Pharmacogenetics of CYP2D6 and CYP2C19 as a pre-prescription tool for drug efficacy and toxicity in a demographically-representative sample of the South African population

by

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DECLARATION

I Tyren Mark Dodgen declare that the entirety of the work contained in this thesis which I am submitting to the University of Pretoria for the degree PhD Pharmacology is my own, original work and that I have not submitted this work to any other tertiary institution for any degree. I am aware of the University of Pretoria's policy regarding plagiarism and due disclosure, acknowledgement and/or reference was made by work contributing to joint research and/or publication.

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SUMMARY

The Cytochrome P450 family of enzymes is responsible for the majority of Phase I metabolism, and has been identified as an important source of pharmacokinetic variation in therapeutic responses. CYP2C19 and CYP2D6, metabolising >35% of commonly prescribed medications, are two of the most important pharmacogenetic markers that have been studied with the aim of improving treatment response and reducing adverse drug reactions.

The Food and Drug Administration (FDA) approved AmpliChip CYP450 Test (AmpliChip) was compared to a previously developed PCR-RFLP platform and a newly developed XL-PCR+Sequencing platform for the ability to identifying genotype and predicting phenotype for CYP2C19 and CYP2D6 respectively. The AmpliChip was found not to be genotypically comprehensive enough for evaluating *CYP2C19* genotype, not robust enough for determining *CYP2D6* genotype and inaccurate in predicting phenotype for both. The XL-PCR+Sequencing method identified three novel alleles and one sub-variant.

Advances in online column-switching solid phase extraction generated a rapid and robust LC-MS/MS method for simultaneously quantifying the probe drugs omeprazole (CYP2C19 substrate), dextromethorphan (CYP2D6 substrate) and their metabolites. Antimodes were identified for phenotypic cut-offs which offered measured phenotype for comparison to predicted phenotype.

Omeprazole metabolism by CYP2C19 correlated well with predicted phenotype in a demographically representative South African cohort. There are concerns regarding the use of omeprazole as a probe drug as participants predicted to be ultrarapid metabolisers for CYP2C19 had similar rates to extensive metabolisers. Regardless of this concern, decreased metabolism was assigned to the *CYP2C19*15* for the first time.

CYP2D6 predicted phenotype correlated very well with measured phenotype, validating the suitability of dextromethorphan use for measuring CYP2D6 metabolism. Substrate modified activity score using 0.5 to predict intermediate metabolisers fine-tuned the XL-PCR+Sequencing platform for phenotype prediction. This finding, along with observations in CYP2C19 metabolism of omeprazole, highlights the importance of substrate specific phenotype prediction strategies.

Controversially, attempts to associate CYP2D6 phenotype prediction with risperidone-related adverse drug reactions has yielded conflicting results. The XL-PCR+Sequencing platform was able to discount this association by predicting a variety of metabolisers in a pilot cohort selected to be experiencing risperidone-related adverse drug reactions. The comprehensive capability of the XL-PCR+Sequencing allowed for the identification of an additional novel allele in this cohort.

The data presented in thisthesis has provided insight into the relationship between predicted and measured phenotype for CYP2C19 and CYP2D6 in the South African population. The XL-PCR+Sequencing platform can be used for future research or can be applied to improve treatment outcome. The LC-MS/MS method developed could be used for future evaluations of predicted and measured phenotype with the ability to be adjusted for therapeutic drug monitoring. This thesis advances pharmacogenetics of CYP2C19 and CYP2D6 for use in the South African population.

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LIST OF SYMBOLS AND ABBREVIATIONS

$	American Dollar (currency)
%	Percentage
&	Ampersand (and)
ęC	Degrees Centigrade
ú	European Euro (currency)
<	Less Than
=	Equals to
>	Greater Than
Ò	Less than or equal to
Ó	Greater Than or Equal to
©	Copyright
3ô	Three Prime End of DNA
5ô	Five Prime End of DNA
5OH	5'-hydroxyomeprazole
Ŭ	Alpha
A	Adenine
A/Ala	Alanine
ABCB1	Gene Coding for P-glycoprotein 1(drug transporter protein)
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ADME	Absorption, Distribution, Metabolism and Elimination
ADR(s)	Adverse Drug Reaction(s)
AmpliChip	AmpliChip CYP450 Test
ANOVA	Analysis of Variance statistical test
AS	Activity Score
ɓ	Beta
BMI	Body Mass Index
C	Cytosine
C/Cys	Cystein
CA	California
cDNA	Complementary Deoxyribonucleic Acid
C_{max}	Maximal Drug Plasma Concentration
CV	Coefficient of Variation
CYP	Cytochrome P450
CYP1A2	Cytochrome P450 gene 1A2
CYP2A6	Cytochrome P450 gene 2A6
CYP2B6	Cytochrome P450 gene 2B6
CYP2C19	Cytochrome P450 gene 2C19
CYP2C8	Cytochrome P450 gene 2C8

CYP2C9	Cytochrome P450 gene 2C9
CYP2D6	Cytochrome P450 gene 2D6
CYP2D7	Cytochrome P450 pseudogene 2D7
CYP2D8	Cytochrome P450 pseudogene 2D8
CYP2E1	Cytochrome P450 gene 2E1
CYP3A4	Cytochrome P450 gene 3A4
CYP3A5	Cytochrome P450 gene 3A5
del	Deletion
DM	Dextromethorphan
DNA	Deoxyribonucleic Acid
Dr.	Doctor of Philosophy (Latin, philosophiae doctor)
DX	Dextrorphan
E/Glu	Glutamic Acid
e.g.	*Exempli gratia*/for example
EDTA	Ethylenediaminetetraacetic Acid
EM	Extensive Metaboliser
EPS	Extrapyramidal Symptoms
ESI	Electronspray Ionisation
et al.	*et alii*/and others
etc.	*et cetera*/and so on
F/Phe	Phenylalanine
FDA	Food and Drug Administration of the United States of America
FISH	Fluorescence *in situ* hybridization
FLD	Fluorescence Detection
g	Grams
g	Gravity
G	Guanine
G6P	Glucose-6-phosphate gene
GCP	Good Clinical Practice
gDNA	Genomic Deoxyribonucleic Acid
GWAS	Genome Wide Association Study
H/His	Histidine
HER2	Human Epidermal Growth Factor 2 Receptor
HPLC	High Performance Liquid Chromatography
I	Roman Numeral for the Number One
I/Ile	Isoleucine
ICH	International Conference on Harmonization
IHC	Immunohistochemistry
II	Roman Numeral for the Number Two
IL	Illinois
IM	Intermediate Metaboliser
Inc.	Incorporation

Indel	Insertion or Deletion
INS	Insertion
K/Lys	Lysine
kb	Kilobase Pairs
L/Leu	Leucine
LC-MS	Liquid Chromatography Single Quadrupole Mass Spectrometry
LC-MS/MS	Liquid Chromatography Coupled to Tandem Mass Spectrometry
LC-TOFMS	Liquid Chromatography Coupled to Time of Flight Mass Spectrometry
LD	Linkage Disequilibrium
LLE	Liquid-Liquid Extraction
LLOD	Lower Limit of Detection
LLOQ	Lower Limit of Quantification
M/Met	Methionine
MD	Medical Doctor
MDR1	Multidrug Resistant Protein 1
mg	Milligrams
mL	Millilitres
MR	Metabolic Ratio
MRM	Multiple Reaction Monitoring
mRNA	Messenger Ribonucleic Acid
ng	Nanogram
NJ	New Jersey
nm	Nano Meters
OME	Omeprazole
P	Probability
P/Pro	Proline
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR based Restriction Fragment Length Polymorphisms
PCSI	Score used by PolyPhen to predict a SNPôs effect on a protein
pg	Picogram
PhD	Doctor of Philosophy (Latin, *philosophiae doctor*)
PM	Poor Metaboliser
PolyPhen	Polymorphism Phenotyping
PPT	Protein Precipitation
Prof.	Professor
RNA	Ribonucleic Acid
RSD	Relative Standard Deviation
RSP	Risperidone
RT-PCR	Real-Time Polymerase Chain Reaction
Q/Gln	Glutamine
R/Arg	Arginine
S/Ser	Serine

SIFT	Sorting Intolerant from Tolerant
SNP	Single Nucleotide Polymorphism
T	Thymine
T/Thr	Threonine
TDM	Therapeutic Drug Monitoring
TFPGA	Tools for Population Genetic Analysis
T_{max}	Time Following Administration to Reach C_{max}
Ê	Trade Mark
µg	micrograms
µl	Microlitres
µm	Micrometer
UK	United Kingdome
UM	Ultra-rapid Metaboliser
USA	United States of America
UTR	Untranslated Region
UV-Vis	Ultraviolet or Visual Spectrum Detection
V/Val	Valine
WI	Wisconsin
X	Stop Codon
X^2	Chi Square

CHAPTER 1. INTRODUCTION

1.1 Pharmacology: a brief history

Quite possibly from the dawn of mankind, extracts of plant material, animal remains and various other materials, have formed the basis of therapeutic relief. Indications were initially founded predominantly on a trial and error basis, postulated by observation. The birth of modern or western medicine late in the 19th century brought with it the promise of antidotal type remedies. This has arisen from proposals of many theories such as Rudolf Virchowôs cell proposal (Povzun, Mal'kov & Frank 2011), Jöns Jacob Berzelius (a Swedish chemist) using structural formulae to described chemical compounds and Louis Pasteur discovering bacteria as a source of disease etc., all of which have contributed to the formation of evidence based therapy. Interdisciplinary understandings of physiology, pathology and chemistry became the cornerstone of understanding in western medicine. Subsequently, the use of these combined disciplines to study the therapeutic effect that a substance has on the body became a distinct discipline to itself, and was named pharmacology.

Although medicinal plants remain a vital component of therapy today, particularly in rural and economically challenged communities (Cook 2009), the true approach to herbal remedies remains an archaic discipline, hinged largely on observations and teachings passed on through generations (Camp et al. 2012). Starting with the isolation of morphine from opium poppy (*principium somniferurri)* by Friedrigh Wilhelm Sertürner in 1806 (Hartmann 2007), many lead compounds have been isolated from plants, fungi, microorganisms and various other sources, thereby contributing significantly to the discipline of pharmacology. Additional examples include digoxin from foxglove (*Digitalis purpurea*), salicylic acid (precursor to the analgesic acetylsalicylic acid which has many other indications as well) from willow bark (*Salix spp.*), penicillin from mould (*Penicillium* rubens), the antipsychotic/antihypertensive reserpine (*Rauwolfia spp*) and lovastatin from the oyster mushroom (*Pleurotum ostreatus*), and the list goes on (Rishton 2008; Li & Vederas 2009; Wachtel-Galor & Benzie 2011). Approximately 70% of the drugs indicated for cancer treatment worldwide have been derived from isolates from natural sources and include

paclitaxel, camptothecin, irinotecan, topotecan, combretastatin etc. (Brower 2008; Wachtel-Galor & Benzie 2011).

The beginning of the 20th century brought with it the breakthrough in synthetic chemistry. In the process of attempting to make arsenical compounds less toxic Paul Ehrlich coincidentally synthesised salvarsan (The Magic Bullet) in 1909. Salarsan proved to be an effective treatment for syphilis (Winau, Westphal & Winau 2004). This humble event revolutionised pharmacology and the pharmaceutical industry, sparking the production and appearance of many different drugs for many different disease indications. Investigating pharmacological activity of herbal compounds has illuminated structural characteristics inspiring the chemical synthesis of new drugs (Vasilevich et al. 2012). Unfortunately, the rate at which new therapeutic compounds were discovered and synthesised in the first half of the 20th century has not been echoed in recent times. The trend seems to be on the decline. With synthetic compounds came answers to many medical mysteries, but this presented new challenges, while establishing pharmacology among the biomedical sciences.

Parallel to the development of modern pharmacology, a deeper understanding of physiology was uncovered. Research into hormones, neurotransmitters and inflammatory mediators helped direct treatment towards pharmacological manipulation of homeostasis. From this, the concept of receptor mediation was uncovered. Further understanding of chemical mediation and pharmacological influence has proved to be pivotal in drug discovery and therapy. Biochemistry was also established during this period. A distinct science emerged as highlighted by the discovery of enzymes and biochemical pathways, dealing in part, with the metabolism and clearance of drugs. This further contributed to the understanding of pharmacology and evolved into the discipline of ADME (absorption, distribution, metabolism and elimination) which aims to understand what happens to a drug once ingested.

1.2 Adverse drug reactions

Along with the therapeutic benefits of pharmacotherapy, physicians started to notice additional effects experienced by their patients. Some were beneficial, but many were unwanted. These unwanted effects are commonly referred to in ñlayman termsò as side effects, but in therapeutic terms the phrase adverse drug reactions (ADRs) has become commonly used,and refers to effects that causes new symptoms requiringintervention. ADRs have a major impact on health, both on an individual and national level (Allison 2008; Wester et al. 2008). Nuckols et al.(2008) observed an average ADR frequency of 10.4% in American patients monitored in both academic and non-academic hospitals. Of those experiencing ADRs, 83% received intravenous medication including opiates, benzodiazepins, anticoagulants and insulin. Intervention to sustain life was as high as 20.0%, in-hospital death at 2.0% and 26.0% of ADRs were preventable, suggesting a need to intervene (Nuckols et al. 2008). A UK cohort analysed by Davis et al.(2009) observed ADR frequency to be 14.7%. Generally the frequencies of ADRs leading to hospitalisation varies extensively in Europe, as figures of 1.8% were observed in the Netherlands (van der Hooft et al. 2006), 2.0% in Romania (Farcas et al. 2010) 3.2% in France (Pouyanne et al. 2000), 3.3% in Germany (Rottenkolber et al. 2011) 3.6% in Italy (Capuano et al. 2004), 6.5% in Great Brittan (Pirmohamed et al. 2004), 8.4% in Denmark (Hallas et al. 1992) and 12.8% in Greece (Alexopoulou et al. 2008). An observational study in South India found that on average, 2.6% of hospitalisations were due to ADRs and 14.5% of patients were documented to experience ADRs while in hospital (Rajakannan et al. 2011). Patients in South India experiencing ADRs tend to spend on average 2 days longer in hospital, compared to unaffected patients (Rajakannan et al. 2011). Other studies found hospital stays to be 2.3 days longer in Germany (Rottenkolber et al. 2011) and as much as 5 days longer in USA hospitals (Nuckols et al. 2008).

Although most studies report similar frequencies for ADRs, there is concern that these observations may be somewhat lower than reality, as under reporting may be common (Hazell & Shakir 2006). In a meta-analysis of data from 37 studies from 12 different countries, the median of under-reporting was found to be as high as 94.0% (interquartile range of 82.0 ï 98.0%). This demonstrates significant widespread under-reporting in both hospitals and general practice (Hazell & Shakir 2006). More recently Lopez-Gonzalez et

al.(2009) estimated under-reporting of ADRs to be somewhat lower at 74%, but still significantly high and clinically concerning. Reasons for under reporting may include ignorance (only severe ADRs reported), lethargy (procrastination or lack of interest etc.), indifference (perceived lack of contribution to knowledge), insecurity (not confident in the diagnosis of ADRs) and complacency (Lopez-Gonzalez, Herdeiro & Figueiras 2009).

There is no doubt that ADRs are an unwanted burden in modern medicine, both clinically and financially, and that it is more cost effective to reduce ADRs which will free up beds in hospitals. Post event ADR costs each patient on average $6647 in the USA (Nuckols et al. 2008). After adjusting their prices according to consumer price indices for hospital and related services, Nuckols et al.(2008)found this cost to be comparable to other studies. In Germany the cost per patient has been estimated at ú2250 (Rottenkolber et al. 2011).

In South Africa, very little is known regarding ADRs. Mehta et al.(2008) estimated that 14.0% of South African hospitalised patients experience ADRs. In addition, there appears to be a 5-10 fold higher fatality rate in South African hospitals compared to those in the USA and UK. Although higher than other reported frequencies, South Africa as a developing country does not have the resources to afford the burden of cost associated with ADRs. What are the ADR cost implications for South Africans? The truth is that no one really knows. South Africaôs health priorities (http://www.doh.gov.za/docs/reports/2010/overview1994-2010.pdf) include infectious diseases as well as diseases of lifestyle commonly seen in developing and developed countries (such as diseases of lifestyle and cancer). The South African health care system should be far more aware of the ADRs, as the State cannot afford the subsequent morbidity/mortality and associated cost implications.

1.3 Combating ADRs

Inter-individual differences in the way people respond to various commonly prescribed drugs often manifest as ADRs. These ADRs often contribute to diminished patient compliance as a result of ADRs and/or ineffective treatment. In order to reduce these risks and associated financial implications, a shift is occurring towards personalised medicine (Burroughs, Maxey & Levy 2002). Personalised medicine influences drug treatment regimens by allowing prescription of optimal drugs and dosages that are appropriate for each individual patientôs

condition and status. In order to personalise treatment regimens, genetic variation is an important avenue that scientists need to pursue. A strong correlation between interpersonal genetic variation and drug response has been identified for several genes and related enzyme substrates (Ingelman-Sundberg 2004; Gardiner & Begg 2006; Daly 2010; Scott 2011). These principles define the field of pharmacogenetics, which many believe will in future contribute significantly to the success of modern medicine (Wolf & Smith 1999; Ingelman-Sundberg 2004; Scott 2011). Ingelman-Sundberg et al. (2004) estimated that pharmacogenetics may reduce ADRs by 10-20% and improve drug efficacy of 10-15%, suggesting that pharmacogenetics would then have a lot to offer South African health care.

One of the prominent focus areas of pharmacogenetics is pharmacokinetics, which offers insight intovariable drug metabolism (Deenen et al. 2011; Niwa, Murayama & Yamazaki 2011; Hiratsuka 2012) and therapeutic response (Wilkinson 2005; Zhou 2009; Johansson & Ingelman-Sundberg 2011; Walko & McLeod 2012). The group of enzymes responsible for a large portion of Phase I metabolism of exogenous and exogenous compounds is the Cytochrome P450 (CYP) family (Wilkinson 2005). This thesis investigates the pharmacogenetics of two CYP enzymes, CYP2D6 and CYP2C19 both for phenotype prediction and measurement. By analysing both predicted and measured phenotype, the relationship can be evaluated as one of the first steps in introducing pharmacogenetics to South African health care. A mixed South African cohort was sampled, representing the diversity of the current South African population. However, no attempt was made to compare the different racial groups in the country.

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CHAPTER 2. LITERATURE REVIEW

2.1 Pharmacogenetics

The discipline of using a specific genetic factor to elucidate inexplicable *in vivo* variation in drug response, both in terms of drug efficacy and toxicity, is called pharmacogenetics. Of course the term ódrugô is used in the broadest sense of the word, as it may encompass both therapeutic and recreational drugs, which may be of synthetic or phytomedicinal origin. Thus the term ódrugô could include almost any xenobiotic (exogenous compound) which an individual may be exposed to. The genetic component of pharmacogenetics pertains to polymorphisms in or around a specific gene and may include single nucleotide polymorphism (SNP), insertion or deletion of nucleosides or even entire genes. These polymorphisms are transcribed to form mRNA containing incorrect or missing code. This incorrect code may then cause the translation of alternate amino acids andtheir incorporated into a protein product. Ultimately the polymorphisms may affect the protein's function (e.g. incorrect folding of protein, protein truncation, etc.) which in turn alters the phenotype.

Once a strong correlation has been established between a variable phenotype and genetic variability, pharmacogenetics has the potential to predict phenotype. The intention of pharmacogenetic analysis would be to assist physicians with optimal drug choice and dosing regimens (personalised medicine). This type of personalised medication would improve therapeutic efficacy and reduce ADRs (Ingelman-Sundberg 2005; Zhou 2009; Johansson & Ingelman-Sundberg 2011; Scott 2011; Teh & Bertilsson 2012). The best areas for application of primary interest in pharmacogenetics would be for drugs with narrow therapeutic windows/indices and debilitating ADRs. Narrow therapeutic index drugs have a smaller margin for error and the extent of the adverse effect will influence whether or not a patient or physician would consider intervention.

The potential for pharmacogenetics is only limited by the ability to accurately identify genes associated with variable drug responses and the changes that occur in these genes.

Therefore, any gene involved in pharmacokinetics or pharmacodynamics (expanded on below) has the potential to influence drug response and may be used as a pharmacogenetic indicator for personalised medicine. However it is important that a strong correlation between genotype and clinical phenotype is established (polymorphisms should be the major influencing factor in drug response).

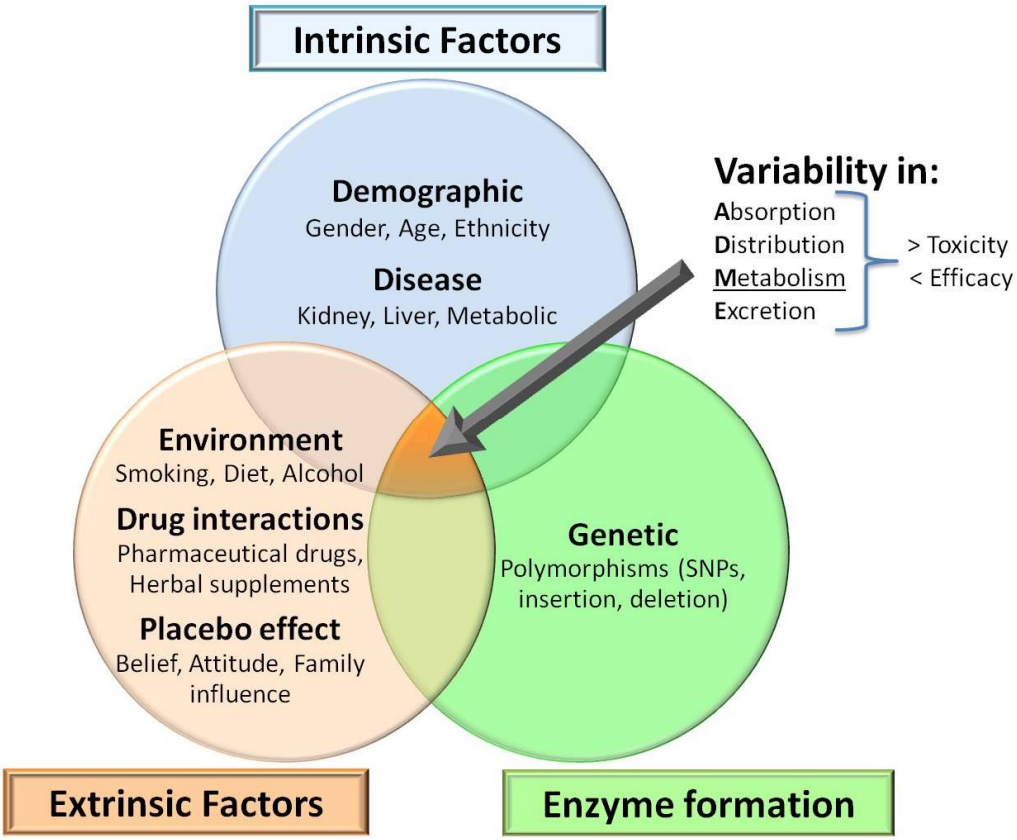

Figure 2-1. Factors that contribute to variability in drug response.
Figure created by Tyren Mark Dodgen, adapted from Burroughs et al. (2002).

Additional influences which may confound the ability to predict phenotype are typically non-genetic. Non-genetic influence include ethnicity, diet, drug-drug interactions, smoking, gender, age, liver size and function and concomitant diseases or physiological parameters (refer to Figure 2-1), all of which may influence drug metabolism but would not be detected by genotyping (Burroughs et al. 2002). Another important influence is epigenetics which has been shown to play a significant role in the up or down regulation of gene expression and translation of mRNA (Gomez & Ingelman-Sundberg 2009).

2.2 Brief history of pharmacogenetics

In a review entitled, ñPharmacogenetics ï five decades of therapeutic lessons from genetic diversityò by Meyer (2004), the author hastraced the history of pharmacogenetics. Since Gregor Mendel observed what has become known as Mendelian Inheritance, scientists have become attentive to the concept of inheritance. Put simply, inheritance pertains to observable characteristics which family members have in common. Therefore the characteristic (phenotype) may have been passed through the generations in an inherent fashion. Characteristics do not have to be exclusive to familial observations, as various distinct communities in multiracial populations have been observed to share common characteristics (McKusick 1998). In this way de novo mutations (mutations which are not inherited) may have an important role in perceived phenotype.

In the 1950ôs, three striking observations were made in the field of medicine. During World War II, soldiers were developing an acute haemolytic response when given a therapeutic dosage of primaquine or other antimalerial drugs. The haemolytic response seemed to be more prominent in African American soldiers compared to their Caucasian colleagues (Meyer 2004). Later, the haemolytic response was found to be linked to a deficiency in erythrocyte metabolism, associated with the glucose-6-phosphate (*G6P*) gene. Located on the X chromosome, any one of 135 defective *G6P* polymorphisms could be responsible for haemolysis. In addition to many different medications, *G6P* defective individuals tend to be susceptible to various chemicals, fava beans, infectious diseases and neonatal jaundice (Tripathy & Reddy 2007; Schuurman et al. 2009). Succinylcholine (suxamethonium) is a neuromuscular junction depolarisation-blocking drug intended for short term muscle relaxation during anaesthesia, and observations made relative to the unintended effect of this drug in certain individuals led to the second observation. When certain patients were treated with succinylcholine a prolonged apnoea (muscular paralysis of the respiratory muscles) occurred which was later associated with polymorphisms in the pseudocholinesterase (butyrylcholinesterase) gene (Goodall & Association of Clinical Biochemists Analytical Investigations Standing Committee 2004). Finally, and perhaps the most well-known example,

particularly in the South African context, is slow acetylation of isoniazide used in the treatment of tuberculosis. Forty years after this phenotype was observed, a strong association with *N*-Acetyltransferase-2 (*NAT2*) polymorphisms was described (Blum et al. 1991; Vatsis, Martell & Weber 1991).

Using the first two observations together with the observations of barbiturate-precipitated porphyria and hereditary hyperbilirubinemia, Motulsky (1957) highlighted ñhow hereditary gene-controlled enzymatic factors determine why, with identical exposure, certain individuals become ósickô, whereas others are not affectedò. Shortly thereafter, Vogel (1959) coined the term ñPharmacogeneticsò, and with the further contribution of Kalowôs (1962) monograph on pharmacogenetics, a new discipline was born. The development of polymerase chain reaction (PCR) in 1985 allowed specific sections of DNA from genetic material to be amplified exponentially and to be distinguish from genomic DNA (gDNA); this contributed significantly to the exponential growth of pharmacogenetics (Meyer 2004). Figure 2-2A demonstrates this exponential growth as a plot of the number of publications (original research and reviews) mentioning the word pharmacogenetics over the last 20 years. The number of articles published annually appears now to have reached plateau. This could result from several reasons, but one opinion is that pharmacogenetics has failed to make old and new medication safer or more effective. Of course this failure could also be the result of medication still being prescribed for most conditions on a óone drug fits allô basis (Gurwitz & Motulsky 2007), but it is also likely that there has not been sufficient adoption of pharmacogenetics by clinicians (Gurwitz et al. 2009). If pharmacogenetics has failed, the number of publications would surely be dropping as scientists and the medical community in general, loose interest. A more plausible reason may be economic recession which was hardest felt around 2009 and the global economy has not recovered adequately to date (Hayden 2011). The dawning of pharmacogenomics is another factor which may have slowed pharmacogenetic publications. Pharmacogenomics first appeared in literature in 1997 (Meyer 2004) and Figure 2-2B demonstrates that, as with pharmacogenetics, the field pharmacogenomics is growing exponentially. Instead of pharmacogenomics being distinctly different from pharmacogenetics, it is really a complementary concept, as pharmacogenomics deals

with multiple genes over an entire genome (a whole-genome approach) affecting response to drugs (Daly 2010; Hulot 2010), where pharmacogenetics can be seen as dealing with a specific gene affecting a drug response. Therefore it could be said that pharmacogenetics explains monogenic drug response and looks at specific genes, whereas pharmacogenomics is used for polygenic drug responses. Advances in knowledge of the human genome in projects such as Human Genome Project (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml) and HapMap (http://hapmap.ncbi.nlm.nih.gov/) as well as advances in high-throughput DNA assessment technologies will contribute significantly to advancing both fields and possibly make personalised medicine a reality.

Meyerôs (2004) time line notes that between 1988 and 1990, the first cloning and characterisation of *CYP2D6* was described. Polymorphisms in *CYP2D6* were linked to defective debrisoquine/sparteine metabolism identified in a collaborative effort between Gonzalez and Meyer (Distlerath et al. 1985; Skoda et al. 1988). Conversely Johansson, Ingelman-Sundberg and Bertilsson discovered that over expression of *CYP2D6* would result in an ultra-rapid metaboliser phenotype, attributed to repetition of functional copies of the *CYPD6* gene (Bertilsson et al. 1985; Johansson et al. 1993). De Morais et al. (1994) with the help of Goldstein, cloned and characterised *CYP2C19*. Many other genes have been scrutinised for their pharmacogenetic potential. In 2000 the first comprehensive pharmacogenetic knowledge data base, PharmGKB was established as a repository to harbour this information (Altman 2007; Whirl-Carrillo et al. 2012).

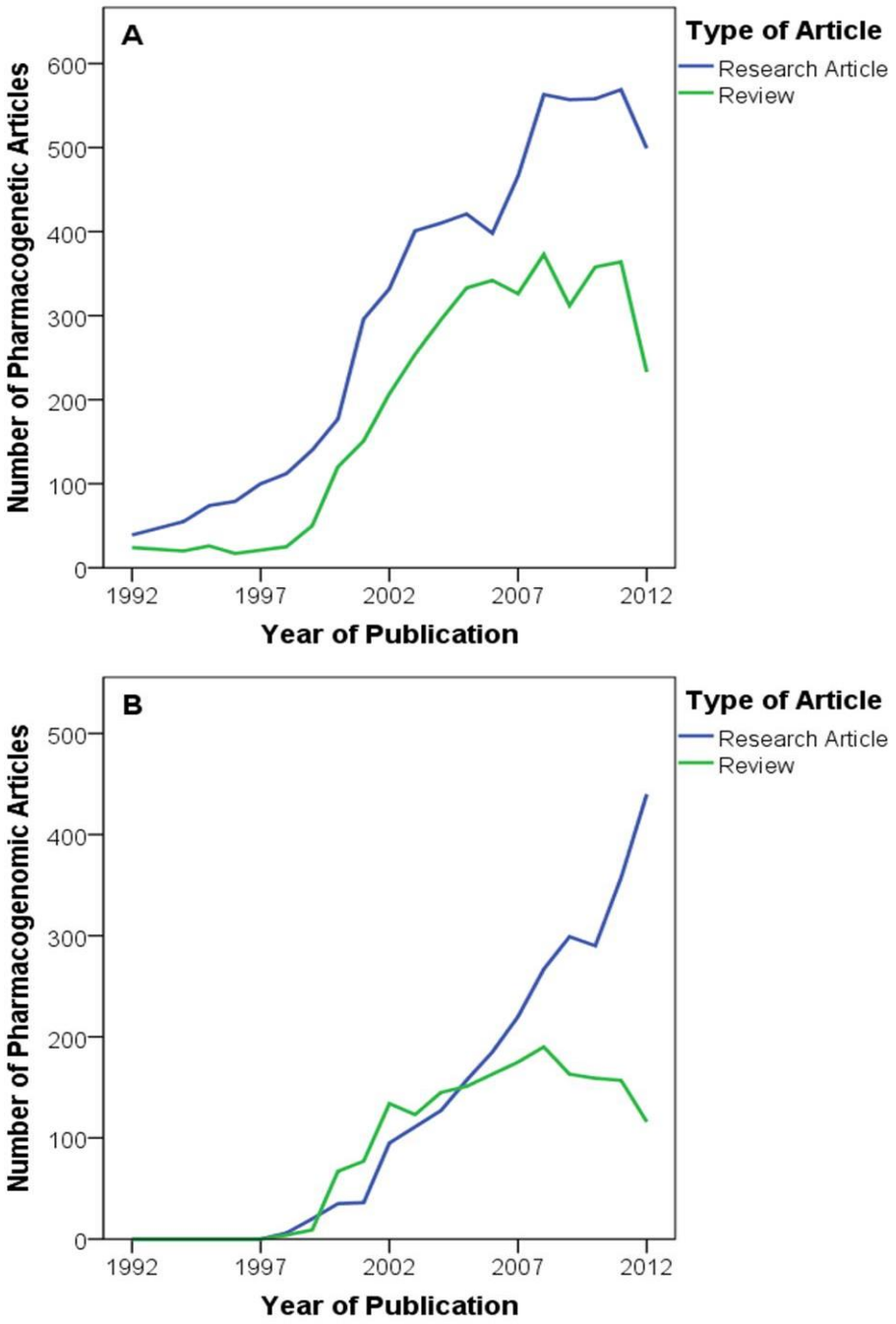

Figure 2-2. The growth of A) pharmacogenetic and B) pharmacogenomic research, as a plot of the number of publications (original research and review) mentioning the word pharmacogenetics or pharmacogenomics over the last 20 years.
Figure created by Tyren Mark Dodgen.

2.3 Pharmacodynamics

Pharmacodynamics is ñthe process of interaction of pharmacologically active substances with target sites, and the biochemical and physiological consequences leading to therapeutic or adverse effects.ò (Meyer 2004). More simply, pharmacodynamics is the drugôs effect on the body. Typically a cell receptor protein or downstream signal mediation proteins are good targets for pharmacogenetic influence. The best known example of pharmacodynamics applied to pharmacogenetics is trastuzumab and HER2 (human epidermal growth factor receptor 2).

HER2 is a cell membrane-bound receptor triggering cell growth, survival and differentiation (Loi et al. 2011) which plays a critical role in aggressive forms of breast cancer but is present in all tissues. As a proto-oncogene, HER2 is responsible for the activation of pathways such as PI3K and ERK (Yarden & Sliwkowski 2001) and over-expression of HER2 gives a significant advantage to tumours to proliferate and for cell survival (Loi et al. 2011). The over expression of HER2 is not only an advantage for breast cancer cells, it also provides a target for treatment. Trastuzumab, marketed as Herceptin (Roche), is a monoclonal antibody which targets an epitope on the extracellular domain of HER2 (Loi et al. 2011). This type of cellular specificity allows for targeted treatment and if a patient is known to be HER2 positive, treatment outcome is likely to be positive. The pharmacogenetic test for HER2 over-expression involves evaluating the *HER2/neu* gene copy number using florescence *in situ* hybridisation (commercial FISH tests are available e.g. PathVysionÊ) having the strongest correlation with treatment response and possibly testing for how much protein is present using immunohistochemistry (commercial IHC tests are available e.g. HercepTestÊ) following breast cancer biopsy (Sauter et al. 2009; Coulson et al. 2010). A known adverse effect however is the cardiotoxic effect of Trastuzumab; this is due to HER2 receptors on cardiomyocytes also being affected by drug dosing.

2.4 Pharmacokinetics

Pharmacokinetics is ñThe process of the uptake of drugs by the body, the biotransformation they undergo, the distribution of the drugs and their metabolites in the tissues, and the elimination of the drugs and their metabolites from the bodyò (Meyer 2004). Simply put, the body's effect on the drug. This includes absorption (i.e. transport proteins), distribution (i.e. transport proteins), metabolism (i.e. enzymes) and elimination (i.e. transporters proteins), commonly abbreviated as ADME-Tox (this includes toxicity related to the compound).

The leading pharmacogenetic interest related to pharmacokinetics is in drug metabolism (Daly 2010), followed to a lesser extent by transporter proteins such as MDR1 (multidrug resistance 1) which is coded for by *ABCB1* (Andersson et al. 2005). The majority of prescribed medications are metabolised in the liver by either Phase I or II reactions, which are mediated by many different catalysing enzymes. Phase I reactions are responsible for the primary transformation of xenobiotics (including therapeutic drugs) which catalyse reactions such as oxidation, reduction or hydrolysis resulting in a drug metabolite with potential sites to add polar conjugates to increase hydrophilicity, thereby facilitating renal excretion. Substrates of Phase II reactions are usually, but not always, the metabolites of Phase I reactions which undergo coupling with a more polar molecule such as glucuronic acid, glycine, glutathione or glutamine as well as conjugation with acetate, sulphate or methyl groups, once again increasing hydrophilicity and renal excretion (Prior & Baker 2003). Enzymes of the haeme-containing CYP family have been estimated to metabolise 80-90% of commonly prescribed medication (Daly 2010) and are largely responsible (70 - 80%) for first pass or Phase I metabolism (Evans & Relling 1999; Eichelbaum, Ingelman-Sundberg & Evans 2006). Therefore the CYP enzymes have become extensively studied for pharmacogenetic relevance.

2.5 Cytochrome P450 (CYP)

CYP enzymes are expressed predominantly in the liver and are responsible for the oxidative biotransformation of exogenous compounds including many drugs, pro-carcinogens and alcohols (van der Weide & Steijns 1999; Daly 2010). In the human genome, 57 active *CYP* genes have been identified as well as 58 pseudogenes. The major human CYP iso-enzymes responsible for drug metabolism include CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 (Rogers, Nafziger & Bertino 2002).

2.5.1 *CYP nomenclature*

In order to better understand the CYP family of enzymes, it is useful to understand the nomenclature system. Firstly the word CYP is an abbreviation, which is used for the mammalian cytochrome P450 family of enzymes (Nelson et al. 1996). The name stems from a combination of ócytoô meaning cellular, chrome from colour, P from pigment and 450 is the wavelength of light (450 nm) the enzyme absorbs when analysed using a spectrophotometer (Wijnen et al. 2007). Figure 2-3 clarifies the remainder of the naming system. When referring to the gene coding for the particular enzyme, the CYP name is written in italics (as in Figure 2-3). Conversely, when the name is written in normal font, this refers to the protein. The first number following CYP indicates the family of iso-enzymes, which are proteins sharing Ó40% sequence homology at the amino acid level. If the homology at the amino acid level is Ó55%, the enzymes fall into the same subfamily, designated by the letter after the family number. The number which follows the subfamily letter indicates a specific gene, a member of the subfamily coding for a specific protein. An asterisk followed by a number indicates the specific allele (a polymorphism, or more commonly, a combination of polymorphisms varying from the wild type which has resulted in amino acid change in the resulting protein). This allele will result in a specific protein variant designated by a dot and a number, or number and letter (e.g. CYP2D6.4A), corresponding with the alleleôs name (Nelson et al. 1996).

In *CYP* nomenclature, **1* commonly depicts the wild type or most frequent allele observed, which often, but not always codes for a functional protein. In order to assign a

new allele, one or more polymorphisms have to be identified causing an amino acid change (a non-synonymous SNP) variation in expression or splices site variation (http://www.imm.ki.se/cypalleles). The allele defining polymorphism or SNP would then be the mutation causing the largest variation in protein function, and is typically used in the primary identification of the specific allele. These alleles may affect the clinical phenotype related to the expression of the gene. Additional polymorphisms which affect the protein function to a lesser extent (often no extent at all as with a synonymous polymorphism) are defined as allelic variants (i.e. the letter after the allele number in the nomenclature). Although these variants often cause no significant difference in clinical phenotype compared to the original allele, they can be important for descriptive purposes. In order to streamline pharmacogenetics for clinical applications, the allele defining polymorphisms for each allele are usually prioritised when establishing pharmacogenetic screening assays.

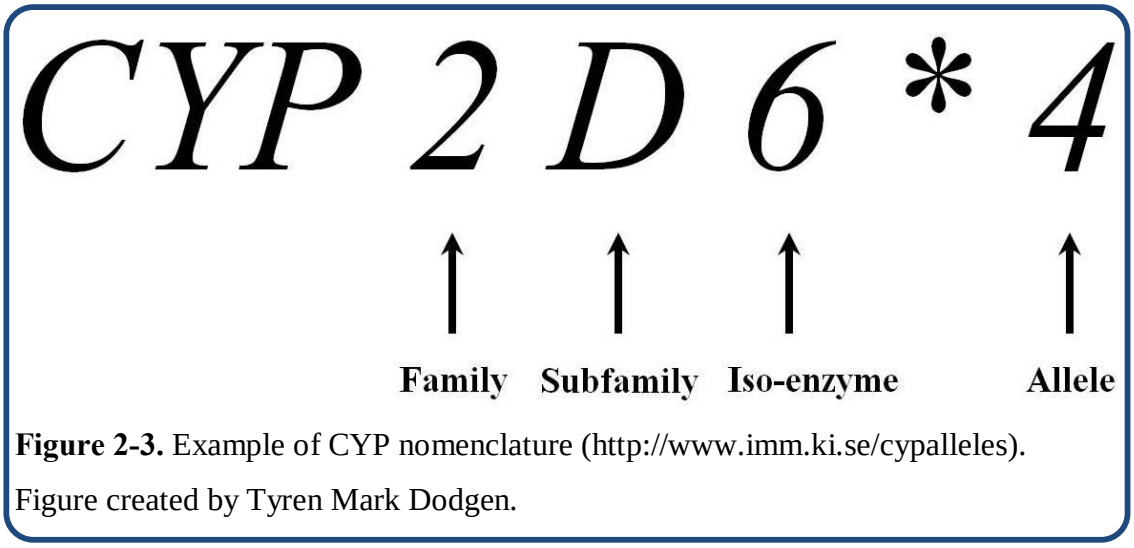

Figure 2-3. Example of CYP nomenclature (http://www.imm.ki.se/cypalleles). Figure created by Tyren Mark Dodgen.

2.5.2 *CYP genes of primary pharmacogenetic focus*

Establishing clinical phenotypic importance or relevance is important when identifying a pharmacogenetic biomarker. Therefore the FDA has categorised a variety of metabolising enzymes based on their ability to predict a phenotypic outcome as well as clinical relevance. Two major categories have been identified for biomarker sorting and include ñvalidò biomarkers referring to those appropriate for decision making to predict variable drug response and ñexplanatoryò biomarkers which are those less well-

developed or established for decision making (http://www.fda.gov/downloads/RegulatoryInformation/ Guidances/ ucm126957).

Three *CYP* genes are among the valid biomarkers on the FDA list, namely *CYP2D6*, *CYP2C9* and *CYP2C19* (Andersson et al. 2005). These biomarkers have the ability to predict phenotypic variation in drug metabolism. Judging by the number of web page hits on The Human Cytochrome P450 (*CYP*) Allele Nomenclature Database (http://www.imm.ki.se/cypalleles), it would appear that that industry and academia are most interested in *CYP2D6*, followed by *CYP2C9* and *CYP2C19* (Ingelman-Sundberg et al. 2007), reiterating the pharmacogenetic importance of these genes.

2.5.3 *Phenotype prediction*

Many polymorphisms and thus alleles have been identified for the highly polymorphic *CYP* genes, which have been catalogued on the P450 Nomenclature Committee website (http://www.imm.ki.se/cypalleles). ñPolymorphicò is a term derived from the word polymorphism, which is used to describe the extent of genetic variability at a specific locus in a genome, compared to what is expected to be present. Along with SNP information for each allele, the database contains information regarding phenotypic effect which has been confirmed by *in vivo* and/or *in vitro* studies. Alleles have thus been classified as non-functional, reduced, normal or higher activity when compared to the wild type reference allele. This enzyme activity, along with genotype, can be used to allocate patients to the following categories based on metabolic activity (Ingelman-Sundberg et al. 2007) (see also Figure 2-4):

- Poor metabolizers (PMs - those who lack functional enzyme activity),
- Intermediate metabolizers (IMs - those who have reduced enzyme activity),
- Extensive metabolizers (EMs - those who have ónormalô enzyme activity) and
- Ultra-rapid metabolizers (UMs - those who have increased enzyme activity resulting from 3 or more functional gene copies)

The numeric Activity Score (AS) designed by Gaedigk et al. (2008) for CYP2D6 prediction is a good example of how metabolism can be predicted (explained in Table 2-

1). As humans are diploid, there are two copies of each gene in a genome. A gene may have various polymorphisms resulting in alternative forms of the gene, reffered to as alleles. Each of the patientôs alleles can be assigned a score based on predicted enzyme activity. The CYP scores for *CYP2D6* alleles were assigned based on information provided by The Human Cytochrome P450 (*CYP*) Allele Nomenclature Database (http://www.imm.ki.se/ cypalleles) for *CYP* genes. Therefore, in terms of activity, none=0, reduced=0.5, normal=1.0 and increased=2.0 (refer to Table 2-1). The summation of the allelic scores would then fall into a category. Gaedigk et al. (2008) found that their Model A predicted phenotype for CYP2D6 most accurately (Refer to Table 2-1). The AS system reduces complication while stratifying cohorts into groups offering insight into quartiles of a cohort as well as minimizing overlap within a group (Gaedigk et al. 2008). Stratifying phenotype prediction using AS allows phenotypic patterns to become apparent. Caution should be taken when implementing AS phenotype prediction as ethnicity, disease state (including liver and kidney function) and concomitant medication may contribute to an unpredictable phenotype (Gaedigk et al. 2008).

Table 2-1. Adapted version of the CYP2D6 and CYP2C19 Activity Score (AS) System for phenotype prediction (Gaedigk et al. 2008).

Estimated metabolic potential of alleles			
CYP2D6	**Allele activity**	**Numeric Activity**	***CYP2C19***
**1xN, *2xN*	Increased	2.0	**17*
**1,*2, *22, *33, *35, *43, *45B, *46*	Normal	1.0	**1+, *28*
**10, *17, *29, *41, *59*	Decreased	0.5	**9, *27*
**4, *5, *14, *16, *40, *56B, *4xN*	Absent	0.0	**2, *3*
**25, *30, *64, *65, *73, *74, *84, *85, *86*	Unknown	1.0	**15*
(Activity according to http://www.cypalleles.ki.se/)			

Phenotype prediction		
AmpliChip	**Prediction**	**Activity Score (AS)**
3 or more functional alleles	UM	> 2.0
1 or 2 functional alleles and increased paired with decreased or absent	EM	1.5-2.0
1 or 2 reduced functional alleles	IM	0.5-1.0
2 absent functional alleles	PM	0.0

Alleles for both genes presented in this table are relevant to this study; additional allele information is available at http://www.cypalleles.ki.se/. The activity of each allele in the genotype is used to predict phenotype. CYP2D6 Activity Score (AS) is assigned according to model A proposed by Gaedigk et al.(2008). As an example, CYP2D6*4/*10 is 0.0+0.5 = 0.5. The AS was adapted to predict CYP2C19 activity.

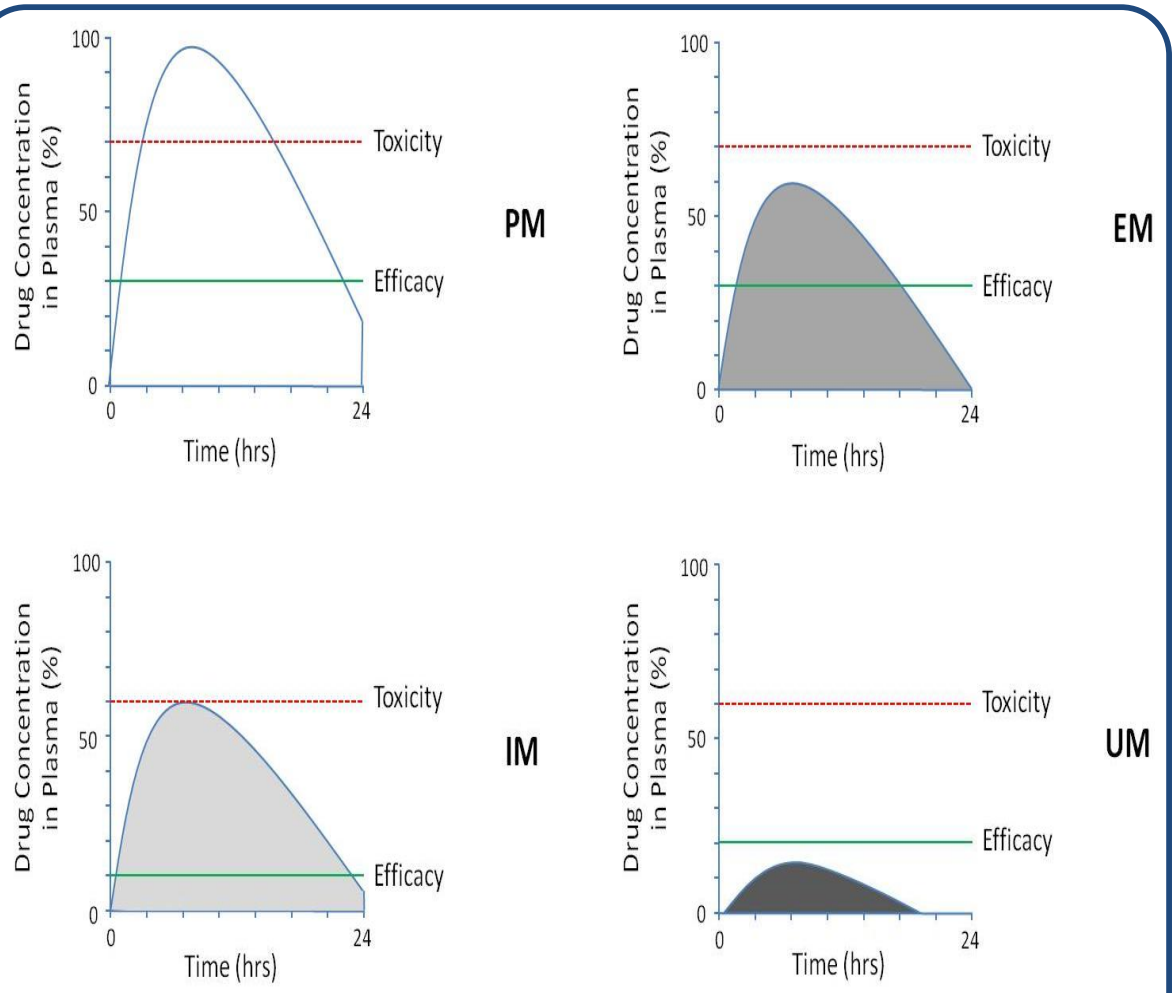

Figure 2-4. Explanation of drug plasma levels in (PM), intermediate (IM), extensive (EM) and ultrarapid metabolisers (UM) as well as drug response.
If a pro-drug is administered the opposite may be expected for the active metabolite. This diagram was created by Tyren Mark Dodgen and is an alternate interpretation of the explanation provided by Evans et al. (2001).

