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Clinical Applications of Pharmacogenetics

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A thesis for submission to University College London for the degree of Doctor of Philosophy (Human Genetics)

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Abstract

The field of pharmacogenetics has grown rapidly over the last few years yet, with a couple of exceptions, pharmacogenetic diagnostics are not yet used in a clinical setting. Progress in the field is discussed and the current state of pharmacogenetic research is assessed in a literature review.

This thesis describes studies to investigate the pharmacogenetics of the anti-epileptic drugs phenytoin, carbamazepine and levetiracetam, using data obtained from the routine clinical use of these drugs, and the pharmacogenetics of the beta-blocker bucindolol in a clinical trial setting.

Evidence is presented of a common polymorphism in the *SCN1A* gene that is associated with the clinical use (dosing) of both phenytoin and carbamazepine. Preliminary results are also presented concerning genetic variation in the *SV2A* and *SV2C* genes which may influence response to levetiracetam. For this study genetic variation was represented using the tagging SNP method applied to HapMap data.

Genetic variation in *ADRB2* may influence response to bucindolol yet the results are not conclusive. More importantly however, they do provide an illustration of how differences in polymorphism frequencies among populations could account for average differences in drug response among populations. It is also shown that there are substantial genetic differences within self-identified racial groups within the context of a clinical trial. The implications of pharmacogenetics for different racial or ethnic groups are discussed in a separate chapter.

In conclusion, common variation in obvious candidate genes does influence drug response, however, rigorous clinical study is required before the use of pharmacogenetic variants to guide choice or dose of drugs becomes part of clinical practice.

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Personal Contribution (experimental chapters)

Chapter 3: I performed the literature search in order to draw up the candidate gene list, I genotyped *CYP2C9**2 and *3, I performed the statistical analyses, interpreted the results and wrote the manuscript for publication[1].

Chapter 4: I designed the experiment, initiated contact with and liaised with collaborators in Taiwan, performed the statistical analyses, interpreted the results and wrote the manuscript for publication[2].

Chapter 5: I performed the literature search for SV2, resequenced *SV2A*, performed preliminary genotyping for many of the polymorphisms (before it was decided to move to an Illumina platform) and undertook DNA standardization and plating. I also selected tags, assisted in the statistical analyses and interpreted the results.

Chapter 7: I designed the experiments, undertook SNP discovery for *ADRB1* (resequencing) and selected tags for *ADRB1* and *ADRB2*. I genotyped the tags and assisting in microsatellite genotyping. I performed all STRUCTURE analyses and directed the B.E.S.T. investigators as to which other analyses to perform (they were not permitted to release raw clinical data to me).

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Thank you to my parents for supporting my decision to return to academia, and thank you to my husband Lionel for putting up with me.

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Chapter 5: John Lynch and Peter Kinirons performed the phenotyping. David Goldtsein, Sanjay Sisodiya and Norman Delanty provided intellectual input and assisted in planning the experiment. Mike Weale assisted with statistical analyses

Chapter 7: Mari Wyn Burley genotyped the microsatellites. The B.E.S.T. investigators performed statistical analyses. David Goldstein provided intellectual input and assisted in planning the experiment.

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AA	African-American
ABCB	ATP-binding cassette protein, family B
AC	Afro-Carribean
ACE	Angiotensin-Converting Enzyme
ADR	adverse drug reaction
ADRB1	gene for adrenergic, beta-1-, receptor
ADRB2	gene for adrenergic, beta-2-, receptor
AED	antiepileptic drug
AI	American Indian and Alaskan Native
APOE	apolipoprotein E
AS	Asian
BEST	Beta-blocker Evaluation of Survival Trial
bp	base pairs
CBZ	carbamazepine
CEPH	Centre d'Etude du Polymorhisme Humain
CHF	congestive heart failure
CNS	central nervous system
CT	computerised (axial) tomography (scan)
CYP	cytochrome
CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4
DME	drug metabolizing enzyme

DNA	deoxyribonucleic acid
EEG	electroencephalography
EU	European ancestry
FDA	(United States) Food and Drug Administration
Hipp	hippocampus
HS	hippocampal sclerosis
HW	Hardy Weinberg
IGE	idiopathic generalized epilepsy
ILAE	International League Against Epilepsy
Indel	insertion/deletion
JME	juvenile myoclonic epilepsy
LD	linkage disequilibrium
LEV	levetiracetam
LVEF	left ventricular ejection fraction
MAF	minor allele frequency
MDR1	(=ABCB) multidrug-resistance protein
$MgCl_2$	magnesium chloride
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NIH	(United States) National Institute of Health
NPV	negative predictive value
PCR	polymerase chain reaction
PGP	(=MDR1 or ABCB1) P-glycoprotein
PHB	phenobarbitone

PHT	phenytoin
PNE	plasma norepinephrine
PPV	positive predictive value
OATP	organic anion transporting polypeptide family
OXC	oxcarbazepine
R&D	research and development
RNA	ribonucleic acid
SCN1A, 2A	genes encoding the α1/α2-subunit of the voltage-gated sodium channel
SCN1B, 2B	genes encoding the β1-subunit of the voltage-gated sodium channel
SNP	single nucleotide polymorphism
SRS	substrate recognition site
SSRI	selective serotonin reuptake inhibitors
SV2A	gene for synaptic vesicle glycoprotein 2A
SV2B	gene for synaptic vesicle glycoprotein 2B
SV2C	gene for synaptic vesicle glycoprotein 2C
TL	temporal lobe
TLE	temporal lobe epilepsy
TPM	topiramate
tSNP	(haplotype) tagging single nucleotide polymorphism
VGB	vigabatrin
VNTR	variable number tandem repeat
VPA	valproate

Chapter 1 Introduction

1.1 An introduction to the field of pharmacogenetics

1.1.1 What is pharmacogenetics?

The term "pharmacogenetics" was defined by Vogel in 1959 as "clinically important hereditary variation in response to drugs"[3]. The intellectual foundations of pharmacogenetics were first articulated two years previously by Arno Motulsky[4]. Referring to examples such as sensitivity to the anti-malarial drug primaquine and the muscle relaxant succinylcholine, Motulsky argued that "otherwise innocuous genetic traits" might underlie variation among individuals in drug response.

Many patients receive medicines to treat or prevent common diseases that do not work as well as they do in other people, do not work at all or even cause harmful adverse reactions. The Physicians' Desk Reference lists typical response rates of patients to a major drug for a selected group of therapeutic areas ranging from 80% for Cox-2 analgesics to just 25% for oncology drugs[5]. In the UK up to 10,000 people a year may die because of adverse drug reactions[6] (ADRs). Pharmacogenetics seeks to reduce the variation in how people respond to medicines by tailoring therapy to individual genetic make-up.

1.1.2 Early pharmacogenetics

For most of its fifty year history, pharmacogenetics focused on describing variation, either genetically or biochemically, in a handful of proteins and genes. Early studies focused on

drug metabolising enzyme variants with Mendelian effects on response. The earliest documented accounts of enzyme deficiencies resulting in drug toxicity were for glucose-6-phosphate-dehydrogenase[7] (e.g. for primaquine), N-acetyltransferase (isoniazide)[8] and butyrylcholinesterase (succinylcholine)[9]. As at that time it was not possible to isolate the genes involved, testing for drug sensitivity was by trial and error, for example, the drug was prescribed, and then the patient's urine was examined to assess the rate of the drug metabolism. Sometimes racial or ethnic information was used to assess likelihood of an adverse drug reaction. For example, it was found that 5 to 10 percent of individuals of Mediterranean and African ancestry lack the glucose-6-phosphate dehydrogenase enzyme and thus risk breakdown of red blood cells from over 200 drugs including primaquine[10].

Another important early example is the classical debrisoquine poor-metabolizer phenotype. This was discovered by Mahgoub and colleagues whilst investigating debrisoquine metabolism. In most people, debrisoquine is quickly broken down to inactive metabolites and eliminated through the urine. Several volunteers took debrisoquine, and within hours, one collapsed with severe hypotension. His was found not to contain any metabolites of debrisoquine[11]. The study was extended to his family members and then an unrelated population that consisted of medical students. This work ultimately led to the molecular cloning of the gene that is primarily responsible for the metabolism of debrisoquine, cytochrome P450 subfamily IID polypeptide 6 (*CYP2D6*) and to the characterization of polymorphisms that eliminate CYP2D6 activity.

1.1.3 A candidate gene approach to pharmacogenetics

It is striking how often obvious candidate genes carry variants that appear to influence drug response. The examples in 1.1.2 identified as candidate genes for variable drug response those genes that encode drug metabolizing enzymes. Drug-metabolizing enzymes (DMEs) evolved to neutralize xenotoxins and/or to control concentrations of signalling molecules in endogenous pathways[12]. The principal site of drug metabolism is in the liver, although many DMEs are also active in other tissues; for example, some DMEs form a component of the blood–brain barrier[13]. In humans most drugs are primarily metabolised by the cytochrome P450 enzymes. The CYP3A family of DMEs collectively comprise the largest portion of liver and intestinal cytochrome P450 enzymes and contribute to the metabolism of 45-60% of currently used drugs[14], as well as dietary compounds, steroid hormones and toxins[15]. They are followed in importance by CYP2D6 (20%) and CYP2C9/19 (15%) with the remaining metabolism mainly carried out by CYP2E1, CYP2A6, CYP1A2, and unidentified P450s[16]. Other important families of DMEs in pharmacogenetics include the glutathione S-transferases, UDP-glucuronosyltransferases and N-acetyltransferases.

The importance of metabolism in adverse reactions in particular was highlighted by Phillips and colleagues[17], who studied the metabolism of 27 drugs that are relatively prone to produce adverse reactions. Of these, 59% are metabolized by at least one enzyme that is known to have low-activity forms, whereas less than 22% of randomly selected drugs satisfy this criterion.

Early progress in the study of drug metabolism, the large number of common variants in DMEs with large functional effects and the fact that most DMEs have several substrates

(often including harmless "probe" drugs which can be taken safely by volunteers to study metabolism) has made DMEs the best-studied group of pharmacogenetic candidate genes to date.

There are other obvious groups of candidate genes that might have variation that is relevant to drug response. Drug transport is another process of obvious importance. Drugs are actively moved between or out of body compartments by specialized transporters and functional genetic variation in genes encoding drug transporters could potentially influence drug response. For example the drug transporter P-glycoprotein (encoded by *ABCB1*) is extensively distributed and expressed in the intestine, liver, renal proximal tubular cells, and capillary endothelial cells comprising the blood brain barrier and transports and has extremely broad substrate specificity[18]. It has a putatively functional polymorphism (C3435T, discussed further in 1.2.1) which could potentially influence response to many P-glycoprotein substrates. Other drug transporters, including further members of the ABC-transmembrane transporter family[19] and members of the organic anion transporting polypeptide (OATP) family,[20] have been shown to harbour functional polymorphisms although none has yet been securely associated with variable drug response.

A third group of pharmacogenetic candidate genes are those that encode drug targets. Drug target or target pathway genes is the fastest growing and arguably the most interesting class of pharmacogenetic variants. Drugs physically bind to their targets, such as receptors or enzymes, and modulate their behaviour, so genes that encode proteins that are involved in these processes (including genes that do not encode the actual target of a drug, but instead other proteins in the drug target pathway) might also be involved in variation in drug response. For example, it has recently been reported that a common

polymorphism in *VKORC1*, encoding a subunit of the vitamin K epoxide reductase complex, the drug target for warfarin, is significantly associated with reduced warfarin dose[21]. The polymorphism was not found to be functional. This association has been replicated many times and several additional associated *VKORC1* polymorphisms (all non-coding)[22] and clinically predictive *VKORC1* haplotypes[23] have also been reported, explaining up to 30% and 25% of variance in dose, respectively. It now appears that the molecular mechanism of this warfarin dose response is regulated at the transcriptional level[23], however, further work will be needed to isolate the causative variant(s).

Similarly, the arachidonate 5-lipoxygenase (*ALOX5*) gene encodes an enzyme that is involved in the production of leukotrienes, which promotes bronchial constriction. A promoter-region variable number of tandem repeats (VNTR) that affects *ALOX5* expression has been associated with the efficacy of both inhibitors of ALOX5 and antagonists of its receptor[24].

Pharmacogenetic variants can also be viewed as belonging to one or other of two broad groups of genes that may influence drug response: the first contains genes such as those that encode DMEs and drug transporters, which control the pharmacokinetic properties (including disposition) of the drug; and the second group contains genes that encode drug targets (plus elements of the associated pathways) that influence drug pharmacodynamics.

Most pharmacogenetics studies to date have followed a candidate gene approach. Although they have not always been comprehensive (often focussing on just one or a few candidate genes from the categories described above) it is nevertheless a rational approach to take and has proved successful.

1.1.4 Pharmacogenetics and Genomics

Like many other branches of the biomedical sciences pharmacogenetics has been invigorated by recent advances in genomics. The human genome project has made it possible to search for relevant genetic differences in a systematic manner. Technological advances in genotyping have greatly lowered the average cost per genotype. Haplotype data from the HapMap project has the potential to improve the efficiency of polymorphism selection.

Today, it is possible to assess entire pathways that might be relevant to drug response. Whole genome scans for pharmacogenetic variants are now realistic, if expensive. At the time of writing, no results from pharmacogenetic whole genome scan studies have been published, however, it is not unreasonable to expect the first studies to be published within the next couple of years. Results from the first whole genome scans for pharmacogenetics should begin to answer the question of whether pharmacogenetic variants are mainly found in obvious candidate genes or whether variation in unexpected genes also affects drug response.

1.2 Where are we now?

1.2.1 Current state of pharmacogenetics research

To assess the state of pharmacogenetics research I have identified from the literature a set of variants that have been significantly associated with drug response in at least two

studies (Table 1.1). They include examples of direct replication, in which the same drug response was studied in the first and at least one subsequent study, for example, the dopamine transporter (*SLC6A3*) variable number tandem repeat polymorphism has been associated with poor response to methylphenidate for treatment of attention deficit/hyperactivity disorder in two studies[25, 26]. They also include examples in which a similar drug response (that is, the same class of drug) was studied, for example the C allele of the tryptophan hydroxylase (*TPH1*) A218C polymorphism is associated with better response to two SSRI class of antidepressant drugs, paroxetine and fluvoxamine. Finally, examples are also included of functional replication; that is, where the polymorphism was associated with a different drug response but there was apparently a common underlying physiological cause for the associations. For example, the *GSTT1* gene deletion is associated with susceptibility to hepatotoxicity in response to both tacrine and troglitazone treatment. Despite these two drugs being in different classes, the common mechanism is that individuals lacking glutathione S-transferase theta 1 enzyme cannot adequately detoxify either of these drugs[27, 28]. Considering the variants in drug metabolizing enzyme genes listed in Table 1.1, all except those in *CYP2B6* and *GSTM3* have large functional, often Mendelian, effects, and would thus be strong candidates for influencing the response of any drugs predominantly metabolised by those enzymes.

The associations were compiled from review papers and from PubMed literature searches using the following keywords and phrases: pharmacogenetics OR pharmacogenomics, association study AND drug response, polymorphism AND drug response.

This exercise was originally performed towards the end of 2003 for inclusion in a review paper[29]. At that time there were 42 pharmacogenetic variants in the list. The list has since grown by another 19. I have noted in the final column of Table 1.1 which variants were included in the 2003 review in order to compare the two groups of variants. Although most of the variants in the post-2003 group were in fact identified earlier (it is the replication study which is post-2003), and the year 2003 is a somewhat arbitrary cut-off date, nevertheless, comparison of the two groups may reveal some trends.

The field is growing rapidly and the list no doubt omits some polymorphisms. It should therefore be considered a snapshot of the field rather than a definitive review. It probably includes some false positives (compare with [30, 31]) and undoubtedly includes some controversial examples. The exon 26 C3435T polymorphism of the *ABCB1* gene has been associated with the efficacy of anti-epileptic drugs and response to anti-retroviral drugs[32, 33]. Many groups have tried to replicate the association with anti-epileptic drug response (the original study found that TT patients were less likely to have drug-resistant epilepsy than CC patients) yet whereas there have been three subsequent reports of an association between this polymorphism (or a haplotype including this polymorphism) and antiepileptic drug response[34-36], four further studies, including a prospective study of 503 patients[37], have not found an association[37-40]. Many of the variants listed in Table 1.1 have well-studied functional effects that are consistent with the observed drug response (for example, thiopurine S-methyltransferase (*TPMT*) and many other examples from metabolism in particular). Before an association is considered to be real one criterion is that it should be demonstrated that the variant does indeed have functional effects.

Table 1.1 Pharmacogenetic variants that have been significantly associated with drug response in at least two studies

Gene	Name	Allele* (common name)	Allele** (Variant, where applicable, or ref)	Type of Mutation***	Type of replication ? #	Associated phenotype	Type of drug response	No?	No?	Pharmacokinetic / *in vitro*	Refs	Post-2003 review ?
Drug target / pathway proteins												
ACE	angiotensin I converting enzyme	Ins/Del	287bp Ins/Del[41]	intronic ins/del	Same	DD associated with decreased proteinurea in response to ACE inhibitor treatment for renal disease	efficacy	21	36	not clear, although DD caucasians have highest plasma levels of ACE	42-44	No
ADRB1	ß-1 adrenergic receptor	Arg389Gly	rs1801253	Missense	Same	Arg/Arg homozygotes have greater response to beta-adrenergic receptor antagonists (metaprolol in hypertensives, atenolol in healthy volunteers)	efficacy	34	40	Basal adenylyl cyclase activity nearly two-fold higher for Arg form	45-47	No
ADRB2	ß-2 adrenergic receptor	Arg16Gly	rs1042713	Missense	Same	Gly allele associated with decreased response to albuterol in asthmatics and salbutamol in healthy volunteers	efficacy	269	57	Gly has increased agonist-promoted downregulation of receptor expression *in vitro*	48-50	No

AGT	angiotensinogen	Met235Thr	p.Met235Thr	Missense	Similar	Thr allele associated with greater reduction of blood pressure and greater decrease in left ventricular mass with antihypertensive treatment	efficacy	125	41	Unclear although Thr allele associated with higher plasma AGT levels	[51-53]	No
AGTR1	angiotensin II receptor, type 1	A1166C	rs5186	3' UTR	Similar	C allele associated with greater response to Angiotensin-II receptor antagonists in hypertensives patients and in healthy volunteers	efficacy	66	41	Not known	[52, 52, 54]	No
ALOX5	arachidonate 5-lipoxygenase	promoter VNTR (WT = 5; non-WT=3,4 or 6)	GGGCGG 3-6 repeats (as described in	promoter VNTR	Same	Homozygotes for non-WT alleles have decreased response to 5-lipoxygenase inhibitor and leukotriene receptor antagonist in asthmatics	efficacy	114	N/A	Non-WT alleles have diminished activity in promoter-reporter constructs	[55-57]	No
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	Thr116Ser (C347G)	rs2372536	non-synonymous	Same	Good clinical status (high number of tender and swollen joints, high disease activity) associated with a high pharmacogenetic index (that is, Ser/Ser homoygous in combination with other putatively functional genetic	efficacy	108	226	N/A	[58, 59]	Yes

						variants).						
BDKRB2	bradykinin receptor B2	C-58T	rs1799722	Promoter	Same	T allele associated with angiotensin-converting enzyme inhibitor-related cough	adverse events	60	190	-58T has higher in vitro transcription rate than -58C	[60-62]	No
CETP	cholesteryl ester transfer protein	TaqIB polymorphism B1/B2	see [63, 64]	Intronic	same	B1B1 associated with increased response to pravastatin and atorvastatin	efficacy	807	217	Not clear although B1B1 carriers also have a more atherogenic lipid profile, including low HDL.	[65, 66]	No
DRD2	dopamine D2 receptor gene	3'UTR Taq1A A1/A2	see [67]	3'UTR	same	A1 associated with greater positive response to antipsychotics haloperidol and nemonapride	efficacy	57	25	Not clear, although A1 allele assoc with decreased DRD2 density in the striatum and decreased binding potential.	[68-71]	No
DRD3	dopamine D3 receptor gene	Ser9Gly	rs6280	Missense	same	Gly associated with response to clozapine; Ser/Ser with non-response	efficacy	133	32	Not clear, although a mutation in this region could disturb membrane insertion. Higher binding affinity for DRD3 selective ligands and dopamine has been demonstrated for the Gly/Gly.	[72-74]	No
DRD4	dopamine D4 receptor gene	Exon 3 VNTR (2- to 7-fold repeat)	48bp repeat (3-11 repeats) (as described	exon VNTR	similar	7 allele significantly less frequent than 4 allele in patients responding to typical	efficacy	60	80	Repeat number affects pharmacologic activities of receptor	[77-79]	No

		4-fold 7-fold	in [75, 76]			neuroleptics (compared to patients responding to clozapine); 4/4 homozygotes have higher rate of good response to neuroleptics.						
ESR1	estrogen receptor 1 (alpha)	Pvu II	See [41]	Intronic	different	PP associated with worse response in bone mass in response to HRT	efficacy	81	108	N/A	[80, 81]	Yes
GNB3	guanine nucleotide binding protein (G protein), beta polypeptide 3	exon 10 C825T	rs5443	synonymous	similar	TT associated with response to antidepressants	efficacy	76	490	Not clear, although T allele form shows enhanced signal transduction in vitro via pertussis toxin (PTX)-sensitive G proteins	[82-84]	No
GRIN2B	glutamate receptor, ionotropic, N-methyl D-aspartate 2B	C2664T	rs1806201	synonymous	Same	CC associated with higher mean clozapine dosage for schizophrenia	efficacy	100	193	not known	[85, 86]	No
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	T102C	rs6313	synonymous	similar	CC or C associated with susceptibility to tardive dyskinesia in response to antipsychotic drugs	adverse events	121	221	Not known	[87, 88]	No
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	His452Tyr	rs6314	Missense	Same	Tyr associated with poorer response to the antipsychotic clozapine	efficacy	185	153	Not known	[89, 90]	No

HTR6	5-hydroxytryptamine (serotonin) receptor 6	C267T	rs1805054	synonymous	similar	TT associated with better response to clozapine and risperidone	efficacy	99	123	N/A	[91, 92]	Yes
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	A1/A2 Leu33Pro	rs5918	non-synonymous	similar	A2 associated with aspirin resistance in healthy volunteers	efficacy	24	80	The A2-expressing cells exhibit increased adhesion, enhanced spreading, more extensive actin polymerization, and enhanced fibrin clot retraction, reflecting A2-related differences in outside-in signaling	[93-95]	Yes
LIPC	hepatic lipase	C-514T	see [96]	promoter region	Same	CC genotype associated with increased response to statins	efficacy	49	693	C allele associated with higher hepatic lipase activity, denser LDL and lower HDL2 cholesterol	[97-99]	No
LTC4S	leukotriene C4 synthase	A-444C	rs730012	Promoter	same	C allelic frequency was significantly higher in aspirin intolerant asthmatic patients as compared with aspirin-tolerant asthmatics	efficacy	186	160	C allele is an additional target for a transcription factor of histone H4 consensus. Transfection of COS-7 with promoter construct increased expression of beta-galactosidase reporter for the C variant.	[100, 101]	Yes

MAOA	monoamine oxidase A	3 and 4 alleles	see [63]	VNTR	different	3/3 patients with major depressive disorder had a significantly better response to fluoxetine treatment when compared to carriers of the 4 allele; frequency of allele 3 was significantly higher in fluvoxamine-treated patients without nausea than in ones with nausea	both	257	66	Alleles with 3.5 or 4 copies of the repeat sequence are transcribed 2-10 times more efficiently than those with 3 or 5 copies of the repeat	[102-104]	Yes
MTHFR	5,10-methylenetetrahyrdrofolate reductase	C677T	rs1801133	Missense	same	TT patients have increased toxicity for methotrexate therapy	adverse events	61	43	TT homozygotes have reduced enzyme activity (30% less than heterozygotes)	[105-107]	No
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	BclI RFLP, C/G	see [69]	Intronic	different	GG individuals with ALL and treated with corticosteroids have reduction in survival probability, G-allele was associated with hypersensitivity to glucocorticoids in healthy volunteers	efficacy	222	191, 196 3, 370	N/A	[108, 109]	Yes
PPARG	peroxisome proliferative activated receptor, gamma	Pro12Ala	rs1801282	non-synonymous (*isoform* 2-specific exon	different	Ala associated with increased response to rosiglitazone treatment for diabetes and improved insulin	efficacy	198	333	N/A	[110, 111]	Yes

				B) and synonymous (CT substitution in exon 6 in isoform 1)		sensitivity in healthy volunteers						
SCN1A	sodium channel, voltage-gated, type 1, alpha	IVS5-91 G>A	rs3812718	splice variant	similar	Polymorphism is associated with maximum dose of the antiepileptic drugs phenytoin and carbamazepine, both of which target the alpha subunit of the sodium channel, with AA>AG>GG	efficacy	425	242	Polymorphism disrupts the consensus sequence of the 5' splice donor site of a highly conserved alternative exon (5N) and significantly affects the proportions of the alternative transcripts in individuals with a history of epilepsy.	[1]	Yes
SLC6A3	dopamine transporter	3'VNTR 10 repeat allele	40bp repeat (3-13 repeats) (as described in [112])	3' UTR VNTR	same	10/10 genotype associated with poor response to methylphenidate for treatment of attention deficit/hyperactivity disorder	efficacy	50	30	not clear, although variability in the length or the sequence of the 3'-UTR may influence levels of the protein in the brain	[25, 26, 113]	No
SLC6A4	serotonin transporter (5-HTT)	promoter VNTR (long/short)	44bp deletion (as described in [114])	promoter VNTR	same	l/l better response to fluoxetine or paroxetine than s/s	efficacy	121	58	Homozygosity for the long variant (ll-genotype) is associated with a two times more efficient 5-HT uptake compared to the s/l-	[115-117]	No

										or s/s-genotype		
TPH1	tryptophan hydroxylase 1	A218C	rs1800532	Intronic	similar	AA and AC associated with poorer response to paroxetine treatment; A/A associated with slower response to fluvoxamine (in subjects not taking pindolol)	efficacy	121	217	Not known	[118, 119]	No
TYMS	Thymidylate synthase	TSER*2/3	28bp repeat (2-3 repeats) (as described in [120].)	promoter VNTR	same	*3 poorer response to 5-Flurouracil treatment than *2; *3/*3 require higher dose	efficacy	65	115	3/3 homozygotes have 3.6 times higher TYMS mRNA and protein levels compared to 2/2 homozygotes	[121-123]	No
VDR	vitamin D (1,25-dihydroxyvitamin D3) receptor	TaqI	see [124-126]	synonymous	different	T allele associated with bone mineral density reduction in response to gonadotropin-releasing hormone agonist therapy, TT genotype assoc with higher percentage change in bone mineral density per year than heterozygotes	efficacy	43	82	Polymorphisms probably nonfunctional, linkage disequilibrium with one or more truly functional polymorphisms elsewhere in the VDR gene is assumed to explain the associations observed	[124-126]	Yes
VKORC1	vitamin K epoxide reductase complex, subunit 1	various [21, 22]	various [21, 22]	intronic, UTR	same	Several *VKORC1* SNPs significantly associated with warfarin dose	efficacy	205	147	N/A	[21, 22]	Yes

