GENETIC POLYMORPHISM OF DRUG METABOLIZING ENZYMES IN THE SHONA AND THE SAN IN ZIMBABWE AND THE CLINICAL IMPACT OF *CYP2D6* POLYMORPHISM ON SAFETY IN THE USE OF PSYCHOTROPICS

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BPharm. Hons

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ABSTRACT

INTRODUCTION: Interindividual variability to drug response is a well known phenomenon and dates back to the 1950s with isoniazid. When the same dose of the same drug is given to different individuals, some may respond well, while others may respond but with side effects, whilst others may not even respond at all. In a number of instances, this variability has been shown to be due to genetic differences. The best documented are pharmacokinetic differences due to polymorphism of drug metabolising enzymes, in particular CYP2D6 which metabolises about 25% of clinically used drugs. The frequencies of these mutations have been studied to a great extent in Caucasians and Orientals, and some have shown to be variable among different ethnic groups. Africans have also been studied but to a much lesser extent, yet they may be even more diverse. We sought to investigate the frequencies of mutations of some drug metabolising enzymes in Zimbabwean ethnic groups and the impact of *CYP2D6* polymorphism in psychiatric patients on routine drug therapy in a naturalistic setting.

MATERIALS AND METHODS: The study was approved by the Medical Research Council of Zimbabwe and the volunteers gave written informed consent. Seventy-eight Shona volunteers were recruited from the University of Zimbabwe Medical School whilst 63 San volunteers were recruited from the Plumtree district in Zimbabwe. Fifty psychiatric patients were interviewed at the Parirenyatwa Hospital Psychiatric unit. Five mL blood samples were drawn from all volunteers and stored at minus 20 °C until required for genotyping. An additional 5 mL blood sample was drawn from each of the psychiatric patients and the plasma was stored at minus 20 °C until required for analysis. PCR-RFLP methods were used to genotype for *CYP2C19*2, CYP2B6*6, CYP2D6*4, *17* and *29* mutations. LC-MS was used for the measurement of plasma concentrations of chlorpromazine, thioridazine, fluphenazine, haloperidol, amitriptyline and nortriptyline. Drug plasma levels were then correlated against *CYP2D6* genotype.

RESULTS: Frequencies for *CYP2C19*2, CYP2B6*6, CYP2D6*4, *17* and *29* in the Shona were 0.16, 0.38, 0.05, 0.22, and 0.16, whilst in the San they were 0.12, 0.40, 0.10, 0.22 and 0.02 respectively. Differences between drug concentrations stratified by *CYP2D6* genotype were observed for thioridazine versus *CYP2D6*29* and for fluphenazine versus *CYP2D6*17* genotypes. No significant correlations were found for the other drugs.

DISCUSSION: Genetic polymorphism of the drug metabolising enzymes CYP2B6, CYP2C19 and CYP2D6 was similar between the San and the Shona, except for the frequency of, *CYP2D6*29*, an African-specific mutation, which was virtually absent in the San. The most prevalent mutation in both populations was *CYP2B6*6, which has implications for a lower population-specific dose of the antiretroviral drug efavirenz to avoid central nervous system side effects. Carriers of *CYP2D6*29* and *CYP2D6*17* mutations need lower doses of thioridazine and fluphenazine respectively to avoid side effects related to high drug plasma levels.

CONCLUSION: We investigated frequencies of mutations of the following drug metabolizing enzyme genes; *CP2D6, CYP2C19* and *CYP2B6* in the Shona and the San. Although clear relationships between *CYP2D6* genotype and thioridazine and fluphenazine concentrations were observed, these did not reach statistical significance due to the limited sample size.

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACN	Acetonitrile
ADME	Absorption, Distribution, Metabolism and Elimination
AIDS	Acquired Immuno-deficiency syndrome
AIMS	Abnormal Involuntary Movements Scale
AMT	Amitriptyline
ARV	Antiretroviral
BNF	British National Formulary
CPZ	Chlorpromazine
CYP	Cytochrome P450 enzyme
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetate
EFV	Efavirenz
EM	Extensive metaboliser
EPS	Extrapyramidal symptoms
FD	Fluphenazine Decanoate
FDA	United States Food and Drug Administration
HIV	Human Immune deficiency Virus
HPLC	High Performance Liquid Chromatography
IM	Intermediate metaboliser
LC-MS	Liquid Chromatography with Mass Spectroscopic detection
MDR	Multi Drug Resistance protein
MEC	Minimum Effective Concentration
mRNA	messenger RNA
MSC	Maximum Safe Concentration

NAT	N-acetyltransferase enzyme
NEB	New England Biolabs
nM	Nanomolar
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
ORF	Open reading frame
PCR	Polymerase Chain reaction
P-gp	P-glycoprotein
PM	Poor metaboliser
QT	measure of the time interval between the start of the Q wave and the end of the T wave in the heart's electrical cycle
RNA	Ribonucleic acid
SLE	Systemic Lupus Erythematosus
SNP	Single nucleotide polymorphism
TAE	Tris-acetate EDTA
TD	Tardive dyskinesia
TPMT	Thiopurine S-methyltransferase
UM	Ultra-rapid metaboliser
UV	Ultraviolet
VKORC	vitamin K epoxide reductase complex subunit 1
WHO	World Health Organisation

LIST OF DEFINITIONS

Allele – Alternative or variant form of the same gene. Human beings usually have 2 alleles of each gene, one paternal and one maternal.

Extensive metabolizer (EM) – individuals who express normal enzyme in normal amounts, hence have normal (extensive) drug metabolism.

Fast acetylator – ultrarapid metabolizer of isoniazid.

Genetic polymorphism – mutation in the population existing at a frequency of at least 1%.

Genotype – the DNA sequence based at a particular gene locus. This represents the heritable trait which can be associated with the observed functional trait (phenotype).

Heterozygous – an individual with different genotypes for the 2 alleles.

Homozygous – an individual with the same genotype for both alleles.

Intermediate metabolizer (IM) – an individual with decreased enzyme activity and diminished drug metabolism.

Phenotype – the observed functional measure of a variable trait. In the pharmacogenetics of drug metabolism, it would be the capacity to metabolize the drug of interest.

Poor metabolizer (PM) – an individual with significantly decreased, or no capacity to metabolize the probe drug. This usually occurs due to an inactive or a dysfunctional metabolic enzyme.

Slow acetylator – poor metabolizer of isoniazid.

SNP – single nucleotide polymorphism. The most common genetic variability at a gene locus is usually a single base pair change referred to as a SNP.

Ultrarapid metabolizer (UM) – individuals with higher than normal amounts of enzyme (or higher than normal enzyme catalytic activity), usually due to gene duplications (this definition is for pharmacogenetic usage, hence enzyme induction is not considered). The consequence will be subtherapeutic drug concentrations.

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

1. INTRODUCTION

The practice of medicine has undergone major advances in the past two decades. This has mainly been driven by the realization that variability in drug response is not only due to traditional variables such as gender, age, weight and environmental factors but also due to genetic factors. Starting with the early studies on the metabolism of the anti-tubercular drug, isoniazid in the 1950s (Bonicke and Reif 1953), inhibition of cholinesterase by the local anaesthetic, dibucaine in the 1950s (Kalow and Genest 1957) and the metabolism of the muscle relaxant, suxamethonium by pseudocholinesterase (Liddell 1962; Liddell *et al.* 1963; Liddell *et al.* 1968), more genetically based variabilities in drug exposure and response were discovered in the 1970s through to the 1990s. As will be discussed later, these include those of beta-blockers and antipsychotic drugs due to genetic variability of the enzyme CYP2D6, that of mephenytoin due to genetic variability of CYP2C19 and that of warfarin due to variability of CYP2C9. The increasing realization of the clinical relevance of genetic variability partly gave impetus to the Human Genome Project which culminated in the complete sequencing of the human genome in 2003 (United States National Human Genome Research Institute, 2003). With that, Genomic Medicine was officially born. Its research and clinical realization is being explored through the sciences and technologies of pharmacogenetics and pharmacogenomics

Pharmacogenetics versus pharmacogenomics

Pharmacogenetics is the study of interindividual variability to drug response. The United States Food and Drug Administration (FDA) defines pharmacogenetics as the study of variations in deoxyribonucleic acid (DNA) sequence as related to drug response and defines pharmacogenomics as the study of variations of DNA and ribonucleic acid (RNA) characteristics as related to drug response (FDA Guidance for Industry, 2008).

Pharmacogenomics is more recent and is broader than pharmacogenetics but there is no internationally accepted consensus depicting any semantic differences between pharmacogenetics and pharmacogenomics, and in practice the two terms are used interchangeably. However it seems that most would use pharmacogenetics to depict the study of single genes and their effects on interindividual differences in drug metabolising enzymes, and pharmacogenomics to depict the study of not just single genes but the functions and interactions of all genes in the genome in the overall variability of drug response, whether this is caused by pharmacokinetics, pharmacodynamics or both (http://www.iuphar.org/pdf/hum_27.pdf). We therefore consider our study to be in the field of pharmacogenetics, although there is significant overlap between the two terms.

Drug variability is not new but has been known for many years now. Many drug targets contain SNPs that translate to amino acid changes that can result in secondary and tertiary structure change of proteins that can affect protein function (Chasman and Adams 2001). In 1953 it was noted that the antitubercular drug isoniazid was excreted in a metabolized form in different amounts in some people, with some excreting twice as much as others and distinguished slow acetylators (SA) and rapid acetylators (RA) (Bonicke and Reif 1953). A few years later, genetic control of isoniazid metabolism in humans was noted. The acetylator trait was one of the first human hereditary traits affecting human drug response to be identified. It has been known since 1963 that acetylation of isoniazid is catalyzed by N-acetyltransferase (NAT) (Evans and White 1964). In the late 1970s and 1980s clinical problems were noted, particularly systemic lupus erythematosus (SLE), associated with polymorphic acetylation of procainamide, which is metabolized by NAT-2. Slow acetylators were found to be at increased risk of developing SLE, compared to rapid acetylators (Reidenberg and Drayer 1986; Woosley *et al.* 1978). These earlier phenotyping studies have now been complemented by genotyping studies. The sequence and analysis of the human

genome has provided a map for gaining a better understanding of the genetic basis of human diversity (Liggett 2001). A genetic basis for interindividual variability with respect to drug response is now known, giving rise to what is known as pharmacogenetics. Until the late ninety's, focus on pharmacogenetics research was mainly on metabolic proteins and especially cytochrome P450s (CYPs). With the knowledge of the molecular mechanisms of diseases, polymorphic targets of drug action are also now under investigation. Environmental factors can also lead to variability in drug response (Gonzalez and Idle 1994). From the pharmacogenetic studies done so far, it is clear that a 'one size fits all' method of drug dosing is not always applicable for some drugs.

Before genotyping can be used as a screening tool for extensive metabolizers (EMs) and poor metabolizers (PMs), a relationship between the genotype and the phenotype must exist i.e. a gene-dose relationship. In other words, a PM based on a genetic test must also be a PM based on the metabolic ratio and this was shown by Barclay *et al,* for perhexiline (Barclay *et al.* 2003). It must however be noted that there can be a discordance between the genotype and the phenotype due to environmental factors modifying the phenotype. For example, enzyme inhibitors can show a person to be a PM whilst the genotype classifies them as EM, a phenomenon refered to as phenocopying (Gardiner and Begg 2006).

1.0.1   THE CLINICAL IMPACT OF PHARMACOGENETICS

In a clinical setting, there are three main types of patients:

- o Those who respond to treatment with no adverse effects. We would like most patients to lie within this group as this is the desired effect. Such patients would be expected to have drug concentrations within the normal therapeutic range and will be EMs with respect to the drug concerned.

- o Those who respond to treatment with adverse effects. Such patients are likely to be PMs or intermediate metabolizers (IMs). Therefore, the drug of interest is expected to

accumulate and may reach toxic levels resulting in side effects. An example is the oral antidiabetic drug phenformin which had to be withdrawn from the market due to lactic acidosis (Roberts *et al.* 2002a).

o Those who do not respond to treatment, with or without adverse effects. These patients are probably ultra-rapid metabolizers (UMs). Consequently, they will rapidly eliminate the drug such that the minimum effective concentration may not be reached and therefore no therapeutic benefit can be expected. This has been demonstrated for the antidepressant drug nortriptyline whereby a dose 3-4 times the normal daily dose had to be given to an UM to achieve therapeutic drug concentrations (Bertilsson *et al.* 1985). UMs might also generate a non-therapeutically active metabolite which is however toxic and can lead to the patient having increased adverse effects. This is particulary common with the metabolism of procarcinogens to reactive metabolites that result in mutagenesis (Shimada and Nakamura 1987; Yamazaki *et al.* 1995).

A number of factors govern the absorption, distribution, metabolism and elimination (ADME) processes. Some of these factors are based on the properties of the drug, and others based on the physiological properties of the human body. Variations in drug ADME and pharmacodynamics may result in one of the three scenarios listed above. At one level or another of ADME, proteins are involved. This leads us to the 'central dogma' of molecular biology, i.e. proteins are polypeptides translated from mRNA which is transcribed from DNA. It is now appreciated that the genes that are responsible for some of the processes of drug ADME can exist polymorphically. Examples include multi-drug-resistance (MDR-1) gene, *NAT-2* and the *CYPs* among others which will be described later. Some mutations in these genes lead to altered drug response and/or plasma concentrations. Ultimately then, genes (sequence of DNA with an open reading frame (ORF) coding for a specific protein) will affect the ADME processes.

At the level of absorption from the gastrointestinal system as well as intracellularly, p-glycoprotein (P-gp), among other transport proteins may play a role. This efflux protein is encoded by the *MDR-1* gene (Ueda *et al*. 1986). Interest in this gene arose when it was discovered that tumour cells which were resistant to cytotoxic drugs had an over-expression of the gene (Roninson *et al*. 1986). The high levels of P-gp resulted in effluxing the cytotoxic drug to such an extent that it could not achieve a high enough intracellular concentration. Some studies have shown that tipifarnib, a potent inhibitor of P-gp synergistically improved treatment of some leukaemias when combined with the cytotoxic anthracyclines (Medeiros *et al*. 2007). Because of the importance of P-gp's role in drug absorption and brain uptake, many pharmaceutical companies have begun to incorporate P-gp drug transport screening into their drug discovery process. Over the last 15 years or so, our understanding of the efflux function of P-gp has progressed significantly, and recent results from animal and clinical studies have called into question the overriding importance of P-gp in pharmacokinetics (Lin and Yamazaki 2003a, 2003b). It has also been proposed that P-gp polymorphisms may have a bearing on the intracellular concentration of some HIV protease inhibitors and hence their therapeutic benefit as will be discussed later (Choo *et al*. 2000; Jones *et al.* 2001).

The major drug metabolising enzymes are the CYP superfamily which has many sub-families. They are mainly located in the liver but are also found in other parts of the body e.g. the intestine, kidney and brain. They are responsible for phase I drug metabolism, which mainly serves to make the drug molecule more hydrophilic, or to prepare it for phase II metabolism, with the ultimate purpose of eliminating it from the body. As aforesaid, the enzymes are polypeptides translated from mRNA transcribed from DNA. Hence each enzyme sub-family is regulated by the relevant *CYP* gene. CYP2D6 has an abundance of

only 2% in the human liver. The CYP2C family has an abundance of 20%. The most abundant CYP in the liver is CYP3A4 (30%) (Rendic and Di Carlo 1997). The relative abundances of these enzymes may be variable, depending on the level of enzyme induction or inhibition present. Over 90% of human drug oxidation can be attributed to CYP1A2 (4%), 2A6 (2%), 2C (12%), 2E1 (2%), 2D6 (30%) and 3A4 (50%). It is interesting that CYP2D6 is present in a relatively low amount but metabolises almost one-third of drugs metabolised by the CYPs (Rendic and Di Carlo 1997). This could be explained by the fact that medicinal chemists once exploited a chemical space for beta blockers and anti-psychotics which had a pharmacophore for CYP2D6 substrates (Wolff *et al.* 1985). Because of the problems associated with the genetic polymorphsim of CYP2D6, medicinal chemists have learnt to avoid chemistries that are associated with metabolism by this enzyme. As a result most new drugs are not substrates of this enzyme.

It can be postulated that, for a drug whose main route of elimination is metabolism by a particular enzyme, an absolute or relative deficiency of that enzyme will lead to increased plasma drug levels. An absolute deficiency can result from total absence or disruption of the gene responsible for coding the enzyme, or hepatic failure e.g. liver cirrhosis, the consequence of which would be decreased enzyme levels. A relative deficiency can result from the enzyme being present in normal or even high amounts, but the activity of the enzyme is decreased for various reasons including enzyme inhibition and mutations. Individuals with a deficient enzyme may metabolize the drug at a slower rate leading to a decreased elimination rate. It follows that when the drug levels are higher than the 'therapeutic window', toxicity results. Side effects can also be more severe, or occur more frequently at higher plasma concentrations than at lower concentration, even within the therapeutic range. For pro-drugs however, PMs would not achieve the same therapeutic benefit as EMs since the active drug will be produced in lower amounts. An example is the

N-demethylation of codeine to morphine (Poulsen *et al.* 1996; Susce *et al.* 2006), and the metabolism of proguanil to cycloguanil (Birkett *et al.* 1994; Wright *et al.* 1995). It must be stressed that these findings would most likely occur with a drug whose main route of elimination depends upon metabolism by that particular enzyme. In other words, the more the drug depends upon the enzyme of interest, the greater the clinical significance of the enzyme being deficient. Conversely, if an individual has a normal drug metabolising enzyme, but in higher than normal amounts, drug metabolism will occur at a faster rate and may lead to sub-therapeutic concentrations and treatment failure. Such people would benefit from higher drug doses in the case that the metabolite is inactive. However, these individuals are at risk of toxicity if a toxic metabolite is being produced, since it will be produced at a faster rate.

1.1 RATIONALE

Pharmacogenetics is a rapidly evolving field and a lot of information regarding genetic polymorphism is being generated for many ethnic groups, particularly Caucasians and Orientals. However, data on African populations and Zimbabweans is still sparse. African samples used for genetic analysis from one location or country may not be adequately representative of all Africans because of the diversity of the African continent. There is a need to fill the gap in pharmacogenetic knowledge pertaining to our Zimbabwean population and ethnic groups. The Shona have been studied to some extent (Masimirembwa *et al*. 1995; Masimirembwa *et al*. 1996b; Masimirembwa *et al*. 1993) hence we studied them as a reference group since they are a major population. In our effort to understand previous phenotypic studies in the San (Sommers *et al.* 1988, 1989), we sought to genotype them as well and compare them to the Shona. Since the previous studies in the Shona found a high frequency of a detrimental polymorphism (*CYP2D6*17*), we sought to investigate its effect in

patients on CYP2D6 substrates, such as psychiatric patients on psychotropic medication, most of which are CYP2D6 substrates.

In Zimbabwe, all the registered medicines we use have been developed and optimised in Caucasian populations. Using Caucasian-based dosing regimens found in drug package inserts may not always give the desired therapeutic outcome because of some mutations, particularly of drug metabolising enzymes which alter drug response in our population e.g. CYP2D6 which metabolises a variety of antipsychotics and antidepressants, and CYP2B6 which metabolises efavirenz. There is a need to carry out studies to determine the extent of variability of drug response in Zimbabweans in an effort to optimise our drug doses and hence therapeutic benefit whilst minimising side effects to drugs.