2.5.4 *CYP2C19*

CYP2C19 is a member of the CYP2C family of enzymes, clustered with genes between chromosome 10q24.1 and 10q24.3 and ordered *CYP2C8-CYP2C9-CYP2C19-CYP2C18* (Gray et al. 1995). These enzymes have been estimated to metabolise approximately 20% of commonly prescribed drugs. Eight percent can be attributed to CYP2C19 (Kashuba & Bertino 2005).

2.5.4.1 *CYP2C19* genotyping

CYP2C19 (NCBI accession number NG_008384.1, OMIM 124020) is a 90,208 base pair gene with 9 exons (section of DNA which is expressed to form a protein) and 8 introns (sections of non-coding DNA which are found between the exons) (Nelson et al. 2004). *CYP2C19*2* and **3* are the most common defective alleles contributing to PM status (refer to Table 2-2), with **3* found predominantly in Asians (Xie et al. 2001). It has been reported in that approximately 5% of Caucasians and Africans and 20% of Asians are PM for CYP2C19 (i.e. have 2 defective alleles) (Desta et al. 2002). Currently there are 28 distinct alleles and various allelic variants identified for *CYP2C19* according to the P450 Nomenclature Committee (http://www.cypalleles.ki.se/; last viewed 23 April 2013). Some of the *CYP2C19* alleles have been confirmed to cause unstable protein, abolish, decease or increase enzyme activity,as defined by*in vivo* and/or *in vitro* studies. *CYP2C19*17*, the allele responsible for increased enzyme activity, has been observed at relatively high frequencies of up to 18.0% in several different populations (Sim et al. 2006). *CYP2C19*9* is an allele found almost exclusively in Africans and appears to be responsible for decreased enzyme activity (Blaisdell et al. 2002). Drögemöller *et al*. (2010) described the novel *CYP2C19*27* allele as having a promoter region allele defining SNP (-1041G>A, rs7902257) causing decreased expression *in vitro*. This SNP was discovered in the South African Xhosa population at relatively high frequency and may be responsible for reduced metabolism. Allele frequencies observed in South African cohorts are summarised in Table 2-2 and frequencies of cohorts sampled throughout African have been summarised by Alessandrini et al. (2013).

Table 2-2. Frequencies of *CYP2C19* alleles in major race/ethnic groups in South African cohorts as compiled by Hicks et al . (2013) .

	Dandara et al. (2001)	Dandara et al. (2011)	Drögemöller et al. (2010)		Ikediobi et al. (2011)		Matimba et al. (2009)	Hicks et al.(2013)						
CYP2C19 Allele	SA Venda	SA Bt20 (mixed Black)	SA Xhosa	SA Coloured	SA Xhosa	SA Coloured	SA Venda	African	American	East Asian	European	Middle Eastern	Oceanian	South/ Central Asian
**1+*	78.0	84.0	17.0	41.0	63.0	22.0	77.00	68.0	69.0	6.0	63.0	87.0	24.0	62.0
**2*	22.0	16.0	21.0	17.0	71.0	20.0	17.00	15.0	12.0	29.0	15.0	12.0	61.0	35.0
**3*	0.0	0.0	0.0	7.0	ND	ND	0.00	<1.0	<1.0	9.0	<1.0	1.0	15.0	2.0
**4*	ND	ND	ND	ND	ND	ND	ND	<1.0	<1.0	<1.0	<1.0	ND	ND	0.0
**5*	ND	ND	ND	ND	ND	ND	ND	ND	0.0	<1.0	<1.0	ND	ND	0.0
**6*	ND	ND	ND	ND	ND	ND	ND	0.0	0.0	0.0	<1.0	ND	ND	0.0
**8*	ND	ND	ND	ND	ND	ND	ND	0.0	<1.0	0.0	<1.0	ND	ND	ND
**9*	ND	ND	9.0	4.0	ND	ND	6.00	ND	ND	ND	ND	ND	ND	ND
**15*	ND	ND	9.00	8.0	ND	ND	0.00	ND	ND	ND	ND	ND	ND	ND
**17*	ND	ND	10.0	14.0	15.0	9.0	ND	16.0	18.0	0.03	0.21	ND	ND	ND

**1+*, represents the wild type allele as well as any additional allele which could not be identified by the genotyping platform.ND; not determined.

2.5.4.2 CYP2C19 phenotyping

Phenotype is defined as an observed characteristic which is controlled in part by genotype but which is also intertwined with environmental influence (Fairbanks & Andersen 1999; Aklillu et al. 2002). Therefore phenotyping is an *in vivo* measure of CYP enzyme activity (Streetman, Bertino & Nafziger 2000). One of the major advantages of phenotyping is that genotype as well as various non-genetic factors are monitored simultaneously (Burroughs et al. 2002), allowing for an accurate comparison between predicted and measured phenotype. To evaluate phenotype, probe drugs are commonly used. Probe drugs are predominantly metabolised by a specific enzyme with the potential to emulate the metabolism of other drugs metabolised by the same enzyme.

Two drugs have been commonly used for the phenotypic analysis of CYP2C19 (Streetman, Bertino & Nafziger 2000):

1. (*S*)-mephenytoin, metabolised into 4ô-hydroxymephenytoin
2. Omeprazole (OME), metabolised into 5ô-hydroxyomeprazole

Due to its relatively low side effect profile and increased ability to discriminate between EMs and PMs compared to mephenytoin (Streetman, Bertino & Nafziger 2000),OME has become the probe drug of choice for CYP2C19 (Kanazawa et al. 2002; Frerichs, Zaranek & Haas 2005; Narimatsu et al. 2006). OME is hydroxylated by CYP2C19 forming 5ô-hydroxyomeprazole (5OH), but is also metabolised by CYP3A4 to form omeprazole sulfone (Figure 2-5). Although OME is metabolised by both of these enzymes, CYP2C19 it thought to be the primary enzyme responsible (Lasker et al. 1998). CYP2C19 has higher selectively and affinity, but lower capacity compared to CYP3A4, which suggests that once CYP2C19ôs capacity has been exceeded the excess will be metabolised by the low affinity high capacity CYP3A4 enzyme (Wijnen et al. 2007). Since CYP2C19 is the major enzyme metabolising omeprazole, a metabolic ratio (MR) of OME and 5OH concentrations can be used to measure metabolic activity.

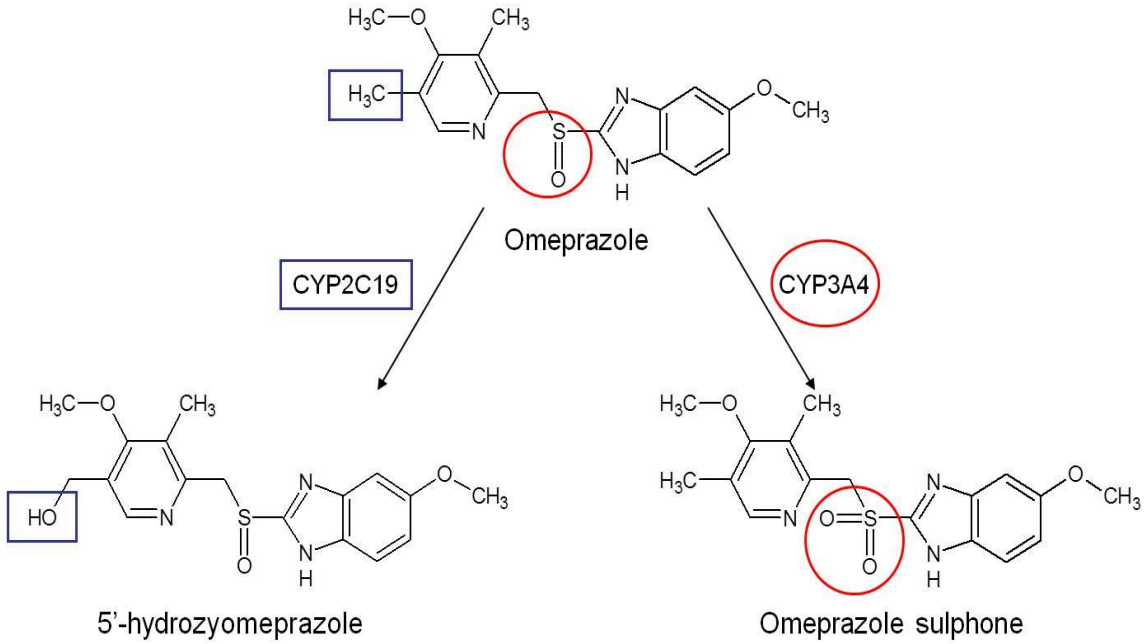

Figure 2-5. Metabolic breakdown of omeprazole by CYP2C19 and CYP3A4.
This diagram was created by Tyren Mark Dodgen and is an adaptation of the one published by Hofmann et al. (2006).

2.5.4.3 Substrates of CYP2C19

CYP2C19 has various drug substrates (Refer to Table 2-3 for well documented examples) namely proton pump inhibitors (PPI), antidepressants, monoamine oxidase inhibitors (MAOI), tricyclic antidepressants (TCA), the selective serotonin reuptake inhibitors (SSRI) and the platelet aggregation inhibitor clopidogrel (Gardiner & Begg 2006; Johansson & Ingelman-Sundberg 2011). Of these citalopram, clopidogrel, omeprazole and proguanil have been extensively researched in the field of pharmacogenetics.

Citalopram

CYP2C19 plays a partial, but important role in the metabolism of SSRIs.Examples include citalopram, fluoxetine and sertraline (Gardiner & Begg 2006). SSRIs are used to treat an array of psychological diseases including depression, anxiety disorders, panic attacks and obsessive-compulsive disorder (Rang et al. 2003). Citalopram acts by inhibiting the neuronal reuptake of serotonin (Rang et al. 2003) and it has been found that the *S*-enantiomer is responsible for citalopramôs pharmacological activity(Johnson

et al. 2006) even though it is administered as a racemic mixture. Citalopram is N-demethylated by CYP2C19 into the inactive desmethylcitalopram, and therefore enzyme activity largely determines citalopramôs elimination half-life. A second CYP, CYP2D6 also plays a role in citalopram clearance by N-demethylating desmethylcitalopram into didesmethylcitalopram. In the case of an individual being a PM for both CYP2C19 and CYP2D6, citalopramôs half-life is extended by 37 hours compared to an EM (Herrlin et al. 2003). A PM patient who is prescribed a therapeutic dose of an SSRI may commonly experience nausea, anorexia, insomnia and organ failure, although the likelihood of overdose is low except when used in combination with MAOIs (Rang et al. 2003). Ranking third in the DALYôs ranking of burden of disease for South Africa, neuropsychiatric conditions should be considered as a priority for pharmacogenetic intervention (Norman et al. 2006)

Clopidogrel

Currently, clopidogrel appears to be dominating *CYP2C19* pharmacogenetic research (Myburgh et al. 2012). In patients undergoing percutaneous coronary intervention (PCI) and those experiencing acute coronary syndromes, clopidogrel has become first line treatment (Gray et al. 1995; Mega et al. 2010). Clopidogrel (prodrug) requires several bioactivation steps largely mediated by CYP450 enzymes (CYP2B6, CYP2C9, CYP2C19, CYP3A4 and CYP3A5) (Kazui et al. 2010; Kurihara A, 2005; Taubert et al. 2006). The active metabolite (currently marketed as paliperidone) binds irreversibly to the $P2RY_{12}$ platelet surface receptor, inhibiting adenosine diphosphate (ADP) induced platelet aggregation (Hollopeter et al. 2001; Savi et al. 2000). If the pharmacogenetics of pharmacodynamics and pharmacokinetics are compared, clopidogrel metabolism appears to be predominantly associated with variation in clopidogrel response (Myburgh et al. 2012). Of the CYP450 enzymes, several articles have discounted genetic variations in CYP2B6, CYP3A4 and CYP3A5 as having a correlation with variations in clopidogrel response (Brandt et al. 2007; Hulot et al. 2006; Simon et al. 2009). The first reported genome wide association study (GWAS) investigating clopidogrel response identified the *CYP2C18–CYP2C19–CYP2C9–CYP2C8* cluster on chromosome 10 as the most likely correlation with a variable clopidogrel response (Shuldiner et al. 2009). Agreeing with this observation, several studies summarised by Myburgh et al. (2012)

have demonstrated a strong association between *CYP2C19* loss of function alleles (**2*, **3*, **4*, or **5*) and variable clopidogrel response. The loss of function alleles appear to result in lower levels of the active clopidogrel metabolite and reduced platelet inhibition. The FDA has recognised the clinical relevance of CYP2C19 pharmacogenetics, and has recommended that health care professionals undertake pre-prescription genotyping prior to treating with prescription (http://www.fda.gov/drugs/drugsafety/postmarketdrugsafetyinformationforpatientsandproviders/ucm203888.htm). A study that sampled South Africans residing in Soweto observed that 78.0% of 1691 individuals sampled had Ó1 major risk factor for heart disease (Tibazarwa et al. 2009). Cardio vascular disease is the most common western world disease experienced in Africa (Sliwa, Damasceno & Mayosi 2005) and ranks seventh in the estimated DALYs ranking of burden of disease for South Africa (Norman et al. 2006).

Omeprazol

PPIs, in particular OME, are used as part of a triple therapy in the treatment of gastric ulcers associated with *Helicobacter pylori* infection and are one of the most extensively prescribed drugs globally (Gardiner & Begg 2006). OME is a commonly prescribed proton pump inhibitor and is used to reduce acid secretion in the treatment of acid related gastrointestinal disorders. The drug exerts its action by irreversibly inhibiting the H^+/K^+ - ATPase pump, also known as the proton pump, at the terminal step of the acid secretion pathway in gastric parietal cells. At neutral pH, OME is inactive, but because it is a weak base it tends to accumulate in acidic environments such as the canaliculi of stimulated parietal cells, where it is activated (Rang et al. 2003; Sweetman 2007). *Helicobacter pylori* causes gastritis, ulceration (duodenal and gastric) and is a gastric carcinogen (Sapone et al. 2003). Triple treatment involves a PPI, amoxicillin and clarithromycin or metronidazole; in some cases colloidal bismuth is used instead of a PPI. PPIs act by reducing acid production within the stomach and thus increasing antibiotic efficacy (Rang et al. 2003). PMs experience a greater response compared to EMs, as the PPI is metabolised at a slower rate (Rogers, Nafziger & Bertino 2002).

Table 2-3. Summary of substrates metabolised by CYP2C19 selected from the PharmGKB website with intervention recommendations (Hicks et al. 2013).

Drug	Class	Metaboliser Status Affected	Response	PharmGKB recommendation
Amitriptyline	Tricyclic Antidepressant	PM	Higher plasma concentrations causing ADRs and lack of compliance	50% starting dose
Citalopram	SSRI	UM	Suboptimal response	Titrate dose to max 150%/consider alternative drug
Clopidogrel	Platelet Aggregation inhibitor	IM/PM	Reduced platelet inhibition causing adverse cardiovascular events	Prasugrel or alternative therapy
Esomeprazole, lansoprazole, omeprazole & pantoprazole	PPI	UM	Suboptimal Helicobacter pylori eradication	Increase dose
Imipramine	Tricyclic Antidepressant	PM	Higher plasma concentrations causing ADRs and potential lack of compliance	Reduce dose by 30%/consider alternative drug
Sertraline	SSRI	PM	Higher plasma concentrations causing ADRs and potential lack of compliance	Reduce dose by 50%

ADR-adverse drug reaction; IM-intermediate metaboliser; PM-poor metaboliser; PPI-proton pump inhibitor; SSRI-selective serotonin reuptake inhibitor; UM-ultrarapid metaboliser. This is not a complete list of CYP2C19 substrates.

In this case it would be much more cost effective to treat a PM with a PPI and amoxicillin, which results in a >90% success rate. On the other hand, EMs will have to undergo full triple therapy and perhaps even increase their PPI dosage (Gardiner & Begg 2006).

Proguanil

In tropical Africa, one of the leading causes of death is malaria, and proguanil is at present one of the drugs of choice for prophylactic treatment in several countries including Nigeria (Bolaji et al. 2002). Proguanil is a pro-drug which is metabolized to its active metabolite cycloguanil and an inactive metabolite 4-chlorophenylbiguanide (Gardiner & Begg 2006) by CYP2C19. The metabolism of proguanil is reduced in PMs (Hoskins, Shenfield & Gross 1998) which thus leads to ineffective treatment. Knowledge of CYP2C19 metabolising status is important in order to avoid inefficient prophylaxis.

2.5.5 *CYP2D6*

CYP2D6 constitutes only 1.5% of total CYP gene expression, yet it is estimated to metabolise approximately 25.0% of commonly prescribed drugs (Gardiner & Begg 2006; Johansson & Ingelman-Sundberg 2011). In addition to the liver, CYP2D6 is also expressed in the brain (Hiroi et al. 1995), prostrate (Finnstrom et al. 2001) and heart (Thum & Borlak 2000).

2.5.5.1 CYP2D6 Genotyping

Located on human chromosome 22, *CYP2D6* is a 4,378 base pair gene with 9 exons and 8 introns. The commonly accepted wild type allele (*CYP2D6*1*) is represented under the NCBI accession number AY545216.1 and polymorphisms or alleles observed have been described to differ from this (Gaedigk et al. 2005). Research on *CYP2D6* has uncovered some unusual challenges in genotyping. Firstly, two pseudogenes, *CYP2D7* and *CYP2D8* have extensive homology with *CYP2D6*, which can easily be mistaken as novel alleles (Andersson et al. 2005). *CYP2D6* can be completely absent in which case the assigned allele is*CYP2D6*5* (Gaedigk et al. 1991). Finally,*CYP2D6* has been found

to have copy number variation, with up to 13 copies described in a Swedish family (Lundqvist, Johansson & Ingelman-Sundberg 1999). *CYP2D6* is one of the most highly polymorphic CYP genes. The P450 Nomenclature Committee has accounted for over 100 distinct *CYP2D6* alleles. The most common *CYP2D6* alleles include: functional alleles with normal enzymatic activity, *CYP2D6*1* and **2*; alleles with reduced enzyme activity, **10*, **17*, **29* and **41*; and alleles with no functional enzymatic activity, **3*, **4*, **5* and **6*. Duplications of a variety of alleles are common alleles including, **1Xn, *2Xn, *4Xn, *10Xn, *41Xn,* etc. (n = 3 to 13), with varying enzymatic activity (refer to Table 2-4 for frequencies of alleles affecting enzyme activity and Alessandrini et al. (2013) for frequencies in Africa). The prevalence of particular alleles has been reported to vary significantly between different ethnic groups (Table 2-4). *CYP2D6*3*, **4*, **5*, **6*, **9*, **10* and **41* have been found to be most frequent in Caucasians (Sistonen et al. 2009). Populations of African origin appear to have relatively high frequencies of *CYP2D6*17* and **29*, and these alleles appear to be almost exclusive to these populations (Wennerholm et al. 2001; Gaedigk et al. 2002; Sistonen et al. 2009). The *CYP2D6*10* allele is found in many populations, but has been observed in excess of 50% frequency in Oriental populations (Johansson et al. 1994; Sistonen et al. 2009). In the admixed South African Coloured population, a particularly high frequency of the *CYP2D6*5* allele (17.2%) has been observed (Gaedigk & Coetsee 2008).

Table 2-4. Frequencies of *CYP2D6* alleles in major race/ethnic groups compared to South African cohorts as compiled by Crews et al. (2012) .

	Dandara et al. (2001)	Gaedigk et al. (2008)	Wright et al. (2010)		Crews et al. (2012)							
CYP2D6 Allele	SA Venda n=76	SA Coloureds n=99	SA Xhosa Control n=53	SA Xhosa Schizophrenia n=51	African	African American	Caucasian (European + North American	Middle Eastern	East Asian	South/ Central Asian	Americas	Oceanian
**1+*	50.0	26.80	23.6	24.5	39.23	41.70	48.40	59.46	33.09	53.70	60.57	70.15
**2d*	17.8	15.20	12.3	15.7	20.12	12.34	26.93	24.10	12.29	31.90	24.12	1.20
**3*	0.0	0.00	0.0	0.0	0.03	0.34	1.27	0.13	0.00	0.00	0.42	0.00
**4*	3.3	7.10	1.9	1.0	3.36	6.03	18.32	7.60	0.46	6.56	11.57	1.13
**5*	4.6	17.20	14.2	18.6	6.07	5.86	2.74	2.34	5.66	2.54	1.98	4.95
**6*	ND	0.00	0.0	0.0	0.00	0.27	0.96	0.96	0.03	0.00	0.74	0.00
**7*	ND	0.00	0.0	0.0	0.00	0.00	0.13	ND	0.00	ND	0.00	0.00
**8*	ND	0.00	0.0	0.0	0.00	0.00	0.03	ND	0.00	ND	0.15	0.00
**9*	ND	0.00	0.0	0.0	0.10	0.54	1.99	0.00	0.09	1.43	1.33	0.00
**10*	ND	2.50	1.9	2.0	6.77	4.32	2.76	3.53	44.58	19.76	4.14	1.60
**14*	ND	0.50	0.0	0.0	0.13	0.00	0.00	ND	0.95	0.00	0.47	0.00
**17*	24.0	12.60	13.2	16.7	19.98	18.32	0.29	1.40	0.02	0.38	2.01	0.05
**36*	ND	0.00	0.0	0.0	0.00	0.56	0.00	ND	2.19	ND	ND	0.00
**41*	ND	0.00	1.9	1.0	10.94	10.31	8.69	22.95	2.54	10.50	6.36	0.00
**1xN*	ND	0.00	0.0	1.0	1.47	0.44	0.70	3.81	0.29	0.50	0.84	11.83
**2xN*	ND	0.00	2.8	2.9	6.41	1.61	2.24	4.93	1.54	1.25	2.42	0.00
**4xN*	ND	0.0	1.9	2.9	1.40	2.07	0.22	0.00	0.00	0.00	0.47	0.00

**1+,* represents the wild type allele as well as any additional allele which could not be identified by the genotyping platformND; not determined.

2.5.5.2 CYP2D6 phenotyping

As with CYP2C19, probe drugs are used to evaluate CYP2D6 metabolism. Five probe drugs have been used predominantly for the phenotypic analysis of CYP2D6 enzyme activity (Frank, Jaehde & Fuhr 2007):

1. Debrisoquine, metabolised to 4-hydroxydebrisoquin
2. Dextromethorphan, metabolised to dextrorphan
3. Metoprolol, metabolized to Ŭ-hydroxymetoprolol
4. Sparteine, metabolised to 2- and 5-dehydrospartiene
5. Tramadol, metabolized to O-desmethyltramadol

Of the five, dextromethorphan (DM) is the most commonly used and most extensively validated probe drug for CYP2D6 metabolism (Frank, Jaehde & Fuhr 2007). DM-containing formulations are safe, freely available over-the-counter and can be obtained without prescription (Küpfer et al. 1984). DM is an antitusive agent which acts by depressing the medullary centre of the brain to decrease the involuntary urge to cough (Sweetman 2007). After oral ingestion and absorption, DM is selectively metabolised by CYP2D6 into dextrorphan (DX) and to a lesser extent to 3-methoxymorphinan by CYP3A4 (Figure 2-6). CYP2D6 has higher affinity for its substrates than CYP3A4 (Wijnen et al. 2007).

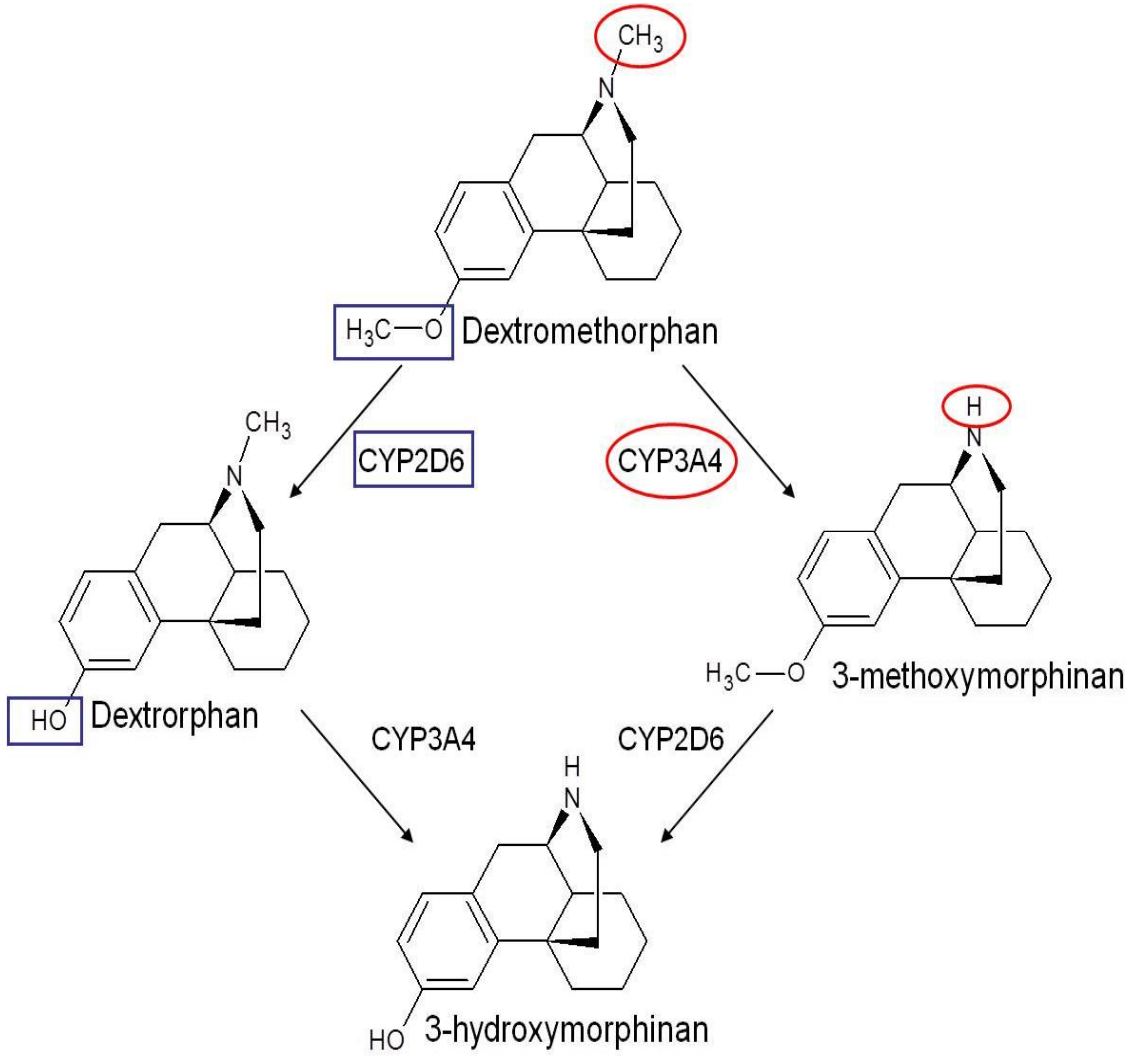

Figure 2-6. Metabolic breakdown of Dextromethorphan by CYP2D6 and CYP3A4. This figure was created by Tyren Mark Dodgen and is an adaptation(Vengurlekar et al. 2002).

2.5.5.3 Substrates of CYP2D6

Clinical research with regard to pre-prescription CYP2D6 testing has covered many areas including oncology (e.g. breast cancer), analgesia, cardiology and psychiatry (e.g. depression and schizophrenia) and others (Gardiner & Begg 2006; Johansson & Ingelman-Sundberg 2011). Several examples are explained in Table 2-5. *β*-blockers, resperidone, tamoxifen and tramadol have been extensively studied for pharmacogenetic applications.

β-blockers

β-adrenoreceptor blockers are commonly used in the treatment of cardiovascular diseases (hypertension, coronary heart disease and heart failure). Carvedilol, nebivolol, propranolol, alprenolol and metoprolol are all *β*-blocker substrates of CYP2D6 (Myburgh et al. 2012), of which metoprolol is extensively broken down by CYP2D6. In a study conducted by Bijl et al. (2009) with a cohort size of 6,348 individuals (1993-1996), at the third and final visit 2,878 (2002-2004) patients using metoprolol who were homozygous for the *CYP2D6*4* allele had a significantly lower diastolic blood pressure and lower heart rate than EMs. The authors suggested that bradycardia (lower heart rate) was of greatest concern in the PMs and could be life threatening. Recommendations were suggested that clinicians should reduce the dose of metoprolol for PMs (Table 2-5) to prevent the possibility of an adverse event, or alternatively change to atenolol, which is eliminated largely unchanged (Bijl et al. 2009). As mentioned in 2.5.1.3, cardiovascular disease is very common in Africa (Sliwa, Damasceno & Mayosi 2005) and ranks seventh in the estimated DALYs ranking of burden of disease for South Africa (Norman et al. 2006).

Risperidone

Treatment variation of psychiatric conditions such as depression and schizophrenia has been associated with CYP2D6 metabolic status (Wright et al. 2010). CYP2D6 PMs are particularly sensitive to the side effects of tricyclic antidepressants such as venlafaxine, typical (classical) antipsychotics and risperidone (RSP). It has been recommended that clinicians should not prescribe antipsychotics that are not substrates of CYP2D6 (although this may be expensive) to CYP2D6 PM patients or that dosage should be adjusted accordingly (Table 2-5). CYP2D6 UMs who are unlikely to respond to antipsychotic CYP2D6 substrates should be prescribed alternate antipsychotics (de Leon, Armstrong & Cozza 2006). Side effects of antipsychotics include extrapyrimidal motor disturbances and tardive dyskinesia. These are involuntary movements (muscle spasms, protruding tongue, movements of the trunk and limbs etc.) similar to those seen in Parkinsonôs disease, which can be extremely debilitating. In addition, RSP can cause uncontrollable weight gain (Rang et al. 2003; Sweetman 2007). Neuropsychiatric

conditions rank third in the DALYs ranking of burden of disease for South Africa (Norman et al. 2006).

Tamoxifen

In the United States, about 180,000 women are diagnosed with invasive breast cancer per annum (Henry et al. 2008). Women who have hormone dependent breast cancer and are yet to reach menopause benefit greatly tamoxifen treatment (Goetz, Kamal & Ames 2008). However, women who are PMs for CYP2D6 are less likely to benefit from tamoxifen treatment. Tamoxifenôs metabolites, endoxifen and 4-hydroxytamoxifen, are up to 100 times more efficient than tamoxifen itself, which makes the metabolism of this pro-drug essential for effective treatment (Henry et al. 2008; Henry et al. 2009). Goetz *et al*.(2008) recommend that alternative forms of treatment should be considered for CYP2D6 PM patients. Malignant neoplasms are ranked ninth in the DALYs ranking of burden of disease for South Africa (Norman et al. 2006).

Tramadol

The synthetic opioid tramadol, used as a racemic mixture, is effective in the management of post-operative pain. Compared to other opioids it is less likely to cause respiratory depression, development of tolerance, dependence or abuse. Tramadol is a pro-drug which is metabolised into 11 metabolites, of which *O*-desmethyltramadol possesses 200-fold higher affinity for the µ-opioid receptor than tramadol itself. CYP2D6 is the enzyme responsible for the metabolism of tramadol into *O*-desmethyltramadol and thus a PM will have a reduced analgesic effect. It is common practice to concomitantly administer a non-opioid analgesic such as dipyrone, which acts synergistically with tramadol to increase efficacy and reduce side effects. Although this strategy was not initiated with CYP2D6 PMs in mind, it significantly improves analgesia in these patients (Stamer et al. 2003). In a study by Stamer et al. (2003) on 300 patients recovering from abdominal surgery who were treated with concomitant tramadol and dipyrone, it was found that CYP2D6 PM patients self-administered a higher dosage of the analgesic due to poor treatment response to tramadol compared to EMs. Thus the hypothesis of reduced tramadol efficacy in CYP2D6 PMs was confirmed. In a separate study (Levo et al. 2003), 33 post-mortem individuals were

analysed for CYP2D6 PM as well as tramadol/*O*-desmethyltramadol blood levels. There was a correlation between PM (4 patients) and increased tramadol intake, and the authors alluded to the possibility of a correlation between PM and accidental overdose as opposed to intentional suicide or premeditated murder. However, caution must be exercised when interpreting small data sets particularly in a setting such as this.

Table 2-5. Summary of substrates metabolised by CYP2D6 selected from the PharmGKB website with intervention recommendations (Hicks et al. 2013).

Drug	Class	Metaboliser Status	Response	PharmGKB recommendation
Amitriptyline & Nortriptyline	Tricyclic antidepressant	PM	Higher plasma concentration - possible ADRs	Alternative antidepressant /50% reduction of dosage (TDM)
		IM	Higher plasma concentration - possible ADRs	25% reduction of dosage - (TDM)
		UM	Low plasma concentrations – sub-therapeutic activity - poor compliance	Consider alternative antidepressant /dosage adjustment (TDM)
Aripiprazole	Second generation atypical antipsychotic	PM	Higher plasma concentration - possible extrapyrimidal ADRs	Use 67% of recommended daily dosage
Clomipramine	Tricyclic Antidepressant	PM	Higher plasma concentration - possible extrapyramidal ADRs	Alternative antidepressant /50% reduction of dosage (TDM)
		UM	Low plasma concentrations – sub-therapeutic activity - poor compliance	Consider alternative antidepressant /dosage adjustment (TDM)
Codeine	Opioid Analgesic	PM	Greatly reduced morphine formation – sub-therapeutic activity – ADRs	Consider alternative analgesic
		UM	Increased formation of morphine - possible ADRs	Consider alternative analgesic
Doxepin	Tricyclic Antidepressant	PM	Higher plasma concentration - possible ADRs i.e. arrhythmia & myelosuppression	Alternative antidepressant /60% reduction of recommended dosage (TDM)
		IM	Higher plasma concentration - possible ADRs i.e. arrhythmia & myelosuppression	20% reduction of recommended dosage - (TDM)
		UM	Low plasma concentrations – sub-therapeutic activity - poor compliance	Consider alternative antidepressant not metabolised by CYP2D6/dosage adjustment use TDM

Table 2-5. Continued...

Drug	Class	Metaboliser Status	Response	PharmGKB recommendation
Flecainide	Antiarrhythmic Agent	PM	Higher plasma concentration - possible QTc prolongation & INR increase	Reduce dose by 50% (TDM)
		IM	Higher plasma concentration - possible QTc prolongation & INR increase	Reduce dose by 25% (TDM)
Haloperidol	Typical Antipsychotic	PM	Higher plasma concentration - possible extrapyramidal ADRs	Alternative antipsychotic /50% reduction of recommended dosage (TDM)
Imipramine	Tricyclic Antidepressant	PM	Higher plasma concentration - possible extrapyramidal ADRs	70% reduction of recommended dosage use TDM
		IM	Higher plasma concentration - possible extrapyramidal ADRs	30% reduction of recommended dosage - use TDM
		UM	Low plasma concentrations – sub-therapeutic activity - poor compliance	Consider alternative /increase recommended dosage by 70% & (TDM)
Metoprolol	*β*1- adrenergic blocker	PM	Higher plasma concentration - possible bradycardia	Consider alternative β-blocker/ reduce dose by 75%
		IM	Higher plasma concentration - possible bradycardia	Consider alternative *β*-blocker/ reduce dose by 50%
		UM	Low plasma concentrations – sub-therapeutic activity - possible heart failure	Titrate dosage to a maximum of 250% of recommended dosage/select alternative *β*-blocker
Propafenone	Antiarrhythmic Agent	PM	Higher plasma concentration - possible ADRs	Reduce dose by 70% use TDM
Tamoxifen	Oestrogen receptor antagonist - breast cancer treatment	PM/IM	Low levels of active metabolite – sub-therapeutic activity - increased risk for relapse	Consider aromatase inhibitor in postmenopausal women or alternative anti-cancer agent

Table 2-5. Continued...

Drug	Class	Metaboliser Status	Response	PharmGKB recommendation
Tramadol	Opioid Analgesic	PM	Greatly reduced levels of O-desmethyltramadol (potent metabolite) - insufficient pain relief - possible ADRs i.e. bradycardia	Consider alternative analgesic not metabolised by CYP2D6
		UM	Increased levels of O-desmethyltramadol (potent metabolite)active metabolite – sub-therapeutic activity - increased risk for relapse	Reduced dose by 30% and be aware of ADRs
Zuclopenthixol	Typical Antipsychotic	PM	Higher plasma concentration - possible ADRs	Reduced dose by 50%/consider alternative antipsychotic
		IM	Higher plasma concentration - possible ADRs	Reduced dose by 25%/consider alternative antipsychotic

ADR-adverse drug reaction; IM-intermediate metaboliser; INR international normalised ratio (measure of coagulation); PM-poor metaboliser; QTc, QT interval (a measure of time between the Q and T wave in the heart's electrical cycle); TDM, therapeutic drug monitoring; UM-ultrarapid metaboliser. This is not a complete list of all CYP2D6 substrates.

2.6 Genotyping technologies

Diagnostic genotyping is the ultimate goal of pharmacogenetics. Ideally diagnostic genotyping requires relative ease of assay, robustness (work with potentially suboptimal samples), relatively automated with minimal steps (reduce human error) and should be relatively inexpensive. Pharmacogenetic screening a of patientôs gene would then allow for phenotype to be predicted from the genetic profile. Detection of polymorphisms can be accomplished using a variety of techniques including denaturing high performance liquid chromatography (DHPLC), restriction fragment length polymorphism (PCR-RFLP) analysis, Sanger sequencing, pyrosequencing, real-time polymerase chain reaction (RT-PCR), DNA/RNA/cDNA microarray-based assays and next generation sequencing (Linder, Prough & Valdes 1997; Meyer 2000; Brockmoller & Tzvetkov 2008; Rebsamen et al. 2009; Ware, Roberts & Cook 2012). PCR-RFLP, RT-PCR and sequencing are cost effective platforms. The former two platforms allow for identification of known polymorphisms and the latter both unknown and known polymorphisms. Unlike many of the other techniques, oligonucleotide microarray-based

methods and next generation sequencing provide the capacity to analyse many polymorphisms simultaneously in a single assay (Rebsamen et al. 2009; Ware, Roberts & Cook 2012). Microarray has the ability to identify many known polymorphisms and next generation sequencing the ability to identify both known and unknown polymorphisms. One of the major disadvantages of both these platforms is cost. If only one gene is being investigated they would potentially be unrealistic.

Several companies supply DNA assays to detect known sequence variations in several selected polymorphic CYP genes. Some of these companies include Roche Diagnostic Corp. (AmpliChip P450 Test), General Electric Health Care (CodeLinkTM P450 SNP Bioarray), Tm Bioscience Corp. (Tag-itTM Mutation Detection Kit microspheres developed by Luminex), Third Wave Molecular Diagnostics (Invader® Technology), Jurilab LTD (DrugMetÊ Genotyping Test) and Seegene Institute (CYP2C19 DPO test) (de Leon, Armstrong & Cozza 2006; Chun et al. 2007). Genotyping tests such as these allow diagnostic laboratories to implement diagnostic tests, providing physicians with vital information to implement personalised medicine.

2.6.1 *AmpliChip CYP450 test*

The AmpliChip combines Roche PCR amplification technology and Affymetrix GeneChip CYP450 oligonucleotide microarray instrumentation. There are five major processes involved (Rebsamen et al. 2009):

(i) PCR amplification of isolated patient DNA
(ii) Fragmentation and labelling of the amplified products
(iii) Hybridization of the amplified product to the microarray and staining
(iv) Scanning of the microarray
(v) Determination of the CYP450 genotype using Roche software

The genotypic information is then used to predict an individualôs CYP2D6 and CYP2C19 enzymatic activity (predicted phenotype) based upon published studies (Roche diagnostics technical notes). The predicted phenotypes (as indicated above) are PM, IM, EM andUM. This technique has the unique advantage of simultaneously assaying for 33 variant alleles in *CYP2D6* and 3 in *CYP2C19* including complete gene

deletions (e.g. *CYP2D6*5*) and gene duplications (e.g. *CYP2D6*1Xn*, *CYP2D6*2Xn*, etc.). The AmpliChip CYP450 test has several advantages over other techniques. RT-PCR for example requires many different probes specified by the investigator (Wright et al. 2010). Gene sequencing is another tool which can be used to genotype an individual. However, compared to the AmpliChip CYP450 test, sequencing has the disadvantage of requiring at least 3 different assays (polymorphism, gene deletion and gene duplication identification) to provide equal coverage (Rebsamen et al. 2009).

2.6.2 *DNA sequencing*

Gene sequencing is a very powerful and comprehensive technique, as one acquires base by base information for the entire gene (i.e. one is not limited to previously identified variations as would be the case with an oligonucleotide based microarray). Sanger sequencing has become the platform of choice used by many geneticists due to its high-throughput capability and its low error rate (when compared to next generation sequencing). However, in some circumstances this technique may not be viable due to the sheer length of the gene (e.g. CYP2C19 >90 000 bp), in which case ñhot spotsò (areas of interest) or only exons (the ñexomeò) are typically examined. Although many of the commercially available techniques are rapid and in some cases more convenient, the sequence of each individualôs gene can be analysed for novel mutations and polymorphisms which are not detected by the other techniques (the AmpliChip CYP450 Test for example). To date little work has been done regarding *CYP2D6* and *CYP2C19* on the South Africa population and therefore sequencing can be a valuable research tool for identifying genetic variations before the use of rapid diagnostic tests. There are two disadvantages of gene sequencing compared to the AmpliChip CYP450 test mentioned above, namely the inability to simultaneously identify (i) compete gene deletions and (ii) multiple copies or gene duplications. However these limitations can be overcome by using several separate complimentary assays. Should the focus only be on known polymorphims, PCR-RFLP and RT-PCR may be more cost effective.

2.7 Phenotyping technologies

Phenotyping (the assessment of phenotype) has been used to assess an individualôs rate of metabolism, and this technique is valuable as a comparative tool. One of the major advantages of measuring phenotype over predicting phenotype is the ability to account for genotypic variation as well as various additional influencing (genetic and non-genetic) factors which may influence phenotype. Non-genetic influences include ethnicity, diet, drug-drug interactions, smoking, gender, age, liver size and function and concomitant disease or physiological conditions (Figure 2-1), all of which may influence drug metabolism but would not be detected by genotyping (Burroughs et al. 2002). Genetic influence may include other proteins which may be causing variable response in pharmacokinetic ADME and pharmacodynamics.

2.7.1 *Principe of the platform*

In phenotyping, a probe drug is carefully selected to evaluate variability in enzyme metabolism of one or more enzymes (Streetman, Bertino & Nafziger 2000). This is designed to emulate the metabolism of a drug with a narrow therapeutic window without exposing the patient or volunteer to unwanted ADRs (Marzo & Balant 1996). The probe drug is administered to the individual and after an appropriate time period (usually T_{max}) the concentration of the parent and metabolite is evaluated. A metabolic ratio (MR) (probe drug metabolite or metabolite to parent drug) is calculated by determining plasma, saliva, serum or urine concentrations. The MR is the phenotypic indication of rate of metabolism (Linder, Prough & Valdes 1997) and is a more efficient way of measuring metabolism, compared to measurement of clearance of a compound over several time points in a kinetic type evaluation (Frank, Jaehde & Fuhr 2007). Phenotyping is typically represented graphically as a $\log_{10}$ transformation of MR as metabolic elimination (pharmacokinetics) tends to be exponential. In order for phenotyping to be effective, the probe drug must be ñselective for the enzyme being studied, be sensitive to changes in enzyme content or activity, require minimally invasive sampling, be non-toxic, and not directly affect the activity of the enzymeò (Frerichs, Zaranek & Haas 2005).

2.7.2 *Instrumentation: selecting the optimal tool for phenotyping*

Researchers have opted for high performance liquid chromatography (HPLC) to measure pharmaceuticals in biological fluids as chromatography allows for separation of complex mixtures to resolve each molecule before detection. Traditionally, detection based on light absorbance or emission by ultraviolet/visual spectrum (UV-Vis) or fluorescent (FLD) detector respectively, would be used to detect pharmaceuticals in various sample matrices (Swartz 2010). If matrix interference or other analytes are eluted at the same time, UV-Vis detection has the disadvantage of not being able to differentiate between the interference and the analytes of interest. FLD detection has one level of selectivity over UV-Vis spectrometry, as the interfering molecule will have to both absorb at the same wavelength of light as the analyte of interest and emit the same wavelength of light. FLD also has the advantage of being, in some cases, 1000 times more sensitive than UV-Vis (Swartz 2010). This selectivity is advantageous, but not absolute, as interference may still be present. FLD detection may suffer from quenching (co-eluting interference which absorbs at the emitted wavelength) and there may still be uncertainty in identification of the analyte of interest. In addition, FLD can only be used if the analyte of interest fluoresces.

To offer more selectivity for the analyte of interest, detection of an ionised analyte by a mass spectrometer offers detection based on mass. Single quadruple mass spectrometry (LC-MS) offers the next level in selectivity as mass is used to discriminate eluting compounds. In this case a specific mass can be extracted from a chromatogram or the mass can be used to exclude interference. LC-MS may select based on mass, but there may still be co-eluting interference which has the same mass as the analyte of interest or may suppress the ionisation process in the source. In addition scan data generated by a LC-MS tends to lack sensitivity in low concentration samples. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) offers a greater level of selectivity, as this mass spectrometer selects for the mass of the analyte of interest, fragments the molecule and evaluates the ratio between precursor and fragment ions (Vogeser & Seger 2008; Himmelsbach 2012). In this way LC-MS/MS has the ability to select for an analyte of interest with a very high level of confidence. Monitoring both a precursor and several fragment ions for selectivity is known as multiple reaction monitoring (MRM)

and LC-MS/MS can monitor many MRMs simultaneously. This simultaneous ability to monitor MRMs makes this detector a powerful quantitative tool for simultaneous quantification of many different analytes in a single sample. As this selectivity is built in to an LC-MS/MS method, these detectors tend to have sensitivity down to sub-picomole/ml concentration ranges. Unfortunately LC-MS and LC-MS/MS suffer from matrix effects inducing ion suppression which can interfere with both sensitivity quantification reproducibility (Matuszewski, Constanzer & Chavez-Eng 2003; Vogeser & Seger 2010). Fortunately these disadvantages can be largely compensated for by the use of an internal standard (Matuszewski, Constanzer & Chavez-Eng 2003). The next level in selectivity is high resolution time of flight mass spectrometry (LC-TOFMS). LC-TOFMS selects for the analyte of interest using very high mass accuracy and spectral data based on isotopic distribution. This selectivity can be taken to the next level by performing MS^n, where the analyte of interest is fragmented at different energy levels allowing for different ratios of fragments and identification of structural components of the analyte. Unfortunately, LC-TOFMS does not have a wide linear dynamic quantification range and is therefore seen as more of a qualitative rather than a quantitative instrument.

LC-MS/MS would be perfect for quantification of each probe drug and their respective metabolites (Frank, Jaehde & Fuhr 2007) as it is far more sensitive, selective, accurate than the other detectors for HPLC.

2.8 South African population

Africaôs population is highly diverse, with Sub-Saharan Africans displaying the highest degree of genetic diversity on the planet (Tishkoff et al. 2009). Genetic diversity in Africa is thought to be a result of greater environmental pressure compared to non-Africans (Tishkoff et al. 2009). Of the African countries, South Africa has one of the most complex population structures. This complexity is evidenced by the many indigenous and ethnically distinct cultural groups residing in South Africa. Commonly, this diversity is recognised by the 11 official languages spoken in South Africa namely Afrikaans (13.3%), English (8.2%), Ndebele (1.6%), Sepedi (9.4%), Setswana (8.2%),

Sotho (7.9%), Swazi (2.7%), Tsonga (4.4%), Venda (2.3%), Xhosa (17.6%), and Zulu (23.8%) (http://www.statssa.gov.za/census01/HTML/CInBrief/CIB2001.pdf). In the interest of simplifying complexity, census documents ask for voluntary disclosure of belonging to one of four major groupings, namely Black African (79.02%), White/Caucasian (9.58%), Coloured/Mixed Ancestry (8.91%) and Indian/Asian (2.49%) populations.

Coloured is a South African term, officially used to describe a complex and uniquely admixed group of South Africans predominantly, but not exclusively, residing in the Western Cape. Coloureds have many different ethnic ancestries including European, Asian and African; admixture was favoured due to South Africaôs position in major trade routes during the fifteenth to nineteenth century (de Wit et al. 2010; Patterson et al. 2010).

