2**
5	$\theta_{CL}*WT*(1+\theta_{Pol1}*P1)*(1+\theta_{Pol2}*P2)*e^{\eta_{CL}}$	$\theta_V*WT*e^{\eta_V}$	θ_{Ka}	Proportional	235.2
6	$\theta_{CL}*WT*(1+\theta_{Pol2}*P2)*e^{\eta_{CL}}$	$\theta_V*WT*e^{\eta_V}$	θ_{Ka}	Proportional	236.0

CL/F, oral clearance; V/F, volume of distribution; K_a, absorption rate constant; θ, population value; η, interindividual variability; WT, body weight (kg); Pol1, polymorphism of ABCB1 c.3435C>T; Pol2, polymorphism of ABCB1 c.1236C>T. P1 and P2 are the conditional instructions that allow the program to incorporate the Pol1 and Pol2 into the model.

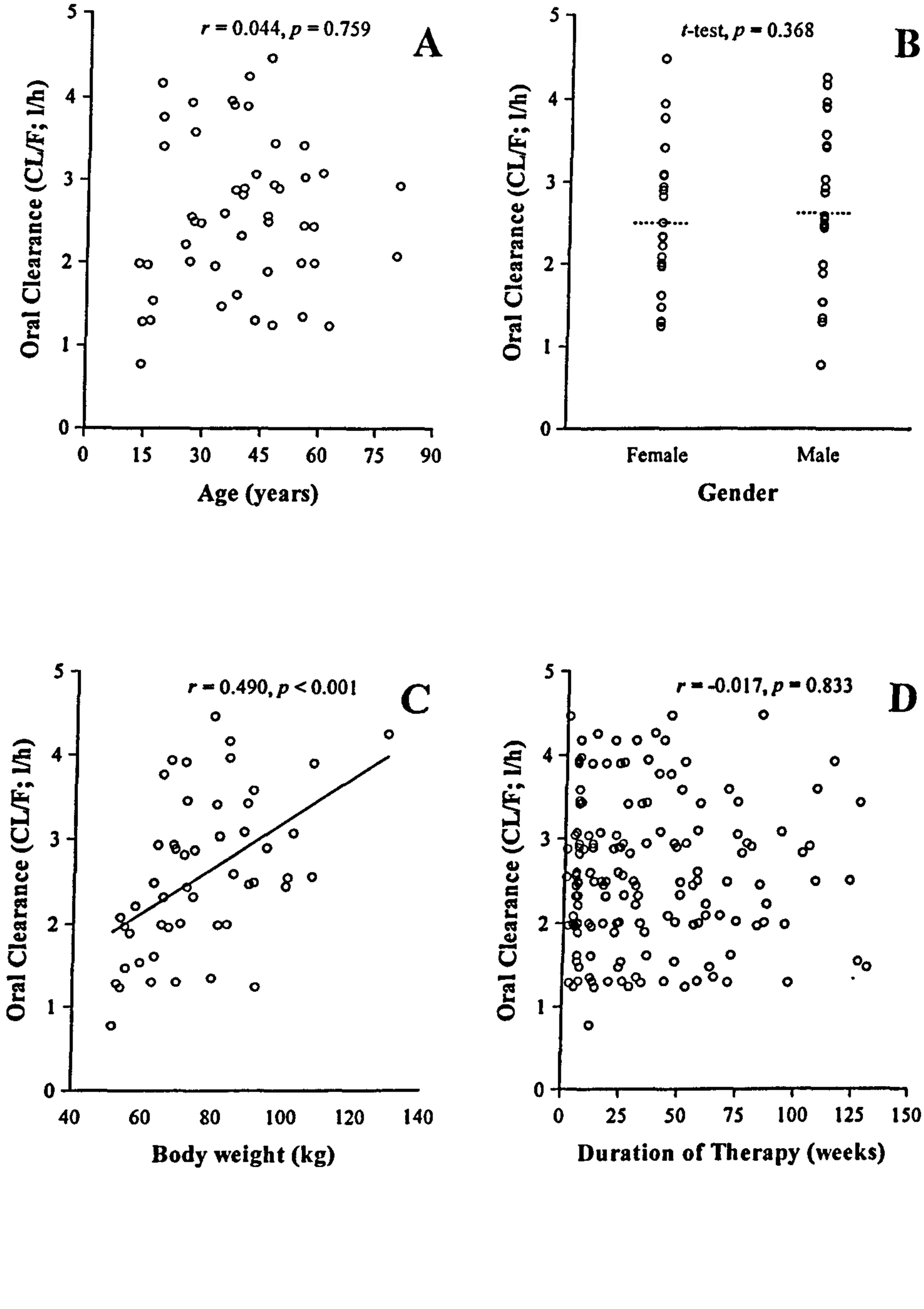

FIGURE 5.5. Relationship between individual estimates of oral clearance and clinical covariates; age (A), gender (B), body weight (C) and duration of LTG therapy (D).