1.1.1 NULL HYPOTHESES

- there is no difference between the frequencies of polymorphisms of drug metabolizing enzymes between the San and the Shona in Zimbabwe
- there is no difference in the drug plasma concentrations between patients with and without mutations of *CYPD6* who are taking psychotropic medication.

1.2 RESEARCH AIM

To investigate genetic variations of drug-metabolising enzymes (*CYP2D6, CYP2C19* and *CYP2B6*) between the San and the Shona in Zimbabwe, and to determine the impact of *CYP2D6* polymorphisms on drug concentrations of some psychotropic drugs.

1.3 OBJECTIVES

1. To investigate the frequency of *CYP2D6*, *CYP2C19* and *CYP2B6* genetic polymorphism in the Shona population, who are the vast majority, and San population (some of the earth's earliest inhabitants) of Zimbabwe.

2. To investigate the frequency of *CYP2D6* mutations in psychiatric patients on routine drug therapy in Zimbabwe.

3. To determine whether there is a link between *CYP2D6* genotypes and drug plasma concentrations in psychiatric patients on selected antipsychotic or antidepressant drugs.

2. LITERATURE REVIEW

The literature on the pharmacogenetic aspects of enzymes of importance and affected drugs will be reviewed in this section.

2.1 CYP2D6 VARIABILITY

CYP2D6 has 497 amino acids. It accounts for about 2% of all hepatic P450s but its role in drug metabolism is extensively higher than its relative content, metabolizing about 20-30% of all drugs on the market. In other words, it can be called a high-affinity low capacity enzyme. The polymorphism of *CYP2D6* was independently discovered in three different labs (Mahgoub *et al.* 1977; Tucker *et al*. 1977; Eichelbaum *et al*. 1979). When the same dose of nortriptyline, debrisoquine and sparteine was given to individuals, 2 distinct phenotypes were discovered in the late 1970s. The genetic basis was elucidated 10-15 years later. In 1988, Gonzalez *et al* characterized the genetic defect in PMs (Gonzalez *et al.* 1988).

CYP2D6 is a highly polymorphic gene locus with more than 70 variant alleles (http://www.cypalleles.ki.se/cyp2d6.htm), which lead to a wide range of enzymatic activity. So-called PMs are carriers of any two non-functional alleles of the *CYP2D6* gene and usually have a metabolic ratio (MR) of urinary debrisoquine-4-OH debrisoquine greater than 12.6 (Masimirembwa *et al*. 1996a; Llerena *et al*. 2004a). Those with the wild-type enzyme are known as EMs. Dextromethorphan, metoprolol and sparteine can also be used as probe drugs for in vivo phenotyping (Eichelbaum *et al.* 1979; Frank *et al.* 2007). When it comes to CYP2D6 we get a sub-group of individuals known as IMs in whom the MR of both debrisoquine and sparteine is somewhat between that of EMs and PMs (Bertilsson *et al*. 2002). The IMs usually have an enzyme that is defective, but still partially active. We also have UMs who may have multiple copies of the *CYP2D6* gene, and an individual with 12 copies has been described (Johansson *et al*. 1993). Null alleles do not encode a functional

protein, and in the case of CYP2D6 there is no detectable residual enzyme activity (Daly *et al*. 1995). Null alleles result in the PM phenotype usually if present in a homozygous fashion (Dahl *et al*. 1992). Most null alleles are a result of single base pair insertions or deletions that interrupt the reading frame or that interfere with correct splicing leading to prematurely terminated proteins (*CYP2D6*3, *4, *6, *8) (Stuven *et al*. 1996). *CYP2D6*5 results in deletion of the entire *CYP2D6* gene (Steen *et al.* 1995).

There are several clinical examples of the clinical relevance of CYP2D6. One which we discussed earlier, is the N-demethylation of codeine to morphine, which is the compound that provides the greater analgesic effect. In this case, PMs may not experience adequate analgesia because they do not form as much morphine (which is about six times more potent than codeine) as EMs (Desmeules *et al.* 1991). Phenformin is an oral antidiabetic drug that was abandoned from the market many years ago due to lactic acidosis. It has now been replaced by the renally excreted metformin. The lactic acidosis is now thought to be due to increased phenformin concentrations due to PM status (Roberts *et al.* 2002a).

A study carried out by Llerena *et al* (2004) under steady state conditions showed that the dose corrected plasma concentrations of fluoxetine were overall significantly related to the number of active *CYP2D6* genes i.e. the more the number of active *CYP2D6* genes, the lower the plasma concentrations of fluoxetine. The authors however did not find a relationship for norfluoxetine, the active metabolite, though they also found a correlation between the fluoxetine/norfluoxetine ratio and *CYP2D6* metaboliser status. When fluoxetine levels were compared to *CYP2C9* genotype status, individuals with a *CYP2C9*2 or *CYP2C9*3 mutation had higher plasma levels of fluoxetine and the active moiety than wild-type patients. There was however no difference in the metabolic ratios, casting doubt over the significance of these findings with respect to *CYP2C9* (Llerena *et al.* 2004b). Another study also showed

lower plasma concentrations of the acetylcholinesterase inhibiting drug donepezil in *CYP2D6* UMs but this was not of statistical significance, probably due to their small sample size (total of 42 patients with 2 UMs) (Varsaldi *et al*. 2006). *CYP2D6* UM metaboliser status has traditionally been linked to duplication of the *CYP2D6* gene. However, there have been some individuals found with a UM phenotype but no duplication of *CYP2D6* was found in them. A role for 31G>A and -1584C>G has been investigated and these alleles were found at a high frequency in UMs (Lovlie *et al.* 2001). A study involving the antiarrythmic drug flecainide in 58 Japanese patients showed that *CYP2D6* genotype (including *CYP2D6**10 which has a frequency of 39% in Japanese and leads to intermediate metaboliser status), age, sex and serum creatinine influenced its pharmacokinetics, with age having the greatest impact. Even encompassing all these factors, the authors could only explain 50% of the variability in pharmacokinetics of the drug (Doki *et al.* 2006).

A number of the HI-antihistamine drugs are metabolized either mainly (e.g.promethazine) or partly (e.g. chlorpheniramine) by CYP2D6. PMs have been found to have lower clearance and higher AUCs than EMs (Yasuda *et al.* 2002). *CYP2D6**10 mutations resulted in more cases of sleepiness in a Japanese study and thus was shown to be a risk factor for hypersomnia associated with use of H1-antihistamines (Saruwatari *et al.* 2006). *CYP2D6**10 has also been shown to result in increased plasma concentrations of the beta blocker metoprolol, and to a much lesser extent for mexiletine (Otani *et al.* 2003; Taguchi *et al*. 2003).

In a study on Japanese psychiatric patients, it was found that there was no significant difference in haloperidol plasma levels between patients without any *CYP2D6**10 allele, those with one or those with two *CYP2D6**10 alleles (Someya *et al*. 2003). This is interesting because there is a small but significant difference in the metabolic ratios of *CYP2D6**10 and

CYP2D6*1 when debrisoquine is used as a probe drug (Dalen *et al*. 2003; Roh *et al*. 1996). This may suggest that *CYP2D6**10, like *CYP2D6**17 and *CYP2D6**29 is a mutation that leads to an enzyme with altered substrate affinity (Wennerholm *et al*. 2002). However, at a higher dosage (>10mg per day) patients with two *CYP2D6**10 alleles had significantly higher plasma levels of reduced haloperidol than those with *CYP2D6**1 (Someya *et al*. 2003). This could mean that CYP2D6 is of greater importance in the metabolism of reduced haloperidol than of haloperidol per se. *CYP2D6**5 was however associated with higher plasma levels of haloperidol as well as reduced haloperidol (Someya *et al.* 2003).

Functional alleles have a frequency of about 71% in Caucasians from the studies done so far (Ingelman-Sundberg 2005). Between 5-10% of Caucasians are PMs, reflecting the 26% frequency of non-functional alleles in this population. *CYP2D6*4* is the most common non-functional allele present at a frequency of up to 20%, being responsible for 75% of PMs in Caucasians. *CYP2D6*3*, **4* and **5* account for 93-98% of Caucasian PMs (Ingelman-Sundberg 2005). Below is a table showing some allele frequencies in different ethnic groups:

Table 1: *CYP2D6* Allele frequencies in Caucasians, Asians and Africans (Adapted from (Ingelman-Sundberg 2005)

CYP2D6 ALLELE	CAUCASIANS %	ASIANS %	AFRICANS%
*1 or *2	71	52	86.5
*3	2	0	0
*4	12-20	<1	2.5
*5	5	4-6	3.8
*10	2	40-50	5.6
*17	0.1	0	22
Duplications	1.1	0.5(Japanese)	2.5
*29	0.1	0	20

*CYP2D6*29* has been found to occur at a frequency of 20% in Tanzanians (Wennerholm *et al.* 2001), bringing to a median frequency of 41% total non-functional and reduced function alleles for black African populations. It can be seen from the above table that allele frequencies differ amongst different populations. The fact that the *CYP2D6*29* allele was identified in only one out of 672 subjects (0.1%) of European descent (Marez *et al.* 1997) shows that it is African specific, and the same is true for *CYP2D6*17, whilst *CYP2D6*10 is Asian specific and *CYP2D6*4 is Caucasian specific. This has implications in the average drug dose that is given in a particular population. For example, the Oriental populations generally use lower doses of some psychotropic drugs than Caucasians (Lou 1990). Few studies have been carried out in African populations. The clinical significance of drug metabolising enzyme polymorphisms in Africans has also not been studied to any significant extent. In 2003, two novel haplotypes of the *CYP2D6* gene were identified in a Japanese population (Yamazaki *et al.* 2003). Both *CYP2D6*44* and *21B alleles caused splicing errors leading to impaired CYP2D6 function. The frequencies of the two mutations were 0.6% and 1.7% respectively. Twenty nine percent of Ethiopians (Aklillu *et al.* 1996), 10% Spaniards and 10% of the populations in Italy and Turkey are UMs, whereas UMs are 1-2% in Northern Europe, 5.5% in Western Europe and essentially absent in Asia. This shows that there is heterogeneity among the Caucasians themselves. Mean CYP2D6 activity in Asians of the EM phenotype is lower than in Caucasians. This is mainly due to *CYP2D6*10 C100T mutation leading to a Pro34Ser amino acid substitution and the formation of an unstable enzyme with decreased activity (Nakamura *et al*. 2002). *CYP2D6*10* allele has been found to have an effect on the pharmacokinetics of loratadine in Chinese subjects with homozygotes having higher AUC and Cmax than heterozygotes or wild type individuals (Yin *et al*. 2005). The results of this study suggest the presence of CYP2D6 dependent pharmokinetics of loratadine in Chinese subjects. Despite some overlap between wild type, heterozygotes and homozygotes in clearance and metabolic ratios, significant correlations between the number

of *CYP2D6**10 alleles versus loratadine clearance or MR were observed, indicating a gene-dose effect (Yin *et al.* 2005). It must however be noted that there were no significant differences in pharmacokinetic parameters between wild type and heterozygotes. This may mean genotyping may only be beneficial for homozygotes unless the drug of interest has a really narrow therapeutic window.

The *CYP2D6**17 and *CYP2D6**29 variants lead to an enzyme with altered substrate specificity (Wennerholm *et al.* 2002). This means the binding of certain drugs to the enzyme and their metabolism is altered for some drugs but not for others. It is hence not very accurate to classify individuals with this allele as IMs or PMs for all drugs that depend upon the *CYP2D6* gene for their elimination from the body.

It would be expected that UMs would need higher doses of active drug to achieve therapeutic plasma concentrations than EMs. This would apply more to drugs with an inactive metabolite. If the metabolite is active, they may actually need a lower dose of the parent compound since they will be forming more of the active metabolite than EMs. On the other hand, IMs as well as PMs would need lower doses of a drug whose main route of elimination is by CYP2D6 metabolism. Conversely, they may experience treatment failure if it concerns a pro-drug whose therapeutic effect depends upon metabolism to an active metabolite.

Since CYP2D6 is a highly polymorphic enzyme and exhibits wide variability, drug design schemes that avoid compounds from being high affinity substrates for CYP2D6 would be key to a successful product on the market. This means studies to determine whether a drug is a CYP2D6 substrate or not are necessary in the early steps of the drug development process. Drug companies nowadays screen drugs in the early development process and drop likely

CYP2D6 substrates where they have alternatives that are equipotent and have reasonable pharmacology (Ingelman-Sundberg 2005).

2.2 CYP2C9 VARIABILITY

CYP2C9 is the major enzyme responsible for the metabolism of the anticoagulant drug, warfarin (Joffe *et al*. 2004). A cytosine to thymine (C>T) transversion at nucleotide 430 encodes for a cysteine substitution at amino acid residue 144, producing the Arg144Cys (*CYP2C9*2*) a variant allele (Stubbins *et al*. 1996). An adenine to cytosine transversion at nucleotide 1075 encodes for a leucine substitution at amino acid residue 359, producing Ile359Leu (*CYP2C9*3*) (Stubbins *et al*. 1996). Also, a null polymorphism (*CYP2C9*6)* containing an adenine base-pair deletion at nucleotide 818 has been identified, which leads to a premature stop codon and a truncated, inactive protein (Kidd *et al*. 2001). *CYP2C9*2* and *CYP2C9*3 are reported to show 12% and 5% lower activity respectively, of wild-type enzyme activity apparently as a result of the amino acid substitutions altering the activity of the enzyme with CYP oxidoreductase (Aithal *et al*. 1999). A study on 150 Africans revealed that 94% of them were homozygous wild type, 4% had *CYP2C9*2*, 2% had the *CYP2C9*3 genotypes (Scordo *et al*. 2001). None of them had the homozygous *CYP2C9*2* or *CYP2C9*3* or *CYP2C9*2/*3* mutation. In contrast, the frequency of CYP2C9*2 in and CYP2C9*3 in Caucasians is 15% and 8% respectively, whilst the polymorphisms exist at low frequencies in the Chinese, similar to Africans (Yang *et al.* 2003)

Phenytoin is metabolised to a great extent by CYP2C9 and partly by CYP2C19 and it is one of the drugs that normally requires therapeutic drug monitoring (TDM) to maintain plasma concentrations within the therapeutic window (Giancarlo *et al*. 2001). This is because it has a narrow therapeutic window with a small concentration difference between sub-therapeutic

and toxic drug plasma levels. Van der Weide *et al* (2001) studied the effect of *CYP2C9* genotype on phenytoin dose requirement in relation to phenytoin serum concentration on 60 Dutch epileptics receiving long term phenytoin treatment.   The highest mean plasma concentration was found in the two patients with the *CYP2C9*2/ *3* genotype, and yet they were actually given the lowest dose of phenytoin. Even though 47 of the patients were on concomitant anti-convulsant medication (phenobarbitone, carbamazepine or sodium valproate), the authors claim the mean required phenytoin dose for carriers and non-carriers of *CYP2C9* mutant alleles did not markedly differ between the different comedication groups, including those on phenytoin alone (van der Weide *et al*. 2001b).   It however seems that CYP2C9 enzyme activity is much more dependent on the genotype than enzyme induction or inhibition. The impact of the CYP2C family on the metabolism and plasma concentration of phenytoin has been investigated in Japanese patients with epilepsy by Mamiya *et al* (1998). Decreased rates of metabolism were observed in patients with *CYP2C9* or *CYP2C19* mutations (Mamiya *et al*. 1998). However, the statistical power of observing the effect of the *CYP2C9* mutations was limited because the frequency of the mutations was found to be very low (just over 2%), yet the sample size was 134 patients (Mamiya *et al.* 1998).

Based on *CYP2C9* genotype, PMs have been shown to require lower doses to achieve the required amount of anti-coagulation with warfarin. A number of warfarin studies have demonstrated significant associations between the possession of one or more *CYP2C9* alleles and low dose requirements (Lindh *et al*. 2009; Oner Ozgon *et al*. 2008; Sconce *et al*. 2005). In most cases, the dose was lowest when *CYP2C9*3* was present. However, findings related to adverse effects of anti-coagulation are less clear. Alternatively, warfarin resistance has also been documented, with some individuals requiring very high doses to achieve the necessary anti-coagulation (Nevruz *et al.* 2009). A number of possible explanations exist, one of which is that the metabolism of warfarin is higher in such individuals, or

malabsorption may be taking place (Sabol *et al.* 2009). Indeed, low plasma concentrations have been found in some people, even when given high doses. Another explanation is a variation in vitamin K epoxide reductase complex subunit 1 (VKORC), the enzyme which warfarin inhibits as a means of exerting its anticoagulant effect (Hildebrandt and Suttie 1982). It is thus well known that any individual on warfarin therapy needs to be monitored, as is indeed the case with the international normalised ratio (INR) being used as an indicator of the extent of anti-coagulation achieved. Some warfarin dosing algorithms have been proposed, encompassing CYP2C9 and VKORC, age and height (Sconce *et al.* 2005).

Irbesartan is a potent antihypertensive drug which acts as an angiotensin II receptor blocker. It is primarily metabolized through oxidation by CYP2C9 and its pharmacokinetics and therapeutic effect were examined in Chinese hypertensive patients (Hong *et al.* 2005). It was shown that there was no significant interaction between environmental factors (age, gender, smoking status, body mass index) and the plasma concentration of irbesartan (Hong *et al.* 2005). However, the *CYP2C9*3* allele was found to result in significantly higher drug plasma levels. On the other hand, this did not translate to therapeutic differences, as there was no significant difference in blood pressure reduction between *CYP2C9*1* carriers and *CYP2C9*3* carriers (Hong *et al.* 2005). Also, the relationship between CYP2C9 phenotype and genotype was investigated in vitro as well in healthy Turkish subjects. The authors found that the *CYP2C9*3* genotype, but not the *CYP2C9*2* genotype significantly impaired the metabolism of losartan (thus impaired enzyme activity) (Babaoglu *et al.* 2004).

2.3 CYP2C19 VARIABILITY

CYP2C19 has been shown to be polymorphic, with populations being divided into poor and extensive metabolizers. Ethnic variations exist, with 2-5% of Caucasians being PMs, 6% Africans being PMs and 13-23% Asians being PMs (Goldstein *et al.* 1997). One of the major

molecular defects in poor metabolizers is a single base pair (G>A) mutation at position 681 in axon 5. The mutation (*CYP2C19*2*) creates an aberrant splice site which produces a premature stop codon and a truncated 234 amino acid protein which lacks the heme-binding region and is therefore inactive. About 60% of PMs are homozygous for this allele. A second allele, *CYP2C19*3* consists of a G>A transition at position 636 which changes the codon for tryptophan at position 212 to a premature stop codon. The result is a truncated 211 amino acid polypeptide containing only the first 4 exons of the *CYP2C19*. This also leads to an inactive enzyme (De Morais *et al*. 1994a; de Morais *et al.* 1994b). Other null mutations include a mutation in the initiation codon (CYP2C19*4) and a splice mutation in intron 5 (CYP2C19*7). Many other mutations most of which lead to an enzyme with lowered catalytic activity have been discovered to date (up to *CYP2C19*17*). Most, if not all of these mutations were discovered by sequencing *CYP2C19* in individuals who had been classified as PMs with either S-mephenytoin or omeprazole, which has been investigated as a possible probe drug.