Drug transporter											
ABCB1	MDR1, P-glycoprotein 1	C3435T	rs1045642	synonymous	different	TT patients less likely to have drug-resistance epilepsy than CC, and have increased immune recovery after initiation of antiretroviral treatment	efficacy	315	123	TT associated with more than 2-fold lower MDR1 expression levels compared with CC. T allele carriers have higher plasma levels of digoxin.	32, 33, 127	No
Metabolism											
BCHE	butyrylcholinesterase	several mutations including: Asp70Gly (dibucaine or atypical variant) Ala539Thr (K allele)	p.Asp70Gly p.Ala539Thr	various missense	same	variants associated with adverse effects in response to succinylcholine	adverse events	1247	65	K variant shows 30% reduction in enzyme activity; dibucaine mutation reduces the binding affinity of BChE enzyme for succinylcholine and dibucaine, resulting in ineffective hydrolysis	128-131	No
COMT	catechol O-methyltransferase	Val158Met	rs4680	Missense	similar	Met associated with significantly higher daily neuroleptic dosage required for schizophrenic patients and poor response	efficacy	94	100	Met variant low activity, low thermal stability; Val has high activity, high thermal stability	132-134	No
CYP2B6	cytochrome P450, family 2, subfamily B,	Gln172His (*6)	rs3745274	non-synonymous	similar	His allele associated with toxicity in response to efavirenz therapy for HIV	adverse events	154	167	*6 allele has increased catalytic activity but may not be causal as in LD with	135-137	Yes

	polypeptide 6									other coding variants		
CYP2C19	cytochrome p450 2C19	*2 *3	variants include: 681G>A (*2 alleles) 636G>A (*3 allele)	nonsense	same	extensive metaboliser (WT) homozygotes have decreased response to the proton-pump inhibitor omeprazole for H. pylori infection	efficacy	153	62	splice site mutation (*2) and premature stop codon (*3) result in non-functional enzyme	[138, 139]	No
CYP2C9	cytochrome p450 2C9	*2 *3	variants include: rs1799853 rs1057910	Missense	same	non-WT alleles associated with reduced warfarin daily dose requirement	efficacy	531	184	Low enzyme activity	[140-142]	No
CYP2D6	cytochrome p450 2D6	many inactive alleles e.g. *3 *4 *5	variants include 2549A>del (*3 alleles) 1846G>A (*4 alleles) *5 is whole gene deletion	nonsense and others	same	non-WT alleles associated with susceptibility to tardive dyskinesia in response to antipsychotics	adverse events	45	N/A	No enzyme activity	[143-145]	No
CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	*3, A6986G	rs776746	splice variant	different	CYP3A5*1 allele associated with higher tacrolimus dose requirements	efficacy	100	180	Severely decreased activity	[146-148]	Yes
DPYD	dihydropyrimidine dehydrogenase (DPD)	DPYD*2A (IVS14+1G>A)	rs3918290	splice variant	same	*2A associated with severe toxicity and fatal outcomes for 5-Fluorouracil treatment	adverse events	20 cases	114	Low/absent enzyme activity	[149-151]	No

GSTM1	glutathione S-transferase M1	GSTM1 null	entire gene deleted	gene deletion	different	Null carriers have increased survival time and progression-free interval following paclitaxel and cisplatinum treatment for ovarian cancer; decreased risk of relapse for cytotoxic therapy for leukemia	efficacy	N/A	128	No enzyme activity	[152, 153]	No
GSTM3	glutathione S-transferase M3 (brain)	GSTM3*A / GSTM3*B	rs1799735	intronic ins/del	different	GSTM1*0/GSTM3*A haplotype less likely to show a beneficial response to D-penicillamine in rheumatoid arthritis; *3A has increased risk of cisplatin totoxicity	both	81	39	not known	[154, 155]	No
GSTP1	glutathione S-transferase pi	Ile105Val	rs947894	Missense	similar	Val associated with increased survival for 5-Fluorouracil and oxaloplatin therapy for colorectal cancer, and following therapy for multiple myeloma	efficacy	107	222	Val form has lower enzyme activity	[156-158]	No
GSTT1	glutathione S-transferase theta 1	GSTT1 null	entire gene deleted	gene deletion	different	GSTT1 associated with susceptibility to tacrine hepatotoxicity and troglitazone hepatotoxicity in combination with GSTM1 null allele	adverse events	110	141	No enzyme activity	[27, 28]	No

NAT2	N-acetyltransferase 2	slow acetylator alleles include: NAT2*5B NAT2*6A NAT2*7A or B NAT2*14A or B	polymorphisms include: rs1801280 (*5 alleles) rs1799930 (*6 alleles) rs1799931 (*7 alleles) rs1801279 (*14 alleles)	Missense	same	slow-acetylator status of NAT2 is a significant susceptibility risk factor for antituberculosis drug-induced hepatotoxicity	adverse events	124	77	0	159, 160	No
NQO1	NAD(P)H dehydrogenase, quinone 1	Pro187Ser	rs1800566	non-synonymous	same	Ser allele is more frequent in patients with therapy-related leukemia	adverse events	104	469	No enzyme activity	161-163	Yes
TPMT	Thiopurine methyltransferase	TPMT*2 TPMT*3A TPMT*3C	include: rs1800462 (T*2 allele) p.Ala154Thr (*3A allele) rs1142345 (*3A and *3C alleles)	Missense	same	Homozygotes for non-WT alleles at very high risk of severe hemapoietic toxicity after thiopurine treatment; heterozygotes intermediate risk of dose-limiting toxicity	adverse events	180	67	lower enzyme activity due to enhanced proteolysis of mutant proteins	164-166	No
UGT1A1	UDP-glucuronosyltransferase 1A1	UGT1A1*28	promoter TA [167]	promoter VNTR	same	*28 associated with increased chance of developing diarrhea and leukopaenia during irinotecan therapy	adverse events	20	118	UGT1A1*28 allele associated with reduced UGT1A1 expression leading to reduced glucuronidation of the	123, 168, 169	No

										irinotecan metabolite SN-38		
Other												
ADD1	adducin 1 (alpha)	Gly460Trp	rs4961	missense	same	Trp associated with increased response to diuretics in hypertensives (hydrochlorothiazide)	efficacy	58	143	Transfection of variants in rat kidney cells showed that only the Trp variant increases the surface expression and maximal rate of the Na–K pump; also directly stimulates the isolated Na–K pump at significantly lower concentrations.	170-172	No
APOE	Apolipoprotein E	E4	*APOE* alleles have combinations of various polymorphisms including rs429358 for APOE*4	missense	same	E4 allele associated with lesser response to statins (wrt to lowering total cholesterol and LDL-cholesterol levels)	efficacy	189	232	Not clear, although E4 allele also associated with higher plasma LDL cholesterol levels	173-175	No
CLOCK	clock homolog (mouse)	T3111C	rs1801260	3'UTR	different	TT patients undergoing SSRI treatment for major depression relapsed within 6 months after recovery more	both	185	171	Affects mRNA stability and half-life	176-178	Yes

						than TC and CC subjects. C allele associated with daytime hypersomnolelence for clozapine monotherapy.						
FCGR3A	FcγRIIIa receptor protein	Phe158Val	p.Phe158Val	missense	different	Val/Val patients greater response to rituximab for non-Hodgkin's lymphoma and higher complete remission (CR) rate with immunosupressive medication for idiopathic thrombocytopenic purpura	efficacy	49	104	Fc gammaRIIIa-158F natural killer cells bind significantly less IgG1, IgG3, and IgG4 than did Fc gammaRIIIa-158V natural killer cells	[179-181]	No
FKBP5	FK506 binding protein 5	C/T	rs1360780	intronic	same	TT genotype associated with antidepressant response	efficacy	233	83	Lymphocytes of individuals who are TT homozygous at rs1360780 had FKBP5 levels twice as high as those of individuals with the two other genotypes (P = 0.024). No rs1360780 genotype−dependent effects on mRNA levels.	[182]	Yes
HLA-B	major histocompatibility complex, class I, B	HLA-B*5701	N/A	N/A	same	associated with hypersensitivity to abacavir	adverse events	200	185	not known	[183, 184]	No

IL10	interleukin 10	A-1082G	see [185]	promoter region	different	GG has better response to prednisone in leukemia patients and sustained response in antiviral therapy for chronic hepatitis C infection (as part of haplotype (108bp) - (-2575T) - (-2763C) - (-1082A) - (-819T) - (-592A))	efficacy	133	104	GG have higher IL-10 plasma levels (clinical significance contradictory)	[186-188]	No
NOS3	nitric oxide synthase 3 (endothelial cell)	Glu298Asp (G894T)	rs1799983	non-synonymous	similar	Asp allele associated with decreased response to hydrochlorothiazide	efficacy	585	119	influences endothelial function	[189-191]	Yes
PLCG1	phospholipase C, gamma 1	PLCG1-8, and-5	see [192-194]	intronic dinucleotide repeat	different	8 and 5 alleles associated with response to lithium	efficacy	61	136	N/A although 5- repeat may be risk factor for bipolar disorder	[192-194]	Yes
SOD2	superoxide dismutase 2, mitochondrial	Ala-9Val	rs1799725	coding, mitochondrial targeting sequence (MTS)	same	Val, and combination of the Val and DRD3 Ser9 alleles, associated with susceptibility to TD in schizophrenics	adverse events	192	95	predicted to cause a secondary structure alteration from an α-helical structure to a β-sheet conformation, may affect the cellular allocation	[195-197]	Yes
TNF	tumor necrosis factor (TNF superfamily, member 2	G-308A	see [198]	promoter region	different	A associated with good response to immunosuppressive therapy in patients with aplastic anemia and carbamazepine hypersensitivity	efficacy	39	373	associated with elevated TNF levels	[199-201]	No

XRCC1	DNA repair protein XRCC1	Arg399Gln	rs25487	missense	different	gln associated with resistance to 5-Fluoruracil and oxaliplatin chemotherapy, gln/gln individuals less likely to develop therapy related acute myeloblastic leukemia	both	61	168	Gln form has decreased DNA repair capacity	202-204	No

Legend

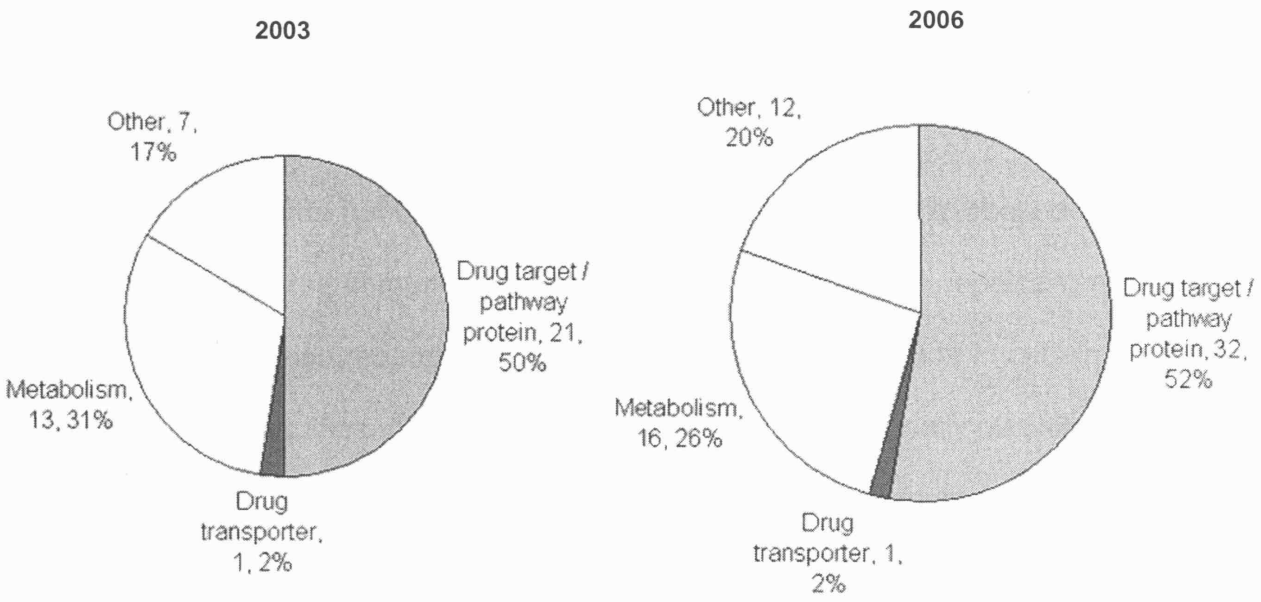
* Name of common alleles

** Allele locus information. Where the available information is sufficiently unambiguous the variant has been described using standard notation (see http://www.genomic.unimelb.edu.au/mdi/mutnomen/) or a dbSNP reference (see http://www.ncbi.nlm.nih.gov/SNP/). For cytochrome P450 alleles also see http://www.imm.ki.se/CYPalleles/ . Where there is insufficient information see references.

*** Indicates, where known, the molecular bases of the common allelic variants

\# Indicates whether the second study was of the same drug response, as described in the text.

Figure 1.1 Types of genes in Table 1.1



1.2.2 Types of pharmacogenetic variants

In the original 2003 survey, half of the polymorphisms were found either in the drug target (or a drug target pathway protein) or in a protein that is in the pathway in which the target acts, whereas nearly one-third were in DMEs (Table 1.2). The proportion of variants in each category today is similar, however the largest growth (in absolute terms) has been in the drug target/pathway category. There have been no new examples of drug transporter polymorphisms; the *ABCB1* variant remains the only representative and as previously discussed, this is a controversial example.

Table 1.1 confirms an important role for the two categories of candidate genes listed in 1.1.3: genes that influence pharmacokinetics, for example, DMEs and transporters; and genes that influence pharmacodynamics, for example, targets and other elements of the pathway in which the target acts. However, there is an obvious ascertainment bias as studies have concentrated mainly on genes that are expected to influence drug response.

A fifth of the variants do not fall into an obvious category and so are labelled "other". They include variants in three immune system-related genes and a DNA repair protein. With hindsight these are also reasonably obvious types of gene to include in certain pharmacogenetics studies. Given their pathogenesis, it is biologically plausible for immune system-related genes to be involved in certain rare drug hypersensitivity reactions, for example[205]. Polymorphisms which affect the repair capacity of DNA repair enzymes could conceivably influence response to those chemotherapeutic agents which are designed to damage cell replication machinery either directly at the level of DNA or by inhibiting enzymes involved with DNA repair and synthesis[206].

The entries in Table 1.1 also highlight another category of candidate genes for drug response: genes that are involved in the underlying disease condition. For example, it is thought that the *ALOX5* regulatory variant determines whether leukotrienes have a role in asthma in a particular patient, and only if they do will inhibitors of *ALOX5* be effective[24]. The variants in *CETP* and *LIPC* are also associated with an altered patient lipid profile. Not yet listed in the table is a first report of the Apolipoprotein E *4 allele, famously associated with increased risk of Alzheimer's disease[207], also predicts decreased cognitive and functional improvement in Alzheimer's patients in response to rosiglitazone (a drug usually used in the treatment of type II diabetes)[208]. Historically, many pharmacogenetic studies

have been designed as "add-ons" to disease predisposition studies and in most cases the same candidate genes or polymorphisms were considered in both. Again, this probably resulted in an ascertainment bias.

1.2.3 Limitations of pharmacogenetic studies to date

Table 1.1 clearly illustrates that genetic factors do play an important role in drug response. It also demonstrates a major limitation of many studies to date. Around half of the studies listed have fewer than 100 patients, with a fifth of the total having less than fifty patients. This means that these studies are generally underpowered, even for detecting variants of relatively large effect (compare with[30]).

To detect even relatively strong associations between genetic variants at a specific locus and variation in drug response, many cases and controls are needed. For example, to detect the effect of a gene variant that explains 5% of the total phenotypic variation in a quantitative response to a drug by typing 100 independent single nucleotide polymorphisms (SNPs) would require 500 patients to provide an 80% chance of detection, assuming an experiment-wide false-positive rate (type I error threshold) of 5% (using the power calculation of Sham and colleagues[209]). Only four of the studies in Table 1.1 are conducted on more than 500 patients. This raises the question of how the other studies detected associations. In fact as previously noted, many of the variants in Table 1.1 have large, Mendelian, effects thus explaining a high proportion of the total phenotypic variation, and as many examples are small candidate gene or even candidate polymorphism studies,

positive P values only require Bonferroni correction for a limited number of tests (e.g. less than the 100 tests given in this example). However, as pharmacogenetics moves into the genomic era, it is increasingly important to ensure that studies are designed to be sufficiently well-powered.

Complications such as stratification[210, 211] have mostly been ignored in the pharmacogenetic literature. Stratification occurs when a patient population is genetically structured, and can create spurious associations between gene variants and drug responses. Few studies in pharmacogenetics have used genetic methods to rule out stratification as an explanation of the association[1, 32]

Most of the studies listed would be considered candidate polymorphism studies rather than candidate gene studies, as they do not seek to systematically represent all the genetic variation in the genes considered. More recent studies have moved beyond this and do fully represent genetic variation[2, 212] often utilising HapMap data.

1.3 Getting to the clinic

1.3.1 Potential clinical applications of pharmacogenetics

There are three major ways that a pharmacogenetic diagnostic could be used in a clinical setting:

1. To improve drug safety: If a polymorphism is found to be associated with an adverse reaction, doctors could avoid prescribing the medicine to patients with this genotype .

2. Adjusting dosage: Variants which influence the optimum dose of a drug could reduce the trial-and-error approach to determine the most effective dose. These will usually, though not always[1, 22] be DME variants.

3. Increasing efficacy: As noted earlier, many drugs are not effective in a large proportion of the population[5]. Pharmacogenetics may identify responsive and non-responsive patients or offer new medicines designed on the basis of the genetics of the disease.

Currently the only examples of pharmacogenetic diagnostics routinely tested in the clinic are those for low-activity variants of the drug-metabolizing enzyme (DME) gene *TPMT* (although monitoring TPMT enzyme activity in red blood cells is still more commonly used than a genetic test and for the *HLA-B**5701 variant and abacavir hypersensitivity[213]. Testing for reduced function *TPMT* genotypes is used to modify doses of thiopurines such as 6-mercaptopurine and azathioprine that are used to treat acute lymphoblastic leukaemia and inflammatory bowel disease[214, 215]. Individuals with deficient or intermediate *TPMT* activity risk toxicity, including fatal myelosuppression, at standard thiopurine doses. Testing for low-activity variants for TPMT has been in clinical practice for more than 10 years in the United States and has been shown to be cost-effective in certain health-care settings[216]. The next pharmacogenetic variant to be routinely tested for in the clinic is likely to be the *UGT1A1*28* variant which predicts toxicity to another oncology agent, irinotecan[168]. The US Food and Drug Administration approved label changes for 6-mercaptopurine and

irinotecan, to include pharmacogenetic testing as a potential means to reduce the rate of severe toxic events in 2004 and 2005, respectively[217], http://www.fda.gov/ .

1.3.2 Scientific barriers to use in the clinic

There are numerous reasons why the variants listed in Table 1.1 are not yet ready to be used as diagnostics in the clinic. I will briefly outline some of the major scientific reasons below.

Is the association real? There are no clear rules for assessing whether an association is real or not. If an association is more or less consistently replicated in several studies this increases confidence in the association. Likewise, the causal variant should be determined and its functional effects should be biologically consistent with the phenotype under study. The *VKORC1* association with warfarin dosing discussed earlier is securely replicated yet the causal variant (s) has not been determined.

Variants have not been prospectively studied. All of the studies in Table 1.1 are retrospective. Whilst there are now increasingly more prospective pharmacogenetics studies (e.g. the recent studies [58, 218-221]) in general, prospective studies are still not adequately designed to provide clear guidelines as to how a pharmacogenetic should be used in the clinic.

Guidelines for clinical use. Without appropriate prospective studies, it is not clear how pharmacogenetics should be used in clinical practise. For example, Roche's AmpliChip for

the rapid genotyping of *CYP2D6* and *CYP2C19* variants was approved by the FDA for diagnostic use in the USA in January 2005. 35% of antipsychotics and 40% of antidepressants, are estimated to require dose adjustments on the basis of polymorphisms in these genes[222]. However, neither Roche nor the FDA has provided appropriate dose schedule recommendations and recommendations will not be available until large-scale prospective trials have been undertaken.

Usefulness of a pharmacogenetic test. To assess the usefulness of a pharmacogenetic test in general, it is important to have a reliable estimate of positive and negative predictive values (PPV and NPV, respectively[223]). The PPV for a certain drug response is defined as the proportion of people with a positive test result who have that response, and NPV is the proportion of people with a negative test who do not have the drug response X. The relative importance of PPV versus NPV is determined in part by the nature of the drug response. One would wish to predict a serious ADR with high confidence, for example, so would require a high NPV.

Education. Clinicians will need to be informed about relevant pharmacogenetic findings, how a given test can actually be ordered for their patients and the actual pharmacogenetic contribution to variable drug response in specific diseases. The general public needs to be educated about pharmacogenetics so that patients can make informed choices about their treatment.

1.3.3 Economic and barriers to use in the clinic

Economic considerations will influence both the directions taken in pharmacogenetic research and its clinical application; with different concerns for health-care providers and drug companies. In general, health-care providers should (eventually) be willing to pay for genetic tests to improve efficacy and reduce adverse effects of medicines. Various factors will influence the cost-effectiveness of pharmacogenetic testing with some classes of drug being more immediate candidates for testing. Efficacy tests will be used for expensive drugs, drugs that only work in a minority of patients, drugs whose effects cannot be assessed immediately, and drugs for chronic diseases. For example, anticancer drugs typically have just 25% efficacy[5] and often have high toxicity. Switching from an ineffective drug regimen wastes time and money. Advance testing would enable doctors to choose the optimal drug for each patient. Pharmacogenetic testing to avoid adverse reactions would also result in immediate cost savings. Aside from the health implications, adverse events cost hospitals up to $5640 per patient[224]. Pharmacogenetic testing will be most cost-effective for drugs with clinically severe and expensive adverse effects, such as 6-mercaptopurine for childhood acute lymphoblastic leukaemia[225]. At the other end of the scale, there is low tolerance for adverse effects of preventative interventions for healthy populations (e.g. vaccines, cholesterol-lowering drugs).

Drug development is a lengthy and expensive process. It takes around 15 years from initial target identification to develop a drug and bring it to marketplace. Only one in 1000 compounds will get that far. DiMasi and colleagues[226] estimate the total R&D cost for a new drug to be $802 million, rising to nearly $900 million including post-approval R&D.

Pharmacogenetics has the potential to streamline the drug development process[227]. Integrating pharmacogenetic testing into drug discovery and early development could halt development of compounds that would be abandoned later anyway. The prospect of rescuing previously failed drugs is also often cited as a potential benefit of pharmacogenetics, however, elapsed patent protection time may prove a disincentive[228].

In the clinical stage of development the aim is to use pharmacogenetics to predict drug response. The nature of pharmacogenetic test will affect the size of the market for a drug. An efficacy test identifying a small population of optimal responders could lead to market segmentation. However, a test to select the correct dosing regimen would not necessarily decrease the market size and a test to predict adverse events would perhaps exclude just a small proportion of the population. Drug companies will accordingly be more interested in predicting adverse effects than efficacy. Thirteen drugs were taken off the US market between 1997 and 2001 due to unacceptable side effects http://www.fda.gov/fdac/features/2002/chrtWithdrawals.html and safety issues are a major reason for early termination of drug development. Efficacy pharmacogenetics will have application in some circumstances. Stratifying patients by genotype may allow identification of response that would have been missed in an unselected cohort, resulting in approval for drugs that would otherwise be rejected. Drugs would then be indicated for a subset of patients, as Herceptin is for the subpopulation of breast cancer patients who express HER2 and Gleevec is for those patients with chronic myeloid leukaemia resulting from Philadelphia chromosome translocation. Pharmacogenetics also has important implications for drugs already on the market, including generic drugs. Academia has an important role to play in taking on those pharmacogenetic projects that will not be addressed by industry.

Whether a pharmacogenetic test makes economic sense will eventually depend on many factors including the cost of performing the actual test, the cost of ADRs (for example) associated with a drug and the prevalence of those ADRs and the cost of the drug in question and its alternatives. A full discussion of the economics of pharmacogenetic testing is beyond the scope of this thesis however a 2004 review found seven of the eleven pharmacogenetic diagnostics considered to be cost-effective[229], which provides some encouragement.

1.3.4 Some ethical considerations for pharmacogenetics

Pharmacogenetics could change the way new drugs are developed, approved and prescribed. Various ethical issues accompany these changes. Early identification of a marker for drug response would lead to smaller Phase III trials involving those patients more likely to respond. This could result in patients with unfavourable genetic profiles being excluded from trials, even though a proportion may actually respond to the drug. There is a risk of creating 'orphan populations' who are left untreated for either scientific (difficult to treat) or economic (too small to be economically viable) reasons. It is possible US and EU orphan drug laws could be applied to provide an incentive in these situations. Many pharmacogenetic variants vary in frequency among populations[29]. If a marker for efficacy has low prevalence in a certain ethnic population, that population may be excluded from research or treatment.

Pharmacogenetics of existing medicines does not share these concerns to the same extent. Here, when the information is used accurately, it simply allows patients to be matched to the best medicines. There are however, other issues relevant to all pharmacogenetic research. One is the accuracy of pharmacogenetic diagnostics, and their sensitivity and specificity across different ethnic groups. There is considerable variation in the pattern of linkage disequilibrium among populations. If the pharmacogenetic marker is not the causal variant then the performance of a test may also vary across different populations. It is therefore desirable to use causal variants diagnostically as opposed to using markers that are in association with causal variants. There is also the clinical validity of test, namely how well a variant predicts phenotype. Drug response is influenced by polygenic and environmental factors. Tests are unlikely to predict drug response with certainty; instead they will predict the likelihood of response. It is likely that patients designated non-responders will be denied access to drugs which may actually benefit some of them.

Variants that predict drug response may also influence disease predisposition, raising serious privacy issues. Around half of common variants listed in Table 1.1 have also been associated with common diseases (in three or more association studies for each[30]) and in many instances they are associated with a common disease other than that being treated. For example, the apolipoprotein E4 allele (ApoE4) is associated with lesser response to statin treatment for lowering of cholesterol[173, 230] and with an increased risk of Alzheimer's disease[231, 232]. If a marker carrying other significant or sensitive information is used to predict drug response then there are potential medical, family and insurance implications.

These concerns need to be addressed by promoting a greater understanding of pharmacogenetics and its implications among the health services and the general public.

1.4 Conclusions and aims of this thesis

Arguably, most of the excitement about recent advances in genomics has focused on its application to understanding common diseases, as this may point to new directions for drug development. Pharmacogenetics, however, could offer more immediate clinical applications. This is in part because many drug responses seem to be genetically and physiologically simpler traits than are common diseases. Another reason that pharmacogenetics might progress more quickly than the genetics of common disease is that an association between genotype and drug response might be of direct diagnostic use; for example, in using genetic predictors to avoid rare ADRs[232] or to select which of several alternative drugs has the highest efficacy[227]. Over the last few years pharmacogenetic studies have started to evolve from underpowered "add-on" studies, secondary to disease predisposition studies, to larger scale and genetically more thorough investigations. However, most pharmacogenetics variants have not yet been assessed prospectively in a manner which would facilitate their introduction into a clinical setting.

At the initiation of this thesis work in September 2002, the field of pharmacogenetics was on the cusp of undergoing a rapid expansion. At the same time, population geneticists were using information about linkage disequilibrium in the human genome to develop novel

methods for representing genetic variation. It was an exciting and challenging time to begin this work.

In the first and second experimental chapters of this thesis I describe work on the pharmacogenetics of carbamazepine and phenytoin dosing. These studies use a candidate gene approach for investigating the genetic bases of interindividual variation in drug doses. The third experimental chapter concerns the pharmacogenetics of a different anti-epileptic drug, levetiracetam. In contrast, this is an efficacy study and considers the possibility that genetic variation in the drug target (and its two isoforms) may influence the effectiveness of levetiracetam.

In the fourth experimental chapter, I change the focus and explore a different theme, that of race/ethnicity and drug response. Using the bucindolol evaluation of survival trial (B.E.S.T.) as an example I explore how best to represent race/ethnicity in the context of a clinical trial in addition to an investigation of how variation in the genes encoding the drug targets of bucindolol may influence response.

Chapter 2 Methods

2.1 Sample collection and storage

2.1.1 Institute of Neurology, London Cohort

This is the main cohort used in the work on epilepsy pharmacogenetics. This cohort was used for the work described in chapter 3 on carbamazepine and phenytoin dosing and in chapter 5 on levetiracetam pharmacogenetics.

2.1.1.1 Patient recruitment

Patients were recruited from the National Hospital for Neurology and Neurosurgery, Queen's Square, London, United Kingdom (incorporating the National Society for Epilepsy at Chalfont, London).

All patients with a diagnosis of a common, non-Mendelian form of epilepsy were eligible for inclusion in the study. Patients were provided with information on the project and those interested provided a blood sample for DNA extraction after signing the informed consent form.

The study protocol was approved by Joint Research Ethics Committee of the National Hospital for Neurology and Neurosurgery and Institute of Neurology and by the ethics panel of University College London. All patients self-identified, or were determined by the treating clinician, to be of European ancestry.