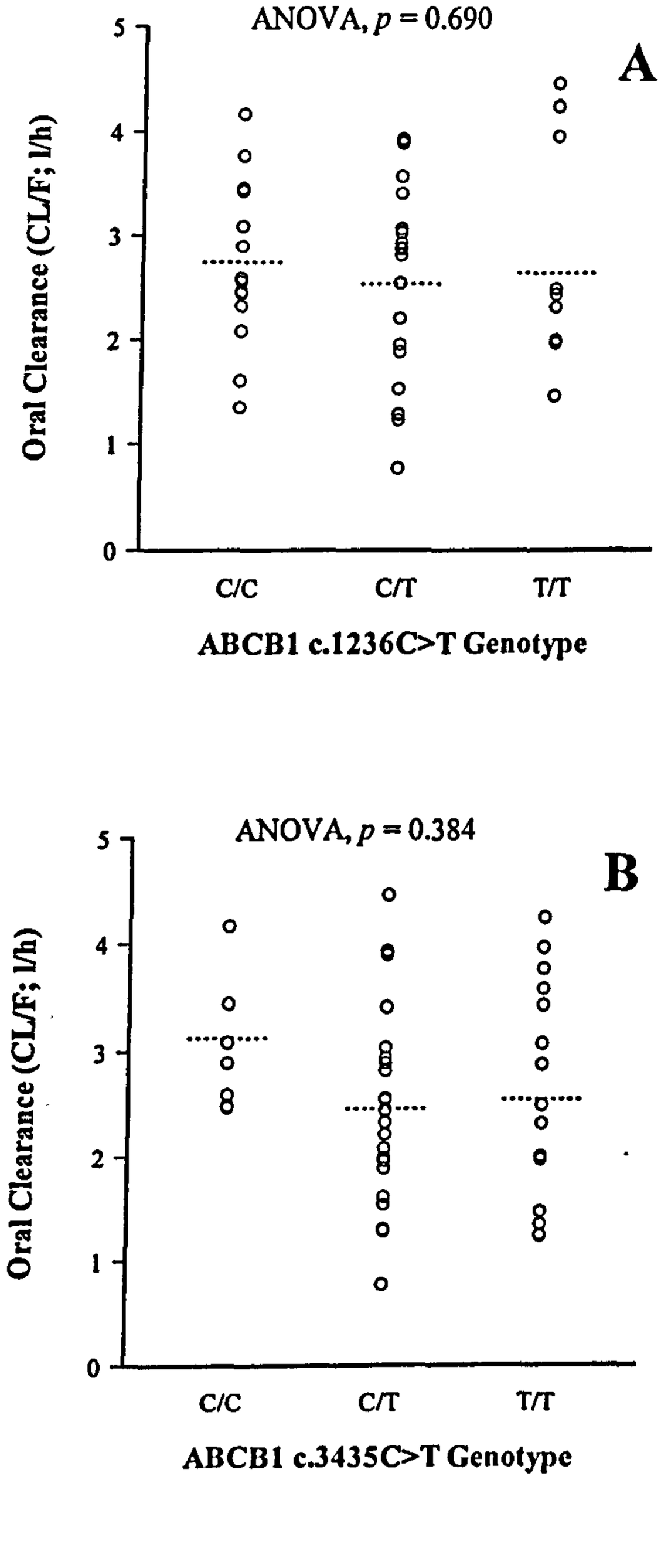

FIGURE 5.6. Relationship between individual estimates of oral clearance and genotypes of ABCB1 c.1236C>T (A) and c.3435C>T (B) polymorphisms.

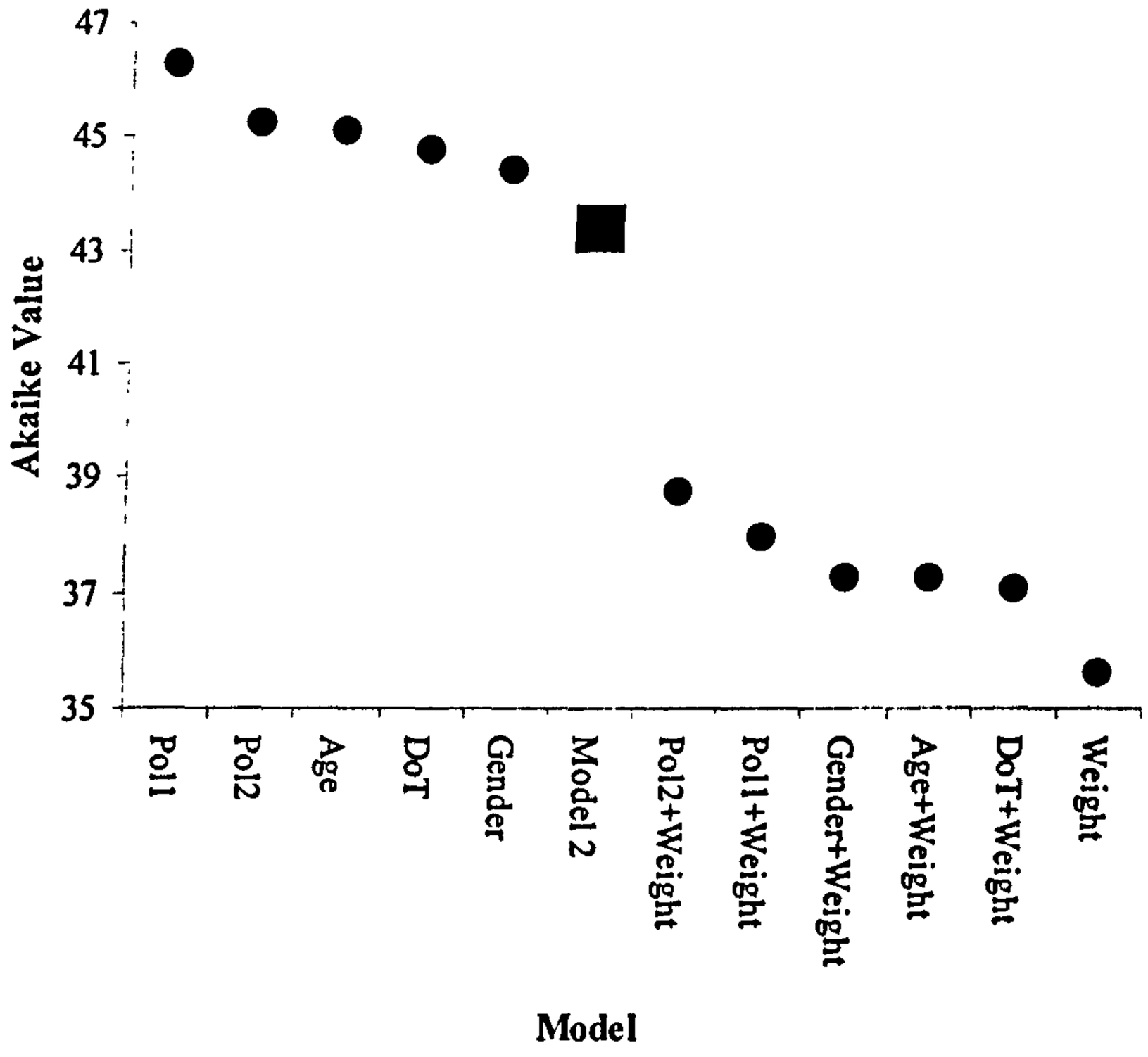

FIGURE 5.7. A plot of Akaike Information Criterion value versus univariate/multivariate inclusion in Model 2. A lower Akaike value indicates a better model fit. Pol1 = ABCB1 c.3435C>T; Pol2 = ABCB1 c.1236C>T; DoT = duration of therapy.

The optimal pharmacokinetic model for LTG provided the following estimates of population values (mean value with relative standard error expressed as a percentage; TABLE 5.13):

θ_{CL} = 0.0334 l/h/kg (6.4%) cv_{CL} = 37.8% (22.4%)

θ_{V} = 0.515 l/kg (37.0%) cv_{V} = 136% (46.4%)

θ_{Ka} = 0.681 /h (37.0%)

Both the estimates of V_d/F and K_a were poorly characterised with relative standard errors of 37% and the interindividual variability in V_d/F had an estimated cv of 136%, which again indicates wide interindividual variability.