In a study conducted on 84 Shona black Zimbabweans, it was found that 77% (n=65) were homozygous for the wild type gene, 19% were of the *CYP2C19*1/*2* genotype and 4% were homozygous *CYP2C19*2* (Masimirembwa *et al*. 1995). The *CYP2C19*3* allele was not found in this population, and neither is it found in Caucasians. Three out of 251 (0.4%) Tanzanian subjects were however heterozygous for the allele. *CYP2C19*3 on the other hand, accounts for about 20% of Asian PMs. About 2% of Japanese people were found to be homozygous for the *CYP2C19*3 allele, and 25% had the *CYP2C19*1/*3 genotype. A meta-analysis showed that subjects of African ancestry have a low frequency of the CYP2C19 PM phenotype and genotype; that the defective *CYP2C19* alleles are uncommon, and that a small proportion of heterozygotes exists in the EM subpopulation (Xie *et al*. 1999a; Xie *et al*. 1999b). Interestingly, 5538 individuals from 24 populations on 16 different islands of

Vanuatu were genotyped in one study. Of these, 61% had a PM genotype (*CYP2C19*2/*2, *2/*3* or *3/*3*) with substantial variation among the populations (38-79%). The overall frequencies of *CYP2C19*1* (wild-type), *CYP2C19*2*, and *CYP2C19*3* were 0.223, 0.633, and 0.144, respectively. This suggests that the majority of Pacific Islanders metabolize a wide variety of clinically important drugs e.g. proguanil to a significantly lower degree than the average European (Kaneko *et al.* 1999). Interestingly in that study, the CY2C19 PM had comparable or better therapeutic response to proguanil. This further supports the suggestion that proguanil itself also possesses antimalarial effects through a mechanism not similar to the antifolate cycloguanil. This negates the proposition that CYP2C19 PMs would be at risk of reduced therapeutic response or promote the selection drug resistant malaria parasites. A novel allele (*CYP2C19*17*) carrying -806C>T and -3402C>T has recently been found with a frequency of 18% in both Swedes and Ethiopians and 4% in Chinese subjects. This allele was found to be associated with ultra-rapid metabolism and predictions revealed that *CYP2C19*17* homozygotes would attain 35% to 40% lower omeprazole area-under-the-curve (AUC) than subjects homozygous for *CYP2C19*1* taking standard doses of omeprazole (Sim *et al.* 2006).

The effects of the *CYP2C19*2* heterozygote genotype on nelfinavir pharmacokinetics is discussed later on (Burger *et al.* 2006). In a study of the AUC of the proton pump inhibitors omeprazole, rabeprazole and lansoprazole in the Chinese, several fold higher AUCs were noted in PMs compared to EMs with a 7.5 fold difference for omeprazole (Qiao *et al.* 2006). *CYP2C19* polymorphism has also been said to affect cure rates of *Helicobacter pylori* infection when omeprazole was used in combination with 2 antibiotics. Individuals who were *CYP2C19*2* heterozygotes and homozygous for MDR-1 3435TT mutation had better cure rates than other patients (Gawronska-Szklarz *et al.* 2005). The effect of the *CYP2C19* genotype on omeprazole bioavailability has also been studied in the Japanese and they once

again found that PMs had a much higher absolute bioavailability than EMs (Uno *et al*. 2007). A *CYP2C19* gene-dose effect for the oxidation of proguanil to cycloguanil and 4-chlorophenylbiguanide in the Chinese was observed by Hoskins *et al* (2003). Their results confirm an important role for *CYP2C19* in the formation of both cycloguanil and 4-chlorophenylbiguanide in vivo (Hoskins *et al.* 2003).

2.4 PHARMACOGENETICS IN PSYCHIATRY AND SIDE EFFECTS OF DRUGS

Antipsychotics are drugs used in the management of psychoses but the conventional antipsychotics are associated with side effects due to blockade of some receptors as shown in the table below:

Table 2: Side effects of antipsychotics due to receptor blockade (Lynn Crismon and Dorson, 1997).

Receptor type	Side effects
Histamine H_1	Sedation, weight gain
Muscarinic	Urinary retention, cognition effects, sinus tachycardia, dry mouth, blurred vision
Alpha1 adrenergic	Orthostatic hypotension, reflex tachycardia, potentiation of antihypertensives
Dopamine D2 receptor	Extrapyramidal side effects, prolactin elevation

A potential area for the clinical application of pharmacogenetics is psychiatry. This is expected as a number of psychoactive drugs are metabolised mainly by CYP2D6 e.g. amitriptyline, whose half life is known to range from 9-27 hours, the basis of which is not well documented. Diminished CYP2D6 activity has been proposed to explain why Orientals experience side effects to psychotropic medications (Lin *et al*. 1991) when prescribed in doses established in Caucasians (Lou 1990). From empirical observations of drug efficacy and side effects, clinicians in Oriental populations have been prescribing some psychotropic drugs at doses lower than in Caucasians. It is now postulated that, besides possible differences in other pharmacokinetic parameters or receptor-drug interactions, the difference

in metabolism of the drugs is the basis of lower doses in the Chinese. As a result of such observations, the Japanese regulatory authorities do not depend on pharmacokinetic studies done in Caucasians to optimize drug doses but insist they be done with Japanese subjects (Shah 1993).

Prolonged sedation as an adverse effect of diazepam is a well known occurrence and this may be due to polymorphism in *CYP2C19*, the major enzyme responsible for diazepam metabolism (Bertilsson *et al*. 1989). This may also be surprising; when one considers the fact that diazepam is N-demethylated to desmethyldiazepam, which is an active metabolite with a half life of about 100 hours (Hoffman-La Roche, Valium product monograph, 2008). Phenytoin is a commonly prescribed anticonvulsant drug that has a narrow therapeutic window and actually requires therapeutic drug monitoring during treatment (Burt *et al*. 2000). An association between *CYP2C9* genotype and phenytoin dose requirements had been shown, with patients carrying at least one mutant *CYP2C9* allele having a mean phenytoin dose required to achieve a therapeutic serum concentration about 37% lower than the mean dose required by wild-type individuals (van der Weide *et al. 2*001a, 2001b).

In clinical practice, the antipsychotic drug thioridazine is contraindicated in patients known to have a genetic defect leading to decreased levels of activity of CYP2D6 (British National Formulary, 2001). A number of antipsychotics have narrow therapeutic ranges and some studies have shown a link between high plasma concentrations and incidences and severity of adverse effects. One study by showed that PMs with respect to *CYP2D6* had a substantially increased risk of being prescribed antiparkinsonian medications to counter EPS. This was only observed in drugs whose major route of elimination is CYP2D6 i.e. haloperidol, zuclopenthixol, perphenazine, and thioridazine (Schillevoort *et al.* 2002). A major setback in antipsychotic drug administration is the development of tardive dyskinesia (TD), which

occurs to a similar extent with most conventional antipsychotics but less so with atypical antipsychotics (Margolese *et al*. 2005). It may occur due to up-regulation of dopamine receptors in the nigrostriatal pathway (Blin *et al*. 1989; Casey 2004). However, the incidence of extrapyramidal symptoms (EPS) varies depending on the chemical class of the drug and its potency. The more potent antipsychotics have been shown to cause more incidences of EPS. TD is characterized by involuntary movements predominantly in the orofacial region and develops in about 20 % of patients (5% cumulative annual incidence) during long term treatment with typical antipsychotics (Jeste and Caligiuri 1993). A number of variables including ageing, duration of treatment, female gender are associated with an increased propensity to develop TD. The high prevalence of TD and its potential irreversibility are an important limitation of treatment with typical antipsychotics (Quitkin *et al*. 1977). The identification of factors contributing to development of TD may be beneficial for individualized treatment with antipsychotics (Margolese *et al*. 2005). It has been shown in several studies that there is a genetic basis for person-to-person variability in the risk for TD e.g. dopamine D3 receptor polymorphisms (Basile *et al*. 1999; Lovlie *et al*. 2000; Steen *et al*. 1997), serotonin receptors (Segman *et al*. 2001; Segman *et al*. 2000) i.e. at pharmacodynamic levels as well as at pharmacokinetic level. At the pharmacokinetic level, higher drug plasma concentrations may put patients at higher risk for developing TD, hence this depends on CYP2D6 activity. It must however be noted that the contribution of CYP2D6 to disposition of antipsychotics and other substrates is comparatively reduced during multiple versus single dose drug administration and considerable overlap in the distribution of antipsychotic serum concentrations between EMs and PMs at steady-state has been noted. Some studies have actually failed to demonstrate any impact of *CYP2D6* or dopamine D3 receptor polymorphism on the likelihood of developing TD (Garcia-Barcelo *et al*. 2001; Mihara *et al*. 2002).

In 2000, a study by Basile *et al* found that the Bsp120I(C>A) polymorphism in the first intron of the *CYP1A2* gene was significantly associated with typical antipsychotic induced TD. A higher mean AIMS (Abnormal Involuntary Movements Scale) score in patients homozygous for the C allele was found compared to heterozygotes and homozygotes for the C allele. The mean AIMS score in patients with the CC genotype (associated with reduced CYP1A2 inducibility) was 2.7 and 3.4 fold greater than those with the A/C or AA genotype respectively. The same pattern was observed when the groups were divided by ethnicity (Caucasian or African-American) or by smoking status (smokers or non smokers). Patients with the CC genotype for *CYP1A2* were at an increased risk to develop more severe TD compared with heterozygotes or homozygotes for the A allele (Basile *et al.* 2000).

Of particular concern is torsades de pointes due to prolongation of the QTc interval as a result of high plasma levels of thioridazine. This potentially fatal side effect has led to the withdrawal of thioridazine from the market in several countries by its manufacturer, Novartis in 2005. This is because the drug has been associated with prolongation of the QTc interval in a dose-dependent manner (World Health Organisation Pharmaceuticals Newsletter No. 4, 2005). An association has been found between the drug plasma levels, prolongation of the QTc interval and CYP2D6 debrisoquine hydroxylation status. This means PMs are at a greater risk of cardiotoxicity when given the same dose of thioridazine as EMs because they achieve higher plasma concentrations (Llerena *et al.* 2002b). Interestingly though, another study failed to find an association between *CYP2D6* genotype and frequency of adverse drug reactions to nortriptyline or fluoxetine, showing little clinical relevance of genotype in this regard (Roberts *et al.* 2004). Neither was an association between the selective serotonin reuptake inhibitors (SSRIs) fluoxetine or paroxetine induced hyponatraemia and *CYP2D6* genotype (Stedman *et al.* 2002). Polymorphism of *MDR-1* has been linked to a risk for nortriptyline-induced postural hypotension. Individuals homozygous for the C3435T

polymorphism had a greater frequency of postural hypotension than those heterozygous for this allele, who also had a higher frequency than those without the allele (Roberts *et al.* 2002b). The authors did not find an association between *CYP2D6* genotype and postural hypotension, even though nortriptyline is primarily metabolized by CYP2D6. Neither did they find an association between nortriptyline dose or plasma concetrations and *CYP2D6* genotype. The postural hypotension is attributed to α-1 receptor blockade in both the autonomic and central nervous systems. The authors propose that the mutation affects the activity of P-gp such that an accumulation of nortriptyline or its metabolites occurs in the brain, leading to the greater postural hypotension (Roberts *et al.* 2002b).

2.5 HIV PHARMACOGENETICS

The one-size-fits-all regimen of antiretroviral (ARV) therapy results in important interpersonal variation in drug concentrations and differences in susceptibility to drug toxicity. As much as one third of patients on various antiretrovirals (protease inhibitors or non nucleoside reverse transcriptase inhibitors) have been found to have drug plasma levels outside the therapeutic range i.e. either above or below. Inappropriate adherence could only explain 35% of sub-therapeutic drug concentrations which means that there are many other factors to consider, such as genetic factors (Molto *et al.* 2007).

A good example of particular relevance to Africans is the non-nucleoside reverse transcriptase inhibitor (NNRTI), efavirenz. This drug is mainly metabolized by CYP2B6 and partly by CYP3A4. The *CYP2B6*6* (G516T) mutation leads to decreased expression levels and function of the enzyme (Lang *et al*. 2001) and results in high plasma concentrations of efavirenz. This mutation occurs at a frequency of about 49% in Zimbabweans and has been shown to be associated with high drug levels, above the therapeutic margin, in a routine

clinical setting. A reduction of the 600mg daily dose by about 35% has been suggested for homozygous carriers of this mutation to maintain their drug concentrations within the therapeutic window (Nyakutira *et al.* 2008). One study showed that 40% of patients with the 516TT genotype had efavirenz concentrations above the therapeutic limit of 4µg/mL, compared to 19% heterozygotes and 5% wild type carriers (Rodriguez-Novoa *et al.* 2005). Additionally, this mutation has been shown to be associated with greater incidences of central nervous system side effects of efavirenz, such as sleep disorders and fatigue, due to the high plasma concentrations (Haas *et al.* 2004; Rotger *et al.* 2005). Other *CYP2B6* mutations have been identified but occur at frequencies of only 1-2% at most (Klein *et al.* 2005). Another CYP2B6 mutation, *CYP2B6*16* (983T>C) has been found in Africans at a frequency of 7%, and 4% in Turks, whilst it was absent in Swedes. This mutation leads to decreased expression of CYP2B6, although catalytic activity is not greatly affected. Individuals with this mutation were found to have impaired metabolism and thus higher plasma concentrations of efavirenz (Wang *et al.* 2006).

Another study investigated nelfinavir and its active metabolite, nelfinavir hydroxyl-t-butylamide (M8). Burger *et al* (2006) found a significant difference between the wild type and heterozygotes for *CYP2C19*2* with respect to the metabolic ratio but indicated that further studies were needed to check for differences in virological responses since both are pharmacologically active. The heterozygotes had a lower M8/nelfinavir ratio, indicating higher nelfinavir plasma levels than the wild-type (Burger *et al.* 2006).

Some *MDR-1* allelic variants have predicted the rate of immunorecovery (increase in CD4 cells) after the start of antiretroviral therapy which underscores the relevance of transporters for access of ARVs to privileged pharmacological compartments. Patients with a TT3435 genotype showed a greater rise in CD4-cell count after treatment with nelfinavir, a substrate

for P-gp, for 6 months than patients with CC3435 or CT3435 (Fellay *et al.* 2002). On the other hand, another study reported no influence of *MDR* G2677T/A and C3435T or the haplotype on the virological and immunological response in treatment naïve HIV positive patients (Winzer *et al.* 2005). It must be noted that some of the antiretrovirals that the patients were on were not substrates of P-gp. Nasi *et al* (2003) analyzed data of 149 treatment naïve patients who were treated with a protease inhibitor containing regimen (n=106) or NNRTI-containing regimen (n=46) and found no association between *MDR-1* genotype at position 3435 and the CD-4 cell count increases or plasma viral load decreases during the first 6 months of treatment (Nasi *et al.* 2003). These conflicting results show that *MDR-1* polymorphism is not yet fully understood and requires further investigation.

Certain genotypes have also been shown to increase the risk of individuals getting side effects to ARVs. APOC3 gene, which codes for apolipoprotein CIII, polymorphisms have been associated with hyperlipidemia with some protease inhibitors e.g. ritonavir. Tarr *et al* (2005) conducted a study and found that the effects of variant alleles of APOE on plasma cholesterol and triglyceride levels, and of APOC3 on plasma triglyceride levels, were comparable to those in the general population. However, when treated with ritonavir, individuals with unfavourable genotypes of APOC3 and APOE were at extreme risk of triglyceridaemia. They had median plasma triglyceride levels of 7.33 mmol/L compared with 3.08 mmol/L in the absence of ART. Therefore, genetic analysis may identify patients at high risk for severe ritonavir-associated hypertriglyceridaemia (Tarr *et al.* 2005).

A genetic link to abacavir hypersensitivity has also been shown. The presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 genotypes had a positive predictive value of 100% for hypersensitivity to abacavir (Mallal *et al.* 2002). Genotyping for the HLA-B*5701 prior to

prescribing abacavir appeared to be cost-effective in some instances in one study (Hughes *et al.* 2004).

2.7 THE FUTURE OF PHARMACOGENETICS

Pharmacogenetics is slowly paving its way into routine clinical practice, although it is better appreciated and understood by pharmaceutical companies. The companies have moved from thinking about whether or not they should include pharmacogenetics in drug development, to how they can actually implement it. Some companies are now performing clinical pharmacogenetics trials e.g. Daiichi pharmaceuticals of Japan ran clinical trials of genetic tests to identify patients most likely to experience severe adverse reactions to the anticancer drug, irinotecan. The issue of population specific prescribing is gaining increasing recognition as evidenced by clinical trials and drugs that have been registered for specific groups. A good example is Bidil®, a combination of isosorbide dinitrate and hydralazine for treatment of heart failure in black patients (Taylor *et al.* 2004). The FDA recently approved labelling changes for Coumadin® (warfarin) to include a dosing algorithm that encompasses genotyping for *CYP2C9* and *VKORC-1*. It approved the Nanosphere Verigene Warfarin Metabolism Nucleic Acid Test which detects some variants of both genes (FDA 2007, http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2007/ucm108984.htm).

In addition to concentrating on single genes, research on all pathways affecting drug response is required to understand the causes behind pharmacokinetic and pharmacodynamic inter-individual variation. Also, pharmacogenetics researchers in the past have focused on inherited genetic variation and ignored somatic cell mutations and other epigenetic effects e.g. DNA methylation or histone modification that can alter gene expression of the cells (Nakajima *et al.* 2003; Sissung *et al.* 2006). The future of pharmacogenetic and genomic research will be a mixture of genome-wide SNP and expression analysis in appropriately

designed clinical studies, and this will have to be combined with in vitro and ex vivo pharmacogenomic research with human cells and model organisms and with human pharmacological research. Finding the right combination of research tools may be the most important demand. Due to the ever-increasing mass of pharmacogenetic information, medical information technologies including bioinformatics will be essential in the future of clinical pharmacogenetics (Brockmoller and Tzvetkov 2008). The FDA has also recently approved changes to the package labels for 6-mercaptopurine (metabolised by TPMT) and irinotecan (metabolised by UGT1A1) but the information falls short of specific dose reduction strategies for individuals who are found to be heterozygous or homozygous PMs, due to lack of clear cut clinical data, showing the need for further clinical studies. The revised labels also did not specifically require genotyping patients before administering drugs because there was little information pertaining to clinical outcomes of individuals without dose adjustments, particularly heterozygotes who may not require dose reduction (Haga *et al.* 2006).

An important development required to bring pharmacogenetics to the clinic, apart from the SNP demonstrating clinical effect, is a reliable diagnostic tool that can be used routinely e.g. the Roche Amplichip® (Jain 2005) for *CYP2D6* and *CYP2C19* mutations, Elucigene™ manufactured by Orchid Cellmark for cystic fibrosis mutation testing, among others (Feldmann *et al.* 2001). These tests must then be marketed appropriately by the manufacturers. FDA approval of the diagnostic test would also be an added advantage (Jain 2005). Genotpying before drug prescription is easier to implement when relevant for chronic diseases where the patient can afford to wait for a day or two before the genotyping results become available. For acute cases where treatment must be given as soon as possible, pharmacogenetics may have to wait until an age when genotyping results can be made available instantly.