In addition to the Coloureds and numerous indigenous cultures and tribes, South African populations are also characterised by immigration (ongoing) from many neighbouring countries as well as Europe and sub-continental and East Asia. As a consequence, South African populations may be highly admixed and complex, making genotype analyses challenging (Warnich et al. 2011). Adding to the complexity are subpopulations within the South African population who have very little admixture, which may be genetically very distinct from one another. These subpopulations are kept relatively homogeneous for many reasons such as religion, language, culture and geographical location to name a few. Certainly, the complexity of South African populations needs to be taken into account when designing a pharmacogenetic screening strategy.

2.9 Study Motivation

Very little is known about South African pharmacogenetics in terms of CYP2C19 and CYP2D6. For both these genes, limited genetic studies have been carried out investigating South African cohorts. No CYP2C19 phenotype studies have been conducted to date and the studies conducted to investigate CYP2D6 are contradictory as large differences in ratios of PMs were observed. No study has investigated the relationship between measured and observed phenotype in a South African study and very few studies have used a comprehensive genotyping platform to investigate pharmacogenetic potential to improve treatment outcome in South Africa. This studyinvolved investigating each of these aspects, contributing to pharmacogenetic knowledge with the vision of applying pharmacogenetics in South Africa to improve drug therapy and reduce ADRs.

2.10 Aims and objectives:

2.10.1 *Aim*

1. To compare predicted phenotype and measured phenotype in a demographically representative cohort and to describe the relationship between genotype (using predicted phenotype) and phenotype for CYP2D6 and CYP2C19 in the South African population.

2. To apply pharmacogenetic analysis to predict phenotype (ADRs) in a small South African cohort clinically diagnosed with schizophrenia and treated with risperidone.

2.10.2 *Objectives:*

Cohort recruiting

1. Recruit 100 apparently healthy South African volunteers as 70 Black African, 10 Caucasian, 10 Coloured and 10 Indian to generally represent the current demographics of the South African population for evaluation of genetic platforms and to compare predicted and measured phenotype.
2. Recruit schizophrenic South African patients experiencing ADRsfrom risperidonetreatment.

Genotyping

3. Utilise the Roche CYP450 AmpliChip Microarray to genotype *CYP2C19* and *CYP2D6* in the demographically representative South African cohort.
4. Utilise a previously validated PCR based restriction fragment length polymorphism (PCR-RFLP) method developed by Drögemöller et al. (2010) to genotype *CYP2C19* in the demographically representative South African cohort.
5. Compile a gene sequence strategy for a base by base analysis of the *CYP2D6* gene with the ability to identify new polymorphisms and novel alleles.
6. Assess the *CYP2D6*gene for polymorphisms using a gene sequencing strategy using the demographically representative South African cohort.
7. Compare the AmpliChip results with the PCR-RFLP and sequencing strategy for their ability to genotype *CYP2C19* and *CYP2D6* respectively.
8. Implement the Activity Score (Gaedigk et al. 2008) model or an adaptation of this numeric system to predict phenotype for *CYP2C19* and *CYP2D6* using genotypic data and metabolic activity information available on The Human Cytochrome P450 (*CYP*) Allele Nomenclature Database (http://www.imm.ki.se/cypalleles) website.
9. Use the *CYP2D6* sequencing strategy to genotype and predict phenotype in the risperidone treated schizophrenic South African cohort experiencing ADRs.

Phenotyping

10. Improve the technique of online solid phase extraction (SPE) as sample preparation for liquid chromatography, with emphasis on simplicity and efficiency.
11. Develop and validate an efficient LC-MS/MS method to simultaneously measure plasma concentrations of omeprazole, dextromethorphan, 5ô-hydroxyomperazole and dextrorphan.
12. Use the developed semi-automated online SPE LC-MS/MS to quantifyomeprazole, dextromethorphan, 5ô-hydroxyomperazole and dextrorphan at two, three and four hours following probe drug administration to the demographically representative South African cohort.
13. Statistically establish the optimal time point for simultaneous drug concentration measurement for phenotype assessment.

14. Use metabolic ratios (MRs) calculated from plasma concentrations of probe drug and metabolite to measure phenotype for *CYP2C19* and *CYP2D6*.
15. Establish phenotypic cut-offs/antinodes to distinguishing different classes of drug metaboliser for *CYP2C19* and *CYP2D6*.
16. Phenotype the resperidone schizophrenic South African cohort in terms of ADRs (movement disorders and weight gain).

Genotype vs. phenotype

17. Determine which of the tested pharmacogenetic screening platforms could most accurately predict phenotype for CYP2C19 and CYP2D6 using data from the demographically representative South African cohort.
18. Statistically evaluate the correlation between predicted and measured phenotype for CYP2C19 and CYP2D6 using data from the demographically representative South African cohort.
19. Utilise a *CYP2D6* gene sequencing strategy in a pilot study, to genotype patients being treated with risperidone displaying ADRs, examining the relationship between poor metabolism genotype and side effects.

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CHAPTER 3. INTRODUCTION OF THE AMPLICHIP CYP450 TEST TO A SOUTH AFRICAN COHORT: A PLATFORM COMPARATIVE PROSPECTIVE COHORT STUDY

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3.1 Abstract

Background: Adverse drug reactions and lack of therapeutic efficacy associated with currently prescribed pharmacotherapeutics may be attributed, in part,to inter-individual variability in drug metabolism. Studies on the pharmacogenetics of Cytochrome P450 (CYP) enzymes offer insight into this variability. The objective of this study was tocompare the AmpliChip CYP450 Test® (AmpliChip) toalternative genotyping platformsfor phenotype prediction of CYP2C19 and CYP2D6 in a representative cohort of the South African population. **Method:**The AmpliChip was used to screen for thirty-three*CYP2D6* and three*CYP2C19* alleles in two different cohorts. As a comparison cohort 2 was then genotyped using a *CYP2D6* specific long range PCR with sequencing (*CYP2D6* XL-PCR+Sequencing) platform and a PCR-RFLP platform for seven *CYP2C19* alleles. **Results:** Even though there was a low success rate for the AmpliChip, allele frequencies for both *CYP2D6* and *CYP2C19* were very similar between the two different cohorts. The *CYP2D6* XL-PCR + Sequencing platform detected *CYP2D6*5* more reliably and could correctly distinguish between *CYP2D6*2* and **41*in the Black African individuals. Alleles not covered by the AmpliChip were identified and four novel *CYP2D6* alleles were also detected. *CYP2C19* PCR-RFLP identified *CYP2C19*9,*15,*17* and **27*in the Black African individuals, with **2, *17* and **27* relatively frequent in the cohort. Eliminating mismatches and identifying

additional alleles will contribute to improving phenotype prediction for both enzymes. Phenotype prediction differed between platforms for both genes. **Conclusion:** More comprehensive genotyping of *CYP2D6* and *CYP2C19*, such as the platforms used in this study, would be more appropriate for pharmacogenetics in the South African population. Pharmacogenetically important novel alleles may remain undiscovered in the South African population when using assays that are designed according to Caucasian specific variation, unless alternate strategies are utilised.

3.2 Background

In order for a pharmacogenetic screening assay to be effective, it must be able to deal with highly polymorphic genes with high throughput capability in an efficient and cost effective way. The Roche AmpliChip CYP450 Test® (AmpliChip) was created with this in mind. In 2005, this Affymetrix platform (Roche Molecular Systems, Inc., Branchburg, NJ) became the first DNA based microarray to be approved by the Food and Drug Administration (FDA) for*CYP2C19* and *CYP2D6* pharmacogenetics (de Leon et al. 2009). The AmpliChip is a high-throughput, comprehensive screening assay designed to simultaneously identify thirty-three *CYP2D6* and three*CYP2C19* alleles from whole blood-derived DNA (http://www.amplichip.us/documents/CYP450_P.I._US-IVD.pdf). In an initial assessment of the AmpliChip, de Leon et al. (2006)said that, ñthis new technology is a major step in ushering ópersonalized prescriptionô into the clinical environment.ò Rebsamen et al. (2009)observed that the AmpliChip is good at predicting PMs and EMs, satisfactory in predicting IMs, but not as efficient at predicting UMs. In summarising, Rebsamen et al. (2009) stated that, ñthis microarray technology could be an excellent tool to improve phenotype prediction.ò The AmpliChip has been validated for *CYP2D6* on German Caucasians (n=158, (Heller et al. 2006)), female Swiss Caucasians (n=165, (Rebsamen et al. 2009)) and a combined Caucasian (n = 3779) and African American (n =452) (de Leon et al. 2009). Heller et al. (2006) concluded that the AmpliChip was fast, accurate and comprehensive in its identification of *CYP2D6* genotype and predicted phenotype. A summary of these articles can be found in Table 3-1 and notably there are more PMs in Caucasians compared to Black Africans and

Koreans .The only group to report results for *CYP2C19* was de Leon et al. (2009). This study found that 98.0% of American Caucasians were EM and 2.0% were PM (cohort: n = 3938), with and allele frequency of 14.2% for *CYP2C19*2*and 0.0% for **3*. In comparison 96.0% of African Americans (cohort size = 478) were predicted to be EM and 4% were predicted to be PM, with allele frequencies of 18.3% for *CYP2C19*2* and 0.1% for **3* (de Leon et al. 2009).

Although several European descent populations have been investigated using the AmpliChip, this assay has not been used to genotype an African population residing in Africa. Considering that novel alleles have been found in African cohorts (Gaedigk & Coetsee 2008; Matimba et al. 2009; Drögemöller et al. 2010; Wright et al. 2010; Warnich et al. 2011), it is important to evaluate these genetically diverse population when considering pharmacogenetic implementation. This requires addressing, considering that ADRs occur in an estimated 14% of hospitalised South African patients resulting in a 5-10 fold higher fatality compared to USA and UK hospitals (Mehta et al. 2008). The implementation of a pharmacogenetic assay may assist in reducing the socio-economic burdens associated with this non-optimal treatment in South Africa. The objective of this study was therefore to evaluate the AmpliChip for use in the South African population as a pharmacogenetic screening tool for *CYP2D6* and *CYP2C19*.

Table 3-1. Summary of reported *CYP2D6* genotyping studies using the AmpliChip CYP450 test.

Article	Nikoloff et al. (2002)		Ishida et al. (2002)	Heller et al. (2006)	Rebsamen et al.(2009)	de Leon et al. (2009)		Ramón y Cajal et al. (2010)
Cohort	**TD+ Schizophrenic Koreans**	**TD- Schizophrenic Koreans**	**Japanese**	**German Caucasians**	**Swiss female Caucasians**	**American Caucasians**	**African Americans**	**Tamoxifen treated Spanish Caucasians**
Varian				***Allele frequency (%)***				
****1***	38.2	40.7	46.9	36.2	35.5	37.4	59.7	35.7
****2***	9.1	9.2	19.8	10.5	15.5	15.9	6.0	16.5
****3***	0.0	0.0	0.0	3.0	0.6	1.8	0.2	0.5
****4***	0.0	0.0	0.0	12.2	20.6	21.0	5.5	14.8
****5***	3.6*	1.6*	ī	8.2	2.4	2.3	2.8	4.9
****6***	0.0	0.0	0.0	2.6	1.2	1.1	0.2	0.5
****7***	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0
****8***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
****9***	0.0	0.0	0.0	0.7	2.7	2.9	0.4	7.1
****10***	47.3	46.1	33.3	1.6	2.7	1.0	3.8	4.4
****11***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
****14***	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
****15***	ī	ī	ī	0.0	0.0	0.0	0.0	0.0
****17***	ī	ī	ī	0.0	0.3	0.3	18.4	0.0
****18***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
****19***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
****20***	ī	ī	ī	0.0	0.0	0.0	0.0	0.5
****25***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
****26***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
****29***	ī	ī	ī	0.0	0.3	0.2	7.7	0.0
****30***	ī	ī	ī	0.0	0.0	0.0	0.0	0.0
****31***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
****35***	ī	ī	ī	6.6	7.3	4.8	0.9	4.4
****36***	0.0*	0.0*	ī	0.0	0.0	0.0	0.6	0.0
****40***	ī	ī	ī	0.0	0.0	0.0	0.3	0.0
****41***	1.4	2.2	0.0	6.3	7.3	9.8	14.9	9.3
****1xn***	ī	ī	ī	6.6	0.6	0.7	0.8	0.5
****2xn***	ī	ī	ī	4.9	1.5	0.5	1.2	0.5
****4xn***	ī	ī	ī	0.3	0.3	0.1	2.4	0.0
****6xn***	ī	ī	ī	0.0	0.0	0.0	0.0	0.0
****10xN***	ī	ī	ī	0.0	0.0	0.0	0.0	0.0
****17xN***	ī	ī	ī	0.0	0.0	0.0	0.2	0.0
****29xN***	ī	ī	ī	0.0	0.0	0.0	0.2	0.0
****35xN***	ī	ī	ī	0.3	0.0	0.0	0.0	0.0
****41xN***	ī	ī	ī	0.0	0.9	0.1	0.0	0.0
Total alleles	220	184	162	304	330	7558	904	182
				Predicted phenotype				
PM	0 (0.0)	0 (0.0)	Not reported	10.5 (16)	9.1 (15)	8.2 (311)	1.8 (8)	6.6 (6)
IM	23.6 (26)	25 (23.0)		4.0 (6)	7.9 (13)	9.7 (365)	32.7 (148)	11.0 (10)
EM	76.4 (84)	75 (69.0)		71.7 (109)	81.8 (135)	80.7 (3048)	63.5 (287)	81.3 (74)
UM	ī	ī		13.8 (21)	1.2 (2)	1.5 (55)	2.0 (9)	1.1 (1)
Cohort	110	92	81	152	165	3779	452	91

Alleles not covered by AmpliChip at the time of reporting are represented by ñð ò. The microarray used in the Nikoloff et al. (2002) paper did not cover *CYP2D6*5*; however, a separate assay was used for **5* detection and the frequency was reported. Predicted phenotype reported or adjusted to represent AmpliChip test calls. TD+; patients with tardive dyskinesia, TD- patients without tardive dyskinesia.

3.3 Materials and Methods

3.3.1 *Study subjects and sampling*

Ethical approval was obtained from the Research Ethics Committee, Faculty of Health Science, University of Pretoria (Approval numbers: Cohort 1 - 102/2005and Cohort 2 - S132/2009) and the study was conducted in accordance with the Declaration of Helsinki, using GCP guidelines. All participating volunteers wereÓ18 years of age,South African citizens and resided in the city of Pretoria during the sampling period. These cohorts were chosen to be demographically representative of the general population of South Africa (http://www.statssa.gov.za/). It should be noted however, that it is not the authorsô intention to use this study for inter-ethnic comparisons. Informed consent was obtained from all participants along with general demographic information including place of birth and voluntary disclosure of ethnic group (Black African, Caucasian, Coloured and Indian). The term Coloured, also referred to as Mixed Ancestry in the South African context, is used officially to describe an admixed group of people predominantly residing in the Western Cape (de Wit et al. 2010; Patterson et al. 2010). The admixture present in this population is derived from several different ancestries including European, Asian and Africa, primarily Khoisan and Bantu influence. The high level of admixture can be attributed to the presence of the major trade routes in South Africa during the fifteenth to nineteenth centuries (de Wit et al. 2010; Patterson et al. 2010).

Cohort 1

Diabetic individuals (n = 83): 57 Black African; 6 Caucasian; 10 Coloured and 10 Indian. These individuals were attending the Diabetic Clinic at the Steve Biko Academic Hospital in Pretoria. This cohort was genotyped using the AmpliChip.

Cohort 2

Apparently healthy volunteers (n=100) were recruited from several different sites in Pretoria. This cohort consisted of 70 Black African, 10 Caucasian, 10 Coloured and 10 Indian individuals. This cohort was used to comparatively evaluate the AmpliChip

platform using PCR-RFLP and XL-PCR+Sequencing for *CYP2C19* and *CYP2D6* respectively.

3.3.2 *Genomic DNA (gDNA) extraction*

Venous blood samples collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes(Becton-Dickinson, Franklin Lakes, NJ, USA) were used for gDNA extraction. Extraction was performed using the Genomic DNA Purification Kit (Fermentas Life Science, Lithuania) or the automated Maxwell® 16 system (Promega, Madison, WI, USA),and extraction was performed according to the manufacturerôs instructions.

3.3.3 *AmpliChip CYP450 Test*

Each sample was simultaneously evaluated for *CYP2D6* and *CYP2C19* using the AmpliChip CYP450 Test® (Roche Molecular Systems Inc., Pleasanton, CA, USA) according to the manufacturerôs protocol (AmpliChip CYP450 Test package insert). In brief, *CYP2D6* and *CYP2C19* were amplified in two separate reactions. Both reactions were monitored for amplification using 1.0% agarose gel electrophoresis for 1 hour (not in protocol). Reactions were pooled and subjected to *DNase* I (Roche Molecular Systems Inc.) fragmentation, following which the fragments were 3ô-end labelled using Terminal Transferase (Roche Molecular Systems Inc.) and TdT Labelling Reagent (supplied in the AmpliChip kit).

Using a pre-programmed protocol, the labelled fragments were hybridised onto the AmpliChip CYP450 microarray, stained with streptavidin-conjugated phycoerythrin (Invitrogen Corp., Carlsbad, CA, USA) and washed in an Affymetrix GeneChip Fluidics Station 450Dx (Affymetrix, Santa Clara, CA, USA). Each AmpliChip was then scanned with an Affymetrix GeneChip Scanner 3000Dx (Affymetrix). The resulting image was orientated using GeneChip Operating Software (Affymetrix) and transferred to AmpliChip CYP450 Data Analysis Software (Roche Molecular Systems Inc.) to determine *CYP2D6* and *CYP2C19* genotypes and predicted phenotypes.

3.3.4 *CYP2C19 Genotyping*

The same gDNA samples analysed by the AmpliChip was also analysed by a PCR-RFLP platform designed for South African Xhosa individuals (Drögemöller et al. 2010). This assay was used to evaluate the ability of the AmpliChip to genotype the *CYP2C19* variation present in the South African population.Alleles identified and assayed were named according the *CYP* Allele Nomenclature Committeeôs online database (http://www.cypalleles.ki.se/). This platform focuses on identifying allele defining SNPs for *CYP2C19*2, *3, *9, *15, *17, *27* and **28* alleles (method summarised in Supplementary Table S1 and S2).

3.3.5 *CYP2D6 Long Range PCR with Sequencing*

A *CYP2D6* Long Range PCR with Sequencing (XL-PCR+Sequencing) strategy was designed to genotype the Cohort 2 and assess the ability of the AmpliChip to successfully genotype the functionally significant alleles present in the South African population. This alternate approach included a series of long range PCR (XL-PCR) amplifications for detection of *CYP2D6*5* (complete gene deletion), *CYP2D6* duplication (increased copy number) and to amplify a *CYP2D6* product for sequencing (introns and exons). All primers (Supplementary Table S3 and S4) utilised for amplification were manufactured by Inqaba Biotechnical Industries (Pretoria, South Africa). The amplification reactions (in detail below) were performed using a Gold-plated 96-Well GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA), followed by electrophoresis using 1.0% agarose gels for 1 hour.

*XL-PCR reactions detection of CYP2D6*5 and duplications*

*CYP2D6*5*detection was based on a duplex XL-PCR assay described by Hersberger et al. (2000). This reaction was performed using Long-Range Taq polymerase (Fermentas Life Science). PCR reaction conditions were optimised for primer concentration and denaturing time to ensure equal amplification of the *CYP2D6*5*deletion fragment (3.2 kb) and the whole*CYP2D6* gene fragment (5.1 kb). Heterozygous samples were

repeated using only the *CYP2D6*specific primers in order to generate the 5.1kb amplicon for sequencing.

The XL-PCR duplex amplification reaction described byGaedigk et al. (2007b) was used to detect the presence of *CYP2D6*duplications (primers and conditions in Supplementary Table S3). A separate XL-PCR reaction amplified a duplication-specific product allowing amplification and characterisation of allelic status of the duplicated gene (Gaedigk et al. 2007b). Theduplication-specific product was characterised by re-sequencing (primers and conditions in Supplementary Table S3).

CYP2D6 re-sequencing

Prior to re-sequencing, amplified PCR products were purified using Exonuclease I and FastAPÊ Thermosensitive Alkaline Phosphatase (Fermentas Life Science) (Werle et al. 1994). Sanger sequencing was then performed by Inqaba Biotechnological Industries, Pretoria, South Africa using the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 and 3130XL and 3500XL sequencersystems (Applied Biosystems Inc.) using primers provided in Supplementary Table S4.

Electropherograms were edited using FinchTV version 1.4.0 (Copyright © 2004-2006, Geospiza Inc.). Following editing, sequences were imported into CLC DNA Workbench version 5.5 (CLCBio, Aarhus, Denmark), assembled and compared to the *CYP2D6* reference sequence AY545216 (GenBank). As with the AmpliChip, *CYP2D6* sequence variations were numbered and assigned alleles according the P450 Nomenclature Committee website.

Evaluation of exon 9 gene conversion

The presence of non-functional *CYP2D6*4N* and **36* allelic variants where evaluated by assaying for the presence of a *CYP2D7* gene conversion in exon 9. The PCR reaction (primers and conditions in Supplementary Table S3) was performed as described by Gaedigk et al. (2006) using BIOTAQ^{TM} DNA Polymerase (Bioline, London, UK). The amplicon was analysed using 3% agarose gel electrophoresis.

Characterisation of novel alleles

To characterise haplotypes associated with novel non-synonymous SNPs, a 6.6 kb long PCR product was amplified using *CYP2D6* specific primers described previously (Gaedigk et al. 2007b). This product was cloned using the CloneJET™PCR Cloning Kit (Fermentas Life Science) according to manufacturerôs instructions and transformed into DH5Ŭ cells (Zymo Research, Orange, CA, USA). Colonies were screened by amplifying the region of interest (where the novel SNP was located) using relevant sequencing primers followed by sequencing. Once the correct colony was identified, colony extraction was performed using Zuppy™ Plasmid Miniprep Kit (Zymo Research) and sequenced as previously described. The haplotype of the novel allele was determined by comparing the sequence obtained from the cloned allele and sequence of the XL-PCR product representing both alleles. Novel allele defining non-synonymous SNPs were analysed using ñsorting intolerant from tolerantò (SIFT) and PolyPhen prediction software estimating*in silico*effect on CYP2D6 activity (Ramensky, Bork & Sunyaev 2002; Ng & Henikoff 2003). Potential splice site variation was evaluated *in silico* using NetGene2 (Brunak, Engelbrecht & Knudsen 1991; Hebsgaard et al. 1996). Novel allelesequences were submitted to the *CYP* Allele Nomenclature Committee for *CYP2D6* allele designation.

3.3.6 *Phenotype prediction*

The AmpliChip software predicted phenotype based on principles explained in Table 2-1 (Gaedigk et al. 2008) (AmpliChip CYP450 Test package insert). The Activity Score (AS) model (Gaedigk et al. 2008) was used to predict phenotype from data generated by *CYP2D6* re-sequencing and the AmpliChip. AS were calculated using model A (Gaedigk et al. 2008). Novel alleles were assigned an AS of 1.0 to allow for phenotypic comparison, since actual enzyme activity has not yet been confirmed. The exception was *CYP2D6*4P*; its novel non-synonymous SNP was linked with 1846G>A, the *CYP2D6*4*-defining SNP that causes a splice defect thereby obliterating activity (AS = 0). The AS was also adopted to predict CYP2C19 phenotype, which is explained in Table 2-1.

3.3.7 *Statistics*

Tools for Population Genetic Analysis (TFPGA) software v1.3 (Miller, 1997: http://www.marksgeneticsoftware.net/tfpga.htm) was used to test allele deviation from Hardy-Weinberg equilibrium using a Fisherôs exact test for each ethnic group within each cohort and for comparing platforms using Fisherôs exact test. Linkage disequilibrium was evaluated using Haploview software v3.31 (Barrett et al. 2005). A *P* value of <0.05 was considered significant.

3.4 Results

3.4.1 *Success rate of the AmpliChip CYP450 Test*

Cohort 1 (n=83) had a success rate of 75.9% for *CYP2D6* and 98.8% for *CYP2C19* for the AmpliChip. There were five ñNo Callsò (all hybridisation positions were occupied and identified by the AmpliChip software, but based on the hybridisation pattern a genotype could not be generated, nor a phenotype predicted) for *CYP2D6*, raising the success rate of the AmpliChip to 81.9% with only 75.9% generating pharmacogenetically relevant data. None of the failed AmpliChips were repeated for this group.

Cohort2 (n=100) had a success rate of 71.0% for *CYP2D6*. Of the AmpliChip micorarrays which failed to generate a genotype, 4.0% were ñNo Callsò. Therefore, 75.0% of the micoroarrays were successful, of which only 71.0% gave pharmacogenetically relevant results. The most frequent hybridisation failures in both cohorts were at the 1758G locus, which is associated with *CYP2D6*8* (1758G>T) and **14* (1758G>A) alleles. The AmpliChip information leaflet mentioned that this would be the most likely hybridisation locus to fail. For *CYP2C19*, 100.0% of the AmpliChips generated a genotype, and a predicted phenotype could thus be assigned in all cases.

Thirteen failed samples and two successful samples (positive controls) were repeated in order to estimate user error. The two samples which had succeeded previously were again successful. Of the thirteen failures, two succeeded, three failed (but hybridised at

additional loci), one failed at different loci and the balance failed as they did before (missing the same hybridisation loci).

3.4.2 *CYP2C19genotype analysis*

AmpliChip

Using the AmpliChipto evaluate genotype, it was found that there were no statistically significant differences in *CYP2C19*allele frequencies between the two sampled cohorts ($P_{\text{¸}}$ 0.08) and all alleles were in Hardy-Weinberg equilibrium in both cohorts. (Table 3-2). Typically rare, *CYP2C19*3* only occurred in Cohort 1, but was relatively infrequent and not statistically significant. .

PCR-RFLP

The PCR-RFLP platform identified significantly high frequencies (refer to Table 3-2; *P*Ò0.002) of *CYP2C19*15* (unknown), **17* (increased metabolism) and **27* (decreased metabolism). Although not significant when combining the ethnicities *P*=0.058,

Table 3-2. *CYP2C19*allele and predicted phenotype frequency in a demographically representative South African (SA) cohort (n=100) compared to other cohorts sampled in SA.

		Cohort 1 AmpliChip				Compare	Cohort 2 AmpliChip				Compare	PCR-RFLP				Dandara et al. (2001)	Dandara et al. (2011)	Drögemöller et al. (2010)		Ikediobi et al. (2011)		Matimba et al. (2009)
Allele	Activity	Black African	White Caucasian	Coloured	Indian	Fischer's Exact	Black African	White Caucasian	Coloured	Indian	Fischer's Exact	Black African	White Caucasian	Coloured	Indian	SA Venda	SA Bt20 (mixed Black)	SA Xhosa	SA Coloured	SA Xhosa	SA Coloured	SA Venda
Allele Frequency (%)																						
1+	Normal	85.7	75.0	75.0	60.0	1.000	82.1	95.0	65.0	65.0	0.000	32.1	70.0	40.0	40.0	78.0	23.0	17.0	41.0	63.0	71.0	77.0
2	Absent	116.0	25.0	25.0	40.0	0.784	17.9	5.0	35.0	35.0	1.000	17.9	5.0	35.0	35.0	22.0	61.0	21.0	17.0	22.0	20.0	17.0
3	Absent	2.7	0.0	0.0	0.0	0.084	0.0	0.0	0.0	0.0	1.000	0.0	0.0	0.0	0.0	0.0	16.0	0.0	7.0	ī	ī	0.0
9	Decreased	ī	ī	ī	ī	ī	ī	ī	ī	ī	0.058	3.6	0.0	0.0	0.0	ī	ī	9.0	4.0	ī	ī	6.0
15	Unknown	ī	ī	ī	ī	ī	ī	ī	ī	ī	0.002	5.7	0.0	10.0	0.0	ī	ī	9.0	8.0	ī	ī	0.0
17	Increaased	ī	ī	ī	ī	ī	ī	ī	ī	ī	0.000	16.4	25.0	0.0	5.0	ī	ī	10.0	14.0	15.0	9.0	ī
27	Decreased	ī	ī	ī	ī	ī		ī	ī ī	ī	0.000	24.3	0.0	15.0	20.0	ī	ī	33.0	8.0	ī	ī	ī
28	Unknown	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	0`0	0`0	0`0	!	!	0`Ɩ	0`Ɩ	!	!	0`0
Alleles identified (n)		112.0	12.0	20.0	20.0		140.0	20.0	20.0	20.0		140.0	20.0	20.0	20.0	152.0	1964-1970	200.0	150.0	218.0	134.0	18.0
Predicted Phenotype Frequency (%)																						
PM		3.5	16.7	10.0	30.0		7.1	0.0	10.0	10.0		5.7	0.0	20.0	50.0	5.3	1.5	3.0	8.0	ī	ī	ī
IM		0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		21.4	10.0	50.0	40.0	32.9	13.0	49.0	40.0	ī	ī	ī
EM		94.7	83.3	90.0	70.0		92.9	100.0	90.0	90.0		45.7	90.0	30.0	0.0	61.6	85.5	39.0	35.0	ī	ī	ī
UM		0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		27.1	0.0	0.0	10.0	0.0	0.0	9.0	17.0	ī	ī	ī
Failure		1.8	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	1.1-0.8	0.0	0.0	0.0	0.0	0.0
Cohort (n)		57.0	6.0	10.0	10.0		70.0	10.0	10.0	10.0		70.0	10.0	10.0	10.0	76.0	982-985	100.0	75.0	109.0	67.0	9.0

Allele frequencies for each allele were compared between cohorts and platforms. Allele frequencies for different ethnicities were summed for comparison. Genetic material for cohort 2 was genotyped using PCR-RFLP to evaluate AmpliChip CYP450 Test® (AmpliChip) genotype calls. *CYP2C19*1+* : wild type and any unidentified alleles; ð :alleles not identified by assay or predicted phenotype not reported

*CYP2C19*9* (decreased metabolism) is significantly high frequency over the whole cohort, when only Black Africans are compared between platforms (*P*=0.029). Interestingly, four samples (three Black Africans and one Indian) were homozygous for *CYP2C19*2*, but were also heterozygous for the **27* allele. This suggests that the 19154G>A and -1401G>A SNPs used for *CYP2C19*2* and**27* detection respectively, may be in partial LD with one another, forming an additional allele. The combination was listed as *CYP2C19*2*, as the presence of the 19154G>A splicing defect would be the allele-defining SNP since this causes a non-functional gene product.

Predicted Phenotype

The only difference between the two cohorts for AmpliChip predicted phenotype was PM for White Caucasians as there were more identified in cohort 1. Caution should be taken when making this comparison, as this 16.7% frequency is only one individual in the cohort and the sample size is not statistically large enough to make this comparison. The adoption of AS combined with *CYP2C19* PCR-RFLP allows IMs to be assigned and also changes the identification profile of PMs (refer to Table 3-2). EM and PM predicted phenotype for Black Africans using *CYP2C19* PCR-RFLP well with the Xhosa individuals screened by Drögemöller et al. (2010), but IM and UM seems to be different.

3.4.3 *CYP2D6 genotype analysis*

AmpliChip

Table 3-3 summarises the *CYP2D6* allele frequencies for the sampled cohorts and compares allele frequencies between cohorts and platforms . The only allele which was out of Hardy-Weinberg equilibrium was *CYP2C19*10* (*P* = 0.006) in cohort 1. *CYP2D6*17* was the only allele with significantallele frequency differences (*P* = 0.045) between the two cohorts which is likely to be a result of the larger number of Black Africans in cohort 2. The AmpliChipfound Black African individuals in both cohorts to have a relatively high frequency of *CYP2D6*17* and *CYP2D6*41*. *CYP2D6*4* and **41* were relatively frequent in the Caucasian, Coloured and Indian populations in both cohorts and could be a source for potential PM (refer to Table 3-3).

Table 3-3. CYP2D6 allele and predicted phenotype frequency in a demographically representative South African (SA) cohort (n=100) compared to other cohorts sampled in SA

		Cohort 1 AmpliChip				Compare	Cohort 2 AmpliChip				Compare	XL-PCR+Sequencing				Dandara et al. (2001)	Gaedigk et al. (2008)	Wright et al. (2010)	
Allele	Activity	Black African	White Caucasian	Coloured	Indian	Fischer's Exact	Black African	White Caucasian	Coloured	Indian	Fischer's Exact	Black African	White Caucasian	Coloured	Indian	SA Venda	SA Coloureds	SA Xhosa Control	SA Xhosa Schizophrenia
											Allele Frequency (%)								
**1*	Normal	30.7	40.0	38.9	30.0	0.301	20.0	31.0	37.5	60.0	0.715	25.7	30.0	30.0	45.0	50.0	26.8	23.6	24.5
**2*	Normal	4.5	20.0	0.0	30.0	1.000	20.0	18.8	18.8	30.0	0.107	8.6	15.0	15.0	30.0	17.8	15.2	12.3	15.7
**4*	Absent	2.3	10.0	16.7	10.0	0.795	0.0	31.3	12.5	0.0	0.586	0.0	20.0	15.0	0.0	3.3	7.1	1.9	1.0
**5*	Absent	5.7	10.0	0.0	0.0	0.523	4.0	0.0	0.0	0.0	0.037	10.7	5.0	5.0	0.0	4.6	17.2	14.2	18.6
**10*	Decreased	6.8	0.0	0.0	10.0	1.000	6.0	6.3	6.3	0.0	0.808	5.7	5.0	5.0	0.0	ī	2.5	1.9	2.0
**14*	Absent	0.0	0.0	0.0	0.0	1.000	0.0	0.0	0.0	0.0	1.000	0.0	0.0	0.0	0.0	ī	0.5	0.0	0.0
**16*	Absent	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	0.0	0.0	0.0	ī	0.5	0.0	0.0
**17*	Decreased	13.6	0.0	11.1	0.0	0.045	31.0	0.0	0.0	0.0	0.584	25.7	0.0	10.0	0.0	24.0	12.6	13.2	16.7
**22*	Normal	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	0.0	0.0	5.0	ī	ð	ð	ð
**25*	Unknown	0.0	0.0	0.0	0.0	1.000	1.0	0.0	0.0	0.0	0.416	0.0	0.0	0.0	0.0	ī	ð	ð	ð
**29*	Decreased	13.6	0.0	0.0	0.0	1.000	6.0	0.0	6.3	0.0	0.598	4.3	0.0	5.0	0.0	ī	4.6	13.2	6.9
**30*	Unknown	1.1	0.0	0.0	0.0	0.461	0.0	0.0	0.0	0.0	1.000	0.0	0.0	0.0	0.0	ī	ð	0.0	0.0
**33*	Normal	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	5.0	0.0	0.0	ī	ð	ð	ð
**35*	Normal	0.0	0.0	0.0	0.0	1.000	0.0	0.0	6.3	0.0	1.000	0.0	5.0	0.0	0.0	ī	ð	ð	ð
**36*	Reduced	0.0	0.0	0.0	0.0	1.000	0.0	0.0	0.0	0.0	1.000	0.0	0.0	0.0	0.0	ī	0.0	0.0	0.0
**40*	Absent	1.1	10.0	5.6	0.0	1.000	3.0	0.0	0.0	0.0	1.000	3.6	0.0	0.0	0.0	ī	0.0	1.9	2.9

Table 3-3. Continued...

		Cohort 1 AmpliChip				Compare	Cohort 2 AmpliChip				Compare	XL-PCR+Sequencing				Dandara et al. (2001)	Gaedigk et al. (2008)	Wright et al. (2010)	
Allele	Activity	Black African	White Caucasian	Coloured	Indian	Fischer's Exact	Black African	White Caucasian	Coloured	Indian	Fischer's Exact	Black African	White Caucasian	Coloured	Indian	SA Venda	SA Coloureds	SA Xhosa Control	SA Xhosa Schizophre nia
										Allele Frequency (%)									
**41*	Decreased	20.5	10.0	27.8	20.0	0.883	26.0	12.5	12.5	10.0	0.000	0.7	15.0	5.0	10.0	ī	0.0	1.9	1.0
**43*	Normal	ī	ī	ī	ī	ī	ī	ī	ī	ī	0.272	0.7	0.0	5.0	5.0	ī	ð	0.9	1.0
**45B*	Normal	ī	ī	ī	ī	ī	ī	ī	ī	ī	0.020	5.7	0.0	0.0	0.0	ī	0.0	10.4	1.0
**46*	Normal	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.7	0.0	0.0	0.0	ī	0.0	0.0	0.0
**56B*	Absent	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.7	0.0	0.0	0.0	ī	0.0	ð	ð
**59*	Decreased	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	0.0	0.5	0.0	ī	ð	ð	ð
**64*	Unknown	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	0.0	0.0	0.0	ī	0.0	0.0	0.0
**65*	Unknown	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	0.0	0.0	0.0	ī	0.0	0.0	0.0
**73*	Unknown	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	0.0	0.0	0.0	ī	ð	0.0	1.0
**74*	Unknown	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	0.0	0.0	0.0	ī	ð	0.0	1.0
**84*	Normal	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.7	0.0	0.0	0.0	ī	ð	ð	ð
**85*	Normal	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.7	0.0	0.0	0.0	ī	ð	ð	ð
**86*	Normal	ī	ī	ī	ī	ī	ī	ī	ī	ī	1.000	0.0	0.0	0.0	5.0	ī	ð	ð	ð
**1xN*	Increased	0.0	0.0	0.0	0.0	1.000	0.0	0.0	0.0	0.0	1.000	0.0	0.0	0.0	0.0	ī	0.0	0.0	1.0
**2xN*	Increased	0.0	0.0	0.0	0.0	1.000	1.0	0.0	0.0	0.0	1.000	0.7	0.0	0.0	0.0	ī	0.0	2.8	2.9
**4xN*	Absent	0.0	0.0	0.0	0.0	1.000	0.0	0.0	0.0	0.0	0.147	2.9	0.0	0.0	0.0	ī	0.0	1.9	2.9
Hybrid alleles		ī	ī	ī	ī	ī	ī	ī	ī	ī	ī	ī	ī	ī	ī	ī	0.0	0.0	0.0
Alleles identified (n)		88	10	18	10		100	16	16	10		140	20	20	20	152	198	106	102

Table 3-3. Continued...

	Cohort 1 AmpliChip				Compare	Cohort 2 AmpliChip				Compare	XL-PCR+Sequencing				Dandara et al. (2001)	Gaedigk et al. (2008)	Wright et al. (2010)	
Predicted phenotype	Black African	White Caucasian	Coloured	Indian	Fischer's Exact	Black African	White Caucasian	Coloured	Indian	Fischer's Exact	Black African	White Caucasian	Coloured	Indian	SA Venda	SA Coloureds	SA Xhosa Control	SA Xhosa Schizophrenia
	Predicted Phenotype Frequency (%)																	
PM	3.5	83.3	10.0	0.0		0.0	10.0	0.0	0.0		0.0	10.0	0.0	0.0	2.6	3.0	3.8	7.8
IM	28.1	0.0	20.0	0.0		42.9	10.0	20.0	0.0		42.9	30.0	50.0	0.0	56.6	53.0	47.2	37.3
EM	43.9	0.0	60.0	50.0		27.1	60.0	60.0	50.0		57.1	60.0	50.0	100.0	40.8	39.0	43.4	47.1
UM	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	4.0	5.7	7.8
Unknown	1.8	0.0	0.0	0.0		1.4	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
No call	7.0	0.0	0.0	0.0		5.7	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Failure	15.8	16.7	10.0	40.0		22.9	20.0	20.0	50.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cohort (n)	3.5	83.3	10.0	0.0		0.0	10.0	0.0	0.0		0.0	10.0	0.0	0.0	2.6	3.0	3.8	7.8

Allele frequencies for each allele were compared between cohorts and platforms for statistical comparison. Allele frequencies for different ethnicities were summed for comparison. Genetic material for cohort 2 was sequenced to evaluate AmpliChip CYP450 Test® (AmpliChip) genotype calls. Lower allele numbers were reported for the AmpliChip due to "No Calls" and failed chips. Individuals could be predicted as poor (PM), intermediate (IM), extensive (EM) and ultrarapid metabolisers using AmpliChip or activity score prediction models. xN: multiple copies of the allele detected; ð , alleles not identified by platform.

XL-PCR+Sequencing

*CYP2D6*re-sequencing not only contributed to a comprehensive assessment ofknown *CYP2D6* sequence variations, but also allowed identification of novelallelic variants. A total of 92 sequence variations were identified including 88 SNPs, two insertions and two deletions (Supplementary Table S5). Electropherograms for non-synonymous SNPs can be viewed in Supplementary Figure S2. Additional novel SNPs were identified, but not assigned to alleles as no apparent clinical relevance was observed (Supplementary Table S5). None of these SNPs were predicted to impact splicing based on the NetGene2 prediction.

Twenty one distinct alleles were identified in Cohort 2 (Supplementary Figure S1). Of the clinically relevant alleles identified by this platform, *CYP2D6*17*and**5* were frequently observed in the Black population. In contrast *CYP2D6*4* and**41* alleles were frequent in Caucasians and *CYP2D6*4*in the Coloured population groups.The alleles identified by gene re-sequencing (n = 200 successful identifications) 17.0% (n = 34) had absent, 31.5% (n = 63) decreased, 51.0% (n = 102) normal and 0.5% (n = 1) increased enzyme function in the sampled cohort.All alleles described by sequencing were in Hardy-Weinberg Equilibrium.

Allele comparison between platforms

The most noticeable discrepancy between the two platforms was that the AmpliChip identified less *CYP2D6*2* alleles (not significant; P = 0.107), but in contrast more **41* alleles (P = 0.000). The normal function *CYP2D6*45B* (n=8) and **46* (n = 1) alleles are not identified by the AmpliChip and were incorrectly assigned as reduced function **41*. Similarly, the AmpliChip identified the non-functional *CYP2D6*56B* (n = 1) as a reduced function *CYP2D6*10B* allele. Of the 200 alleles tested, the AmpliChip identified nine *CYP2D6*5* alleles compared to the seventeen identified by the Hersberger et al. (2000) assay, of which only five subjects were accurately identified by both assays. This difference was significant (P = 0.037). When investigated further, the Hersbergeret al. (2000) assay predicted nine individuals to be heterozygous *CYP2D6*5*while the AmpliChip reported homozygous **1* (n = 1), **2*(n = 1), **4* (n = 1),**17* (n = 5) or **41* (n = 1) genotypes. Eighteen alleles identified by the XL-

PCR+Sequencing platform were not identified by the AmpliChip. In addition to *CYP2D6*45B*, **46* and **56B* mentioned above, *CYP2D6*59* (reduced function) was misidentified as *CYP2D6*2* or **22* (both normal function). *CYP2D6*33* and **43*(both normal function) were identified as *CYP2D6*1* (normal function), respectively.

Predicted phenotype

There were more PMs identified in Cohort 1 compared to Cohort 2. The XL-PCR+Sequencing platform did not identify more PMs, but It did increase prediction of IMs. XL-PCR+Sequencing compared well with the cohort described by Wright et al. (2010), however there were fewer PMS.

3.4.4 *CYP2D6 novel alleles*

Figure 3-1 displays the four novel alleles we identified with the XL-PCR+Sequencing in comparison to other similar alleles. The *CYP2D6*4P* allele was found in a Caucasian individual and had a *CYP2D6*4*. However, due to the detrimental 1846G>A SNP that causes aberrant splicing, *CYP2D6*4P* received an AS of 0.0 classifying it as non-functional.

*CYP2D6*84* has a **2A* backbone and was found in a Black African individual. In addition to those found on *CYP2D6*2A*.The allele-defining SNP 2574C>A in exon 5 resulting in an amino acid change (P267H) was predicted to be benign by PolyPhen (PSCI score of 0.871), but SIFT predicted it to affect protein function (SIFT score of 0.03). The amino acid change was from a non-polar proline to a basic histidine, a charge change, supporting the potential for altered activity. As there is a discrepancy between the *in silico* prediction tools, an AS score of 1.0 was given to this allele for comparative purposes.

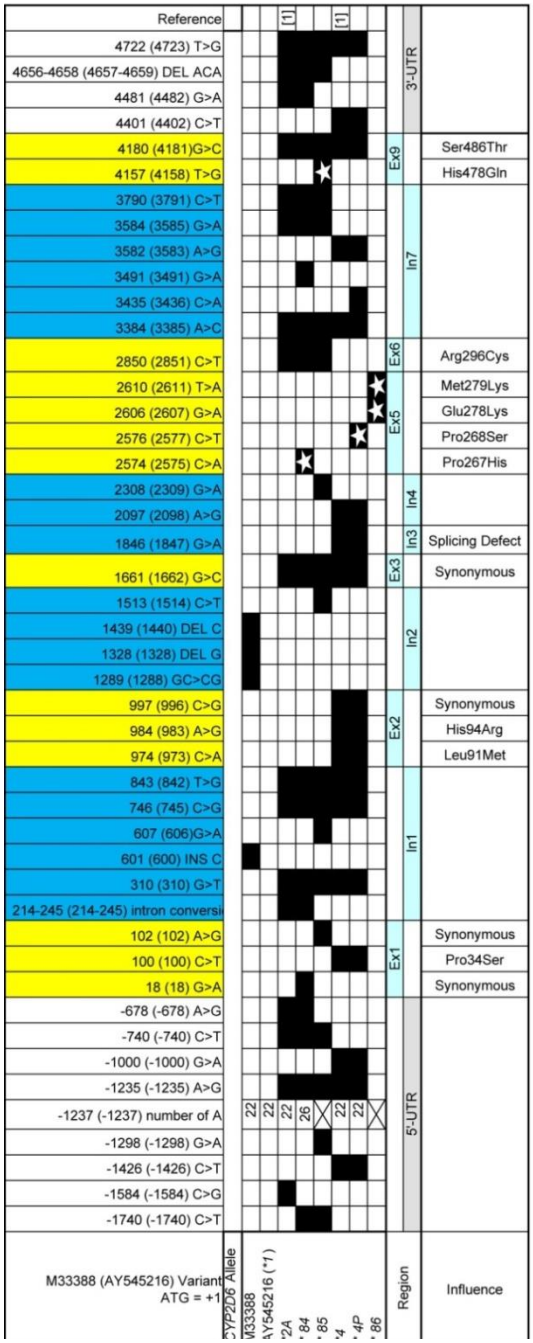

Figure 3-1. Schematic diagram representing novel*CYP2D6*4P,*84,*85*and*86*alleles identified in the South African cohort described in this study.

Polymorphisms are represented by black blocks. Allele defining polymorphism are indicated by a white star. These alleles have been accepted by the *CYP* Allele Nomenclature Committee for *CYP2D6* (http://www.imm.ki.se/CYPalleles/). *CYP2D6*2A* and **4* used for comparison, were adopted from [1] Gaedigk et al. (2008).; UTR: untranslated region; Ex: exon; In: intron. This figure was created by Tyren Mark Dodgen.

*CYP2D6*85* was also found in a Black African individual. The allele defining SNP for *CYP2D6*85* was 4157T>G that results in a H478Q amino acid change. According to PolyPhen (PSIC=0.419) and SIFT (SIFT = 0.58) this change is unlikely to affect activity. Therefore, we assigned an AS of 1.0 to this allele. *CYP2D6*85* also has a *CYP2D6*2* backbone.

The final novel allele, *CYP2D6*86*,was discovered in an Indian individual. Only two SNPs 2606G>A and 2610T>A were observed, and both caused an amino acid changes, i.e. E278K and M279K. However, only 2610T>A was predicted to likely affect protein function by PolyPhen, (PSIC=1.905, SIFT=0.01) due to a hydrophobicity change from a non-polar to a basic amino acid in a buried site. The other SNP, 2606G>A, was unlikely to affect enzyme activity (PSIC=0.205, SIFT = 0.07). However, because the 2610T>A was not confirmed to alter activity, *CYP2D6*86* allele was assigned an AS score of 1.0 for comparative purposes. Both SNPs have been described previously, but not within a defined allele (Tandon et al. manuscript in preparation, http://www.cypalleles.ki.se/cyp2d6.htm).

3.5 Discussion

The AmpliChipôs ability to simultaneously assay for *CYP2D6* gene duplications, gene deletions as well as 33 *CYP2D6* and 3 *CYP2C19* variants simultaneously, characterises it as high-throughput. However, several limitations were identified which question the use of the AmpliChip in the South African population.

First, the AmpliChip performed poorly in terms of reliability. For *CYP2D6* the average failure rate in both groups was 22.4%. In addition, only 2 out of the 13 samples that failed on first attempt succeeded after a second attempt, raising an additional concern of cost effectiveness. Possible explanations for the poor success rate of the AmpliChip in this population include (i) suboptimal transportation conditions and mishandling during transfer possibly damaging the microarrays; (ii) concerns regarding the length of the amplification - this has previously been suggested to be a weak point in the assay (de Leon et al. 2009). Rebsamen et al. (2009) supported this proposal and identified gene

duplication errors. However, each amplification reaction was tested for product using 1.0% agarose gel electrophoresis prior to proceeding to the fragmentation step. The failures observed are therefore unlikely to be due to the lack of a PCR product; (iii) inadequate fragmentation, which in turn impacts on hybridisation, thereby rendering the test a failure. In 2007, the FDA reported that the DNase I recommended in the AmpliChip information leaflet was of reduced quality, resulting in low specific activity (http://www.fda.gov/Safety/Recalls/EnforcementReports/2007/ucm120450.htm); (iv) lack of standardisation of the strepravidin R-phycoerythrin conjugate. Roche has stopped supplying the recommended reagent and has not recommended a suitable replacement.