The measured *versus* population predicted and individual predicted LTG concentration plots of the base and optimal models are presented in FIGURE 5.8. The plots indicate a slightly better fit with the optimal model for measured, compared to population predicted, concentrations, with less scatter around the line of identity. There was no difference between the models when the individual predictions were used. This is expected since the structural model was identical.

5.3.5 Discussion

Given the wide variability in interindividual pharmacokinetics, these findings may improve current understanding of the clinical pharmacology of LTG. This investigation complements previous traditional-type studies that have been performed in standardised populations receiving monotherapy or polytherapy regimens (Cohen *et al.*, 1987; Ramsay *et al.*, 1991; Bartoli *et al.*, 1997; Hussein & Posner, 1997; Armijo *et al.*, 1999), but, in addition, has uniquely examined the effects of ABCB1 gene polymorphisms on LTG pharmacokinetics.

TABLE 5.13. Lamotrigine pharmacokinetic parameter estimates and their relative standard errors (RSE) using various models.

	Model				
Parameter	**2**	**3**	**4**	**5**	**6**
	Base model		**Optimal model**		
θ_{CL}	2.46	0.033	0.0334	0.0324	0.0329
RSE θ_{CL}	7.5%	6.3%	6.4%	8.5%	7.4%
θ_{Vd}	51.5	42.1	0.515	0.529	0.508
RSE θ_{Vd}	41.0%	38.0%	37.0%	37.0%	37.0%
θ_{Ka}	1.57	0.709	0.681	0.657	0.691
RSE θ_{Ka}	73.0%	35.0%	37.0%	40.0%	38.0%
θ_{Add}	NG	0.0974	-	-	-
RSE θ_{Add}	-	428%	-	-	-
θ_{Pro}	0.276	0.262	0.265	0.267	0.264
RSE θ_{Pro}	8.5%	10.0%	7.7%	7.7%	7.7%
θ_{Pol1}	-	-	-	0.132	-
RSE θ_{Pol1}	-	-	-	136%	-
θ_{Pol2}	-	-	-	0.0552	0.0319
RSE θ_{Pol2}	-	-	-	237%	398%
cv_{CL}	45.3%	37.3%	37.8%	36.9%	37.8%
RSE cv_{CL}	22.4%	22.9%	22.4%	22.2%	21.9%
cv_{Vd}	132%	141%	136%	138%	134%
RSE cv_{Vd}	62%	24.5%	46.4%	49.6%	47.7%

θ, population value; CL, oral clearance; V_d, volume of distribution; Ka, rate constant of absorption; cv, coefficient of variation; Pol1, polymorphism of ABCB1 c.3435C>T; Pol2, polymorphism of ABCB1 c.1236C>T; Add, additive error; Pro, proportional error; NG, negligible.

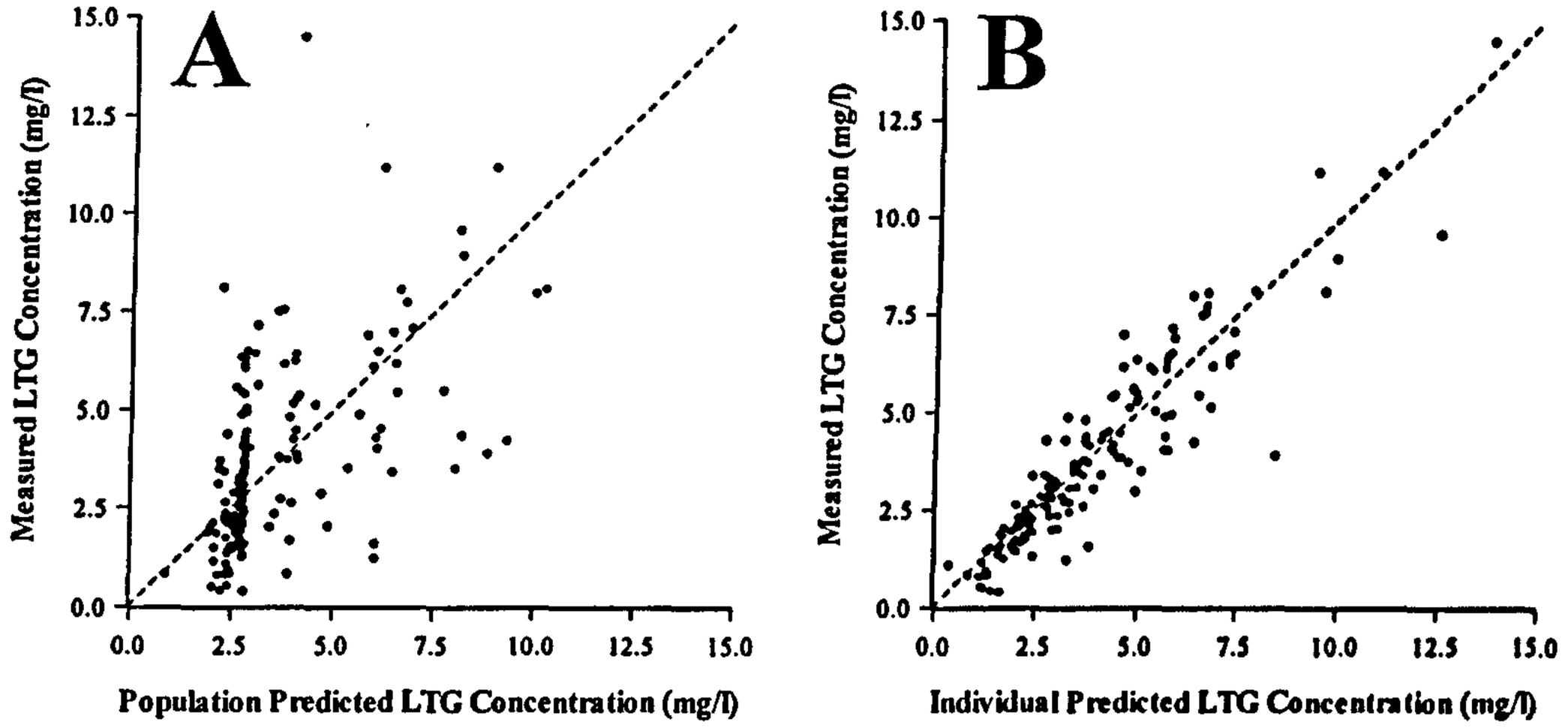

FIGURE 5.8. Plots of measured lamotrigine concentration *versus* population and individual predicted lamotrigine concentration for base model (A and B) and optimal model (C and D).