In summary, there are several examples showing the potential for the application of pharmacogenetics but few studies have been carried out in routine clinical settings, let alone in African patients. Most studies have been single dose studies carried out healthy Caucasian volunteers. Such results must be interpreted with caution when it comes to routine clinical settings, and Africans in particular bearing in mind the genetic differences with Caucasians mentioned above. In addition, there may be several environmental factors that may influence drug response, apart from genetic factors. Hence studies in the relevant population in the relevant setting are required to determine the clinical significance of pharmacogenetics. This is especially so considering the high prevalence of the *CYP2D6*17* polymorphism in Africans. Africans may also differ among themselves because of the great diversity of the African continent. Hence different ethnic groups amongst Africans may have different frequencies of polymorphisms and this also needs investigation.

2.8 TECHNOLOGICAL PLATFORM

PCR is a revolutionary technology developed by Kary Mullis in the 1980s based on using the ability of DNA polymerase to synthesize a new strand of DNA complementary to the offered template strand (http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechPCR.shtml). PCR technology has advanced over the years. Nowadays real-time PCR is available which permits the analysis of the products while the reaction is actually in progress. This is achieved by using various fluorescent dyes which react with the amplified product and can be measured by the instrument. This also facilitates the quantitation of the DNA (http://www.horizonpress.com/pcr/, (Arikawa *et al*. 2008; Pagliarulo *et al*. 2004). Hotstart technology that prevents DNA polymerase extension until sufficiently high temeperatures are reached is another advancement in PCR technology. This approach enables higher specificity of the PCR amplification and reduce formation of off-target products (Koukhareva *et al*.

2008; Lebedev *et al.* 2008). DNA sequencing is another method developed for determine the sequences of bases in DNA. Its origins date back to the 1970s where by the Maxam-Gilbert methods (Maxam and Gilbert 1977) and the chain-termination methods (Sanger and Coulson 1975; Sanger *et al.* 1977) were described. Nowadays new sequencing technologies have enabled discovery of novel polymorphisms and high throughput DNA sequencing for both human and microbial genetic material (Chan 2005; Hall 2007). Microarray technology for the analysis of tens to thousands of SNPs on chips is the current platform of choice to multiplex the simultaneous analysis of some SNPs. Various types exist from the medium size and subject specific chips e.g the Roche Amplichip to the high density Ilumina chip which analyzes over 6000 SNPs (Jain 2005; Kirov *et al.* 2006; Li *et al.* 2008).

High performance liquid chromatography (HPLC) is one of the most widely used analytical techniques in the pharmaceutical industry because of its reliability and superior performance (Zhang *et al.* 2008). Several types of detectors for HPLC are used, depending on the type of assay including atomic absorption detectors, fluorescence detectors, ultraviolet light (UV) detectors, electrochemical detectors and mass spectrometric detectors (MS) (LaCourse 2002). The most widely used is probably the UV detector. However, MS is nowadays the premier tool for *in vitro* and *in vivo* assays that are part of new drug discovery (Korfmacher 2005). MS detection has allowed analysis times to be reduced by up to 80% making it an attractive choice in industry (Plumb *et al*. 2008) where high throughput assays are essential.

3. MATERIALS AND METHODS

The thermocyclers used for the PCR reactions were GeneAmp 2700® from Applied Biosystems (Singapore) and a PTC-100™ Programmable Thermal Controller from MJ research (Watertown, MA, USA). Viewing of the agarose gels was done using the GelPhotoSystem GFS1000 from Fran Techtum Lab (AB, Sweden), as well as GeneFlash from Syngene Bioimaging (Cambridge, United Kingdom). The UV-Visible spectrophotometer (Model UV-160) was manufactured by Shimadzu Corporation (Kyoto, Japan).

Ethidium bromide, EDTA and JumpstartRedAccutaqLA DNA polymerase were bought from SIGMA (St Louis, MO, USA). Agarose gel, Trizma base, 25 mM magnesium chloride, SmaI restriction enzyme and Molecular Weight Marker VIII were purchased from Roche Diagnostics (Mannheim, Germany). The Fermentas DNA ladder mix was sourced from Fermentas Life Sciences (Inqaba Biotechnical Industries, South Africa). The PCR primers and other restriction enzymes were from Eurogentech (Geneva, Switzerland) and New England Biolabs, NEB (Beverly, Massachusetts, USA) respectively. For some nested PCRs, Taq DNA polymerase used was obtained from the Tobacco Research Board (Harare, Zimbabwe). dNTPs were purchased from Boeheringer Mannhein (Mannheim, Germany). All reagents used were PCR grade. The reference standards for chlorpromazine, thioridazine, fluphenazine, haloperidol, amitriptyline and nortriptyline were a generous donation from AstraZeneca, Gothenburg, Sweden. The drug analysis was done on an API4000 LC-MS-MS machine (Applied Biosystems, USA). Mobile phase solvents used were of HPLC grade.

3.1 ETHICAL APPROVAL

Ethical approval to carry out the study was obtained from the Joint Parirenyatwa Hospital and College of Health Sciences Ethics Committee as well as the Medical Research Council of Zimbabwe.
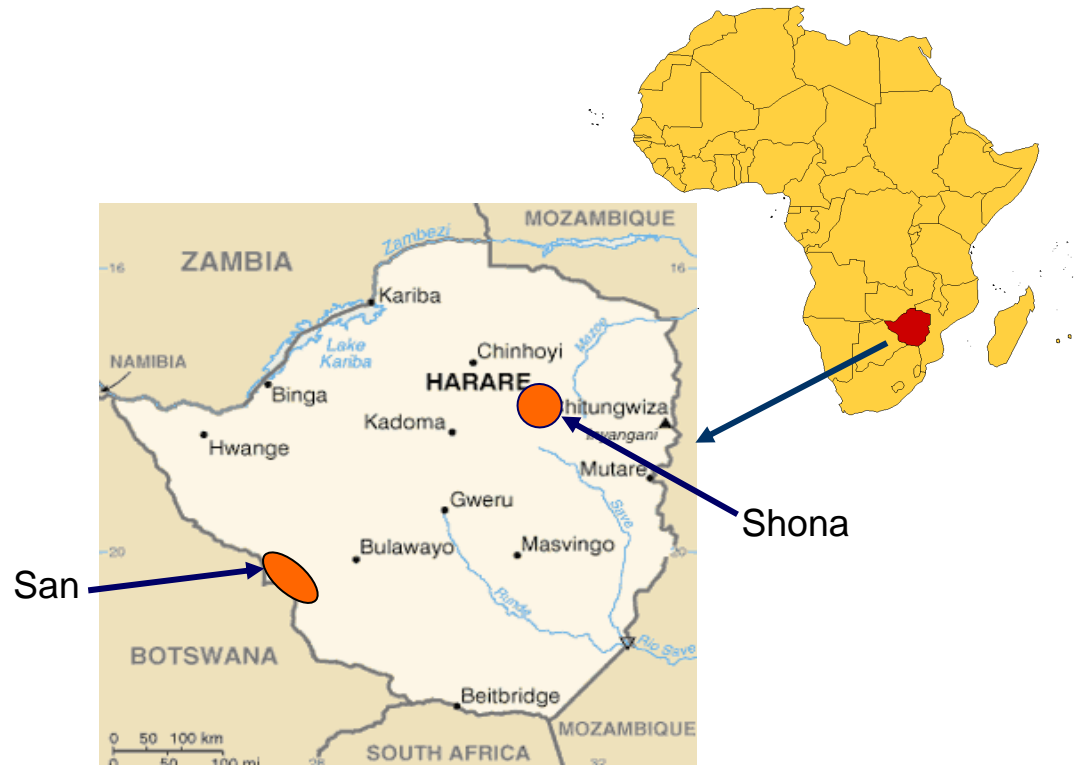
Figure 1: Geographic recruitment locations of the Shona and San volunteers in Zimbabwe

3.2 RECRUITMENT OF VOLUNTEERS

As mentioned above, a genetic polymorphism is defined as a variation in a DNA sequence occurring in at least 1% of the population. Hence using the equation:

$$ n \ = \ \frac{t^2 \text{ x } p(1-p)}{m^2} $$

where n = required sample size

t = confidence level at 95% (standard value of 1.96)

p = estimated prevalence of genetic polymorphisms (at least 1% i.e. 0.01)

m = margin of error at 1.5% (value of 0.015),

it can be seen that a sample size of about 169 alleles will enable us to state the presence/absence of genetic polymorphisms in the ethnic groups. Since each individual has 2 alleles, we need about 85 patients in each ethnic group.

The Shona are the majority of Zimbabwe's population (>70%). Blood samples for the Shona participants were obtained from 78 University of Zimbabwe medical students after they gave written informed consent. Medical students were chosen because of their convenient location at Parirenyatwa hospital. This was very close to the collaborating African Institute of Biomedical Science and Technology (AIBST) laboratory, where all samples were processed. The close proximity reduced transport costs and enable maintenance of the integrity of the samples. Self identification as Shona was the inclusion criteria. The subjects also had to confirm that both their maternal and paternal grandparents were Shona. Failure to meet the above requirements was the exclusion criteria. A phlebotomist would draw blood samples into vacutainers, containing EDTA as the anticoagulant, which were then put in a cooler box with ice. The samples were then transported to the laboratory, where they were aliquoted into cryotubes and stored at -20 °C until needed.

The San live as isolated communities in the western parts of Zimbabwe. Apart from the above-mentioned bodies, approval to visit the San in Plumtree district was obtained from the Provincial Medical Director for Matebeleland South as well as the Plumtree District Administrator and the village heads. Although this study was driven by ethnicity and not geographical location, we went to Plumtree district because we were interested in the San population, and consultations with historians led us to find the San in Plumtree. In selecting the San volunteers, self identification of being a San, including both maternal and paternal grandparents was used as inclusion criteria. Although the possibility of dishonesty and intermarriages cannot be ruled out, the isolated lifestyle of the San minimizes the possibility

of intermarriages. We gathered from our visit to their community that they do not even go to schools or clinics in surrounding areas and they generally do not mix with anyone outside their villages. A nurse from the district hospital served as the translator between the study team and the San people. The blood samples were treated just like those of the Shona. We recruited 63 San volunteers from 2 villages in Plumtree district. These were the only villages reported to have the San residing in them, hence there were no more San volunteers in the area we could obtain to reach our target of 85.

We tried very hard to obtain volunteers from the coloured community of Zimbabwe. After approaching more than 200 different individuals, only 13 volunteered to donate 5ml of their blood for genotyping, giving a response rate of less than 6.5%. We tried several methods of getting to talk with the coloureds including visits to several shopping centres, going to churches in coloured neighbourhoods (Arcadia and Braeside), going to clinics in these areas, high schools, old peoples homes etc, but none of these avenues were successful. Our trouble here was the exact opposite of what we experienced with the Shona, where we had a long queue of medical students (mainly in their fourth or fifth year) waiting to donate their blood and we were literally overwhelmed by their response. The coloureds seemed to understand and agree with most of what we were saying with respect to the need for a database on genotypes of Zimbabwean ethnic groups, but when it came to the part about drawing a 5 mL blood sample, most of them flatly refused. Their refusal perhaps may have been because of fear of getting tested for HIV, even though we stressed that we were not going to test their blood for HIV as well as anonymising the blood samples. There were also no coloureds amongst the medical students or the psychiatric patients.

Our failure to obtain enough coloureds for a meaningful analysis was a major setback as we believe we would have found interesting results because of their admixture. Novel mutations

and unique allele distributions have been found in African-Americans (Gaedigk *et al.* 2005) and very recently in South African coloureds (Gaedigk and Coetsee 2008).

3.3 PSYCHIATRIC PATIENTS

Apart from the above-mentioned bodies, approval was also obtained from the Parirenyatwa Hospital Department of Psychiatry. Once again, Parirenyatwa Hospital was chosen because of its close proximity to AIBST. This was even more critical because blood samples containing drug for drug concentration determination have to be processed and frozen within the shortest possible time.

The patients were from a routine health care setting so our sample was representative of the general patient population in that particular setting in terms of the average age and gender distribution. 70% of our patients (35 out of 50) were outpatients and these were patients who were in a generally stable condition and who will have come for a monthly review and refill of their prescriptions. We excluded those whom the psychiatrist deemed were unable to make an informed decision on their own and/or whose next of kin were either unavailable or refused to give consent. In a few instances we excluded patients because we failed to draw blood from them due to extremely violent behaviour and some were just not cooperative. Blood samples from 50 psychiatric patients were collected over a period of about 10 weeks. Time did not permit the study team to go beyond 10 weeks since the drug concentrations needed to be measured thereafter, as well as the genotyping to be done. A total of 66 individuals were interviewed but only fifty agreed to take part in the study, giving a response rate of 76%. Each volunteer had blood drawn on one occasion. The study team went to the hospital 4 days a week when the psychiatrists would be present for evaluating the patients. After the patient's routine medical examination, and when the doctor was through with his

routine job, we would approach the patient for their consent to take part in the study. Written informed consent was given either by the subject or their next-of-kin if the subject was unable to make their own decision. The time that the patient last took their medication and the time the blood sample was drawn were recorded. The blood samples were then drawn by the doctor into two 5 mL vacutainers per patient and transported to the lab. One of each was then centrifuged and the plasma was stored at -20 °C within 2 hours of drawing the blood, until required for drug analysis. The other blood would also be stored at -20 °C until required for genotyping.

Since the psychiatric patients were from a routine clinical setting, various dosing regimens were used and it was necessary to adjust for these dosage variations for our analysis of results. The dose-corrected plasma drug concentrations were obtained by dividing the total daily dose of drug for each patient, by the plasma concentration i.e. a concentration/dose ratio was used (Grasmader *et al*. 2004). The patients underwent a routine medical examination as per current practice and no active liver or kidney malfunction was recorded by the medical personnel. None of the patient records had any information about hepatic or renal function thus we therefore assumed the patients had normal hepatic and renal function.

Figure 2 below is a flow diagram showing experimental proceduress for the ethnic group volunteers as well as the psychiatric patients.
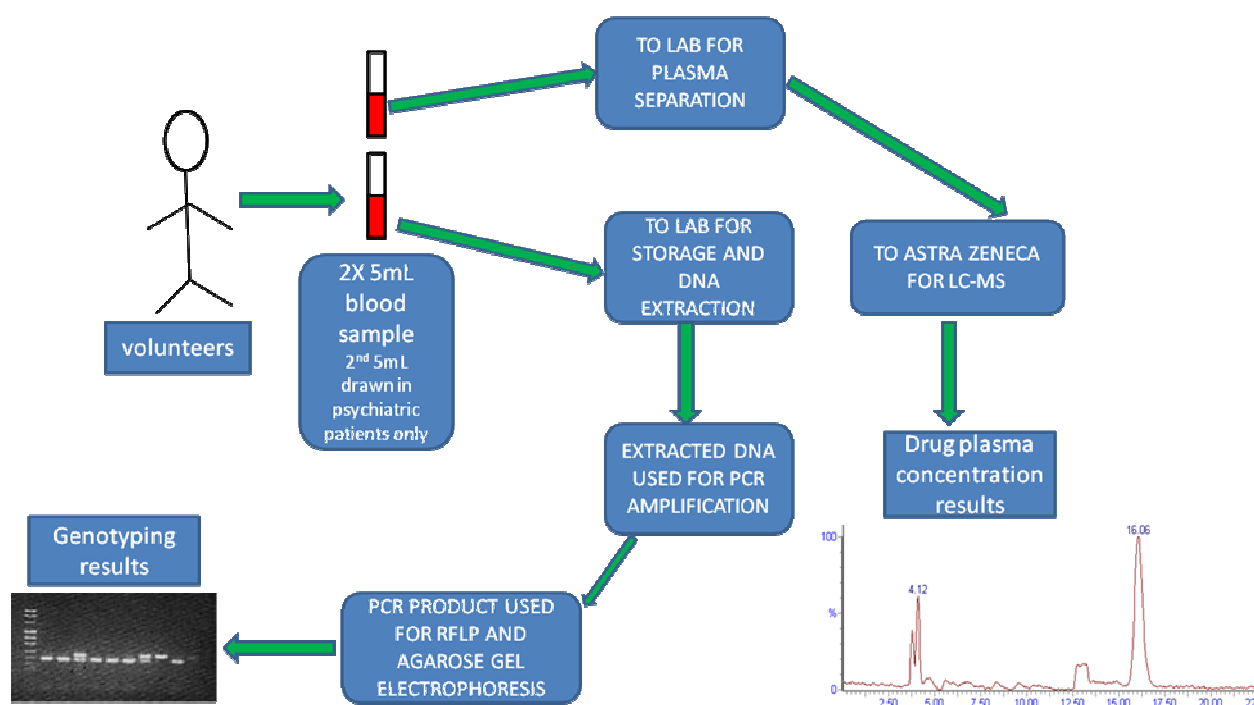
Figure 2: Flow chart of sample processing

3.4 GENOTYPING PROCEDURES

DNA was extracted from the blood samples using the Qiagen Inc. QIAamp DNA blood mini kit (Hilden, Germany). The DNA would then be stored either at 4 °C (short term storage) or -20 °C (long term storage) depending on how long it would be stored before being used. After each extraction, DNA concentration and purity for a few samples from the batch was measured using a UV spectrophotometer (Shimadzu Corporation, Model UV-160). The ratio of absorbance at 260 nm to 280 nm was used to determine the purity.

3.4.1 PREPARATION OF AGAROSE GEL FOR ELECTROPHORESIS

A 50X stock solution of tris acetate EDTA was prepared by mixing 242.28 g trizma base and 18.6 g EDTA. These were dissolved in about 900 mL water. The pH was then adjusted to 8.0 with acetic acid and the volume made up to 1000 mL with distilled water. This TAE buffer would be diluted 1:50 with water to prepare a 1X working solution.

To check amplification of the 5.1 kb *CYP2D6* gene, 60 mL of a 0.7% agarose gel was prepared by mixing 0.42 g agarose with 1X TAE buffer in beaker to a total volume of about 60 mL. For smaller fragments like the nested *CYP2D6* or *CYP2C19* or *CYP2B6* amplification, a 3% gel was prepared by mixing 9 g agarose with 300 mL 1X TAE buffer. The volume of the gel prepared depended on the number samples to be analyzed, larger gels being used for a greater number of samples. The agarose was then dissolved by boiling in a microwave. In labs without a microwave, a hotplate can be used. We chose to use a microwave because it is faster and more convenient. After the solution had cooled to about 50-60 °C, 10 mg/mL of ethidium bromide was added in a volume corresponding to between 5-10 µL per 100 mL of gel. The solution would then be poured into a suitable mould to solidify. The gel would then be placed into the electrophoresis tank so that samples can be loaded and then electrophoresis can take place.