The high frequency of unknown predicted phenotypes called by the AmpliChip is a serious limitation for routine implementation in the South African population. Approximately 7.7% were ñUnknownò predicted phenotypes even though the AmpliChip was successful. These individuals would not have benefited from pharmacogenetic screening by AmpliChip for *CYP2D6*. This questions the use of this pharmacogenetic screening assay in the South African population as the frequency of the ñUnknownò predicted phenotype is higher than the frequency of PMs identified (1.0-9.6%). With the AmpliChip being more expensive that the other platforms and having a low success rate, the AmpliChip will not assist in reducing the finantial burden associated with CYP2C19 and CYP2D6 associated ADRs.

3.5.1 *AmpliChip compared to CYP2C19PCR-RFLP platform*

The ability to cover population-specific alleles is another limitation. The AmpliChip may have had a high success rate for *CYP2C19*,and the frequencies compared well with previously reported values in various African populations (de Leon et al. 2009; Sistonen et al. 2009), but there may be several mutations which the AmpliChip was unable to identify (i.e. *CYP2C19* alleles other than**2* and **3* were discovered) . Thus, there may be a higher frequency of alternative polymorphisms resulting in absent (*CYP2C19*4*, **5*, **6*, **7* and **8*) or increased (**17*) enzyme function (http://www.cypalleles.ki.se/). Additionally, a glimpse into the South African Xhosa and Cape Mixed Ancestry (Coloured) populations has revealed a novel mutation in the promoter region -1041G>A

(*CYP2C19*27*) which was found to be present at a relatively high frequency of 33.0% (Drögemöller et al. 2010). *In silico*analyses and luciferase expression assays suggest that this polymorphism may result in reduced expression of *CYP2C19* (Drögemöller et al. 2010). The absence of these important alleles from the AmpliChip highlights the need to develop a more specific and/or comprehensive assay for this population.

The more comprehensive PCR-RFLP genotyping method identified 83 alleles out of 158 that were wrongly assigned as ñ*CYP2C19*1*ò (i.e. wild type and unidentified alleles), by the AmpliChip. This is significant for the accuracy of downstream phenotype prediction and agrees with concerns that the *CYP2C19* alleles identified by the AmpliChip, would not be comprehensive enough for the South African population. The incorrect assignment of ñ*CYP2C19*1*ò was especially relevant to the Black South African cohort,as 48.6% of the alleles initially assigned as ñ*CYP2C19*1*ò by the AmpliChip, were assigned other alleles after PCR-RFLP genotyping (Table 3-2). However, the effect of these alleles needs to be carefully considered before drawing any conclusions.

The variation in the LD pattern observed for the *CYP2C19*2* and **27* defining SNPs, identified in the three Black Africans and one Indian individual, but was not observed in the small Caucasian cohort. This alternative LD was identified previously in a Black African population (Drögemöller et al. 2010) and one should be aware of the clinical implications of this. For example, if *CYP2C19*27* was responsible for decreased metabolism, an individual testing positive for both the **2*and **27* alleles could be **2/*27* (AS=0.5) or **2+*27/*1* (AS=1). The low LD observed predominantly in Africans may complicate the assignment of alleles and may necessitate the genotyping of multiple SNPs before allele assignment.

Considering the high frequencies observed for *CYP2C19*17* in a variety of populations (Kurzawski et al. 2006; Sim et al. 2006; Rudberg et al. 2008; Ragia et al. 2009; Kearns, Leeder & Gaedigk 2010) and the identification of other high frequency alleles such as **27* (Drögemöller et al. 2010), which may have clinical implications, it could be argued that the AmpliChip is not comprehensive enough for any population. In addition, the

AmpliChip is a relatively expensive assay for prediction of CYP2C19 phenotype and a population specific, reasonably priced assay such as the PCR-RFLP assay is advised for future phenotype prediction, especially in developing countries where resources are limited.

3.5.2 *AmpliChip compared to the CYP2D6 XL-PCR +Sequencing platform*

As our cohort represented a diverse population it was not surprising to find a large number of*CYP2D6*allelic variants as well as four novel alleles. Nine *CYP2D6*2* alleles were miss-called as **41*, resulting in an over estimation of *CYP2D6*41*/**41* homozygotes (also demonstrated by Gaedigk et al. 2005). The AmpliChip-derived frequency of *CYP2D6*41*among our Black subjects was therefore higher when compared to similar cohorts (Sistonen et al. 2009), which detected the *CYP2D6*41* alleles by its key SNP (2988G>A). The AmpliChip designates *CYP2D6*41* using the -1584C>G variation and linkage disequilibrium with other SNPs, which generally hold true in Caucasians, but not in subjects of Black African ancestry (Gaedigk et al. 2005). Furthermore, using the *CYP2D6*41* key SNP will also allow differentiation of *CYP2D6*41* from the **45B* and **46* alleles which are not identified by the AmpliChip.

*CYP2D6*1* was incorrectly assigned as **41* by the AmpliChip five times. This could possibly be due to the lack of hybridisation. These inaccurate genotype assignments affect the prediction of subjectsô phenotypes to various extents. In addition, the AmliChip does not contain identifying, or key SNPs, for *CYP2D6*45*, **46*, **56* and **59*, which we have discovered by the XL-PCR+Sequencing platform and hence, defaulted these alleles to *CYP2D6*2* or **10* according to the SNPs tested and AmpliChip algorithm. Inaccurate results in combination with alleles that are not captured by the AmpliChip could have serious pharmacogenetic and clinical implications.

3.5.3 *Predicted phenotype*

There was a noticeable difference in phenotypic prediction between the AmpliChip and the AS for both CYP2D6 and CYP2C19. This was apparent when comparing each

system on both a group to group and combined level. Accurate phenotype prediction appears to be a limitation of the AmpliChip which supports the use of a numeric method for phenotype identification (Gaedigk et al. 2008; de Leon et al. 2009; Rebsamen et al. 2009). In addition, a 93% CYP2C19 EM prediction may be an overestimate. Use of the numeric AS allows for CYP2C19 IM to be predicted, which is a subset of the cohort that could potentially benefit from pharmacogenetic screening. Articles comparing clopidogrel (prodrug) response to *CYP2C19* variability have demonstrated reduced metabolism in individuals who have *CYP2C19*1/*2* or **1/*3* allele combinations. These genotypes were associated with normal or only slightly reduced platelet aggregation, as clopidogrel needs to be metabolised into its active metabolite in order to affect platelet aggregation (Hulot et al. 2006; Shuldiner et al. 2009; Mega et al. 2010). It may therefore be more appropriate to split this EM group into EM and IM. In this way **1/*2*and **1/*3*individuals could potentially benefit from pharmacogenetic screening. Measured phenotype would needed to fully understand and evaluate phenotype prediction by the various platforms. With the AmpliChip not identifying the increased function *CYP2C19*17* tailoring of clopidogrel dosage would be difficult.

3.5.4 *Pharmacogenetic relevance for the South African population*

The possible existence of additional functionally relevant alleles unique to the South African population will need to be considered if *CYP2D6* and *CYP2C19* pharmacogenetics are to be applied in this population. With the large amount of genetic variation observed in this South African cohort it would be essential to use more comprehensive platforms for pharmacogenetic screening to ensure a more accurate predicted phenotype. Predicted phenotype may not be clinically relevant if genotyping is incomplete and inaccurate, highlighting the importance of establishing novel approaches for predicting phenotype (Gaedigk et al. 2008; de Leon et al. 2009). It will also be important to compare genotype and measured phenotype in this population, to assess the accuracy of the predicted phenotype called by the AmpliChip (Gaedigk & Coetsee 2008) as well as other prediction strategies. Due to the high failure rate and high cost of the AmpliChip it was not feasible to repeat these AmpliChips to evaluate error.

3.6 Conclusion

When applied to a demographically-representative sample of the South African population, the AmpliChip had a low success rate and a high number of unknown predicted phenotype calls were observed. This platform would need to be refined before being applied as a pre-prescription pharmacogenetic screening tool in this, and possibly other genetically-diverse African populations. Alternative platforms for genotyping, such as the ones used in this study, would be more clinically appropriatefor pharmacogenetic screening of *CYP2D6* and *CYP2C19*. With sequencing technologies advancing read lengths are improving which will allow sequencing to form the basis of pharmacogenetics in the future. this will facilitate simultaneous identification of novel alleles in complex populations. In addition the comparison between predicted phenotype and measured phenotype will also need to be considered.

3.7 Referances

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CHAPTER 4. QUANTITATIVE PLASMA ANALYSIS USING AUTOMATED ONLINE SOLID PHASE EXTRACTION WITH COLUMN SWITCHING LC-MS/MS FOR CHARACTERISING CYTOCHROME P450 2D6 AND 2C19 METABOLISM.

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Dodgen, T.M., Cromarty, A.D. & Pepper, M.S. 2011, "Quantitative plasma analysis using automated online solid-phase extraction with column switching LC-MS/MS for characterising cytochrome P450 2D6 and 2C19 metabolism", *Journal of separation science,* vol. 34, no. 10, pp. 1102-1110.

4.1 Abstract

Background: In this study an easy and efficient assay for simultaneous quantitation of plasma concentrations of probe drugs and their metabolically relevant metabolites for the phenotypic analysis of cytochrome P450 2D6and 2C19 respectively has been established. **Method:** This sensitive method makes use of a simple initial sample preparation, followed by a six minute automated analysis that includes online solid phase extraction, column switching and tandem mass spectrometry. **Results:** Validation over a concentration range of 1.3 - 2500 ng/mL for dextromethorphan, omeprazole, dextrorphan and 5ô-hydroxyomeprazole was performed with LLOQ between 215 pg/mL and 1145 pg/mL. Intra- and inter-day precision and accuracy over the calibration ranges were within 15% for all analytes with recoveries of greater than 85%. Advantages are small sample volumes required, a robust, sensitive and highly selective method suitable for pre-prescription metabolic screening. **Conclusion:** This method could compliment or offer an alternative to DNA mutation analysis for determining appropriate dosage regimens for personalised medicine.

4.2 Background

LC-MS/MS has become the analytical instrument of choice for the analysis of biologically active small molecules (in particular pharmacotherapeutics) because of high sample throughput capability, sensitivity, selectivity, accuracy of quantification and wide linear dynamic range of detection (Smyth & Brooks 2004; Stoob et al. 2005; Deng & Sanyal 2006; Bakhtiar & Majumdar 2007). One area where online solid phase extraction LC-MS/MS has not been extensively applied as yet is in the field of enzyme metabolic profiling. DNA analysis is presently the primary prediction model to explain variability in the Cytochrome P450 (CYP) enzymes.

A rapid, comprehensive and reliable phenotypic platformfor enzyme activity can expose both genetic and environmental influences, which may be valuable as a pre-prescription metabolic screening technique (Fairbanks & Andersen 1999; Aklillu et al. 2002). An LC-MS/MS method sensitive enough to monitor *in vivo* metabolic ratios of probe drugs can be used to predict the metabolic rate of other drugs with a narrow therapeutic index thus providing a tool for personalised medicine (Marzo & Balant 1996).

Despite extensive Phase II metabolism, dextromethorphan (DM) is the preferred probe drug for *in vivo* phenotypic analysis of CYP2D6 as it is a selective substrate, freely available and has a relatively safe side effect profile (Küpfer et al. 1984; Sweetman 2007; Wojtczak et al. 2007). DM is metabolised to dextrorphan (DX) by CYP2D6 (Figure 2-6) (Vengurlekar et al. 2002; Wijnen et al. 2007). Omeprazole (OME) is the preferred probe drug for CYP2C19 analysis, having similar beneficial attributes and is metabolised to 5ô-hydroxyomeprazole (5OH) by CYP2C19 (Figure 2-5) (Balian et al. 1995; Wijnen et al. 2007). A ñplasma metabolic ratioò of the relevant concentration of probe drug to drug metabolite can be used to simultaneously measure each enzymes activity in patients dosed with a single administration of both probe drugs (Linder, Prough & Valdes 1997).

A number of quantitative mass spectrometric methods have been reported for pharmaco -genetic or -kinetic measurements for either DM or OME with their relevant metabolites (Vengurlekar et al. 2002; Frerichs, Zaranek & Haas 2005; Hofmann et al. 2006; Eichhold et al. 2007). However, few methods have assayed these analytes simultaneously and those that have

involved a multidrug ñcocktailò combining several other probe drugs and used urine concentrations to assess the total CYP450 enzyme family activity. This approach can be advantageous, as combination analysis is a cost-effective approach to metabolic screening, however, these methods use an inappropriate 8 hour sampling period for the urine, extensive sample workup and relatively long analytical methods (Streetman et al. 2000; Tamminga et al. 2001; Jerdi et al. 2004; Ryu et al. 2007). Metabolic ratios generated from urine samples also give debatable results in patients with compromised renal function and is highly influenced by Phase II metabolism. Plasma sampling at the approximate reported maximal drug plasma concentration (C_{max}) overcomes many of these concerns. The three hour post dosing values are reported in this study which appears to be after T_{max} (time following administration to reach C_{max}) for all the plasma analytes.

Sample preparation of complex biological matrices suitable for LC-MS/MS analysis must have efficient analyte recovery, overcome drug-protein binding and avoid matrix related ion-suppression in the ESI source (Kragh-Hansen 1981; Kremer, Wilting & Janssen 1988; Bakhtiar & Majumdar 2007). Common plasma preparation techniques for LC-MS/MS analysis include solvent protein precipitation (PPT), solid phase extraction (SPE) and liquid-liquid extraction (LLE). These techniques all involve several manual working steps and require relatively large solvent volumes that must be reconcentrated, potentially contributing to contamination, recovery errors and can lead to large variability. As a result there is a growing trend to use automated online sample preparation methods to facilitate the use of LC-MS/MS for routine testing (Vogeser & Seger 2008).

To be time efficient, ñrapid LCò methods warrant the use of ñrapid efficientsample preparationò (Bolden et al. 2002; Trenholm, Vanderford & Snyder 2009). On-line analyte capture with column switching techniques improves assay efficiency by reducing required sample volumes and minimising manual sample preparation steps. Column switching methods have been reported for the analysis of OME (Shim, Bok & Kwon 1994; Cass et al. 2003; Uno et al. 2005; Shimizu et al. 2006), DM (Liu et al. 1993; Härtter et al. 1996; Lutz et al. 2008) and other drugs (Stoob et al. 2005; Suenami et al. 2006; Ansermot et al. 2008; Subramanian, Birnbaum & Remmel 2008), yet these reported methods generally have relatively long run times. None were validated for the simultaneous measurement of DM , OME and their

metabolites as automated online solid phase extractionwith column switching coupled to tandem mass spectrometry. Furthermore, no automated online solid phase extraction combined with a sub-2 µm particle column for analysis of these analytes by tandem mass spectrometry has been reported.

This report describes a validated, low sample injection volume, automated on-line SPE method coupled to a triple quadrupole mass spectrometer. The emphasis is on a robust, rapid method combining automated analyte extraction and sensitive quantification using a UPLC type sub-2 µm particle analytical column in a six minute analysis.

4.3 Materials and methods

4.3.1 *Chemicals*

DM hydrobromide, OME, DX and imipramine (internal standard) were obtained from Sigma-Aldrich (St. Louis, USA). Two different samples of 5OH were obtained: one kindly donated by AstraZenica (Mölndal, Sweden) and the other purchased from Cayman Chemical Company (Michigan, USA). Pyrogen free deionised water was produced in-house using an ELGA ñGeneticsò water purification unit (ELGA, Wycombe, UK). Methanol, acetonitrile and 25% ammonium hydroxide solution were obtained from Merck (Darmstadt, Germany) and mass spectrometer grade formic acid from Fluka (Buchs, Switzerland). All solvents used in mobile phase and sample preparation were of HPLC grade.

Plasma for preparation of standards was drawn in-house from healthy drug naïve volunteers and pooled. The probe drugs dextromethorphan and omeprazole were purchased as Benylin Cough Syrup (Pfizer Inc, New York, USA) and Altosec (Aspen Pharma, Durban, South Africa) respectively from a retail pharmacy. The study was carried out under international GCP guidelines and was approved by the University of Pretoria Research Ethics Committee of the Faculty of Health Sciences, (Protocol number: S132/2009).

4.3.2 *Instrumentation*

The triple quadrupole LC-MS/MS system consisted of an Agilent 1100 series autosampler, binary pump, degasser, column oven equipped with a 6 port switching valve, an additional

Agilent 1200 series binary pump (Waldbronn, Germany) and a Shimadzu LC-20AT isocratic pump (Kyoto, Japan) coupled to an AB Sciex 4000 QTrap mass spectrometer with a Turbo ñVò electrospray ionisation (ESI) source (Applied Biosystems/MDS Sciex, Concord, Canada). The complete system was centrally controlled with data collected and analysed using Analyst Software, version 1.4.2 (Applied Biosystems/MDS Sciex).

4.3.3 *Optimisation of the MS/MS detection parameters*

The mass spectrometer was individually tuned for each of the five analytes to achieve the optimal detection parameters. Each analyte was dissolved at 1µg/mL in 50/50 (v/v) methanol and 0.1% formic acid in water for positive mode optimisation and 50/50 methanol and 0.025% ammonium formate in water for negative mode optimisation.

The dissolved analytes were directly infused into the ion source using a Harvard syringe pump (Harvard Apparatus,Hollinston, Massachusetts, USA) at a flow rate of 10 µL/min. Multiple reaction monitoring (MRM) tuning was carried out to optimise fragmentation conditions and to identify the best precursor/product transition for quantification. Analyst Softwareôs óQuantitation Optimisationô was finally employed to fine tune the optimal mass spectrometer parameters under chromatographic flow conditions.

4.3.4 *Chromatographic conditions*

Online solid phase extraction of pre-prepared samples (5 µL) was accomplished using a BDS Hypersil-C_{18} 10 x 4 mm with 5 µm particle size guard column (Thermo Fisher Scientific, MA, USA) fitted between ports 1 and 4 of the six port switching valve. Analytes were transferred at 100 εL/min onto this capture column using an isocratic flow from binary Pump 2 of 90% mobile phase C (0.1% formic acid adjusted to pH 9 with ammonium hydroxide) and 10% mobile phase D (0.1% formic acid in acetonitrile). Remaining polar proteins, ionic ion-suppressing compounds and polar contaminants passed through the capture column to waste while the analytes were strongly retained. Simultaneously, an Agilent XDB C_{18} ñrapid resolutionò analytical column of 50 x 4.6 mm with 1.8 µm particles (Waldbronn, Germany) was equilibrated using binary Pump 1 flowing at 0.5 mL/min with 80% mobile phase A (water) and 20% mobile phase B (0.1% formic acid in acetonitrile) and 0.5 mL/min water from Pump 3. Two minutes after sample injection, the valve was switched to Position 2 and the

trapped sample analytes were eluted from the capture column by Pump 1 using a short change to 50% mobile phase A and 50% mobile phase B timed to accommodate the delay volume of the pump. Direction of flow through the capture column was maintained post valve switching. A high pressure ñmixing Teeò (Upchurch Scientific, USA) was used between the switching valve and analytical column, to efficiently dilute the mobile phase with water pumped at 0.5 mL/min by Pump 3. This resulted in the analytes re-accumulating in a sharp band at the inlet of the analytical column. At 2.25 minutes post injection the flow from Pump 3 was stopped, and the flow of Pump 1 was increased to 1.0 mL/min. At the same time a gradient of increasing mobile phase B was initiated, resulting in separation of the analytes. At 4.2 minutes the valve switched back to its start position allowing first washing, then re-equilibration of the capture column using Pump 2. Re-equilibration of the analytical column using Pump 1 took place during the loading phase of the capture column of the following sample run. The eluted analytes were directed into the ESI source of the mass spectrometer without any flow splitting and were identified and quantified by MRM analysis under the optimised conditions. Details of the plumbing and mobile phase gradient timing for each pump are summarised in Figure 4-1 and Table 4-1 respectively.

Autosampler carry-over was assessed for each of the analytes using two blank injections following the highest concentration in the calibration curves. An autosampler sample needle wash was used to minimise carry-over. The autosampler tray was set to 10ęC to ensure sample stability.

4.3.5 *Preparation of stock, standard and internal standard solutions*

Stock solutions of DM hydrobromide, OME, DX, 5OH (analytes) and imipramine (internal standard) were accurately weighed and diluted in methanol to a concentration of 0.1 mg/mL and stored at -20ęC. Imipramine was further diluted to a concentration of 25.0 ng/mL in methanol for addition to each sample. Stock solutions prepared from separate weighings were used for inter-day analysis. Separately prepared stock solutions were used to prepare high and low quality control standards. Methanol was used as a stock solution diluent as it does not freeze at -20ęC. Calibration standards were prepared by diluting stock solutions at ambient temperature into water at a concentration range of 1.3 - 2500 ng/mL. Plasma-based standards

were prepared in an identical manner as the aqueous calibration standards for recovery assessment studies (1.3 ï 50.0 ng/mL).

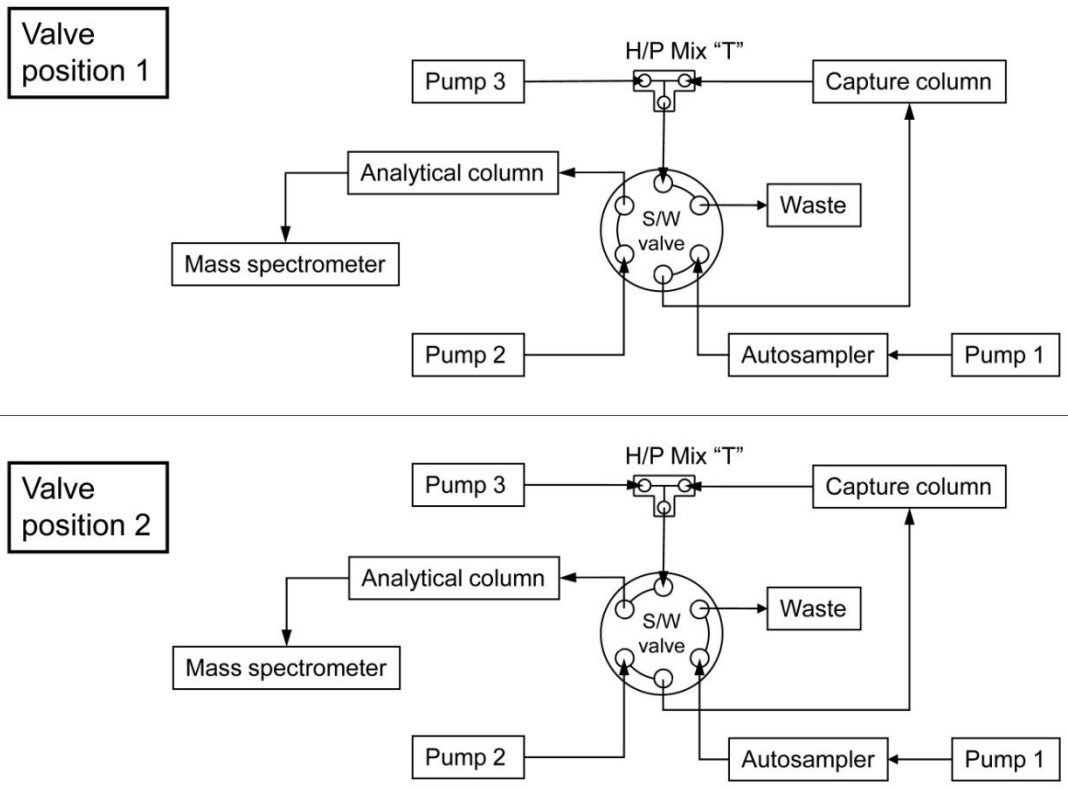

Figure 4-1. Diagrammatic representation of the plumbing for the automated online SPE column switching method.
This figure was created by Tyren Mark Dodgen.

4.3.6 *Sample preparation*

On the day of analysis the samples were thawed and 200 µL of internal standard in methanol added to 100 µL of sample. Each sample was then vortex mixed at maximum speed for 30 seconds. Samples were then sonicated in an ultrasonic bath for 10 minutes and vortex mixed once again at maximum speed for 30 seconds. These were then centrifuged at 14000 x *g* for 10 minutes to remove precipitated plasma proteins. The supernatant was transferred to tapered glass autosampler vial inserts for analysis.

Table 4-1. Programs controlling each of the pumps and the switching valve.

Binary pump 1				
Step	Time (min)	Flow Rate (εL/min)	A (%)	B (%)
0	0.00	500	80	20
1	1.59	500	80	20
2	1.60	500	50	50
3	1.80	500	50	50
4	2.10	500	70	30
5	2.15	500	70	30
6	2.20	500	70	30
7	2.25	1000	85	15
8	2.45	1000	85	15
9	3.20	1000	44	56
10	4.00	1000	20	80
11	5.35	1000	20	80
12	5.40	1000	20	80
13	5.55	1000	80	20
14	6.00	1000	80	20

Binary pump 2				
Step	Time (min)	Flow Rate (εL/min)	C (%)	D (%)
0	0.00	100	90	10
1	0.80	100	90	10
2	0.90	1000	90	10
3	3.00	1000	90	10
4	3.30	1000	90	10
5	3.90	1000	15	85
6	4.60	1000	15	85
7	4.75	1000	90	10
8	6.00	1000	90	10

Isocratic pump 3			
Step	Time (min)	Flow Rate (εL/min)	E (%)
0	0.00	500	100
1	2.20	500	100
2	2.25	0	100
3	6.00	0	100
4	6.01	500	100

Column Switching Valve Program	
Time (min)	Valve Position
0.00	Position 1
2.00	Position 2
4.20	Position 1
6.00	Position 1

Mobile phases: A: H2O; B: CH3CN/0.1% formic acid. C: H2O with 0.1% NH4HCO2, pH 9; D: CH3CN/0.1% formic acid; E: H2O

4.3.7 *Assay validation parameters*

Validation parameters were assessed according to guidelines of the International Conference on Harmonization (ICH) as accepted by numerous regulatory authorities (Peters, Drummer & Musshoff 2007).

During method development the capture column retention and elution conditions were tested by removing the analytical column and monitoring the capture columnôs eluent by the mass spectrometer during both the capture and elution phases. Mixed analyte samples diluted in water and plasma were used for optimisation of the capture and elution conditions. Analyte recovery from plasma was determined using spiked plasma prepared as described in Section 2.5. After initial sample preparation, each spiked plasma sample was analysed in triplicate and compared to the unprocessed water based calibration curve. The percentage difference

between the measured plasma concentrations compared to unprocessed water based calibrants was used to calculate the analyte recoveries.

Lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were defined as three times and ten times the average baseline noise of an unspiked blank at the retention time of each analyte respectively. Calibrations were performed using standards prepared as described in Section 2.5. Five replicates of each standard were used to establish the linear calibration equation (y=mx+c) and analysed using the ratio of analyte peak area over the internal standard peak area after quantitative integration by Analyst 1.4.2 Software.

Linearity was measured as the coefficient of determination (R^2) measured from the five calibration replicates. Precision was assessed from the relative standard deviation from the mean (RSD) and calculated separately for each concentration level of the calibration curve. Similar to precision, accuracy was calculated using each concentration level of the calibration curve and reported as a percentage of the actual known concentration. Five separate calibration curves were analysed each day and on different days to establish the intra- and inter-day precision and accuracy respectively. Both precision and accuracy were deemed acceptable if the %CV was less than 15%.

4.3.8 *Evaluation of ion suppression*

Ion suppression was assessed by direct infusion at the ESI source inlet of an aqueous solution of all five analytes at 1 ng/εL each at a continuous 15 εL/min into the eluent of a double blank plasma sample (without addition of internal standard) injected using the complete analysis method. A signal intensity change of any of the five analytes would be indicative of ion suppression (or enhancement) related to sample matrix or solvent gradient during the analysis (Bakhtiar & Majumdar 2007).

4.3.9 *Clinical sample evaluation*

Apparently healthy, probe-drug naïve volunteers participated in the evaluation of this method. Two 5 mL blood samples were collected into EDTA containing blood tubes prior to drug administration to assess baseline analyte levels. All participants then received a combined oral dose of approximately ±60 mg Benylin Dry Cough Syrup (DM) and 40 mg Altosec (OME). At

2, 3 and 4 hours after administration, two un-coagulated 5 mL blood samples were collected, centrifuged at 850 x *g* for 10 minutes and the plasma aliquoted into 1.5 mL centrifuge tubes for storage at -70°C.

4.4 Results

Table 4-2. Automated online solid phase extraction LC-MS/MS: optimized parameters and calibration parameters for all analytes.

	Analytes					IS
	Dextromethorphan	Omeprazole 1	Omeprazole 2	Dextrorphan	5'-hydroxy-omeprazole	Imipramine
Optimisation						
Q1 m/z	272.2	346.3	346.3	258.0	362.4	281.4
Q3 m/z	147.0	198.0	136.2	199.0	214.3	86.3
Declustering potential (Volts)	81.0	25.0	25.0	81.0	41.0	41.0
Collision energy (Volts)	41.0	17.0	40.0	37.0	17.0	12.0
Calibration parameters						
Range (ng/mL)	1.3-2500	1.3-2500	1.3-2500	1.3-2500	1.3-2500	ð
Linearity (r2)	0.9991	0.9923	ð	0.9971	0.993	ð
Equation of calibration (y = mx + c)	y = 0.135x - 0.000439	y = 0.083x - 0.0344	ð	y = 0.119x - 0.0126	y = 0.0386x - 0.0299	ð
LLOD (pg/mL)	53 .0	229 .0	ð	43.0	136.0	ð
LLOQ (pg/mL)	226.0	1145.0	ð	215 .0	680.0	ð

IS, internal standard. Calibration parameters for omeprazole 2 (used for crosstalk monitoring) and imipramine (IS) were not calculated (ð).

4.4.1 *Optimisation of MS/MS detection parameters*

Positive ionisation generated the highest sensitivity by a factor of at least three for all the analytes making this the ionisation mode of choice. Method optimisation used the three most intense precursor/product transitions for each of the analytes. The MRM transition yielding the highest intensity was used to further fine tune the method. The optimised mass spectrometer parameters are summarised in Table 4-2. The mass spectrometerparameters that remained constant for all analytes were: curtain gas; 27, ionisation voltage; 4500 V, ion source temperature; 450 ęC, gas 1; 35.0, gas 2; 45.0, collision gas; medium, entrance potential 10.0 V; collision cell exit potential; 12.0 V and dwell times; 50.0 msec. Two transitions for omeprazole were monitored to assess the possibility of cross-talk of the most intense fragment at 198.0 with the dextrorphan fragment at 199.0. No crosstalk was observed as sufficient chromatographic resolution was achieved.

4.4.2 *Optimisation of chromatography*

Complete analyte capture was achieved on the capture column at the highest concentrations tested with no analyte breakthrough before 2.4 minutes. The analytes were rapidly eluted after switching the valve at 2.0 minutes with a ñslugò of 50% acetonitrile in a sharp band, then recaptured as a narrow band on the analytical column due to dilution of the eluent with water from Pump 3. The analytical column separation provided identification and accurate quantification of all the analytes and internal standard. Figure 4-2 shows a typical chromatogram. Retention times were very stable at 3.30 ± 0.01 min for DX, 3.35 ± 0.01 min for 5OH, 3.92 ± 0.03 min for OME, 4.23 ± 0.01 min for DM and 4.57 ± 0.02 min for imipramine as measured for all calibration curves (intra- and inter-day) and recovery assays. Although DX and 5OH partially co-elute, MRM analysis allows for accurate identification and quantification of both analytes without any interference. The online SPE took 2 minutes followed by a 4 minute analytical separation after the column switch.The capture column washing and re-equilibration was during the last 2 minutes of the separation giving a 6 minute run time. No noticeable carry-over for any analyte was seen as assessed in blank samples measured directly following the highest levels of each calibration curve. The sample needle wash step and efficient capture column elution contribute to this.

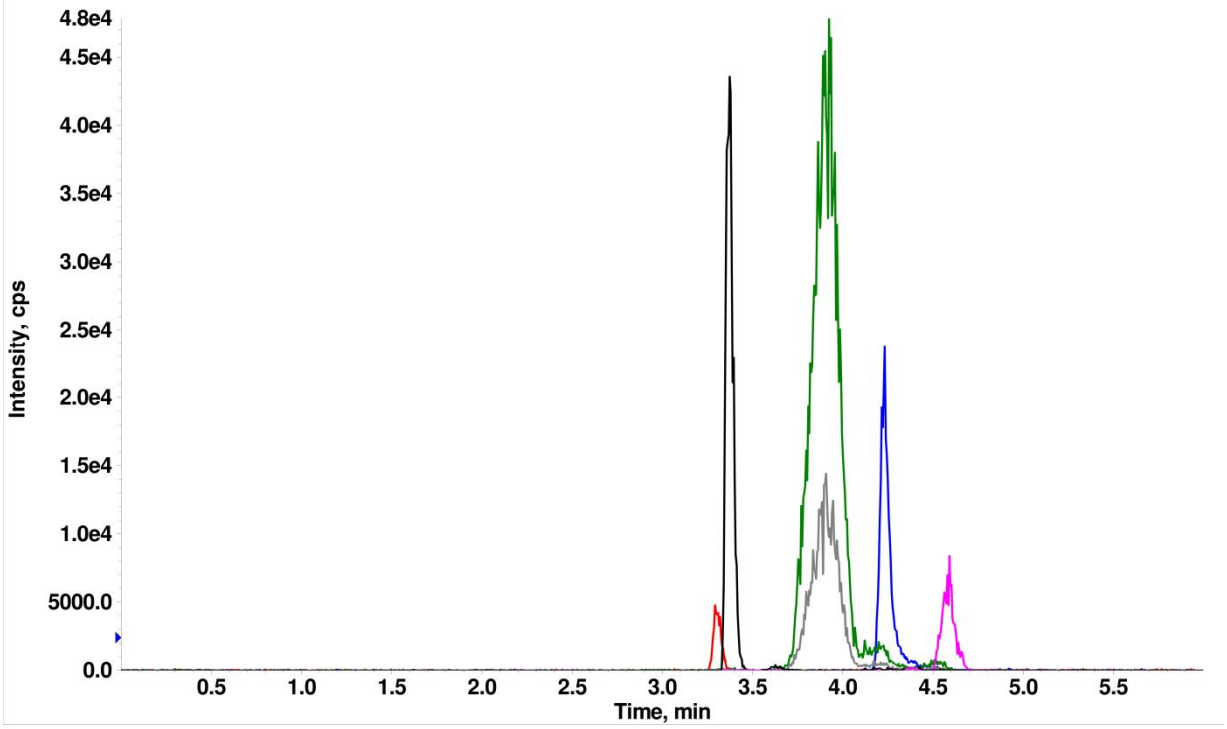

Figure 4-2. The MRM chromatogram using automated online SPE, column switching liquid chromatography tandem mass spectrometry of a patient plasma sample collected 3 h post probe drug administration.

Peak order is dextrorphan (3.30 min), 50-hydroxyomeprazole (3.35 min), omeprazole (3.92 min ï two transitions monitored), dextromethorphan (4.23 min) and imipramine (4.57 min ï the IS) This figure was created by Tyren Mark Dodgen.

4.4.3 *Validation parameters*

Without the ultra-sonication step in the preliminary sample workup, analyte recoveries were low (38% for dextrorphan). The ultra-sonication step in the initial PPT increased the analyte recoveries to above 85% for all analytes (Table 4-3). Imipramine recovery from plasma samples was 85.8%. Intra- and inter-day precision measured as %CV was less than 15% for all analytes. A slight positive bias was observed for the recovery of the lowest concentrations of the metabolites. Precision, accuracy and other performance parameters of this method are summarised in Table 4-3. In a sampled cohort (n=78) at 3 hour post-dosing the mean concentrations of the analytes (SD) were: dextromethorphan 5.39 (± 6.8ng/mL), omeprazole 676.00 (± 514.1 ng/mL), dextrorphan 5.04 (± 3.7 ng/mL)and 5ô-hydroxyomeprazole 224.00 (± 128.3 ng/mL).

Table 4-3. Precision (CV) and accuracy validation for intra-day, inter-day and recovery

	Intra-day (n = 5)			Inter-day (n = 5)			Recovery (n = 3)		
Concentration (ng/mL)	Found (mean± S.D.)	Accuracy (%)	CV(%)	Found (mean± S.D.)	Accuracy (%)	CV (%)	Found (mean ± S.D.)	Recovery (%)	CV (%)
				Dextromethorphan (DM)					
1.3	1.23 ± 0.07	98.2	5.7	1.34 ± 0.09	107.1	7.0	1.09 ± 0.07	87.0	6.5
2.5	2.43 ± 0.04	97.2	1.6	2.52 ± 0.12	100.7	4.8	2.57 ± 0.21	102.9	8.1
5.0	5.05 ± 0.30	100.9	5.9	5.22 ± 0.32	104.3	6.1	5.19 ± 0.20	103.9	3.8
25.0	25.18 ± 1.65	100.7	6.6	25.19 ± 2.42	100.7	9.6	23.69 ± 0.57	94.8	2.4
50.0	49.92 ± 0.80	99.8	1.6	49.82 ± 4.28	99.6	8.6	44.16 ± 0.65	88.3	1.5
250.0	259.42 ± 8.19	103.8	3.2	284.93 ± 6.75	114.0	2.3			
500.0	556.04 ± 38.14	111.2	6.9	469.39 ± 46.29	93.9	9.9			
2500.0	2474.11 ± 162.03	99.0	6.5	2464.95 ± 211.14	98.6	8.6			
				Omeprazole (OME)					
1.3	1.16 ± 0.05	93.2	4.4	1.25 ± 0.15	100.3	11.7	1.06 ± 0.07	84.6	6.8
2.5	2.51 ± 0.11	100.5	4.4	2.64 ± 0.33	105.4	12.5	2.11 ± 0.17	84.6	7.9
5.0	5.24 ± 0.32	104.9	6.0	5.44 ± 0.42	108.7	7.7	4.71 ± 0.37	94.2	7.9
25.0	23.42 ± 0.80	93.7	3.4	23.45 ± 1.87	93.8	8.0	20.27 ± 0.93	81.1	4.6
50.0	51.32 ± 1.46	102.6	2.8	51.23 ± 4.75	102.5	9.3	42.57 ± 2.89	85.1	6.8
250.0	243.86 ± 14.34	97.5	5.9	276.94 ± 17.69	110.8	6.4			
500.0	508.96 ± 39.51	101.8	7.8	486.48 ± 67.87	97.3	14.0			
2500.0	2407.00 ± 112.41	96.3	4.7	2442.83 ± 182.45	97.7	7.5			
				Dextrorphan (DX)					
1.3	1.41 ± 0.06	112.9	4.3	1.42 ± 0.07	113.7	5.2	1.40 ± 0.11	112.3	8.0
2.5	2.64 ± 0.07	105.7	2.7	2.74 ± 0.27	109.5	9.9	2.82 ± 0.13	112.9	4.1
5.0	5.18 ± 0.30	103.6	5.9	5.47 ± 0.20	109.5	3.6	5.27 ± 0.15	105.3	2.9
25.0	23.81 ± 0.49	95.3	2.1	25.46 ± 1.21	101.9	4.7	24.60 ± 0.42	98.4	1.7
50.0	50.86 ± 1.38	101.7	2.7	49.58 ± 1.68	99.2	3.4	46.98 ± 0.84	94.0	1.8
250.0	276.20 ± 9.37	110.5	3.4	280.86 ± 28.99	112.3	9.8			
500.0	496.09 ± 22.51	99.2	3.7	496.09 ± 26.77	99.2	5.4			
2500.0	2414.33 ± 74.68	96.6	3.1	2433.69 ± 262.05	97.3	10.8			
				5'-hydroxy omeprazole (5OH)					
1.3	1.12 ± 0.10	89.5	9.0	1.17 ± 0.15	93.5	13.2	1.49 ± 0.02	118.8	1.3
2.5	2.35 ± 0.13	93.8	5.5	2.30 ± 0.21	92.1	9.2	2.90 ± 0.16	116.2	4.8
5.0	4.75 ± 0.14	95.0	2.9	5.06 ± 0.43	101.1	8.5	5.86 ± 0.14	117.2	2.5
25.0	21.32 ± 1.14	85.3	5.4	23.00 ± 1.18	92.0	5.1	26.18 ± 0.43	104.7	1.6
50.0	52.42 ± 1.35	104.8	2.6	51.37 ± 5.68	102.7	11.0	51.14 ± 1.49	102.3	2.9
250.0	268.07 ± 14.14	107.2	4.8	266.85 ± 34.09	106.7	12.8			
500.0	561.66 ± 34.77	112.3	5.5	560.60 ± 72.34	112.1	12.9			
2500.0	2388.91 ± 68.28	95.6	2.9	2421.21 ± 211.53	96.8	8.7			

3.4. Evaluation of ion suppression

The ion suppression test using a constant post column infusion of a mixed analyte standard and injection of blank plasma samples after standard sample preparation showed no matrix effect at the retention times of any of the analytes. A small transient signal drop was observed in both water and plasma samples which corresponded to the switching valve movement times.

4.5 Discussion

The method reported here is a semi-automated method to simultaneously quantitate several analytes in plasma by positive ion ESI mode LC-MS/MS. Several enhancements over reported methods for quantitating the same analytes were introduced. The volume of sample required after addition of solvent precipitant was only 5 µL.The effect of protein binding of the analytes was greatly reduced by incorporating an ultra-sonication step after addition of the internal

standard in methanol which liberated the analytes from the proteins prior to centrifugation. The high recovery of the analytes from spiked plasma samples endorses this vital ultra-sonication step to release the bound drug fraction from the protein. Initial solvent PPT eliminated column blocking protein sufficiently as the capture column could tolerate more than 300 injections before any significant pressure increase occurred. Maintaining the capture column temperature at ambient and the direction of flow through the capture column after the valve also contributed to the longevity of the capture column. Recoveries were comparable to several reported methods, including SPE, LLE and column switching (Härtter et al. 1996; Vengurlekar et al. 2002; Cass et al. 2003; Frerichs, Zaranek & Haas 2005; Hofmann et al. 2006; Shimizu et al. 2006; Eichhold et al. 2007; Kumar, Mann & Remmel 2007). Recovery for 5OH (Hofmann et al. 2006; Shimizu et al. 2006), OME (Frerichs, Zaranek & Haas 2005), DX (Eichhold et al. 2007) and DM (Vengurlekar et al. 2002; Eichhold et al. 2007) was better than several reported LLE based methods.

The use of an online capture column has several advantages. Ion suppression was almost totally eliminated especially at the early elution times as no significant decrease in signal intensity was seen during the post column infusion study. The effective extraction (facilitated by the high pH of the initial sample transfer mobile phase) resulted in pre-concentrationof the analytes, increased method sensitivityandpermitted comprehensive washing of the capture column without significant analyte loss. This efficient analyte capture could accommodate greatly increased injection volumes for samples with sub-nanogram per millilitre analyte concentrations to increase sensitivity several fold without compromising the analytical separation (volumes of up to 100 εL).

Although the binary Pump 1 program appears to be complicated, it is automated and incorporates changes in flow rate and mobile phase composition including a timed short high acetonitrile ñbumpò to elute the captured analytes in a sharp band. This high organic solvent mobile phase needed to elute the analytes from the capture column in a sharp band would normally compromise the separation on a short analytical column, but the use of a third pump and an efficient low volume high pressure ñmixing Teeò to substantially dilute the organic solvent with water resulted in the analytes being retained and re-concentrated at the inlet of the short sub-2 µm high resolution analytical column, improving sensitivity and robustness. This

dilution with water avoided peak broadening and potential unretained chromatographic elution from the analytical column. A short increasing acetonitrile gradient combined with the cessation of the water flow from the third pump facilitated chromatographic separation of the analytes. The change in pH during the elution appeared to be reproducible as the analyte retention times were observed to be stable with small standard deviations.

One rate determining parameter of any chromatographic method is the length of the analytical column. Choice of a short sub-2 µm particle size analytical column reduced assay times considerably without compromising resolution. Run times of 6 minutes are significantly shorter than the 20-30 minutes reported for several methods for equivalent separations (Streetman et al. 2000; Tamminga et al. 2001; Vengurlekar et al. 2002; Jerdi et al. 2004; Frerichs, Zaranek & Haas 2005; Hofmann et al. 2006; Eichhold et al. 2007; Ryu et al. 2007). The increased relative concentration of analyte in each narrowed peak increased sensitivity and was easy to integrate, which is a further advantage of this rapid method. The LLOQ for this method was lower for 5OH (Cass et al. 2003; Shimizu et al. 2006), OME (Shimizu et al. 2006), DX (Härtter et al. 1996) and DM (Härtter et al. 1996) compared to other reported column switching methods despite the low injection volume. The increased operating pressure of the HPLC due to the small particle size of the stationary phase was effectively reduced by maintaining the temperature stable analytical column at 60ęC to reduce the viscosity of the mobile phase. The capture column was however plumbed outside of the column oven to avoid damage as a result of the high temperature. This proved a vital component in the capture column longevity.

Evaluation of actual patient samples revealed that this method is applicable for simultaneously phenotyping CYP2D6 using dextromethorphan and CYP2C19 using omeprazole at a 3 hour once off sampling. Due to the high plasma concentration of OME at the times of sample collection, the administered dosage for routine screening could be lowered.

Although not tested during this study, the simple initial sample preparation used in combination with online solid phase extraction allows this method to be applied to the quantitation of these and similar analytes in a variety of different biological matrices such as saliva and cerebrospinal fluids without any modification. To extend the method to urine

analysis the samples would need to be diluted with water to reduce salt content prior to ultra-sonication and a larger sample volume injected. The use of the capture column accommodates increased injection volumes as discussed earlier.

4.6 Conclusion

A rapid, highly sensitive, automated online solid phase extraction tandem mass spectrometric method for the simultaneous quantification of DM, OME, their respective metabolites and an internal standard has been developed and validated to assess the metabolic status of two pharmacogenetically important CYP enzymes. A simple initial sample preparation followed by automated online SPE in combination with column switching and post capture column eluent dilution with water effectively reduces sample preparation time, analysis time, reduces solvent requirements, and is cost-effective. Sensitivity, high selectivity and no interferences were added benefits. The robust nature and limited manual sample preparation steps reduced preparation time, operator errors, increased confidence in the results and could make routine laboratory phenotypic metabolic screening feasible. This cost effective and rapid method could potentially complement or replace genotyping as the metabolic screening assay for pharmacogenetic variability due to the added epigenetic influences monitored simultaneously.

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CHAPTER 5. EVALUATION OF PREDICTIVE *CYP2C19* GENOTYPING ASSAYS COMPARED TO MEASURED PHENOTYPE IN A SOUTH AFRICAN COHORT

A version of this chapter will be published shortly.

5.1 Abstract

Development of appropriate pharmacogenetic platforms may reduce adverse drug reactions (ADRs) that are debilitating and have cost implications in current health care. **Aims:** This study was designed to direct future pharmacogenetic screening of *CYP2C19* in South Africa and to compare predictedand measured phenotype in a demographic cohort of the South African population. **Methods:** Genotyping of the *CYP2C19 *2*, **3*, **9*, **15*, **17*, **27* and **28*alleles was performed using PCR-RFLP.An Activity Score (AS) system was used for CYP2C19 phenotype prediction. Actual phenotype was measured*in vivo* using plasma concentrations of omeprazole (OME-probe drug), and 5ô-hydroxyomperazole (5OH-CYP2C19 specific metabolite) three hours post administration. Concentrations were simultaneously quantified using semi-automated online solid phase extraction, coupled to tandem mass spectrometry. **Results:** Of the alleles genotyped, *CYP2C19*9* and **15*(reduced enzyme activity) contributed to improving phenotype prediction. *CYP2C19*17* did not increase OMEmetabolism and *CYP2C19*27*did not reduce OME metabolism. The adapted AS system improved phenotype prediction of CYP2C19, but required further adjustment to improve OME metabolismprediction. Partial genotype-phenotype discrepancies highlight the need for comprehensive *CYP2C19*genotyping to predict OME metabolism.*CYP2C19* variation may not be the only factorcontributing to variable OME metabolism. **Conclusion:** Substrate adjusted AS systems would be valuable for future pharmacogenetic screening. Additional *CYP2C19* alleles may yet be identified in this complex population.

5.2 Background

*CYP2C19*2* and **3* (non-functional alleles) are the most commonly genotyped *CYP2C19* alleles (Sistonen et al. 2009). Unfortunately, the these alleles have been prioritised based on frequent observations in predominantly Caucasian and Asian cohorts.Such genotyping platforms may not be comprehensive enough for Africans who possess the greatest degree of genetic diversity. *CYP2C19**17, responsible for increased CYP2C19 expressionand metabolism (Sim et al. 2006). This allele has been observed relatively frequently in several populations of different ethnicity (Kurzawski et al. 2006; Sim et al. 2006; Rudberg et al. 2008; Ragia et al. 2009; Kearns, Leeder & Gaedigk 2010)including a South African cohort (Drögemöller et al. 2010b) and is important in the prediction of UMs. Genotyping strategies designed to a variety of alleles would be more valuable for the South African population. A good example of an African-specific allele is *CYP2C19*9* (Blaisdell et al. 2002) and genotyping platforms used on multi-ethnic cohort would need to include these alleles.