2.1.1.2. Patient phenotyping and databasing

Clinical details of participating patients were stored in a database at the Institute for Neurology, Queen's Square, London. The database covers in detail a wide variety of clinical phenotypes including syndromic diagnosis (according to ILAE classification), seizure diagnosis, seizure frequency and antiepileptic drug history

Table 2.1 Patient clinical characteristics – epilepsy syndrome type

Epilepsy Syndrome	Institute of Neurology cohort	Beaumont Hospital cohort
mTLE + HS	133 (16.6%)	139 (17.4%)
mTLE - HS	160 (19.9%)	35 (4.4%)
Non-JME IGE	64 (8.0%)	71 (8.9%)
IGE – GTCS only	2 (0.2%)	0 (0.0%)
JME	41 (5.5%)	75 (9.4%)
Other focal	324 (40.3%)	370 (46.2%)
Unclassifiable	79 (9.8%)	111 (13.9%)
Total	**803**	**801**

2.1.1.3. Control cohort

Controls were assembled from the National Twin Research Unit at Guy's and St Thomas' Hospitals, London, United Kingdom. To be considered for the control cohort, individuals must have been of British ancestry and unrelated to any other member in the selected cohort. Only one member of each sibship was included.

The use of these samples as controls is covered by ethics approval held at the Twin Research Unit, Guy's and St Thomas' Hospitals, London, United Kingdom.

2.1.1.4. DNA extraction from whole blood

DNA extraction from whole blood was carried using an Autogen extractor (AutoGenPrep® NA-2000). Each blood sample was split in to 2x50ml Falcon tubes. To these tubes was added a cell lysis solution. In order to obtain a nuclei pellet, the samples were then spun at 2600 rpm for 20 minutes. The supernatant was discarded and the pellet washed with 10ml PBS and centrifuged again at 2600 rpm for 10 minutes. The supernatant was discarded and the pellet re-suspended in 5ml PBS. The samples were again spun at 2600 for 10 minutes and the pellet discarded. To digest protein and lyse the white blood cell nuclei 500ml proteinase K and SDS was added to each sample. The samples were then incubated overnight at 55°C. On completion of digestion the samples were transferred to the Autogen machine which performs an automated phenol/choloroform extraction. Extracted samples were placed in labeled tubes and stored at -70°C.

2.1.1.5 DNA quantitation and standardization

DNA was quantified using the Picogreen® method. Briefly, picogreen® is a fluorescent dye that undergoes a dramatic fluorescence enhancement upon binding with DNA. This fluorescence can be measure using a microplate fluorimeter. The principle advantages of the picogreen® method are increased sensitivity (10,000 times more sensitive than ultra violet absorbance methods), the linear nature of the fluorescence over three orders of magnitude and robustness to protein contamination.

The "Quant-iT™ PicoGreen® dsDNA Assay Kit" from Molecular Probes (part # P7589) was used for DNA quantitation. Manufacturer's instructions were followed.

To 19.9ml TE solution was added 100 µl of Picogreen®. A standard curve was set up as follows. To 45 µl of the TE/Picogreen® solution was added 5 µl of standard concentration DNA (100 µg/ µl, delivered with Quant-iT™ PicoGreen® kit). A standard curve was constructed by filling the first five wells of a 96 well plate with the following volumes of the TE/Picogreen mix (solution A in Table 2.2) and the TE/Picogreen/DNA mix (solution B in Table 2.1).

Table 2.2 Dilution factors for picogreen quantitation

Position	Solution A (µl)	Solution B (µl)	Concentration (ng/ul)
A1	76	24	2.4
B1	88	12	1.2
C1	94	6	0.6
D1	97	3	0.3
E1	98.5	1.5	0.15

The remaining 91 wells on the plate were filled with 99ul of solution A. To each well was added 1ul of DNA of unknown concentration. Each well was thoroughly mixed and allowed to stand for 5 minutes. The fluorescence of each well was measured using a fluorimeter (FluoroCount^{TM}, Packard) and the concentration of each unknown sample calculated from the standard curve.

Once the concentration of each sample was known, it was possible to create 96 well "working" plates at a standardized concentration much lower than that of the stock tubes. In general we aimed to standardize working plates at a concentration of 10ng/ul.

2.1.2 Beaumont Hospital, Dublin, Ireland, Cohort

This cohort was used in the work described in chapter 4 on levetiracetam pharmacogenetics. Patients were recruited from the epilepsy clinic at Beaumont

Hospital, Dublin, Ireland. Inclusion criteria were the same as for the Institute of Neurology cohort and similar clinical details were stored in a database at Beaumont Hospital, Dublin. The study protocol was also approved by Beaumont Hospital Research Ethics Committee. All patients self-identified or were determined by the treating clinician, to be of European ancestry.

Controls were obtained from a cohort of current and retired bank employees and their spouses, (all of European ancestry) who were free of diagnosed hypertension and vasoactive drugs, when recruited to the Allied Irish Bank Study[233].

2.1.3 National Taiwan University Hospital, Taiwan, Epilepsy Patient Cohort

This cohort was used in the work described in chapter 4 on phenytoin serum levels at maintenance dose. Patients were recruited from the epilepsy clinic at National Taiwan University Hospital, Taiwan. The study was approved by the ethics committee of the Department of Neurology, National Taiwan University Hospital. Detailed patient demographic data have been previously described[234]. Patients self-identified, or were determined by the treating physician, as being of Chinese ancestry. All patients have provided written informed consent.

Patients were phenotyped as follows: On attendance of clinic a set of detailed examinations were performed. Past history was taken, including birth history, meningitis, brain injury, and family history. Clinical history including age of onset of seizure and the seizure pattern was also taken. Brain EEG, CT scan and MRI scans were performed to identify the seizure focus and possible brain lesions. After examination, the etiology of the patients was identified and epilepsies and epilepsy syndromes were classified according to the ILAE (1989). MRI was used in all to explore the etiology. If there was no particular focus, the patient would be classified as unidentified.

2.2 DNA sequencing

2.2.1 PCR amplification

Amplification of genomic DNA by PCR was performed using Applied Biosystems 9700 thermal cycler under the following conditions:

PCR reaction mixture:

10ng DNA, 2.5 mM $MgCl_2$, 0.2 mM of each primer, 1.6ul 10X Qiagen PCR buffer and 0.25 units of Qiagen HotStartTaq polymerase. The mixture is made up to 10µl using H_2O.

PCR cycling conditions:

Initial denaturation at 95°C for 15minutes.

35 cycles of:

Denaturation at 94°C for 45 seconds,

Primer annealing at 60°C* for 60 seconds,

Primer extension at 72°C for 60 seconds,

Final extension at 72°C for 10 minutes

*Or temperature advised by manufacturer of primers

A negative control (H_2O instead of DNA) was included in all experiments.

If amplification did not occur under these conditions then the primer annealing temperature would be altered in a "touchdown" reaction where the temperature would drop by 0.5 °C every two cycles until it reached 55 °C.

2.2.2 Agarose gel electrophoresis

Before proceeding to the PCR cleanup and sequencing stages, the outcome of the PCR reaction was determined by running products on agarose gels. One µl Orange G® loading buffer (Trevigen) was added to 2 µl of each PCR product. DNA fragments were loaded on a 1% agarose gel containing 2.5 µg/25 µl ethidium bromide. A 100bp Hyperladder® (Midwest Scientific) size marker was added alongside the samples to allow identification of DNA fragments size. The gels were cast in 1x TB* buffer and run at a constant voltage of 100V. Gels were viewed over an UV light box.

*Tris-borate buffer (TB) is made from Tris-base and boric acid. 10 X TB stock solutions is made by dissolving 54 g Tris-base and 27.5 g boric acid in 1.0L water.

2.2.3 PCR clean up for sequencing reaction

In order to remove single stranded DNA (primers) and unincorporated dinulceotides from each reacted PCR making them suitable for a sequencing reaction, the following protocol was applied.

An equal volume of MicroClean® (Microzone Ltd.) was added to each cycled PCR reaction. The sample was left at room temperature for 15 minutes then spun at 3750 rpm for 60 minutes. The supernatant was discarded. 150µl of ethanol was added to each sample and the plate spun at 3750 RPM for 10 minutes and the supernatant discarded. The samples were allowed to air dry for

30 minutes at room temperature. Once drying was complete, 5µl of water was added to each well.

2.2.4 Sequencing Reaction

Sequencing was performed using the dye terminator cycle sequencing technology as incorporated in the Applied Biosystems BigDye™ reaction. Cycle sequencing utilizes successive rounds of denaturation, annealing and extension in a thermocycler to create a linear amplification of extension products. With dye terminator labeling, each of the four dideoxy terminators is tagged with a different fluorescent dye. The growing chain is simultaneously terminated and labeled with the dideoxy terminator dye that corresponds to the terminal base. All reactions are carried out in the same tube, all four colours are assessed within the same capillary and false stops go undetected because no dye is attached. The ABI Prism Terminator Reaction Kit® contains the following reagents: didexoynucleotide terminators, each based type labeled with different fluorescent dyes, deoxynucleotides, AmpliTaq DNA polymerase FS (with thermally stable pyrophosphatase), $MgCl_2$ (2.0 mM) and Tris-HCl buffer (pH 9.0). They are premixed into a single "ready reaction" kit and concentration

The following reagents and conditions were used for all sequencing reactions:

Sequencing reaction mix:

1 µl cleaned PCR product, 1 µl Ready Reaction mix, 2 µl 5x buffer, 3.2 µM primer. The mix was made up to a volume of 10µl with distilled H_2O.

Sequencing cycling conditions:

Initial denaturation at 96°C for 10 seconds

25 cycles of:

Primer annealing at 50°C for 5 seconds

Primer extension at 60°C for 4 minutes

2.2.5 Sequencing reaction clean up

40µl of 75% ethanol was added to each sequencing reaction. The sample was mixed thoroughly and left at room temperature for 15 minutes. The plate was then centrifuged at 3750 RPM for 60 minutes and the supernatant discarded. Each pellet was then washed with 150µl of 70% ethanol. The pellets were then allowed to air dry for 30 minutes at room temperature.

10µl of hi-dye formamide was added to each cleaned sequence reaction before loading on either an ABI Prism® 3100 or 3700 automated capillary electrophoresis DNA sequencer. The output traces were analyzed using the Sequencher® software.

2.3 *Genotyping by TaqMan*

ABI TaqMan is a PCR based system on quenching one of two allele specific fluorescent dyes (TAM and VIC). A TaqMan assays contains locus specific primers used to target a typical PCR reaction to the region containing the polymorphism of interest. However, the assay also contains two allele specific probes, each composed of a short stretch (ca. 20-25 bases) of oligonucleotides labeled with a specific fluorescent dye (TAM for one allele, VIC for the other). On the 5' terminus of each probe is a reporter dye and on the 3' terminus is a quenching dye. When the probe is intact, energy transfer occurs between the two dyes and emission from the reporter is quenched by the quencher. During the extension phase of PCR, the probe is cleaved by 5' nuclease activity of Taq polymerase thereby releasing the reporter from the oligonucleotide-quencher and producing an increase in reporter emission intensity. Fluorescence is detected using a CCD camera. If only one allele is present (i.e. the individual is homozygous for the SNP in question) then only that allele specific probe fluoresces. If on the other hand the individual is heterozygous (i.e. a copy of each allele present) both probes will fluoresce. Fluorescence can be plotted in two dimensions, each axis representing a fluorescent dye (or allele). Measurement takes place directly in the well without post-PCR processing. This reduces the time of analyses, minimizes the risk of error, reduces the risk of cross-contamination and eliminates the labor and supply costs of post-PCR steps.

What follows is a description of the standard TaqMan sequencing protocol used for the work described in this thesis.

Manufacturers recommended TaqMan reaction mix:

1 µl (10ng) DNA, 0.125 µl TaqMan assay, 2.5 µl 2X TaqMan Universal Master Mix. The mix was made up to a volume of 5µl with distilled H_2O.

Manufacturers recommended cycling conditions:

Initial denaturation at 95°C for 10 seconds
60 cycles of:
Denaturation at 92°C for 15 seconds
Primer extension at 60°C for 1 minute

The assays were run on an ABI Prism® 7900HT machine and scored with SDS sequence Detection System) software.

2.4 Genotyping of microsatellite markers

Microsatellite markers and structure inference was carried out as part of BEST study described in chapter 7.

We genotyped the following X-linked microsatellites: DXS984, 996, 1036, 1053, 1062, 1203, 1204, 1205, 1206, 1211, 1212, 1220, 1223, 7103, 8014, 8061, 8068, 8073, 8085, 8086, 8087 and 8099 and the following chromosome 1 microsatellites: D1S196, 206, 213, 249, 255, 450, 484, 2667, 2726, 2785, 2797, 2800, 2836, 2842, 2878 and 2890. The chromosome 1 markers form part of the ABI Prism linkage mapping panel 1.

A 10× primer master mix was made in advance; the PCR master mix was then made up of:

Primer master mix (1.55.0 pmol for each primer)
10 × Super Taq PCR buffer 1 (HT Biotechnologies)
0.2 mM of each dNTP (Advanced Biotechnologies),
Super Taq (HT Biotechnologies)
TaqStart (Clontech) (premixed in a 2:1 ratio, neat Taq: neat TaqStart)
dH2O to a total of 10 l per reaction.

Meanwhile, 1020 ng of DNA were pipetted into a 96-well microtiter plate. After being vortexed, the master mix was aliquoted into microtiter platestrip lids. After a centrifugation step, the samples were cycled in a Perkin-Elmer 9700 PCR machine (PE Biosystems). Thermal profiles were 38 cycles of 30 s at each of 95°C, 55°C, and 72°C, with a 4-min 95°C initial denaturation step and a 10-min

72°C final extension. In some cases, a touchdown procedure was used, decreasing the annealing temperature by 0.5°C/cycle for the first eight cycles.

PCR products were diluted and pooled, prior to being loaded onto a 96-lane ABI 377 sequencer (PE Biosystems). Size calling was performed using GENESCAN software. A sample from a control individual was run at least twice on each gel to standardize for gel-to-gel shifts in migration.

These panels of microsatellites were chosen as they have previously been evaluated and found effective in the assessment of population genetic structure[235]. Different methods of evaluating population genetic structure are discussed in section 6.4.

2.5 Screening for variation across SV2A

The following primers pairs were reacted using standard protocol (described in section 2.2.1) to produce template for sequencing. To increase the sensitivity of SNP discovery, forward and reverse primers were reacted using the standard sequencing protocol (described in section 2.2.4).

All primers below are in 5'-3' direction

SV2A-1F TGTTACCTGTGTGGTCTCTTCG

SV2A-1R TTCTCCACTGCTCTCAAACTCC

SV2A-2F CGACCCTTCCTGAGTTATCC

SV2A-2R CACTTCACTGGGTCTTCTCC

SV2A-3F CTGTTTGACTGCCTGCCTTC

SV2A-3R TAAGGGAAATGATGGGCAAG

SV2A-4F GTCATTCAGGTGAGGAGATGG

SV2A-4R GACAATGGAAACAGACAACAGG

SV2A-5F CTCCACCTACATCCAATTCTCC

SV2A-5R CTGGAGGAACAGAGATCAATCC

SV2A-6F ATGAGGTTCCCTCAAACTCCTATC

SV2A-6R TCTTTCAGAACAGCTCATCCAG

SV2A-7F ACTTCTTCCCTGTTCCTTCTACCT

SV2A-7R CTGTCCGTGCTAGCTGACTATAAG

SV2A-8F TGAGCGAGTGTTCTCAGTAAGC

SV2A-8R ATAAACTCCAGGAGGACCTTGG

SV2A-9F TATGACTGCCTGCTTATCTTGG

SV2A-9R TTCCAACTACCTCTTGTCTGAGG

SV2A-10F ACTTGGAATAGGAGCACTCTGG

SV2A-10R GGATAGGAGTTTGAGGGAACC

SV2A-11F TATTCTACATCCGCCTCCTACG

SV2A-11R TTCCAGAGTCACAAAGACATGG

SV2A-12F GTCCTGTGTCTCCTGCTTCTTC

SV2A-12R CAGCTTACACAGGGCATTCAG

SV2A-13F CACGCTTCATCTAAGTAACTGTGG

SV2A-13R CCTCTGTCACATTCAACTTCACC

SV2A-14F GACATGTGCCTGTCCGACTC

SV2A-14R TCCACTCTCTTCGGTCTGTCTC

SV2A-15F ATCTTCCTGGACTTCACTTCC

SV2A-15R ACATACACATTCACACACAGTCC

SV2A-16F CCACAGGTATGCAATTATGCTAGG

SV2A-16R CAACACGTCACAGCTACAGTGG

SV2A-17F GAGAGGCTCCACCCACTACA

SV2A-17R GATCAAGGTAGAGAGGCCAATTT

SV2A-18F TTCCTAACCTTGGTAGGATGTGG

SV2A-18R AGAGGTGATGACTGTCCACTGC

SV2A-19F ACCCTAAAGAGCATCCAAGATAGC

SV2A-19R AGGAAAGAGAGGAGCTTTGACC

SV2A-20F TTACTCTGCTTCAACTCCAAGCTC

SV2A-20R GTGGGAATGTCTCTTGAGCTGTAA

SV2A-21F GAGATTTCCTGGGAAGATGAAATG

SV2A-21R AAGAGACTCCATCTCACACATAAAGC

SV2A-22F CATCCTGGCTAACACAGTGAAAC

SV2A-22R AGTGAGAGGGAATATGGTGATTTG

SV2A-23F TGCATATAACCTAGGCACATCCTC

SV2A-23R ATCTCAGCTCACTGTAACCTCCAC

SV2A-24F ATCATTGCATGAATCCACTCCT

SV2A-24R GAGCAGGGCAGACACGATATT

SV2A-25F CTTCTGTGTTCAAACACCAACTG

SV2A-25R ATCACCCACACACATAGACTTCAG

SV2A-26F AAAGTATAGTGGCTGCTGTGC

SV2A-26R TCAAAGGACACTGACTTGAGC

SV2A-27F CTGAGGTCAGGAGTTTGAGACC

SV2A-27R CACAGCTAAGACACCAAACACG

2.6 Selection and assessment of tagging SNPs

2.6.1 Introduction to tagging

In association studies it is generally neither possible nor desirable to exhaustively test every SNP. As many SNPs will carry redundant information because they are in strong linkage disequilibrium (LD) with other SNPs, it is possible to use haplotypes that contain a subset of non-redundant loci in association studies. There are several methods of measuring LD. One of the most useful is r^2, which is a measure of the association between pairs of alleles. This is most relevant to association studies as it is related to power. If the sample size required for a specific level of statistical power is N in typing the causal variant directly, then the sample size required for the same level of power is N/ r^2, where r^2 is the LD between the causal variant and the typed associated marker[236].

The term "tagging" was introduced by Johnson and colleagues who suggested the method to capture the variance in commonly observed haplotypes across a gene or region[237]. However, tagging common haplotypes is only one of many possible ways to select a subset of SNPs that retain as much information as possible about the other SNPs. Broadly speaking the approaches that have been proposed so far can be divided into 2 groups[238], those based on maximizing the haplotype diversity present in the tagging set compared to the tagged set (*diversity based*) and those based on establishing as high an

association as possible between the "tagging" and "tagged" set (*association based*).

2.6.2 Selecting tagging SNPs

Haplotype r^2 is defined as the proportion of variance in a "tagged" SNP of interest that is explained by an Analysis of Variance based on the *G* haplotypes formed by the set of tSNPs. :

$$Yi = x_{i1}b_1 + x_{i2}b_2 + \ldots. + x_{iG}b_G$$

Where *Yi* is the predicted state of the tagged SNP of interest on the *i*th chromosome, $x_{i1}\ldots x_{iG}$ are indicator variables for the *G* haplotypes and $b_1\ldots b_G$ are coefficients estimated by standard least squares from the observed data.

Allelic r^2 only differs in that the tSNP alleles, as opposed to the haplotypes defined by the tSNPs as the indicator variables in the above regression equation. Although less efficient than haplotype r^2, allelic r^2 is attractive in that haplotype inference, a complicating factor when dealing with large genomic regions, is not required.

All tags discussed in this thesis were selected using either the haplotype r^2 method or the allelic r^2 method, depending on the size of the genomic region

being tagged. Relying on pairwise measures of association (i.e. the r^2 value between two SNPs) is less efficient as pairs of SNPs will only have high pairwise association when their minor allele frequencies are very closely matched, thus meaning that SNPs that exhibit a full range of frequencies will need to be selected as tags.

2.6.3 The HapMap project

The HapMap project website (www.hapmap.org) allows the user to select any region of the genome of interest in a manner similar to previously established genome browsers (UCSC, NCBI etc). Genotypic data is generated from 30 trios of a specific ethnic origin and can be downloaded either as a large batch (i.e. all HapMap data or a chromosome), or for any defined region of the genome. HapMap project data was used for tagging SNP selection in chapter 5.

2.6.4 TagIT

All tagging SNPs described in this thesis were selected using the TagIT package (available from http://www.genome.duke.edu/centers/pg2/tagit), implemented in the Matlab programming environment. TagIT allows tag selection using a wide range of variations of either diversity or association based methods (summarized in TagIT user guide). TagIT also offers functions allowing the calculation, and

display, of various measures of LD that aid in judging the suitability of the technique for a give gene or region.

Using the haplotype r^2 method focuses on the coefficient of determination (measured as haplotype r^2 or Hr^2) from a regression model involving the tagging and tagged SNPs. This provides a formal measure as to how well each individual SNP is being captured by the set of tSNPs. Increasing the number of tags improves performance. However, whilst improvement in tagging performance asymptotes, the cost of genotyping an increasing number of tags does not. A compromise is thus required and a consensus seems to be emerging that a Hr^2 value above 0.8 is acceptable[238, 239]. This implies that increasing the sample size to *n*/0.8, would be comparable to exhaustive typing of the hypothesised causal variant captured by a tag with a Hr^2 of 0.8.

2.6.5 Evaluating the ability of tagging SNPs to detect unseen variation

The tagging method, when applied to incomplete data (such as that generated by the HapMap project), raises the question of how well unknown variation is being captured. In order to address this point the SNP dropping procedure was developed[240]. The approach entails taking the global set of known SNPs and for each SNP *'i'*, dropping it from the analysis in turn. For each reduced set of *N-1* SNPs new tags are selected, and their ability to represent the dropped SNP *'i'* is assessed (by Hr^2). In this way, a table of Hr^2 values for dropped SNPs is built,

providing a statistical estimate of how well the tSNPs can represent SNPs that are not observed (for example SNPs which are not yet discovered) in the region.

The trade off between tagging performance and genotyping cost necessitates the definition of a threshold for SNP dropping performance. At present no consensus exists as to what this value should be. Indeed it is difficult to define as any threshold would depend on the context of the region. However, a value somewhere between 0.5-0.7 as the lower threshold would seem acceptable.

When dealing with incomplete data such as that provided by HapMap, dips in SNP dropping performance can indicate regions of insufficient SNP coverage. If however, the region indicated by the dip in SNP dropping performance does not correspond to a drop in LD, it then becomes very difficult to nominate a region in which to focus resequencing efforts as genealogically informative loci could be located anywhere across the LD block.

2.7 Statistical methods used in association analysis

2.7.1 Allelic and genotypic association

For single SNP analysis we assessed the significance of genotypic and allelic contingency tables using Pearson's chi^2 distribution. For tables with insufficient

cell counts (i.e. <5) we used an exact probability test as implemented in the program, RxC (available at http://bioweb.usu.edu/mpmbio/rxc.asp).

2.7.2 Haplotypic association

We assessed the distribution of haplotypes across case and control groups by calculating score statistics using the package haplo.score [18] in R [19]. Briefly, this method uses generalized linear models to generate score statistics which can be used to examine the correlation between expectation-maximization generated haplotypes and the trait of interest. We coded cases and controls as a binomial trait. When generating score statistics, we only considered haplotypes observed at a frequency of 1% or greater as spurious inference increases with lower frequency haplotypes.

Chapter 3 Genetic predictors of clinical use of the anti-epileptic drugs phenytoin and carbamazepine

3.1 Introduction

3.1.1 Clinical use of phenytoin and carbamazepine in epilepsy

Phenytoin and carbamazepine are important first-line anti-epileptic drugs (AEDs) widely prescribed throughout the world. Control of epilepsy with phenytoin can be a difficult and lengthy process due to the drug's narrow therapeutic index and the wide inter-individual range of doses required. Similarly, appropriate doses for carbamazepine take time to determine because of autoinduction of metabolism and neurologic side effects generally assumed to necessitate slow dose increases.

Starting doses of phenytoin and carbamazepine and the rate of dose increase vary by clinic. Adverse drug reactions (ADRs) may lead to a reduction in dose, whereas insufficient seizure control may require dose to be increased. Therefore, a broad range of doses of phenytoin and carbamazepine are used, with the final maintenance dose normally determined by trial and error. Empirical calibration of

dose may entail significant clinical harm in terms of delayed efficacy or adverse drug reactions (ADRs). ADRs are relatively common for both drugs but phenytoin in particular. Dose-related side effects include cognitive impairments, ataxia, poor concentration, diplopia and lethargy[241]. Central nervous system-related ADRs can be partly attributed to a direct effect of the drugs on their targets whereas certain other adverse drug reactions, for example cosmetic side effects related to phenytoin treatment, are not thought to be dose-related[241].

3.1.2 Candidate gene selection

Phenytoin is metabolised by the hepatic cytochrome P450 enzymes CYP2C9 and CYP2C19, is transported by P-glycoprotein and targets the alpha subunit of the sodium channel. CYP2C9 is estimated to be responsible for up to 90% of phenytoin inactivation[242].

Substantial *in vitro* data demonstrate that both the *2 and *3 alleles[243] result in significant reductions in the metabolism of various CYP2C9 substrates, with *3 showing consistently greater reductions in intrinsic clearance than *2. There have been numerous reports on phenytoin pharmacokinetics (Table 3.1), but no large studies of response. Prior to this study, the largest dosing study involved 60 phenytoin-treated patients and found that carriers of either the *CYP2C9**2 or *3 allele required on average a 30% lower maintenance dose than individuals with

two functional (*1) alleles[244]. The *CYP2C9*2 and *3 alleles have frequencies of 0.11 and 0.08 in individuals of European ancestry[245].

Phenytoin acts by blocking voltage-sensitive sodium channels in neurons, and binds to the alpha subunit encoded by the brain-expressed genes *SCN1A, 2A, 3A,* and *8A*. Here we have focused on variation in *SCN1A*, a gene implicated in many Mendelian forms of epilepsy[246].

Finally, passage of phenytoin across the blood-brain barrier is probably affected by P-glycoprotein. The *ABCB1* gene carries a silent polymorphism in exon 26 (3435C>T, or rs1045642) that has been associated with altered expression levels of P-glycoprotein[127] and also with a range of drug responses and clinical conditions[247]. In particular, this polymorphism has been weakly correlated with both response to AEDs[32] and phenytoin plasma levels[248]. Although there is evidence that 3435C>T may not be causal, it is likely to be a marker for one or more causal variants[247].

Carbamazepine is metabolised by the hepatic cytochrome P450 enzyme CYP3A4. Carbamazepine also induces CYP3A4 via activation of the pregnane X receptor (*PXR*, or *NR1I2*). This may contribute to the requirement of dose being increased over time after the drug is initiated. There is great interindividual variability in CYP3A4 expression and activity has been shown to vary up to at least 20-fold in vivo[249]. Overall, it is not thought likely that variation in the

CYP3A4 gene itself (either coding or regulatory) is a primary contributor to inter-individual variability in CYP3A4 enzyme activity[250-254]. We have therefore not included *CYP3A4* in this study. Since this study, it has been reported that the *CYP3A5**3 allele influences carbamazepine dose and pharmacokinetics in Japanese patients with epilepsy[255] however, given the low frequency of the *1*1 homozygous genotype in individuals of European ancestry we did not include it in our study.

Carbamazepine also acts by binding to the alpha subunit of voltage-sensitive sodium channels in neurons, blocking high frequency discharges, and is a possible substrate of P-glycoprotein[256]. We have therefore associated variation in both *SCN1A* and *ABCB1* with dosing of carbamazepine.