Population pharmacokinetic analysis methods, such as NONMEM®, were originally designed for the analysis of the type of sparse data that is routinely collected in a clinical setting. However, these approaches have also been shown to be useful in analyzing rich data sets collected in an experimental setting. The advantages of using NONMEM® include the ability to estimate pharmacokinetic parameters for individuals, to correctly handle differing numbers of data points per patient, to investigate the influence of clinical factors on pharmacokinetic parameters, and to separate random effects into interindividual variability in pharmacokinetic parameters and residual error in concentration measurements.

When compared with published data, only the population estimates of CL/F were consistent with previous results. The estimate of V_d/F was half that of previous reports and K_a was one third of the value that has been reported elsewhere (Cohen *et al.*, 1987; Ramsay *et al.*, 1991; Hussein & Posner, 1997; Wooton *et al.*, 1997). Both of these latter parameters were poorly characterised (relative standard error 37%) and interindividual variability in V_d/F was high. Poor quality data obtained during the absorption phase up to the time of peak concentration might have contributed to the inadequate estimation. The time to peak concentration for individual LTG doses in a chronic treatment regimen has been reported to range between 0.5 and 4.0 hours (Cohen *et al.*, 1987). In this study, the majority of samples (67.3%) were collected within 4 hours of the last dose and would thus have been influenced by variability in CL/F, V_d/F and K_a. To estimate all parameters effectively with sparse data collection in the absorption and distribution phases is difficult. In addition to the high interindividual variability and narrow range of time to peak concentration, any small inaccuracy in recording the time of last dose and the time of blood sampling could, in theory, have influenced the estimates of V_d/F and K_a.

In this study, the optimal population estimate for CL/F was 0.033 l/h/kg or 2.5 l/h based on a mean population body weight of 75 kg. This is in agreement with previously published values (Cohen *et al.*, 1987; Hussein & Posner, 1997; Wooton *et al.*, 1997). The magnitude of interpatient variability in CL/F was 38%, which indicates significant unexplained interindividual variation.

Although the estimate of CL/F was consistent with a previous report (Hussein & Posner, 1997), it failed to demonstrate any association with the duration of therapy. This is probably because most of the blood samples (73%) were taken after at least 8 weeks on therapy, which is beyond the expected time of completion of the autoinduction process with LTG. It is believed that the autoinduction process is complete within 2 weeks after LTG initiation (Hussein and Posner, 1997), and that the short duration of autoinduction with LTG is unlikely to be clinically significant (Rambeck & Wolf, 1993).

The interindividual variability in CL/F was reduced slightly when body weight was included in the model. This finding is consistent with a previous study by Hussein & Posner, (1997) who found that a higher body weight was associated with a higher CL/F. This may be explained by the proposed correlation between body weight and liver size. Some studies have demonstrated a significant correlation between body weight and liver size (Andersen *et al.*, 2000) and for drugs that are principally metabolised in the liver, there is a clear association between systemic clearance and lean body mass (Morgan & Bray, 1994). Studies exploring the relationship between liver size and AED clearance are limited. A single study in children and adolescents receiving CBZ demonstrated that clearance was correlated with liver volume (Reith *et al.*, 2000). The present study lends further weight to the proposed association between body weight and the clearance of AEDs which undergo hepatic metabolism.

The wide tissue distribution and disparate list of compounds that are transported by P-gp indicate its potentially important role in drug absorption, distribution, and elimination. Genetic polymorphisms that alter the drug transport capacity of P-gp therefore have the potential to affect the pharmacokinetics of many therapeutic agents. The most commonly studied ABCB1 polymorphisms are c.1236C>T, c.2677G>T/A and c.3435C>T substitutions. Significant linkage disequilibrium between these polymorphisms has been reported in both Oriental and Caucasian populations (Kim *et al.*, 2001; Tang *et al.*, 2002). Although the functional significance of ABCB1 variants remains controversial, genotypes and haplotypes of ABCB1 polymorphisms have been associated with alterations in the pharmacokinetics of drugs such as digoxin (Hoffmeyer *et al.*, 2000), fexofenadine (Kim *et al.*, 2001) and cyclosporin (Yates *et al.*, 2003).

The current study suggests that ABCB1 c.1236C>T and c.3435C>T polymorphisms do not significantly influence the clearance of LTG. The most obvious explanations for this observation include the possibility that LTG may be not a substrate for P-gp, or that the polymorphisms investigated do not significantly influence the expression or activity of P-gp. However, it is reasonable to speculate that the level of expression of P-gp in the intestine and any genetic variability therein may not be sufficient to influence the pharmacokinetics of a drug with high oral bioavailability such as LTG (Patsalos, 1999). Equally, P-gp efflux transport capacity may be saturable, leading to substrate overload, and absorption of LTG by passive diffusion or active influx transport (Cascorbi, 2006). Either of these alternative mechanisms could potentially mask any deficiency in P-gp transport capacity resulting from genetic polymorphisms. A study with cyclosporin has shown that the effect of the ABCB1 3435C>T polymorphism on CL/F can be largely attributed to altered absorption rather than hepatic clearance (Yates *et al.*, 2003). In addition, a study with digoxin has also highlighted the important effect of the ABCB1 3435C>T polymorphism on the extent of absorption rather than the rate of renal

clearance (Johne *et al.*, 2002). Accordingly, the current study may indirectly indicate that the effect of ABCB1 c.1236C>T and c.3435C>T polymorphisms on the absorption of LTG is negligible.

There was a large variation in LTG CL/F within each ABCB1 genotypic group. This might indicate the presence of other confounding factors, including as yet undetected SNPs or dietary and environmental contributors. It is also possible that the overall rate of LTG clearance is predominantly influenced by physiological factors, rather than genetic polymorphisms. Other than the sample size issue, the variability in estimated pharmacokinetic parameters can be attributed collectively to model mis-specification, assay variability, intraindividual variability and non-compliance with medication. Some of this variability may also be related to the assumption of a 12-hour dosing interval in subjects with incomplete dosing records.

5.3.6 Conclusion

The population pharmacokinetic parameter estimate of CL/F during LTG monotherapy was in good agreement with the findings of previous studies. Steady-state plasma LTG concentrations can be affected by the influence of body weight on clearance. There was no clear evidence to support the functional involvement of ABCB1 c.1236C>T and c.3435C>T polymorphisms in the CL/F of LTG. The current study findings need to be verified in a large, prospective, purposely-designed pharmacokinetic study.