3.4.2 CYP2D6 GENE AMPLIFICATION

The *CYP2D6* gene and the upstream *CYP2D7*pseudogene are highly homologous. Therefore to avoid interference and unspecific amplification from the *CYP2D7* gene, it is necessary to specifically amplify the *CYP2D6* gene first. Since the number of *CYP2D6* copies will now have by far outnumbered the few unamplified *CYP2D7* copies, the subsequent PCRs to detect specific *CYP2D6* mutations can be successfully carried out, without interference from *CYP2D7*. The 5.1 kb *CYP2D6* gene was amplified using a method modified from Gaedigk, Marcucci *et al*, 2003 (unpublished). The primers 5'-CCAGAAGGCTTTGCAGGCTTCAG-3' and 5'-ACTGAGCCCTGGGAGGTAGGTAG-3' were used to amplify the 5.1 kb fragment using the following conditions: initial denaturation at 94 ºC for 3 minutes, followed by 35 cycles at 94 ºC for 20 seconds, 58 ºC for 20 seconds and 72 ºC for 10 minutes. A final extension step at 72 ºC for 15 minutes completed the reaction. The PCR mixture consisted of 1 µL of DNA, 2.6 µL Accutaq LA buffer, 0.75 mM dNTP mixture, 0.5 mM of each primer,

3.5% DMSO, 2.5 mM magnesium chloride and 1.2 U Jumpstart RedAccutaq LA DNA polymerase. The above were made up to a total volume of 20 µL with water.

After the amplification was over, 3 µL of the PCR product was loaded onto a 0.7% agarose gel in an electrophoresis tank with 1X TAE buffer. The gel was then run at about 4.0 V/cm until the molecular weight marker was well resolved. When good amplification was observed, the PCR product was diluted one hundred fold by mixing 1 µL of the product with 99 µL of water. This diluted product was then stored at 4 °C to be used for the subsequent PCRs. The undiluted product was stored at -20 °C until required for another dilution. In some instances, the amplification was not visible on the gel. However, the nested PCRs were still visible and successful, whether or not the initial PCR product had been diluted or not. However, using undiluted product resulted in non-specific amplification being seen on the nested PCR product, though it did not interfere with results. The typical gel photo for this reaction is shown in Fig 4 in the results section.

3.4.3 CYP2D6 ALLELE GENOTYPING

The subsequent nested PCRs used to detect *CYP2D6* mutations *4, *10, *17 and *29 were performed using methods slightly modified from Gaedigk, Marcucci *et al*, 2003. The primers and annealing temperatures used in each PCR were different and specific for each mutation being investigated as shown in table 3 below. The primers were used to amplify the various fragments using the following conditions: initial denaturation at 94 ºC for 3 minutes, followed by 35 cycles at 94 ºC for 20 seconds, annealing for 10 seconds and 72 ºC for 20 seconds. A final extension step at 72 ºC for 6 minutes completed the reaction. In addition to 0.8 µL of diluted PCR product from the *CYP2D6* gene amplification, each PCR reaction contained 1.5 µL 10X PCR buffer, 0.16 mM dNTP mixture, 0.2 mM of each primer, 2.5 mM

magnesium chloride and 0.04 U Taq DNA polymerase. The above were made up to a total volume of 16 µL with water

To check if amplification was successful, 2 µL of the PCR product were loaded and ran on a 3% agarose gel. If the amplification was successful, digestion with restriction enzymes was then performed to detect mutations. The reaction mix for the digestions consisted of 1.35 µL of 10X buffer and 0.08-0.2 U of the restriction enzyme made up to 12 µL with water. At times the volume of restriction enzyme would be increased if the digestions were not complete. The above mixture would then be added to the PCR product and incubated overnight at the required temperature as shown in table 3.

Table 3: Primer sequences used for PCR, mutations to be detected, annealing temperatures, primer positions and incubation temperatures for digestion.

Primer Sequences	Mutation	Annealing temp/°C	Primer positions (Kimura)	Incubation Temp/°C
5'-CCAGAAGGCTTTGCAGGCTTCAG-3' and 5'-ACTGAGCCCTGGGAGGTAGGTAG-3'	*CYP2D6* entire gene	58	1279-1302 and 6350-6372	N/A
5'-AGATGCGGGTAAGGGGTCGCCTTCC-3' and 5'-TATGGGCCAGCGTGGAGCGAGCAGAGGCGC-3'	*CYP2D6*29	58	3242-3277 and 3430-3454	60
5'GTCGTGCTCAATGGGCTGGCGGCCGTGCGCGAGGCG-3' and 5'-GGTTTCTTGGCCCGCTGTCCCCACTC-3'	*CYP2D6*17	58	2557-2592 and 2785-2810	37
5'-CAGAGACTCCTCGGTCTCTCG-3' and 5'-AGAGGCGCTTCTCCGTGTCCA-3'	*CYP2D6 *4	58	3263-3284 and 3635-3654	60

The primers were designed according to the work done by Gaedigk, Marcucci *et al* and were then ordered from Eurogentech. The positions of the primers are as shown in the table above.

Genotyping for the *CYP2D6**4 allele required incubating the PCR product with BstNI restriction enzyme at 60 °C for at least 5 hours. The digestion product was then run on an agarose gel to yield results as shown in Fig 5 in the results section. The 392 bp PCR product would be digested in wild type individuals to 194, 161 and 37 bp fragments to give two visible fragments as shown above. A *CYP2D6**4 mutation would result in digestion to 355 and 37 bp fragments only, leading to one visible band. The 37 bp fragment was not visible on our gels due to its very small size.

Genotyping for the *CYP2D6**17 allele required incubating the nested PCR product with FokI restriction enzyme overnight at 37 °C. Wild type individuals remain with undigested product as can be seen from the 254 bp fragment (Fig 6 in results section), whilst a mutation results in digestion to two fragments of 180 bp and 74 bp size respectively.

Genotyping for the *CYP2D6**29 allele required incubating the nested PCR product with BstUI restriction enzyme at 60 °C for at least 5 hours. Wild type individuals have PCR product digested to two fragments 178 bp and 35 bp in size. Only the 178 bp fragment was visible and the 35 bp fragment could not be seen due to its small size (Fig 7 in results section). A mutation resulted in the 213 bp PCR product fragment remaining undigested as can be seen in Fig 7.

3.4.4 CYP2C19*2 GENOTYPING

The *CYP2C19**2 genotyping was performed according to a method modified from de Morais *et al*, 1994. The primers 5'-AATTACAACCAGAGCTTGGC-3' and 5'-TATCACTTTCCATAAAAGCAAG-3' were used to amplify a 169 bp fragment using the following conditions: initial denaturation at 94 ºC for 3 minutes, followed by 35 cycles at 94 ºC for 10 seconds, 53 ºC for 10 seconds and 72 ºC for 20 seconds. A final extension step at

72 ºC for 6 minutes completed the reaction. In addition to 1 µL of DNA, each PCR reaction contained 2.5 µL 10X PCR buffer, 0.3 mM dNTP mixture, 0.4 mM of each primer, 2.5 mM magnesium chloride and 0.05 U Taq DNA polymerase. The above were made up to a total volume of 25 µL with water.

Successful amplification would be followed by digestion at room temperature (about 25°C).The digestion mixture was comprised of 2 µL 10X buffer A (from Roche) and 0.5 U SmaI restriction enzyme made up to 5 µL with water. The mixture would then be added to the PCR mix in each tube, mixed and then incubated. The samples were then loaded on a 3% gel which would then be run at around 4-6 Volts/cm until the molecular weight marker was adequately resolved. The typical gel photo following digestion of PCR product with SmaI restriction enzyme at 25 °C overnight is shown in Fig 8 in the results section. The wild type results in digestion of the 169 bp PCR product into two fragments of 120 bp and 49 bp, whilst the mutant remains undigested.

3.4.5 CYP2B6*6 GENOTYPING (Modified from Rotger *et al*, 2005)

The *CYP2B6*6* genotyping was performed according to a method modified from Rotger *et al*, 2005. The primers 5'-GGTCTGCCCATCTATAAAC and 5'-CTGATTCTTCACATGTCTGCG-3' were used to amplify a 526 bp fragment using the following conditions: initial denaturation at 94 ºC for 3 minutes, followed by 35 cycles at 94 ºC for 20 seconds, 58 ºC for 20 seconds and 72 ºC for 1 minute. A final extension step at 72 ºC for 6 minutes completed the reaction. In addition to 1 µL of DNA, each PCR reaction contained 1.5 µL FastStart® 10X PCR buffer, 0.2 mM dNTP mixture, 0.2 mM of each primer, 3.3 mM magnesium chloride and 0.05 U FastStart® Taq DNA polymerase. The above were made up to a total volume of 15 µL with water.

Successful amplification was followed by BsrI digestion for 5 hours at 60 °C. To make the digestion reaction mixture, 0.3 U of BsrI were mixed with 1.5 µL of 10X NEB buffer 3 and made up to 5.9 µL with water. Digestion of the 526 bp PCR amplification product for the wild-type allele resulted in three fragments of 267 bp, 236 bp and 23 bp in size. The 23 bp fragment was not visible. A mutation resulted in digestion to 2 fragments only, of 503 bp and 23 bp, but only the 503 bp band was visible. In the gel photo (Fig 9 in results section), the last three wells show 516GG, 516GT and 516TT genotypes respectively.

3.5 DRUGS PATIENTS WERE RECEIVING

The table below shows the drugs that are commonly prescribed for psychiatric conditions in Zimbabwe, and that some of our study participants were receiving. The table also shows the common dosage, indication and typical side effects of the drugs.

Table 4: Dosages, indications and side effects of drugs commonly prescribed for psychiatric conditions (adapted from the British National Formulary, September 2001)

Drug	Dose	Indication	Typical side effects
Amitriptyline	25-200 mg daily	Depressive illness	Dry mouth, sedation,, blurred vision, constipation
Chlorpromazine (CPZ)	75-300 mg daily	Schizophrenia and other psychoses	Extrapyramidal symptoms, drowsiness
Thioridazine	50-600 mg daily	Schizophrenia	Extrapyramidal symptoms, drowsiness, QT interval prolongation
Haloperidol	5-30 mg daily	Schizophrenia and other psychoses	Extrapyramidal symptoms
Fluphenazine Decanoate	12.5-100 mg monthly	Schizophrenia and other psychoses	Extrapyramidal symptoms, systemic lupus erythematosus

Figure 3 below shows the chemical structures of the drugs, as well as those of nortriptyline (active metabolite of amitriptyline) and mesoridazine (active metabolite of thioridazine).

AMITRIPTYLINE

NORTRIPTYILINE

H₃C—N
CH₃

H₃C—N
H

CHLORPROMAZINE

N

N

S

Cl

FLUPHENAZINE

O

NH2

N

N

N

S

F
F
F

HALOPERIDOL

REDUCED HALOPERIDOL

THIORIDAZINE
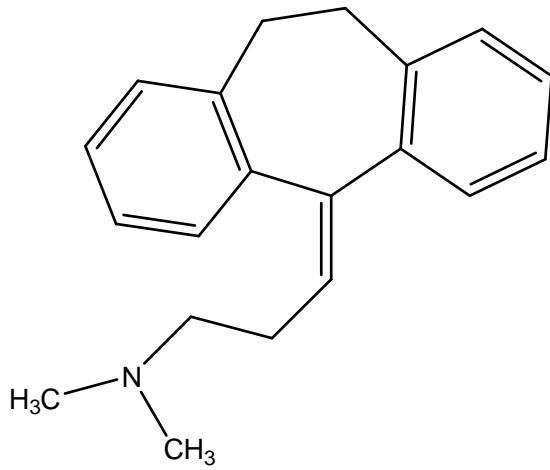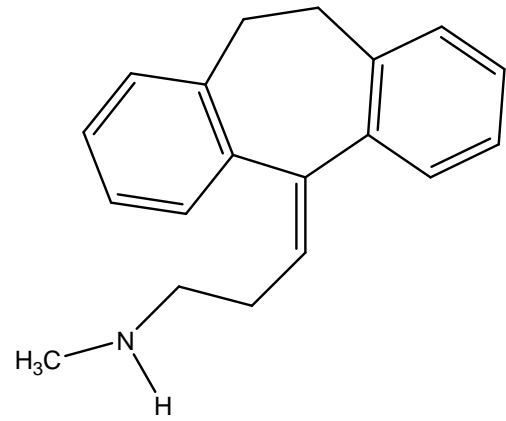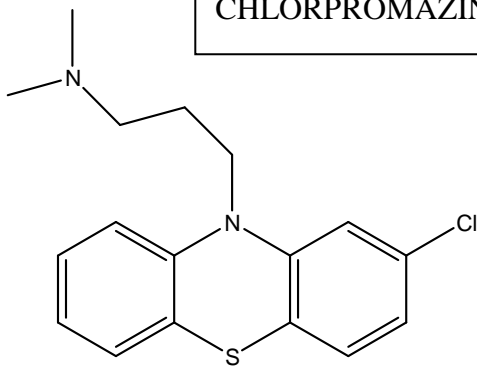
MESORIDAZINE
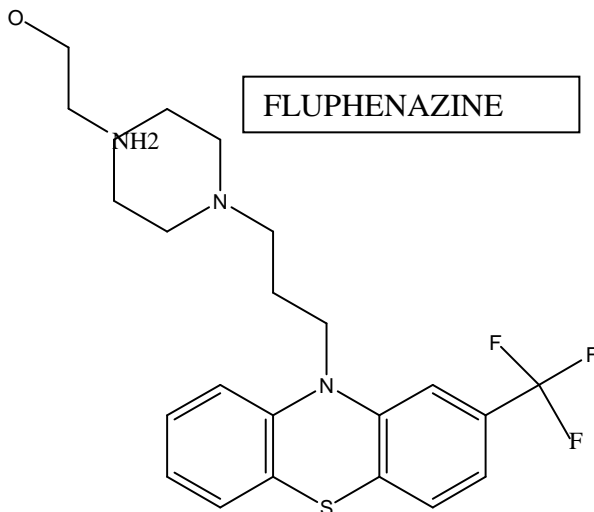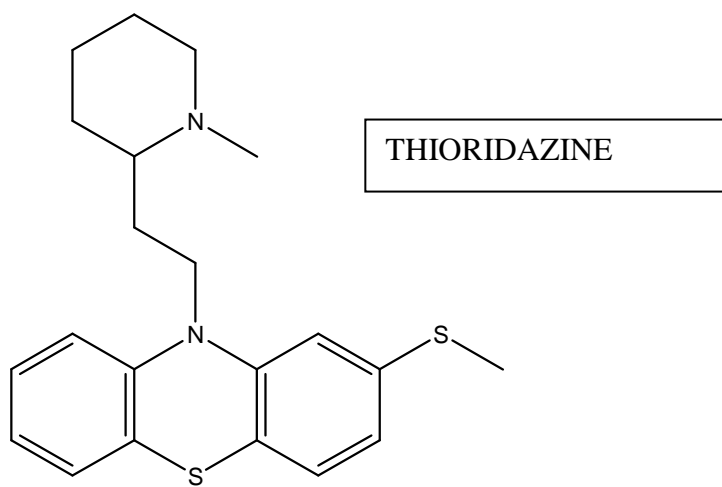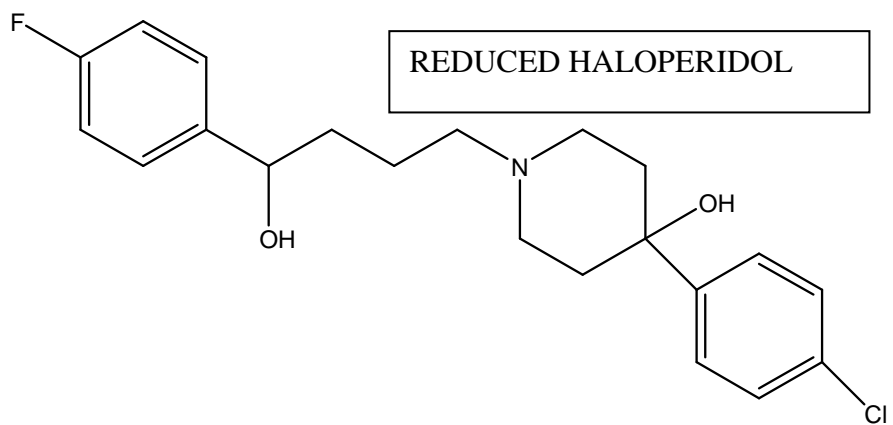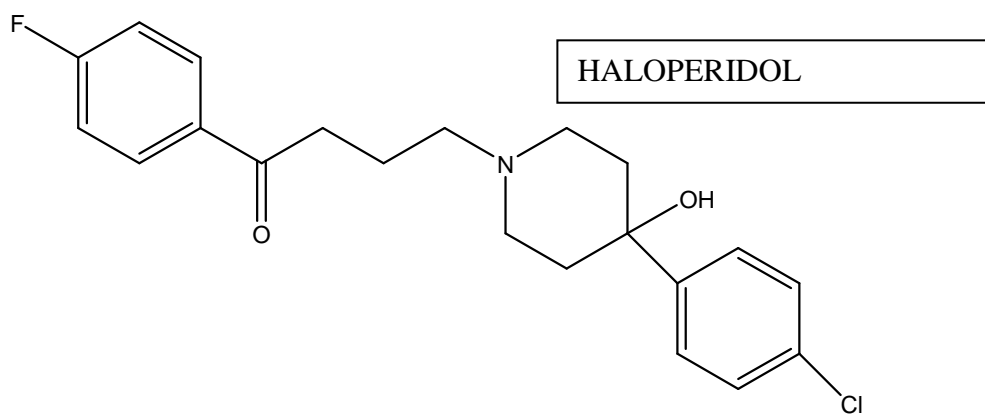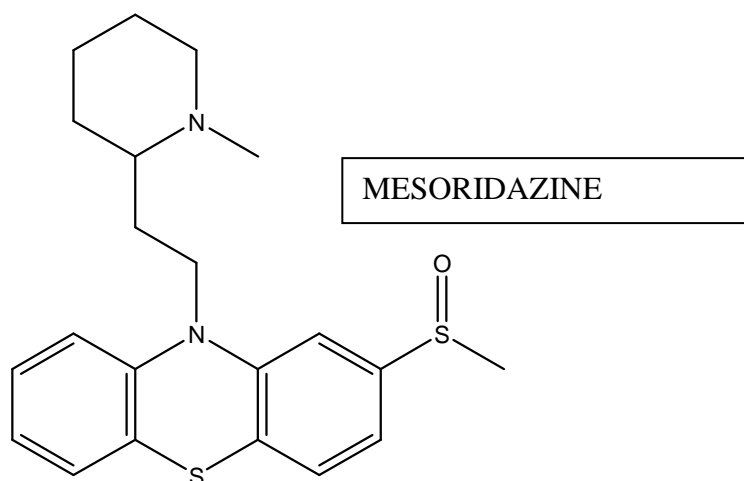
Figure 3: Chemical structures of the psychotropic drugs and some metabolites

3.6 LC-MS METHOD FOR MEASURING DRUG PLASMA CONCENTRATIONS

Plasma samples were sent to AstraZeneca, Sweden for high performance liquid chromatography with mass spectrometry detection (HPLC-MS) to determine the plasma concentrations of the psychotropic drugs in samples from patients and the method is outlined below.

3.6.1 Preparation of the standards:

Drug standards were diluted into the same tube with 10% acetonitrile (ACN) to obtain solution A of the following concentrations: 30 µM amitrptyline, 30 µM nortriptyline, 3 µM haloperidol, 30 µM CPZ, 150 µM thioridazine and 0.3 µM fluphenazine.

Two hundred microlitres of solution A was then diluted to 2000 µL and labelled solution B. Solution B was then serially diluted by a factor of 3 with blank plasma to obtain 6 levels of concentration of each of the drugs to be analyzed.