Table 3.1 Functional effects of *CYP2C92 and *3 polymorphisms**

CYP2C9 allele	*In vitro* expression	Pharmacokinetic	Mechanism
*2	29% reduction in phenytoin clearance compared to *1[257]	*2 carriers have increased serum concentrations of phenytoin, following a single dose in healthy volunteers[258]	*3 mutation is located in substrate recognition site (SRS) 5, accounting for reductions in binding capacity and intrinsic clearance[259].
*3	93 to 95% reduction in phenytoin clearance compared to *1[257, 260].	*3 carriers have significantly lower maximal elimination rates than *1/1 patients[261, 262] and increased serum concentrations of phenytoin, following a single dose in healthy volunteers[258]	*2 allele is not located in an SRS. Mechanism responsible for reduction of metabolism rate is unclear[263].

3.1.3 Aims

The broad range of doses used and the clear sets of candidate genes make phenytoin and carbamazepine excellent candidates for pharmacogenetic diagnostics. If gene variants could be identified that predict appropriate doses, or that identify individuals more likely to suffer ADRs, these important AEDs could be used more effectively and safely. In this study we have considered the known

functional alleles *2 and *3 at the *CYP2C9* gene, and the putatively functional 3435C>T polymorphism in the *ABCB1* gene. As no common functional variants are known for *SCN1A*, we used a haplotype tagging strategy[238]. We have related variation in all three genes to the maximum dose of phenytoin in 281 patients treated with phenytoin. For carbamazepine, we related variation in both *SCN1A* and *ABCB1* to maximum dose in 425 patients. Finally, we tested for association with presence or absence of ADRs. In most, but not all cases the maximum dose used here will also be the maintenance dose, since starting doses tend to be lower than what is required (see discussion).

3.2 Methods

3.2.1 Subjects

The patient cohort is described in section 2.1.1. We identified 448 patients who were treated with phenytoin, of whom 119 were continuing treatment at the time of recruitment and the remainder had stopped treatment. DNA and dose information was available for 281 patients (Figure 3.1).

When an ADR occurred, the type of ADR, its severity (mild, modest or severe) and the degree of certainty with which the adverse reaction can be attributed to phenytoin was recorded. One or more ADRs were recorded in 20.6% of patients.

The most common ADRs were: gum hypertrophy (40%); unsteadiness, dizziness or ataxia (19%); sleepiness (15%); other, largely cosmetic, adverse drug reactions (9%) and rash (6%). No other single adverse drug reaction contributed to more than 5% of the total.

We identified 533 patients who were treated with carbamazepine; DNA and dose information was available for 425 (Figure 3.2). One or more ADRs were recorded in 24% of patients. The most common ADRs were: sleepiness and fatigue (20%); diplopia and other visual disturbances (17%); ataxia, unsteadiness or dizziness (15%); rash (11%); and gastrointestinal symptoms (8%). No other single adverse drug reaction contributed to more than 5% of the total. Of the 425 carbamazepine patients with DNA and dose information, 240 were not included in the phenytoin analyses. This overlap means that the two full cohorts cannot be considered independent.

The following clinical details were recorded whenever available: date when phenytoin / carbamazepine was started and stopped, maximum dosage reached, response and occurrence of ADRs. Although more details are available in the relevant clinical notes, it has not been possible in all cases to review these notes.

Figure 3.1 Distribution of Maximum Phenytoin Doses

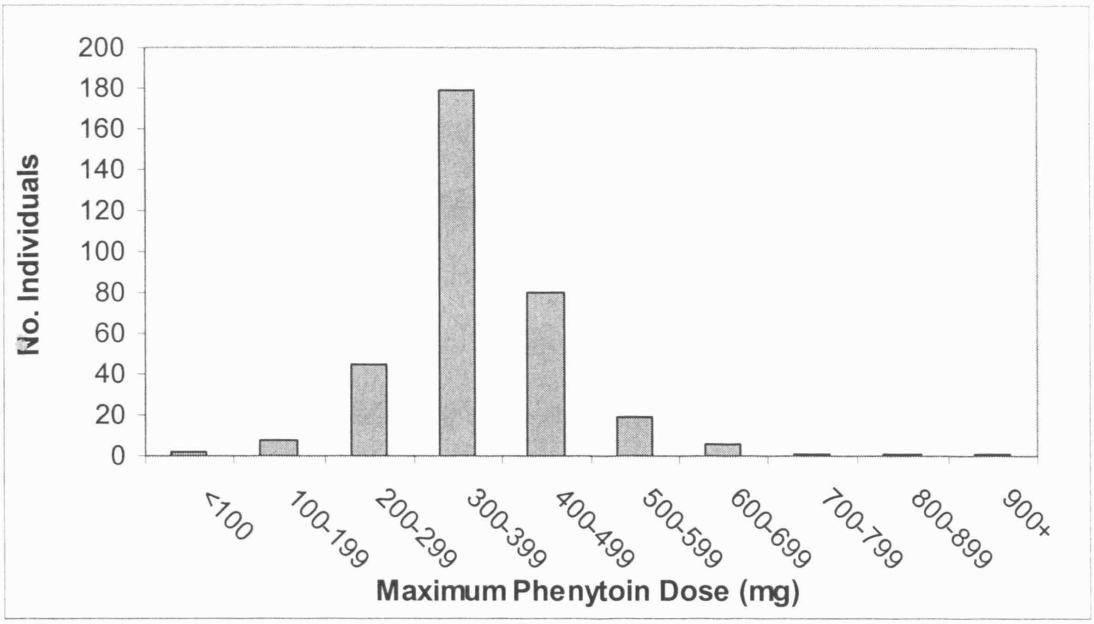
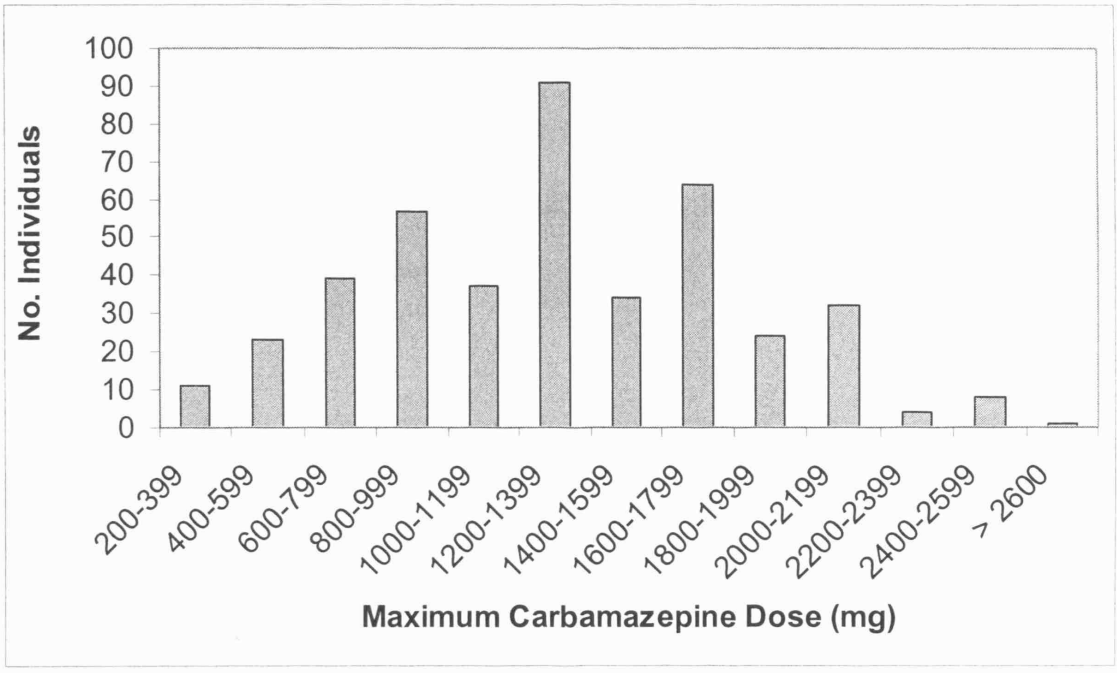


Figure 3.2 Distribution of Maximum Carbamazepine Doses



3.2.2 Brain Tissue

Thirty-five pairs of surgically resected peri-lesional temporal neocortex and hippocampus brain tissue were selected at random from the archives of frozen tissue at the National Hospital for Neurology and Neurosurgery. In each case therapeutic surgery had been undertaken to relieve chronic drug resistant epilepsy. All tissue had been flash-frozen in liquid nitrogen within 30 minutes of resection, and stored at -80C until use. Routine detailed histological examination of the fixed (unaffected) temporal lobe and hippocampus from each case had shown hippocampal sclerosis and the absence of epileptogenic pathology in the temporal lobe. In each case, written informed consent had been obtained from the patient for the use of resection material for research approved by the institutional ethics committee; all samples were irreversibly anonymised prior to analyses. Twenty-three brain samples from patients with Parkinson's Disease (PD) were obtained from the Brain Bank at the Institute of Neurology. Samples were anonymised, and ethical permission for this study was obtained from the joint research ethics committee of the National Hospital for Neurology and Neurosurgery and Institute of Neurology.

3.2.3 Genotyping

*CYP2C9*2* and *CYP2C9*3* genotyping was performed using pre-developed Taqman assay reagents for allelic discrimination (Applied Biosystems, see section 2.3)

The four *SCN1A* tSNPs (rs590478, rs8191987, rs3812718 and rs2126152) had been previously genotyped using Taqman assays (Depondt and colleagues, in preparation). These tSNPs correspond to SNP1, SNP5, SNP7 and SNP8 in [238] and have frequencies of 0.24, 0.13, 0.45 and 0.32 in 384 unrelated control individuals from the British twin registry[264]. The authors found the proportion of haplotype diversity explained by these tSNPs to be 94% (criterion 2 in TagIT, http://www.genome.duke.edu/centers/pg2/tagit), and the average haplotype r^2 (criterion 5 in TagIT) to be 0.8 (using data from[238]). These values also mean that common SNPs (minor allele frequency > 8%) in the gene are generally predicted well by the subset of tSNPs, and that little loss of power is therefore expected in typing the tSNPs instead of typing directly a causal SNP, assuming its minor allele frequency is sufficiently high.

ABCB1 3435C>T genotypes had been previously determined using direct sequencing[247].

3.2.4 Nucleic acid purification from brain samples and RT-PCR

Genomic DNA was extracted from approximately 25 mg of brain tissue using a Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's conditions. Total RNA was isolated from approximately 30 mg of the same brain tissue using the Lipid Tissue Purification Kit (Qiagen), according to manufacturer's conditions. RNA quality was checked by denaturing agarose gel electrophoresis and ethidium bromide staining according to manufacurer's suggestion. The RNA was quantified spectrophotometrically at 260 nm, and 1 μg RNA of each sample was reverse-transcribed to cDNA using the High Capacity cDNA Synthesis Kit (Applied Biosystems), in standard conditions. RT-PCR was carried out on a volume of cDNA corresponding to 10 ng starting RNA.

RT-PCR was used to determine that exon 5N is present in human mRNAs using commercially obtained human foetal total brain mRNA (Stratagene). Adult human cDNA was obtained from brain tissues and amplified with primers flanking exon 5 designed to amplify exons 5A and 5N equally (both 5'-3' direction):

F - CCACCTCTGCCCTGTACATT

R - CTCCCACAATGGTTTTCAGG

The resulting fragment was digested with AvaII, which cuts only copies containing exon 5N, for >2 hours and separated on 3% agarose gels in the presence of ethidium bromide. The relative intensity of the 5N product and 5A

product was measured for each sample using the Syngene package. The ratio of 5A to 5N was corrected for the molecular weight of the two products.

3.2.5 Statistical Analyses

All regression analyses were implemented in the usual way using STATISTICA (StatSoft Inc, Tulsa, USA) with phenytoin / carbamazepine dose as the dependent variable and genotype score(s) as the independent predictor(s).

Multi-locus association analysis was carried out using the score test[265] (described in section 2.9.2)

Standard χ^2 analyses were used to compare genotype frequencies between individuals with and without ADRs.

A Student's *t*-Test was used to compare ratios of SCN1A with 5A to SCN1A with 5N by SCN1A IVS5-91 G>A genotype. Data was normally distributed.

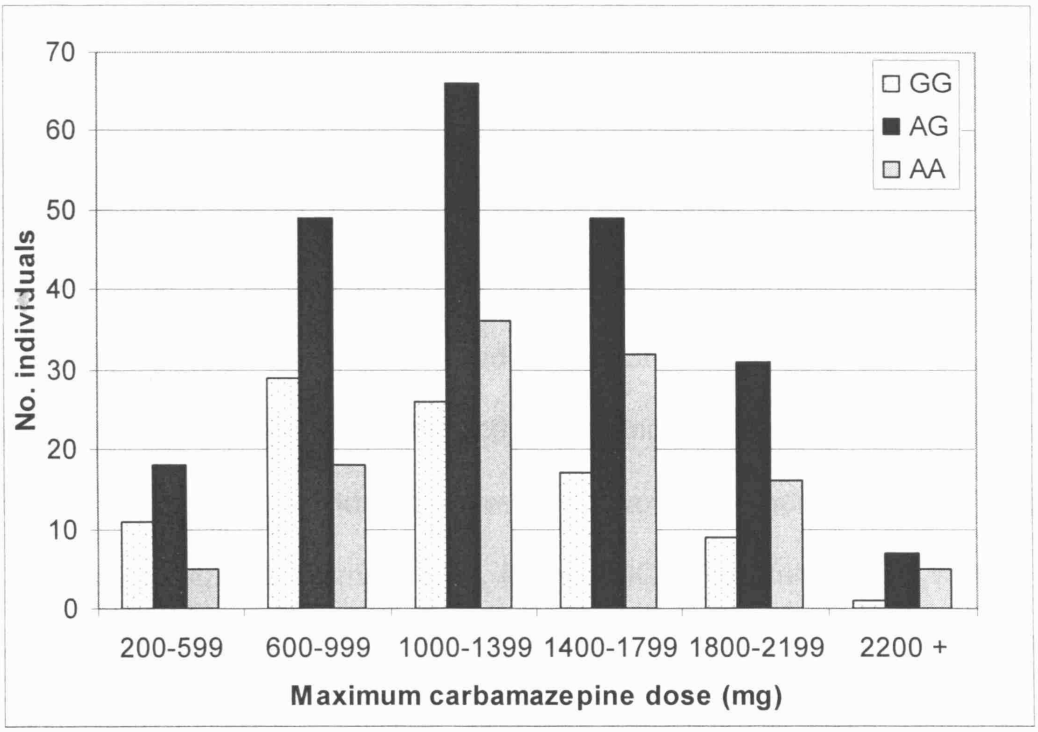
3.3 Results

3.3.1 Genetic association analyses

There were no significant violations of Hardy Weinberg equilibrium after Bonferroni corrections for multiple comparisons.

For carbamazepine we find that one of the *SCN1A* tagging SNPs (SNP7, IVS5-91 G>A, or rs3812718) is highly associated with maximum dose. A regression model allowing arbitrary effects for each genotype is significant at the level P=0.0051 (uncorrected). The genotypic effects however are consistent with additive effects, and under a regression model restricted to additive effects the significance is P=0.0014 (uncorrected). These results remain significant after Bonferroni correction for five tests (four in the *SCN1A* gene, and one in the *ABCB1* gene). Maximum doses averaged 1313mg, 1225mg and 1083mg for AA, AG and GG individuals (with genotype counts of 112, 220 and 93 individuals respectively, Figure 3.3). A weighted linear haplotype regression, using all the tSNPs, does not increase significance.

Figure 3.3 Distribution of maximum carbamazepine doses (mg) for each *SCN1A* IVS5-91 G>A genotype



For phenytoin we find that the *CYP2C9**3 allele shows significant association with maximum dose, with P= 0.0066 (uncorrected). As there was only one individual homozygous for the *3 allele in our cohort we excluded this genotype from our regression model (though this single observation follows the same trend of reduction in maximum dose). This value remains significant after Bonferroni correction for 7 independent genotyping tests (four tests in the *SCN1A* gene, two in *CYP2C9*, and one in *ABCB1*). Mean phenytoin doses for individuals with 0, 1 or 2 copies of the *3 allele were 354mg, 309mg and 250mg, respectively (but notice numbers for these three genotypes are 229, 39, 1 respectively).

*CYP2C9*2* did not show a significant association with dose. A multiple regression analysis on combined *2 and *3 genotype did not support a significant role for *2.

The *SCN1A* IVS5-91 G>A polymorphism is also associated with the dosing of phenytoin with P=0.014 (uncorrected) under an unrestricted regression model and P=0.0045 (uncorrected) under an additive model. The latter model would appear to be indicated from the apparent additive effect of the *SCN1A* genotype on carbamazepine dosing explained above. Under the additive model significance is retained after correcting for 7 independent tests. The unrestricted model shows only a trend after correction. Maximum phenytoin doses averaged 373mg, 340mg and 326mg for AA, AG and GG individuals, respectively (genotype counts of 73, 109 and 60, respectively). A weighted linear haplotype regression, using all the tSNPs, does not increase significance. When the combined *CYP2C9*3* and *SCN1A* IVS5-91 G>A are considered, the doses range from a mean of 250mg for the single *3*3/GG individual to 297mg for (18) *1*3/AG individuals and 377mg for (the 62)*1*1/AA individuals, P= 0.014, uncorrected under unrestricted model. (The single *3*3/GG individual has again been excluded however it followed the same trend).

Of the patients included in the carbamazepine analysis, 185 also had been included in the phenytoin analysis. When these patients are not included, the result remains significant under an additive model with P=0.0063 (uncorrected). An unrestricted model gives P=0.020 (uncorrected). The phenytoin and

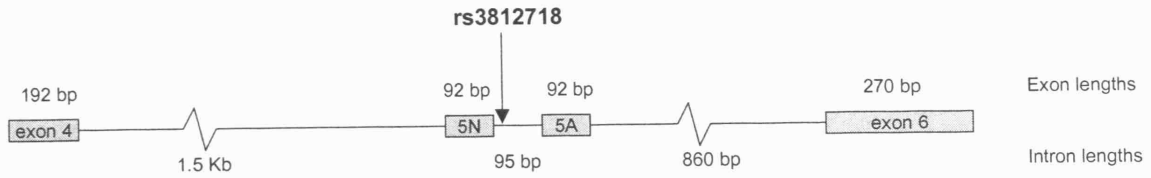
carbamazepine results therefore provide a functional replication of the effect of the *SCN1A* variant.

The *ABCB1* 3435C>T polymorphism shows no association with dosing for either phenytoin or carbamazepine.

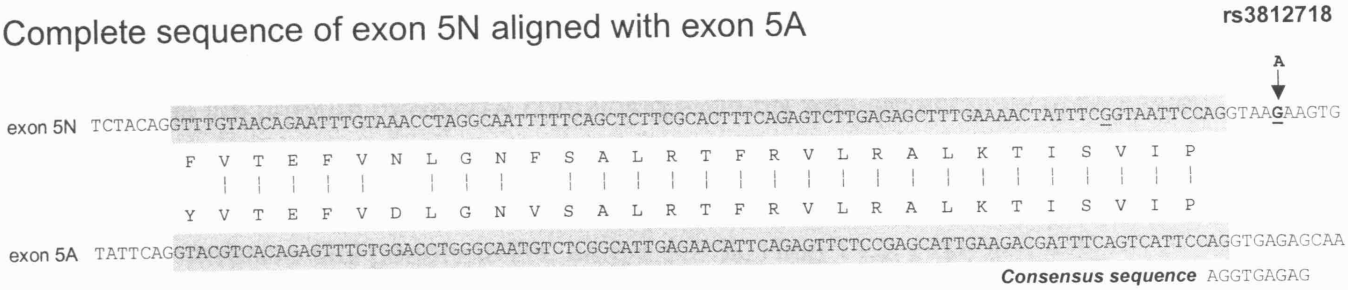
3.3.2 IVS5-91 G>A polymorphism in *SCN1A* affects alternative splicing

We also find for the first time that the IVS5-91 G>A polymorphism in *SCN1A* affects the alternative splicing of exon 5 (Figure 3.4). This polymorphism is located in the 5' splice donor site of a highly conserved alternatively spliced exon apparently expressed mainly in foetuses (5N)[266]. The major allele (A) disrupts the consensus sequence of the foetal exon (5N) possibly reducing the expression of this exon relative to the adult exon (5A). A similar splicing event occurs in the German cockroach sodium channel gene, *para*^{CSMA}, but in a different domain (domain III). Substituting aspartic acid into the S3-S4 linker is associated with altered voltage-gating and sensitivity to the insecticide deltamethrin (although there are other substitutions between exons)[267].

Figure 3.4 Genomic Structure of *SCN1A* surrounding exons 5N and 5A and regulation of exon 5N in epileptic tissues



Complete sequence of exon 5N aligned with exon 5A

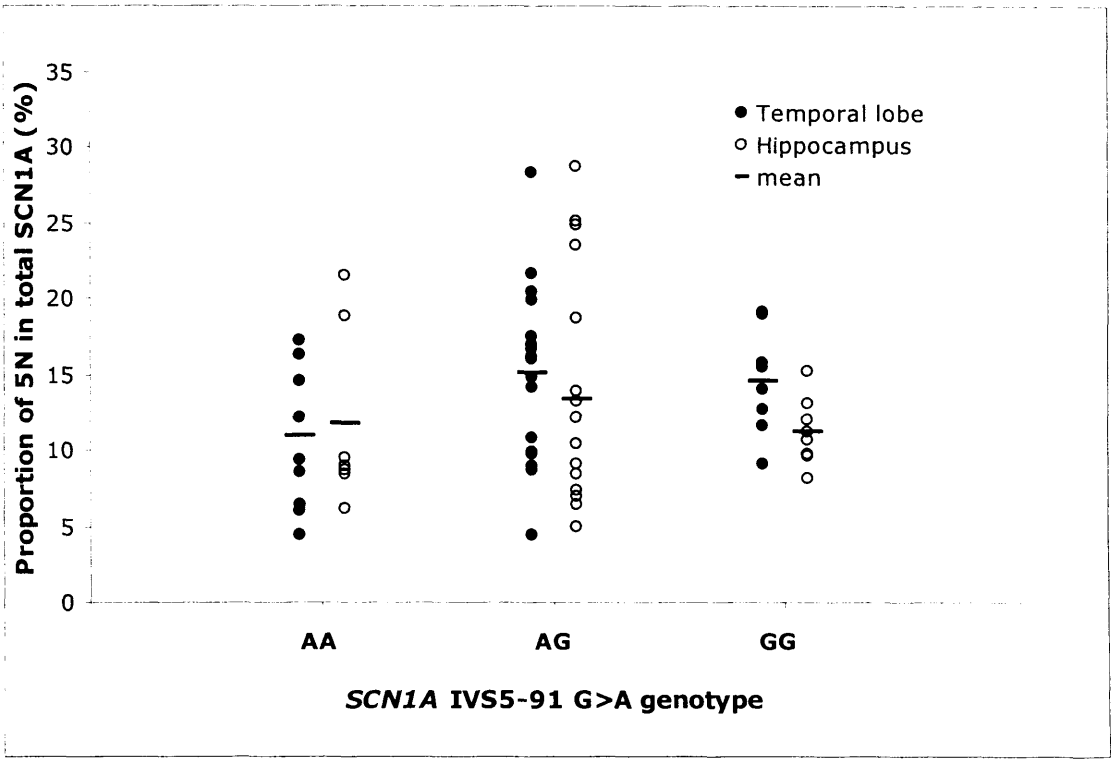


We first studied human foetal whole brain mRNA and confirmed that exon 5N is present (data not shown). Next we amplified the region including exon 5, in adult human cDNA samples derived from brain tissue, with primers flanking that exon. The product of this PCR was digested with the restriction enzyme Ava II, which cuts only in copies containing exon 5N. In the mRNA purified from foetal brain (the genotype is unavailable from the commercial mRNA) more than 60% of the amplified *SCN1A* mRNA contained exon 5N. We also looked in adult brains derived from a Parkinson's disease brain bank. In these adult brains without a history of epilepsy the levels of *SCN1A* with exon 5N were much lower with 9.5 ±

0.7% (n = 23) over all genotypes with individuals with the AA genotype having slightly, but not significantly less 5N (8.6 ± 0.75%, n = 5) than individuals of either AG (9.94 ±1.09% n = 14) or GG (9.2 ± 0.96 % n = 4).

There is evidence that seizures can upregulate the inclusion of exon 5N in neuronal sodium channels in rodents[268]. We therefore also assessed the percentage of *SCN1A* mRNA containing exons 5A and 5N in brain resection tissue derived from patients undergoing surgery for refractory epilepsy. In these tissues the amount of *SCN1A* containing exon 5N was significantly upregulated in the temporal lobe (TL) relative to the hippocampus (Hipp) in individuals with the permissive GG genotype (TL 14.6 ± 1.2 %, Hipp 11.2 ± 0.8% n = 8), P=0.023, but not in individuals with the AA (TL 11.0 ± 1.5% Hipp 11.7 ± 2.2 % n = 10) or AG (TL 15.1 ± 1.3 % Hipp 13.4 ± 1.9 n = 8) genotypes (Figure 3.5).

Figure 3.5 Proportion of *SCN1A* 5N transcript in brain tissue from patients with a history of epilepsy



Taken together, these results show that seizures influence the proportions of alternative transcripts of the *SCN1A* gene. We also show that the influence of seizures is dependent on genotype with the GG permissive genotype resulting in a significant increase of the 5N form in the temporal lobe relative to the hippocampus. These results do not make clear how the IVS5-91 G>A splice site polymorphism influences sensitivity to carbamazepine and phenytoin. Future work focused on precise expression patterns in subregions of the hippocampus and functional assays of drug sensitivity of the channels encoded by 5A and 5N

may eventually help to clarify this. Finally, it is also possible that the presence/absence of 5N during development leads to changes in other sodium channels, which change adult sensitivity to sodium channel blockade. This work however demonstrates a novel phenomenon in humans of how seizures influence neuronal function as neither rats nor mice possess a functional copy of exon 5N in SCN1A (Schorge, S., unpublished observation).

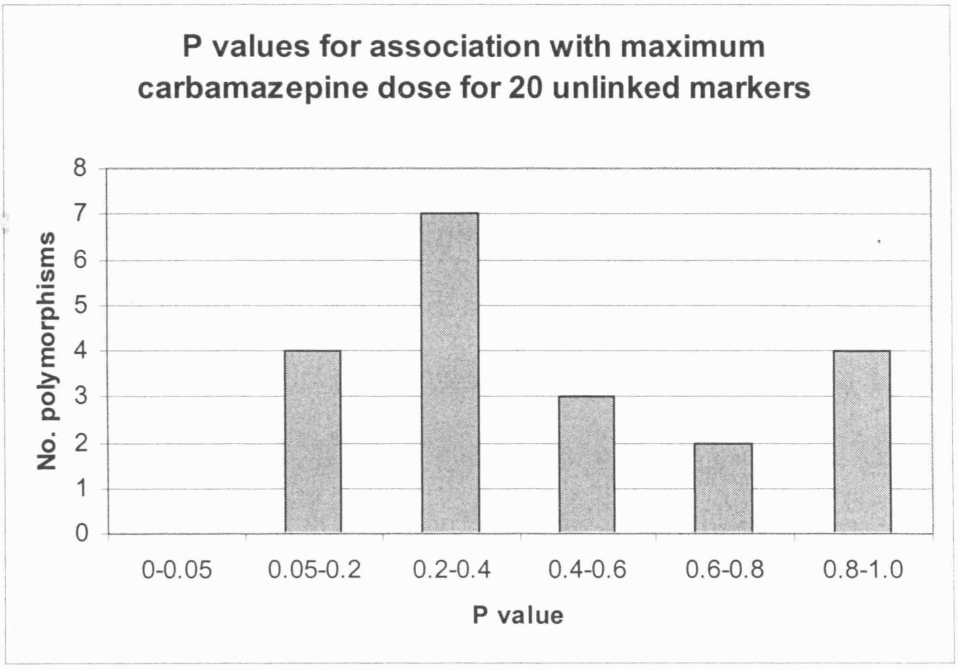
Given the demonstrated association of the IVS5-91 G>A polymorphism and 5N and 5A levels, and its presence in a splice donor consensus site, it is very likely that this polymorphism is itself the causal polymorphism for altered sensitivity to phenytoin and carbamazepine. Because of the extensive LD throughout the gene it is formally possible that the causal variant lies elsewhere[238]. We have however, undertaken exhaustive screening of the *SCN1A* exons and intron-exon boundaries and have not found any other common variants that are predicted to have functional effects (Depondt and colleagues, in preparation).