CHAPTER 6

GENERAL DISCUSSION & CONCLUSION

6.1 General Discussion

The objective of this programme of work was to examine the influence of genetic variability in DME, DTP and voltage-gated sodium channel genes on the clinical pharmacology of CBZ and LTG. The individual projects which comprise this thesis are to a certain extent diverse, although not unrelated. While the general introduction endeavours to summarise present understanding of epilepsy, its clinical treatment, variation in human genetics, the pharmacogenetics of AEDs, the clinical pharmacology of CBZ and LTG, and the genes and SNPs of interest, the individual results chapters developed particular aspects of the pharmacogenetics of CBZ and LTG in more detail.

Chapter 3 addressed the prevalence of common polymorphisms in the genes of interest and the validity of the methods used to identify these genetic variants. Next, in chapter 4, an attempt to study the influence of common polymorphisms in DME and sodium channel genes on the optimal dose and adverse effect profile of CBZ (sections 4.1 and 4.2). Finally, in chapter 5, a series of studies was conducted to investigate the influence of common polymorphisms in ABCB1 and sodium channel genes on the treatment response, optimal dose and pharmacokinetic parameters of LTG (sections 5.1 – 5.3).

Individual results are discussed in detail in the relevant sections, both in relation to their particular limitations and in their consistency with previously published work. This general discussion attempts to summarise individual aspects of this project and to highlight those of particular interest.

Interindividual variability in the response to drug treatment is the fundamental basis of pharmacogenetic studies. Pharmacogenetics offers a systematic approach to the evaluation of genetic influences on the response to individual drug treatments. With this

in mind, this project has attempted to improve the current understanding of the clinical pharmacology of AEDs. Previous studies in this regard have tended to focus on the association between genetic variation and pharmacokinetic mechanisms influencing AED disposition. Less emphasis has been given to the potential influence of variation in genes encoding AED targets (Ferraro & Buono, 2005). Although this project has principally concentrated on pharmacokinetic factors, efforts were also made to examine pharmacodynamic influences.

The ability of the body to eliminate AEDs and their metabolites affects the magnitude and duration of efficacy (Perucca, 1999). Responsiveness to AEDs can be influenced by drug metabolism in several ways. Under normal circumstances, when the parent drug is the principle active compound, DME variants that have relatively low activity may be associated with higher plasma drug concentrations than might otherwise be expected under standard dosing regimens. This may result in the occurrence of adverse effects. On the other hand, DME variants with relatively high enzymatic activity may be associated with lower plasma drug concentrations than might otherwise be expected under standard dosing regimens. This may result in a lack of efficacy. In the case of AEDs that are metabolically activated, such as oxcarbazepine (Perucca, 2001), relatively lower biotransformation capacity may result in treatment failure due to inadequate plasma concentrations of the active compound, whereas, relatively higher bioactivation may result in the elevation of plasma concentrations and the risk of adverse effects. For AEDs that are active as both parent and metabolite, for example CBZ, the influences of variable DME activity are more complex and dependent upon the specific nature of the metabolic pathway and the relative contribution of individual compounds to the efficacy and tolerability profiles. Thus, there are a variety of potential mechanisms by which polymorphisms in DME genes can influence AED responsiveness.

In addition to the above, the extent by which AEDs are absorbed and distributed to various regions of the brain influences their potency and adverse effect profile (Browne, 1998; Kim, 2002b; Ramachandran & Shorvon, 2003). The absorption of AEDs and their distribution to organs and tissues is determined not only by the physicochemical properties of the drugs themselves, but also by endogenous molecules such as DTPs and plasma proteins (Kim, 2002b; Patsalos & Perucca, 2003). Several DTP systems have been described that are responsible for moving substrates both in and out of cells, which would suggest that DTPs can be involved in drug pharmacokinetics from multiple mechanistic standpoints (Kim, 2002a; Kim, 2002b). The most extensively studied DTP in the epilepsy field is P-gp, which has been proposed to transport several AEDs (Potschka *et al.*, 2002). Like DMEs, the expression and activity of P-gp may also be affected by polymorphisms in the encoding gene (Hoffmeyer *et al.*, 2000).

Most AEDs have multiple mechanisms of action (Kwan *et al.*, 2001), and definitive evidence linking specific molecular targets to individual drugs is lacking. It is likely that the effects of any given AED are a composite of its entire spectrum of cellular actions. The most common AED target is the voltage-gated sodium channel (Kwan *et al.*, 2001), an observation which may be consistent with the premise that epilepsy can be viewed as arising from group of diverse ion channelopathies. There is increasing evidence to support the notion that several forms of epilepsy are associated with common allelic variants in SCN genes (Ceulemans *et al.*, 2004; Kamiya, 2004). Thus, it is possible that disease susceptibility variants may influence therapeutic activity of AEDs and potentially contribute to the phenomenon of pharmacoresistant epilepsy. This should be considered in the design and analysis of drug target pharmacogenetic studies.

In section 3.1, there were significant differences in the prevalences of common polymorphisms in DME, DTP and sodium channel genes when these were compared

between the 400-strong study population and those observed in previously published non-Caucasian populations. The genotype distributions of these polymorphisms were consistent with those predicted by HWE. Deviation from HWE is often associated with systematic error in the genotyping methodology (Xu *et al.*, 2002; Hosking *et al.*, 2004; Salanti *et al.*, 2005; Wittke-Thompson *et al.*, 2005). Thus, HWE can be useful as an initial test to evaluate assay reliability. In section 3.1, the PCR-RFLP assay designed for the identification of EPHX1 c.337T>C polymorphisms produced a genotype distribution that was inconsistent with HWE. Re-analysis was conducted for this polymorphism using direct sequencing. The genotype distribution identified by direct sequencing was consistent with HWE. If one assumes that this discrepancy was a reflection of the genotyping methodology and not a characteristic of the population itself, then it is reasonable to suggest that direct sequencing is a more reliable technique than PCR-RFLP for the identification of the EPHX1 c.337T>C polymorphism. Indeed, several studies employing a PCR-RFLP assay to identify this polymorphism have reported a similar problem (Takeyabu *et al.*, 2000; Gsur *et al.*, 2003; Godderis *et al.*, 2004).

Given that two of the study subjects were not European, population stratification can be useful to eliminate the diversity in drug response between ethnics. Population stratification refers to differences in allele frequencies between cases and controls due to systematic differences in ancestry rather than association of genes and disease. It has been proposed that false positive associations due to stratification can be controlled by genotyping a few dozen unlinked genetic markers. This available method is also known as genomic control. Further studies should utilise this approach to address the population stratification issue.