In general, genetic variation in Africans has been poorly characterised. This is ironic considering that African populationshave increased genetic diversity compared to non-Africans.The reported site of origin for modern humansis in Africa and African individuals have been exposed to greater environmental pressures over a longer period of time resulting in greater genetic diversity (Campbell & Tishkoff 2008; Tishkoff et al. 2009; Henn et al. 2011). South Africa encompasses some of the most diverse and unique African populations (Warnich et al. 2011), illustrated by 11 official languages as well as many more distinct ethnic groups and cultures (http://www.statssa.gov.za/Publications/P03014/P030142011.pdf). This needs to be kept in mind when establishing pharmacogenetically-guided treatment plans in South Africa.

To date, very few studies have been published examining *CYP2C19* variation in South African populations and there is no data assessing the relationship between predicted and measured phenotype. The available studies regarding genetic variation have been performed in the Venda (Dandara et al. 2001; Matimba et al. 2009), Xhosa and Cape

Mixed Ancestry (Coloured) populations (Drögemöller et al. 2010b; Ikediobi et al. 2011), which comprise 2.3%, 17.6% and 8.9% of the South African population, respectively (Statistics South Africa Census 2011 ï http://www.statssa.gov.za/Publications/P03014/P030142011.pdf). With regard to the Venda cohorts, the data for this group was obtained from two studies, utilising a small cohort (n=9) (Matimba et al. 2009) and a larger cohort (n=76) (Dandara et al. 2001). The first employed a re-sequencing approach identifying a number of SNPs in *CYP2C19*, while the other examined only *CYP2C19*2* and **3*. More recently, Drögemöller et al. (2010b) looked at the Xhosa (n=100) and Cape Mixed Ancestry (Coloured, n=75) populations and detected the*CYP2C19*2*, **3, *9, *15,*17*alleles, as well as the novel **27* and **28* alleles (the majority of which remain uncharacterised *in vivo*).In a separate study, Ikediobi et al. (2011) also examined two similar cohorts (n=109 and n=67, respectively) looking at *CYP2C19*2* and **17*, as well as additional intronic and 5ô upstream variants. A study by Dandara et al. (2011) looking at the Birth to Twenty (Bt20) cohort was recently published, but once again only *CYP2C19*2* and **3* frequencies were reported. Additional allele frequencies can be found on the PharmGKB website (Whirl-Carrillo et al. 2012).

From these studies, a number of interesting observations can be made. First, the predominantly Asian allele, *CYP2C19*3*,was only detected in the Coloured population, reflecting the level of admixture in this population (Drögemöller et al. 2010b). Second, expression of the novel *CYP2C19*27* allele was described to be reduced *in vitro* due to the -1041G>A (rs7902257) SNP in the promoter which could potentially alter the associated metabolic phenotype. Third, the absence of *CYP2C19*10* and an alternative linkage disequilibrium (LD) pattern found for *CYP2C19*17*, emphasises apparent differences between African-Americans in the USA and the Xhosa from South Africa. This,together with allelic frequency differences seen between the Venda, Xhosa and Coloureds, reiterates the importance of acknowledging diversity within Africa and those of the African Diaspora (Drögemöller et al. 2010b). More comprehensive African-specific genotyping strategies are therefore required when evaluating South Africans.

Furthermore, CYP2C19 phenotype has not been measured in the South African population using *in vivo* metabolic analysis, and a genotype-phenotype comparison would therefore be valuable.

In this chapter, predicted and measured metabolism for CYP2C19 was investigated in a demographically representative South African cohort.

5.3 Materials and methods

5.3.1 *Study subjects*

The study was approved by the Student Research Ethics Committee, Faculty of Health Science at the University of Pretoria (S132/2009) and was carried out under good clinical practice (GCP) guidelines. Apparently healthy, unrelated volunteers (n = 100) were recruited to approximately represent the demographics of the current South African population and consisted of 70 Black African, 10 Caucasian (White), 10 Coloured and 10 Indian volunteers (http://www.statssa.gov.za/). The sample was not chosen to compare inter-ethnic differences, but to represent the demographics that could potentially be seen in clinical practice in South African. Volunteers of both genders were included in the study if they were South African by birth, older than 18 years of age and if they were not pregnant or breast-feeding. An informed consent form was completed by each volunteer, including particulars such as age, height, weight, waist circumference, any medication being taken at the time of the study, place of birth, and voluntary disclosure of ethnic grouping (Black African, Caucasian, Coloured or Indian).

5.3.2 *Genomic DNA (gDNA) extraction*

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) for baseline quantitation of analytes during the phenotype assays, and were used for gDNA extraction using the Genomic DNA Purification Kit (Fermentas Life Science, Lithuania) or the automated Maxwell® 16 system (Promega, Madison, WI, USA), according to manufacturerôs instructions.

5.3.3 *CYP2C19 genotyping*

CYP2C19 variation was evaluated using PCR-RFLP analysis described previously (Drögemöller et al. 2010b).Alleles identified and assayed were named according the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committeeôs online database (http://www.cypalleles.ki.se/). Allele defining SNPs for the *CYP2C19*2*, **3*, **9*, **15*, **17*, **27* and **28* alleles were assayed as described previously for this cohort (Dodgen et al. 2013).

5.3.4 *Phenotype prediction*

The Activity Score (AS) concept, initially developed to compare genotypic and phenotypic data to establish a more accurate CYP2D6 phenotype prediction (Gaedigk et al. 2008), was adapted for CYP2C19 phenotype prediction.The adapted AS was applied to PCR-RFLP genotypes (Dodgen et al. 2013). The AS reported here is based on known enzyme activity reported in the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committeeôs online database for *CYP2C19* alleles (http://www.cypalleles.ki.se/cyp2c19.htm). Table 5-1 explains how the AS system was adapted to predict CYP2C19 metabolism. The model was then modified based on omeprazole metabolism for more accurate prediction (Table 5-1).

Table 5-1. CYP2C19 phenotype prediction.

Estimated metabolic potential of alleles			
Allele activity	Numeric Activity	AS	Modified AS
Increased	2.0	*17	
Normal	1.0	*1+, *28	*1+, *17, *27, *28
Decreased	0.5	*9, *27	*9, *15
Absent	0.0	*2, *3	*2, *3
Unknown	1.0	*15	
(Activity according to http://www.cypalleles.ki.se/)			
Phenotype prediction			
Activity Score (AS)		Prediction	
> 2.0		UM	
1.5-2.0		EM	
0.5-1.0		IM	
0.0		PM	

Alleles for both genes present in this table are relevant to this study, additional allele information is available at http://www.cypalleles.ki.se/. Activity of each allele in a genotype is used to predict. The AS was adopted from Gaedigk et al. (2008) to predict CYP2C19 activity. AS was modified based on omeprazole metabolism.

5.3.5 *CYP2C19 omeprazole phenotyping*

Invivo CYP2C19 enzyme activity was determined using omeprazole (OME) as the probe drug and 5ô-hydroxyomeprazole (5OH) as the CYP2C19 specific metabolite. Plasma concentrations of OME and 5OH were quantified simultaneously in plasma samples taken at two, three, and four hours following probe drug administration, using an automated on-line solid phase extraction tandem mass spectrometry (LC-MS/MS) method described previously (Dodgen, Cromarty & Pepper 2011). A metabolic ratio ($MR_{(5OH/OME)}$) of plasma concentration of metabolite over probe drug was used to indicate CYP2C19 enzyme activity. In this manuscript we used a 5OH/OME ratio as opposed to the traditional OME/5OH ratio. By reporting MR in this format predicted phenotype was proportional to the metabolic ratio (instead of inversely proportional). The $\log_{10}$ transformation of the ratio would then simply be a change of sign and would still be easy to compare (i.e. $\log_{10}(10/1) = 1$ and $\log_{10}(1/10) = -1$).

5.3.6 *Statistical analysis*

Statistical analyses were performed using Statistical Package for the Social Sciences version 20.0 (SPSS Inc., Chicago, Il). To establish the best time point for phenotype measurement, the metabolic ratios for each time point were correlated to one another using Pearsonôs Correlation Coefficient. Optimal $MR_{(5OH/OME)}$ antimodes/cut-offs were evaluated using receiver-operator characteristic (ROC) to distinguish between PM, IM and EM. Antimode values were assigned based on obtaining 100% specificity for PMs and by comparing sensitivity and specificity values to find the optimum for both to discriminate between IMs and EMs. Baseline characteristics (age, BMI and waist circumference) were compared to phenotype using ANOVA and gender was compared to phenotype using Chi-square analysis. In addition to the Kappa statistical test for conformation, a custom ñDiscrepancy Analysisò calculation was used to calculate ñinter-rater discrepanciesò using absolute differences to test for conformation. The four categories are ordinal according to metabolism (UM > EM > IM > PM) and the calculation measures the average deviation from perfect conformation. This can also be explained as the closeness of the predicted phenotype to measured phenotype over the entire cohort. Perfect conformation would receive a value of 0.0, while one, two and three deviating categories would receive values of 1.0, 2.0 and 3.0, respectively. All values were added and subsequently divided by the number of individuals in the cohort (n). Sensitivity, specificity, and positive and negative predictive values were calculated as a second measure of predictive accuracy. *P*-values less than 0.05 were considered statistically significant.

5.4 Results

5.4.1 *Subject demographics*

At the time of sampling, the cohort had an average age of 34.4 ± 9.8 years (range 19-58), BMI of 26.1 ± 5.8, an average waist circumference of 86.9 ± 13.3 and 55% were female. The Black African volunteers sampled in this study covered all of the 11 official languages.

5.4.2 *CYP2C19 in vivo phenotyping with omeprazole as probe substrate*

The two hour sampling had a correlation of 0.849 and 0.789 with the three and four hour samplings, respectively. The correlation between the three and four hour phenotyping was 0.910. This suggests that the three and four hour samplings were most similar and that either of these should be used. The three hour sampling was selected as the phenotyping time point of choice, as it would be the more convenient time point for future analysis.

Figure 5-1 illustrates the log $MR_{(5OH/OME)}$ distribution in the sampled cohort. Predicted phenotype evaluated using ROC analysis copaired modified AS generated antimode cut-offs. $MR_{(5OH/OME)}$ = -1.152 (66.7% sensitivity and 100.0% specificity) was established to discriminate between PM and IM. A second cut-off could be established at $MR_{(5OH/OME)}$ = -0.447 (81.8% sensitivity and 77.3% specificity) to discriminate between IM and EM. The superimposed QQ-plot over the histogram in Figure 5-1 is in agreement with the use of these antimodes as cut-offs to differentiate metaboliser status. The slight plateau coinciding with each antimode is evidence of agreement. UMs were not identified in this cohort. Using these cut-offs and modified AS four individuals were measured to be PM, 40 IM and 55 EM.

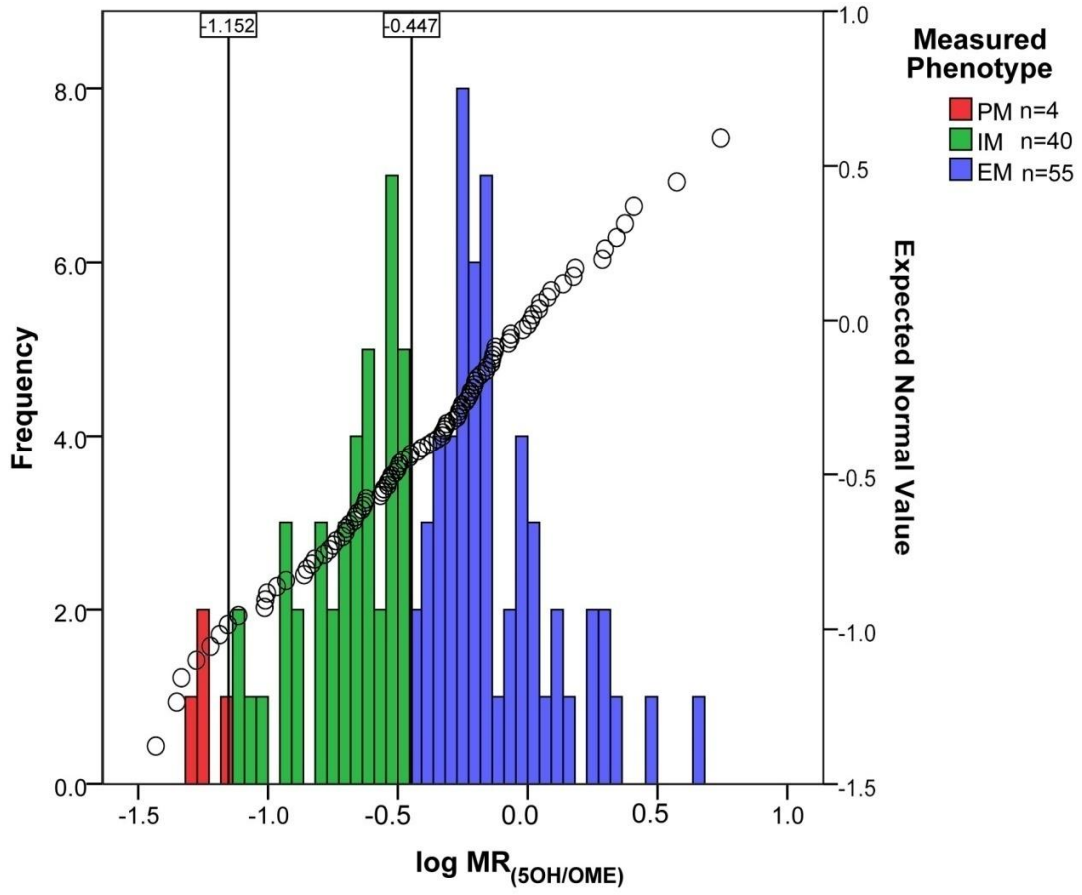


Figure 5-1. Phenotypic distribution of omeprazole metabolism by CYP2C19 in a demographically representative sample of the South African cohort.

Phenotype was measured as the metabolic ratio of the concentration of 5'-hydroxyomeprazole (5OH, metabolite) over omeprazole (OME, probe drug) in a plasma sample taken 3 hours after probe drug administration.The graph has been stratified into poor (PM), intermediate (IM) and extensive metabolisers (EM).The solid X-axis division lines represents the proposed antimodes to discriminate PM from IM and IM from EM individuals. The superimposed QQ-plot agrees with this antimodes. The number in parenthesis indicates the number of individuals in each phenotypic group. This figure was created by Tyren Mark Dodgen.

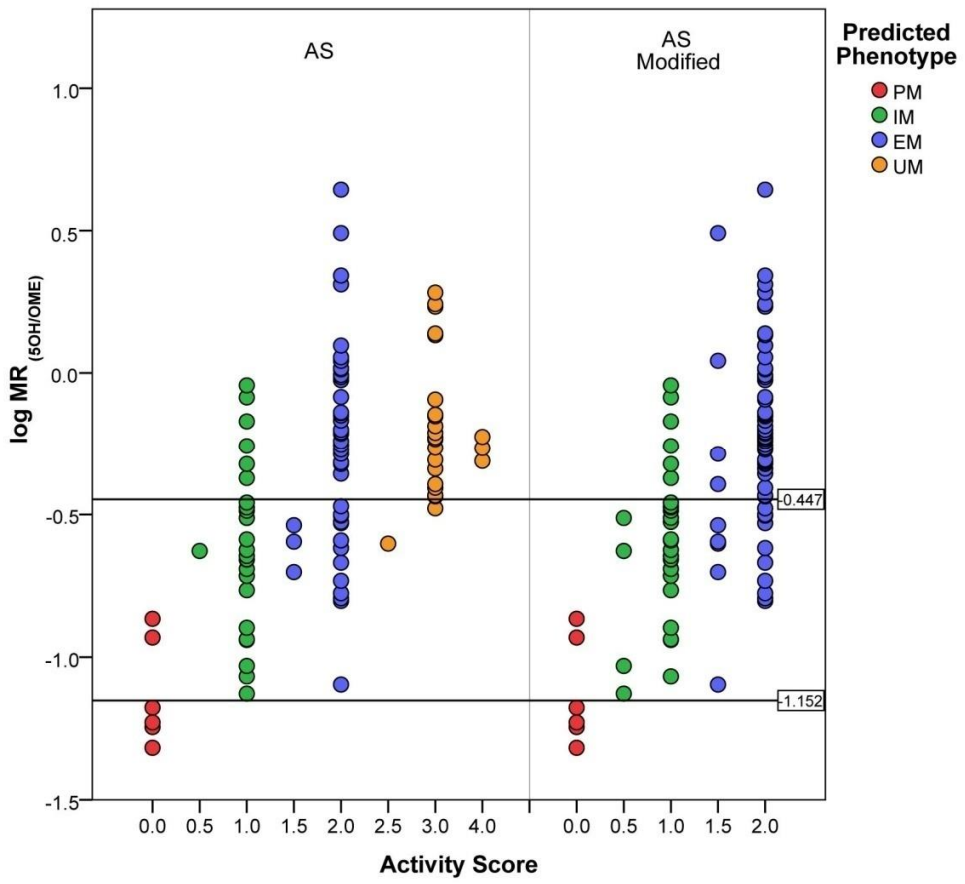


Figure 5-2. Comparison of predicted phenotype generated from *CYP2C19* genotype (using traditional and modified prediction systems) to CYP2C19 measured phenotype. Phenotype prediction included an adapted activity score system (Gaedigk et al. 2008) and an activity score system modified based on omeprazole metabolism (RFLP + AS mod.). Phenotypic groupings included poor (PM), intermediate (IM), extensive (EM) and ultra-rapid (um) metabolism depending on the capability of the platform and system used for prediction.The solid line represents phenotypic cut-offs separating phenotypic pm, im and em from bottom to top.This figure was created by Tyren Mark Dodgen.

5.4.3 *Comparison between predicted and measured phenotype for CYP2C19*

A graphic representation of alternate models for phenotype prediction compared to measured phenotype is illustrated in Figure 5-2. In general, there is a wide range of metabolic ratios within the different predicted phenotypic groups and fairly extensive overlapping of each of the groups is seen. Each grouping was, however, visually distinct from the other, suggesting that *CYP2C19* genotype is a major factor in OME metabolism and can be used to aid in the prediction of CYP2C19 metabolic activity. The UM group predicted by PCR-RFLP using the original AS system appeared to have metabolic activity similar to the EM group. Therefore it appears that *CYP2C19*17*did not increase OME metabolism in this cohort. Figure 5-3 evaluates the*in vivo* activity of *CYP2C19*15* and **27*. The unknown metabolic activity of these alleles paired with various alleles with known metabolic potential suggests that *CYP2C19*15* had reduced and **27* normal enzyme activity. This evaluation demonstrated that *CYP2C19*15* and **27* should have AS values of 0.5 and 1.0 respectively. Additionally, the decreased functionality of *CYP2C19*9* was confirmed in this study (results not shown).

Cross-correlation of ordinal phenotypic groups allowed further evaluation of the relationship between predicted and measured phenotype (Figure 5-3). The modified version of the AS system was the most accurate predictor of *in vivo* OME phenotype. Both Kappa and Inter-Rater Discrepancy scores favoured the modified AS. The modified AS was also superior in sensitivity, specificity and both positive and negative predictive value. IM prediction sensitivity at 57.5% was the lowest value, followed by EM specificity at 65.9%. This confirms that the modified AS predicted phenotype was the most accurate predictor of phenotype when examining the OME metabolism. Discordance was found in all of the race groups (refer to Supplementary Table S6).

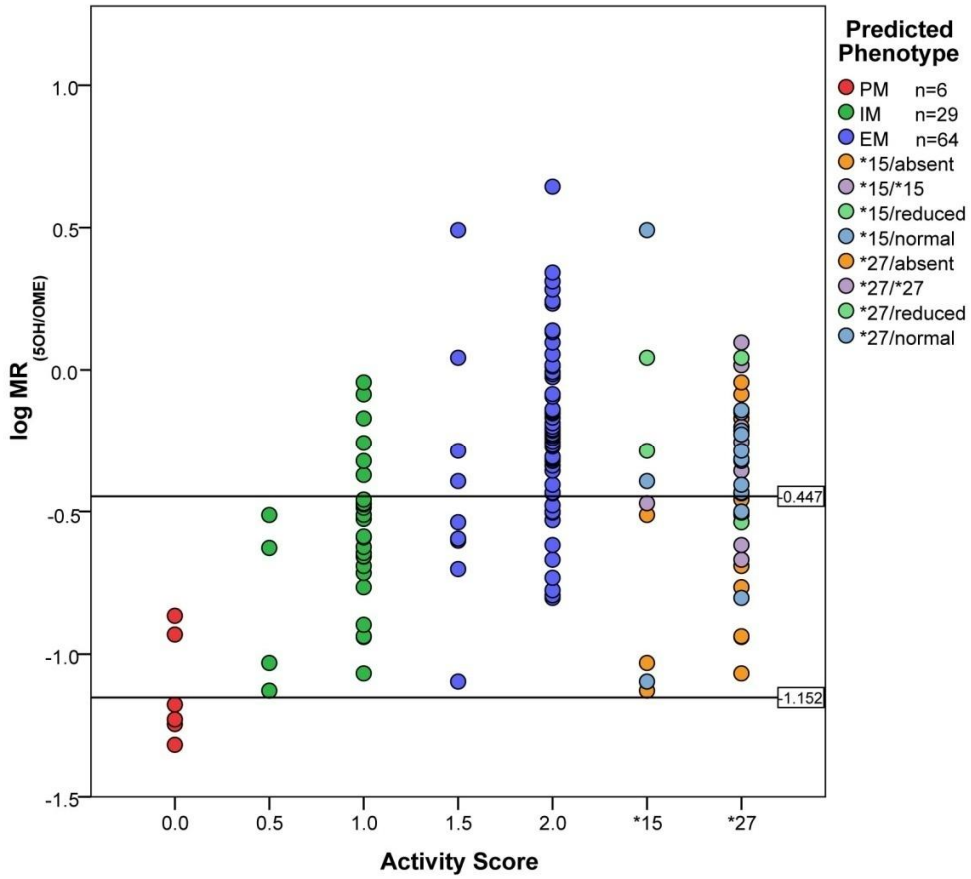
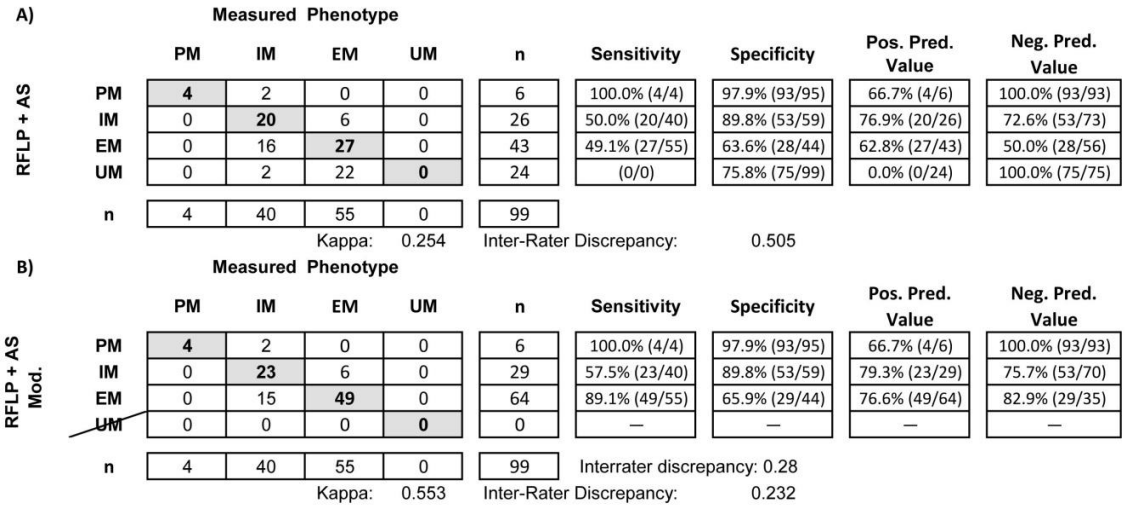


Figure 5-3. *In vivo* elucidation of the metabolic activity of *CYP2C19*15* and **27* (unknown activity) using the comparison of predicted phenotype (as determined by the modified Activity Score system) to measured metabolic activity.

Lines represent antimodes which indicate phenotypic cut-offs from bottom to top separating poor (PM), intermediate (IM) and extensive (EM)metabolism.This figure was created by Tyren Mark Dodgen.



A)

RFLP + AS	Measured Phenotype: PM	IM	EM	UM	n	Sensitivity	Specificity	Pos. Pred. Value	Neg. Pred. Value
PM	4	2	0	0	6	100.0% (4/4)	97.9% (93/95)	66.7% (4/6)	100.0% (93/93)
IM	0	20	6	0	26	50.0% (20/40)	89.8% (53/59)	76.9% (20/26)	72.6% (53/73)
EM	0	16	27	0	43	49.1% (27/55)	63.6% (28/44)	62.8% (27/43)	50.0% (28/56)
UM	0	2	22	0	24	(0/0)	75.8% (75/99)	0.0% (0/24)	100.0% (75/75)
n	4	40	55	0	99				

Kappa: 0.254 Inter-Rater Discrepancy: 0.505

B)

RFLP + AS Mod.	Measured Phenotype: PM	IM	EM	UM	n	Sensitivity	Specificity	Pos. Pred. Value	Neg. Pred. Value
PM	4	2	0	0	6	100.0% (4/4)	97.9% (93/95)	66.7% (4/6)	100.0% (93/93)
IM	0	23	6	0	29	57.5% (23/40)	89.8% (53/59)	79.3% (23/29)	75.7% (53/70)
EM	0	15	49	0	64	89.1% (49/55)	65.9% (29/44)	76.6% (49/64)	82.9% (29/35)
UM	0	0	0	0	0	—	—	—	—
n	4	40	55	0	99				

Interrater discrepancy: 0.28

Kappa: 0.553 Inter-Rater Discrepancy: 0.232

Figure 5-4. Evaluation of the ability to predict phenotype using *CYP2C19* genotype when compared to measured phenotype (*in vivo* metabolic ratios) using omeprazole as a probe drug.

Phenotype was predicted from **(A)** an adapted Activity Score system (Gaedigk et al. 2008)based on known *CYP2C19* allele activities (http://www.imm.ki.se/CYPalleles.htm) and **(B)**using a modified Activity Score system based on omeprazole metabolism. The grey blocks represent perfect correlation.A diagonal line through the metaboliser group represents the inability of the prediction system to predict a specific metaboliser group. The Inter-Rater Discrepancy statistical test was calculated to measure agreement. Sensitivity, specificity, positive predictive value (pos. pred.) and negative predictive value (neg. pred.) were calculated to measurethe ability to predict phenotype. ñð ò represents inability to calculate the measure of performance as the metabolic class was not predicted by the system. This figure was created by Tyren Mark Dodgen.

5.4.4 *Potential confounding factors for CYP2C19 metabolism*

None of the measured potential confounding factors appeared to influence OME metabolism. Using the one-way ANOVA test, *P* values of 0.694, 0.689 and 0.624 were calculated for age, BMI and waist circumference, respectively. Pearsonôs Chi-square

analysis of the influence of gender on OME metabolism gave a *P* value of 0.186, showing no significant influence.

5.5 Discussion

CYP2C19 genotype has been associated with variable pharmacokinetic metabolism of drugs such as clopidogrel (anticoagulant), citalopram (and many other antidepressants), proguanil (prophylactic anti-malarial), OME (and other proton pump inhibitors) and several others (Andersson et al. 1992; Hoskins, Shenfield & Gross 1998; Herrlin et al. 2003; Gardiner & Begg 2006; Brandt et al. 2007; Mega et al. 2010; Myburgh et al. 2012). A comprehensive pharmacogenetic assay for dosage tailoring in the South African population would therefore be valuable. As South Africa is made up of ethnically diverse population groups, it is important to evaluate various pharmacogenetic assays, population specific polymorphisms and targeted re-sequencing technologies to identify the best strategy for future implementation of pharmacogenetics and genomics in the country. This study evaluated the PCR-RFLP platform for pharmacogenetic screening of a demographically representative sample of the South African population.

5.5.1 *Phenotyping*

The reduced time needed for sampling a single plasma concentration for phenotyping is more convenient for the volunteer/patient than the alternative four to eight hour urine accumulation. It also avoids the problems associated with Phase II metabolism, preferential urinary accumulation of the metabolite and variation in urinary pH (Dodgen, Cromarty & Pepper 2011). The use of the three hour time point is in accordance with what has been used previously to phenotype CYP2C19 using OME and 5OH concentrations in plasma (Tamminga et al. 2001; Isaza et al. 2007). Three hour MR was employed previously to evaluate the effect *CYP2C19*17* would have on OME metabolism (Sim et al. 2006). Sim et al. (2006) observed an increase in metabolism, which is contradictory to what was observed in this study. In addition, phenotyping should be taken after T_{max} to avoid the increase in OME seen after absorption from

influencing phenotype. The T_{max} for OME is two to three hours, although it may vary from a half to six hours depending on the formulation and various other factors (Howden et al. 1984; Thomson et al. 1997; Sharma et al. 2000; Calabresi et al. 2004; Baldwin et al. 2008). Capsule based formulation tends to have an earlier T_{max} than tablet formulation, which supports the use of capsule formulation in this study (Thomson et al. 1997).

Previous studies have reported phenotypic cut-offs separating PMs from IMs/EMs to range between log $MR_{(5OH/OME)}$-0.6 and -3.04 (Kortunay et al. 1997; Isaza et al. 2007; Ramsjö et al. 2010). Interestingly, Ramsjö et al. (2010) reported a large difference in $MR_{(5OH/OME)}$ separating PMs from IMs/EMs between Koreans (-3.04) and Swedes (-0.86). The cut-off values established in the present study are within the range of MR observed in other studies. The cut-off separating PM from IM was -1.152, which is lower than what was reported in Swedes. This could potentially be due to the large component of Black Africans in this cohort.

5.5.2 *Comparison of CYP2C19 predicted and measured phenotype*

As has already been highlighted in this chapter, a more comprehensive genotyping approach is required to improve the accuracy of phenotype predictionin the South African population. To achieve this, additional alleles such as the decreased function *CYP2C19*9* and **15* alleles needed to be taken into account. The lack of data pertaining to *CYP2C19*9* in previous studies may be due to the fact that, to our knowledge, this allele is African-specific (Blaisdell et al 2002) and therefore not of great relevance to the more frequently studied Caucasian and Asian populations. The absence of this allele in all population groups examined in this study, with the exception of the Black African group, once again confirms that this allele may be of more relevance to individuals of African descent.

Even though previously identified functional alleles that are thought to be of relevance to South African populations were included in the study, the predictive ability of the PCR-RFLP assay remained weaker than expected. This emphasises the importance of adapting

the AS system used in this study, according to the substrate of interest, as demonstrated by the large improvement in predictive ability (Figure 5-4). This reduces the complication of phenotype prediction,as the numeric based AS system allows a cohort to be stratified into additional groups which may offer insight into quartiles of a cohort as well as minimizing overlaps with in a group (Gaedigk et al. 2008). Stratification allows phenotypic patterns to become apparent. Caution should be taken when implementing AS phenotype prediction as ethnicity, disease state (including liver and kidney function) and concomitant medication may contribute to an unpredictable phenotype (Gaedigk et al. 2008).

This data also illustrates the importance of substrate modified AS for accurate phenotype prediction. The need to modify the AS to predict OME metabolism was particularly relevant for the well studied and validated *CYP2C19*17* allele, which suggests that OME is not a sensitive enough probe drug for assessing CYP2C19 metabolism. Perhaps CYP3A4 metabolism may be a confounding factor. Omeprazole sulphone (CYP3A4 metabolite) plasma concentration could be measured to evaluate the extent of CYP3A4 metabolism of OME, but this cannot contribute to the genotyping evaluation techniques or the prediction of CYP2C19 phenotype. Kearns et al. (2010) highlight this dilemma and suggest pantoprazole (also a proton pump inhibitor) as a more selective probe drug for CYP2C19 phenotypic analysis. Similarly, escitalopram metabolism appears to be unaffected by *CYP2C19*17*, which illustrates that metabolism predictions may be substrate specific (Rudberg et al. 2008). Substrate specificity has also been proposed for another CYP enzyme, CYP2D6. For example, *CYP2D6*17* metabolises risperidone with full function. A shift of AS value of 0.5 to 1.0 for *CYP2D6*17* would therefore be appropriate when predicting risperidone metabolism (Gaedigk et al. 2008; de Leon et al. 2009).

Although this study did not validate the functionality of the 5ô upstream variants, it provided, for the first time, phenotypic data for *CYP2C19*15*, demonstrating reduced metabolism (Figure 5-2). The allele was first described in a Black African by Blaisdell et

al. (2002), where the allele defining SNP 55A>C, (rs17882687) resulting in amino acid change Ile19Leu, was found to be in linkage disequilibrium with 80161A>G (rs3758581), resulting in an Ile331Val amino acid change. The Ile19Leu mutation was not evaluated for activity, because the change is near the N-terminus which is thought not to alter activity. The *CYP2C19*15* allele defining SNP, 55A>C, has subsequently been found to be in complete LD with -2030C>T (rs11316468) (Drögemöller et al. 2010a). In silico analyses of the -2030C>T SNP, utilising three separate programs, consistently predicted the removal of a GATA factor binding site, and although dual reporter luciferase assays did not detect a significant change in expression as a result of this SNP (*P*=0.0928), these results do suggest a trend towards decreased expression (Drögemöller et al. 2010b). The role that GATA binding sites play in CYP2C19 expression is further validated by Mwinyi et al. (2010). Therefore, the removal of a GATA factor binding site, as a result of -2030C>T, could provide an explanation for the reduced metabolic activity seen with*CYP2C19*15* in this cohort.

Initially, *CYP2C19*27* appeared to have reduced phenotypic activity (Figure 5-2). When some of the volunteers were homozygous for the allele they appeared to have an IM phenotype. This was sporadic as others appeared to be EM. However, once AS was adjusted, the Kappa score dropped and the Inter-Rater Discrepancy value increased. This *in vivo* demonstration contradicts the *in vitro* dual luciferase results that suggest reduced expression as a result of -1041G>A promoter variant. Decreased expression as a result of this variant may exist, but this decrease in expression is not enough to have a phenotypic effect on OME. Unknown confounding factors may also be masking of the marginal reduced activity of *CYP2C19*27*. Evaluation of this allele with other substrates may result in different metabolic activity. As this allele is frequent, 16.4% in Black South Africans (Dodgen et al. 2013), 10% in Xhosa and 14% in South African Coloureds (Drögemöller et al. 2010b), it may still contribute to improving predicted phenotype in the South African population.

Although examining a greater number of alleles and modifying the AS system according to OME did improve the predictive ability of genotyping assays, this system remains imperfect. The wide range of metabolic ratios within each predicted phenotype group, the overlap of these groups and the lack of perfect correlation (Figure 5-1 & 5-2), suggest a partial predicted-measured phenotype discrepancy for CYP2C19. These discrepancies could be explained, in part, by environmental factors such as diet, which was not considered in this study (Poolsup, Li Wan Po & Knight 2000), and could either induce or inhibit CYP2C19 activity. Disease status such as HIV/AIDS has been found to affect CYP2D6 metabolic activity (O'Neil et al. 2000; Jones et al. 2010) and could potentially have a similar effect on CYP2C19 activity, although HIV status of the sampled volunteers was unknown . With approximately 10.5% of South Africans estimated to be HIV positive this may be an important confounding factor to consider in future studies (http://www.statssa.gov.za/publications/P0302/P03022010.pdf). Epigenetics may also play a significant role as *CYP2C19* has a confirmed CpG island in intron 1 (Ingelman-Sundberg et al. 2007) as well as the potential for histone modification and microRNAs which may prove to be significant in the future.

A more tangible explanation for the observed discrepancy could be that the PCR-RFLP method only accounts for known alleles, occurring at significant frequencies, and may miss important rare unknown alleles which may be phenotypically relevant. The presence of rare alleles has previously been reported in African individuals for genes with pharmacogenetic relevance (Matimba et al. 2009; Wright et al. 2010) , as *CYP2C19*28* was observed at a frequency of 0.005 in the Xhosa population (Drögemöller et al. 2010b), but absent in this cohort. Re-sequencing of phenotypic outliers and using re-sequencing in the poorly characterised and largely under-represented African populations may assist in identifying important low frequency alleles. With advances in pharmacogenomics, such as a whole genome approaches to identify genetic markers which may explain inter-individual variation, pharmacogenetics may well advance to a genomic approach where drug response (both in terms of ADRs and lack of efficacy) may be linked to a variety of variants. As next generation sequencing technologies improve, the re-sequencing of the complex *CYP* genes at an affordable price, may address the concerns associated with rare

alleles and low LD in African populations and may provide comprehensive genotyping of these individuals for application in pharmacogenomics.

This study highlights the need to use comprehensive and population specific genotyping methods when predicting phenotype. Adjustment of the predictions based on the substrate of interest can be both valuable in developing and in validating comprehensive pharmacogenetic screening assays in the future. Substrate specific modification of phenotypic predictions (i.e. the OME modified AS in this study) could contribute to ñpersonalised medicationò of potentially toxic drugs. Additional comparisons between predicted and measured phenotype in cohorts of individuals receiving different CYP2C19 substrates are therefore required.

5.6 Conclusion

Comprehensive genotyping is essential when predicting phenotype, and re-sequencing may be the best approach to uncover additional *CYP2C19* variations affecting phenotype in understudied population such as this one. Predicted phenotype compared well with observed phenotype once AS was modified. Substrate specific AS prediction models would be valuable in tailoring dosage regimens with accuracy. Alternatively, a phenotyping approach may also prove beneficial when tailoring dosage regimens. These results contribute to the ongoing quest to improve and implement pharmacotherapy in South Africa and provide valuable insight into the complex nature of the poorly characterised African genomes.

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CHAPTER 6. PHARMACOGENETIC COMPARISON OF CYP2D6 PREDICTIVE AND MEASURED PHENOTYPE IN A SOUTH AFRICAN COHORT

A version of this chapter will be published shortly.

6.1 Abstract

The relationship between genetic variation in *CYP2D6* and variable drug response represents a potentially powerful pharmacogenetic tool. However, little is known regarding this relationship in the diverse South African population. **Aims:** To direct future pharmacogenetic screening for *CYP2D6* in this population, the relationship between predicted and measured phenotype was evaluated. **Methods: A** XL-PCR+Sequencing platform was used to determine *CYP2D6* genotype in 100 healthy South African volunteers. Phenotype was predicted using the Activity Score (AS) model. Three hour plasma metabolic ratios of dextrorphan (DX)/dextromethrophan (DM) were measured simultaneously, using semi-automated online solid phase extraction coupled with tandem mass spectrometry. Metabolic ratios served as a surrogate measure of *in vivo* CYP2D6 activity with DM administered as the probe drug. **Results:** An antimode for distinguishing between IM and EM was set at log $MR_{(DX/DM)}$ = -0.271. AS = 0.5 was used to identify IMs instead of AS = 0.5-1.0. No antimode could be set to distinguish PM using this cohort. There was a strong association between genotype and phenotype with a kappa value of 0.792, an Inter-Rater Discrepancy of 0.051 and sensitivity >72.7%, reiterating the dominant role CYP2D6 plays in DM metabolism. **Conclusion:** Comprehensive *CYP2D6* genotyping reliably predicts CYP2D6 activity and can be utilised as a valuable pharmacogenetic tool for screening in South Africa.

6.2 Background

The *CYP2D6* gene is highly polymorphic and harbours numerous sequence variations including SNPs, insertions, deletions (including the entire gene) and hybridisation with the *CYP2D7* pseudogene. To date over 100 distinct alleles and sub-variants have been catalogued by the P450 Nomenclature Committee (http://www.cypalleles.ki.se/cyp2d6.htm). *CYP2D6* allele frequencies have been described to differ considerably among ethnically distinct populations (Jakobsson et al. 2008; Sistonen et al. 2009; Alessandrini et al. 2013).The non-functional *CYP2D6*3*, **4*, **5* and **6* alleles and the reduced function *CYP2D6*9*, **10* and **41* alleles are commonly observed in Caucasian populations (Sistonen et al. 2009). Populations of African origin have relatively high frequencies of the reduced function alleles*CYP2D6*17* and **29*, which are rare or absent in other populations (Wennerholm et al. 2001; Gaedigk et al. 2002; Gaedigk et al. 2008; Sistonen et al. 2009). The reduced function *CYP2D6*10* has been found in many population at frequencies of about 1-5%, but it is the most common allele in East Asian populations,where frequencies in excess of 50% have been described (Johansson et al. 1994; Sistonen et al. 2009). The non-functional *CYP2D6*5* allele, characterised by the deletion of the entire *CYP2D6* gene, is another allele that is globally found at frequencies between 2-5%. Interestingly *CYP2D6*5* was very prominent in South African Coloureds, an admixed South African population,at 17% (Gaedigk & Coetsee 2008). Additional information regarding allele frequencies can be found on the PharmGKB website (Whirl-Carrillo et al. 2012).

Africaôs populations are highly diverse, with sub-Saharan Africans displaying the highest degree of genetic diversity. As shown by Sistonen et al. (2007) and Alessandrini et al. (2013), the *CYP2D6* gene locus is no exception. Genetic diversity in Africa is thought to be a result of greater environmental pressure compared to non- Africans (Tishkoff et al. 2009). Of the African countries, South Africa has one of the most complex population structures. This complexity is evidenced by the many indigenous and ethnically distinct cultural groups residing in the country. Certainly, the complexity of South African populations needs to be taken into account when designing a pharmacogenetic platform for phenotype prediction. The complexity of the *CYP2D6*gene locus combined with inter-ethnic differences clearly poses a challenge to designing

comprehensive and accurate pharmacogenetic screening strategies/platforms (Dodgen et al. 2013).

Few studies have investigated *CYP2D6*genotype in South African populations. Two studies, namely a Coloured (Gaedigk & Coetsee 2008) and a Xhosa cohort (Wright et al. 2010) were comprehensively investigated in sufficiently large cohorts. For phenotype, Sommers et al. (1988; 1989a; 1989b; 1990; 1991) conducted a series of phenotyping studies using several different probe drugs (not dextromethorphan) independent of genotype. These studies were conducted on the genetically homogenous Barakwena Bushmen, San Bushmen and Venda cohorts, respectively. Although important, these observations represent a relatively small proportion of the South African population and would therefore not be an accurate reflection of phenotype in the general population.

Little is known about *CYP2D6*genotype to phenotype relationships in South African populations, as no such data exist to date. This investigation provides the first comprehensive data set consisting of extensive genotype data obtained on two different platforms and *in vivo* phenotype data, in a demographically representative South African population.

6.3 Materials and methods

6.3.1 *Study subjects and sample collection*

The study was carried out under GCP guidelines and was approved by the Student Research Ethics Committee of the Faculty of Health Science at the University of Pretoria (S132/2009). Apparently healthy volunteers were recruited from the Pretoria area. This study cohort represented South African population demographics and included 70 Black, 10 Caucasian (White), 10 Coloured and 10 Indian subjects. The aim of this study was not to compare inter-ethnic variability, but to represent the diverse Southern African population that utilises national and public health care. Participants born in South Africa were included irrespective of gender. Volunteers were Ó18 years of age and were neither pregnant nor breast feeding. A written, informed consent form was completed by each volunteer, which included questions regarding

age, height, weight, waist circumference, medication/food supplements utilised at the time of the study, place of birth, and voluntary disclosure of ethnic grouping (Black African, Caucasian, Coloured or Indian).

6.3.2 *Genomic DNA (gDNA) extraction*

Phenotypic baseline blood samples collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) were used for gDNA isolation. The Genomic DNA Purification Kit (Fermentas Life Science, Lithuania) or the automated Maxwell® 16 system (Promega, Madison, WI, USA) were used according to manufacturerôs instructions to isolate high quality gDNA for genetic analysis.

6.3.3 *CYP2D6 genotyping*

The study cohort was genotyped employing a *CYP2D6* XL-PCR+Sequencing platform that was designed to predict phenotype in the South African population with high accuracy (Dodgen et al. 2013). This approach included a series of long-range PCR (XL-PCR) amplifications to detect the *CYP2D6*5* gene deletion and the presence of duplications or multiplications and to amplify a *CYP2D6* product for sequence analysis of the entire gene. All primers utilised for this approach were manufactured by Inqaba Biotechnical Industries (Pretoria, South Africa) and have been published along with a detailed description of the platform (Dodgen et al. 2013). Briefly, the amplification reactions were performed using the GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA), followed by agarose gel electrophoresis. Sequencing was performed by Inqaba Biotechnological Industries using the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 and 3130XL and 3500XL genetic analyser systems (Applied Biosystems Inc.). The Activity Score (AS) model (Gaedigk et al. 2008) was applied to predict phenotype from genotypes generated by our *CYP2D6* XL-PCR+Sequencing platform.

6.3.4 *CYP2D6 phenotyping with dextromethorphan*

In vivo CYP2D6 enzyme activity was measured using dextromethorphan (DM) as the probe drug and dextrorphan (DX) as the CYP2D6-specific metabolite. Each volunteer received 60-62.5mg DM (Benylin Dry Cough Syrup; Pfizer containing DM) with 250ml of water. Blood samples were collected prior to DM administration and at two, three and four hour intervals post-drug administration. Plasma was prepared from each sample and analysed using automated column-switching online solid phase extraction coupled to tandem mass spectrometry according to a validated method (Dodgen, Cromarty & Pepper 2011). A metabolic ratio of the concentration of DX over DM ($MR_{(DX/DM)}$) was used as ameasure of CYP2D6 enzyme activity. In this manuscript we utilise a DX/DM ratio as opposed to the more traditional DM/DX ratio. By reporting MR in this format, predicted phenotype is proportional to the metabolic ratio (ie $\log_{10}$ (10/1) and not inversely proportional ($\log_{10}(1/10) = -1$).

6.3.5 *Statistical analysis*

The Statistical Package for the Social Sciences version 20.0 (SPSS Inc., Chicago, IL) was used for statistical evaluation. Metabolic ratios per volunteer were individually correlated at each time point using Pearsonôs Correlation Coefficient to establish the best time point for phenotypic measurement. Baseline characteristics (age, BMI and waist circumference) were compared to phenotype using ANOVA while gender was analysed using Chi-square (X^2) analysis. Optimal $MR_{(DX/DM)}$ antimode/cut-off was evaluated to distinguish between IM and EM using receiver-operator characteristic (ROC). Cut-off values were assigned based on obtaining 100% specificity for PMs and by comparing sensitivity and specificity values to find the optimum for both to discriminate between IMs and EMs. The Kappa statistical test in combination with a custom ñdiscrepancy analysisò calculation (using absolute differences to test for conformation) was used to calculate ñInter-Rater Discrepanciesò for each predictor of phenotype from genotype. Since the four categories of metabolism are ordinal (UM>EM>IM>PM), the calculation measured the average deviation from the perfect conformation (predicted vs. measured phenotype). This can also be explained as the closeness of the predicted phenotype to measured phenotype over the entire cohort. Perfect conformation would receive a value of 0.0, while one, two and three deviating categories would receive values of 1.0, 2.0 and 3.0, respectively.The closer the Inter-

Rater Discrepanciesvalue was to 0.0, the closer the conformation (conversely, increasing values represent greater discordance). Sensitivity, specificity, positive predictive value and negative predictive value were calculated as a second measure of predictive accuracy. Statistical significance was considered as *P*-values less than 0.05.

6.4 Results

6.4.1 *Subject demographics*

One hundred healthy volunteers were recruited for CYP2D6 phenotype and genotype analysis. The cohort had an average age of 34.4 ± 9.8 years (range 19-58), BMI of 26.1 ± 5.8, waist circumference of 86.9 ± 13.3 and 55% were female. All 11 official South African home languages were represented.

6.4.2 *CYP2D6 in vivophenotyping with dextromethorphan as probe substrate*

To establish the sample collection time point that best discriminates PM from subjects with IM and/or EM phenotypes, a Pearsonôs Correlation Coefficient was calculated comparing the metabolic ratios for each individual at each time point. Coefficients of correlations were 0.924 (between 2 and 3 hours), 0.899 (between 2 and 4 hours) and 0.954 (between 3 and 4 hours), respectively. Although all values indicated strong correlations, the strongest was between 3 and 4 hours suggesting that either the 3 or 4-hour time points would be most suitable for phenotype assignment.