3.6.2 Extraction

One hundred microlitres of the spiked plasma standard and 100 µL of the samples were separately precipitated by addition of 600 µL of chilled ACN. Samples were then left for 30 mins at 4 °C to ensure complete precipitation. The samples were then centrifuged at 20000g for 10 mins at 4 °C. Two hundred microlitres of the supernatant liquids was drawn and then diluted with 200µl purified water for analysis of the rest of the drugs except for thioridazine which was further diluted. For thioridazine 200 µL supernatant was diluted with 800 µL of 50% ACN.

3.6.3 CHROMATOGRAPHIC CONDITIONS

The HPLC system model was Agilent HP1100 and the MS detector model was API4000. A 10 cm, C18 column was used. The mobile phase comprised of 45 parts 1% glacial acetic acid in water, and 55 parts acetonitrile. The flow rate was 1mL per minute. The acquisition parameters are as shown in table 5 below:

Table 5: Acquisition parameters

DRUG NAME	Q1(m/z)	Q3(m/z)	DP(V)	CE(V)	CXP(V)	RT(mins)
Amitriptyline	278.2	233.2	50	2	15	3.46
Nortriptyline	264.3	233.1	42	2	15	3.42
Haloperidol	375.9	165.2	50	34	15	3.12
CPZ	319.2	86.2	50	33	17	3.55
Thioridazine	371.2	126.3	60	33	10	3.82
Fluphenazine	438.1	171.2	80	36	12	3.62

Q1=parent ion; Q3=daughter ion; DP=Declustering potential; CE= Collision energy; CXP= collision cell exit potential; RT=retention time.

4   RESULTS

4. 1 TYPICAL PCR-RFLP RESULTS FOR *CYP2D6, 2C19* AND *2B6*

Samples in 0.7% agarose gel

MWM
Fermentas DNA
Ladder mix

5.1kb 2D6 fragments
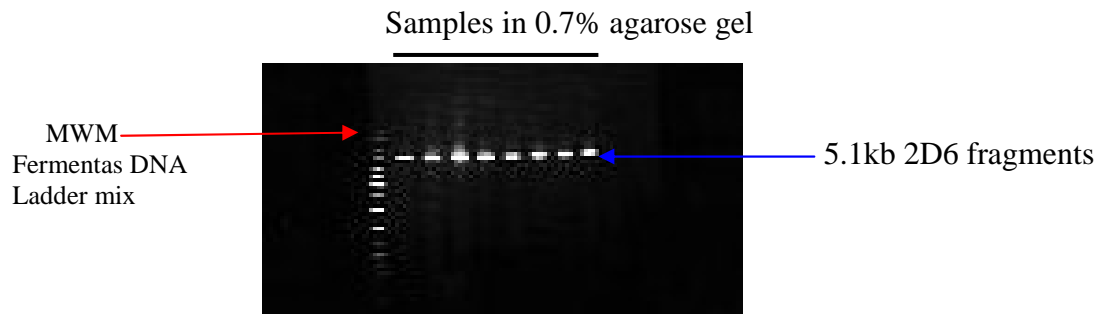
Figure 4: *CYP2D6* gene amplification gel photo.

Samples on 3% agarose gel

Roche MWM VIII

355 bp

161 bp

194bp
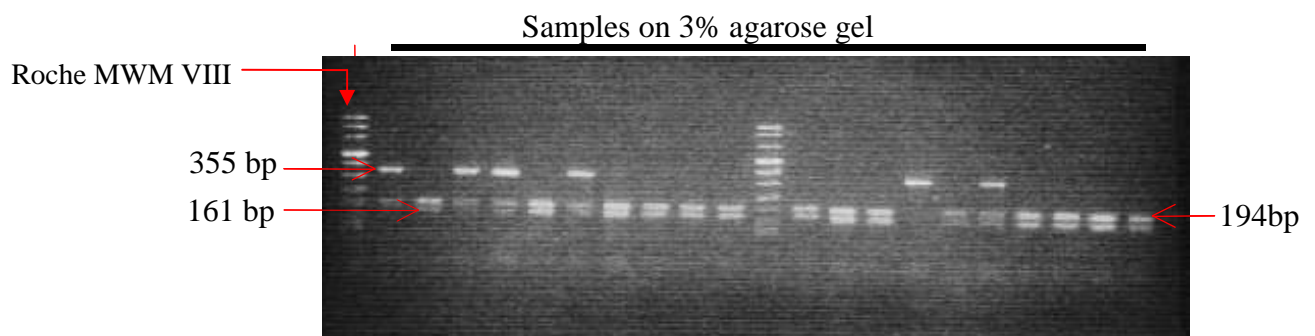
Figure 5: *CYP2D6*4 BstNI digestion results gel photo

Roche MWM
VIII

Samples on 3% agarose gel

254bp

180bp

74bp
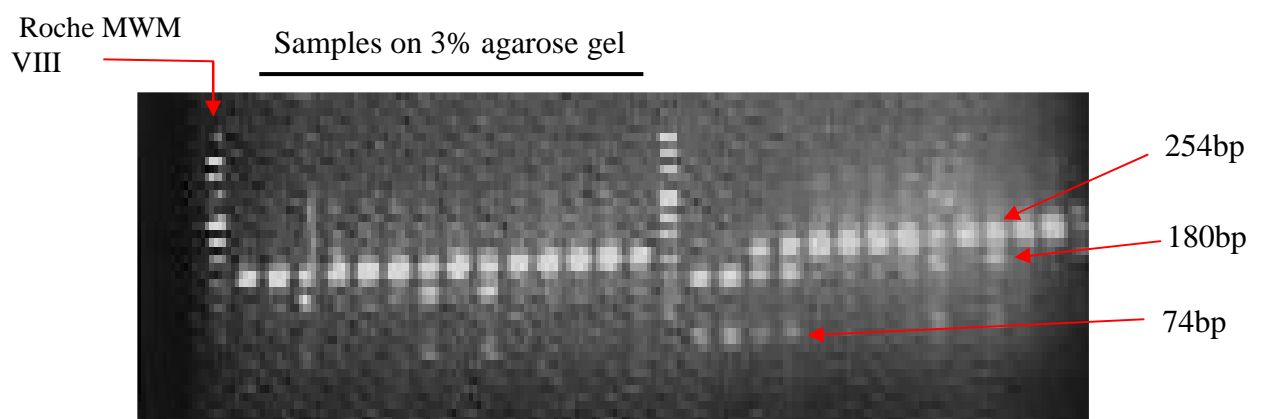
Figure 6: *CYP2D6*17 digestion results gel photo

49

Samples on 3% agarose gel
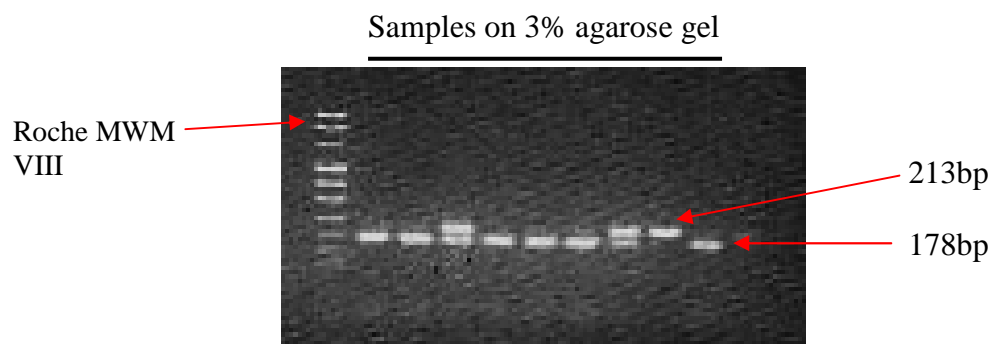
Figure 7: *CYP2D6*29 digestion results gel photo*

Samples on 3% agarose gel
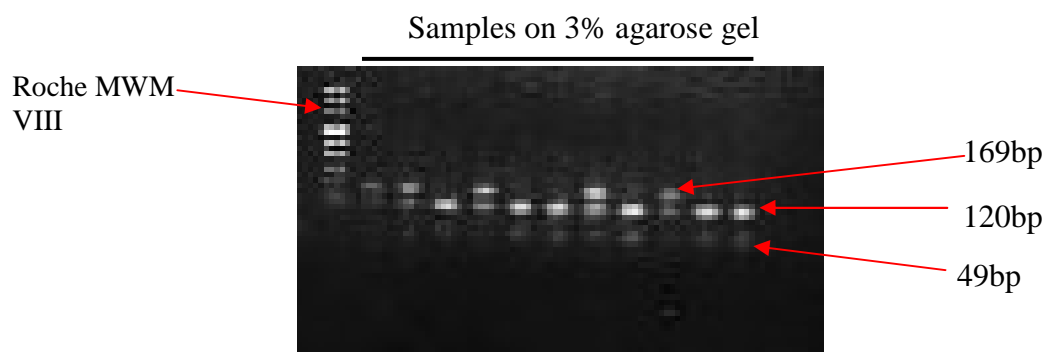
Figure 8: *CYP2C19*2 digestion results gel photo*

Samples on 3% agarose gel
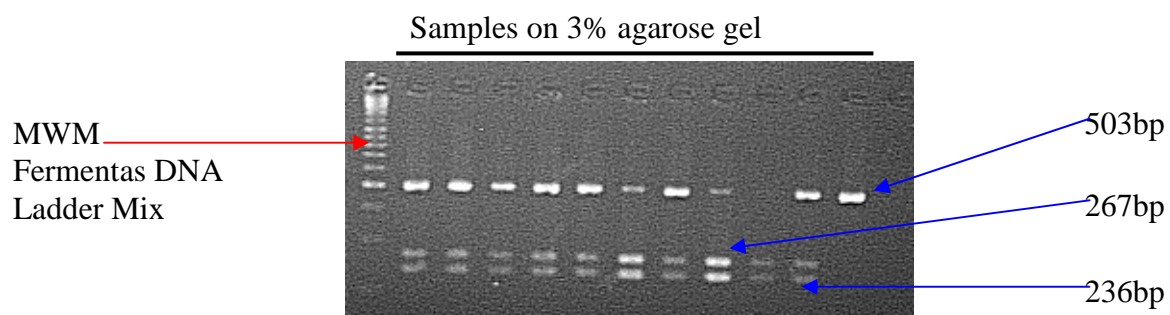
Figure 9: *CYP2B6*6 digestion results gel photo*

50

4.2 FREQUENCIES OF MUTATIONS IN THE SHONA AND SAN

The table below shows the actual numbers of volunteers with each genotype in the Shona and the San. The frequencies are shown in table 7.

Table 6: Numbers of individual genotypes in the Shona and San

	CYP2D6									CYP2C19			CYP2B6		
Ethnic group	*17 -/-	*17 +/-	*17 +/+	*29 -/-	*29 +/-	*29 +/+	*4 -/-	*4 +/-	*4 +/+	*2 -/-	*2 +/-	*2 +/+	*6 -/-	*6 +/-	*6 +/+
Shona	51	21	6	58	15	5	71	5	2	57	18	3	29	38	11
San	40	18	5	61	2	0	52	106	1	49	13	1	20	36	7

KEY
-/- = homozygous wild type
+/- = heterozygote
+/+ = homozygous mutant

From the table above, all the genotype frequencies were in Hardy-Weinberg equilibrium using the equation:

$$p^2 + 2pq + q^2 = 1$$

where p^2 is the frequency of the homozygous wild type,

$2pq$ is the frequency of the heterozygotes,

and q^2 is the frequency of the homozygous mutant.

(www.anthro.palomar.edu/synthetic/synth_2.htm, 2003).

Table 7: Frequencies of the *CYP2C19*2*, *CYP2D6*4*, *CYP2D6*17*, *CYP2D6*29* and *CYP2B6*6* mutations from 78 Shona and 63 San volunteers.

Allele	Frequency in Shona	Frequency in San	p-value <0.05
CYP2C19*2	0.16	0.12	No
CYP2D6*4	0.05	0.10	No
CYP2D6*17	0.22	0.22	No
CYP2D6*29	0.16	0.02	Yes (<0.0001)
CYP2B6*6	0.38	0.40	No

For the medical students and the San, demographic information was not collected because it does not affect genotype

4.3 PSYCHIATRIC PATIENTS

Information pertaining to the patient demographics (that may affect drug plasma concentrations), genotypes, and drug plasma concentrations can be found in appendices 3 to 8. It must be stressed that environmental factors (e.g. age, sex, diet) do not affect the genotype, since it is hereditary and does not change in an individual. Hence we did not collect information about the geographical origin of the patients. The mean age was 39 years (Standard Deviation = 15 years) and the age range was 18-73 years. Twenty-three patients were female and 27 were male. Twenty-seven patients were on the antiparkinsonian drug, benzhexol to counter the extrapyramidal side effects of the antipsychotics. Of these, for the 23 where we had the genotypes, 16 (70%) had either a *CYP2D6*17 or *CYP2D6*29 mutation. For those 22 patients who were not on benzhexol, 12 (55%) had a mutation.
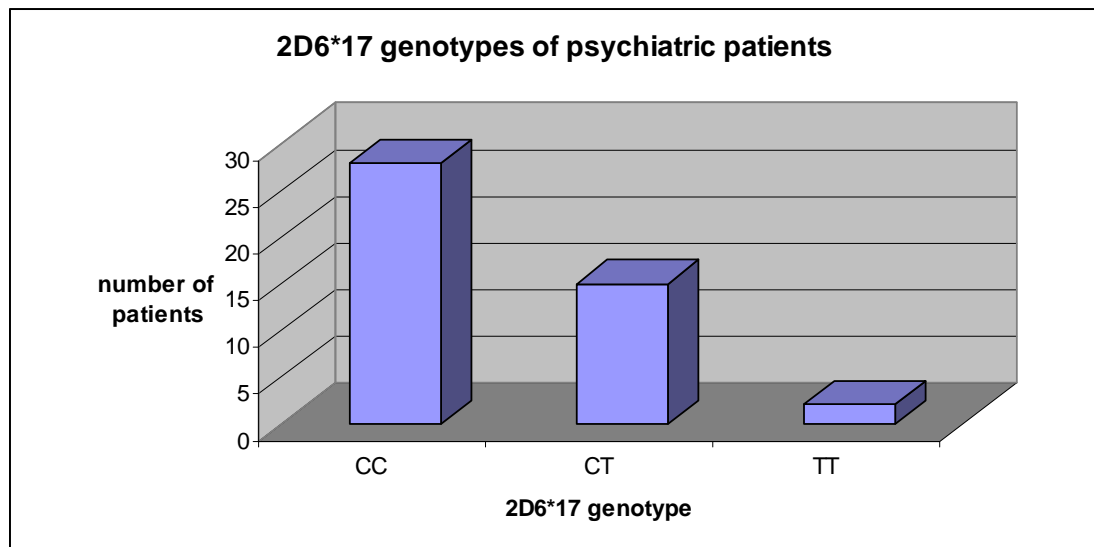
Figure 10: Distribution of the *1023 C/T* (*CYP2D6*17) genotypes in psychiatric patients

Twenty-three of the 50 patients had the wild-type CC genotype. Fifteen of the 50 patients were heterozygous for the *CYP2D6*17 mutation whilst 2 were homozygotes. The genotype of 5 patients (3 males and 2 females) could not be determined due to low PCR amplification. This is probably due to the presence of inhibitory components from the DNA extraction process interfering with the PCR amplification process in these particular subjects. Thus the

frequency of the *CYP2D6**17 mutation was 21%, consistent with what we observed in the medical students (Shona) in this study.
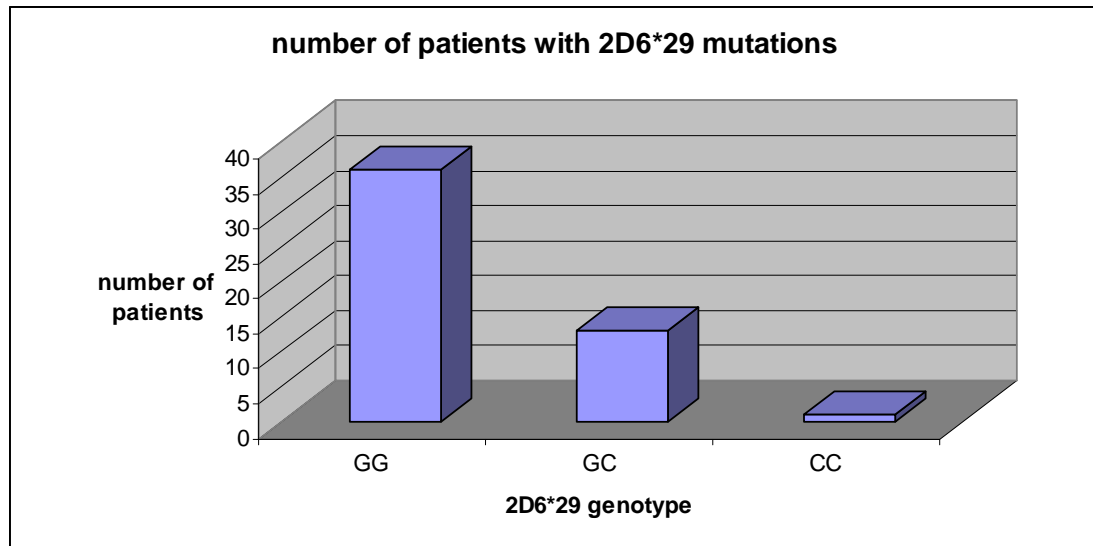
Figure 11: Distribution of *1661G/C* (*CYP2D6**29) genotypes in psychiatric patients

Thirty-six of the 50 patients had the wild-type GG genotype. Thirteen were heterozygous for the *CYP2D6**29 mutations whilst one patient was homozygous. The frequency of the *CYP2D6**29 mutation was 15%, consistent with what we observed in the Shona population in this study.