Response to AED treatment can be defined in terms of efficacy (abolition of seizures or reduction in their frequency) or in terms of tolerability (prevalence and/or severity of adverse effects). However, it is a challenge to establish a definite association between genotype and treatment response phenotype because non-genetic confounders, such as drug-drug interactions, treatment compliance and patient lifestyle, are difficult to control. The AED response phenotype is highly complex and represents a classic example of the interaction between genetic and environmental factors. As a result, understanding genetic variation may not fully explain the outcome of pharmacological treatment. When faced with such difficulties in defining responsiveness, it may be more prudent to employ pharmacokinetic parameters as the principal phenotypic definition in AED pharmacogenetic studies. These are more quantifiable and more reliable than measures of efficacy and/or tolerability and a more easily extrapolated from patient to patient, investigator to investigator and from research centre to research centre. Even then, a multitude of events and protein interactions occur from the moment that an AED is ingested. The fundamental issues of absorption, distribution, metabolism and excretion all involve interaction with multiple endogenous and exogenous factors, binding of drug to target proteins, and interactions of those proteins with both other proteins and DNA (Spear, 2001; Clancy & Kass, 2003; Ramachandran & Shorvon, 2003). Despite this enormous complexity, it is possible that identifying the relative contribution of genetic variation to the phenotype of AED response will permit preliminary progress in the field of pharmacogenetics.

Using a comprehensive drug response phenotype definition and a well-defined study population can improve the quality of pharmacogenetic findings, and result in a better understanding of the association between genotypes and phenotypes. In the current project, for studies involving drug responsiveness and clinical outcome, inter-investigator variability in clinical practice and in the assessment of individual patients

was largely eliminated by the selection of subjects from research projects within a single centre and which employed a similar procedure for the initiation of treatment and clinical follow-up. This ensured that all patients were treated equally. The potentially confounding effects of drug-drug interactions on drug response phenotypes were similarly eliminated by employing only those patients who were treated with AED monotherapy. Drug-drug interactions can affect the dose and concentration of each individual compound in the therapy regimen (Patsalos & Perucca, 2003), with a consequent influence on treatment response. CBZ and LTG were evaluated individually as each drug has different pharmacokinetic and pharmacodynamic characteristics. Categorising patients according to the severity of their seizures may have helped to optimise the phenotype definition. However, given the limited number of patients and the retrospective nature of this project, no sub-analysis was performed in terms of seizure severity.

To identify a pure association between genotype and drug response phenotype, one should also consider the clinical relevance of each phenotypic definition. Drug response phenotypic definitions that are clinically relevant have more practical application and thus, increase the value of pharmacogenetic findings. With this in mind, in Chapters 4 and 5, OD was employed as a composite measure of both efficacy and tolerability of CBZ and LTG, respectively. The OD is equivalent to the maintenance dose and is more advantageous than maximal tolerated dose. Previously, association between genetic markers and maximal tolerated doses of PHT and CBZ has been reported by Tate *et al.* (2005). The maximal tolerated dose phenotype has clinical implications with regard to safety but does not provide any measure of efficacy that might have added to the clinical applicability of the findings. Many patients, even in a tertiary referral setting, become seizure free on a modest dose and may never reach their personal limit of tolerability (Kwan & Brodie, 2001). In these individuals, estimating maximal tolerated

dose on the basis of genotype is of limited and non-existent value. In the current study, clinical response phenotypes focused on two relevant outcomes, namely seizure freedom and intolerable adverse effects. These outcome measures demonstrate the efficacy and safety of AEDs, respectively. The probability of achieving seizure freedom varies greatly depending on the effectiveness of treatment and also on seizure severity, pre-treatment seizure frequency and the duration of the assessment period (Perucca, 1997; Kwan & Brodie, 2000). Intolerable adverse effects of AEDs are the most common reason for early treatment withdrawal. In many patients, the health-related quality of life is affected more by adverse effects than by seizures themself (Perucca, 1997). As such, evaluation of seizure freedom and adverse effects represents an important component in the assessment of overall clinical outcome.

In section 4.1, the influence of genetic variability on CBZ dose requirement was evaluated. Like most AEDs, CBZ doses are predominantly determined by the level of response and the occurrence of adverse effects. Inadequate seizure control necessitates an increase in CBZ dose, whereas the emergence of adverse effects limits the amount of drug that can be prescribed. Dosage adjustment of CBZ is complicated due to the potential for drug-drug interactions and the phenomenon of autoinduction. As a result of autoinduction and the onset of adverse effects, the maintenance dose of CBZ will be higher than the initial dose, but often lower than maximum dose. Typical CBZ maintenance doses have been reported between 200 to 1600 mg/day (Kwan & Brodie, 2001), similar to the range of doses observed in the current project.

This project identified polymorphisms in the EPHX1 gene as potential predictors of the OD of CBZ. The influence of DME variants on the pharmacokinetics of CBZ is important because CBZ is predominantly metabolised through hepatic enzyme biotransformation (Pelkonen *et al.*, 2001). There is limited genetic variability in the

CYP3A4 and CYP3A5 genes (Gracia-Martin *et al.*, 2002) and the most common of these had no influence on CBZ dose. This might suggest that any genetically determined variability in CBZ metabolism occurs at other stages of the pathway. It is possible that the rate of CBZ-E hydrolysis could determine the variability in CBZ metabolism. This hydrolysis process is predominantly mediated by mEH (Pelkonen *et al.*, 2001). Polymorphisms in the EPHX1 gene are known to affect the activity of mEH (Nakajima *et al.*, 2005) and therefore have the potential to influence plasma concentrations of CBZ and its dose requirement. Other common DME polymorphisms examined did not predict the OD of CBZ. This might suggest that these polymorphisms have limited functional consequences or that the enzymes in question are not fundamental contributors to CBZ metabolism. On the basis of this study, it would appear that genetic variants of the EPHX1 gene are the strongest candidate markers of inter-individual variability in CBZ dosing.