No associations were found between any genotype and presence of an ADR or presence of the subset of ADRs that are central nervous system-related.

3.3.3 Stratification does not explain the association

Although all patients in this study self-identified, or were determined by the treating physician, as being of North Western European origin, cryptic stratification can still drive spurious associations between polymorphisms and phenotypes, including drug responses[269]. One approach for checking whether stratification is present for a given phenotype, termed genome control, is to assess the association of that phenotype with markers from elsewhere in the genome that are not linked to the associated polymorphism under study[211]. As part of other projects in the laboratory not related to this work on dosing requirements for carbamazepine and phenytoin we have typed a series of unlinked polymorphisms. We have assessed the degree of association of each of these polymorphisms with carbamazepine dosing. We find none of these polymorphisms is significantly associated with maximum dose (Fig 3.6). We may therefore set a threshold on the probability that the significant association for the *SCN1A* polymorphism is influenced only by stratification. Under this null hypothesis the distribution is as described by our set of genome control markers, and the *SCN1A* polymorphism is a significant outlier in this distribution with $p<0.05$. As noted previously this formulation of genomic control is conservative[211].

Figure 3.6 Association of 20 unlinked markers with maximum carbamazepine dose



We also note that some of the polymorphisms considered were chosen for relevance to epilepsy and so could possibly influence dose requirements, but in the context of using them for genome control this is a conservative effect. This analysis therefore effectively rules out stratification as an explanation of our results.

3.4 Discussion

We have identified functional polymorphisms that are highly associated with the dose used in regular clinical practice for two leading AEDs, phenytoin and carbamazepine. For phenytoin, a well-known low-activity variant in the *CYP2C9* gene associates with dose, as does a newly described functional variant in the *SCN1A* gene, encoding the target of phenytoin. The *SCN1A* variant is also highly associated with dosing of carbamazepine, thus providing functional replication of the effect of this variant. This *SCN1A* variant is the first polymorphism in a drug target associated with the use of an AED, and one of only a handful of target polymorphisms for which there is strong evidence of an effect on clinical drug use[29]. Furthermore this is the first demonstration of pharmacologic significance of alternative splicing in a human sodium channel. Although there are other examples of alternative splicing in other human sodium channel genes, none has been associated with functional effects[270].

This polymorphism is potentially of more general importance because of the prominence of sodium channel blockade in the treatment of epilepsy (and other neurological conditions). For example, more than half of epilepsy patients treated pharmacologically in the UK receive a drug which principally targets the sodium channel[271].

With respect to carbamazepine and phenytoin, this study provides a direction for a dosing scheme to be used in a prospective study to assess how pharmacogenetic diagnostics can improve dosing decisions. In particular, it may be clinically relevant to determine whether some individuals can safely be given more rapid dose increases. Although these polymorphisms explain relatively little of the total variation in the unselected cohort (6.5%, and 2.5% for phenytoin and carbamazepine, respectively), it is likely that in more controlled settings they will have a much larger proportionate effect. Our study did not take into account, for example, other AEDs taken together with phenytoin or carbamazepine, some of which are known to induce (e.g. phenobarbitone) or inhibit (e.g. sodium valproate) cytochrome P450 enzymes. Nevertheless, in our cohort we see average dose ranges across genotypes from 127mg to 230mg for phenytoin and carbamazepine respectively, indicating there is an important effect of these variants on dose. Presumably in a selected cohort the effect would be stronger. These results therefore suggest the possibility that genetic diagnostics could reduce the time it takes, on average, to control seizures using phenytoin and carbamazepine.

One limitation of the current study is that the database upon which our analyses were based records only the maximum dose received by each patient, rather than maintenance dose. The implications of this limitation are somewhat different for phenytoin and carbamazepine.

When the starting dose is insufficient to control seizures it may be increased until control is achieved. If the initial dose produces ADRs, on the other hand, it may be lowered. On balance, therefore, upward adjustment of dose is due to lack of efficacy and downward adjustment due to ADRs. This means, for example, that the effect of *3 on phenytoin dose is probably estimated conservatively here: any effect of *3 on dose reduction due to ADRs is not visible in our data. It would appear, however, that any effect of *3 on dose because of ADRs is small as we have observed no direct association between *3 and ADRs. For carbamazepine recording maximum dose seems less of a limitation as starting doses are virtually always less than what is finally necessary to control seizures.

It should also be noted that the issue of compliance was not addressed in this study and it is not possible to confirm that all patients were taking the drugs correctly and at the doses prescribed. Also, we decided to use Bonferroni correction for multiple statistical comparisons rather than a less stringent method (e.g. permutation methods) in order to demonstrate that our results remain significant even after a conservative form of statistical correction.

Our results support the view that the major target, transporter, and drug metabolizing enzyme are good starting points to study drug response and that pharmacogenetic traits are therefore more tractable for genetic analyses than those for common disease predisposition[29]. We also emphasize that a haplotype tagging strategy[238] identified a previously unknown functional variant in the

SCN1A gene. This functional variant was found 91 bp away from the nearest exon known at the time of the study, illustrating the need for exhaustive tagging.

Overall, our findings suggest that using genotype data may make it possible to safely reduce the time required to reach an effective dose. It is therefore also a priority to assess the utility of dose-adjustment on the basis of genotype for these medicines in a prospective clinical study. Prospective studies of carbamazepine and phenytoin, informed by a detailed retrospective study, would also serve as a useful model for future pharmacogenetic studies[272].

Chapter 4 A common polymorphism in the *SCN1A* gene associates with phenytoin serum levels at maintenance dose

4.1 Introduction

4.1.1 Pharmacokinetic v pharmacodynamic predictors of drug response

In the previous chapter I described how we found a common polymorphism in *SCN1A* (IVS5-91 G>A, or rs3812718) to be highly associated with the maximum doses that patients received of phenytoin or carbamazepine in addition to confirming that genetic variation in *CYP2C9* associates with phenytoin maximum dose[1].

The effect of genetic variation in *CYP2C9* on phenytoin pharmacokinetics[234, 258, 261, 262]or dose[1, 244] has been well documented. There are likely to be other, non-genetic factors, for example body mass, age, and concomitant medication, which also affect phenytoin pharmacokinetics[273-275]. In contrast, the *SCN1A* IVS5-91 G>A polymorphism is a pharmacodynamic variant, as it is predicted to alter the effect of phenytoin on its target, without changing the concentration of drug present at the target. In order to eliminate the variation between drug doses and

serum levels (likely to be caused by pharmacokinetic factors), one can use serum concentration at maximum or maintenance dose, as serum concentrations already reflect variation due to pharmacokinetic factors.

Although traditionally viewed as influenced by pharmacokinetic variants, serum levels at maintenance dose may be influenced by pharmacodynamic variants because of the dosing decisions that the treating physician makes on the basis of observed patient responses (in terms of both efficacy and side effects). To isolate the effect of a pharmacodynamic variant it could be useful to look at the serum levels as opposed to dose, if the serum levels have been collected in a sufficiently consistent way. This should result in a more clear relationship with pharmacodynamic determinants (e.g. target sensitivity) because the variation associated with pharmacokinetic determinants (e.g. rate of metabolism) has been reduced or eliminated. However, there may be additional factors, for example time since drug administration, which contribute to variation in serum levels and the extent to which they contribute may vary between cohorts.

4.1.2 Aims

In this study we have related variation at the *SCN1A* IVS5-91 G>A polymorphism to maximum dose and to maintenance dose of phenytoin in 168 patients treated with phenytoin (dosage distributions are illustrated in figures 4.1 and 4.2). We noted that one limitation of the previous study was that analyses were based only

on maximum dose and not on maintenance dose. We have also related genotype to phenytoin serum levels at maximum dose and at maintenance dose of phenytoin in the same 168 patients. As there is no standard definition of maintenance dose, we have proposed the following working definition: "The maintenance dose of a given drug is a dose which has not been changed for two or more consecutive visits in the history of the patient's treatment (provided the neurologist reviewing the records agrees that they themselves would not have altered the patient's dose)". Maximum dose is as defined previously[1], that is, it is the maximum dose patients were exposed to during their regular treatment of epilepsy.

Figure 4.1 Distribution of Maintenance Phenytoin Doses

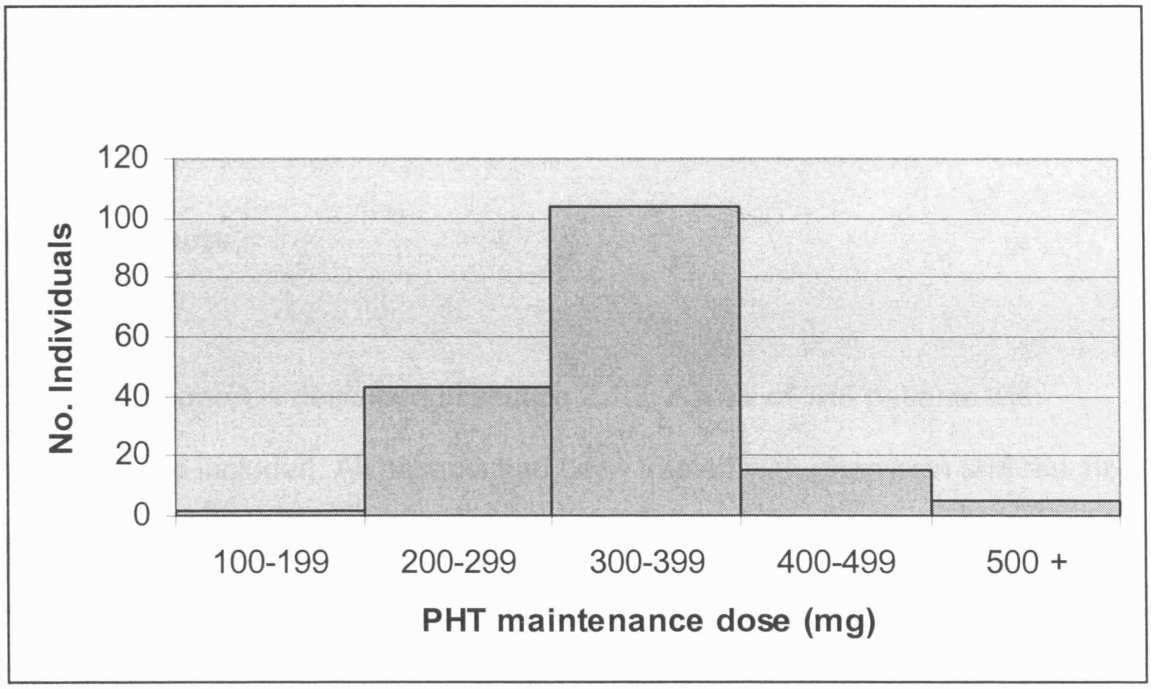
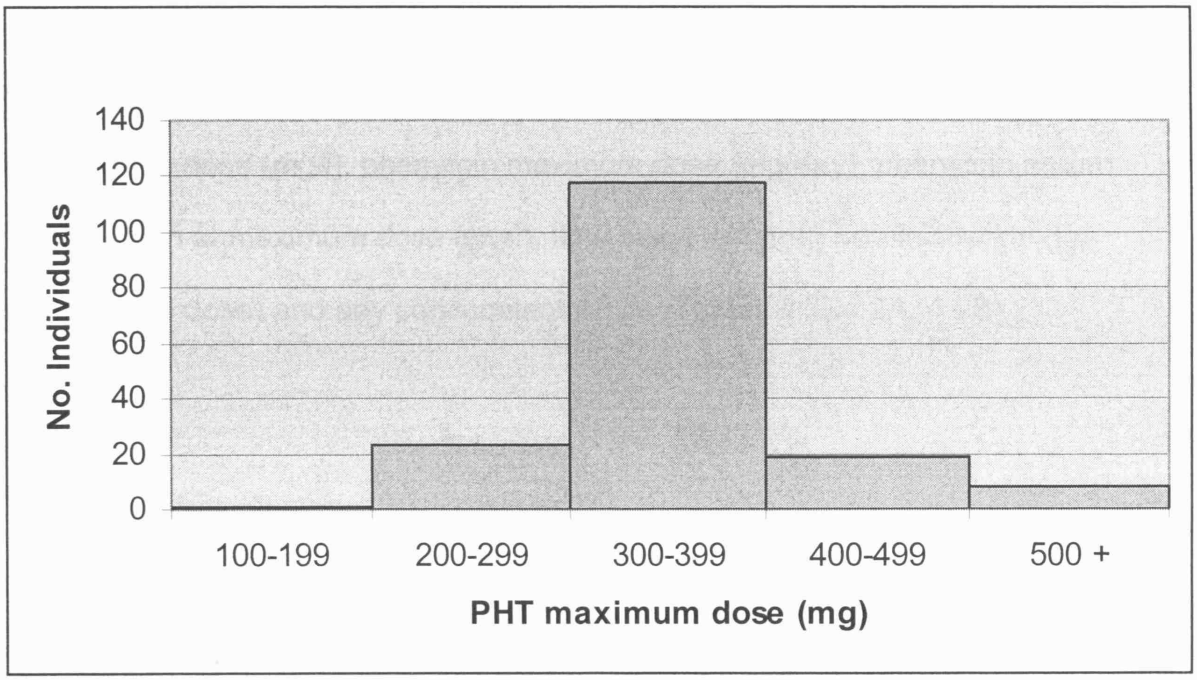


Figure 4.2 Distribution of Maximum Phenytoin Doses



4.2 *Methods*

4.2.1 Subjects

The patient cohort is described in section 2.1.3. A total of 168 patients with epilepsy were included. All patients had been treated with phenytoin and reached a maintenance dose. Patients self-identified, or were determined by the treating physician, as being of Chinese ancestry. All patients have provided written informed consent. Patient demographic data have been previously described[234].

For each individual the following were recorded: gender, weight (kg), epilepsy classification (according to ILAE guidelines), etiology, treatment outcome, phenytoin maintenance dose (mg/day), phenytoin serum concentration at maintenance dose (mg/l), phenytoin maximum dose (mg/day), phenytoin serum concentration at maximum dose (mg/l), time since last drug administration (for maintenance dose) and any concomitant AEDs (Tables 4.1, 4.2A, 4.2B).

Table 4.1. Patient Characteristics

Gender		**%**
Male	95	57
Female	73	43
Weight (kg)		
Min	40	
Mean	62	
Max	125	
Phenytoin maintenance dose (mg/day)		
Min	100	
Mean	299	
Max	600	
Phenytoin maximum dose (mg/day)		
Min	100	
Mean	317	
Max	600	
Phenytoin serum concentration at maintenance dose (mg/l)		
Min	1.1	
Mean	15.2	
Max	49.2	
Phenytoin serum concentration at maximum dose (mg/l)		
Min	1.1	
Mean	17.6	
Max	49.2	
Time since last drug administration (maintenance dose)(h)		
Min	1.0	
Mean	8.9	
Max	21.5	
Concomitant AEDs		
Carbamazepine	49	29.0
Valproate	36	21.3
Phenobarbital	19	11.2
Lamotrigine	24	14.2
Topiramate	15	8.9
Vigabatrin	4	2.4
Clonazepam	4	2.4
Gabapentin	3	1.8
ANY of the above	98	58.0

Table 4.2A Patient Characteristics – Epilepsy Classification

Classification	Total	%
Localized-related epilepsies and syndrome		
Temporal lobe epilepsy	125	74.4
Frontal lobe epilepsy	21	12.5
Parietal lobe epilepsy	5	3.0
Occipital lobe epilepsy	1	0.6
	152	**90.5**
Generalized epilepsies and syndrome		
Idiopathic	2	1.2
Cryptogenic	14	8.3
	16	**9.5**

Table 4.2B Patient Characteristics – Etiology

Etiology	Total	%
Unidentified	67	39.9
Mesial temporal sclerosis	23	13.7
Post-traumatic	20	11.9
Infection	14	8.3
Vascular	13	7.7
Tumour	11	6.5
Perinatal	7	4.2
Developmental lesion (malformation)	7	4.2
Focal atrophy	4	2.4
Idiopathic	2	1.2

4.2.3 Genotyping

SCN1A IVS5-91 G>A was genotyped using an Applied Biosystems Taqman assay:

Forward Primer Sequence: GATTTTGGTACATTCATATCCTTTTTCAAGTGA

Reverse Primer Sequence: CAAAGATGCAAAATGAGAGTGATGAAAACT

Reporter 1 Sequence: CAACTTAATTTGATATTTAGC

Reporter 2 Sequence: CAACTTAATTTGATGTTTAGC

*CYP2C9*2* and **3* and *CYP2C19*2* and **3* have been previously genotyped.

4.2.3 Statistical Analyses

All regression analyses were implemented in the usual way using STATISTICA (StatSoft Inc, Tulsa, USA) with phenytoin maintenance/maximum dose or phenytoin serum concentration at maximum/maintenance dose as the dependent variable and genotype score as the independent predictor.

4.3 *Results*

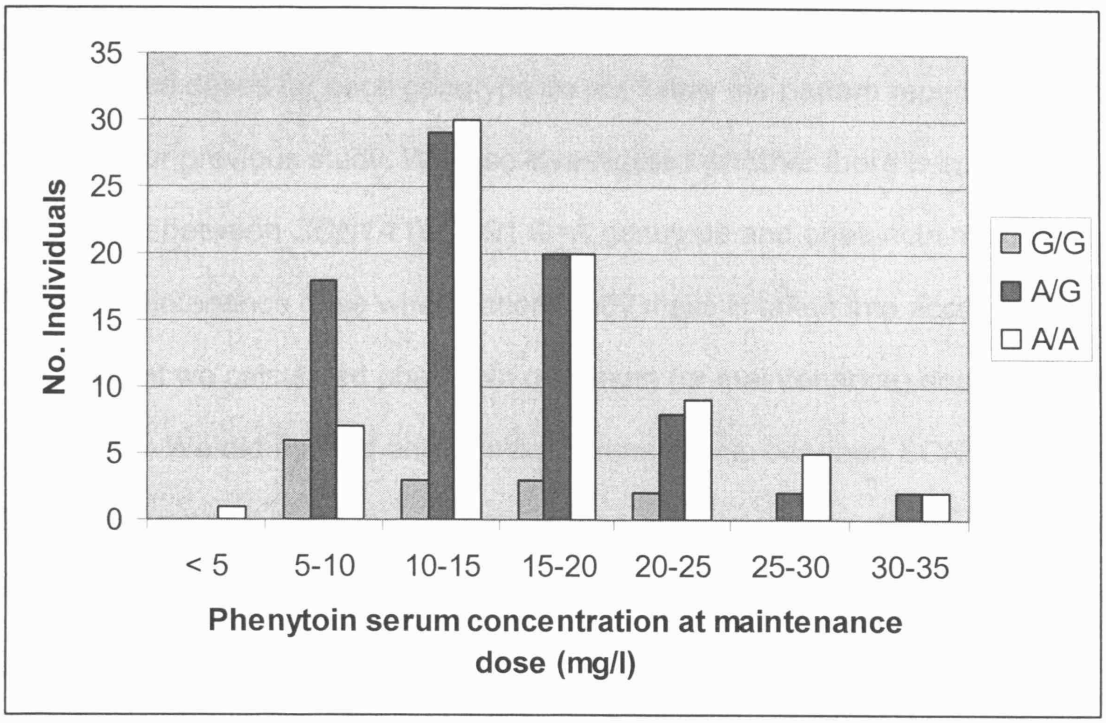
4.3.1 *SCN1A* IVS5-91 G>A polymorphism and phenytoin serum concentration

The *SCN1A* IVS5-91 G>A polymorphism is in Hardy-Weinberg equilibrium. For all analyses we have fitted an additive model rather than an unrestricted model as we have previously found the genotypic effects to be consistent with additive effects[1]. All P-values reported are uncorrected for multiple testing. We find that the *SCN1A* IVS5-91 G>A polymorphism is associated with phenytoin serum concentration at maintenance dose (P=0.03, uncorrected, explaining 2.8% of the total variation, figure 4.3). This association does not remain significant after Bonferroni correction for multiple testing. There is a non-significant trend between *SCN1A* IVS5-91 G>A genotype and phenytoin serum concentration at maximum dose (P=0.06, uncorrected). In both cases (i.e. for maximum and maintenance dose), the mean serum concentrations for each genotype follow the pattern expected from the previous results[1] (i.e. AA > AG > GG).

As various AEDs are known to inhibit or induce phenytoin drug-metabolizing enzymes[275], we repeated the above analyses for the subset of 71 individuals on phenytoin monotherapy. The data generally mirror those for the entire cohort and despite the reduced cohort size, the value for phenytoin serum concentration at maintenance dose remains significant (P=0.03, uncorrected, explaining 7.1% of

total variance). This association does not however, remain significant after Bonferroni correction for multiple testing. As with the full cohort there is a non-significant trend between *SCN1A* IVS5-91 G>A genotype and phenytoin serum concentration at maximum dose (P=0.07, uncorrected).

Figure 4.3 Distribution of phenytoin serum concentrations at maintenance dose for each *SCN1A* IVS5-91 G>A genotype



4.3.2 *SCN1A* IVS5-91 G>A polymorphism and phenytoin dose

We next investigated any association between *SCN1A* IVS5-91 G>A genotype and phenytoin maximum dose and phenytoin maintenance dose. We do not find a significant relationship between *SCN1A* IVS5-91 G>A genotype and phenytoin maximum or maintenance dose. Furthermore, the mean phenytoin maximum or maintenance doses for each genotype do not follow the pattern reported for the cohort in our previous study. We also investigated whether there is any association between *SCN1A* IVS5-91 G>A genotype and phenytoin maximum dose or maintenance dose when patient body mass is taken into account. For each patient we calculated phenytoin maximum (or maintenance) dose per kg body mass. We did not find any significant relationship between *SCN1A* IVS5-91 G>A genotype and phenytoin maximum dose or maintenance dose per kg body mass. However, the average values by genotype now fit the expected pattern for phenytoin maximum dose (i.e. AA > AG > GG).

There were no associations across the cohort between time since drug administration and phenytoin serum concentration at maximum or maintenance dose. Inclusion of time since drug administration as a covariate did not alter the value of the significant associations between *SCN1A* IVS5-91 G>A genotype and

phenytoin serum concentration at maintenance dose for either the full cohort or the reduced monotherapy cohort.

4.4 Discussion

We have associated a functional[1] polymorphism in the *SCN1A* gene with phenytoin serum concentration at maintenance dose. The association appears stronger when considering a reduced cohort of patients receiving phenytoin monotherapy, in the sense that a smaller sample size gives a similar significance level. There is also a non-significant trend between *SCN1A* IVS5-91 G>A genotype and phenytoin serum concentration at maximum dose (for the full cohort and the 71 patients receiving phenytoin monotherapy).

We found no significant association however between the *SCN1A* IVS5-91 G>A polymorphism and phenytoin maintenance or maximum dose. Formally, therefore, these results are a failure to replicate our previous observation described in chapter 3. However, the results do provide support for a pharmacodynamic effect of the *SCN1A* IVS5-91 G>A polymorphism. In the smaller sample size used in this study relative to the previous one[1] (242 patients with *SCN1A* IVS5-91 G>A genotype data), we do see an association with serum level at maintenance dose.

There are several reasons why a relationship between *SCN1A* IVS5-91 G>A and maximum dose may not exist in this cohort. There are important differences between the two study populations. For example, there are higher proportions of seizure free patients, and patients sensitive to phenytoin, in the current cohort (Table 4.3) compared to the cohort originally studied[1]. There are also substantial differences in disease etiology and classification (Tables 4.2A and 4.3B, 4.4 and 4.5). The range of phenytoin doses observed was narrower in this cohort than in our previous study (100 to 600 mg per day compared to 90 to 900 mg per day in the original study[1]). It is possible that a dosing strategy where doses are usually increased to the maximum clinically tolerated by the patient, is required to reveal a relationship between genotype and maximum dose. The frequency of *SCN1A* IVS5-91 G>A is significantly higher in the current cohort (0.68 v 0.53, $P<0.0001$) which would result in a reduction in power to detect any effect.

An attempt to separate pharmacodynamic and pharmacokinetic factors can be complicated by sources of variation that might affect serum levels particularly. For example serum levels vary partly as a function of interval since dose, whereas a given dose remains fixed. A pharmacodynamic variant could in principle show its effect in either type of data set (i.e. dose or serum levels). The weight given to different factors (for example eliminating the pharmacokinetic effects, or eliminating the variation associated with time since administration) will depend on the particular data set. In addition, taking account of time since

administration in the analysis only eliminates that part of the variation if the times since administration are accurate and this is not always known.

In the case of the current cohort there is only an association between *SCN1A* IVS5-91 G>A genotype and serum level at maintenance dose, which may be because the pharmacokinetic variability has been sufficiently reduced in this dataset. It is also possible that genetic or non-genetic factors affecting phenytoin pharmacokinetics are more important for this cohort and obscure any role for *SCN1A* IVS5-91 G>A in dosing. The frequency of the *CYP2C9**3 allele is not significantly different between the two cohorts, however, the frequencies of the *CYP2C19**2 and *3 alleles are significantly higher in this cohort than those reported for a population of European ancestry. The sodium channel alpha-subunit is encoded by the brain-expressed genes *SCN1A, 2A, 3A*, and *8A* and there are also other potential targets for phenytoin action within the CNS. Therefore a large prospective study investigating phenytoin response (including maximum or maintenance does) would ideally include a larger candidate gene list.

We only found a significant association between *SCN1A* IVS5-91 G>A genotype and phenytoin serum concentration at maintenance dose, not phenytoin serum concentration at maximum dose. This might be expected for reasons analogous to those outlined previously for maintenance dose being a more desirable phenotype than maximum dose (i.e. maximum dose was a more conservative

phenotype)[1]. As for the previous chapter, it was not possible to evaluate patient compliance.

Overall our findings are functionally consistent with the result described in chapter 3, though they are not an exact replication. The results provide further motivation to assess the biological mechanism by which the *SCN1A* polymorphism influences patient response to anti-epileptic drugs[1]. It also remains a priority to assess the usefulness of phenytoin dose adjustment on the basis of genotype in a prospective clinical study, which should include monitoring of phenytoin serum concentration.

Table 4.3 AED and PHT response for Taiwan cohort and Institute of Neurology cohort

	Taiwan cohort		Institute of Neurology cohort	
	Total	**%**	**Total**	**%**
AED response*				
Seizure-free	113	67.3	46	17.5
Intractable	52	31.0	145	55.1
U	3	1.8	72	27.4
Phenytoin response**				
Refractory	17	10.1	73	27.8
Partially refractory	15	8.9	9	3.4
Sensitive	99	58.9	29	11.0
Partially sensitive	37	22.0	7	2.7
Neither	0	0.0	19	7.2
U	0	0.0	126	47.9

* Intractable is the occurrence of at least for seizures over the year with at least 3 AEDs tried. Seizure free is freedom from seizures for at least one year with two or less AEDs tried. U= unclassified (neither intractable nor seizure free)

** 1) 'Sensitive': a 50% or greater reduction in seizure frequency following initiation of phenytoin; 2) 'Refractory': no change or increase in seizure frequency; 3) 'Neither': less than 50% decrease in seizure frequency. Patients having been treated with the same drug on more than one occasion are classified as sensitive if they responded on each occasion, partially sensitive if they responded at least once but not on every occasion, refractory if they never responded, and partially refractory if they were refractory but not on every occasion and never showed a response which could be classified as sensitive. Patients showing a response to an phenytoin started after epilepsy surgery were classified as U (unknown) instead of sensitive, as in such cases one cannot tell for sure whether the decrease in seizure frequency can be attributed to phenytoin or rather is a result of surgery.