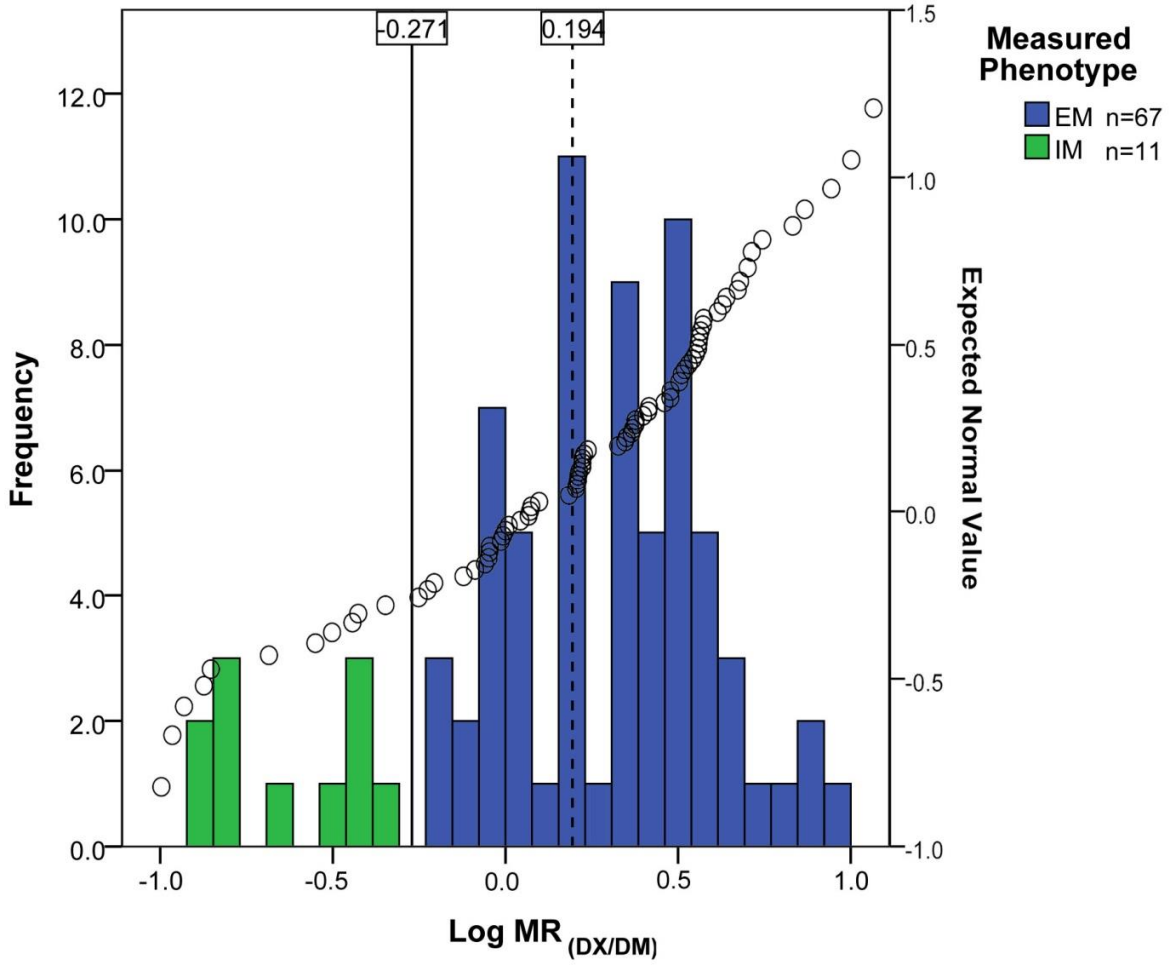

Figure 6-1. Phenotypic distribution of *in vivo* CYP2D6 metabolism in a demographically representative South African cohort.

Phenotype was measured as the metabolic ratio of the concentration of dextrorphan (DM, metabolite) over dextromethrophan (DX, probe drug) in a plasma sample taken 3 hours after probe drug administration. The graph has been stratified into intermediate (IM) and extensive metabolism (EM). The solid X-axis division line represents the proposed antimode to discriminate IM from EM individuals using AS = 0.5. The superimposed QQ-plot agrees with this antimode. The broken line represents an antimode using AS = 0.5-1.0. The only PM subject was excluded because this individual had a metabolic ratio of 0 which cannot be $\log_{10}$ converted.

MRs were available for 79 individuals at the 3-hour time interval as several individuals either did not take enough of the probe drug or did not participate at the 3-hour sampling time point. Figure 6-1 demonstrates the distribution of log $MR_{(DX/DM)}$ for the sampled cohort. Using ROC analysis the cohort was divided into a bimodal distribution with the assistance of XL-PCR+Sequencing predicted phenotype. An antimode was set at $logMR_{(DX/DM)}$ = -0.271 (sensitivity = 88.9%, specificity = 95.7%), a $MR_{(DX/DM)}$ of 0.54 separating IM and EM. Based on this cut-off, 11 subjects were classified as IM and 67 as EM. The broken line in Figure 6-1 represents the cut-off if AS = 0.5 and 1.0 were combined to define IM status. The superimposed QQ-plot over the histogram in Figure 6-1 is more in agreement with using an AS = 0.5 for IM prediction rather than AS = 0.5-1.0. We observed a single PM individual with a *CYP2D6*4/*5* genotype who had no measurable DM metabolites after three hours. The slight plateau coinciding with the antimode is evidence of agreement. This data point could not be plotted as log_{10} (0/6.55) could not be calculated. No subjects with UM genotypes were observed.

6.4.3 *Comparison between predicted and measured phenotype for CYP2D6*

Figure 6-2 is a graphic representation of the comparison between genotype-predicted phenotype (categorised by AS) and measured phenotype. Notably, a wide range of metabolic ratios were observed among the subjects within each AS class. There was also overlap between the AS classes, but AS = 0.5 (IM) appeared notably different to the other AS classes (t-test *P*-value = <0.01). The increase in metabolism as AS increases demonstrated the strong influence *CYP2D6* variation has on metabolism. The XL-PCR+Sequencing genotype approach was a reliable predictor of phenotype. Discordant individuals were found among each of the race groups (Supplementary Table S7).

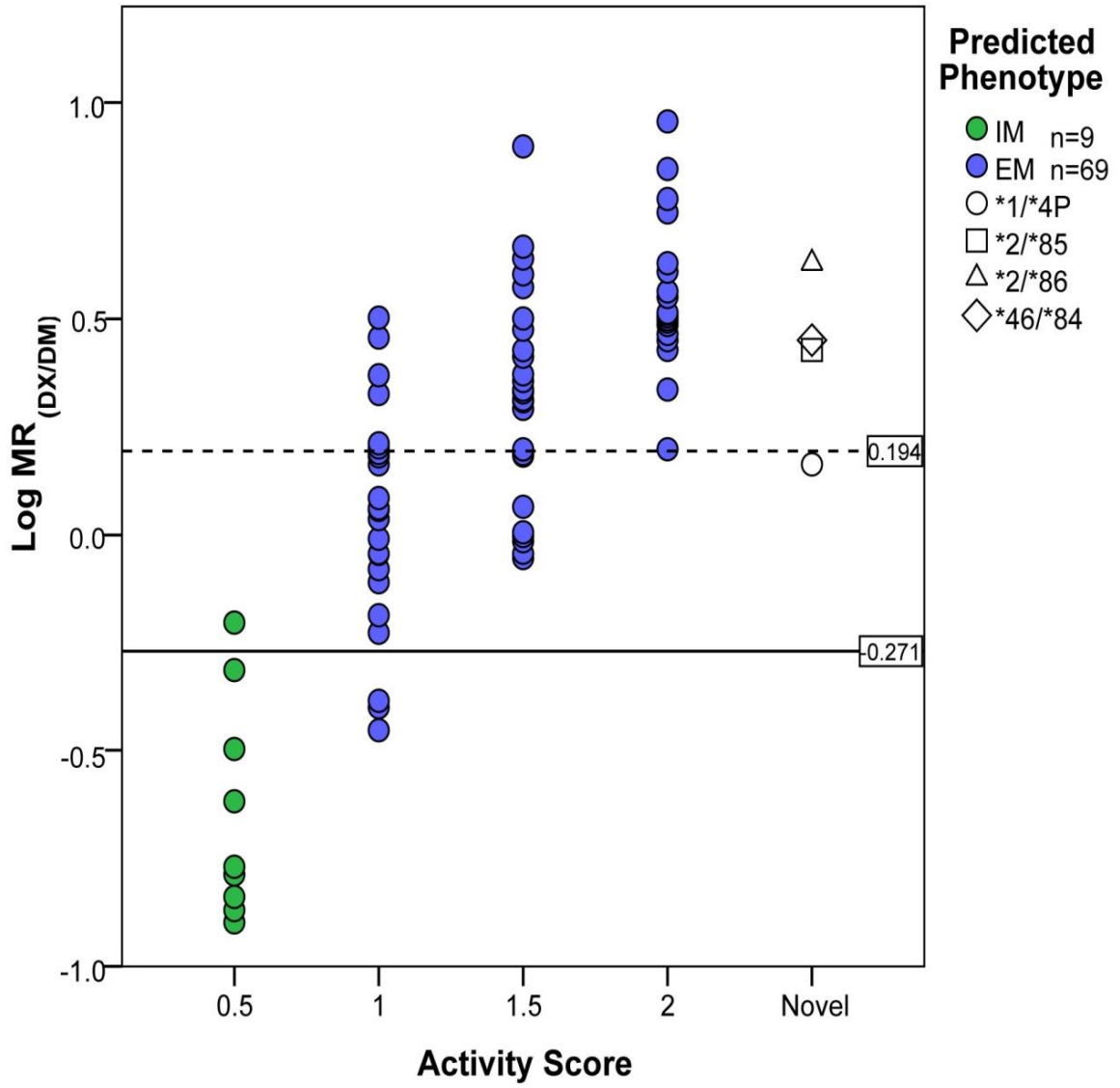

Figure 6-2. Comparison between predicted and measured phenotype in a demographically representative South African cohort.

Metabolic classes of poor (PM), intermediate (IM) and extensive metabolisers (EM) were grouped for comparison. Genotypes containing novel SNPs have been represented separately for preliminary phenotypic evaluation. The dashed line represents an IM-EM antimode established using ROC analysis and conventional AS. The solid line represents a more appropriate antimode, using AS = 0.5 to divide IM and EM. This figure was created by Tyren Mark Dodgen.

A)

Predicted Phenotype IM=0.5-1.0	Measured Phenotype: PM	IM	EM	n	Sensitivity	Specificity	Pos. Pred. Value	Neg. Pred. Value
PM	1	0	0	1	100.0% (1/1)	100.0% (78/78)	100.0% (1/1)	100.0% (78/78)
IM	0	26	6	32	74.3% (26/35)	86.4% (38/44)	81.3% (26/32)	80.9% (38/47)
EM	0	9	37	46	86.0% (37/43)	75.0% (27/36)	80.4% (37/46)	81.8% (27/33)
n	1	35	43	79				

Kappa: 0.623 Inter-Rater Discrepancy: 0.190

B)

Predicted Phenotype IM=0.5	Measured Phenotype: PM	IM	EM	n	Sensitivity	Specificity	Pos. Pred. Value	Neg. Pred. Value
PM	1	0	0	1	100.0% (1/1)	100.0% (78/78)	100.0% (1/1)	100.0% (78/78)
IM	0	8	1	9	72.7% (8/11)	98.5% (67/68)	88.9% (8/9)	95.7% (67/70)
EM	0	3	66	69	98.5% (66/67)	75.0% (9/12)	95.7% (66/69)	90.0% (9/10)
n	1	11	67	79				

Kappa: 0.792 Inter-Rater Discrepancy: 0.051

Figure 6-3. Evaluation ofCYP2D6 predicted phenotype ability to predict measured phenotype. Phenotype was predicted using (A) ASmodel A (Gaedigk et al. 2008) and (B) AS = 0.5 represents intermediate metaboliser (IM). Poor (PM), IM and extensive metabolism (EM) represents distinct and ordinal phenotypic groupings. The grey blocks represent perfect conformation. Sensitivity, specificity, positive predictive (pos. pred.) value and negative predictive (neg. pred.) value were calculated to measure the assayôs ability to predict phenotype. The Kappa and Inter-Rater Discrepancy statistical tests were calculated to measure statistical phenotypic agreement. This figure was created by Tyren Mark Dodgen.

Ability to predict phenotype was further scrutinised by comparing predicted phenotype to measured phenotype by cross-correlation of the ordinal phenotypic groups (Figure 6-3). Concordance between predicted and phenotype are represented by grey blocks and discordant individuals, i.e. those with genotypes that did not match with their phenotype, are shown as white blocks. XL-PCR+Sequencing predicted 16 discordant individuals out of 79 when IM was defined as AS = 0.5-1.0. In contrast, when IMs were identified as AS = 0.5 there were only 4 discordant individuals. The *CYP2D6* XL-PCR+Sequencing platform achieved a low Inter-Rater Discrepancy score and acceptable Kappa score. Sensitivity, specificity, positive predictive values

and negative predictive values were ≥ 72% throughout. The lowest value was observed for sensitivity of IM status (72.5%). The identification of IMs using AS = 0.5 improved Kappa scores from 0.623 to 0.792 and Inter-Rater Discrepancy value from 0.190 to 0.051.

using ROC analysis and conventional AS. The solid line represents a more appropriate antimode, using AS = 0.5 to divide IM and EM.

6.4.4 *Metabolic activity of novel alleles or sub-alleles*

Three of the volunteers carried novel alleles (Dodgen et al. 2013) and presented with DX/DM ratios indicative of EM (n = 3) phenotypes (Figure 6-2). This was expected as each individual had a functional allele (i.e. *CYP2D6*1, *2* and **45B*) in combination with the novel allele. The volunteer with a *CYP2D6*1/*4P* (**4P* being a sub-allele) genotype exhibited a log $MR_{(DX/DM)}$ = 0.163 which agreed with an AS = 1.0 classification and measured as EM, which was expected.

6.4.5 *Potential confounding factors influencing CYP2D6 metabolism*

The influence of age, BMI and waist circumference on plasma $MR_{(DX/DM)}$was calculated using One-way ANOVA. None of these factors were found to influence plasma $MR_{(DX/DM)}$ with *P*-values of 0.434, 0.816 and 0.371 respectively. Similarly, gender did not influence plasma $MR_{(DX/DM)}$ as a *P*-value of 0.350 was calculated using a Pearsonôs Chi-square analysis.

6.5 Discussion

This is the first assessment of the relationship between genotype and phenotype for CYP2D6 in the South African population and contributes to fill the knowledge gap for this understudied population. Since South Africa is an ethnically diverse population it is important to evaluate a variety of pharmacogenetic assays to identify a plausible strategy for future pharmacogenetic and genomic personalised medicine in the country. We compared the *CYP2D6* XL-PCR+Sequencing platform with observed *in vivo* activity using a $MR_{(DX/DM)}$ plasma ratio in samples collected 3 hours after the probe substrate was administered.

6.5.1 *Novel CYP2D6 allelic variants*

Three novel alleles and one sub-allele were identified in subjects belonging to three different ethnic/racial groups represented in the study cohort (Dodgen et al. 2013). This highlights the need to investigate previously under-investigated populations using platforms which allowing for identification of new sequence variations. The individuals carrying novel alleles presented as EM (*CYP2D6*1/*4P, *46/*84*, **2/*85* and **2/*86*) with $MR_{(DX/DM)}$ ratios being comparable to those seen for subjects with AS ranging from 1.0-2.0 (Figure 6-2). This suggests that the novel alleles encode functional proteins that do not cause appreciable reduction in the $MR_{(DX/DM)}$ ratio. The observed reduced activity of the subject with the *CYP2D6*4P* was concordant with an AS = 1.0 classification as this allele posses the 1846G>A SNP that causes aberrant splicing and obliterates enzyme function. Further *in vitro* studies are warranted to further characterise the pharmacokinetic profiles of the novel alleles to determine their metabolic potential towards DM metabolism. Additional insights could also be obtained from subjects carrying these alleles in combination with a non-functional variant, but because these are rare, a large number of individuals may have to be screened to identify subjects with informative genotypes. The presence of the novel alleles described here and the potential for the existence of additional variants that have not been yet discovered, and may prove to be important in explaining variation in drug response in South Africa (Matimba et al. 2009).

6.5.2 *Phenotyping using dextromethorphan*

The utilisation of a plasma sample taken 3 hours after DM administration may be more convenient for both the investigator/clinician and the volunteer/patient in order to determine the phenotype compared to4 or 8 hour urine collection protocols. Potential Phase II metabolism complications such as varying glucuronation, preferential urinary accumulation of the metabolite due to compromised renal function (Dodgen, Cromarty & Pepper 2011) and variability due to urine pH are also avoided by sampling plasma. Serum $MR_{(DM/DX)}$ have been shown to be comparable to urine $MR_{(DM/DX)}$ (Jurica et al. 2012). Although plasma and serum are likely to be comparable as a measure of phenotype (rate of metabolism), protein binding could interfere with

the accurate determination of $MR_{(DM/DX)}$ when measuring DM and DX in serum. With serum, the bound fraction (likely to be the majority of the molecules in the blood) could be removed when preparing serum (Dodgen, Cromarty & Pepper 2011). Therefore, plasma may be a more sensitive measure of metabolism than serum.

Similar to previous observations, the 3-hour sampling time point was determined to be optimal for the determination of CYP2D6 activity with DM (Tamminga et al. 2001; Jurica et al. 2012). Previously, Abdul Manap et al. (1999) estimated T_{max} for DM in EMs to be about 2 hours (ranging from 0 to 8 hours), compared to 3 hours for PMs (Desmeules et al. 1999). Based on these studies and observations during the present study, the DX/DM ratio in plasma samples collected 3 hours after probe drug administration was used as a surrogate measure for CYP2D6 activity and phenotype assignment.

The phenotypic antimode used to differentiate IM from EM, log $MR_{(DX/DM)}$ = -0.271 ($MR_{(DM/DX)}$ = 0.536) was lower compared to the cut-off value of 1.678 ($MR_{(DM/DX)}$ = 0.021) used by Jurica et al. (2012). A possible explanation for this could be that there is a difference between plasma and serum/urine. Only one PM was observed in our cohort and because no DX could be measured, a metabolic ratio could not be calculated. A tentative cut-off value of -1.000 could be used to separate IM from PM, but this would have to be investigated in the future.

The incidence of 1.2 % PMs in this cohort is lower than previous estimations of 5-10% in Caucasians and 7.1% in African Americans (Gaedigk et al. 2002; Gaedigk & Coetsee 2008). It was also lower than the 4.1-7.4% observed in South African populations investigated previously using a variety of probe drugs (Sommers, Moncrieff & Avenant 1989a; Sommers, Moncrieff & Avenant 1989b). A high frequency of 19.8% PMs was observed in South African San Bushmen (n = 96) when phenotyped with debrisoquine (Sommers, Moncrieff & Avenant 1988), but it is unlikely that PM frequencies will be this high in a cross-section of the South African population. This suggests that this cohort may need to be expanded to further evaluate the relationship between predicted and measured phenotype in PM South Africans.

6.5.3 *CYP2D6 comparison between predicted and observed phenotype*

The numeric based AS system reduces complication of phenotype prediction while stratifying the population into additional groups which may offer insight into quartiles of a cohort as well as minimizing overlaps with in a group (Gaedigk et al. 2008). By stratifying phenotype prediction using the numeric AS, various additional phenotypic patterns may become apparent. Caution should be taken when implementing AS phenotype prediction as ethnicity, disease state (including liver and kidney function) and concomitant medication may contribute to an unpredictable phenotype (Gaedigk et al. 2008). Another concern when applying the AS for the prediction of CYP2D6 drug metabolism activity is that some alleles may have varying activity for a given drug substrates (Whirl-Carrillo et al. 2012). Although not conclusive with DM metabolism, *CYP2D6*17* has been found to metabolise risperidone at a level comparable to that observed for the fully functional *CYP2D6*1*-encoded protein (de Leon et al. 2009). Furthermore, *CYP2D6*10* and **17* have been described to have varying affinity and clearance rates for a variety of substrates compared to *CYP2D6*1* (Wennerholm et al. 2002; Shen et al. 2007). Investigation into the ability of different *CYP2D6* allelesto metabolise relevant substrates may prove valuable when customising the AS for more accurate phenotype prediction.

The correlation between predicted and measured phenotype for CYP2D6 in this cohort appears to be strong, but not absolute. The observed discrepanciesare illustrated by the wide range of metabolic ratios within each of the predicted metabolic groups as well as the overlap between the groups (Figure 6-2). AS = 0.5 however, has a narrower ranged between log $MR_{(DX/DM)}$ -0.898 and -0.205. This AS group, only marginally overlapped with the AS = 1.0 group which had a log $MR_{(DX/DM)}$ range of -0.454 to 0.503. AS = 1.0 and 1.5 overlapped substantially, with AS = 1.5 having a log $MR_{(DX/DM)}$ range of -0.056 to 0.899. The same was seen for AS = 1.5 and 2.0 with log $MR_{(DX/DM)}$ ranging between 0.198 and 0.957. Therefore AS = 0.5 should be used to distinguish IM from EM. Variation within the promoter region, which could potentially contribute to the observed discordance, was not evaluated in this study.

Interestingly, both the predicted IMs who measured IM, had *CYP2D6*10B* as the reduced function allele combined with **4* or **4Xn* (refer to Supplementary S7). This suggests that **10B* may have slightly higher activity than other reduced function alleles. The promoter region should be investigated in future studies. CYP3A4 also metabolises DM and genetic variation as well as extensive induction in this gene may influence or compensate for CYP2D6-mediated metabolism or lack thereof, respectively. In this way they may not be independent from one another, and thus should be measured simultaneously (Funck-Brentano et al. 2005). Various non-genetic factors could also influence phenotype, contributing to the variability of the data and observed discordances (Poolsup, Li Wan Po & Knight 2000). However, factors such as age (Tamminga et al. 2001), BMI, gender and waist circumference showed no significant correlation towards the phenotype measure (DX/DM). Although Tamming et al. (2001) also observed that age does not affect CYP2D6 metabolism, they observed gender (under 40 years of age) to play a role in metabolism. Frank et al. (2007) however concur with the lack of gender influence on CYP2D6 activity. This appears to be the first time that waist circumference was compared to CYP2D6-mediated DM metabolism. It would be important to re-evaluate these potential confounding factors in a cohort in which PM individualsare also represented.

Several potential confounding factors were not investigated in this study. Epigenetic factors such as regulatory methylation, microRNAs and CpG islands may have a significant influence on CYP activity (Gomez & Ingelman-Sundberg 2009). A putative CpG island has been found between the 5ô-flanking region and intron 4(GenBank reference sequence ï NC_000022.9) which was identified using Methyl Primer Express (version 1.0, Applied Biosystems) (Ingelman-Sundberg et al. 2007). Pharmaco-epigenetics may indeed become an integral component of pharmacogeneticscreening for CYP2D6 activity in the future. Concomitant herbal remedies have also been demonstrated to affect CYP2D6 activity (Hellum & Nilsen 2007). This might be significant as approximately 72% of Black South Africans consume such remedies, amounting to approximately 20,000 tons of mostly indigenous plant material per annum (Mander et al. 2007). This factor is not exclusive to South Africa as the WHO estimates that approximately 80% of the African and Asian populations rely on traditional medicine as therapy (http://www.who.int/mediacentre/factsheets/fs134/en/). An estimated 10.5% (5.24 million

people) of the South African population is HIV positive (http://www.statssa.gov.za/publications/P0302/P03022010.pdf), which could be another important influence of non-genetic variation. HIV has been found to reduce baseline CYP2D6 activity causing a risk of phenotype switching. The phenotype switch between IM to PM has for example been described in a cohort of HIV-positive Caucasian patients. Neither the disease itself, nor its interaction with the drug could explain this observation (O'Neil et al. 2000; Jones et al. 2010). This variable was not assessed in this cohort as it was not within the scope of this project and disclosure of HIV status in South Africa is strictly voluntary. Due to a fear of discrimination it is unlikely that South Africans will disclose their HIV status.

6.6 Conclusion

The combination of duplex XL-PCR with gene sequencing and AS-based phenotype prediction proved to be a comprehensive and reliable strategy for *CYP2D6* genotyping in the South African population. This strategy can thus be incorporated as is into future pharmacogenetic studies or personalised medication concerning CYP2D6. The phenotyping strategy used may also be beneficial as the one-time phenotype measure in a 3-hour plasma sample was found to be optimal and convenient. This study provides the next step incomplex *CYP2D6* pharmacogenetics in the South African population.

6.7 References

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CHAPTER 7. RELATIONSHIP BETWEEN RISPERIDONE-RELATED ADVERSE DRUG REACTIONSAND*CYP2D6* POLYMORPHISMS IN THE SOUTH AFRICAN POPULATION: A PILOT STUDY

A version of this chapter will be published shortly.

7.1 Abstract

Contradictory information regarding the influence of *CYP2D6* polymorphisms on adverse drug reactions (ADRs), extrapyramidal symptoms (EPS) and weight gain related to risperidone (RSP) treatment,prompted an investigation into the potential use of pharmacogenetics for personalising RSP treatment in South Africa. **Aim:** To evaluate the influence of *CYP2D6* genetic variation on RSP-related ADRs in a South African cohort. **Methods:** EPS was evaluated using the Abnormal Involuntary Movement Scale (AIMS), the Barnes Akathisia Scale (BAS) and the Simpson-Angus Scale (SAS). Comprehensive *CYP2D6*sequence variations were evaluated using XL-PCR+Sequencing. **Results:** No statistically significant association was found between *CYP2D6* variation and RSP-related ADRs. Confounding variables and concomitant medication did notappear to be associated with RSP ADRs either. RSP-related EPS and weight gain do notappear to occur simultaneously. A novel *CYP2D6* allele was identified which is unlikely to affect metabolism based on *in silico* evaluation. **Conclusion:** *CYP2D6* variation appeared not a good pharmacogenetic marker for predicting risperidone-related ADRs in this South African cohort. Evaluation of a larger cohort would be useful to confirm these observations. Additional clinically relevant *CYP2D6* alleles may be present in the South African population.

7.2 Background

Atypical antipsychotic medication has largelysuperseded typical or classical antipsychotics for the treatment of schizophrenia in the last two decades. This is largely due to improved efficacy in treating negative symptoms of schizophrenia as well as a more favourable side effect profile (less extrapyramidal symptoms and tardive dyskinesia). Demonstrating antagonism at 5-hydroxytryptamine (5-HT_2) and dopamine (D_2) receptors (Janssen et al. 1988; Leysen et al. 1988), risperidone is an atypical antipsychotic which improves both positive and negative symptoms of schizophrenia (Singam, Mamarde & Behere 2011). RSP is also registered for the treatment of manic episodes of bipolar disorder and irritability associated with autistic disorder. Off-label uses include behavioural problems in dementia, refractory or psychotic major depressive disorder, refractory obsessive-compulsive disorderand Tourette's disorder, among others (Shekelle et al. 2007). The range of patients receiving atypical antipsychotictreatment (including RSP) is vast and includes children (Malone & Waheed 2009; Vitiello et al. 2009; Lazzeretti et al. 2011) as well as the elderly (Jeste et al. 2008; Burke & Tariot 2009; Trifiro, Spina & Gambassi 2009) for a variety of different indications.

Although the atypical antipsychotics have an improved side effect profile, adverse drug reactions (ADRs) still pose a challenge. Genetic polymorphisms and environmental influences are typically implicated in ADRs. A study on 500 French patients taking RSP revealed that dosage adjustments were needed in 61% of patients due to the effects of co-medication and 10% as a result of genetic factors (Martin et al. 2004). Understanding the contribution of environmental factors, particularly drug-drug interactions resulting from polypharmacy (common in psychiatry treatment), and also of genetic influences, may assist in reducingADRs. Environmental factors will persist so long as the factor is present and can often be modified. However, genetic influence is permanent and would need be accounted for (de Leon, Sandson & Cozza 2008a).

One of the most important genetic factors influencing RSP pharmacokinetics is Phase I metabolism mediated predominantly by *CYP2D6* (Fleeman et al. 2011). *CYP2D6* is a highly polymorphic gene with over 100 alleles identified to date (http://www.cypalleles.ki.se/cyp2d6.htm).

RSP is metabolised (but not exclusively) by CYP2D6 into 9-hydroxyrisperidone (a metabolite recently marketed as paliperidone) which is equipotent in terms of receptor-binding activity (Schotte et al. 1995). Although the plasma concentration of RSP has been found to be higher in PMs and 9-hydroxyrisperidone is higher in EMs, the total active moiety (the summation of both) appears not to varymuch between the metaboliser classes (Riedel et al. 2005; Vermeulen, Piotrovsky & Ludwig 2007; Wang et al. 2007; Kang et al. 2009; Novalbos et al. 2010). Therefore it has been proposed that CYP2D6 metabolism is unlikely to be of clinical relevance in terms of efficacy. In support of this, Kang et al. (2009)demonstrated that *CYP3A5* polymorphisms are more likely to influence the RSP active moiety. Conflicting results have also been reported, where an increased RSP active moiety was found in *CYP2D6* predicted PMs compared to EMs (Hendset et al. 2009; Jovanovic et al. 2010; Locatelli et al. 2010). This, combined with the suggestion by de Leon et al. (2008a; 2008b) that RSP and 9-hydroxyrisperidone are not equipotent (risperidone having higher activity), may in part explain the ADRs experienced by PMs versus EMs. In this case ADRs may be due to reduced rate of elimination of the active moiety in PMs resulting accumulation of plasma levels with repeated dosing. The extended plasma half-life for RSP in *CYP2D6* PMs from 2.9 hours in EM to 15.1 hours in PMs (Novalbos et al. 2010) combined with a typical dosage of 3.0 to 8.0 mg taken at two time intervals daily, could resultin increasing plasma levels towards toxicity. In addition, both higher plasma concentrations of the active moiety (Yoshimura, Ueda & Nakamura 2001; Locatelli et al. 2010; Yasui-Furukori et al. 2010) and the *CYP2D6* PM genotype (de Leon et al. 2005a) have been associated with an increased incidence of extrapyramidal symptom (EPS) ADRs. Weight gain is another important ADR of RSP (Nasrallah 2008).Weight gain manifests in reduced patient compliance irrespective of symptomatic improvement (Weiden, Mackell & McDonnell 2004; Nasrallah 2006). Although there is very little known about the association between *CYP2D6* polymorphisms and weight gain, Lane et al. (2006) observed a significant correlation between the **10* allele and weight gainin RSP-treated patients.

In light of contradicting data on*CYP2D6* pharmacogenetics and RSP associated ADRs (Fleeman et al. 2011), this pilot study is aimed to addressshed light on this relationship in a small cohort of

South African patients. As very little information exists on *CYP2D6* polymorphisms in the South African population, a fully comprehensive XL-PCR+Sequencing approach described by Dodgen et al. (2013) was adopted to obtain genotype and predict phenotype. *CYP2D6* genotype was analysed in a RSP treated cohort selected on the basis of RSP ADRs including movement disorders (EPS) and weight gain. A predicted *CYP2D6* PM phenotype was expected as patients were selected for ADRs.

7.3 Materials and methods

7.3.1 *Patient cohort*

Ethics approval was obtained from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (approval number: 24/2007) and the study was carried out under GCP guidelines. Inpatients or outpatients at Weskoppies public hospital (Pretoria, South Africa) receiving RSP treatment were recruited if they experienced RSP-related ADRs. ADRs included weight gain and/or movement disorders. This pilot cohort included 24 patients older than 18 years of age, who gave informed consent, of any raceand either gender irrespective of their psychiatric diagnosis. Patients suffering from neurological disorders which may have been mistaken for RSP-related ADRs were excluded from the sampled cohort. On the day of recruitment, one of the collaborating psychiatrists (MD level) documented clinical signs (ADRs) and drew two venous blood samples in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) for *CYP2D6* genotypic evaluation.

7.3.2 *Phenotypic evaluation*

ADRs resulting from RSP treatment were measured as clinical phenotype in this study. Two distinct and prominent ADRs were measured: weight gain and movement disorders. The former was simply the weight gained from the onset of RSP treatment until the time of recruitment. Dyskinesiaand dystonia were measured using the Abnormal Involuntary Movement Scale (AIMS, Munetz & Benjamin 1988), akathisia using the Barnes Akathisia Scale (BAS, Barnes 1989) and Parkinsonism using the Simpson-Angus Scale (SAS, Simpson & Angus 1970).

7.3.3 *CYP2D6genotyping*

Genomic DNA (gDNA) was extracted from whole blood in each EDTA vacutainer, using theautomated Maxwell® 16 system (Promega, Madison, WI, USA) according to instructions. Genotyping was performed using a *CYP2D6*XL-PCR+Sequencing strategy described previously(Dodgen et al. 2013). Briefly, the strategy makes use of two duplex long-range PCR (polymerase chain reaction) assays, onefor *CYP2D6*5* (complete gene deletion)and the other for duplication detectionfollowed by *CYP2D6* gene sequencing for allele determination. *CYP2D6* gene sequencing was performed by Inqaba Biotechnological Industries (Pretoria, South Africa) using 3130XL and 3500XL (Applied Biosystems). PCR products were purified using FastAPThermosensitive Alkaline Phosphatase (Fermentas Life Science) according to manufacturerôs instructions. ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems) and appropriate *CYP2D6* sequencing primers were used for sequencing reactions.

Resulting electropherograms were edited using FinchTV version 1.4.0 (Copyright © 2004-2006, Geospiza Inc.) and compared to the AY545216 (GenBank) in CLC DNA Workbench version 5.5 (CLC bio, Aarhus, Denmark) software for polymorphism identification. Single nucleotide polymorphisms (SNPs) were numbered according to M33388 (GenBank) and alleles identified according to the Human CYP Nomenclature website for *CYP2D6* (http://www.cypalleles.ki.se/index.htm).

The novel polymorphism was cloned using the CloneJET^{TM}PCR Cloning Kit (Fermentas Life Science) according to manufacturerôs instructions and transformed into DH5Ŭ cells (Zymo Research, Orange, CA, USA). Colonies were screened by amplifying the region of interest (where the novel SNP was located). Once the novel SNP was found the clone was amplified and re-sequenced. The novel SNP was evaluated using *in silico* software, Sorting Intolerant from Tolerant (SIFT) and PolyPhen prediction software to estimate the potential effect on CYP2D6 activity (Ramensky, Bork & Sunyaev 2002; Ng & Henikoff 2003). The novel allele was submitted to the Human CYP Nomenclature committee for *CYP2D6* allele designation.

7.3.4 *Phenotype prediction*

An adjusted version of the Activity Score (AS) system (Gaedigk et al. 2008) was used to predictCYP2D6 phenotype. The adjusted phenotype prediction system was adopted from genotype-phenotype comparisons described by Dodgenet al. (Dodgen et al. 2013), where activity is Increased = 2.0, Normal = 1.0, Decreased = 0.5 and None = 0.0 using information from the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committeeôs online database for *CYP2D6*. *CYP2D6*17*, traditionally assigned an AS of 0.5, was given a score of 1.0, as this allele has been found to metabolise RSP with full function (de Leon et al. 2009). If no information on phenotypic activity was available the allele was assigned a score of 1.0. Summation of genotypic scores (e.g.**2/*41*= 1.0+0.5 = 1.5) predicted phenotype as PM = 0.5, IM = 0.5-1.0, EM = 1.5-2.0 and UM >2.0.

7.3.5 *Statistical analyses*

Tools for Population Genetic Analysis (TFPGA) software v1.3 was used to test allele deviation from Hardy-Weinberg equilibrium using Fisherôs exact test(Miller 1997). Additional statistical evaluations were calculated using SPSS version 20.0 (SPSS Inc., Chicargo, Ill). The Mann-Whitney test was used following cross tabulation to compare sex and race (Black African or White Caucasian) to ADRs. As the distribution of data in all continuous data sets was not normal, nonparametric tests were used for comparison. The Kruskal-Wallis test, using Chi-square, compare predicted phenotype with each risperidone EPS experienced as well as weight gained (ADRs). Kendallôs tau-b was used to compare age, number of cigarettes smoked, RSP dosage, and each ADR to the other. Concomitant medication, anticholinergic medication, sex and race were evaluated as confounding influences of RSP-related ADRs using the Mann-Whitney Test. *P* values <0.05 were considered significant.

7.4 Results

7.4.1 *Patient characteristics*

Anticholinergic medication prescribed concomitantly included orphenadrine (n=5) and biperidine (n=3). Additional concomitant medication at the time of sampling included sodium valproate (n=8), lithium (n=4), oxazepam (n=4), clonazepam (n=3), cyproteroneacetate(n=2), fluoxetine (n=2), propranolol (n=2), thyroxin (n=2), venlafaxine hydrochloride (n=2), carbamazepine (n=1), citalopram (n=1), hydroxyzine (n=1), imipramine (n=1), metformin (n=1), omeprazole (n=1), paroxetine (n=1) and perindopril (n=1). Eight patients were prescribed RSP monotherapy. The observed characteristics for the cohort of 24 RSP-treated patients experiencing movement disorders and/or weight gain are presented in Table 7-1. Parkinsonism (SAS, n=18) appears to be the most commonly experienced ADR followed closely by dyskinesiaand dystonia (AIMS, n=17). Weight gain was experienced in eight of the RSP-treated patients, of which only two experienced EPS and weight gain simultaneously, but in both cases the weight gain was less than 5 kg. None of the patients admitted to the use of cannabis at the time of sampling.

7.4.2 *CYP2D6 genotype and predicted phenotype*

All *CYP2D6* alleles identified were in Hardy-Weinberg equilibrium. Of the 12 different *CYP2D6* alleles identified (Table 7-2), three were responsible for absent enzyme function (**4,*5* and **6B*), four were responsible for reduced enzyme function (**10B*, **17*, **29* and **41*) and one novel allele **10B* was identified. No duplications (functional or non-functional) were identified. Table 7-2 also showsthe genotypes observed, the AS scores and predicted phenotype. PM was predicted in one patient (4.2%), 12 were IM (50.0%) and 11 were EM (45.8%). There were no UMôs predicted in this cohort.

Table 7-1. Descriptive statistical data for a South African risperidone-treated cohort (n=24) experiencing ADRs and evaluation of related and confounding factors.

	Descriptive Statistics				Comparative Statistics *P* value (Correlation Coefficient if applicable)			
	Number	Mean	SD	Range	AIMS	BAS	SAS	WG
Female/Male	8/16				0.637	0.182	0.038	0.928
Black/White	9/15				0.086	0.656	0.694	0.227
Age		32.9	12.4	18-61	0.331 (0.150)	0.709 (-0.062)	0.289 (0.161)	0.699 (0.063)
Cigarettes (per day)	11[a]	9.0	9.1	0-20	0.124 (-0.257)	0.670 (0.077)	0.175 (-0.224)	0.259 (0.200)
Dosage (mg/day)		3.9	1.8	1-7	0.714 (-0.060)	0.703 (-0.067)	0.642 (0.074)	0.903 (0.021)
Adverse drug reactions (ADRs)								
AIMS (Max = 40)		8.0	7.6	0-22	ð	0.291 (0.182)	0.233 (0.189)	0.004 (-0.484)
BAS (Max = 14)		2.1	3.6	0-11		ð	0.752 (0.054)	0.458 (-0.136)
SAS (Max = 40)		7.2	6.7	0-20			ð	0.010 (-0.435)
Weight gained (WG)		6.9	13.3	0-45				ð
CYP2D6 predicted phenotype								
PM	4							
IM	9				0.841	0.797	0.335	0.855
EM	11							

[a]More than 10 cigarettes per day. ADRs were compared to sex and race using the Mann-Whitney test. ADR occurrences were evaluated for correlation with other ADRs, age, cigarettes smoked and dosage using Kendall's tau-b. The Kruskal-Wallis test was used to evaluate whether predicted phenotypes significantly influence each ADR. This test does not identify where the difference is, but whether or not there is a statistical difference at all. In this case a *P*-value is generated per ADR and not per predicted phenotype.

7.4.3 *Novel allele*

Figure 7-1 illustrates the novel allele identified in this cohort. The non-synonymous 3877G>A SNP was identified in exon 8 with a**1* backbone. This SNP resulted in amino acid change E418K. No functional difference was found by *in silico* PolyPhen software, predicting the amino acid change to be benign with a PSIC score of 1.178. A second *in silico* prediction by SIFT software agreed with PolyPhen predicting that this mutation would be tolerated with a SIFT score of 0.13. An electropherogram for this SNP can be viewed in Supplementary Figure S2.

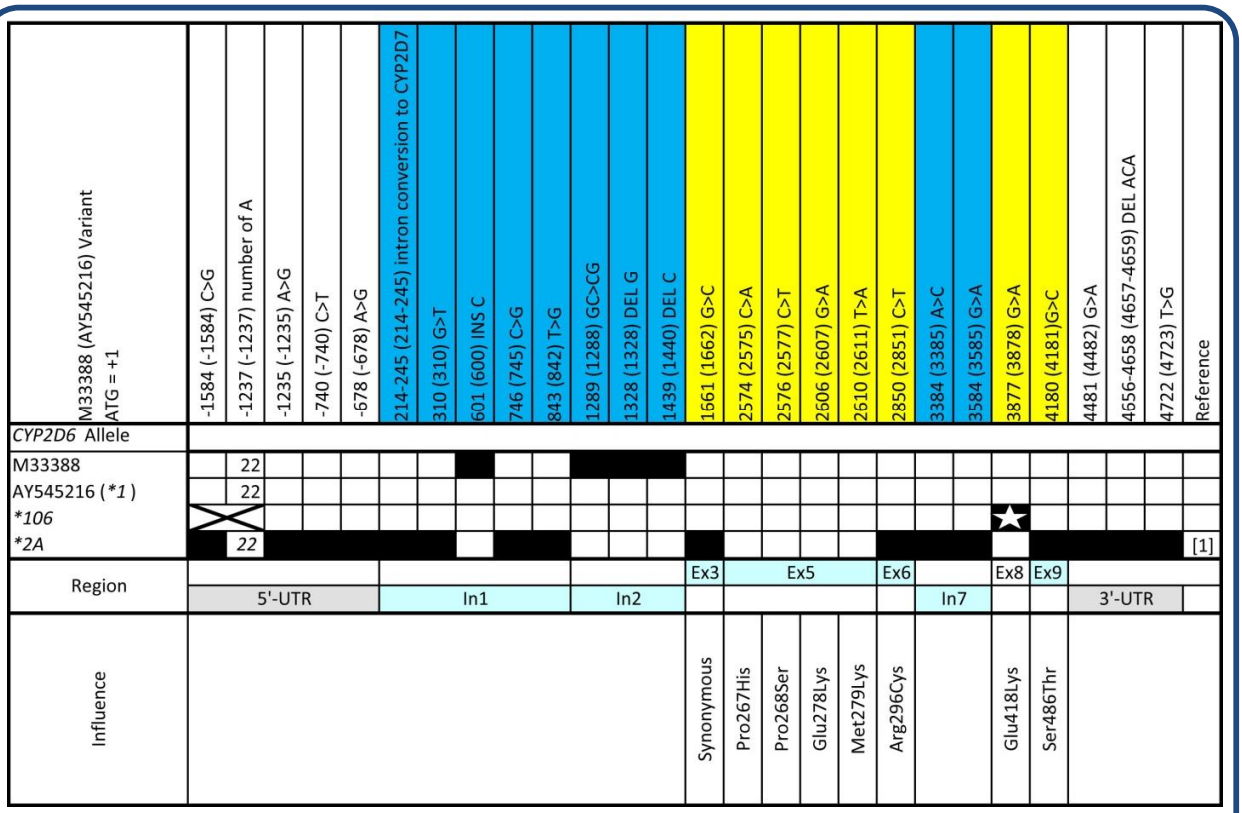

Figure 7-1. Comparison of the novel CYP2D6 alleles identified in this cohort to similar alleles. M33388 and AY545216 reference sequences were used for numbering and as *CYP2D6*1* respectively.

Black boxes represent sequence variation at a specific locus. A star in a black box represents novel snp resulting in non-synonymous amino acid change and thus allele defining SNPs and a X represents regions not sequenced. M33388, AY545216 and CYP2D6*2A are depicted as presented on the nomenclature committee website (www.cypalleles.ki.se) or as represented by Gaedigk et al., (2007).This figure was created by Tyren Mark Dodgen.

Table 7-2. *CYP2D6* genotype and predicted phenotype frequencies in a South African risperidone treated cohort experiencing ADRs.

CYP2D6 Genotype	AS	Predicted Phenotype	Number (n = 24)	Ethnicity
**1/*1*	2.0	EM	1	White
**1/*17*			1	Black
**1/*2*			2	Black
**2/*106*			1	Black
**2/*2*			2	White
**2/*43*			1	Black
**1/*29*	1.5		1	Black
**2/*41*			1	White
**35/*41*			1	White
**1/*4*	1.0	IM	5	4 W / 1 B
**2/*4*			1	White
**2/*6B*			1	White
**4/*35*			1	White
**5/*17*			1	Black
**5/*41*	0.5		1	White
**5/10B*			2	Black
**4/*4*	0.0	PM	1	White

ADRs, adverse drug reactions; AS, risperidone modified version of the activity score system (Gaedigk et al. 2008), EM, extensive metabolism; IM, intermediate metabolism; PM, poor metabolism.

7.4.4 *Risperidone ADRs and CYP2D6 influence*

None of the individual RSP-related EPSs were found not to correlate with other EPSs, as correlation coefficients were poor and *P* values were all above 0.23 (refer to Table 1). This suggests that tardive dyskinesia, akathisia and parkinsonismsymptoms experienced as a result of RSP therapy are independent of one another. Strong negative correlations of -0.484, was found between weight gain and akathisia (P = 0.004) and -0.435 between weight gain and parkinsonismsymptoms (P = 0.010). This suggests that when weight gain is experienced, the patient is unlikely to experience akathesia or parkinsonism and vice versa. Tardive diskinesia had a weak correlation of -0.136 with weight gain (P = 0.458), and therefore may not share the same relationship with weight gain, as the other RSP-related EPSs. When *CYP2D6* predicted phenotype was compared to RSP-related ADRs, no statistical association was found, with *P* values Ó0.335 (Table 7-1).

7.4.5 *Confounding variables which may be associated with risperidone ADRs*

Age, race, gender and sex appeared not to be associated with RSP-related ADRs with *P* values Ó0.086 (refer to Table 1). Sex correlated well with Parkinsonism symptoms (*P* = 0.038), suggesting that women are more likely to experience Parkinsonism symptoms than men when treated with RSP. Although not statistically significant (*P* = 0.086), the Black African portion of the cohort was apparently more susceptible to tardive diskinesia. Caution is advised regarding the latter two comparisons as the sample size in this cohort was small, and a larger cohort would need to be sampled to confirm the association. Concomitant medication was found not to influence RSP-related ADRs (*P* ≥0.081), as was number of cigarettes smoked (*P* Ó0.124; Table 7-1).

7.5 Discussion

7.5.1 *CYP2D6 genetics*

As all alleles were in Hardy-Weinberg equilibrium, the *CYP2D6* screening assay using XL-PCR+Sequencing appears to be accurate and comprehensive for *CYP2D6* allele identification in this cohort. *CYP2D6*4, *6B* (absent function) as well as **41* (reduced function) were observed inCaucasians, while **17* and **29* (both reduced function) were observed in Black Africans. One Black African individual was heterozygous for *CYP2D6*4*, an allele which is observed at low frequency in African populations (Sistonen et al. 2007; Sistonen et al. 2009). *CYP2D6*10B* (reduced function)has been found at high frequency in African populations (Matimba et al. 2009). Therefore it is not surprising to find *CYP2D6*10B* in Black Africans in this cohort. *CYP2D6*5* was found in both Black Africans and Caucasians which agrees with what has observed in cohortsof varying ethnicity (Sistonen et al. 2007; Sistonen et al. 2009). The novel *CYP2D6*106* allele identified was observed in a Black African individual, emphasising the importance of comprehensive genetic screening methods to accommodate low frequency alleles which may be of clinical relevance (Matimba et al. 2009). Two different SNPs have been described at this the 3877 locus. Marez et al. (1997) identified a 3877G>C causing a E418Q amino acid change which forms part of the *CYP2D6*4H* allele. The second SNP identified at the 3877 locus was the same as *CYP2D6*106*, but formed part of the *CYP2D6*52* allele. As the

sample size of each ethnic groups is small, allele frequencies will not be discussed for this cohort.

7.5.2 *CYP2D6 and risperidoneEPS*

The sampled cohort was selected for RSP-related ADRs (RSP induced EPS and weight gain) experienced by RSP-treated South African patients. Although the significance of *CYP2D6* polymorphisms affecting RSP metabolism in both drug naïve and experienced patients has been well established (Hendset et al. 2009; Jovanovic et al. 2010; Locatelli et al. 2010), it is still unclear whether genetic mutations in *CYP2D6* are associated with ADRs. If the link between reduced RSP metabolism by CYP2D6 PM and EPS was as clear as has previously been described (de Leon et al. 2005a; de Leon et al. 2005b; Bozina et al. 2008), the majority of this cohort would have been expected to be PMs. This was not the case, as only 4.2% (1/24) were PMs. This frequency for PM is similar observed in various volunteer based southern African cohorts (Dandara et al. 2001; Matimba et al. 2009; Wright et al. 2010; Dodgen et al. 2013).

Little research has been published on the association between RSP induced EPS and *CYP2D6* polymorphisms and contradictory findings are apparent. The majority refute the association (Riedel et al. 2005; Plesnicar et al. 2006; Jovanovic et al. 2010), agreeing with what was observed in this study.This lack of association could be similar to the observed poor correlation between *CYP2D6* polymorphisms, RSP active moiety and clinical response measured using prolactin levels released due to dopamine receptor occupation (Wang et al. 2007). Patients receiving higher RSP dosages have previously been found to be more likely to experience EPS (Riedel et al. 2005; Mannheimer et al. 2008), but this was not clear in the present study.

7.5.3 *CYP2D6 and risperidoneweight gain*

An important RSP-related ADR that has received little attention is weight gain. Weight gain has been found to reduce compliance in patients taking antipsychotics even if the psychopharmacological treatment is effective (Weiden, Mackell & McDonnell 2004; Nasrallah 2006). The *CYP2D6*10* polymorphism 188C>T was found be associated with weight gain in RSP-treated Chinese patients (Lane et al. 2006). Interestingly, the association appeared to be

stronger in heterozygotes (C/T) compared to homozygotes (T/T). In the present study,*CYP2D6* defective polymorphisms did not associate with weight gain and only 1 of the 7 patients experiencing weight gain was a PM. In patients who gainedmore than 5 kg, RSP-related EPS was not observed. This is an interesting observation, which may have a genetic predisposition.