In section 4.2, the association between polymorphisms in DME genes and a unique phenotype that may be exquisitely sensitive to CBZ adverse effects was evaluated. This study failed to reveal any association between common genetic polymorphisms and the proposed phenotype in question. Previous studies examining the EPHX1 gene have also failed to associate multiple polymorphisms with the incidence of CBZ hypersensitivity reaction (Gaedigk *et al.*, 1994; Green *et al.*, 1995). Two common polymorphisms (c.337T>C and c.416A>G) in the EPHX1 gene have, however, been associated with variation in the plasma concentrations of CBZ-E (Nakajima *et al.*, 2005). If the functional effect of these polymorphisms is genuine and if they are associated with CBZ dose (as reported in section 4.1), the the current study might question the popular notion that CBZ-E is responsible for precipitating many of the adverse effects associated with CBZ treatment (Ramsay & Wilder, 2002). Although it is dangerous to make such sweeping generalisation on the basis of studies with such low numbers of participants,

several previous investigations have indeed failed to demonstrate any correlation between CBZ-E concentrations and the toxicity of CBZ in epilepsy patients (Theodore *et al.*, 1989; Semah *et al.*, 1994). The current study also suggested that, with the exception of EPHX1, the contribution of other DME polymorphisms to the risk of developing CBZ adverse effects is modest or non-existent. This would support the suggestion that common polymorphisms in these genes do not significantly influence the activity of their respective enzymes.

As with other AEDs, response to LTG treatment is likely to be significantly influenced by the activity of DMEs, DTPs and sodium channels. LTG is predominantly metabolised by UGT1A4 (Magdalou *et al.*, 1992; Vashishtha *et al.*, 2001) and is believed to be transported by P-gp (Potschka *et al.*, 2002). As such, the major determinants of LTG pharmacokinetics are likely to be UGT1A4 and P-gp. The mechanism of action of LTG is similar to that of many other AEDs, such as CBZ and PHT, and is mediated by inhibition of voltage-gated sodium channels. Thus, any alteration in the expression or activity of UGT1A4, P-gp and voltage-gated sodium channels has the potential to impact on LTG pharmacokinetics and pharmacodynamics. Such alterations include polymorphisms in the respective encoding genes. The influence of UGT1A4 variants was not examined in this project because polymorphisms in this gene are rare and no functional variants had been reported in the Caucasian population at the time this project was planned. As a result, only polymorphisms in the ABCB1 and SCN2A genes were evaluated in relation to the LTG treatment response phenotype.

The findings reported in section 5.1 suggest that ABCB1 and SCN2A variants do not significantly influence the response to LTG monotherapy. There are several potential explanations for this observation. Firstly, LTG may not be a strong substrate for P-gp, in which case LTG distribution in the brain might not be significantly influenced by P-gp-

mediated efflux transport. This is plausible because LTG is known to be highly lipophilic (Mashru *et al.*, 2005). Secondly, common polymorphisms in ABCB1 and SCN2A genes may not be functional, and thus may not affect the expression or activity of P-gp or $Na_v1.2$ proteins, respectively. Even if they were functional, the modification in protein structure or function may not be of sufficient magnitude to influence the response to LTG treatment. Finally, it is important to remember that LTG may act on other subtypes of voltage-gated sodium channel and may be transported by other DTPs. One should also be aware that the response to AED therapy in terms of efficacy is easily influenced by other clinical and environmental factors, including seizure severity and pathological causes (Stephen *et al.*, 2001; Brodie, 2005).

In section 5.1, it was reported that patients who are seizure free on LTG monotherapy have a wide distribution of optimal doses. Since the efficacy of LTG treatment does not significantly associate with its plasma concentration (Kilpatrick *et al.*, 1996; Mahmood *et al.*, 1998), it is possible that the diversity in dose requirement might be influenced by inter-individual variability in P-gp function and the resulting ability of LTG to cross the BBB. Polymorphisms in the ABCB1 gene that have the potential to alter the expression and activity of P-gp were postulated to be responsible for the spread of individual LTG dose requirements. This hypothesis was confirmed (section 5.2) with the association between common polymorphisms in the ABCB1 gene and the OD of LTG. This is an interesting finding and one which may help to dismiss concerns about whether LTG is a substrate for P-gp mediated transport.

The influence of ABCB1 variants on LTG pharmacokinetics was further examined in an effort to understand the relationship between polymorphisms in this gene and LTG dose, as reported in section 5.2. This additional investigation failed to identify any association with the clearance of LTG, suggesting that the influence of dose is most

likely related to some other as-yet unidentified pharmacokinetic or pharmacodynamic factors. P-gp is believed to be expressed in a tissue-specific or disease-specific manner (Croop *et al.*, 1989). For example, epileptic brain tissue has been shown to have a higher expression of P-gp than normal brain (Marchi *et al.*, 2004; Volk *et al.*, 2004). It is possible that, in epilepsy patients, the expression of P-gp in the gastrointestinal tract, kidney and liver is relatively lower than that observed at the BBB. The effect of ABCB1 variants may be enhanced in conditions where P-gp is over-expressed whereas genetic variability would have less impact in conditions or in tissues where P-gp expression is low or normal. This would explain how ABCB1 polymorphisms can associate with LTG dose (by influencing BBB transfer) but not LTG clearance, which is a more peripheral phenomenon and not necessarily subject to disease mediated augmentation.

The studies presented in this thesis have revealed some previously unrecognised factors that may influence individual AED dose requirement. The predominant application of these pharmacogenetic findings is in AED dosing strategies. Polymorphisms in the SCN1A gene have previously been identified as useful predictors for CBZ and PHT dose (van der Weide *et al.*, 2001; Tate *et al.*, 2005; Tate *et al.*, 2006). This observation, together with the intriguing findings presented in this thesis, provide a theoretical direction for future clinical investigations to assess whether pharmacogenetic profiling can improve dosing decisions. There is clear clinical benefit in determining the optimal titration rate and target dose for an individual patient. Those who require a lower dose and a slower titration rate can be identified before the initiation of treatment, and any unnecessary exposure to high doses of AEDs can be prevented, thereby minimizing the risk for adverse effects. Similarly, patients with a high dosing requirement can be titrated faster in order to avoid prolonged periods of under-exposure and a consequent lack of efficacy. Pharmacogenetic profiling may also be more useful than therapeutic drug monitoring in determining the dose of AEDs, particularly for those AEDs that have

a poor plasma concentration-response relationship (Patsalos, 2001). Finally, pharmacogenetic testing can be utilised prospectively, before the AED is started and only a small amount of blood sample or a cheek-swab sample is required for genotypic analysis.

These findings represent another step in the ongoing effort to improve understanding of the role of pharmacogenetics in epilepsy management. Knowledge of AED mechanisms of action and awareness of genetic factors that determine treatment response should not only improve the use of AEDs but may also allow AEDs to be designed for specific targets in particular populations or that avoid any detrimental genetic variability in therapeutic response. The preponderance of genetic polymorphisms in the human genome suggests that pharmacogenetic variability will likely be an issue for consideration in almost all drug treatments.