4.4 CHLORPROMAZINE

The figure below shows the CPZ plasma concentrations stratified by genotype in box and whisker plots. Since the patients were on a variety of doses, we divided the plasma concentration by the dose each patient was receiving to obtain the dose-corrected concentrations. These concentrations were then used to generate the box and whisker plots and perform statistical analyses. The y-axis has the concentrations on a logarithmic scale. Logarithmic transformation was necessary to normalize the data for CPZ because of the very wide range of concentrations we observed. This extreme variability may also be responsible

for our failure to observe a significant effect of genotype on CPZ concentrations. From the plots, and from a Mann-Whitney U test, it is evident that there is no difference in CPZ concentration between individuals with and those without a *CYP2D6*17* or *CYP2D6*29* mutation.
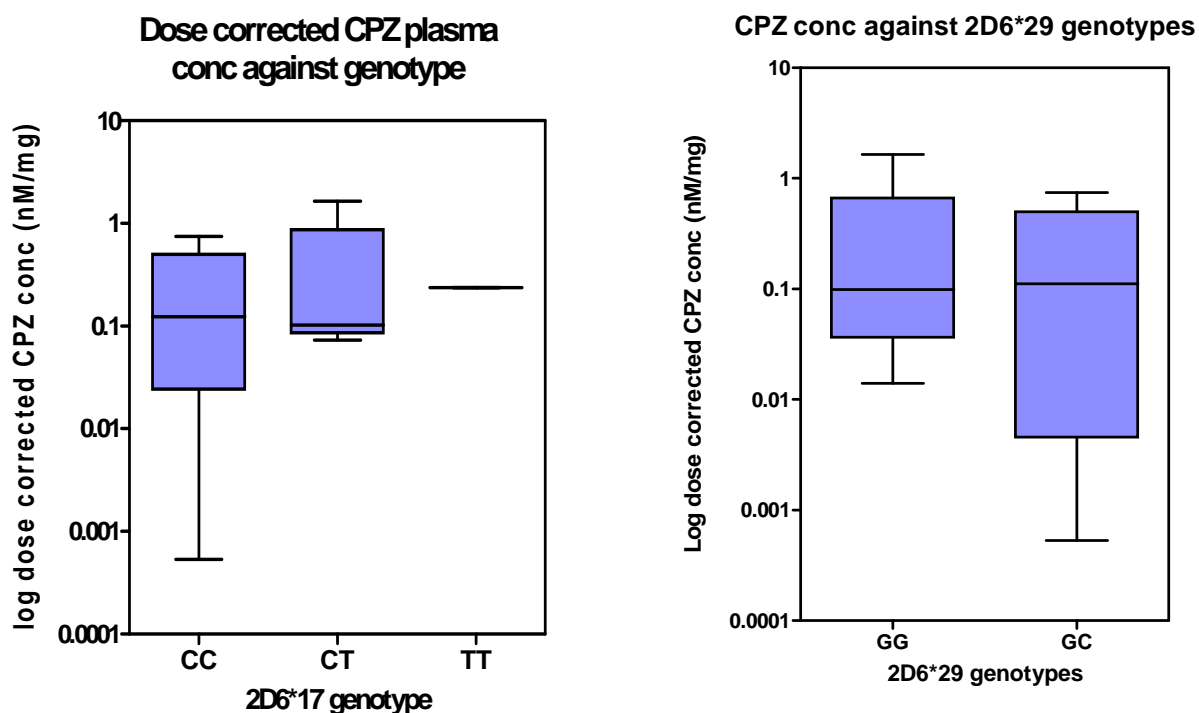
Figure 12: Dose-corrected CPZ plasma concentration against *CYP2D6*17* and *CYP2D6*29* genotypes

Table 8 below shows the CPZ raw plasma concentrations stratified by patient *CYP2D6*17* and *CYP2D6*29* genotype. As mentioned above, the raw concentrations are not as useful as the dose corrected concentrations, hence they were not included in the statistical analysis. The high variability in CPZ concentrations is evident from the ranges (lowest and highest concentrations when concentrations are arranged in ascending order) and the high standard deviation in the table.

Table 8: Chlorpromazine plasma concentration (not dose adjusted) compared by *CYP2D6* genotype

CYP2D6*29 Genotype	Number of patients	Average plasma Concentration (nM)	Range (lowest and highest concentrations, nM)	Std dev
GG	11	132	4.4-515	174
GC	5	124	5.3-466	193
CC	0			
CYP2D6*17 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
CC	8	105	4.4-466	156
CT	5	141	11-515	213
TT	1	74		

4.5 THIORIDAZINE

Figure 13 shows box and whisker plots of the dose corrected thioridazine plasma concentrations. The plot on the left shows a clear genotype-concentration relationship that is proportional to the number of *CYP2D6*29* alleles. For heterozygotes the median concentration is actually double that of patients with no mutation, whilst for homozygous mutants, the concentration was 3 times that of the wild type individuals. This however did not reach statistical significance. A trend was however not observed for *CYP2D6*17*.
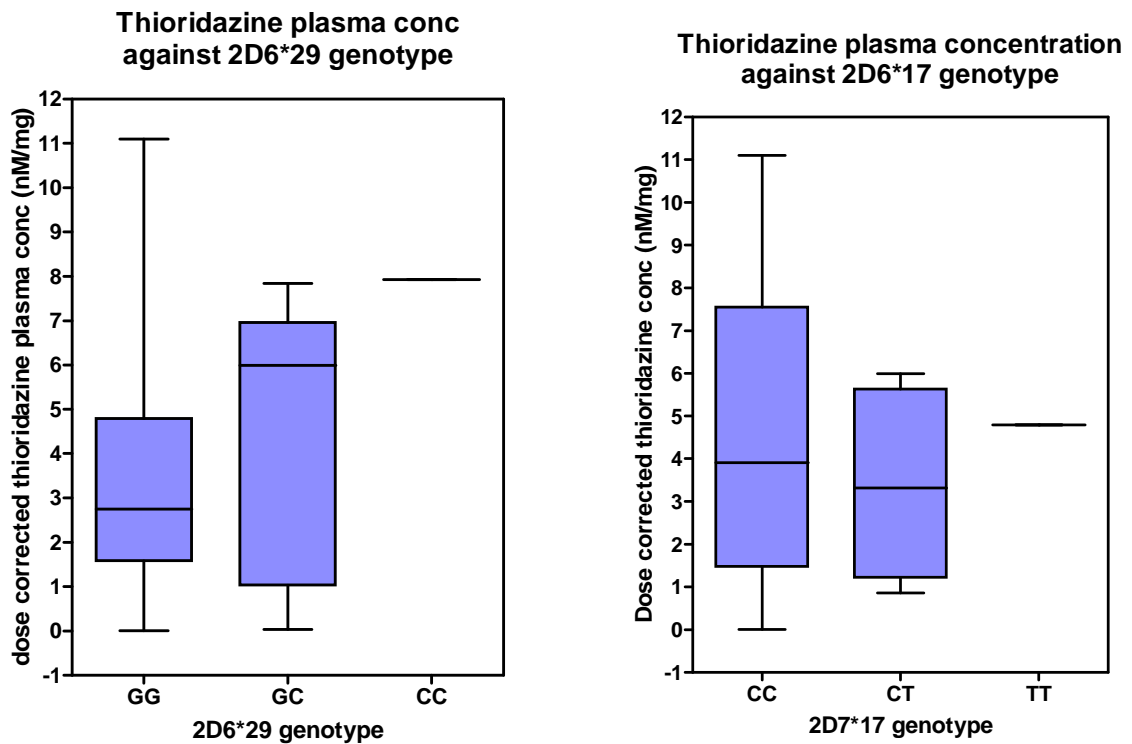
Figure 13: Dose-corrected thioridazine plasma concentration against *CYP2D6**17 and *CYP2D6**29 genotypes

Table 9 shows the average raw plasma concentration stratified by patient *CYP2D6* genotype. From the table, it may appear as though the single individual who was homozygous for *CYP2D6**17 had higher drug concentrations, but this can be attributed to the higher dosage the patient was receiving. This difference fell away when the concentration was adjusted for dose, showing the importance of this procedure for accurate interpretation of results.

Table 9: Thioridazine plasma concentration (not dose adjusted) compared by *CYP2D6* genotype

CYP2D6*17 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
CC	12	2478	5.3-5984	2082
CT	6	2135	464-4264	1430
TT	1	3874		
CYP2D6*29 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
GG	15	2377	9-5984	1789
GC	4	2143	5-4229	2216
CC	1	3670		

4.6 HALOPERIDOL

Figure 14 shows dose corrected haloperidol plasma concentrations stratified by genotype. The box and whisker plot on the left shows that heterozygous individuals for *CYP2D6**17 tended to have lower haloperidol concentrations, a somewhat paradoxical effect. However, this can be expected because haloperidol is metabolized to reduced haloperidol by CYP2D6 as well as CYP3A4 (Fang *et al*. 1997). This reduced haloperidol is also metabolized back to haloperidol by CYP2D6 (Tyndale *et al.* 1991), although CYP1A1 and CYP3A4 have been reported to play a role as well (Fang *et al*. 2001). Nevertheless, the trend was not statistically significant. No trend was observed for an effect of *CYP2D6**29* polymorphism on haloperidol concentrations in our study.
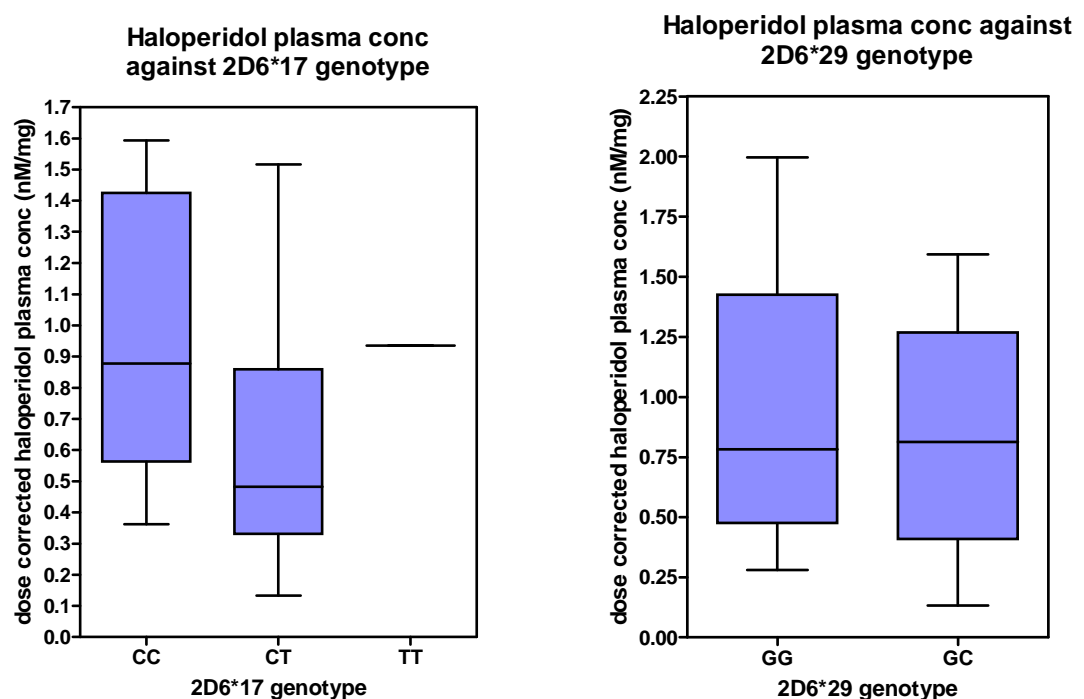
Figure 14: Dose-corrected haloperidol plasma concentration against *CYP2D6**17 and *CYP2D6**29 genotypes

Table 10 below shows the raw haloperidol plasma concentrations in the patients. The trend towards increasing concentrations with *CYP2D6*17* genotype is the opposite of the dose-adjusted trends shown in Figure 14. This is because higher doses were coincidentally given to those with polymorphism. No patient on haloperidol treatment was a homozygote for *CYP2D6*29*.

Table 10: Haloperidol plasma concentration (not dose adjusted) compared by *CYP2D6* genotype

*CYP2D6*17 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
CC	12	24.3	6.8-62.7	14.9
CT	9	30.5	2.5-96.7	35.5
TT	1	49.7		
*CYP2D6*29 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
GG	15	28.3	3.5-96.7	27.3
GC	7	19.7	2.5-49.7	15.4

4.7 FLUPHENAZINE DECANOATE

As can be seen from Figure 15, there is a gene-concentration relationship for fluphenazine with *CYP2D6*17* genotype. The more polymorphisms an individual had, the higher the fluphenzine concentration. This was however not observed for *CYP2D6*29*. This is the opposite of the trend for thioridazine above, which was observed for *CYP2D6*29* but not for *CYP2D6*17*. As for all the drugs, these concentrations were adjusted for the dose the patients were receiving to counteract the variations in dosage that may have had implications on drug plasma concentrations.
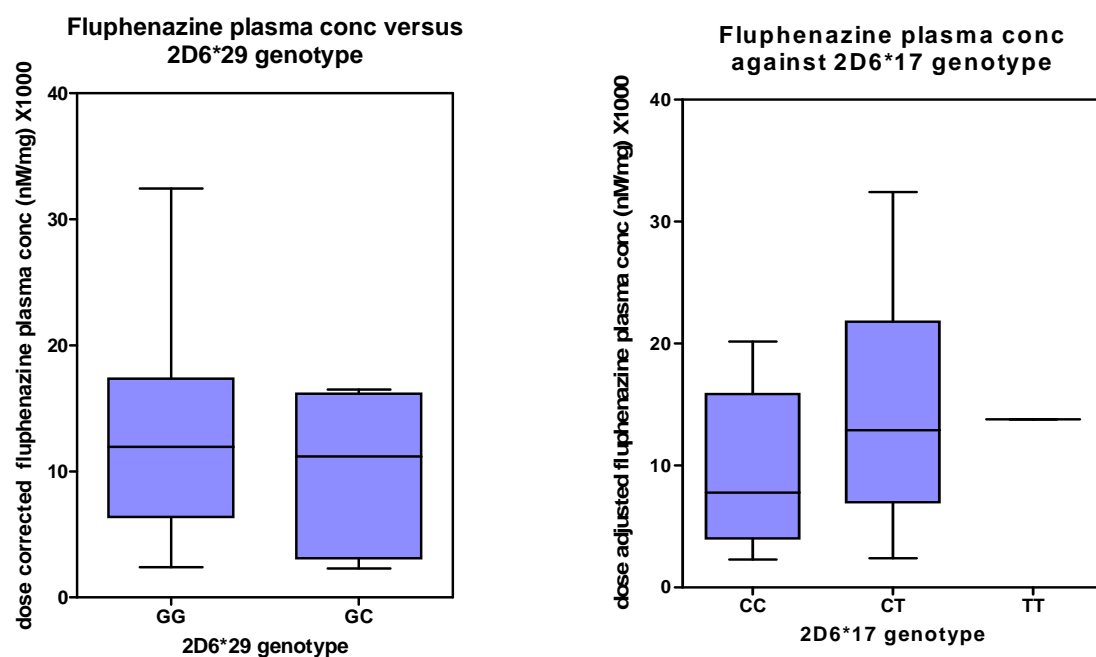
Figure 15: Dose-corrected fluphenazine plasma concentration against *CYP2D6**17 and *CYP2D6**29 genotypes

Table 11 shows the unadjusted fluphenazine concentrations, but there is no consistent trend, probably due to the various doses patients were receiving.

Table 11: Fluphenazine plasma concentration (not dose adjusted) compared by *CYP2D6* genotype

CYP2D6*29 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
GG	15	1.00	0.22-3.70	1.05
GC	6	0.49	0.13-0.94	0.35
CC	1	0.58		
CYP2D6*17 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
CC	11	0.50	0.13-0.94	0.30
CT	8	1.35	0.20-3.70	1.36
TT	1	0.79		

4.8 DRUG CONCENTRATIONS BY GENDER

As drug concentrations may differ by gender, figure 16 show dose adjusted drug concentrations stratified by sex. The median concentrations for fluphenazine and thioridazine were higher in females than in males. No trends were observed for the other drugs.
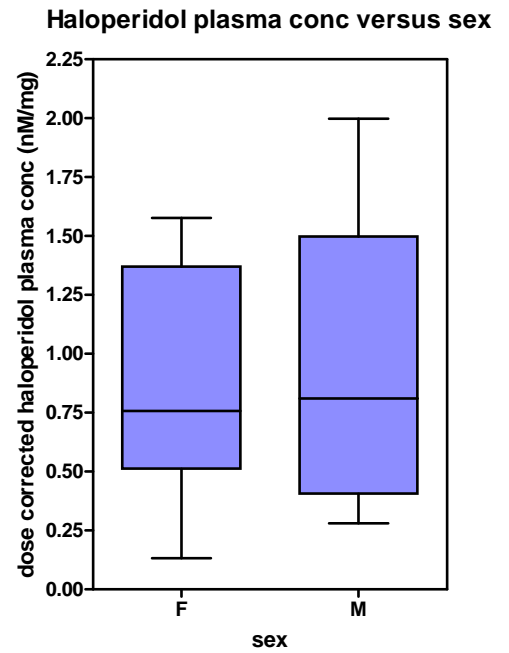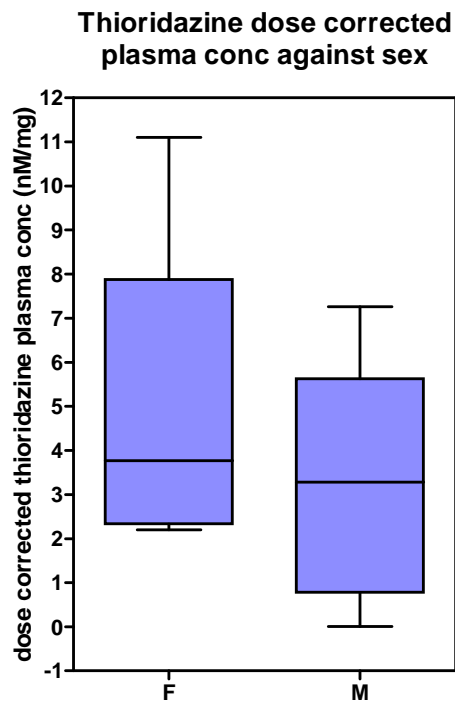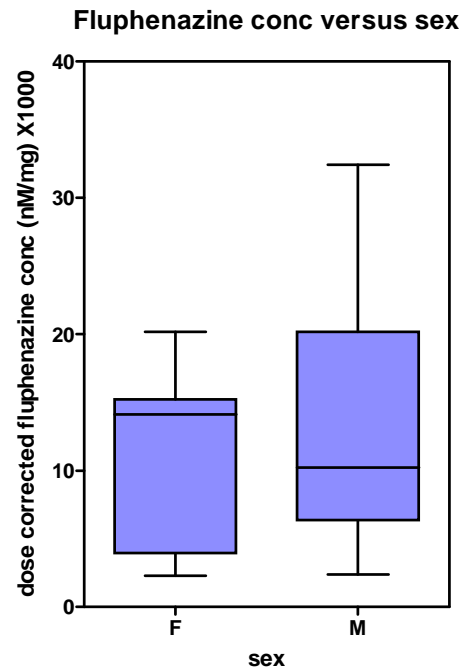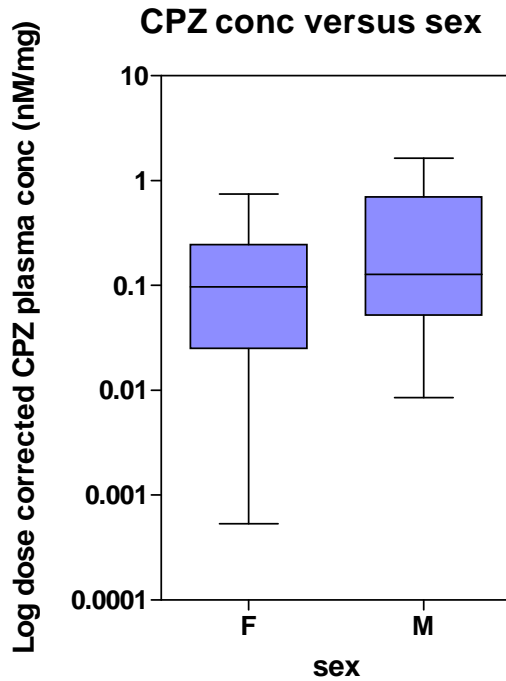
Figure 16: Dose corrected plasma concentrations of CPZ, fluphenazine, thioridazine and haloperidol versus male or female gender.