7.5.4 *Concomitant medication*

Many of the patients sampled in this cohort were taking concomitant medication which may have confounded the ADRs observed. The P450 Drug Interaction Table posted by the Division of Clinical Pharmacology at Indiana University offers valuable information regarding drug substrates, inducers and inhibitors of the CYP enzymes (Flockhart 2009). Applying this information to this cohort offers the potential to evaluate the effect of concomitant medication. Fluoxetine, imipramine, paroxetine and propranolol were prescribed concomitantly to some of the patients in this cohort and are listed as substrates of CYP2D6. More importantly, fluoxetine (Brynne et al. 1999) and paroxetine (Bertelsen et al. 2003) were listed as strong inhibitors of CYP2D6 causing more than a 5-fold increase in the plasma area under the curve (AUC) concentration and/or an 80% decrease in clearance of CYP2D6 substrates (Flockhart 2009). It is therefore recommended that these medications should not be co-administered with RSP, although sometimes this is unavoidable. Three patients were concomitantly prescribed one of these drugs with RSP. The first patient was an EM on fluoxetine and received 4 mg risperidone per day, and had a 15/40 score for SAS. The second patient was a PM on fluoxetine who receive 2mg RSP per day, and scored 9/40 for AIMS and 14/40 for SAS. The third patient was an EM on paroxetine who received 6 mg risperidone daily, and scored 4/14 for BAS, 1/40 for SAS and gained 4 kg. These patients,in theory, should have experienced the worst RSP-induced EPS, but this was not the case. Similarly, a different patient was co-prescribed hydroxyzine, which is listed as an inhibitor of CYP2D6 (Hamelin et al. 1998). This patient received 1mg RSP per day, was an EM and had a higher EPS than the previous three patients, scoring 21/40 for AIMS, 9/14 for BAS and 4/40 for SAS. In this case it would appear that hydrazine was contributing to perceived RSP ADRs, but this would bethe example in this cohort. Finally, citalopram listed as an inhibitor of CYP2D6, which was discounted previously due to lack of supporting documentation (Mannheimer et al. 2008), did not appear to increase EPS when co-prescribed

with RSP in this study. The majority of the patients who gained more than 5 kg while on RSP were not receiving concomitant medication, except one patient who gained 45 kg and who was co-medicated citalopram.

7.5.5 *Additional genes to consider*

Other genes that affect RSP efficacy and cause ADRs have been previously been considered. In terms of RSP EPS, genes which affect metabolism (enzymes), drug and the dopamine clearance as well as the drug receptor could be responsible for EPS and may be important pharmacogenetic markers. For example, *CYP3A5* has been shown to influence RSP active moiety (Kang et al. 2009; Xiang et al. 2010). *ABCB1* polymorphisms haslikewise been shown to influence RSP active moiety levels *in vivo*(Gunes et al. 2008). Genetic variability of Phase II metabolism by a glutathion S-transferase enzyme coded for by *GSTM1* has been associated with tardive dyskinesia experienced in RSP-treated patients (de Leon et al. 2005b). The Ser9Gly mutationin dopamine receptor gene *DRD3* is associated with increased risk of tardive dyskinesia in RSP-treated patients (de Leon et al. 2005b; Gasso et al. 2009). Weight gain as a result of RSP treatment has been linked to polymorphisms in 5-HT2A, 5-HT2C, 5-HT6 and BDNF (Lane et al. 2006).

7.5.6 *Limitations*

Caution is advised when interpreting these results as the numbers are relatively small and there are confounding factors which have been included in the sampling. In some cases large cohorts are needed to find significant association (de Leon et al. 2005b). Perhaps if the cohort size is increased an association will be found, but the question is whether thiswill be a strong pharmacogenetic marker to reduce ADRs. Particularly in view of the important environmental influences including concomitant medication. Although no statistical evidence was found, concomitant medication may have confounded the results in our study and this will need to be considered in future studies.

7.6 Conclusion

CYP2D6 polymorphisms did not contribute to RSP-related ADRs (EPS and weight gain) in this pilot cohort of RSP-treated South Africans. Weight gain and EPS appeared not to be experienced simultaneously when patients were treated with RSP. A larger cohort would be valuable to confirm the results of this study. Additional novel mutations in *CYP2D6* which have clinical relevance are likely to be present in the South African population.

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CHAPTER 8. CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Summary of thesis outcomes

In this thesis the pharmacogenetics of two important drug metabolising enzymes with high frequency of polymorphisms, CYP2C19 and CYP2D6, were evaluated in cohorts sampled from the South African population. Pharmacogenetic evaluation required identifying and evaluating genetic assay platforms which generate genetic information from which phenotype predictions can be determined. Phenotypic platforms were required, which are composed of methods to quantify probe drugs and their metabolites simultaneously in plasma as well as strategies to interpret quantified data to match classifications used in pharmacogenetics . The outcomes of the different platforms could then be compared to establish pharmacogenetic models which could potentially be applied in both research and personalised medicine in the future.

8.1.1 *AmpliChip CYP450 Test*

The AmpliChip CYP450 Test (AmpliChip) approved by the FDA for CYP2C19 and CYP2D6 phenotype prediction, was used to predict the phenotypes of German Caucasian (Heller et al. 2006), female Swiss Caucasian (Rebsamen et al. 2009), Caucasian and African American (de Leon et al. 2009) and tamoxifen treated Spanish Caucasian (Ramђn y Cajal et al. 2010) cohorts with reasonable success. These encouraging results motivated the evaluation of the AmpliChip for use in South African patients.

CYP2C19

Two independent demographically representative South African cohorts were sampled in order to evaluate the AmpliChip's ability to genotype and predict phenotype for CYP2C19 in the South African Population. Both cohorts showed similar results for genotype and predicted phenotype. The AmpliChip results (genotype and predicted phenotype) for Cohort 2 were then compared to the results from a PCR-RFLP genotyping using activity score (AS) for phenotype prediction. This was termed the PCR-RFLP platform. The AS was an adaptation of the model developed for CYP2D6 prediction by Gaedgk et al. (2008). This PCR-RFLP platform for CYP2C19 phenotype

prediction was designed by evaluating genetic sequence variations in South African Xhosa individuals by Drögemölleret al. (2010). Although *CYP2C19* genotype determination by the AmpliChip was successful, additional alleles offered by the PCR-RFLP platform were significantly frequent in the tested cohort (refer to Table 3-2; *P*Ò0.029) and included the following: *CYP2C19*9* (decreased metabolism), *CYP2C19*15*(decreased metabolism,), **17* (increased metabolism) and **27* (reduced expressionby *in silico*analyses and luciferase expression assays (Drögemöller et al. 2010)). An interesting linkage disequilibrium (LD) pattern was identified between *CYP2C19*2* and **27* where 19154G>A and -1401G>A (allele defining SNPs respectively) were in LD in some cases and not in others, confirming what was described by Drögemöller et al. (2010) as the high complexity and diversity of the South African population. Optimal phenotype prediction platforms need to be able to accommodate these types of complexity, which the PCR-RFLP platform did. The introduction of an AS taking a variety allele combinations into account allowed confident identification of IMs and UMs, which is not possible when using the AmpliChip platform.

CYP2D6

The AmpliChip had low success rate for identifying the *CYP2D6* phenotypes of in both Cohorts 1 and 2, as genotype could not be established for many of the participants. As a result the AmpliChip could generate a predicted phenotype for <76.0% of participants. The lack of hybridisation at the locus required for determining the *CYP2D6*8* (1758G>T) and **14* (1758G>A) alleles was the most frequent cause for failure and the possibility of this failure was disclosed in the AmpliChip information leaflet. AmpliChip results for Cohort 2 were compared for both genotype and predicted phenotype to a CYP2D6 XL-PCR+Sequencing comparative platform. This XL-PCR+Sequencing platform was designed to identify known *CYP2D6* polymorphisms in this cohort, in addition to identifying novel sequence variations. This platform also made use of AS for phenotype prediction (Gaedigk et al. 2008). Allele frequency discordance was observed between the AmpliChip and XL-PCR+Sequencing platforms, of which *CYP2D6*5* and **41* were the most significant (*P*Ò0.037,). *CYP2D6*5* was poorly identified by the AmpliChip, as several individuals were identified as homozygous (i.e. *CYP2D6*2/*2*) by the AmpliChip, but the XL-PCR+Sequencing platform identified the

individuals to have a **5* allele (i.e. *CYP2D6*2/*5*). Conversely, the AmpliChip identified *CYP2D6*5* in several individuals who were identified to be homozygous by the XL-PCR+Sequencing platform. This miss-classification has a serious impact on accurate phenotype prediction. The AmpliChip misidentified *CYP2D6*2* as **41* several times in the South African Black sub-set of the cohort. The reason is most likely due to the lack of -1584C>G LD with *CYP2D6*2* being observed in people of Black African ancestry (Gaedigk et al. 2005a; Gaedigk et al. 2005b). This is the first time this discrepancy was observed in a cohort genotyped by the AmpliChip, which is a major concern considering how frequently this mismatch was observed in this cohort. For confident identification of *CYP2D6*41*, the allele defining SNP 2988G>A should be used (Gaedigk et al. 2005b). Several additional alleles were identified by the XL-PCR+Sequencing platform in Cohort 2, included three novel alleles and a novel sub-variant, all of which may be important in accurately predicting phenotype. These additional alleles highlight the importance of identifying low frequency and novel alleles which may have pharmacogenetic relevance in African populations (Matimba et al. 2009). The XL-PCR+Sequencing platform increased the frequency of IM prediction, but did not predict any additional PMs.

Lesson learned

The AmpliChip platform, although approved by the FDA for diagnostic use, was not comprehensive enough to genotype *CYP2C19* and not robust enough for *CYP2D6* genotyping in the South African population (low success rate and miss identified several *CYP2D6* alleles). The AmpliChip would not be suitable for South African pharmacogenetic screening without significant population-specific modification and further validation. The alternative platforms, PCR-RFLP for CYP2C19 and XL-PCR+Sequencing for CYP2D6 phenotype prediction would provide more confident predictions. Exploring CYP2C19 and CYP2D6 for genetic variants in larger cohorts using these more robust platforms also has the potential to uncover additional novel alleles for more accurate phenotype prediction in the genetically complex South African population.

8.1.2 *Online-SPE LC-MS/MS method for phenotype prediction*

Advances in online SPE allowed for the development of a rapid and robust LC-MS/MS method for the simultaneous quantification of the probe drugs omeprazole (OM) and dextromethorphan (DM) as well as their metabolites 5'-hydroxyomeprazole (5OH) and dextrorphan (DX) which are specifically metabolised by CYP2C19 and CYP2D6 respectively. Streamlining as well as fundamental understanding of the sample preparation means that the method could easily be adapted to evaluate alternative probe drugs with only slight modifications. Alternatively, should pharmacogenetics not be established for a variety of drugs with narrow therapeutic windows, methods such as this could serve as a routine therapeutic drug monitoring platform for improving treatment outcome through tailoring dosage regimens.

Lesson learned

Online-SPE coupled to LC-MS/MS allows for rapid evaluation of probe drugs for comparison to predicted phenotype.

8.1.3 *CYP2C19 predicted vs. measured phenotype in a demographic South African cohort*

Now that the PCR-RFLP phenotype prediction platform had been established as more appropriate for South African cohorts, it is important to evaluate the platform's ability to predict phenotype. This comparison requires a phenotyping platform for evaluating the rate of metabolism. The probe drug OME was used to measure CYP2C19 metabolic rate. The automated on-line SPE LC-MS/MS developed for measuring OME metabolism at various time intervals proved rapid and robust for routine analysis and formed the first component of the phenotype measuring platform. Once-off plasma measurement at the 3-hour post drug administration time point proved optimal for comparison to predict phenotype. Rate of metabolism was calculated as a metabolic ratio ($MR_{metabolite/probe}$) from which phenotypic cut-offs/antimodes could be established for grouping individuals into different phenotypic classes. Using MRs and statistically defined cut-offs forms the final component of this platform.

A modified AS proved favourable for phenotype prediction when comparing predicted with measured phenotype (Kappa score of 0.553 and an Inter-Rater discrepancy of 0.232). Although

UM metabolism of OME by *CYP2C19*17* was observed previously (Sim et al. 2006), this was not confirmed in this study. Kearns et al. (2010) recommend the use of pantoprazole which is claimed to be more selectively metabolised by CYP2C19 for comparison between predicted and measured phenotype. Although OME is not regarded by some researchers to be an optimal probe drug for CYP2C19 activity, reduced metabolic efficiency of OME resulting from *CYP2C19*15* was observed for the first time.

An additional explanation for the relatively low correlation between OME predicted and measured phenotype may be additional SNPs not identified by this platform. As seen with *CYP2C19*27*, novel alleles can be frequent as well as indigenous to a specific population and could potentially contribute to a poor predicted versus measured phenotype correlation. A re-sequencing approach would allow identification of unknown alleles, which may improve phenotype prediction and add confidence to the platform.

Lesson learned

OME may not be an optimal probe for evaluating CYP2C19 phenotype, but there appears to be a reasonable correlation between predicted and measured phenotype in this South African cohort. The modification of the AS for phenotype prediction suggests that it could be valuable to create substrate specific custom AS prediction models for phenotype prediction. These custom models should help fine-tune phenotype prediction for clinical use.

8.1.4 *CYP2D6 predicted vs. measured phenotype in a demographic South African cohort*

The same phenotypic platform used for CYP2C19 phenotyping was used to measure phenotype activity for CYP2D6. DM was chosen as the probe drug. This phenotypic platform included the quantification of DM metabolism, calculation of MRs ($MR_{metabolite/probe}$) and statistical establishment of cut-offs/antinodes. The automated on-line SPELC-MS/MS developed for measuring DM metabolism at various time intervals proved rapid and robust for routine analysis. Once again, the 3-hourpost drug administration once-off plasma measurement proved optimal for comparison to predicted phenotype. As the LC-MS/MS method was designed to simultaneously

quantify metabolism of the CYP2C19 and CYP2D6, the finding that the 3-hour sampling point was optimal for both establishes this as a valuable phenotypic platform.

Phenotype prediction using AS=0.0 for PM and AS=0.5 for IM, proved optimal for predicting phenotype with a Kappa sore of 0.792 and an Inter-Rater Discrepancy of 0.051. In contrast to the use of OME as a probe drug for CYP2C19 metabolism, DX was almost exclusively metabolised by CYP2D6 allowing accurate prediction of CYP2D6 metabolism. This strong correlation of predicted and measured phenotype allowed preliminary evaluation of novel alleles. Although these novel alleles did not appear to affect metabolism significantly, their pairing with a fully functional alleles could mask subtle variation in metabolic rates. These novel alleles would have to be investigated further to elucidate their metabolic potential. Evaluation of low frequency alleles may be important in predicting phenotype in the genetically diverse South African population (Matimba et al. 2009). As CYP2D6 is a highly polymorphic gene with many sequence variations, along with structural rearrangements and involved in metabolism of as many as 25% of commonly prescribed medications (Zhou 2009; Johansson & Ingelman-Sundberg 2011), characterisation of this gene is very important for South African pharmacogenetics.

Lesson learned

DX proved to be an optimal probe drug for comparison between predicted and measured phenotype in the South African cohort. The strong correlation suggests that there are few confounding factors influencing CYP2D6 in apparently healthy South Africans. Of course disease status, malnutrition, use of traditional remedies as well as various other potential influencing factors could not be discounted in this cohort and all these factors would need to be kept in mind when establishing pharmacogenetic strategies. This strong correlation does however indicate that DM be recommended for further elucidation of novel allele function. Although DM metabolism compared well with predicted phenotype in this cohort, it is important to evaluate pharmacogenetically relevant substrates individually in order to adjust AS and improve treatment outcomes.

8.1.5 *Risperidone treated patients experiencing ADRs evaluated for CYP2D6 predicted phenotype*

Extrapyramidal symptoms (EPS) and weight gain are well documented ADRs associated with risperidone (RSP) treatment (Nasrallah 2008). Contrasting data exists when comparing CYP2D6 impaired metabolism of RSP with particular ADRs experienced (Arranz & de Leon 2007; Fleeman et al. 2011). A pilot cohort of patients treated with RSP who experienced related ADRs (phenotype) was recruited. The genotypes of these patients were then assessed using the newly designed XL-PCR+Seqencing platformand the phenotypes predicted. The XL-PCR+Seqencing platform worked well in genotyping and predicting phenotype for these patients with one novel allele being identified. The predicted phenotypefor this cohort was expected to be poor metabolisers (PM), but this study observed a lack of correlation between CYP2D6 PM and RSP-related ADRs which appears to be a common observation (Riedel et al. 2005; Plesnicar et al. 2006; Jovanovic et al. 2010). A proposed reason for lack of phenotype prediction is the fact that RSP and the CYP2D6 specific metabolite 9'-hydroxyrisperidone are equipotentin their pharmacodynamic effects (Schotte et al. 1995). It is therefore more likely that a variation in the concentration of the total active moiety would be indicative of ADRs experienced. CYP3A5 metabolic variation has been proposed as possible candidate to predict ADRs (Kang et al. 2009). As ADRs could be caused by excessive concentrations of the active moiety, variation in the absorption, distribution and elimination phases (other aspects of pharmacokinetics) may be more promising to predict ADRs. Alternative pharmacogenetic markers involving both pharmacokinetics and pharmacodynamic aspects such as *CYP3A5*(Kang et al. 2009; Xiang et al. 2010), *ABCB1* (Gunes et al. 2008), *GSTM1* (de Leon et al. 2005), *DRD3* (de Leon et al. 2005; Gasso et al. 2009), 5-HT2A, 5-HT2C, 5-HT6 and BDNF (Lane et al. 2006) should also be investigated. These potential pharmacogenetic markers should be prioritised for further investigation in South African patients. Therapeutic drug monitoring using rapid LC-MS/MS methods may also be considered to evaluate pharmacokinetics of RSP and the active metabolites, which has the potential to influence ASRs experienced.

Lesson learned

Although the XL-PCR+Sequencing platform developed and validated in this thesis worked well for predicting DX metabolism, a lack of correlation was found between CYP2D6 predicted phenotype and RSP associated ADRs. CYP2D6 phenotype prediction using a different drug that is targeted by the same enzyme was not a good pharmacogenetic marker of ADRs experienced by patients using RSP, but several confounding aspects have been identified that would need evaluation.

8.2 Limitations

In addition to the important findings demonstrated in this thesis, certain limitations should be recognised and addressed in future South African cohorts. As a preliminary evaluation into South African pharmacogenetics these studies served their purpose. However, larger cohorts need to be evaluated which would require significant funding. Larger grants generated to focus on individual aspects identified by this research would allow for larger (perhaps ethnically homogenous) cohorts to be recruited, which would be valuable for future studies.

The two cohorts used to evaluate the AmpliChip for use in South African were large enough to identify important flaws in the AmpliChipôs ability to genotype Black South Africans. These limitations in combination with the poor success rate observed in genotyping *CYP2D6* and a comparison with alternative platforms was enough to invalidate the AmpliChip for South African use at present. The PCR-PFLP platform used as an alternative for *CYP2C19* genotyping was considerably more comprehensive than the AmpliChip, but was developed using a select Xhosa cohort (Drögemöller et al. 2010). Further investigation is required into *CYP2C19* sequence variation in South African cohorts (possibly homogenous ethnicity) in order to identify additional novel alleles which may be unique to the South African population, in order to identify which may be important for determining predicted phenotype (this was not the focus of this study). Although *CPY2D6* is considerably more polymorphic than *CYP2C19*, the identification of four novel alleles as well as a new sub-variant demonstrates the importance of not basing platforms on only one ethnically homogeneous cohort. The sample size in Cohort 2 (healthy volunteers) was large enough to identify novel alleles, but appeared to predict relatively

few CYP2D6 PMs. Had the sample size been larger, more representative frequencies might have been observed. *CYP2D6* hybrid allele assays need to be added to the XL-PCR+Sequening platform as many hybrid alleles have been added to theP450 Nomenclature Committee website recently, many of which result in absent metabolic activity (http://www.imm.ki.se/cypalleles).

CYP2C19 predicted phenotype compared well with measured phenotype once AS had been modified (based on observed OME metabolism). Perhaps additional important alleles remain to be identified which could further refine the prediction of the phenotype. Although this is a good example of substrate specific modification for phenotype prediction, *CYP2C19* alleles appear not to be the major influencing factor in OME metabolism. CYP2D6 predicted and measured phenotype using DX correlated considerably better than CYP2C19, but both models would gain value from larger more ethnic specific cohorts for future comparison.

Cohort size in the ADR experiencing RSP treated patients may have been statistically small, but the lack of influence of CYP2D6 variation was apparent. Perhaps patients who are experiencing ADRs to a greater extent could be evaluated for correlation. Also sampling patients prior to dose titration may produce different results. Measuring RSP and 9ô-hydroxyrisperidone (CYP2D6 specific metabolite) in addition to other possible metabolite concentrations at different post dosing intervals may help in evaluating possible correlations between variable CYP2D6 metabolism and ADRs. The presence of other metabolites or secondary metabolites from other metabolising enzymes, both Phase I and II and transporters, could identify the main influence on RSP-related ADRs.

In general, the cohort sizes in this thesis were modest, but data which responds to each aim and objective of the thesis was able to contribute a meaningful results, equating well to independent studies. Perhaps in-depth analysis of ethnically homogenous cohorts with larger cohort sizes may be valuable in verifying these results.

8.3 Future directions

As little is known about *CYP2C19* and *CYP2D6*sequence variation in the diverse South African population, studies focussing on several ethnically homogenous cohorts not yet investigated could assist in uncovering additional novel alleles. It is important to extract as much information from a cohort as possible and it may be an option to genotype large cohorts using technologies capable of generating large amounts of sequence data. Next generation sequencing as well as various other high-throughput DNA technologies are becoming valuable tools for research of human disease (Green, Guyer & National Human Genome Research Institute 2011). With the ability to generate large amounts of sequence data generated from each individual in a cohort, the information generated may be maximised. In this way genetic information of many different pharmacogenetically relevant genes including *CYP2C19* and *CYP2D6* could be identified. Fortunately with innovative and ever improving use of computational analyses, such as bioinformatics, it is becoming possible to process large amount of sequence data generated by high-throughput technologies that can then be used to solve DNA associated clinical challenges (Drögemöller et al. 2011). In this way the information could be used to develop South African specific SNPs for the development of GWAS arrays. The highly polymorphic *CYP2D6* for example was not included in the original human genome sequence (Cole et al. 2008) and is insufficiently characterised by current GWAS microarrays (Drögemöller et al. 2011). GWAS microarrays do not necessarily cover the full genetic diversity of African cohorts, nor the complex LD patterns which are often observed in their genome (Marchini & Howie 2010). The findings of Shuster et al. (2010) are a good example of this, as in just five Southern African individuals over a million novel variants were found in many genes across the genomes. Although GWAS microarrays are a cost effective way of finding clinical associations, next generation sequencing may become a valuable screening tool for making genetic verses clinical associations, as the cost of sequencing is constantly decreasing. In addition, next generation sequencing has the ability to evaluate polygenic influence simultaneously, which will prove valuable when a holistic pharmacogenomic approach for personalised medicine is needed.

Based on the comparison between predicted and measured phenotype, OME appeared not to be the optimal probe drug for evaluating pharmacogenetics in the South African population.

Pantoprazole has been observed to be more selective than OME (Kearns, Leeder & Gaedigk 2010) and should be evaluated as a probe drug for CYP2C19 phenotype measurement in a South African cohort. This would necessitate the development and validation of a new *in vivo* quantitative method for evaluating pantoprazole metabolism to hydroxypantoprazole (the CYP2C19 specific metabolite) and some minor modifications to the online-SPE LC-MS/MS method developed for this study would be required.

The need to adjust phenotype prediction for each substrate was an important outcome of this study and should be extended to pharmacogenetically plausible substrates. In order to do this, studies should be conducted to compare treatment outcome and ADRs experienced to identified pharmacogenetic markers for each substrate. This can be conducted on "drug exposed" patients who are already receiving treatment. A comparative study of treatment success verses failure or ADR free verses ADRs experienced could help tailor phenotype prediction and identify additional genotypic factors (i.e. AS gating or specific allele associated metabolic variation). These comparisons could help fine-tune phenotype prediction for optimal pharmacogenetics and could improve treatment outcome and reduce ADRs. Pharmacogenetic screening strategies for clinical implementation could also be streamlined in this way.

In addition to looking into primary causes of variation in drug therapy and ADRs (i.e. sequence variation of genes) it is important to keep in mind that there may be various non-genetic confounding factors which may result in lack of efficacy and ADRs. Examples of non-genetic factors include environmental, diet, drug, disease and demographics to name a few (Burroughs et al. 2002). Epigenetics has an important influence in treatment response and hence pharmacoepigenetics (Gomez & Ingelman-Sundberg 2009) would also need to be investigated. Epigenetic factors such as regulatory methylation of DNA and proteins, microRNAs and CpG islands may have a significant influence on CYP activity (Gomez & Ingelman-Sundberg 2009). For *CYP2D6*a putative CpG island has been found between the 5ô-flanking region and intron 4(GenBank reference sequence ï NC_000022.9) which was identified using Methyl Primer Express (Ingelman-Sundberg et al. 2007). For *CYP2C19* a CpG island in intron 1 (Ingelman-Sundberg et al. 2007) as well as potential histone modification and microRNAs have been

identified, which may prove significant in the future. This is where rapid and robust LC-MS/MS methods for therapeutic drug monitoring may be a practical alternative, should genetic variation not provide a confident phenotypic prediction.

Ultimately genetic and non-genetic factors would need to be compiled and tested to guide tailored treatment regimens. The use of algorithms could be employed to simplify this extremely complicated approach. A good example of this would be the algorithms which have been designed to guide warfarin dosage (Sasaki et al. 2009). Taking these complexities into consideration, there are still many questions requiring research that need to be conducted before we can implement personalised medication in the South African.

8.4 Contribution to Science

This thesis covers various aspects of a pharmacogenetic investigation by evaluating genotypic and phenotypic platforms for predicting and measuring the CYP2C19 and CYP2D6 phenotypes in South African population cohorts. The relationship between predicted and measured phenotype of these two important enzymes was assessed and strategies for predicting phenotype from genetic information fine-tuned. This is the first time that the relationship between predicted and measured phenotype had been evaluated and reported in a South African cohort. The newly developed CYP2D6 platform for phenotype prediction (XL-PCR+Sequencing using an activity score (AS) prediction model) was then used to evaluate a small pilot cohort aimed to shed light on the controversial pharmacogenetics of adverse drug reactions (ADRs) resulting from CYP2D6 risperidone (RSP)metabolism. This patient based study serves as an example of what applied pharmacogenetics may entail.

8.5 Highlights of this thesis:

1. The AmpliChip was found not to be fit for predicting phenotype for CYP2C19 and CYP2D6 in the South African population.
2. Advances in online-SPE LC-MS/MS allowed the development of a rapid and robust phenotype measuring tool, combined with statistically derived phenotypic cut-offs, created a valuable phenotypic platform. With slight modifications to the assay method alternative probe drugs could be substituted or added.
3. For CYP2C19 phenotype prediction, the PCR-RFLP platform performed reasonably well with a reasonable correlation with measured phenotype (additional novel alleles may need to be found for accurate phenotype prediction).
4. For CYP2D6 phenotype prediction, the XL-PCR+Sequencing platform performed very well, generating a strong correlation with measured phenotype. Four novel alleles and one sub-variant attest to the power of this platform for identifying new alleles. With the addition of an assay to monitor hybrid alleles, this would be the perfect platform for future CYP2D6 pharmacogenetics in South African cohorts and patients.
5. The CYP2D6 predicted phenotype did not correlate with ADRs experienced by RSP treated South African patients, discounting the use of this pharmacogenetic marker to reduce RSP associated ADRs
6. Two articles have already been published from this thesis and three additional manuscripts have been prepared to be submitted for publication shortly.

The aims and objectives of all the aspects of the study were successfully completed, providing new findings as well as fine tuning of phenotype predicting platforms to aid the progression of pharmacogenetics in South African health care.

8.6 References

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APPENDIX 1. CONFERENCE ATTENDANCE AND PUBLICATIONS RELATED TO THIS THESIS

Conference attendance

1. South African Genetics Society (SAGS) congress 2008 (University of Pretoria 27-29 March).
   Oral presentation. Title: Utilization of the Roche AmpliChip platform for pre-prescription genotyping.
   Award: Runner up, Best Oral Presentation.
2. Faculty of Health Sciences, Faculty day 2008 (University of Pretoria 13 August).
   Oral Presentation. Title: Phenotyping of CYP450-mediated drug metabolism in a demographically representative sample of the South African population.
   Award:2nd place, Young Scientist Award.
3. South African Pharmacology and Neurosciences Congress 2008 (Rhodes University 5-8 October).
   Oral Presentation. Title: Drug metabolite phenotyping of CYP2D6 and CYP2C19 enzyme activity in a demographically representative sample of the South African population.
   Award:2nd place, Young Scientist Award.
4. SAAMS/ChromSA Symposium 2008 (Warmbaths Resort, Bela Bela 13-15 October).
   Oral Presentation. Title: Phenotyping of CYP450-mediated drug metabolism in a demographically representative sample of the South African population (Method Development).
5. Faculty of Health Sciences, Faculty day 2009 (University of Pretoria 12 August).
   Oral Presentation. Title: Pre-prescription genotyping of CYP2D6 and CYP2C19 in a demographically representative group of the South African population.
   Award:1st place, Best Junior Researcher.
6. International Congress on Pharmaceutical and Pharmacological Science 2009 (University of the North West 23-26 September).
   Oral Presentation. Title: Genotype vs. phenotype and the allelic variation of CYP2D6 and CYP2C19 in a demographic sample of the South African population.

Award:2nd place, Young Scientist.

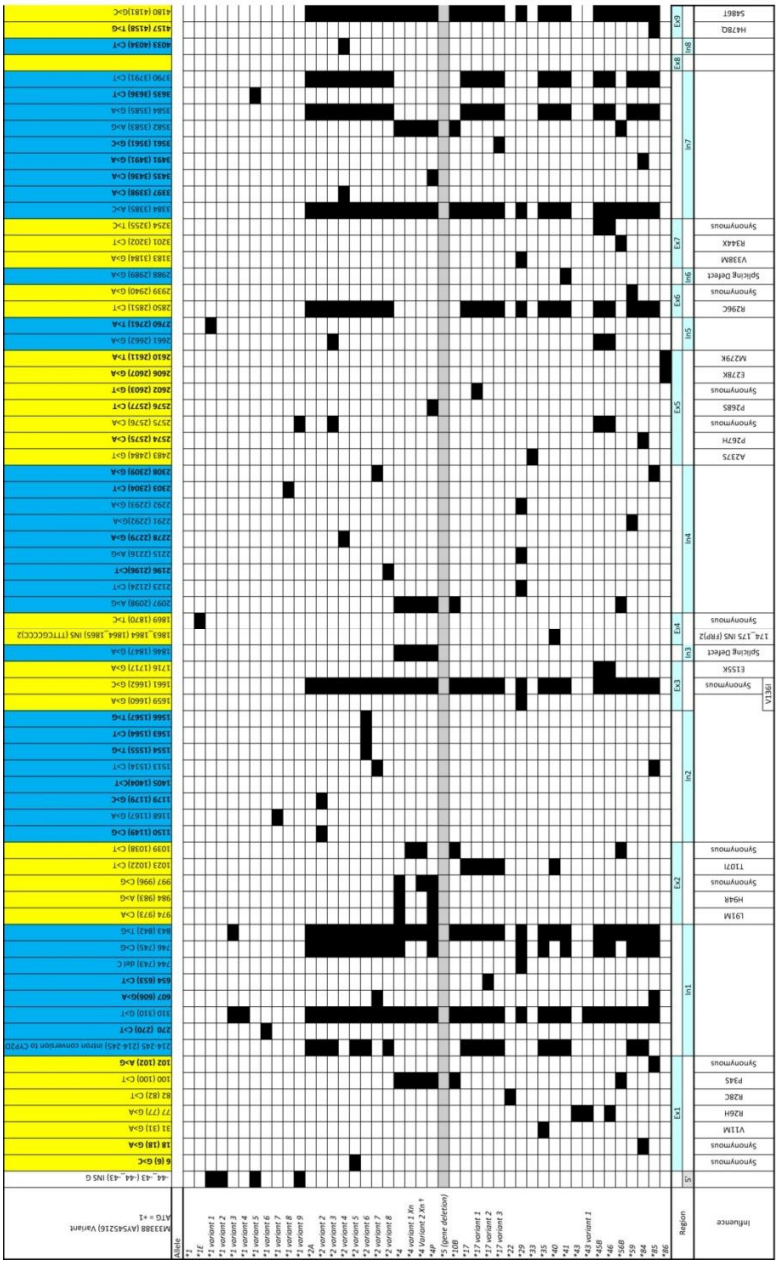
7. South African Genetics Society (SAGS) congress 2010 (University of Free State 9-11 April).
   Oral Presentation. Title: Is the Roche AmpliChip CYP450 Test the correct pharmacogenetic screening assay for CYP2D6 and CYP2C19 in the South African population?
   Award: Runner up, Best PhD presentation.
8. WorldPharma 2010, Copenhagen, Denmark (17-23 July).
   Poster presentation. Title: CYP2D6 and CYP2C19: a comparison of direct versus predicted phenotyping in a South African population.
9. Faculty of Health Sciences, Faculty day 2010 (University of Pretoria 18 August).
   Oral Presentation. Title: Pharmacogenetics of CYP2C19 for personalising medication in the South African population.
   Award:3rd place, Best Junior Researcher.
10. South African Congress for Pharmacology and Toxicology 2010 (Stellenbosch University hosted in Cape Town 3-6 October).
    Oral Presentation. Title: Comparing CYP2C19 genotyping techniques to measured phenotype for use in South African pharmacogenetics.
11. Joint International Conference of the African and Southern African Societies of Human Genetics 2011 (Cape Town, 6-9 March).
    Poster Presentation. Title: Evaluation of CYP2C19 genotype and phenotype in a representative sample of the South African population.
    Poster Presentation. Title: Evaluation of CYP2D6 genotype and phenotype in a representative sample of the South African population.
    Poster Presentation. Title: Risperidone-associated adverse drug reactions and Cytochrome P450 2D6 polymorphisms: a pilot study.
    Bursary: Awarded by the organising committee for attendance

Publications:

1. Dodgen T.M. & PepperM.S. 2009. ñCYP450 Pharmacogenetics.ò Molecular and Metabolic Medicine Update, Ampath, Vol. 5, No. 3.
2. DodgenT.M., CromartyA.D., &PepperM.S. 2011, "Quantitative plasma analysis using automated online solid-phase extraction with column switching LC-MS/MS for characterising cytochrome P450 2D6 and 2C19 metabolism."*Journal of Separation Science*, vol. 34, no. 10, pp. 1102.
3. Myburgh, R., Hochfeld, W.E., Dodgen, T.M., Ker, J. & Pepper, M.S. 2012, "Cardiovascular pharmacogenetics", *Pharmacology & Therapeutics,* vol. 133, no. 3, pp. 280-290.
4. Dodgen, T.M., Hochfeld, W.E., Fickl, H., Asfaha, S.M., Durandt, C., Rheeder, P., Drögemöller, B.I., Wright, G.E., Warnich, L., Labuschagne, C.D., van Schalkwyk, A., Gaedigk, A. & Pepper, M.S. 2013, "Introduction of the AmpliChip CYP450 Test to a South African cohort: a platform comparative prospective cohort study", *BMC Medical Genetics,* vol. 14, no. 1, pp. 20.
5. Alessandrini, M., Asfaha, S., Dodgen, T.M., Warnich, L. & Pepper, M.S. 2013, "Cytochrome P450 pharmacogenetics in African populations", *Drug Metabolism Reviews,* vol. 45, no. 2, pp. 253-275.

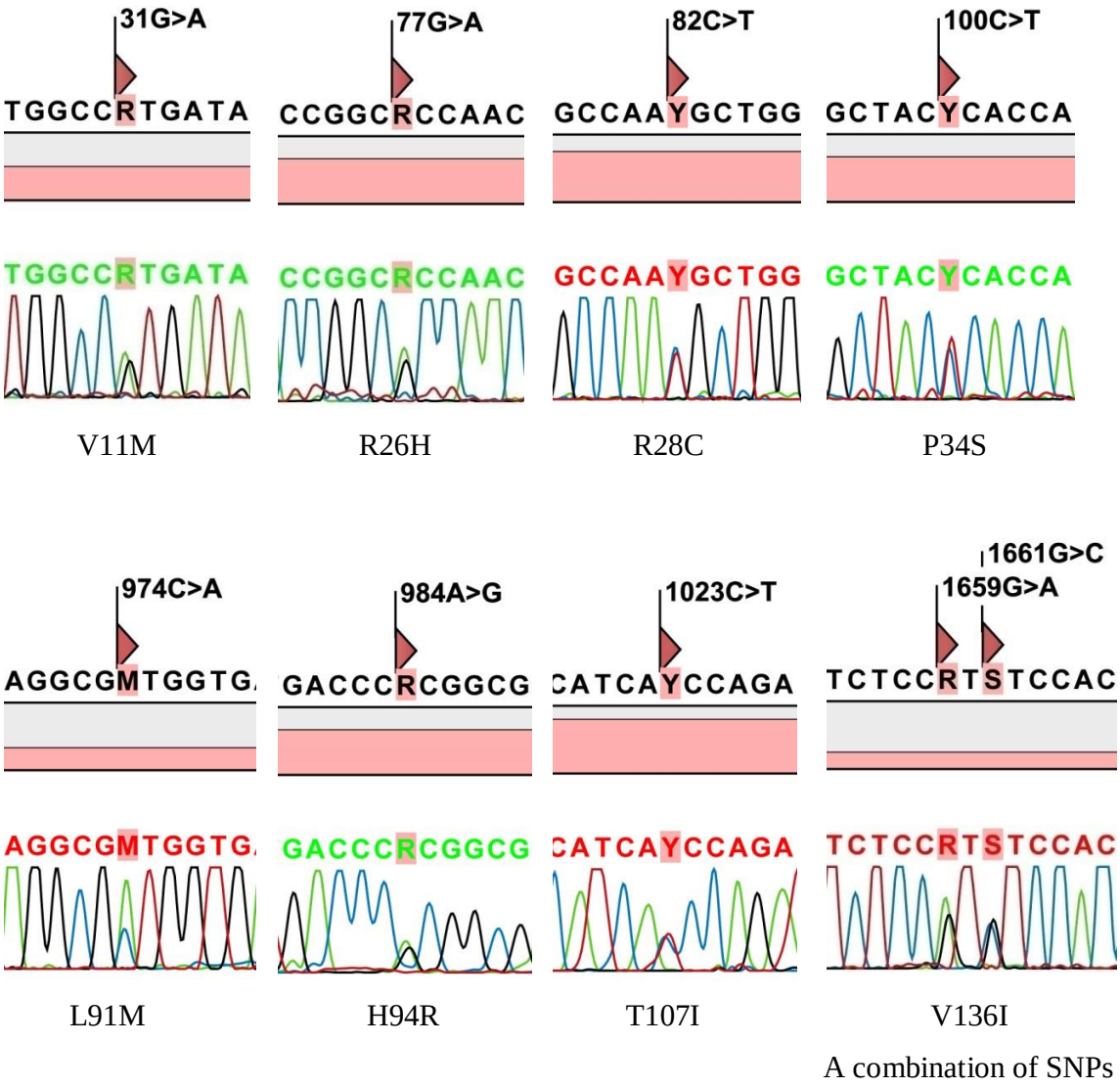
APPENDIX 2 SUPPLEMENTARY INFORMATION

Supplementary Figure

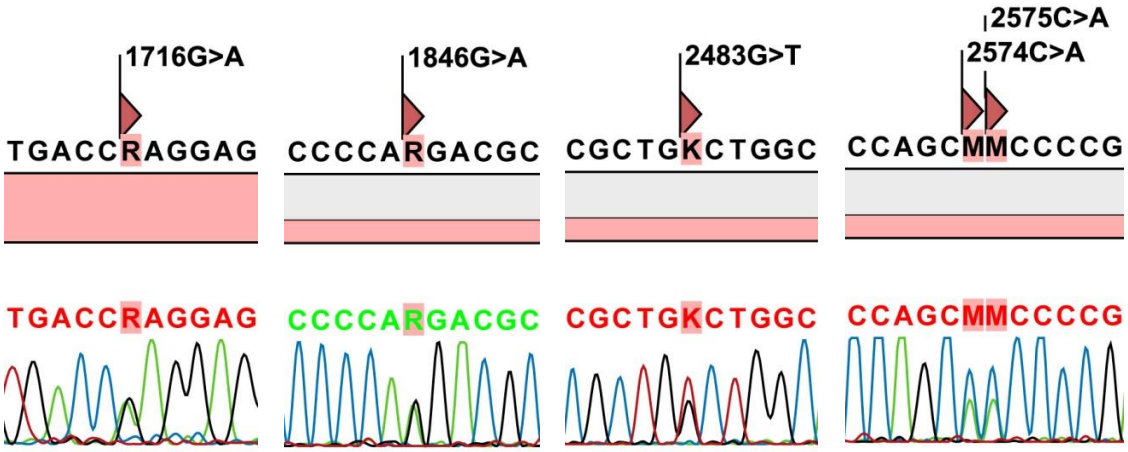


Supplementary Figure S1. Overview of *CYP2D6* alleles identified in Cohort 2.

Black boxes represent sequence variation.This figure was created by Tyren Mark Dodgen.

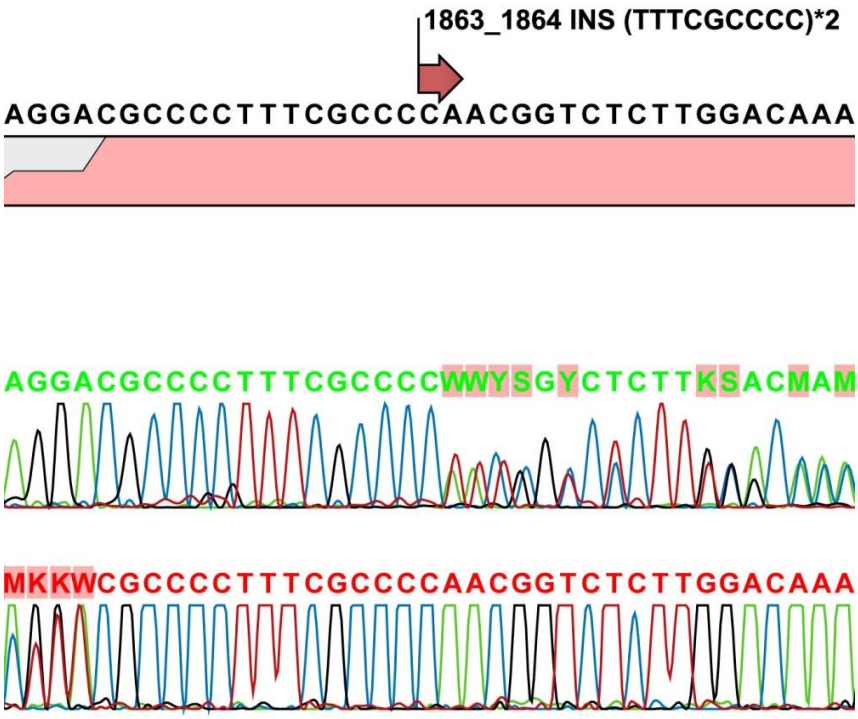


Supplementary Figure S2. Electroferograms of non-synonymous SNPs identified for *CYP2D6.* Figures created by Tyren Mark Dodgen.



E155K Splicing Defect A237S P267H

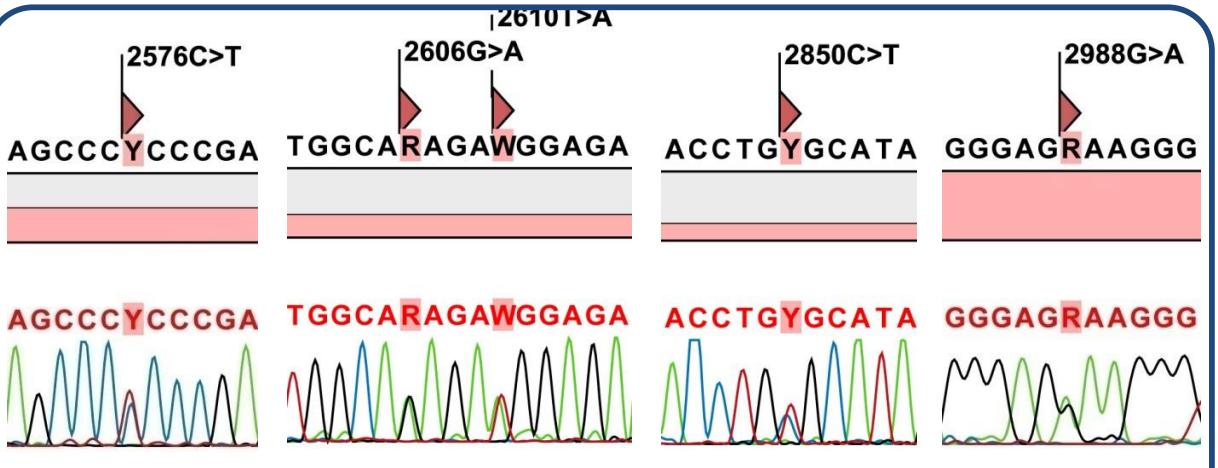
2574 creates *CYP2D6*84*



174_175 INS (FRP)x2

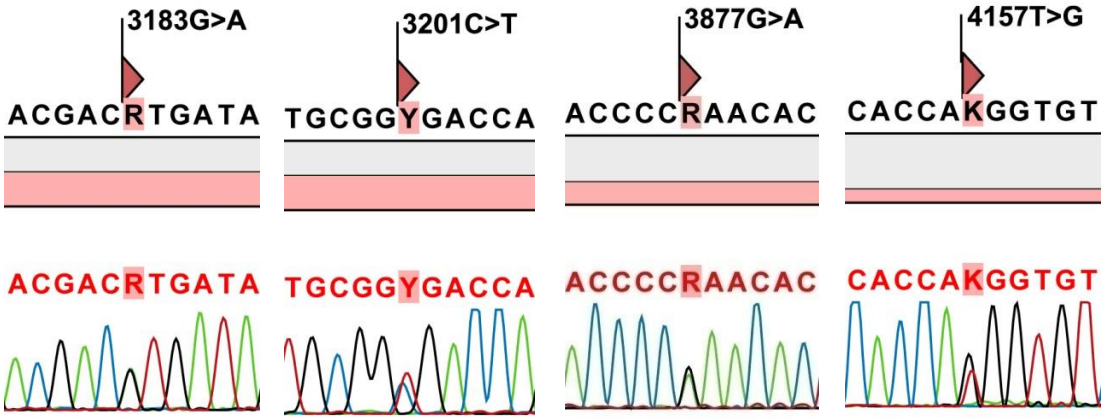
Creates an elongated protein

Supplementary Figure S2. Continued...



P268S E278K & M279K R296C Splicing Defect

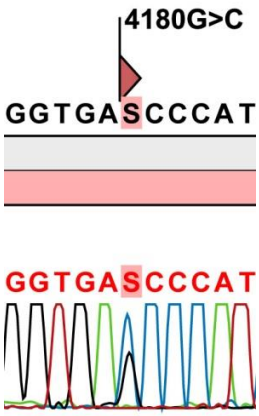
*CYP2D6*4P* Novel *CYP2D6*86*



V338M R344X E418K H478Q

Novel *CYP2D6*106* Novel *CYP2D6*85*

Supplementary Figure S2. Continued...



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Supplementary Figure S2. Continued...

Supplementary Tables

Supplementary Table S1. PCR specifications for amplification of the region of interest specific to each *CYP2C19* allele (Drögemöller et al. 2010).

CYP2C19 Allele	Primer		Primer (mM)	$MgCl_2$ (mM)	No. Cycles	Annealing temp. (°C)	Extension Time (sec)
	Name	Sequence					
**2*	CYP2C19E5:F	CAA CCA GAG CTT GGC ATA TTG T	0.2	2.5	10;30	65;60	30
	CYP2C19E5:R	GCA GAA CAG AGC TTT TCC TAT C					
**3*	CYP2C19E4:F	GCA ACC ATT ATT TAA CCA GCT AGG	0.4	2	10;30	60;55	30
	CYP2C19E4:R	TCA AAA ATG TAC TTC AGG GCT TGG TC					
**9*	CYP2C19E2+3:F	CAT AAA AGA CTG TTG GAC CAG G	0.2	3	40	58	60
	CYP2C19E2+3:R	AGG AGA GCA GTC CAG AAA GG					
**15/*28*	CYP2C19P+E1:mF*15	CTC TCA TGT TTG CTT CTgaTT TCA	0.4	2	25	55	30
	CYP2C19P+E1:R	GAC AGA CTG GAA AAG GCA ACA AAA G					
**17*	CYP2C19P+E1:mF*17	GTG TCT TCT GTT CTC AAT G	0.4	2	25	55	30
	CYP2C19P+E1:R3	CAC CGT CAT AAT TGA GAG CAC TGA AG					
**27*	CYP2C19-1041mF	GCT CTT CCT TCA GTT ACA CTG AaC	0.4	2	25	55	30
	CYP2C19P+E1:R2	GAG ATG CTT TGA GAA CAG AAG ACA C					
**28*	CYP2C19E7:F	GGG CTT CTC TTC CTT CTT TCA TTT CT	0.2	2	10;30	55;50	30
	CYP2C19E7:R	CTC TCA CCC AGT GAT GGT AGA GGG					
P + E1	CYP2C19P+E1:F	CAG AAC TGG AAC ACC TAG CTC TCA	0.2	3.5	40	68	180

P: Promoter, E: Exon, F: Forward primer, R: Reverse primer, m: Mutagenic primer, Lower case letters: Mutagenic bases. Reactions for *CYP2C19*2*, **3* and **28* used a touchdown PCR, with 10 cycles at initial Tm, followed by 30 cycles at 5°C lower than Tm. All reactions contained 0.5 U BIOTAQ™ DNA polymerase enzyme (Bioline, London, UK), with 1X Buffer, 0.4 mM dNTPs, 0.5 εM of each primer and å 15 ng gDNA. For all reactions initial denaturation was performed at 94°C for 3 min; cycles consisted of denaturation at 94°C for 15 sec, annealing for 15 sec and extension at 72°C; and a final extension at 72°C for 5 min.