Pharmacogenetic research has developed rapidly, with recent advances in molecular genetics and genome sequencing. This is largely due to the emergence of new technologies that allow rapid screening for specific polymorphisms, as well as recently gained knowledge of genetic sequence derived from the Human Genome project. Epilepsy-related pharmacogenetic research is beginning to take advantage of this new information in order to enhance current understanding of epilepsy therapeutics. This new found knowledge can be employed effectively in two main areas: identification of specific genes and gene products associated with various forms of epilepsy and which may act as targets for new AEDs and identification of genes and allelic variants of genes that can influence the response to current AEDs.

We are still a long way from having a pharmacogenetic test that clinicians can routinely use to identify the most appropriate AED for any particular patient, however there is

increasing evidence that pharmacogenetics will be important in clinical practice. In the future it may be considered unethical not to perform such tests routinely to avoid exposing individuals to doses of AEDs that could be harmful. The ability to identify sensitive or insensitive individuals before commencing AED treatment would also be of economic importance. AED treatment based on genetic profile might avoid the current empiricism associated with matching the most appropriate AED, at its optimal dose, to each patient. The development of pharmacogenetics as a scientific discipline should offer a departure from current empirical prescribing and a move towards more individualised AED treatment.

6.2 Future Research Directions

The recent advances in genetic technology encourage a rapid development of pharmacogenetic research. The relatively high prevalence of seizure disorders, limited number of drugs, relatively poor success rate and the broad range of interindividual responses make epilepsy an ideal target for pharmacogenetic research and its application in clinical practice. To date, pharmacogenetic studies in epilepsy have predominantly explored the association between genetic polymorphisms and either the pharmacokinetics of specific AEDs (i.e. PHT) or the efficacy of AEDs in general (irrespective of individual treatments). Other established drugs such as CBZ, VPA, PB and ethoxusimide, and newer AEDs such as LTG, topiramate and levetiracetam have not yet been investigated in any significant detail. Drugs with single mechanisms of action (i.e. levetiracetam) or single metabolic pathways may be the best place to start. Levetiracetam does not appear to act via conventional AED mechanisms such as, GABAergic facilitation, inhibition of voltage-gated sodium channels or modulation of voltage-gated calcium channels. In contrast, it would appear to bind to synaptic vesicle protein SV2A, which is ubiquitously found in the mammalian CNS (Lynch *et al.*, 2004).

As such, genetic polymorphisms in the SV2A gene are potential contributors to variability in the response to levetiracetam treatment. This is a single example of the enormous potential which exists for the exploration of pharmacogenetics in epilepsy in an effort to better understand the clinical pharmacology of both established and modern AEDs.

The OD predictive models that were developed for CBZ and LTG in this project explained less than 20% of the dose variation between individuals. More genetic and non-genetic variables are clearly required to improve the predictability of this approach. Polymorphisms in genes which encode voltage-gated sodium channels, other DMEs and other DTPs that are related to the pharmacokinetics and pharmacodynamics of CBZ and LTG may be the most appropriate place to start. Polymorphisms of the SCN1A gene that have been reported to be associated with the maximum dose of CBZ (Tate *et al.*, 2005) should almost certainly be included in any such predictive model of CBZ OD or LTG OD, as both drugs act by blockade of voltage-gated sodium channels. However, other less obvious genes should not be excluded and DME and DTP genes should be subject to further investigation particularly with regard to those SNPs which occur less commonly than those reported here. Haplotype studies may be more appropriate than those based on genotype and whole-genome scans may become available in the near future. However, the genotype-based approach, as presented in this project, has some advantages over the haplotype strategy. Genotyping may permit the identification of causal variants, especially for rare SNPs, which can dramatically alter the expression and/or the activity of their respective proteins. This approach is typically directed by the notion that functional variants are highly associated with SNPs located in the promoter and coding regions. As such, the number of SNPs which need to be typed is limited and this reduces the cost of genotyping. In fact, these are the variants to which random SNPs searches are most likely to lead and such polymorphisms are more likely to be in

linkage disequilibrium with the causal allele (Risch, 2000). Employing a haplotype strategy, on the other hand, is useful for SNPs that have a relatively high allele frequency (>10%; Becquemont, 2003) and is entirely dependent on linkage disequilibrium (Roses, 2000). Linkage disequilibrium occurs when combinations of alleles at different loci present more frequently than would be expected from random association. Variations of several ordered SNPs that are close to, or within, a particular chromosomal region are likely to be inherited together when they are in linkage disequilibrium. As such, SNPs associated with a drug response phenotype can 'mark' the position on the chromosome where a susceptibility gene is located. The whole-genome scan technique may enable the identification of several multiple small susceptibility regions, within which the search for genetic markers can be focused (Roses, 2000). However, the haplotype strategy is not the best choice for low frequency SNPs because it misses rare haplotypes, which might otherwise be clinically relevant.

To generate stronger research data, prospectively designed studies is preferred than retrospectively designed studies, largely because avoidance of confounding variables. A prospective study is designed before the data is collected, which allows the researchers several advantages over the retrospective study. Researchers can design a pharmacogenetic study with a control group, design internal devices to eliminate bias, and follow subject throughout the length of the study to observe development of the outcome in question. The down-sides of prospective studies are that they are more expensive and time-consuming to design and execute, and are difficult to use to study rare polymorphisms as the number of subjects is often too low. Although studies presented in this thesis have produced several intriguing findings, these have to be further explored in prospectively designed studies with formal power calculations included and followed by replication studies.

6.3 Conclusion

In conclusion, this series of pharmacogenetic studies examined the clinical pharmacology of CBZ and LTG and served to identify polymorphisms in genes encoding mEH and P-gp as potential predictors of CBZ and LTG OD, respectively. Common genetic polymorphisms of other DMEs that are responsible for CBZ metabolism, namely CYP3A4 g.-392A>G, CYP3A5 g.6986A>G, CYP1A2 g.5734C>A, and UGT2B7 c.802C>T, did not significantly associate with the OD of CBZ and none were associated with CBZ adverse effects. Polymorphisms in the ABCB1 gene encoding P-gp did not prove useful in predicting the response to LTG treatment and did not appear to influence the oral clearance of LTG.

The experimental work contained in this thesis advances current knowledge of the association between genetic variability and the clinical pharmacology of AEDs. Positive findings require confirmation in a prospectively-designed study employing a larger cohort of newly diagnosed epilepsy patients. Characterisation of polymorphisms in genes that encode proteins related to AED pharmacokinetics and pharmacodynamics may enable us to predict efficacy and tolerability of AEDs and to make informed decisions with regard to titration rates and target doses for individual patients. Fostering a closer relationship between clinical pharmacology and genetics has the potential to make significant progress in the treatment of epilepsy.

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