Table 4.4 Patient characteristics from Institute of Neurology cohort - Epilepsy Classification

ILAE Classification	Total	%
Focal Symptomatic	102	38.8
Focal Cryptogenic	77	29.3
Generalized Idiopathic	23	8.8
U	40	15.2
Other	21	8.0

Table 4.5 Patient characteristics from Institute of Neurology cohort - Etiology

Etiology	Total	%
Unidentified	166	63.1
Hippocampal sclerosis	39	14.8
Other*	24	9.1
Juvenile myoclonic epilepsy	8	3.0
Post-traumatic	7	2.7
Perinatal	5	1.9
Cavernoma	4	1.5
Post-infectious	4	1.5
Cardiovascular diseased	3	1.1
Tumour	3	1.1

*All categories with 1 or 2

Chapter 5 Genetic variation in *SV2A* and *SV2C* influences response to the antiepileptic drug levetiracetam but not epilepsy predisposition

5.1 Introduction

5.1.1 Levetiracetam and the treatment of epilepsy

Levetiracetam is currently licensed in Europe and the US as adjunctive therapy for partial epilepsy with or without secondary generalisation in children and adults and for juvenile myoclonic epilepsy in adults. It is also licensed in Europe as first-line monotherapy in partial epilepsy. Its efficacy has been demonstrated in a number of regulatory clinical trials[276-278] and it is effective in a wide range of seizure types and syndromes[279-281]. It has also been reported to be of benefit in off-licence use in the treatment of symptomatic generalised epilepsies and a range of other neurological conditions, including migraine prophylaxis[282], cerebellar tremor[283], tardive dyskinesia[284, 285] and restless legs syndrome[286]. Levetiracetam is generally well tolerated and displays predictable pharmacokinetics[287].

5.1.2 Candidate gene selection

Levetiracetam targets synaptic vesicle protein 2 (SV2)[288]. SV2 is a membrane glycoprotein common to all synaptic and endocrine vesicles. Three isoforms have been identified to date: SV2A, SV2B and SV2C. SV2A is the most widely distributed and is present in all types of neurons; SV2B is almost as prevalent, whereas SV2C is only expressed in a small subset[289, 290]. There is also a distantly related protein, SVOP[291]. SV2A is the predominant isoform in the brain and it is to this isoform which levetiracetam binds. There are sharp differences among patients in responsiveness to levetiracetam, and it is possible that some of the variation may due to genetic differences in *SV2A* or its isoforms.

SV2 is clearly an essential protein as homozygous *SV2A* knockout mice appear normal at birth but fail to grow, experience severe seizures and die by 3 weeks[292]. Heterozygous knockout mice, although viable, are 10 times more likely to have seizures than wild-type animals[292]. Mice lacking SV2A have increased expression of SV2C[293].

The role of SV2 in synaptic events is not yet fully understood. SV2 is not thought to be directly involved in synaptic vesicle fusion, but instead has a regulatory role. There is evidence that SV2 regulates the size of the readily releasable pool (RRP) of synaptic vesicles available for calcium-stimulated exocytosis[293] and enhances low-frequency neurotransmission by increasing the resting RRP and thus release probability in quiescent neurons[294]. The molecular mechanism of action of SV2 in regulating the RRP is unclear.

5.1.3 Aims

These observations suggest the possibility that the activity level of SV2A may influence predisposition to epilepsy and therefore an assessment of genetic variation in *SV2A* and its isoforms may therefore also contribute to understanding the biological basis of epilepsy. We have therefore used a haplotype tagging strategy[238, 295] to represent genetic variation in *SV2A*, *SV2B* and *SV2C* and have related this genetic variation to levetiracetam response and to epilepsy predisposition in two separate cohorts of epilepsy patients.

5.2 Methods

5.2.1 Subjects

This study was approved by the relevant institutional Ethics Committees. All patients and controls self-identified or were determined by the treating clinician as being of European ancestry.

Institute of Neurology cohort: This cohort is described in section 2.1.1. 725 patients with a diagnosis of epilepsy were included in the predisposition study. Of these 257 levetiracetam -treated patients were included in the levetiracetam

response study. For the predisposition study 357 controls were obtained from the National Twin Research Unit at Guy's and St Thomas' Hospitals, London, United Kingdom. Only one member of each sibship was included

Beaumont hospital, Dublin cohort: This cohort is described in section 2.1.2. 778 patients with a diagnosis of epilepsy were included in the predisposition study. Of these, 334 levetiracetam -treated patients were included in the overall levetiracetam response study with 221 being considered at the moment under the current Irish response classification scheme. DNA from 352 controls were obtained from the Allied Irish Bank Study[233] for the predisposition study.

5.2.2 Response Classification

Institute of Neurology cohort: We calculated each patient's total seizure frequency per month for the six months prior to commencing levetiracetam in order to estimate the "baseline" seizure frequency. In order to graph each individual's response over time, each patient's baseline seizure frequency was set as 100%. We then reviewed patient response to levetiracetam at each subsequent clinic visit. Response was plotted as a percentage change in seizure frequency from baseline. This allowed us to view each individual's change in seizure frequency as a pattern of response.

We considered three groups of response (Table 5.1): 1. Excellent response (>95% improvement in seizure frequency), 2. Partial response (25-95% improvement in seizure frequency, and 3. No response (<25% improvement in seizure frequency (including worsening of seizure frequency).

As there is no consensus on how best to represent response to an AED we also considered three other variations of this classification scheme (see Appendix)

Table 5.1 Institute of Neurology cohort levetiracetam response classification scheme

Category	Description	No. Patients	% Patients
1	Excellent response (>95% improvement in seizure frequency)	36	14.0
2	Partial response (25-95% improvement in seizure frequency.	86	34.0
3	No response (<25% improvement in seizure frequency (including worsening of seizure frequency).	131	51.6
	Total	253	86.1

Beaumont hospital, Dublin cohort: This included all patients with a history of present or previous exposure to levetiracetam who had been unresponsive to at

least two other prior AEDs and recorded their demographic and clinical data. Response was divided into 1. 'seizure-free' (seizure-free for a minimum of 6 months after commencing LEV); 2. 'partial >50%' (greater than 50% reduction in seizures for a minimum of 6 months after commencing LEV); 3. 'honeymoon' (seizure-free for less than 6 months after commencing LEV and then returned towards baseline frequency); and 4. 'no-response'. For our initial analyses we have compared seizure free patients to non-responders only (Table 5.2)

Table 5. 2 Beaumont hospital, Dublin levetiracetam response classification scheme

Category	Description	No. Patients	% Patients in this scheme (% Patients in cohort)
1	Seizure free	58	26.3 (17.4)
2	No response or worsening or response	163	73.8 (48.8)
	Total	221 (334)	100 (66.2)

5.2.3 *SV2A* sequencing

We decided to sequence *SV2A* in order to make a thorough assessment of all common polymorphisms. We designed a series of overlapping amplicons, each

around 1kb, and sequenced them in 16 unrelated CEPH individuals in both forward and reverse directions (see 2.5 for primer details). In total, we sequenced the entire transcribed gene, except for ~1kb of repetitive intronic sequence, plus ~3kb immediately upstream and ~1kb immediately downstream of the gene. We found 18 SNPs with a minor allele frequency greater than 0.05 in the original screen (Table 5.3). Fourteen of these are not in dbSNP. All 18 were resequenced in 32 CEPH trios.

One *SV2A* nonsynonymous SNP has been previously reported; rs1801870 (Ser643Thr). There is no frequency data for this SNP in dbSNP and we did not find any variation at this location in the 16 individuals screened. This SNP therefore does not appear to be common in individuals of European ancestry and was not typed in our cohort.

Table 5.3 *SV2A* polymorphisms from resequencing

SNP	rs number or relative position from ATG where no rs number (i.e polymorphism is not in dbSNP)	Location	Frequency in CEPH
1	-1949	5' flank / promoter	0.11
2	-1598	5' flank / promoter	0.11
3	-59	5' flank / promoter	0.10
4	+1047	Intron 1 (5'UTR)	0.46
5	+3631	Intron 1 (5'UTR)	0.10
6	rs577935	Exon 2 (5'UTR)	0.08
7	+6620	Intron 3	0.11
8	+7472	Intron 5	0.10
9	rs626785	Intron 5	0.19
10	+8248	Intron 6	0.09
11	+8258	Intron 6	0.09
12	+8568	Intron 7	0.11
13	+8781	Intron 8	0.12
14	rs16835135	Intron 8	0.11
15	+9076	Intron 8	0.10
16	+9156	Intron 8	0.08
17	rs7534365	Exon 13 (3'UTR)	0.15
18	+14825	3' flank	0.12

5.2.4 Genotyping

All SNPs were genotyped using Illumina's GoldenGate Assay (San Diego, CA, US).

Tags were selected using "worst locus allelic r^2" (criteria 18 in TagIT http://www.genome.duke.edu/centers/pg2/tagit). The minumum r^2 threshold was 0.7 and the minimum minor allele frequency was 0.05.

Genotype data for *SV2B* and *SV2C* tagging was taken from HapMap CEPH (Phase II). We included 10KB immediately upstream and 1KB immediately downstream of the gene. We also tagged *SV2A* using HapMap CEPH data and included any tags that had not previously been selected based on the sequencing data. *SV2A* tag selection is therefore based on all polymorphism data available to us.

We selected a total of 86 tags, 11 from *SV2A*, 40 from *SV2B* and 35 from *SV2C*.

5.2.5 Statistical Analyses

P values for single marker association were calculated by applying exact tests to genotype*response classification contingency tables. In some cases involving

larger sample sizes, Fisher's Exact test was not possible and the chi-squared test was employed instead.

5.3 Results

There were no significant violations of Hardy–Weinberg equilibrium after Bonferroni corrections for multiple comparisons. All P values reported are uncorrected for multiple testing.

We find that two polymorphisms, rs11205277 in *SV2A* and rs17651293 in *SV2C* (both in HapMap) are highly significantly associated with response to levetiracetam in the UK cohort (most significant P values are P= 0.0008 and P=0.003, respectively) and also show association with response in the Irish cohort (P=0.05 and P=0.02, respectively). None of the associations remains significant after Bonferroni correction for multiple comparisons (conservatively, 86 SNPs x 4 classification schemes = 334 tests for the UK cohort, 86 tests for the Irish cohort).

Although we find a number of polymorphisms to be associated with epilepsy predisposition or mTLE predisposition in either the UK or Irish cohort, none shows association in both cohorts, and no associations remain significant after Bonferroni correction for multiple comparisons.

5.4 Discussion

We have tested 86 common polymorphisms in *SV2A, SV2B* and *SV2C* for association with levetiracetam response. We find two common polymorphisms in *SV2A* and *SV2C* to be associated with response in both cohorts. Currently each cohort is phenotyped differently so the associations cannot be considered true replications. We are currently attempting to reclassify each cohort according to each of the two schemes considered so far in order to attempt a true replication.

The polymorphism rs11205277 is in fact approximately 3kb upstream of *SV2A* and rs17651293 is in intron 3 of *SV2C*. Neither polymorphism appears to fall in a known regulatory element or splice site consensus sequence however, we intend to assess their functional effects initially using in vitro expression assays.

We do not find any polymorphisms to be associated with epilepsy predisposition or mTLE predisposition in both cohorts.

This is the first study relating variation in *SV2A*, *SV2B* and *SV2C* to levetiracetam response. Other studies suggest that genes encoding the target for a drug, or related pathway proteins, are a good starting point for pharmacogenetic association studies[29] and in chapters 3 and 4 I have described a drug target polymorphism (in *SCN1A*) to be associated with clinical use of two other antiepileptic drugs[1]. It is also possible that genetic variation in wider drug target

pathway genes, not considered here, influence response to levetiracetam. Other genetic and non-genetic factors are also likely to play a role.

Chapter 6 Race or ethnicity and drug response.

6.1 Introduction

It is hoped that advances in genetic technologies should improve our understanding of disease etiology and of the factors influencing response to treatment[296]. Although there has been relatively little progress to date in using genetics to improve the treatment of common diseases, there are some encouraging signs of progress in basic research[29, 297, 298]. If genetics does eventually prove relevant to the treatment of common diseases, then to the extent that genetic advances are uneven among racial and ethnic groups, disparities may result. Clinical utility may well emerge sooner from the study of drug response than of disease predispostion[29, 299]. Pharmacogenetics also appears poised to become a common component of the drug development pipeline[300], with proof of concept studies having clearly demonstrated its potential[301].

In this chapter I discuss ways in which genetics could contribute to future health disparities, and outline some general research approaches that could counter this. "Race" or "ethnicity" are not defined in this chapter as the aim is primarily to be able to discuss the evidence to date about differences among groups, and for this purpose I consider mainly how people are distinguished in the current

medical literature, for example in clinical trials. For this reason, in discussing different groups, they are referred to as "racial" or "ethnic" groups. I do however discuss the debate about the extent to which human genetic structure correlates with racial or ethnic groupings

Throughout much of the world "race" and "ethnicity" are major determinants of health. For example, African Americans have by some estimates a two-fold greater incidence of fatal heart attacks and a 10% higher incidence of cancer[302, 303] than European Americans, and South Asian or Caribbean-born British are around 3.5 times as likely to die as a direct result of diabetes than British of European ancestry[304]. The healthcare that people receive also depends on race and ethnicity. African Americans are less likely to receive cancer screening services, and more likely to have late-stage cancer when diagnosed[303] than European Americans. Non-insulin dependent diabetes is up to 40% less likely to be diagnosed in British Asians[304].

There is a reasonably strong consensus that most current health disparities result mainly from socio-economic and other environmental factors[305], including inequalities in the delivery of healthcare. The fact that genetics does not contribute significantly to current health disparities, however, should not allow complacency about the possibility that biological differences associated with "race" or "ethnicity" will contribute to disparities in the future. There are substantial changes underway in how medicines are developed and how they are

used, and some of these changes present risks that medicines will become less inclusive, and that some of the variation in how they work may correlate with racial or ethnic groups, thus exacerbating health disparities.

The concerns about how genetics might influence the inclusiveness of medicines in the future can be broadly divided into three partially overlapping categories: 1) Pharmacogenetic diagnostics, in which individual genotype is used to guide the selection of medicines, 2) Molecular subclassification of disease, in which genetic features of a disease are used to guide therapy choice, and 3) Pipeline pharmacogenetics, in which genetics is used during the evaluation of new chemical entities. The issues raised in each of these areas are considered in turn.

6.2 Inclusiveness of current medicines

Before discussing whether genetics may contribute to future disparities in healthcare it is worth assessing the inclusiveness of current medicines. There are at least 29 medicines (or combinations of medicines) that have been claimed in a peer-reviewed scientific or medical journal to show differences in either safety or, more commonly, efficacy among ethnic or racial groups (Table 6.1). These claims, however, have proven universally controversial[306-308] and there is

currently no clear consensus on how important a factor race/ethnicity is in drug response.

Nevertheless, from 1995-1998, 8% (15/185) of new drug product labels contained a statement about racial/ethnic differences in effectiveness[309]. To get an overview of the relationship between race/ethnic group and drug response I have assembled a set of drugs that have been associated with claims of different effects among ethnic or racial groups based on reviews (for example[310]) and PubMed literature searches using combinations of the following keywords and phrases: ethnic*, race, racial, drug response, pharmacokinetic*, cytochrome P450. We also indicate whether there is any evidence for a genetic or a physiological contribution for the reported racial/ethnic differences in drug response, or indeed any other supporting evidence that the differences are real. In cases where the differences in response is associated with underlying physiological differences (as in the case of ACE inhibitors, see below) then these physiological differences may be themselves environmentally influenced, genetic, or both. Thus an indirect way to assess this is with methods familiar in genetic epidemiology, including migrant studies, studies of admixed populations and comparisons of populations in different geographical locations (e.g. West Africans and African-Americans)[311]. The most direct way to assess the differences in drug response among racial or ethnic groups is to find the causes of the variable drug response, and to investigate how these causes differ among the groups. In most cases, however, the genetic bases of variable drug

responses are too poorly known to allow a direct assessment. The one exception is beta-blockers: a polymorphism in the drug target may contribute to the differences in responses.

Table 6.1 Examples of drugs with different response in different ethnic or racial groups

If the difference between racial or ethnic groups seems to be real, then it is graded according to how likely it is to have a physiological or genetic basis ,as follows A) there is an indication of genetic causation, B) the association has a reasonable underlying physiological basis, C) the difference is consistently shown but no physiological basis has been demonstrated, D) possibly false positive claim. Racial or ethnic groups: AA, African American; AC, Afro-Caribbean; AI, American Indian and Alaskan Native; AS, Asian; EU, of European ancestry; HI, Hispanic; PI, Pacific Islander. ALL, acute lymphoblastic leukaemia; INR, International Normalized Ration (for blood clotting time); N/A, not applicable.

Drug Class	Examples	Difference in Drug Response	Sample size	Is the difference real?	Evidence
ACE inhibitors	Enalapril	Less response in AA than EU with left ventricular dysfunction[312]	1196 EU 800 AA	B	Probably related to lower bioactivity of endogenous nitric oxide (NO) in AA than EU[313]
	Lisinopril	Response in EU, no response in AA (lowering of blood pressure)[314]	N=124		
	Trandolapril	AA with hypertension required 2-4 times dose to obtain similar lowering of blood pressure to EU[315]	207 EU 91 AA		

Combination of two vasodilators	BiDil	Greater efficacy in AA than EU with congestive heart failure[316]	Trial I: 180 AA 450 EU Trial 2: 215 AA 574 EU	B	
Vasodilator antihypertensive	Sodium nitroprusside	Attenuated vasodilation response to methacholine and sodium nitroprusside in normotensive AA compared to EU[317, 318]	11 AA 9 EU 21 AA 19 CAEU	C	Attenuated responses to multiple vasodilators commonly observed in AA[317]. Mechanisms not fully understood
Beta-adrenoceptor blocker (non-selective)	Propranolol	More effective in EU than AA for initial treatment of hypertension[319]	N/A	A	Hypertensives and healthy volunteers homozygous for the Arg allele at Arg389Gly have been shown to have greater response to metoprolol, and metoprolol
	Nadolol	More effective in EU than AA for systemic hypertension[323]	N=365		

Beta-adrenoceptor blocker (B1-selective)	Atenolol	More effective in EU than AA for hypertension[324]	N=1105		and atenolol[45, 46, 320]. The Arg allele is more frequent in EU than AA (0.723 v 0.575)[321]. Higher proportion of low-renin hypertension in AA, and non-adrenergic mechanisms contribute more to blood pressure maintenance in AA than EU[322].
Beta-adrenoceptor blocker (non-selective)	Oxprenolol	Mean blood pressure reduction less for AA than EU[325]	N/A		
	Bucindolol	Only survival benefit in non-AAs[326]	N=2708		
Vasopeptidase inhibitor	Omapatrilat	Increased risk of angioedemas in AA than EU, for hypertension[327]	N/A	D	Basis of ADR not known and study not replicated
Anticoagulant	Danaparoid	Significantly more EU had favourable outcome than AA at 3 months (for ischemic stroke patients)[328]	292 AA 801 EU	D	No significant difference in other response measures. Other measures e.g. age better predictor of outcome[328]. Danaparoid is not hepatically metabolised. Results not

					replicated.
	Warfarin	Average warfarin dose required to maintain the INR between 2.0 and 3.0 greater in AC than EU and EU require higher dose than AS[329]. AC and Indo-AS require more warfarin to hold their INR between 3 and 4.5 than do EU[329]	737 EU 58 AC 72 AS 15 AC 18 AS 95 EU	D	Functional variation in CYP2C9 affects interindividual warfarin dosing[140, 141] but has not been demonstrated to account for between-population differences. Differing average body weight may account for some of the differences between EU and AS[330]
Alpha-adrenoceptor blocker	Prazosin	More effective in EU than AA for hypertension[324]	N=1105	B	Non-adrenergic mechanisms contribute more to blood pressure maintenance in AA than EU[322].
Thiazide (diuretic)	Hydrochlorothiazide	Greater systolic and diastolic blood pressure responses in AA than EU[331]	225 AA 280 EU	B	Likely to be related to lower bioactivity of endogenous nitric oxide (NO) in AA than

					EU[313]
Calcium channel blocker	Diltiazem	More effective in AA than EU for hypertension[324]	N=1105	B	Likely to be related to increased predisposition of AA to the salt sensitive form of essential hypertension[332]
Beta-adrenoceptor agonist	Isoproterenol	Attenuated vasodilation in normotensive AA compared to EU[333-335]. Average dose giving 25beat/min HR increase >2-fold higher in AA than EU[336]	27 AA 27 EU 18 AA 18 EU 9 AA 13 EU N=16	C	Attenuated responses to multiple vasodilators commonly observed in AA[317]. Mechanisms not fully understood
Glucocorticoid	Methylprednisolone	Adverse effects (steroid-associated diabetes) more common in AA than EU[337].	9 AA 9 EU	C	Also altered pharmacokinetics between AA and EU[337].
HepC Antiviral treatment	Ribavirin / Interferon alpha	AA have lower rate of response to treatment than EU[338]	100 AA 100 EU	B	May be due to differing immune abilities. AA produce more cytokine than EU, and EU responders less than non-responders[339].
	Interferon	Poorer response in AA than EU[339, 340].	n=40 31 AA 62 EU		

Prostaglandin analogue	Travoprost	Response greater in AAs than EU, for ocular hypertension[341]	N=1381	D	Not replicated, no supporting evidence.
Cytotoxic agents	6-MP and methotrexate (& other ALL agents)	Significant difference in response by ethnicity for childhood ALL, with AS > EU> HI > AA[342] EU have increased survival compared to AA and HI[343] AA, HI, and AI children with ALL have worse survival than EU and PI children[344].	6703 EU 1071 HI 506 AA 167 AS, 4061 EU 518 AA 507 HI N=4952	D	More likely to be due to differences in quality of healthcare/therapy
	Docetaxol and Carboplatin	Greater response in AS than EU with advanced non-small cell lung cancer[345]	N=68	D	Not replicated. No intermediate phenotypes to support result.
Insulin	Insulin	Insulin sensitivity significantly lower in HI and AA than EU, and greater acute insulin response in AA than HI[346]	14 EU, 15 AA, 28 HI	C	Differences remain after adjusting for body fat. Results well replicated[346].
Antipsychotic	Haloperidol	AS schizophrenic patients had a significantly higher rating for extrapyramidal symptoms at	13 EU, 16 AS	C	No significant difference in haloperidol serum

		fixed dose and significantly lower mean required dose than EU[347] Extrapyramidal side effects higher in Chinese than non-Chinese patients[348]	32 Chinese, 32 non-Chinese		concentrations although EU have greater reduced haloperidol concentrations[348].
	Clozapine	AS showed a greater change than EU in total scores of the 8-item Brief Psychiatric Rating Scale while receiving a significantly lower mean dose of clozapine and were significantly more likely to experience anticholinergic and other side effects[349]	17 Korean-American, 17 EU	D	Although a relevant drug target polymorphism differs in frequency between EU and AS[29] (HTR2A His452Tyr) it has not been reliably associated with response in both populations.
	Chlorpromazine	EU received significantly larger maintenance doses than either AS or HI patients[350]	N/A	D	Overall more likely to be due to different prescribing practices
	Various	AA given higher doses than EU[351-354]	n=442 n=173 76 AA, 88 EU N=293	D	
Analgesic	Morphine	Reduction in blood pressure was greater in	8 Chinese 8 EU	D	Conflicting results[310]

		EU than in Chinese[355]. AI more susceptible to depression of respiratory response than EU[356]	22 EU AI Native Indians		

It should be noted that many of these studies are small, and there is heterogeneity amongst study designs. There is also a clear bias towards studies comparing European Americans and African-Americans, reflecting a focus on the American racial/ethnic context. Certain therapeutic areas are particularly prominent among the list of medicines, most notably those related to cardiovascular disease. For example, there is arguably a consensus in the literature that African-Americans respond less well than European Americans to beta-blockers, angiotensin I converting enzyme (ACE) inhibitors and angiotensin-receptor blockers, some of the main agents now used for heart-related conditions. Conversely African-Americans may respond as well as or better than European Americans to diuretics and calcium channel blockers.

It has been argued that these differences result from underlying differences in the causes of hypertension, on average, in individuals of (west) African ancestry and individuals of European ancestry[357] For example European Americans and African-American hypertensives typically differ in characteristics such as salt sensitivity, plasma volume and renin levels[358] and it is possible that there could be (average) differences in the pathogenesis of this condition.. It has been suggested that decreased sensitivity to beta-blockers may be associated with the higher proportion of low-renin hypertension found in African-Americans, and that non-adrenergic mechanisms contribute more to blood pressure maintenance in African-Americans than European Americans[322].

Differences in the response to ACE inhibitors may be related to lower bioactivity of endogenous nitric oxide (NO) in African-Americans than European Americans[313]. The

increased benefit of nitrates and hydralazine in African-Americans is consistent with this hypothesis. For example BiDil, a drug that combines isosorbide dinitrate (a nitric oxide donor) and hydralazine (an antioxidant and vasodilator agent), showed insufficient efficacy in two large, ethnically mixed clinical trial for congestive heart failure[359, 360] to win regulatory approval. A retrospective analysis of the original trials, however, indicated a greater efficacy for African-American participants in the trial than European Americans[316], and on this basis the FDA approved a trial of BiDil in African Americans[361]. The trial recently terminated early because interim analyses showed the drug to be highly effective, and the makers of BiDil will now seek approval for its use in the African American population. However, any genetic basis to the difference in NO bioactivity between African-Americans and European Americans remains to be elucidated. Furthermore, there have been no comparative studies investigating differences in NO bioactivity between African and African-American subjects and any relationship between endothelial nitric oxide synthase (NOS3 or eNOS) variants and plasma nitric oxide levels or other intermediate or clinical phenotypes is not fully understood[362].

It seems very likely that genetics contributes to some of these average differences reported between different racial/ethnic groups. On the other hand, at least some of the differences can be attributed to confounded environmental factors. A study by Mokwe et al investigated blood pressure response to quinapril, an ACE inhibitor, in 533 African-American and 2046 European Americans with hypertension. They found that while African-Americans had a lower average response to quinapril than European Americans, age, gender, body size and pre-treatment blood pressure significantly

predicted blood pressure response[363]. These factors correlate with race/ethnicity, and when accounted for reduce the effect attributable to race/ethnicity

Some claims for differences among racial/ethnic groups in drug response will be false positives, and probably more likely to be reported in the literature than a negative finding[31]. The Mokwe et al analysis makes the point, however, that even for those differences that are real it is not clear which if any are due to genetic factors and which are due to environmental correlates with race or ethnicity. The analysis also makes clear that there is substantial variation within racial or ethnic groups in environmental correlates of response, as will also be the case for genetic factors.

6.3 Genetic contributions to differences in drug response

6.3.1 Introduction

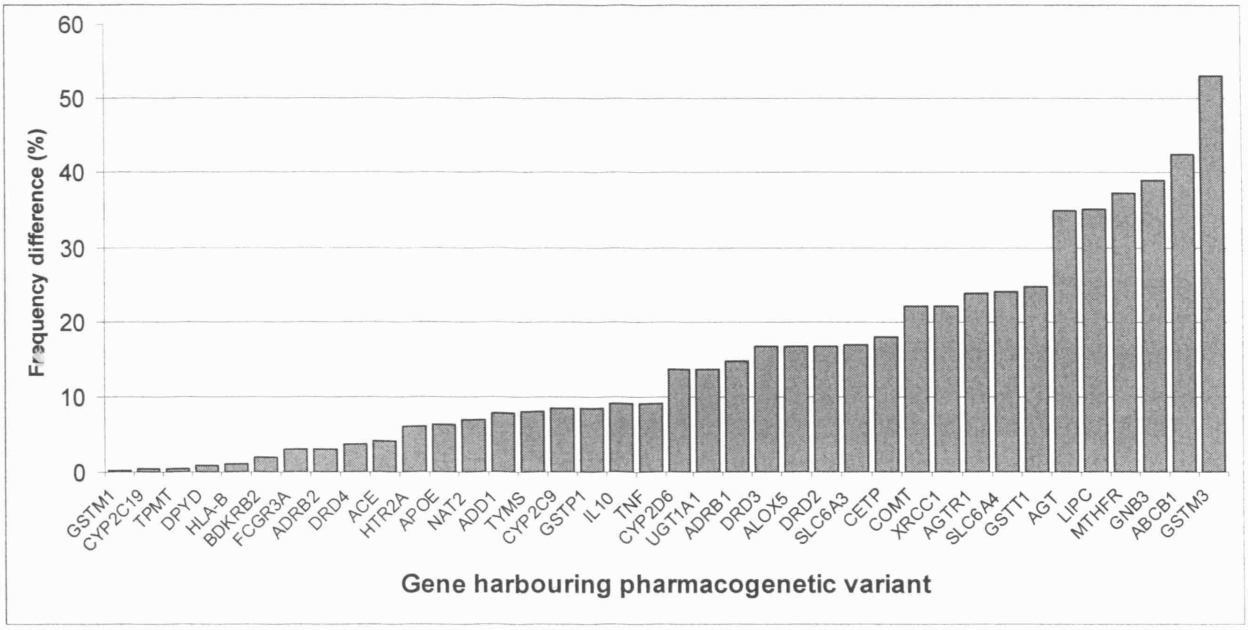
In discussing possible genetic factors it is useful to distinguish genetic factors that are specific to the drug used from those that influence the nature of the condition itself. The drug-specific effects would include variants related to both pharmacokinetics and pharmacodynamics of the drug, for example drug metabolizing enzymes (DMEs) and transporters, and drug targets and target-related proteins, respectively.