Supplementary TableS2. RFLP specifications for specific *CYP2C19* allele identification (Drögemöller et al. 2010).

CYP2C19 Allele	Restriction enzyme	Digestion Temperature (°C)	Genotype	Size of Fragments (bp)	Gel for detection
**2*	*Sma*I	25	GG AG AA	410, 113 523, 410, 113 523	2.0% Agarose
**3*	*BsaJ*I	60	GG AG AA	244, 124, 104 244, 228, 124, 104 244, 228	2.5% Agarose
**9*	*HpyCH4*III	37	GG AG AA	279, 98 377, 279, 98 377	2.5% Agarose
15*/28*	*BsaB*I	60	AA AC CC	264, 22 286, 264, 22 286	2.5% Agarose
**17*	*HpyCH4*V	37	CC AC AA	467, 40, 19 486, 467, 40, 19 486, 40	2.0% Agarose
**27*	*Acl*I	37	GG AG AA	265 265, 242, 23 242, 23	2.5% Agarose
**28*	*HpyCH4*IV	37	GG AG AA	305, 134, 123 305, 257, 134, 123 305, 257	2.0% Agarose

Enzymes were purchased from New England Biolabs Inc., Beverly, USA.

Supplementary Table S3. PCR specifications for *CYP2D6* amplification and characterisation.

Primer Name	Primer sequence 5ô3ô	Reaction conc. (mM)	Ann. (°C)	Product length	Position (Ref. Seq.)
***CYP2D6*5* detection duplex reaction (Hersberger et al., 2000)**					
CYP2D6*5 FW	5ć-CAC ACC GGG CAC CTG TAC TCC TCA	0.06	65.0	3.2 kb	43-66 (X90926)
CYP2D6*5 RW	5ć-CAG GCA TGA GCT AAG GCA CCC AGA C	0.06			9353-9377 (M33388)
CYP2D6 (5.1) FW	5ć-GTT ATC CCA GAA GGC TTT GCA GGC TTC A	0.20		5.1 kb	3854-3881 (AY545216)
CYP2D6 (5.1) RW	5ôGCC GAC TGA GCC CTG GGA GGT AGG TA	0.20			6350-6375 (M33388)
50 ɛL reaction: 1.75 U Long PCR Enzyme Mix (Fermentas Life Science, Lithuania), 5.0 ɛL of 10 x Long PCR Buffer containing 15 mM $MgCl_2$, primers, 2.0 ɛL DMSO, 1.0 ɛL dNTPs (10.0 mM, Fermentas Life Science, Lithuania) and 2.0 ɛL genomic DNA (å 15.0 ng/ɛL).					
***CYP2D6* duplication detection duplex reaction (Gaedigk et al., 2007)**					
CYP2D6 DUP FW	5ć-CCA TGG AAG CCC AGG ACT GAG C	0.16	68.0	3.5 kb	6404-6425 (M33388)
CYP2D6 DUP RW	5ć-CGG CAG TGG TCA GCT AAT GAC	0.16			326-346 (AY545216)
CYP2D6 (6.6) FW	5ć-ATG GCA GCT GCC ATA CAA TCC ACC TG	0.20		6.6 kb	2367-2392 (AY545216)
CYP2D6 (6.6) RW	5ć-ACT GAG CCC TGG GAG GTA GGT AG	0.20			8931-8953 (AY545216)
25.0 ɛL reaction: 0.9 U Long PCR Enzyme Mix (Fermentas Life Science, Lithuania), 2.5 ɛL of 10x Long PCR Buffer containing 15 mM $MgCl_2$, primers, 1.25 ɛL DMSO, 0.5 ɛL dNTPs (10.0 mM, Fermentas Life Science, Lithuania) and 1.0 ɛL genomic DNA (å 15.0 ng/ɛL).					
***CYP2D6* duplication specific amplification for discrimination (Gaedigk et al., 2007)**					
CYP2D6 DUP SEQ FW	5ć-CCA GAA GGC TTT GCA GGC TTC AG	0.40	68.0	8.6 kb	3860-3882 (AY545216)
CYP2D6 DUP RW	5ć-CGG CAG TGG TCA GCT AAT GAC	0.40			326-346 (AY545216)
25.0 ɛL reaction: 0.9 U Long PCR Enzyme Mix (Fermentas Life Science, Lithuania), 2.5 ɛL of 10x Long PCR Buffer containing 15 mM $MgCl_2$, primers, 1.25 ɛL DMSO, 0.5 ɛL dNTPs (10.0 mM, Fermentas Life Science, Lithuania) and 1.0 ɛL genomic DNA (å 15.0 ng/ɛL).					
***CYP2D6*4N* and **36* detection duplex reaction (Gaedigk et al., 2006)**					
CYP2D6*36 FW	5ć-AGC CAC TCT CGT GTC GTC AGC TT	0.40	68.0	579 bp	15430-15452 (M33387)
CYP2D6*36 RW	5ć-CGA CTG AGC CCT GGG AGG TAG GTA G	0.40			6349-6373 (M33388)
CYP3A7 FW	5ć-CAC CTC TGC TAA GGG AAA CAG GCC	0.40		860 bp	4426-4449 (NG_007983)
CYP3A7 RW	5ć-GCC AGC CTG AAC ATC CTT TTT GCT A	0.40			5261-5285 (NG_007983)
25 ɛL reaction: 1.25 U BIOTAQTM DNA Polymerase (Bioline, London, UK), 2.50 ɛL of10x Reaction Buffer, 1.50 ɛL MgCl2 solution (50 mM), primers, 0.5 ɛL dNTPs (10.0 mM, Fermentas Life Science, Lithuania) and 1.0 ɛL genomic DNA (å 15.0 ng/ɛL).					

PCR, Polymerase chain reaction; conc., concentration; Ann. (°C), annealing temperature; Ref. Seq., NCBI GenBank reference sequence accession number; FW, forward primer; RW, reverse primer. Extension was performed at 68°C (except *4N and *36 detection which was 72°C) and times were 1 minute per 1.0 kb calculated on the longer fragment in the duplex reactions. Each reaction was cycled 35 times with denaturation at 94°C for 15 sec and annealing temperature held for 30 sec. If both products were present for the CYP2D6*5 PCR the reaction was repeated using only CYP2D6 specific primers at 0.26 mM each prior to sequencing.

Supplementary Table S4. Primers used to detecte and discribe SNPs associated with *CYP2D6* alleles, ordered according to gene orrientation 5' to 3'.

Primer Name	Sequence 5' to 3'	
2D6 5 prime 4	5’-CTG CCA TAC AAT CCA CCT G	
2D6 5 prime 3	5’-CCT CCT CCA CTG CTT	
2D6 5 prime 2	5’-CCT TGC CCT TTC CCT	
2D6 5 prime 1	5’-TGC TGT GTT GAC TGT G	
2D6 Gene 1	5’-GGA CTT TGT ACT CCA TAA C	
2D6 Gene 2	5’-CAC AGT CAA CAC AGC A	*
2D6 Gene 3	5’-CAC GGA AAT CTG TCT C	*
2D6 Gene 4	5’-GGC ATG AAG GAC TGG A	
2D6 Gene 5	5’-GCT TTG TGC CCT TCT G	*
2D6 Gene 6	5’-TTC CTG GCG CGC TAT G	*
2D6 Gene 7	5’-CAC CTG GAC AAG TCT C	
2D6 Gene 8	5’-GTC AAG CCT GTG CTT G	*
2D6 Gene 9	5’-CTG GAC TCT AGG ATG C	*
2D6 Gene 10	5’-GAG GAT GAT CCC AAC G	*
2D6 3 prime	5’-ACT GAG CCC TGG GAG	

* primers used for screening allelic polymorphisms.

Suplementary Table S5. *CYP2D6* polymorphisms identified in a demographically representitive South African cohort.

	Polymorphism	SNP ID GenBank	Region	Sequence context
1	-1740C>T	rs58188898	Upstream	agctgccctc (C>T) cacaaaagac
2	-1426C>T	rs57087978	Upstream	actgaaaata (C>T) aaaaagctag
3	-1298G>A	rs59099247	Upstream	caatggaggg (G>A) agccaccagc
4	-1235A>G	rs28735595	Upstream	aaaaaaaaag (A>G) attaggctgg
5	-1000G>A	rs1080989	Upstream	ggaggaggac (G>A) accctcaggc
6	-740C>T	rs28624811	Upstream	gaatgtgtgc (C>T) ctaagtgtca
7	-678G>A	rs57485914	Upstream	gattttctgc (G>A) tgtgtaatcg
8	-44_-43insG	rs75085559	5'UTR	ggggtggggg (->G) tgccaggtgt
9	6G>C	none	Exon 1	caggtatggg (G>C) ctagaagcac
10	18G>A	rs148382141	Exon 1	tagaagcact (G>A) gtgcccctgg
11	31A>G	rs769258	Exon1	gcccctggcc (G>A) tgatagtggc
12	77G>A	rs28371696	Exon 1	atgcaccggc (G>A) ccaacgctgg
13	82C>T	rs138100349	Exon 1	ccggcgccaa (C>T) gctgggctgc
14	100C>T	rs1065852	Exon 1	tgcacgctac (C>T) caccaggccc
15	102C>T	rs151226748	Exon 1	cacgctaccc (A>G) ccaggccccc

	Polymorphism	SNP ID GenBank	Region	Sequence context
16	214G>C	rs1080995	Intron 1	cggcagaggt (G>C) ctgaggctcc
17	221C>A	rs1080996	Intron 1	ggtgctgagg (C>A) tcccctacca
18	223C>G	rs1080997	Intron 1	tgctgaggct (C>G) ccctaccaga
19	227T>C	rs1080998	Intron 1	gaggctcccc (C>T) accagaagca
20	232G>C	rs75276289	Intron 1	tcccctacca (G>C) aagcaaacat
21	233A>C	rs28695233	Intron 1	cccctaccag (A>C) agcaaacatg
22	245A>G	rs1021000	Intron 1	gcaaacatgg (A>G) tggtgggtga
23	270C>T	rs29001678	Intron 1	acaggctgga (C>T) cagaagccag
24	310G>T	rs28371699	Intron 1	tttgggggac (G>T) tcctggagaa
25	607G>A	none	Intron 1	actgctcacc (G>A) gcatggacca
26	654C>T	none	Intron 1	ggcctctcgg (C>T) aattttggtg
27	744delC	none	Intron 1	acccccgccc (C>-) acgatcagga
28	746C>G	rs769260	Intron 1	ccccgcccca (C>G) gatcaggagg
29	843T>G	rs28532243	Intron 1	tagtctgggg (T>G) gatcctggct
30	974C>A	rs28371703	Exon 1	gcgcgaggcg (C>A) tggtgaccca
31	984A>G	rs28371704	Exon 1	ctggtgaccc (G>A) cggcgaggac
32	997C>G	rs28371705	Exon 1	gcgaggacac (C>G) gccgaccgcc
33	1023C>T	rs28371706	Exon 1	gtgcccatca (C>T) ccagatcctg
34	1039C>T	rs1081003	Exon 1	tcctgggttt (C>T) gggccgcgtt
35	1150C>G	rs186133763	Intron 2	gggtcgtgga (C>G) atgaaacagg
36	1168G>A	rs1081004	Intron 2	gccagcgagt (G>A) gggacagcgg
37	1179G>C	rs180847475	Intron 2	tggggacagc (G>C) ggccaagaaa
38	1405C>T	none	Intron 2	agtgcaaagg (C>T) ggtcagggtg
39	1513C>T	rs67497403	Intron 2	ccagggactg (C>T) gggagaccag
40	1554T>G	none	Intron 2	gggtggtgga (T>G) ggtggggcta
41	1563C>T	none	Intron 2	atggtggggc (C>T) aatgccttca
42	1566T>G	none	Intron 2	gtggggctaa (T>G) gccttcatgg
43	1659G>A	none	Exon 3	cgcttctcc (G>A) tgtccacctt
44	1661G>C	rs1058164	Exon 3	gcttctccgt (G>C) tccaccttgc
45	1716A>G	rs28371710	Exon 3	gtgggtgacc (G>A) aggaggccgc
46	1788G>A	none	Intron 3	gcacaaagcg (G>A) gaactgggaa
47	1846G>A	rs1800716	Intron 3	cccaccccca G>A) gacgcccctt
48	1863_1864 INS (TTTCGCCCC)X2	none	Exon 4	cctttcgccc (TTTCGCCCC)X2 aacggtctct
49	1869C>T	rs2267448	Exon 4	gccccaacgg (C>T) ctcttggaca
50	1998T>C	rs28371713	Exon 4	agtcgggctt (T>C) ctgcgcgagg
51	2097A>G	rs2267447	Intron 4	caggatttgc (A>G) tagatgggtt
52	2123C>T	rs76327133	Intron 4	aaggacattc (C>T) aggagacccc
53	2196C>T	none	Intron 4	gaggtgtgcc (C>T) gggtcagggg
54	2215A>G	rs80262685	Intron 4	gggcaccagg (A>G) gaggccaagg
55	2278G>A	rs184517596	Intron 4	ttctcctctg (G>A) gcaaggagag
56	2291A>C	none	Intron 4	aaggagagag (A>C) gtggaggctg

	Polymorphism	SNP ID GenBank	Region	Sequence context
57	2292G>A	rs75203276	Intron 4	aggagagagg (G>A) tggaggctgg
58	2303C>T	rs79738337	Intron 4	tggaggctgg (C>T) acttggggag
59	2308G>A	rs188062577	Intron 4	gctggcactt (G>A) gggagggact
60	2358A>T	rs1807313	Intron 4	ccctgggtct (A>T) cctggagatg
61	2483G>T	rs28371717	Exon 5	cccagcgctg (G>T) ctggcaaggt
62	2574C>A	rs148769737	Exon 5	ccagcccagc (C>A) cccccgagac
63	2575C>A	rs28371718	Exon 5	cagcccagcc (C>A) ccccgagacc
64	2576C>T	none	Exon 5	agcccagccc (C>T) cccgagacct
65	2602G>T	rs28371719	Exon 5	aggccttcct (G>T) gcagagatgg
66	2606G>A	rs77913725	Exon 5	cttcctggca (G>A) agatggagaa
67	2610T>A	none	Exon 5	ctggcagaga (T>A) ggagaaggtg
68	2661G>A	rs28371721	Intron 5	ggtgggttga (G>A) cgtcccagga
69	2760 T>A	rs28371723	Intron 5	tggaggtcat (T>A) tgggggctac
70	2850 C>T	rs16947	Exon 6	tgagaacctg (C>T) gcatagtggt
71	2939 G>A	rs79292917	Exon 6	tcctacatcc (G>A) gatgtgcagc
72	2988 G>A	rs28371725	Intron 6	gccgagggag (G>A) aagggtacag
73	3183 G>A	rs59421388	Exon 7	gatcgacgac (G>A) tgatagggca
74	3201 C>T	rs147960066	Exon 7	gcaggtgcgg (C>T) gaccagagat
75	3254 T>C	rs28371726	Exon 7	ccgtgattca (T>C) gaggtgcagc
76	3384 A>C	rs1985842	Intron 7	cagcaccagc (A>C) cctggtgata
77	3397 C>A	none	Intron 7	tggtgatagc (C>A) ccagcatggc
78	3435 C>A	rs28371729	Intron 7	actctaggaa (C>A) cctggccacc
79	3491 G>A	none	Intron 7	tgggtggggg (G>A) tccagagtat
80	3561 G>C	none	Intron 7	accaggacct (G>C) ccagaatgtt
81	3582 A>G	rs2004511	Intron 7	ggaggaccca (A>G) cgcctgcagg
82	3584 G>A	rs28371730	Intron 7	aggacccaac (G>A) cctgcaggga
83	3635 C>T	none	Intron 7	gtgactgcgc (C>T) ctgctgtggg
84	3790 C>T	rs4987144	Intron 7	ccctgcatct (C>T) ctgcccaggg
85	4028 C>T	none	Intron 7	acgcccctcc (C>A) ctccccacag
86	4033 C>T	none	Intron 7	cctcccctcc (C>T) cacaggccgc
87	4157 T>G	none	Exon 8	ccagccacca (T>G) ggtgtctttg
88	4180 G>C	rs1135840	Exon 8	ttcctggtga (G>C) cccatccccc
89	4401 C>T	rs28371738	Downstream	gacaggggta (C>T) gttgaggctg
90	4481 G>A	rs28572577	Downstream	gcccagattg (G>A) tgacaaggac
91	4656-4658 delACA	rs35183748	Downstream	caccatcaca (ACA>del) acagtcacct
92	4722 T>G	rs35028622	Downstream	gggagctata (T>G) gccagggcta

Supplementary Table S6. CYP2C19 phenotype comparison between traditional AS, modified AS and measured phenotype using *in vivo* phenotyping for CYP2C19 in a demographically representative sample of the South African population.

	PCR-RFLP	AS 1		AS 2 (*15 = 0.5, *17 = 1.0)		Phenotyping	
Sample	Genotype	Activity Score	Predicted phenotype	Activity Score	Predicted phenotype	Measured phenotype	log $MR_{(5OH/OME)}$
A-BL-01	*2/*17	2.0	**EM**	1.0	**IM**	IM	-0.591
A-BL-02	*15/*27	2.0	EM	1.5	EM	EM	0.043
A-BL-03	*9/*27	1.5	EM	1.5	EM	IM	-0.538
A-BL-04	*17/*27	3.0	UM	2.0	EM	EM	-0.227
A-BL-05	*2/*27	1.0	IM	1.0	IM	IM	-0.512
A-BL-06	*1/*27	2.0	EM	2.0	EM	EM	-0.141
A-BL-07	*2/*15	1.0	IM	0.5	IM	IM	-1.031
A-BL-08	*2/*2	0.0	PM	0.0	PM	IM	-0.865
A-BL-09	*2/*27	1.0	IM	1.0	IM	IM	-0.691
A-BL-10	*27/*27	2.0	EM	2.0	EM	IM	-0.618
A-BL-11	*27/*27	2.0	EM	2.0	EM	EM	0.017
A-BL-12	*1/*17	3.0	**UM**	2.0	**EM**	EM	0.282
A-BL-13	*2/*2	0.0	PM	0.0	PM	PM	-1.245
A-BL-14	*27/*27	2.0	EM	2.0	EM	EM	-0.356
A-BL-15	*27/*27	2.0	EM	2.0	EM	IM	-0.669
A-BL-16	*27/*27	2.0	EM	2.0	EM	EM	-0.255
A-BL-17	*2/*27	1.0	IM	1.0	IM	IM	-0.765
A-BL-18	*1/*27	2.0	EM	2.0	EM	EM	-0.316
A-BL-19	*27/*27	2.0	EM	2.0	EM	EM	-0.200
A-BL-20	*1/*1	2.0	EM	2.0	EM	EM	-0.271
A-BL-21	*1/*17	3.0	**UM**	2.0	**EM**	EM	-0.093
A-BL-22	*1/*9	1.5	EM	1.5	EM	IM	-0.595
A-BL-23	*1/*27	2.0	EM	2.0	EM	IM	-0.803
A-BL-24	*1/*2	1.0	IM	1.0	IM	IM	-0.897
A-BL-25	*2/*2	0.0	PM	0.0	PM	PM	-1.318
A-BL-26	*1/*17	3.0	**UM**	2.0	**EM**	EM	-0.152
A-BL-27	*9/*17	2.5	**UM**	1.5	**EM**	IM	-0.602
A-BL-28	*1/*2	1.0	IM	1.0	IM	IM	-0.715
A-BL-29	*2/*17	2.0	**EM**	1.0	**IM**	IM	-0.526
A-BL-30	*1/*15	2.0	EM	1.5	EM	IM	-1.096
A-BL-31	*15/*27	2.0	EM	1.5	EM	EM	-0.286

	PCR-RFLP	AS 1		AS 2 (*15 = 0.5, *17 = 1.0)		Phenotyping	
Sample	Genotype	Activity Score	Predicted phenotype	Activity Score	Predicted phenotype	Measured phenotype	log $MR_{(5OH/OME)}$
A-BL-32	*1/*17	3.0	**UM**	2.0	**EM**	EM	-0.187
A-BL-33	*1/*2	1.0	IM	1.0	IM	IM	-0.475
A-BL-34	*17/*17	4.0	**UM**	2.0	**EM**	EM	-0.225
A-BL-35	*1/*1	2.0	EM	2.0	EM	EM	-0.085
A-BL-36	*2/*27	1.0	IM	1.0	IM	IM	-0.940
A-BL-37	*15/*17	3.0	**UM**	1.5	**EM**	EM	-0.393
A-BL-38	*1/*1	2.0	EM	2.0	EM	IM	-0.732
A-BL-39	*2/*2	0.0	PM	0.0	PM	IM	-0.931
A-BL-40	*1/*27	2.0	EM	2.0	EM	IM	-0.500
A-BL-41	*1/*17	3.0	**UM**	2.0	**EM**	EM	-0.233
A-BL-42	*27/*27	2.0	EM	2.0	EM	EM	0.096
A-BL-43	*1/*17	3.0	**UM**	2.0	**EM**	EM	0.139
A-BL-44	*1/*17	3.0	**UM**	2.0	**EM**	EM	0.242
A-BL-45	*2/*9	0.5	IM	0.5	IM	IM	-0.628
A-BL-46	*2/*27	1.0	IM	1.0	IM	IM	-0.937
A-BL-47	*1/*1	2.0	EM	2.0	EM	EM	-0.139
A-BL-48	*1/*17	3.0	**UM**	2.0	**EM**	EM	-0.211
A-BL-49	*17/*17	4.0	**UM**	2.0	**EM**	EM	-0.266
A-BL-50	*1/*27	2.0	EM	2.0	EM	EM	-0.322
A-BL-51	*1/*17	3.0	**UM**	2.0	**EM**	EM	-0.339
A-BL-52	*1/*17	2.0	EM	2.0	EM	EM	0.311
A-BL-53	*2/*27	1.0	IM	1.0	IM	IM	-0.458
A-BL-54	*1/*17	3.0	**UM**	2.0	**EM**	EM	-0.265
A-BL-55	*1/*27	2.0	EM	2.0	EM		
A-BL-56	*15/*15	2.0	**EM**	1.0	**IM**	IM	-0.471
A-BL-57	*17/*27	3.0	**UM**	2.0	**EM**	EM	-0.436
A-BL-58	*2/*15	1.0	IM	0.5	IM	IM	-1.128
A-BL-59	*2/*27	1.0	IM	1.0	IM	EM	-0.086
A-BL-60	*17/*27	3.0	**UM**	2.0	**EM**	EM	-0.433
A-BL-61	*1/*1	2.0	EM	2.0	EM	IM	-0.776
A-BL-62	*1/*27	2.0	EM	2.0	EM	EM	-0.214
A-BL-63	*1/*1	2.0	EM	2.0	EM	EM	-0.209
A-BL-64	*1/*1	2.0	EM	2.0	EM	EM	-0.015
A-BL-65	*1/*9	1.5	EM	1.5	EM	IM	-0.702
A-BL-66	*1/*1	2.0	EM	2.0	EM	EM	-0.241
A-BL-67	*1/*2	1.0	IM	1.0	IM	EM	-0.258
A-BL-68	*1/*17	3.0	**UM**	2.0	**EM**	IM	-0.478

	PCR-RFLP	AS 1		AS 2 (*15 = 0.5, *17 = 1.0)		Phenotyping	
Sample	Genotype	Activity Score	Predicted phenotype	Activity Score	Predicted phenotype	Measured phenotype	log $MR_{(5OH/OME)}$
A-BL-69	*1/*2	1.0	IM	1.0	IM	IM	-0.645
A-BL-70	*1/*17	3.0	**UM**	2.0	**EM**	EM	-0.307
A-CA-01	*1/*17	3.0	**UM**	2.0	**EM**	EM	-0.147
A-CA-02	*1/*1	2.0	EM	2.0	EM	EM	-0.025
A-CA-03	*1/*1	2.0	EM	2.0	EM	EM	0.644
A-CA-04	*1/*1	2.0	EM	2.0	EM	EM	-0.007
A-CA-05	*1/*17	3.0	**UM**	2.0	**EM**	EM	0.233
A-CA-06	*1/*17	3.0	**UM**	2.0	**EM**	EM	0.133
A-CA-07	*17/*17	4.0	**UM**	2.0	**EM**	EM	-0.311
A-CA-08	*1/*2	1.0	IM	1.0	IM	IM	-0.587
A-CA-09	*1/*1	2.0	EM	2.0	EM	IM	-0.794
A-CA-10	*1/*1	2.0	EM	2.0	EM	EM	0.013
A-CL-01	*2/*15	1.0	IM	0.5	IM	IM	-0.512
A-CL-02	*1/*15	2.0	EM	1.5	EM	EM	0.491
A-CL-03	*1/*2	1.0	IM	1.0	IM	EM	-0.372
A-CL-04	*1/*1	2.0	EM	2.0	EM	IM	-0.530
A-CL-05	*2/*2	0.0	PM	0.0	PM	PM	-1.177
A-CL-06	*2/*27	1.0	IM	1.0	IM	EM	-0.171
A-CL-07	*2/*27	1.0	IM	1.0	IM	EM	-0.044
A-CL-08	*1/*2	1.0	IM	1.0	IM	IM	-0.657
A-CL-09	*1/*17	2.0	EM	2.0	EM	EM	0.342
A-CL-10	*1/*27	2.0	EM	2.0	EM	IM	-0.504
A-IN-01	*1/*1	2.0	EM	2.0	EM	EM	0.055
A-IN-02	*2/*27	1.0	IM	1.0	IM	IM	-1.067
A-IN-03	*1/*2	1.0	IM	1.0	IM	IM	-0.624
A-IN-04	*1/*1	2.0	EM	2.0	EM	EM	-0.168
A-IN-05	*1/*27	2.0	EM	2.0	EM	EM	-0.150
A-IN-06	*17/*27	3.0	**UM**	2.0	**EM**	EM	-0.406
A-IN-07	*2/*2	0.0	PM	0.0	PM	PM	-1.229
A-IN-08	*2/*27	1.0	IM	1.0	IM	EM	-0.322
A-IN-09	*1/*2	1.0	IM	1.0	IM	IM	-0.487
A-IN-10	*1/*2	1.0	IM	1.0	IM	IM	-0.658

Activity score (AS) represented as AS1 as well as a modified AS (**15* = 0.5, **17* = 1.0, **27* = 1.0, **28* = 1) represented as AS2. Plasma collected 3 hours after probe drug (omeprazole-OME) administration and used to calculate *in vivo* measured phenotype. 5'-hydroxyomeprazole (5OH) / OME. Phenotypes observed include ultrarapid (UM)extensive (EM), intermediate (IM) and poor metabolism (PM). Areas for which phenotype could not be calculated were left blank. Yellow highlighting indicates discrepancy between predicted and measured phenotype. Bold text indicates discrepency between AS1 and modified AS2 prediction.

Supplementary Table S7. comparison of CYP2D6 XL-PCR+Sequencing predicted phenotype using ASand measured phenotype using *in vivo* phenotyping for CYP2D6 in a demographically representative sample of the South African cohort. IMwas predicted in two different ways in order to compare conformation with measured phenotype to identify the optimal prediction strategy.

		XL-PCR+Sequencing (IM=AS 0.5-1.0)		Phenotyping	XL-PCR+Sequencing (IM=AS 0.5)		Phenotyping	Phenotyping
Sample	Genotype	AS	AS prediction	Measured phenotype	AS	AS prediction	Measured phenotype	3h log $MR_{(Dx/DM)}$
A-BL-01	**1/*17*	1.5	EM	EM	1.5	EM	EM	0.1865
A-BL-02	**2/*10B*	1.5	EM	EM	1.5	EM	EM	0.4276
A-BL-03	**1/*2*	2.0	EM	EM	2.0	EM	EM	0.5061
A-BL-04	**5/*17*	0.5	IM	IM	0.5	IM	IM	-0.4972
A-BL-05	**17/*45B*	1.5	EM	IM	1.5	EM	EM	-0.0023
A-BL-06	**1/*4xN*	1.0	IM	EM	1.0	EM	EM	0.1820
A-BL-07	**1/*2*	2.0	EM	EM	2.0	EM	EM	0.6090
A-BL-08	**2/*5*	1.0	IM	IM	1.0	EM	EM	-0.0439
A-BL-09	**5/*2xN*	2.0	EM	EM	2.0	EM	EM	0.5506
A-BL-10	**1/*17*	1.5	EM	EM	1.5	EM	EM	0.4750
A-BL-11	**1/*10B*	1.5	EM	EM	1.5	EM	EM	0.3299
A-BL-12	**1/*29*	1.5	EM	EM	1.5	EM	EM	0.5732
A-BL-13	**1/*40*	1.0	IM	EM	1.0	EM	EM	0.1898
A-BL-14	**1/*17*	1.5	EM	IM	1.5	EM	EM	-0.0142
A-BL-15	**2/*17*	1.5	EM	EM	1.5	EM	EM	0.3717
A-BL-16	**45B/*84*	2.0	EM	EM	2.0	EM	EM	0.4506
A-BL-17	**1/*29*	1.5	EM	EM	1.5	EM	EM	0.1873
A-BL-18	**1/*2*	2.0	EM	EM	2.0	EM	EM	0.8465
A-BL-19	**1/*5*	1.0	IM	IM	1.0	EM	EM	-0.0086
A-BL-20	**1/*1*	2.0	EM	EM	2.0	EM	EM	0.5142
A-BL-21	**2/*17*	1.5	EM	EM	1.5	EM	EM	0.2917
A-BL-22	**1/*17*	1.5	EM	EM	1.5	EM	EM	0.4124
A-BL-23	**2/*17*	1.5	EM	EM	1.5	EM	EM	0.6667
A-BL-24	**1/*17*	1.5	EM	IM	1.5	EM	EM	-0.0432
A-BL-25	**1/*45B*	2.0	EM	EM	2.0	EM	EM	0.5634
A-BL-26	**5/*17*	0.5	IM	IM	0.5	IM	IM	-0.7874
A-BL-27	**1/*5*	1.0	IM	EM	1.0	EM	EM	0.3261
A-BL-28	**5/*45B*	1.0	IM	IM	1.0	EM	EM	0.0618
A-BL-29	**17/*29*	1.0	IM	IM	1.0	EM	IM	-0.4006
A-BL-30	**17/*17*	1.0	IM	IM	1.0	EM	EM	-0.1114
A-BL-31	**1/*10B*	1.5	EM	EM	1.5	EM	EM	0.3363
A-BL-32	**17/*40*	0.5	IM	IM	0.5	IM	IM	-0.8393
A-BL-33	**1/*17*	1.5	EM	IM	1.5	EM	EM	-0.0555
A-BL-34	**43/*17*	1.5	EM	EM	1.5	EM	EM	0.8987
A-BL-35	**1/*4xN*	1.0	IM	EM	1.0	EM	EM	0.2026
A-BL-36	**2/*17*	1.5	EM	EM	1.5	EM	EM	0.6395

Sample	Genotype	XL-PCR+Sequencing (IM=AS 0.5-1.0)		Phenotyping	XL-PCR+Sequencing (IM=AS 0.5)		Phenotyping	Phenotyping
		AS	AS prediction	Measured phenotype	AS	AS prediction	Measured phenotype	3h log $MR_{(Dx/DM)}$
A-BL-37	*2/*85	2.0	EM	EM	2.0	EM	EM	0.4280
A-BL-38	*1/*45B	2.0	EM	EM	2.0	EM	EM	0.4995
A-BL-39	*17/*29	1.0	IM	EM	1.0	EM	EM	0.0855
A-BL-40	*1/*2	2.0	EM	EM	2.0	EM	EM	0.4651
A-BL-41	*17/*17	1.0	IM	IM	1.0	EM	IM	-0.4538
A-BL-42	*5/*17	0.5	IM	IM	0.5	IM	IM	-0.8698
A-BL-43	*10B/*45B	1.5	EM	EM	1.5	EM	EM	0.3094
A-BL-44	*2/*56B	1.0	IM	EM	1.0	EM	EM	0.3696
A-BL-45	*4xN/*10B	0.5	IM	IM	0.5	IM	IM	-0.3146
A-BL-46	*1/*4xN	1.0	IM	EM	1.0	EM	EM	0.5027
A-BL-47	*1/*5	1.0	IM	EM	1.0	EM	EM	0.4565
A-BL-48	*1/*5	1.0	IM	IM	1.0	EM	EM	0.0369
A-BL-49	*2/*17	1.5	EM	EM	1.5	EM	EM	0.1837
A-BL-50	*1/*17	1.5	EM	EM	1.5	EM	EM	0.1982
A-BL-51	*5/*17	0.5	IM	IM	0.5	IM	IM	-0.7696
A-BL-52	*29/*29	1.0	IM	IM	1.0	EM	EM	-0.0459
A-BL-53	*17/*40	0.5	IM	IM	0.5	IM	IM	-0.8983
A-BL-54	*5/*17	0.5	IM	IM	0.5	IM	IM	-0.6181
A-BL-55	*5/*17	0.5	IM		0.5	IM		
A-BL-56	*17/*17	1.0	IM	IM	1.0	EM	IM	-0.3856
A-BL-57	*1/*45B	2.0	EM		2.0	EM		
A-BL-58	*2/*40	1.0	IM	EM	1.0	EM	EM	0.2121
A-BL-59	*1/*41	1.5	EM	EM	1.5	EM	EM	0.5008
A-BL-60	*1/*40	1.0	IM		1.0	EM		
A-BL-61	*1/*1	2.0	EM		2.0	EM		
A-BL-62	*45B/*17	1.5	EM		1.5	EM		
A-BL-63	*1/*10B	1.5	EM		1.5	EM		
A-BL-64	*1/*17	1.5	EM		1.5	EM		
A-BL-65	*1/*10B	1.5	EM		1.5	EM		
A-BL-66	*1/*17	1.5	EM		1.5	EM		
A-BL-67	*2/*17	1.5	EM		1.5	EM		
A-BL-68	*5/*17	0.5	IM		0.5	IM		
A-BL-69	*5/*17	0.5	IM		0.5	IM		
A-BL-70	*10B/*45B	1.5	EM		1.5	EM		
A-CA-01	*1/*2	2.0	EM	EM	2.0	EM	EM	0.7772
A-CA-02	*1/*41	1.5	EM	EM	1.5	EM	EM	0.3563
A-CA-03	*1/*4	1.0	IM	IM	1.0	EM	EM	-0.2283
A-CA-04	*2/*41	1.5	EM	IM	1.5	EM	EM	0.0063
A-CA-05	*2/*33	2.0	EM	EM	2.0	EM	EM	0.5121
A-CA-06	*35/*41	1.5	EM	EM	1.5	EM	EM	0.6029
A-CA-07	*1/*1	2.0	EM	EM	2.0	EM	EM	0.3364
A-CA-08	*4/*5	0.0	PM	PM	0.0	PM	PM	Can not calculate
A-CA-09	*1/*4P	1.0	IM	EM	1.0	EM	EM	0.1634

Sample	Genotype	XL-PCR+Sequencing (IM=AS 0.5-1.0)		Phenotyping	XL-PCR+Sequencing (IM=AS 0.5)		Phenotyping	Phenotyping
		AS	AS prediction	Measured phenotype	AS	AS prediction	Measured phenotype	3h log $MR_{(Dx/DM)}$
A-CA-10	**4/*10B*	0.5	IM	IM	0.5	IM	EM	-0.2048
A-CL-01	**29/*41*	1.0	IM	IM	1.0	EM	EM	-0.0811
A-CL-02	**2/*4*	1.0	IM	IM	1.0	EM	EM	0.0581
A-CL-03	**1/*1*	2.0	EM	EM	2.0	EM	EM	0.9565
A-CL-04	**1/*2*	2.0	EM		2.0	EM		
A-CL-05	**1/*4*	1.0	IM		1.0	EM		
A-CL-06	**4/*17*	0.5	IM		0.5	IM		
A-CL-07	**1/*59*	1.5	EM		1.5	EM		
A-CL-08	**17/*43*	1.5	EM	EM	1.5	EM	EM	0.3134
A-CL-09	**2/*5*	1.0	IM	IM	1.0	EM	EM	-0.1877
A-CL-10	**1/*10B*	1.5	EM	EM	1.5	EM	EM	0.1977
A-IN-01	**1/*41*	1.5	EM	IM	1.5	EM	EM	0.0653
A-IN-02	**1/*2*	2.0	EM	EM	2.0	EM	EM	0.4937
A-IN-03	**2/*2*	2.0	EM	EM	2.0	EM	EM	0.4858
A-IN-04	**1/*1*	2.0	EM	EM	2.0	EM	EM	0.1984
A-IN-05	**1/*1*	2.0	EM	EM	2.0	EM	EM	0.7461
A-IN-06	**2/*86*	2.0	EM	EM	2.0	EM	EM	0.6286
A-IN-07	**2/*2*	2.0	EM		2.0	EM		
A-IN-08	**1/*22*	2.0	EM		2.0	EM		
A-IN-09	**1/*41*	1.5	EM		1.5	EM		
A-IN-10	**1/*43*	2.0	EM		2.0	EM		

Predicted phenotype was assigned using the activity score (AS) model (Gaedigk et al., 2008). Plasma collected 3 hours after probe drug (dextromethorphan) administration and used to calculate in vivo measured phenotype. Measured phenotype was calculated as log the concentration of dextrorphan (DX) over dextromethorphan (DM). Phenotypes measured include extensive (EM), intermediate (IM) and poor metabolism (PM). Areas for which phenotype could not be calculated were left blank. Yellow highlighting indicates discrepancy between predicted and measured phenotype. Bold font indicates the difference between using AS=0.5-1.0 and AS=0.5 for predicting IM.

APPENDIX 3 ETHICS APPROVALS

100 1908 – 2008

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

26/08/2009

Number	:	**S132/2009**
Title	:	Pharmacogenetics of CYP2D6 and CYP2C19 as a pre-prescription tool for drug efficacy and toxicity in a demographically-representative sample of the South African population
Investigator	:	Tyren Dodgen, Department of Pharmacology, University of Pretoria (SUPERVISORS: Prof. Michael Pepper / Dr. Duncan Cromarty)
Sponsor	:	Department of Immunology & Department of Pharmacology
Study Degree:		**MSc (Pharmacology)**

This Student Protocol has been considered by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 25/08/2009 and found to be acceptable.

Prof AG Nienaber	(female) BA (Hons) (Wits); LLB (Pretoria); LLM (Pretoria); LLD (Pretoria); Diploma in Datametrics (UNISA)
Prof V.O.L. Karusseit	MBChB; MFGP (SA); M.Med (Chir); FCS (SA)
Prof J A Ker	Deputy Dean: MBChB (Pretoria); MMed (Int) (Pretoria); MD (Pretoria)
Prof M Kruger	(female) MBChB.(Pretoria) M. Med.Paed.(Pretoria) M. Phil. (Applied Ethics) (Stell) PhD.(Leuven) (Special Advisory Member)
Dr N K Likibi	MBChB.; Med.Adviser (Gauteng Dept. of Health)
Dr T S Marcus	(female) BSc (LSE), PhD (University of Lodz, Poland)
Mrs M C Nzeku	(female) BSc (NUL); MSc Biochem (UCL,UK)
Snr Sr J. Phatoli	(female) BCur (Et.Al); BTech Oncology
Mr Y M Sikweyiya	MPH (Umea University Umea, Sweden); Master Level Fellowship (Research Ethics) (Pretoria and UKZN); Post Grad. Diploma in Health Promotion (Unitra); BSc in Health Promotion (Unitra)
Dr L Schoeman	(female) BPharm (North West); BAHons (Psychology)(Pretoria); PhD (KwaZulu-Natal); International Diploma in Research Ethics (UCT)
Dr R Sommers	Deputy Chairperson: (female) MBChB; M.Med (Int); MPhar.Med
Prof C W van Staden	CHAIRPERSON: MBChB (Pretoria); MMed(Psych) (Pretoria); MD (Warwick,UK); FCPsych (SA); FTCL (London); UPLM (UNISA)
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path)
Dr AP van der Walt	BChD, DGA (Pretoria)

Student Ethics Sub-Committee

Prof R S K Apatu	MBChB (Legon,UG); PhD (Cantab); PGDip International Research Ethics (UCT)
Dr A M Bergh	(female) BA (RAU); BA (Hons) (Linguistics) (Stell); BA (Hons) (German) (UNISA); BEd (Pretoria); PhD (Pretoria); SED (Stell)
Mrs N Briers	(female) BSc (Stell); BSc Hons (Pretoria); MSc (Pretoria); DHETP (Pretoria)
Dr S I Cronje	BA (Pretoria); BD (Pretoria); DD (Pretoria)
Dr M M Geyser	(female) MBChB (Pretoria); BSc (Computer Science)(Pretoria); BSc Hons (Pharm) (Potchefstroom); MpraxMed (Pretoria); MSc (Clinical Epidemiology) (Pretoria); FCEM (SA); Dip PEC (SA)
Prof D Millard	(female) B.lur (Pretoria); LLB (Pretoria); LLM (Pretoria); AIPSA Diploma in Insolvency Law (Pretoria); LLD (UJ)
Dr S A S Olorunju	BSc (Hons). Stats (Ahmadu Bello University –Nigeria); MSc (Applied Statistics (UKC United Kingdom); PhD (Ahmadu Bello University – Nigeria)
Dr L Schoeman	CHAIRPERSON: (female) BPharm (North West); BAHons (Psychology)(Pretoria); PhD (KwaZulu-Natal); International Diploma in Research Ethics (UCT)
Dr R Sommers	Deputy Chairperson (female) MBChB; M.Med (Int); MPhar.Med

DR L SCHOEMAN; BPharm, BA Hons (Psy), PhD;
Dip. International Research Ethics
CHAIRPERSON of the Faculty of Health Sciences
Student Research Ethics Committee, University of Pretoria

DR R SOMMERS; MBChB; M.Med (Int); MPhar.Med.
DEPUTY CHAIRPERSON of the Faculty of Health Sciences
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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

7/03/2012

Amendment:		**Study Degree to be upgraded from MSc to PhD**
Number	:	**S132/2009**
Title	:	Pharmacogenetics of CYP2D6 and CYP2C19 as a pre-prescription tool for drug efficacy and toxicity in a demographically-representative sample of the South African population
Investigator	:	Tyren Dodgen, Department of Pharmacology, University of Pretoria (SUPERVISORS: Prof. Michael Pepper / Dr. Duncan Cromarty)
Sponsor	:	Department of Immunology & Department of Pharmacology

This Amendment was reviewed by the Faculty of Health Sciences, Student Research Ethics Committee, University of Pretoria on 6/03/2012 and found to be acceptable.

Prof M J Bester	BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc (Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Prof J A Ker	MBChB; MMed(Int); MD – Vice-Dean (ex officio)
Dr NK Likibi	MBB HM – (Representing Gauteng Department of Health) MPH
Dr MP Mathebula	Deputy CEO: Steve Biko Academic Hospital
Prof A Nienaber	(Female) BA (Hons) (Wits); LLB (Pretoria); LLM (Pretoria); LLD (Pretoria); PhD; Diploma in Datametrics (UNISA)
Prof L M Ntlhe	MBChB(Natal); FCS(SA)
Mrs M C Nzeku	(Female) BSc(NUL); MSc Biochem(UCL,UK)
Snr Sr J. Phatoli	(Female) BCur (Et.Al); BTech Oncology
Dr R Reynders	MBChB (Pret), FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)
Dr T Rossouw	(Female) MBChB.(cum laude); M.Phil (Applied Ethics) (cum laude), MPH (Biostatistics and Epidemiology (cum laude), D.Phil
Mr Y Sikweyiya	MPH (Umea University Umea, Sweden); Master Level Fellowship (Research Ethics) (Pretoria and UKZN); Post Grad. Diploma in Health Promotion (Unitra); BSc in Health Promotion (Unitra)
Dr L Schoeman	(Female) BPharm (NWU); BAHons (Psychology)(UP); PhD (UKZN); International Diploma in Research Ethics (UCT)
Dr R Sommers	**Vice-Chair** (Female) - MBChB; MMed (Int); MPharMed.
Prof T J P Swart	BChD, MSc (Odont), MChD (Oral Path), PGCHE
Prof C W van Staden	**Chairperson** - MBChB; MMed (Psych); MD; FCPsych; FTCL; UPLM; Dept of Psychiatry

Student Ethics Sub-Committee

Prof R S K Apatu	MBChB (Legon,UG); PhD (Cantab); PGDip International Research Ethics (UCT)
Mr S B Masombuka	BA (Communication Science) UNISA; Certificate in Health Research Ethics Course (B compliant cc)
Mrs N Briers	(female) BSc (Stell); BSc Hons (Pretoria); MSc (Pretoria); DHETP (Pretoria)
Prof M M Ehlers	(female) BSc (Agric) Microbiology (Pret); BSc (Agric) Hons Microbiology (Pret); MSc (Agric) Microbiology (Pret); PhD Microbiology (Pret); Post Doctoral Fellow (Pret)
Dr R Leech	(female) B.Art et Scien; BA Cur; BA (Hons); M (ECI); PhD Nursing Science
Dr S A S Olorunju	BSc (Hons). Stats (Ahmadu Bello University –Nigeria); MSc (Applied Statistics (UKC United Kingdom); PhD (Ahmadu Bello University – Nigeria)
Dr L Schoeman	CHAIRPERSON: (female) BPharm (North West); BAHons (Psychology)(Pretoria); PhD (KwaZulu-Natal); International Diploma in Research Ethics (UCT)
Dr R Sommers	**Vice-Chair** (Female) MBChB; M.Med (Int); MPhar.Med
Prof L Sykes	(female) BSc, BDS, MDent (Pros)

DR L SCHOEMAN; BPharm, BA Hons (Psy), PhD;
Dip. International Research Ethics
CHAIRPERSON of the Faculty of Health Sciences
Student Research Ethics Committee, University of Pretoria

DR R SOMMERS; MBChB; M.Med (Int); MPhar.Med.
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FWA 00002567, Approved dd 22 May 2002 and
Expires 24 Jan 2009
IRB 0000 2235 IORG0001762 Approved dd Jan 2006 and
Expires 21 Nov 2008

University of Pretoria
Faculty of Health Sciences Research Ethics Committee
University of Pretoria

Soutpansberg Road
MRC-Building
Room 2 - 19

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Pretoria
0001

Date: 10/04/2007

PROTOCOL NO.	**24/2007**
AMENDMENT	None.
PROTOCOL TITLE	Cytochrome P450 2D6 Polymorphisms In Weight Gain And Movement Disorders Associated With Risperidone: A Pilot Study Using The Roche CYP 450 Ampliship.
INVESTIGATOR	Dr C K Mataboge
Sub-INVEST.	Prof C W van Staden, Dr C Durandt, Prof M Pepper, Prof J L Roos **T:**012-3199619 **F:**012-3199617 **E:** connie.matoboge@up.ac.za **C:**0823362735
DEPARTMENT	Dept of Psychiatry; Weskoppies Hospital ; University of Pretoria
STUDY DEGREE	None.
SPONSOR	None.
VAT NO.	None.
MEETING DATE	28/02/2007

This Protocol and Informed Consent have been considered by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 28/02/2007 and found to be acceptable.

Mr P Behari	B.Proc. KZN; LLM – Unisa; (Lay Member)
*Advocate AG Nienaber	(female)BA(Hons) (Wits); LLB; LLM (UP); Dipl.Datametrics (UNISA)
*Prof V.O.L. Karusseit	MBChB; MFGP (SA); M.Med (Chir); FCS (SA): Surgeon
*Prof M Kruger	(female) MB.ChB.(Pret); Mmed.Paed.(Pret); PhDd. (Leuven)
Dr N K Likibi	MB.BCh.; Med.Adviser (Gauteng Dept.of Health)
Mrs E.L. Nombe	(female) B.A. CUR Honours; MSC Nursing – UNISA (Lay Member)
Snr Sr J. Phatoli	(female) BCur (Et.Al) Senior Nursing-Sister
*Dr L Schoeman	(female) Bpharm, BA Hons (Psy), PhD
*Prof J.R. Snyman	MBChB, M.Pharm.Med: MD: Pharmacologist
*Dr R Sommers	(female) MBChB; M.Med (Int); MPhar.Med;
*Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path) Senior Specialist; Oral Pathology

DR R SOMMERS; MBChB; M.Med (Int); MPhar.Med.
SECRETARIAT of the Faculty of Health Sciences Research Ethics Committee - University of Pretoria

* = Members attended the meeting on 28/02/2007.
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