For both the disease-associated (see section below "subclassification of disease") and the drug specific effects, there is clearly scope for inter-group differences; many variants known to influence drug response show important frequency differences among racial/ethnic groups pharmacogenetic variants vary in frequency among populations

(reviewed in[29]). For example *ADRB1* Gly389 variant, associated with decreased response to beta-blockers[45, 46], is more common in African-Americans than in European Americans (0.43 v 0.28). Many DME variants also vary in frequency among populations[245] and for some DMEs (e.g. CYP2D6, CYP2C19, CYP2C9 and NAT2) the proportion of individuals with little or no functional enzyme varies substantially among populations. For example, because of nulls at CYP2D6 up to 10% of individuals of north European ancestry will receive no analgesic effect of the prodrug codeine[364], whereas 98% of the inhabitants of the Arabian peninsula are able to transform codeine into the active form morphine[365].

These examples, and those discussed for disease-specific effects, make clear that gene variants that causally influence how patients respond to treatment often have significant frequency differences among racial/ethnic groups. This makes clear that genetic differences among racial/ethnic groups will often lead to average differences in drug response. These selected examples are completely consistent with the general pattern for variants known to influence drug response. Most genetic variants that have been associated with drug response in two or more studies have highly significant differences in frequency between Caucasians and Africans or African-Americans (Figure 6.1). The fact that the majority of the functional variants that we do know about have significant allele frequency differences between African Americans or Africans and Europeans shows that genetics could contribute to differences.

Figure 6.1 Frequency differences between pharmacogenetic variants (%) in European Americans and African Americans



Overall, analysis of responses to existing medicines and new chemical entities are far from conclusive about the scope for (average) genetic differences among groups to contribute to variable drug response. In most cases it is difficult to separate genetic from environmental reasons. For example whilst one study found a significant difference in response by race/ethnicity for childhood acute lymphoblastic leukemia (ALL), with greatest response in the order Asians > Europeans> Hispanics> African-Americans[344], other investigators found no difference in outcome between African-Americans and European Americans when given equal access to the most advanced therapies[366]. It was suggested that the conflicting findings were "possibly due to the specialized referral base of the unique practice of St Jude's Hospital [where the latter study took place], which attracts patients from an 8-state area and provides therapy at no cost to the patient's family"[344]. Nevertheless, the examples available of drug-specific pharmacogenetic variants make clear the possibility of average differences among

ethnic/racial groups. It is therefore impossible to rule this out as a possibility on first principles, as some authors seem to have implied. For example, Cooper et al have stated "Race-specific therapy draws its rationale from the presumption that the frequencies of genetic variants influencing the efficacy of the drug are substantially different among races. This result is hard to demonstrate for any class of drugs, including those used to treat heart failure"[306]. However, response may differ among racial/ethnic groups either because of average genetic *or* environmental differences. Thus, race-specific therapy does not draw its rational from a presumption of genetic differences. In the case of BiDil it is not currently known whether the reason it works differently in African Americans and European Americans is because of genetics, environment, or both. Furthermore, there is no shortage of gene variants known to influence drug response that have substantial differences in frequency (often greater than 10%) among racial or ethnic groups.

Despite the limited information currently available, three general points are apparent: 1) genetic differences among groups are graded, as opposed to dichotomous; 2) when genetic factors play a role, finding the genetic factors themselves so that they can be considered directly will reduce the need to consider race/ethnicity as a loose proxy for predicting drug response. For example, in the case of *CYP2D6* genotype and codeine response, a simple genetic test however would indicate who would and who would not respond, regardless of geographic ancestry; and finally, 3) many differences in drug response associated with race/ethnicity will be due to environmental correlates as opposed to average population genetic differences. This implies that even when the genetic structure of a test population is taken into account, it may still be appropriate to

consider race/ethnicity as a variable in order to take account of the environmental correlations[311].

6.3.2 Pharmacogenetic diagnostics

One common view of the aim of pharmacogenetics is to provide diagnostics that allow matching of medicines with the genetic makeup of the patient, in order to ensure use of medicines most likely to work and least likely to produce adverse drug reactions (ADRs). For pharmacogenetic diagnostics to be useful they must be sufficiently specific and sensitive.

One concern is whether pharmacogenetic tests will be equally predictive across different ethnic groups. There are two different ways in which a diagnostic might perform differently among ethnic or racial groups. The first reason is that underlying physiology may be different on average among racial/ethnic groups. For example, the variant that influences response to a drug in one ethnic group might not have the same effect, on average, in another ethnic/racial group because of different gene-gene or gene environment interactions. For example, the *ABCB1* C3435T polymorphism is associated with altered drug response[32, 33], pharmacokinetics and P-glycoprotein expression[127], however the correlation between the polymorphism and P-glycoprotein levels is not consistent across ethnic groups[367, 368]. If C3435T is the causal polymorphism (and this has not been proven) then this could be an example of physiological differences across ethnic groups. The second, and perhaps more likely reason is if the diagnostic is a proxy, for example if it is based on linkage disequilibrium

(LD). LD is the non-random association of alleles at different polymorphic sites in the genome and levels are often high across long genomic stretches, making it hard to know whether an associated variant is causal, or just a marker for (i.e., usually inherited with) the causal variant(s). The predictive value of an LD diagnostic will depend on the degree of association between the markers and the underlying causal variants and there is considerable variation in the pattern of LD across the genome and among populations[369]. Unfortunately diagnostics that are based on markers serving as proxies for the causal variants will generally have different predictive properties among different ethnic or racial groups.

One illustration of the potential of pharmacogenetic diagnostics provides a warning about the transferability of diagnostics across ethnic/racial groups. Abacavir is an effective antiretroviral drug used to treat HIV-1 infection. Approximately 5% of patients treated with abacavir develop a hypersensitivity reaction that requires discontinuation of the drug. Pharmacogenetic studies have identified multiple markers in the human leukocyte antigen (HLA)-B chromosomal region, including *HLA-B*5701*, associated with hypersensitivity to abacavir in European American populations[183, 184, 370]. However, the *HLA-B*5701* allele is not associated with hypersensitivity in African-Americans[370]. Therefore a pharmacogenetic diagnostic using this allele would have no predictive value in African-Americans. In this case it is not clear whether abacavir hypersensitivity has different underlying causes in African Americans and Americans of European ancestry. The HLA-B alleles are situated within a tract of approximately 200 kb with extensive LD, meaning that is not clear within this tract which variants are responsible for the association in Europeans/Hispanics. It will require considerably larger association

studies and or functional studies to determine whether it is the HLA-B*5701 allele, or an associated variant, which is responsible for the ADR.

These considerations also suggest that in the near-term at least, pharmacogenetic diagnostics should rely on validated causal variants in preference to either single markers as proxies, or genome-wide SNP profiles, both of which will usually rely on LD to generate associations between the markers and the drug responses. Since the degree and pattern of LD typically varies among populations, LD-marker based tests will often need to be adjusted for different ethnic/racial groups. This requires large, expensive studies of many populations.

6.3.3 Genetic subclassification of disease

Common diseases result from complex interactions between genetic and environmental factors. As more is learnt about the genetic bases of common diseases they may be divided into distinct subclasses with similar phenotypes but different underlying genetic bases. In many cases, specific drugs would then be indicated for specific subtypes of a disease, as are Herceptin for the subpopulation of breast cancer patients who express HER2 and Gleevec for those patients with chronic myeloid leukaemia resulting from the BCR-ABL gene fusion. Similarly, two studies identified mutations in the *EGFR* gene in lung cancers which predict patient response to the tyrosine kinase inhibitor gefitinib [371, 372].

It is currently unclear how often the underlying genetic bases of disease show average differences among racial/ethnic groups, but there are suggestions this may be the case

for some diseases. For example, susceptibility to Crohn's disease is associated with 3 polymorphisms in *CARD15* in European American[298], however none of these variants was present in a sample of Japanese patients[373]. Another example is a *CCR5* variant which protects against HIV infection and progression. Up to 25% of European American are heterozygous for this variant yet it is practically absent in other ethnic groups[374]. The *EGFR* mutations which predict responsiveness to gefitinib are more frequent in Japanese patients, possibly explaining the increased responsiveness of Japanese.

Even when a genetic variant associated with disease susceptibility is present in multiple ethnic groups, it may have different effects. For example the E4 variant of *APOE* is associated with a substantially increased risk of Alzheimer's disease[231], however, a large meta-analysis has shown this effect varies among racial/ethnic groups. Homozygosity for E4 allele increases the risk of Alzheimer's disease by a factor of 33 in Japanese, 15 in Europeans, and 6 in African-Americans[375]. Similarly, a more recent study found differential effects of the E4 allele between Europeans and African-Americans[376]. It is impossible on current evidence to say these differences reflect an interaction of *APOE* with genetic background or with environment.

To the extent that progress is faster in understanding the genetic bases of common disease in some ethnic groups as opposed to others, the ability to genetically subclassify diseases might proceed faster in some groups than others, meaning that treatment can be made more precise in these groups. In this context it may be a concern that the HapMap project is not sufficiently inclusive. The project currently includes European Americans, Africans (the Yoruba in Ibadan, Nigeria), Japanese and Han Chinese. However the HapMap currently excludes Native Americans and Pacific

Islanders, two of the five racial categories indicated by the FDA[311]. Exclusion of Native Americans in particular would seem inconsistent with NIH policy to include minorities in biomedical research (there are approximately 4.3 million Native Americans or Native Americans in combination with one or more other races in the US, making up 1.5 percent of the total population (http://www.census.gov/Press-Release/www/releases/archives/facts_for_features/001492.html). The decision was made after consultation with some representatives of the Native American health-research community, who cited concerns about HapMap data being used to facilitate population history studies and comparisons among populations[377]. From a broader perspective, given the genetic diversity among African populations, it would almost certainly have been more informative to choose a second African population as opposed to a second East Asian one.

6.3.4 Drug pipeline pharmacogenetics

In some ways the use of pharmacogenetics during the development of potential new therapies provides the most serious concerns because of its potential to influence the medicines that are brought to market. In the past the basic model in drug development was to try and find drugs that are as widely applicable as possible, hence the effort to prove efficacy in large and therefore expensive phase III populations. There is now interest in carrying out smaller, less expensive trials using genetics[227]. Although the idea of focusing clinical trials on subgroups of patients is not new, stratification by disease subtype having always been a goal of medical research[378], the use of genetics in this context is new. Pharmaceutical companies have long tended, when possible, not to pursue compounds known to be metabolized largely by highly polymorphic systems

such as CYP2D6, but pharmacogenetics has otherwise played little role in drug development. Increasingly, however, there is interest in the use of systematic genetic analyses in an effort to identify the genetic causes of variable responses during the evaluation of new chemical entities. Widespread use of such reverse-genetic strategies could result in important changes in drug development, including reliance on more focused clinical trials[227].

Early identification of a marker for drug response could lead to smaller Phase III trials involving those patients more likely to respond. Efficacy pharmacogenetics might lower the cost of Phase III clinical trials if randomisation could be applied to a population of patients selected for drug efficacy in Phase II[227]. This would result in patients with unfavourable genetic profiles being excluded from trials, even though a proportion would likely respond to the drug (even if less frequently than the target population). There is a risk of creating 'orphan genotypes' that are left untreated for either scientific (difficult to treat) or economic (too small to be economically viable) reasons. Many genetic variants, including drug metabolizing enzyme polymorphisms and drug target polymorphisms vary in frequency among populations. If a marker for efficacy has low prevalence in a certain ethnic population, that population may be excluded from research or treatment.

6.4 How to represent human population genetic structure

What constitutes an ethnic or racial "group" is a highly contentious issue[308, 311]. There are however, some areas of general agreement. Most importantly, no matter how groups are defined, most of the genetic variation in the species is due to differences

among individuals within groups, not to differences between groups. It is also agreed, however, that individuals with the same geographic ancestry are more similar, on average, than individuals with different geographic ancestries.

The main areas of dispute are how to represent that portion of our overall variability that does correlate with geography, and about how important this portion of our variation is in medicine. Risch and colleagues[311] propose using five major racial groups in biomedical research based on continental ancestry. Whilst this method is easy to implement, it remains unclear how well it captures human population structure. A second method, explicit genetic inference, ignores geographic, racial or ethnic labels and instead groups similar individuals using genetic data[235]. There has been debate about how well self-identified ethnicity corresponds with explicit genetic inference, and more generally how well we currently understand the global pattern of human genetic diversity[235, 311]. Data from Rosenberg and colleagues appears to support the scheme proposed by Risch and colleagues of a small number of major groups corresponding largely to continent of origin; in a sample of 1056 individuals from 52 populations they identified six main genetic clusters, five of which correspond to major geographic regions[379].

Even in situations where there is a generally good correspondence between self identified ethnicity and explicit genetic inference, there may be contexts where it is still advisable to obtain the most precise information possible about genetic structure. For example, in evaluating new medicines it could be straightforward to include explicit genetic inference as part of the overall analyses, with a negligible increase in cost and complexity. In addition to the increased precision afforded by explicit genetic analyses,

groups, identified by racial/ethnic labels or by genetics, will often themselves be genetically structured, and this structure cannot be well captured by self identified ethnicity. For example, estimates of European ancestry proportion in African-Americans average around 21% but there is a wide range of ancestry proportions among individuals[380].

The importance of group differences in medical genetics has been the subject of much debate. Cooper and colleagues argue that race is not an adequate proxy for choosing a drug[377]. While it is certainly true that individual genotype will always be more informative than racial/ethnic labels (for genetic effects), in some cases race and ethnicity may be useful biological proxies for the underlying genetic variation. There are many examples of variants that are known to influence drug response and that differ substantially among racial/ethnic groups. Because there are many other variants that are not known, some drug response will correlate with racial/ethnic groups, some of which may be relevant to the selection of treatment alternatives.

6.5 Discussion

My aim here has been to outline how advances in genetics could contribute, even if modestly, to disparities in the quality of healthcare among different racial/ethnic groups. A straightforward solution would be more and better research in those groups that have been traditionally under-represented in clinical and other biomedical studies. The NIH specifically requires "members of minority groups and their subpopulations must be included in all NIH-supported biomedical and behavioural research projects involving

human subjects" (http://grants2.nih.gov/grants/policy/emprograms/overview/women-and-mi.htm).

More specifically with respect to pharmacogenetics, it is important that basic pharmacogenetics research is carried out in as broad a range of ethnic groups as possible. It is nevertheless inevitable that diagnostics will often be identified in specific ethnic groups, and in this case it is essential that the diagnostic be tested explicitly in other ethnic groups, as scientists from GlaxoSmithKline have recently done for their Abacavir associations[370]. In this context, one valuable tool may be the use of healthy volunteers in those cases where drugs can be safely administered, or where probe drugs may indicate the effect of a gene variant on transport or metabolism. The use of healthy volunteers would also facilitate efforts to ensure that functional variation at relevant genes is equally well described in all major racial/ethnic groups. Finally, even greater efforts are required to expand the diversity of drug trial populations. The results of such trials could also be interpreted with more clarity, and the effects of genetic structure more systematically assessed, if genetic structure were routinely analysed in drug trials[235].

Overall it is hard to say whether advances in genomic medicine will exacerbate or attenuate health disparities. It does appear that no matter how research is performed, most medicines will tend to work similarly among different human populations. Because of our demographic youth as a species, most human genetic variation comes from an ancestral source population, and is present in most current racial/ethnic groups. But rough statistical similarity in how medicines work among racial/ethnic groups may not always be good enough.

Chapter 7 Genetic predictors of response to the beta-blocker bucindolol in a racially/ethnically mixed clinical trial.

7.1 Introduction

7.1.1 The Beta-blocker Evaluation of Survival Trial

The Beta-blocker Evaluation of Survival Trial (BEST) investigated whether the non-selective beta-blocker bucindolol prolongs the life of patients with moderate to severe congestive heart failure (CHF). In common with most U.S. clinical trials, trial participants self-identified as belonging to one of the following racial groups: white (with ethnic sub-categories Hispanic and non-Hispanic); black or African American; Asian; American Indian or Alaskan Native; and Hawaiian or Pacific Islander[311].

A modest survival benefit was observed for patients who self-identified as a category other than African American (of these 91% self-identified as white, non-Hispanic (EA), and 7% self-identified as Hispanic) but not for African-American patients (AA). A nominally significant interaction effect was found for race and treatment, however this disappeared after correction for multiple testing[326].

7.1.2 Beta-blockers and congestive heart failure

The use of beta-adrenergic blocking drugs as routine therapy for CHF is motivated by the demonstration that excessive adrenergic activity contributes to CHF pathogenesis

[381]. The BEST trial was an attempt to evaluate the effect of one such agent on survival. In the BEST trial 2708 patients were randomly assigned to double-blind treatment with bucindolol or placebo (1354 patients in each arm) and followed for the primary end point of death from any cause. Other response measures were hospitalization for CHF (hospital-free survival time), and changes in left ventricular ejection fraction (LVEF) and plasma norepinephrine (PNE) levels after three months of treatment. The trial was designed to include sufficient AA patients to enable subgroup and treatment interaction analyses for race.

The trial was stopped early, after patients had been enrolled for an average of 2.0 years with no significant difference in mortality between the two arms[326]. However, because of the demonstration of beneficial survival effects of other beta-blockers for CHF, response was reanalyzed for racial subgroups. This revealed a survival benefit in non-AA patients only[326].

7.1.3 Selection of candidate genes

Bucindolol targets the beta-1 and beta-2 adrenoceptors, encoded by *ADRB1* and *ADRB2*, respectively. Both *ADRB1* and *ADRB2* carry well-characterized functional variants. Two functional polymorphisms in *ADRB1* have been associated with response to beta-blockers other than bucindolol. The Gly allele at Ser49Gly has been shown to be more sensitive to the beta-1 selective beta-blocker metoprolol *in vitro*[382]. Hypertensives and healthy volunteers homozygous for the Arg allele at Arg389Gly have been shown to have greater response to metoprolol and metoprolol and atenolol (also beta-1 selective), respectively[45, 46, 320].

ADRB2 also has two common functional polymorphisms. Both of these polymorphisms have been associated with response to beta-agonists *in vitro* and *in vivo* (reviewed in [383]), although neither has been associated with response to any beta-blocker. *In vitro* studies show the Gly allele at Arg16Gly has enhanced agonist-promoted down-regulation, while the Glu allele at Gln27Glu is resistant to agonist-promoted down-regulation[50, 384]. The Gly allele at Arg16Gly is associated with decreased response to the beta-agonists albuterol and salbutamol in asthmatics and healthy volunteers, respectively[48, 49]. Healthy volunteers homozygous for the Gln allele at Gln27Glu have greater venodilation in response to the beta-agonist isoprenaline than individuals homozygous for the Glu allele[385, 386]. In addition, a common substitution in the 5'-leader cistron, 5'-LC-Arg19Cys, is associated with increased beta-2 adrenoceptor expression[387]. Drysdale et al suggested that promoter and coding region haplotype is a better predictor of beta-agonist responsiveness than any individual SNP[388]. A rare *ADRB2* polymorphism, Thr164Ile, is also functional. The Ile164 variant has reduced agonist-sensitivity *in vitro* and *in vivo*[389, 390].

The Gly allele at the *ADRB2* Ser49Gly polymorphism also has been associated with increased survival, over 5 years, for patients with CHF[391] (some of whom received beta blockers). The *ADRB2* polymorphism, Thr164Ile, is also functional. The Ile164 allele at the rare *ADRB2* Thr164Ile polymorphism is also associated with decreased survival for CHF (patients received a variety of cardiovascular drugs, approximately 20% received beta-blockers)[392].

Bucindolol is metabolised by the polymorphism drug metabolizing enzyme *CYP2D6* however this was not included as a candidate gene in this initial study.

7.1.4 Race, Population Structure, and drug response

The BEST trial was racially/ethnically mixed, with DNA available from 762 EA and 207 AA. As noted, there is also a suggestion that race or ethnicity has a role in response to bucindolol, with a survival benefit apparently occurring only in non-AA. For this reason, it is important to assess the role of this genetic structure in influencing drug response. If the study population is structured and the subgroups differ in drug response this can lead to stratification, where significant associations occur at loci that are unlinked to any causal sites, but that have frequency differences between the subgroups. As discussed in the previous chapter there are two main ways to describe population structure. Ethnic or racial labeling (as was used in the BEST study) uses racial labels to describe the structure of human genetic variation[311] but whilst this method is easy to implement, it is not clear how well it captures human population structure. Moreover such a scheme misses genetic variation *within* a group, which is particularly important in admixed populations as stratification may confound any associations. Explicit genetic inference ignores geographic, racial or ethnic labels and instead groups individuals using genetic data (e.g.[235]). This has the advantage that it is possible to estimate individual ancestry proportions in admixed groups such as AA. One possible advantage of self-identified racial labeling is that it may serve as a better proxy for possibly relevant cultural and social factors[311]. On the other hand, estimated genetic admixture is a continuous variable, which might contain more information than self-identification, which is usually categorical.

To control for stratification we therefore introduced admixture proportion as a covariate in our models. We genotyped microsatellite markers with no known functional significance to explicitly infer the genetic structure in the sample, estimating the proportion of each individual's genome having ancestry from one of two "parental" populations. We then asked whether an individual's ancestry proportion associated with drug response, and whether inclusion of ancestry affects the associations between variation in the drug targets and drug response.

7.1.5 Aims

In this study we investigated whether variation in the drug target genes *ADRB1* and *ADRB2* influences response to bucindolol. We directly genotyped all four common functional variants at the two targets *ADRB1* and *ADRB2*. In order to represent other variation in these genes that might have a role, we also typed additional SNPs to generate a set that would satisfactorily tag the common variation in the genes giving a total of four tags for *ADRB1* and three for *ADRB2*. We restricted our analyses to EA and AA patients.

Because EA and AA may respond differently to bucindolol, as has been suggested for other cardiovascular-related drugs[393], we also explicitly assessed the genetic ancestry of a subset of the patients to determine whether genetically inferred ancestry is associated with drug response in both the AA and EA subjects.

7.2 *Methods*

7.2.1 Subjects

762 EA and 207 AA individuals who enrolled in BEST. The study was approved by the relevant institutional Ethics Committees. DNA samples were provided by the BEST DNA Bank. Phenotypic data and archived DNA were used from patients who participated in the BEST, who consented for DNA substudies. The study was a multicentre, randomized, long-term, placebo-controlled trial of the -blocker bucindolol in patients with Class III/IV CHF. DNA was extracted from whole blood or myocardium by using standard techniques.

7.2.2 Microsatellite markers and structure inference

29 unlinked chromosome 1 and X-linked microsatellites were genotyped in 192 EA and 192 AA individuals from the BEST population as described in section 2.4. We also genotyped 88 Cameroonian individuals[394] to provide an example of an African population.

7.2.3 *ADRB1* and *ADRB2* tag selection and genotyping

We used information on the haplotype structure for *ADRB2* described elsewhere[388], to select three SNPs which represent a minimum of 90% of haplotype diversity in EA and AA. *ADRB1* haplotype structure had not been previously described so we resequenced

approximately 8kb using amplicons placed throughout the gene and surrounding regions in 24 unrelated CEPH individuals. We genotyped all polymorphisms found in 32 CEPH trios and used this data to select four tags.

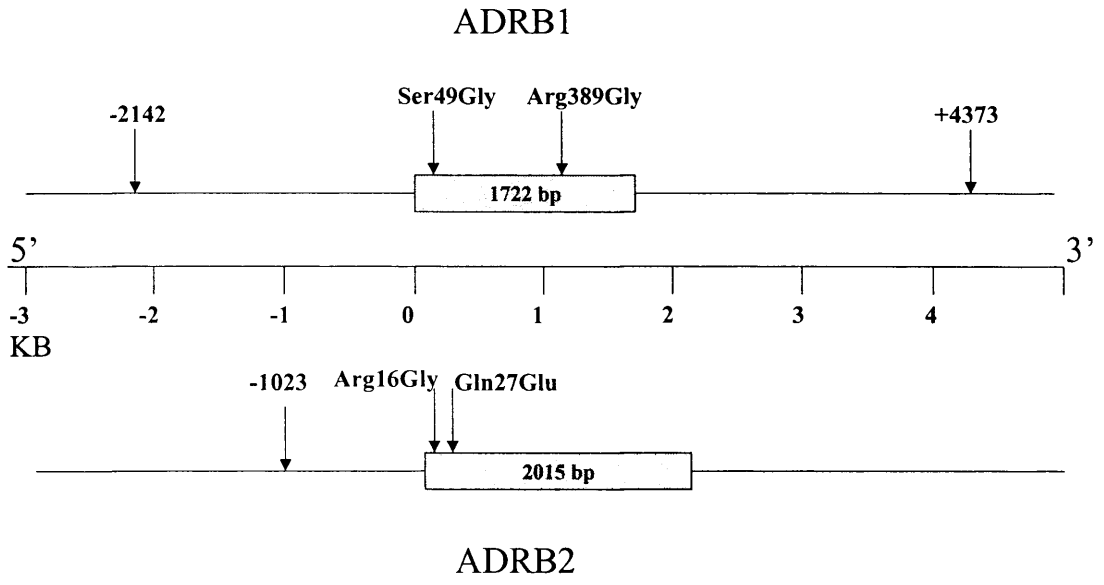
Tags were selected using criterion 2 in TagIT (http://www.genome.duke.edu/centers/pg2/tagit) with a minimum r^2 threshold of 0.9 and a minimum minor allele frequency of 0.05.

Genotyping of *ADRB1* and *ADRB2* polymorphisms (listed in table 7.1, also see figure 7.1) was performed TaqMan assay-by-design reagents for allelic discrimination (Applied Biosystems, as described in 2.3).

Table 7.1 *ADRB1* and *ADRB2* polymorphism frequencies in healthy (i.e. without CHF or hypertension) control individuals and the BEST population

Gene	Polymorphism (or relative position from transcript start)	Frequency in healthy controls[321 245]		Frequency in BEST population	
		EA	AA	EA	AA
ADRB1	T-2142**C**			0.134	0.429
	Ser49**Gly**	0.13	0.13	0.134	0.240
	Arg389**Gly**	0.28	0.43	0.281	0.429
	A4373**C**			0.139	0.249
ADRB2	rs2053044 (G-1023**A**)			0.402	0.427
	Arg16Gly	0.46	0.49	0.380	0.459
	Gln27**Glu**	0.35	0.18	0.405	0.185

Figure 7.1 *ADRB1* and *ADRB2* tags



7.2.4 Study design

We have separated the analyses into two types. Our primary analyses investigated the effect of genotype on response to treatment. Secondary to the tests of genotype effect, we have undertaken subgroup analyses of treatment effect within individual genotypes (for example the effect of treatment on LVEF change within the *ADRB1* T-2142C T/T genotype). We also investigated baseline LVEF and PNE levels, although these are not related to treatment.

For the primary analyses we tested seven polymorphisms (four functional plus three additional "tagging" SNPs) for association with time to death (survival time), time to death or first CHF hospitalization (hospital-free survival time), baseline LVEF and PNE

levels and change in LVEF and PNE levels. This makes for 42 tests in total. All tests were performed separately within EA and AA. We did not make an extra correction for analyses within each ethnic/racial group as the original trial reported only a nominally significant interaction between race and treatment so we would not expect any polymorphism to have an effect on the difference between EA and AA.

For the secondary, subgroup analyses we tested for associations between the seven polymorphisms and with time to death (survival time), time to death or first CHF hospitalization (hospital-free survival time), baseline LVEF and PNE levels and change in LVEF and PNE levels within each of the three genotype groups for each polymorphism. This makes for 252 tests in total. Again all tests were performed separately within EA and AA. For both sets of analyses the resulting multiple testing corrections are conservative as survival time and hospital-free survival time are positively correlated phenotypes and some polymorphisms are in almost complete linkage disequilibrium.

7.2.5 Statistical analysis

Endpoints were time to death (survival time), time to death or first CHF hospitalization (hospital-free survival time), and change in LVEF and PNE levels. Baseline LVEF and PNE levels were also investigated. The log-rank test was used to compare survival distributions (mortality rates) by race/ethnicity, genotype and treatment group. Cumulative survival curves were constructed by Kaplan–Meier methods[395]. The Cox proportional-hazards regression model was used to examine the effects of treatment stratified by the indicated genotype. For the clinical characteristics of the patients,

continuous variables were considered as mean ± SD, and comparisons were by t test for LVEF and Wilcoxon rank-sum tests for PNE (as data non-normal).

7.2.6 STRUCTURE analysis

A model-based clustering method implemented by the program STRUCTURE (available at http://pritch.bsd.uchicago.edu/structure.html) was used to assess the ancestry of individuals in terms of the proportion of their genome coming from specific genetic subclusters[396]. No information other than genotype data was taken into account in this procedure. Briefly, the model implemented in STRUCTURE assumes K clusters, each characterized by a set of allele frequencies at each locus. Under the admixture option the model then estimates the proportion of each individual's genome having ancestry in each identified cluster. This proportion was then included as a covariate in the analyses described above. Using Bayes' theorem, we found K=2 was most appropriate for our data. Other parameters are: Ancestry model = use population info (admixture model), MIGRPRIOR = default value, burnin = 100000, MCMC reps 100000, independent allele frequencies.

7.3 *Results*

7.3.1 *ADRB1* and ADRB2

7.3.1.1 Primary analyses: effects of genotype on response to treatment

Significant results (before Bonferroni correction for multiple comparisons) are summarised in table 7.2. No associations remain significant after Bonferroni correction for multiple comparisons. However, some associations are highly significant before correction for multiple testing and may warrant further investigation. The most interesting of those results is for the known functional polymorphism *ADRB2* Arg16Gly, which is non-significantly correlated with baseline LVEF in AA only (P=0.006, uncorrected). This is the first time this polymorphism has been correlated with LVEF.

Table 7.2 *ADRB1* and *ADRB2* genetic variation and response to bucindolol: Results from primary analyses.

Polymorphism	Outcome	Racial/ethnic subgroup	Responder genotype	P value (uncorrected)	P value (corrected for 42 tests)
ADRB1 A4373**C**	Overall survival	EA	C	0.0073	N/S
ADRB2 rs2053044 (G-1023**A**)	Change in LVEF	AA	A	0.0072	N/S
ADRB2 Arg16**Gly**	Baseline LVEF	AA	Gly	0.0060	N/S

7.3.1.2 Secondary analyses: effects of genotype on response to treatment - within genotype groups

Results are summarised in table 7.3. After correction for multiple testing we find that heterozygosity at the *ADRB2* Gln27Glu and at the *ADRB2* rs2053044 polymorphism is correlated with enhanced survival in response to bucindolol in EA (Figure 7.2). These associations remain marginally significant within EA alone but not within AA, although the AA results follow the same pattern as for EA. The *ADRB2* Gln27Glu and rs2053044 polymorphisms are in very high LD in EA (with only 8 recombinant chromosomes in all individuals genotyped), and so it is not possible to confirm which is driving any associations observed. There is no interaction between genotype and treatment, within each population and no three-way interaction between genotype, treatment and race. The Gln27Glu polymorphism differs in allele frequency between EA and AA (Table 7.1). It is possible that this polymorphism may explain largely or entirely the difference in treatment benefit between the two groups. When considering the two homozygous genotypes in EA there is no effect of treatment. In addition there is no difference in survival proportions between EA and AA heterozygotes taking bucindolol (60%). Although it is not clear whether the effect of this polymorphism is real, since the original result of treatment benefit in non-AA may not be real, these results do follow the same pattern and therefore provide a possible explanation for the disparity between AA and non-AA.

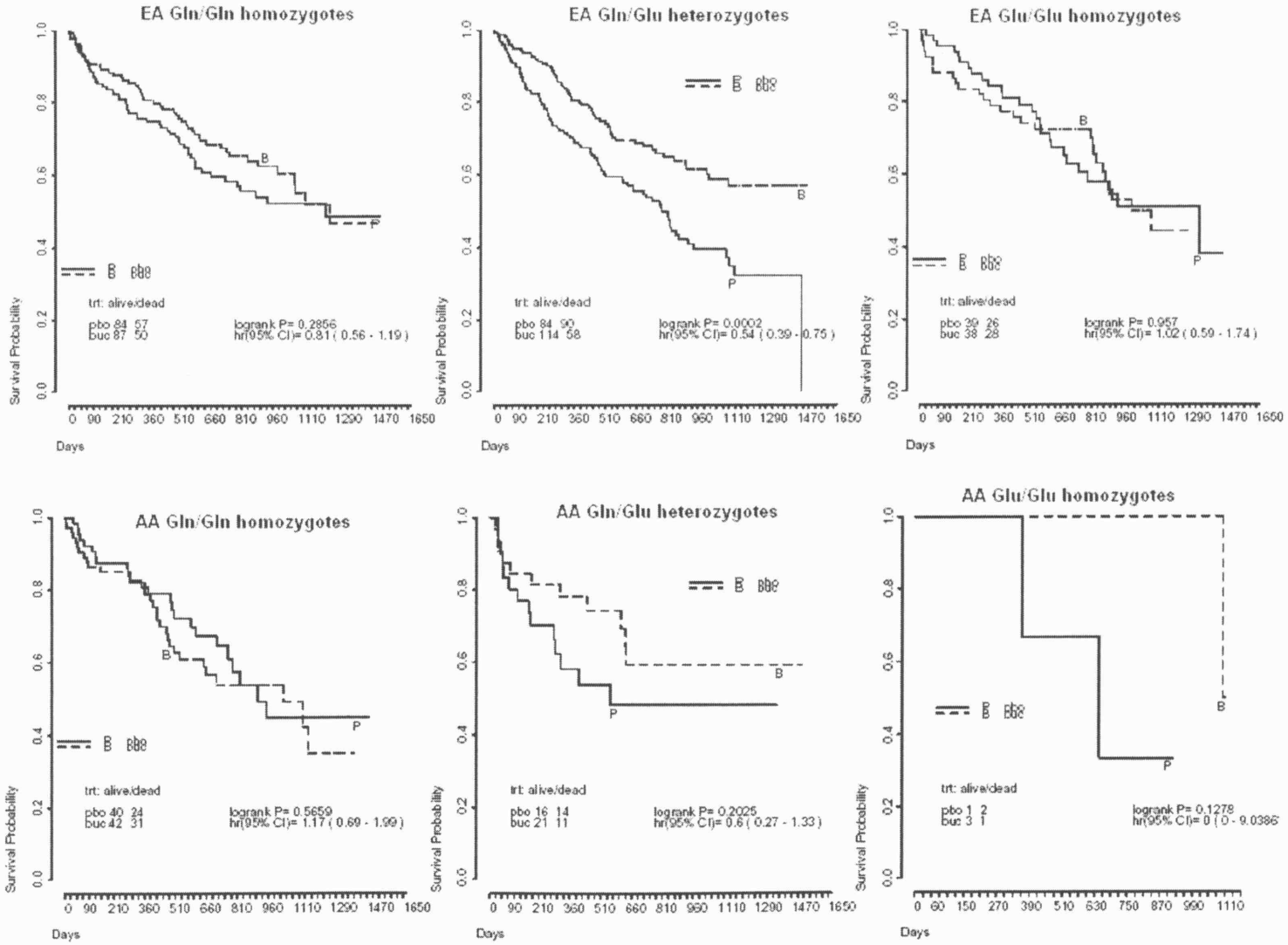
Again some further associations, which do not remain significant after correction for multiple tests, may warrant further investigation. In particular, for the *ADRB1* Ser49Gly and Arg389Gly functional polymorphisms, a non-significant correlation with treatment benefit is only observed in EA individuals with Ser/Ser and Arg/Arg genotypes, respectively (P=0.012 and P=0.0011, uncorrected). In previous studies the Gly allele at Ser49Gly has been associated with beta-blocker response in vitro[382], which is in contrast to our results, as has homozygosity for the Arg allele at Arg389Gly[45, 46, 320], which is in agreement with our results. For both polymorphisms however, it should be noted the 'responder' genotype was the most frequent.

Table 7.3 *ADRB1* and *ADRB2* genetic variation and response to bucindolol: subgroup analyses

Polymorphism	Outcome	Racial/ethnic subgroup	Responder genotype	P value (uncorrected)	P value (corrected for 252 tests)
ADRB1 T-2142C	Hospital-free survival	EA	TT	0.0019	N/S
ADRB1 Ser49Gly A/G	Hospital-free survival	EA	Ser/Ser A/A	0.0023	N/S
ADRB1 Arg389Gly C/G	Hospital-free survival	EA	Arg/Arg C/C	0.0011	N/S
ADRB1 A4373C	Hospital-free survival	EA	AA	0.0033	N/S
ADRB2 rs2053044 (G-1023A)	Hospital-free survival	EA	GA	0.0001	0.0252

ADRB2 Gln27Glu C/G	Hospital-free survival	EA	Gln/Glu CG	0.0001	0.0252

Figure 7.2 ***ADRB2*** **Gln27Glu polymorphism and response to bucindolol**



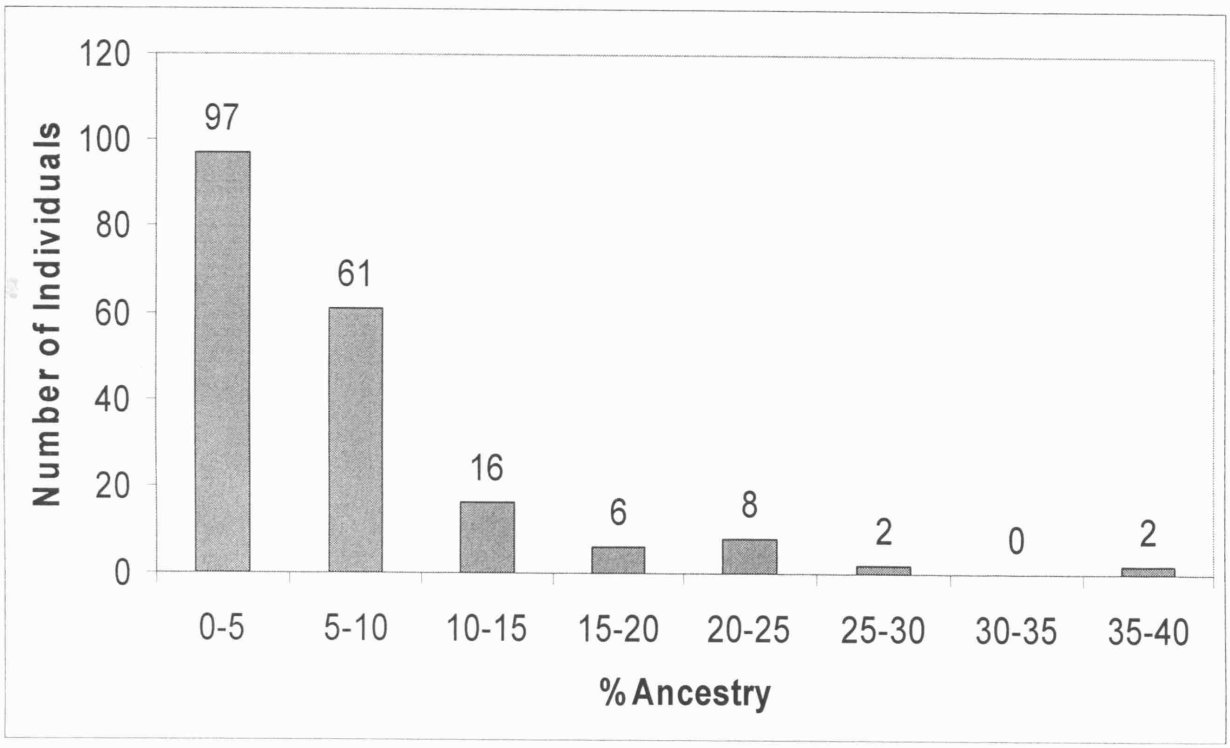
7.3.2 STRUCTURE analyses

As noted, bucindolol appears to improve survival benefit in non-AA and not in AA. In addition, many cardiovascular related drugs have been claimed to show differences in efficacy between AA and EA[393]. For this reason we assessed the genetic structure of the BEST population. The STRUCTURE analyses were carried out in 192 AA, and 192 EA samples (Table 7.4). We find that there are two ancestral groups inferred. The average proportion of European ancestry for AA in the BEST population is 22% with values ranging from 2% to 80%. This is in good agreement with other estimates of European ancestry proportion in AA (e.g.[380]). The STRUCTURE results reveal highly variable levels of genetic admixture within the BEST AA (Figure 7.3) but not within the EA (Figure 7.4).

Table 7.4 Genetic Structure of BEST Population and Cameroonian controls

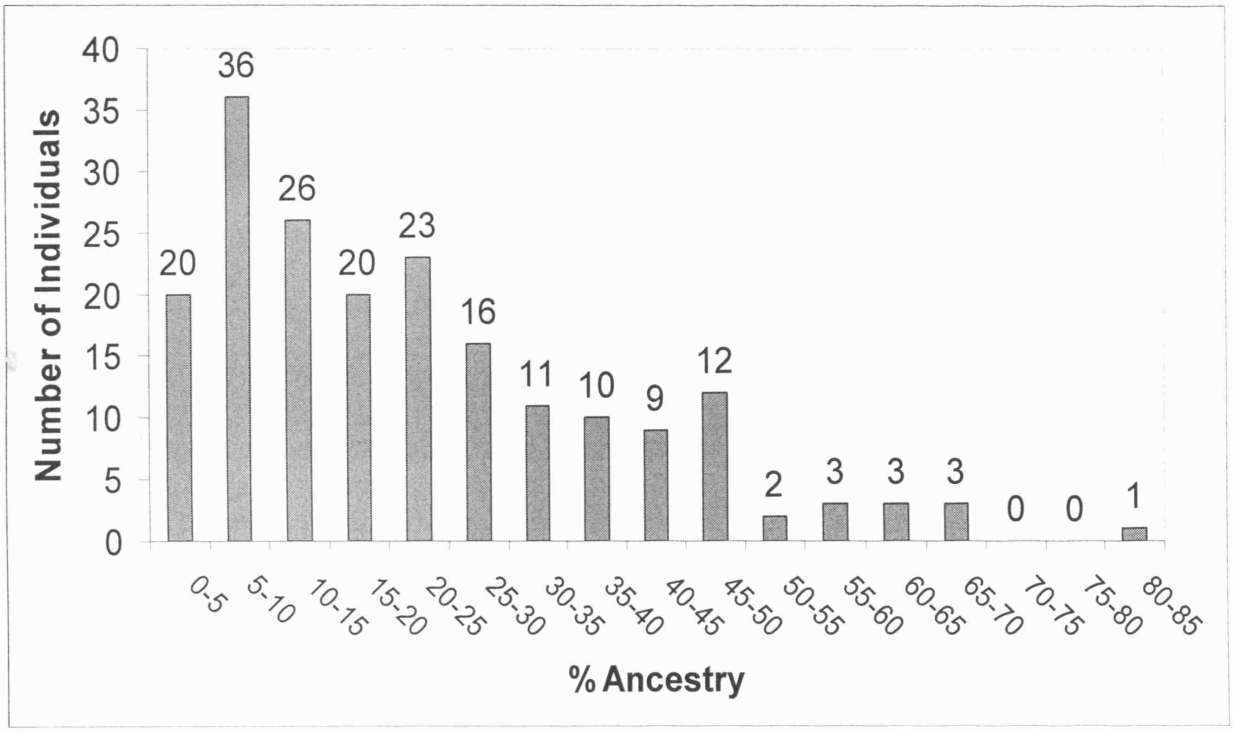
Self-identified population	Average % ancestry in each inferred cluster		No. Individuals
	1	2	
European American	0.93	0.07	192
African American	0.22	0.78	192
Cameroonian	0.09	0.91	98

Figure 7.3 Inferred % African ancestry for BEST European Americans



This figure demonstrates that there is little internal structure within the Americans of European ancestry

Figure 7.4 Inferred % European ancestry for BEST African Americans



This figure shows that the African-Americans have a broad range of ancestry proportions indicating substantial genetic structure. This may be relevant to drug response

We tested first whether admixture proportion explains any of the responses to bucindolol. We find no significant associations between admixture proportion and any measure of survival or drug response.

We next used admixture proportion as a covariate to test whether any of the significant (or approaching significance) effects of genetic variation in the *ADRB1* and *ADRB2* genes were due to stratification. In principle, if stratification due to the underlying West African and European subpopulations is generating a spurious association between a given SNP and an outcome variable, then

treating accurate estimates of admixture proportions as a covariate should eliminate or reduce that association. Unfortunately, this assessment is hard to make based on the reduced data set in which data are available and the significant results do not remain significant.

The results that come closest to significance in reduced sample are for the *ADRB2* rs2053044 polymorphism, where genotype has a borderline significant effect on survival in AA (P=0.06) and a borderline significant effect on LVEF change in all patients (P=0.08).

In both cases introducing admixture proportion into the model has no effect on significance, suggesting that stratification is not driving the results and instead there are some non-genetic aspects of race influencing the results. However, either genotype or phenotype correlations could be stratified.

7.4 Discussion

We have shown that heterozygosity at the *ADRB2* Gln27Glu and rs2053044 loci is associated with response to bucindolol in EA patients. AA heterozygotes appear to have similar survival probabilities as EA heterozygotes. There is no significant treatment benefit for either homozygous genotype, or for ~~all~~ both homozygote groups, within each subpopulation, for either polymorphism.

Assuming that the reported results are actually real, it is possible that the treatment benefit observed in heterozygotes alone is responsible for the observation that bucindolol only benefits non-AA. The Glu allele at *ADRB2* Gln27Glu polymorphism is less frequent in AA than EA (0.185 v 0.405) as are Gln/Glu heterozygotes (0.302 v 0.457). Therefore if this polymorphism were causal then the lower proportion of AA heterozygotes would make the association with treatment benefit more difficult to detect. That treatment benefit is not seen in AA may also be in part due to the smaller number of AA in the BEST population. These results illustrate the importance of investigating the underlying causes of drug response, when basing treatment on race or ethnicity. BiDil, a CHF drug that a combines isosorbide dinitrate (a nitric oxide donor) and hydralazine (an antioxidant and vasodilator agent), was approved for trial only in AA. The trial terminated early as the drug proved so effective. However the basis for differing response in not known. Whether the Glu27Glu polymorphism is responsible for, or contributes to, the ethnic/racial difference in bucindolol response will require further work, however, this result provides an example of how genetic differences could potentially account for average differences in drug response between racial or ethnic groups. These results also highlight how clinical trials are generally underpowered to investigate ethnic or racial differences in drug response, and particularly to investigate the underlying causes, e.g. genetic, of ethnic or racial differences in response.

Selection favoring the heterozygote is one of the mechanisms that maintain polymorphisms and help to explain some kinds of genetic variability. There are several cases in which the heterozygote conveys certain advantages and some disadvantages while both versions of homozygotes are only at disadvantages[397]. A well-established case of heterozygote advantage is that of the human beta-globin gene involved in sickle cell anaemia.

Another important feature of the results is the heterogeneity within AA. There is evidence that certain drugs, including beta-blockers, angiotensin I converting enzyme (ACE) inhibitors and angiotensin-receptor blockers may work differently, on average, in EA and AA[393]. These differences may reside in the interaction between the drug and receptor, to some extent determined by polymorphisms in the receptor, by polymorphisms associated with the severity or pathogenesis of the disease or by variations in genes responsible for drug metabolism that determine the concentration of active drug or the duration of exposure of the drug to its target[29]. Given the significant population structure within AA, it could be important to determine individual ancestry in biomedical research rather than relying on self-identified ethnicity.

Chapter 8 Conclusions

8.1 Directions in pharmacogenetics

It is clear that pharmacogenetics is undergoing a period of tremendous growth at present. A pubmed search using the keyword 'pharmacogen*' returns over 7000 publications, however, it is notable that over a third are review articles. I have attempted to summarize the current state of pharmacogenetics research in Chapter 1, however, it is likely to become increasingly difficult to keep abreast of new developments as the volume of publications increases. Resources such as the Pharmacogenetics and Pharmacogenetics Knowledge Base[398] which aims to "*curate information that establishes knowledge about the relationships among drugs, diseases and genes, including their variations and gene products*" could prove useful, however, it depends on submissions from the scientific community.

It is likely that the many of the most obvious pharmacogenetic variants, namely DME variants with large effects, have already been discovered and characterized. Future research will increasingly focus on searching for common variants with modest effects on drug response. Whilst the candidate gene approach of study design has delivered many successes, future research in pharmacogenetics is more likely to mirror efforts in disease gene mapping and will utilize similar population genetic techniques. Genomic data, including

HapMap data, will become more and more important as pharmacogenetics slowly shifts from a candidate gene model towards whole-genome studies.

8.2 Pharmacogenetics in epilepsy

Three of the four experimental chapters in this thesis concern the pharmacogenetics of anti-epileptic drugs (AEDs). There are many challenges associated with epilepsy pharmacogenetics in general. All three patients cohorts described in these chapters were recruited from tertiary referral centres and most patients were on AED polytherapy. It is thus difficult or impossible to disentangle the effects and interactions of different AEDs. The type and severity of epilepsy also plays a role in drug response. This illustrates the importance of prospective pharmacogenetics, ideally conducted on controlled, monotherapy cohorts. One key advantage of epilepsy pharmacogenetics is the availability (for a limited number of patients) of brain tissue from resection, which can be used in functional follow-up studies. This resource has generally been underutilized in epilepsy genetics, let alone pharmacogenetics.

The association between genetic variation in *SCN1A* and carbamazepine and phenytoin dose was the first example of an association between genetic variation in the target of an AED and drug response[1] and chapter 5 also describes how

genetic variation in the drug target for levetiracetam may influence response. Prior to these studies, most epilepsy pharmacogenetics studies had focused on variation in DMEs[295].

8.3 Drug target pharmacogenetics

In addition to the AED drug target pharmacogenetic associations reported this thesis, the chapter investigating genetic determinants of response to the beta-blocker bucindolol also described a drug target polymorphism in *ADRB2* that may influence drug response. Unlike, the AED results, this has not been replicated and the fact that it is the heterozygous genotype which is associated with response should be treated with caution.

It was noted in chapter 1 that drug target or target pathway pharmacogenetics is the fastest area of growth in this field. This is perhaps to be expected. A limited number of DMEs are responsible for the metabolism of the majority of drugs on the market today. All marketed drugs today target around 500 gene products[399] and the "druggable genome" (the number of genes which encode potential drug targets) is estimated to include between 2000 and 3000 genes[400]. Extending drug target pharmacogenetics to include components of the drug target pathway gives a huge pool of potential candidate genes for investigation.

8.4 Retrospective v Prospective study design

All association genetics studies described in this thesis were of retrospective design. Prospective studies are rare in pharmacogenetics although more common in disease predisposition genetics projects. Prospective studies have many advantages. Specific risk factor measurements can be included in the study and measured throughout its duration. Retrospective studies depend on data already collected without the hypothesis in mind. For example, the AED pharmacogenetic studies here required clinicians to go through historic patients medical records in order to collect relevant phenotype information. As the treating clinician did not record drug response data in order that a future pharmacogenetics study be performed there is a risk of incomplete or inaccurate data. Retrospective studies are also more prone to recall bias. The main reason for not performing prospective studies is cost. They are also more complex and time-consuming to carry out and suffer loss to follow-up. Given the economic cost of prospective studies it is sensible to perform initial retrospective investigations in order to inform the design of a more thorough prospective study.

8.5 Follow-up of associations

The genetic associations reported in this thesis are only the first steps in their respective pharmacogenetic studies. All require further follow-up work. The *SCN1A* IVS5-91 G>A polymorphism association with phenytoin maximum dose

and serum levels at maintenance dose, and with carbamazepine maximum dose, is the most secure. It has been functionally replicated twice, the polymorphism is predicted to have functional effects and preliminary *in vitro* experimental work appears to confirm altered function. However, there has not been an exact replication of an association between this polymorphism and any of the three phenotypes considered in this thesis. Further functional work is required to unambiguously establish an effect. Before this polymorphism can be used as a diagnostic in the clinic it is necessary also to evaluate it in a prospective clinical challenge. This will be challenging and will require careful design and funding.

The reported associations between genetic variation in *SV2A* and *SV2C* and levetiracetam response are less secure. Although a replication, of sorts, exists for each association (the two cohorts considered were phenotyped differently), the P values for the Dublin cohort associations are marginal. Furthermore, neither polymorphism has obvious function. Both are, however, currently being assessed in *in vitro* expression experiments. We are also currently extending this study to consider genetic variation in thirty seven genes associated with the SV2 pathway.

The *ADRB2* association with bucindolol response is tentative and is unlikely to be followed up by this group. More important are the associated issues concerning the conduct clinical trials and pharmacogenetic studies in populations of mixed race/ethnicity.

8.6 Concluding thoughts

The work described in this thesis provides a basis for future research into the pharmacogenetics of three antiepileptic drugs. It also demonstrates a number of principles in pharmacogenetic association studies, including the feasibility and validity of the tagging approach, particularly for genes where functional variants have not yet been characterized, and the candidate gene approach of study design. It is also important that genetic associations be replicated, preferably several times, in order to be considered secure. In the future it will become increasingly important to establish multi-centre collaborations in order to replicate associations and increase cohort sizes, particularly for the study selected cohorts. It is critical that causal variant(s) are determined and studied, in part to ensure transferability of diagnostics across populations.

It is hoped that the identification of genetic variants that influence drug response will lead to more targeted drug therapy. Ultimately, a key aim of pharmacogenetics is to tailor a patient's drug regimen according to his or her individual genetic make-up, resulting in greater efficacy and fewer adverse events. However, there is a long way to go before current pharmacogenetic research is utilized in a clinical setting and it will take the combined efforts of academia, industry and health care providers to make this a reality.

Publications arising from this thesis

Research Papers

Tate, S. K., Depondt, C., Sisodiya, S. M., Cavalleri, G. L., Schorge, S., Soranzo, N., Thom, M., Sen, A., Shorvon, S.D., Sander, J. W., Wood, N. W., Goldstein, D. B. Genetic predictors of clinical use of the anti-epileptic drugs phenytoin and carbamazepine. *Proc.Natl.Acad.Sci.U.S.A* **102**, 5507-5512 (2005).

Tate, S.K. et al. A common polymorphism in the *SCN1A* gene associates with phenytoin serum levels at maintenance dose. *Pharmacogenet Genomics.,***16(10)**, 721-726 (2006)

Tate, S.K. et al. Genetic variation in *SV2A* and *SV2C* influences response to the antiepileptic drug Keppra. [In preparation]

Review Papers

Goldstein, D. B., Tate, S. K., & Sisodiya, S. M. Pharmacogenetics goes genomic. *Nat.Rev.Genet.* **4**, 937-947 (2003).

Tate, S. K. & Goldstein, D. B. Will tomorrow's medicines work for everyone? *Nat.Genet.* **36**, S34-S42 (2004).

Tate, S. K. & Goldstein, D. B. Pharmacogenetics and the treatment of cardiovascular disease. *Handbook of Experimental Pharmacology: Cardiovascular Pharmacogenetics* **160**: 25-37 (2004).

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