4.9 AMITRIPTYLINE (AMT)

Figure 17 shows the dose corrected amitriptyline concentrations for the 9 patients receiving this drug. It may appear as though individuals with a polymorphism have higher concentrations but this was not statistically significant.
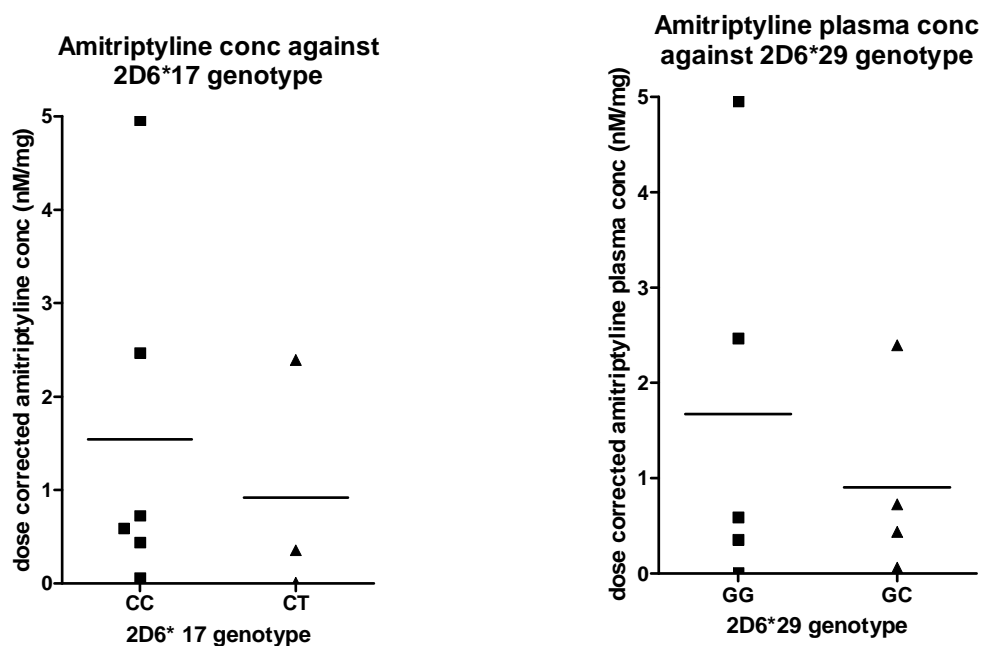
Figure 17: **Dose corrected AMT plasma concentration versus *CYP2D6**17 and *CYP2D6**29 genotypes**

Table 12 shows the unadjusted AMT concentrations in patients with the different genotypes. None of the differences were of statistical significance possibly due to the small numbers of patients receiving AMT.

Table 12: AMT plasma concentration (not dose adjusted) compared by *CYP2D6* genotype

CYP2D6*17 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
CC	6	349	22-893	346
CT	3	453	2.0-1293	728
TT	0			
CYP2D6*29 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
GG	5	463	2.0-1293	526
GC	4	284	22-892	409
CC	0			

We managed to measure the drug concentrations of the active metabolite of AMT nortriptyline. The concentration of the metabolite alone is not very informative; hence table 14 shows the AMT/nortriptyline metabolic ratio. A smaller ratio is indicative of faster metabolism. Unfortunately, few patients were receiving AMT, hence none of the trends was significant. We also do not expect a lower metabolic ratio for patients with *CYP2D6*17* ploymorphism, although this is what the results appear to indicate.

Table 13: Nortriptyline plasma concentration (not dose adjusted) compared by *CYP2D6* genotype

*CYP2D6*17 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
CC	6	187	13-187	181
CT	3	309	0-868	485
TT	0			
*CYP2D6*29 Genotype	Number of patients	Average plasma Concentration (nM)	Range	Std dev
GG	4	354	197-868	344
GC	4	158	13-518	241
CC	0			

Table 14: AMITRIPTYLINE/NORTRIPTYLINE metabolic ratio (not dose adjusted) compared by *CYP2D6* genotype

*CYP2D6*17 Genotype	Number of patients	Average metabolic ratio	Range	Std dev
CC	6	2.1	0.80-4.85	1.18
CT	2	1.3	1.09-1.49	0.28
TT	0			
*CYP2D6*29 Genotype	Number of patients	Average metabolic ratio	Range	Std dev
GG	4	1.73	0.8-3.25	1.06
GC	4	2.07	1.09-3.85	1.22
CC	0			

The two most commonly prescribed drugs were CPZ and thioridazine, hence we performed a secondary analysis to determine whether the presence or absence of a mutation had an effect on the drug concentrations, regardless of its exact type. Once again for CPZ, no trend was observed (Figure 18). For thioridazine, there was a higher median concentration for

individuals with a polymorphism (*CYP2D6*17 or CYP2D6*29*) as shown in figure 19. This

is probably mainly due to *CYP2D6*29* as mentioned above.
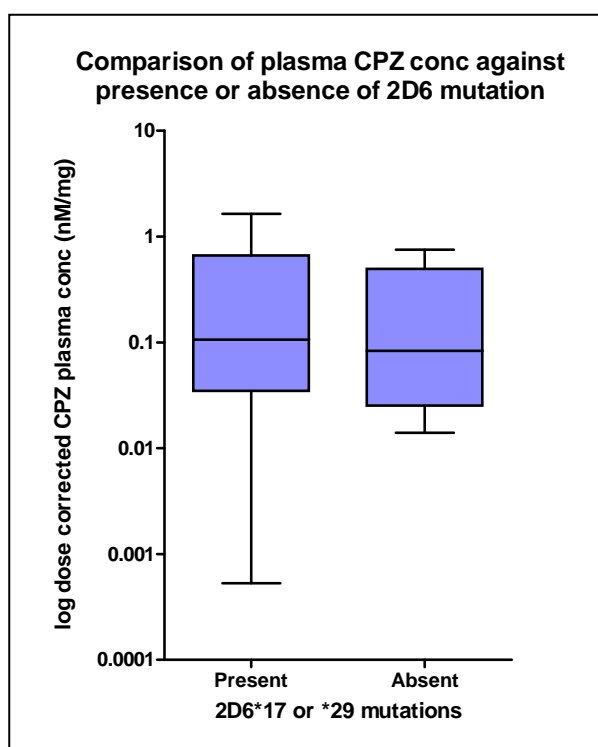
Figure 18: Comparison of dose corrected CPZ plasma concentration against presence or absence of *CYP2D6*17 or *CYP2D6*29 mutations
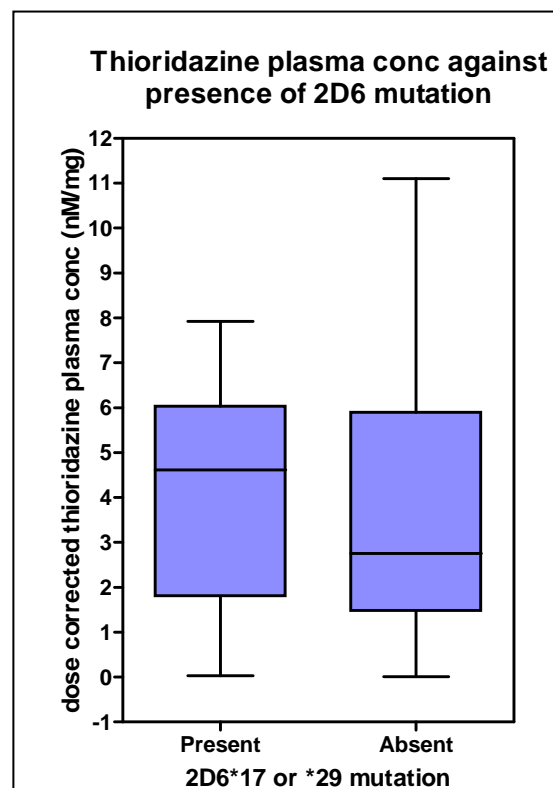
Figure 19: Comparison of dose corrected thioridazine plasma concentration against presence or absence of*CYP2D6*17 or *CYP2D6*29 mutations

5. DISCUSSION

The first part of this thesis is a population study whereby we sought to investigate the frequency of polymorphisms of various drug metabolizing enzymes. Since we observed a high frequency of *CYP2D6*17* and *CYP2D6*29*, we then sought to investigate the clinical impact of these polymorphisms on their substrate drugs. A suitable group of patients on CYP2D6 substrates would be psychiatric patients on psychotropic drugs

5.1 FREQUENCY OF MUTATIONS IN THE SAN AND THE SHONA

CYP2B6, CYP2C19 and *CYP2D6* mutations were genotyped for in the San and Shona in our study, contributing to filling the gap in knowledge of the frequencies of these mutations in Africans, and in particular, Zimbabwe. The Shona are the vast majority of Zimbabwe's population (over 70%) (http://www.questconnect.org/africa_zimbabwe.htm). The Shona volunteers were mainly medical students at the University Of Zimbabwe College Of Health Sciences. Since this is an institution of higher learning that accepts students from all parts of the country, getting unrelated volunteers was an easy task. The San are one of the earliest inhabitants on the earth, where they have lived for at least twenty thousand years (http://www.africaguide.com/culture/tribes/bushmen.htm). In Zimbabwe, only about 200 are left, and we found 63 volunteers from Plumtree district, near the Botswana border. There are more San people in Botswana and Namibia.

The frequency of the *CYP2C19*2*, *CYP2D6*17* and *CYP2B6*6* mutations were similar in the San to what we observed in the Shona people in this study. The frequency of the *CYP2D6*4* mutation was double that found in the Shonas (10% compared to 5%), though statistical analysis showed that this difference was not significant. It is however different from what is seen in Caucasians, where the mutation can be found at frequencies of up to 20% (Gaedigk *et*

al. 1999). Our most striking observation was the frequency of the *CYP2D6*29* mutation in the San. Out of 63 individuals, we found only two heterozygous carriers of this allele, giving an allele frequency of 2%. This is in stark contrast to what we found in the Shona and other Africans (16-20%). This difference was statistically significant. Interestingly, for *CYP2D6*17*, another African specific mutation was found at a frequency of 22% in both the Shona and the San. Previous phenotyping studies in the San using debrisoquine as a probe drug showed 19% poor metabolisers and a low frequency (4%) using metoprolol (Sommers *et al.* 1988, 1989). It has previously been shown that individuals homozygous for *CYP2D6*17* were slower metabolizers when debrisoquine or dextromethorphan were used as probe drugs than when metoprolol or codeine were used, showing a different substrate specificity for *CYP2D6*17*. That same study also found that *CYP2D6*29* led to reduced metabolism of metoprolol and dextromethorphan, but not for debrisoquine or codeine (Wennerholm *et al.* 2002). Thus it can be concluded that metoprolol oxidation is greatly decreased in *CYP2D6*29* carriers whilst debrisoquine oxidation is lower in *CYP2D6*17* mutants. Our genotyping study could therefore contribute to explaining the phenotyping results by De Sommers in the sense that the 4% PMs they found for metoprolol were probably *CYP2D6*29* carriers, whilst the 19% PMs were *CYP2D6*17* carriers. Discordance of debrisoquine and metoprolol hydroxylations was also noted in Shonas where it was found that some individuals who were EMs for debrisoquine hydroxylation were also PMs for metoprolol hydroxylation (Masimirembwa *et al.* 1996a). This dissociation was also noted in a phenotyping study in Zambians (Simooya *et al.* 1993). In 2001, Wennerholm *et al* also showed that bufuralol hydroxylation was greatly reduced by *CYP2D6*29*, but to a lesser extent when debrisoquine was used. The very low frequency of the *CYP2D6*29* polymorphism in the San compared to the Shona allays our fears of the possibility of intermarriages between the San and the Shona majority, or the possibility that they may not

be true San. As mentioned above, our findings are in agreement with the phenotyping studies performed in the San by De Sommers *et al* in 1989.

Our results of frequencies of mutations in the Shona are comparable to those found in other African populations, particularly those found in Zimbabweans in earlier studies (Masimirembwa *et al*. 1995; Masimirembwa *et al*. 1996b; Masimirembwa *et al*. 1993).

As mentioned earlier, the frequencies of the *CYP2D6* mutations we found in the Shona are similar to those we found in the psychiatric patients. The idea was to check the prevalence of these mutations in the general population so that we can extrapolate the magnitude of the importance of the mutation, towards bringing pharmacogenetics into the clinical setting. A mutation which results in marked differences in therapeutic outcome, but which is occurs very rarely, would not justify genotyping thousands of patients to find one with the mutation. On the other hand, if the mutation is more common, then we have better reason to genotype patients routinely, before prescribing the affected drugs.

CYP2B6 is an important enzyme in the metabolism of a commonly prescribed antiretroviral drug, efavirenz. Our study shows that the *CYP2B6*6* allele occurs at a frequency of 38-40% in the general population of Zimbabwe. This has important implications in the prescription of this drug in our African setting because this mutation has been associated with lower clearance and higher drug plasma concentrations in several studies. In a study recently carried out by Nyakutira *et al* (2007), about 50% of patients were found to have plasma concentrations above the MSC of 4 mg/L and *CYP2B6* genotype and sex were major predictors of efavirenz pharmacokinetics.

5.2 PSYCHIATRIC PATIENTS

5.2.1 FREQUENCY OF *CYP2D6* MUTATIONS IN PSYCHIATRIC PATIENTS

The response rate was relatively high, and this may be due to the fact that the only major procedure was a single draw of blood, apart from taking other information pertaining to the patient's medication history.   Unfortunately, when we decided to commence the study, this was a time when the government had just introduced a cash upfront system for payment of hospital charges. This resulted in what used to be an overcrowded psychiatric unit being nearly empty and it took us ten weeks just to get 50 patients. Funds and time available in the study period did not permit us to recruit more patients hence our small sample size as will be discussed in the limitations section.The frequencies of both the *CYP2D6* *17 and *CYP2D6* *29 mutations were consistent with what we observed in the general Shona population of Harare. This means that these mutations had no effect on the likelihood of having mental illness. Also, if one had mental illness, having these mutations probably did not increase the risk of being hospitalised due to side effects. However, the percentage of patients who had mutations who were being given the antiparkinsonian benzhexol was higher than those who were not on benzhexol (70% compared to 55%). Thus it appears that if one had a mutation, one was at higher risk for developing extrapyramidal symptoms (EPS). Observation of this effect may have been dampened by the routine clinical practice in our setting whereby patients on high doses of antipsychotics or on several drugs can be given antiparkinsonian medication as prophylaxis, before they even show signs of developing EPS.

5.3 INDIVIDUAL DRUGS

A few of the psychiatric patients were on concomitant medication including antiretrovirals, antibiotics, antihypertensives (hydrochlorothiazide, HCT) and antidiabetics (glibenclamide). To our knowledge, these drugs do not interact with CYP2D6 or inhibit it, hence drug

interactions with the concomitant medication is unlikely. Unfortunately, none of the psychiatric patients was coloured so this did not help in our efforts to recruit members of the coloured community.

5.3.1 CHLORPROMAZINE

The average plasma concentration of CPZ was higher in heterozygous *CYP2D6**17 carriers, though it was well below the maximum safe concentration (MSC) of 940 nM. The higher drug levels in the patients with the *CYP2D6**17 mutation is consistent with our hypothesis. Only one patient was a homozygous carrier of *CYP2D6**17 but their plasma concentration was actually below the therapeutic range of 94-940 nM. A number of possible explanations exist. The fact that an intermediate or poor metaboliser may experience side effects to the drug such that they no longer adhere to their regimen cannot be excluded. The plasma concentrations of CPZ in this study varied to a great extent (117 fold variation), with most patients below the minimum effective concentration (MEC). Actually, only 5 out of the 18 patients on CPZ had plasma levels above the MEC. Although this may suggest that patients are severely underdosed, this is probably an adherence issue since most of the patients were on at least 100 mg daily. There are many possible explanations for the wide variability, one of which is that, absorption of the drug is itself erratic, with 10-80% of the oral dose reaching the systemic circulation due to considerable first pass metabolism (Sanofi-Aventis, 2005). This means that a number of patients were supposedly on medication yet in actual fact they probably were not benefiting from it. Added to this is the issue of concomitant administration of antipsychotics and possible drug-drug interactions. Out of 18 patients given CPZ, only one patient was not on other antipsychotics showing the high frequency of prescription of this antipsychotic drug. Thus only one patient was on CPZ alone (patient ID number 7 in appendix 8). In other words, patients were rarely given CPZ on its own, but rather in combination with other antipsychotics. That CPZ was studied in its real clinical

usage rather than controlled trials that do not translate to reality is a major strongpoint of our study. The other 17 patients were usually given haloperidol and/or fluphenazine decanoate as well. Thus if a patient was getting better, it cannot be assumed that CPZ played a significant role, except perhaps for boosting the plasma concentration of the co-administered drug by inhibiting its metabolism by CYP2D6. For CPZ, we failed to observe a statistically significant relationship between the *CYP2D6* genotype and drug plasma concentration,

No significant difference was found between patients with zero or one *CYP2D6*29* mutation in terms of the CPZ plasma concentration. The same explanations as those for *CYP2D6*17* can be offered. In addition, the *CYP2D6*29* mutation leads to altered substrate specificity, such that the mutation affects some, but not all drugs metabolised by *CYP2D6*, and it may affect some drugs more than others. It is possible that the *CYP2D6*29* mutation does not greatly affect the efficiency of *CYP2D6* in metabolising CPZ. We then analysed drug plasma levels compared to the combined presence or absence of a *CYP2D6* mutation. We found a very slight difference, which was not statistically significant, whether an individual had a *CYP2D6*17* or *CYP2D6*29* mutation or not, as shown in Fig 18, contrary to our hypothesis that individuals with mutations leading to diminished 2D6 activity would have higher drug levels. Apart from the adherence issue, it is probable that CPZ was metabolised by alternative routes (particularly CYP1A2) in these patients, since over 75 different metabolites have been identified in blood and urine. *CYP2D6*29* may have actually dampened the results of *CYP2D6*17* since *CYP2D6*29* did not previously show any effect. There was no patient homozygous for *CYP2D6*29* who was on CPZ.

5.3.2 THIORIDAZINE

Thioridazine is a drug that has been withdrawn from a number of markets due to the potentially fatal side effect of torsades de pointes, which has been associated with high drug

plasma levels. Thioridazine causes prolongation of the QTc interval in a dose dependent manner. It is possible that those at the highest risk are CYP2D6 poor metabolisers. In 2002, Llerena *et al* reported a correlation between thioridazine dose, plasma concentrations and CYP2D6 hydroxylation capacity, with the risk for QTc interval prolongation (Llerena *et al.* 2002a, 2002b).

In our study, for thioridazine, there was no significant difference between plasma concentrations for wild-type patients and heterozygotes for *CYP2D6*17. However, for the single homozygous carrier, their plasma level was much higher than the other two means. Since it was only one patient, they could not be included in the statistical calculations. Looking at the box and whisker plot for *CYP2D6*29, it is clear that the median dose corrected concentration for heterozygotes is more than twice that of the wild-type, whilst that for the homozygous *CYP2D6*29 carrier is almost 3 times that of the wild-type. It is therefore possible that thioridazine metabolism is greatly affected by the *CYP2D6*29 mutation but much less so by *CYP2D6*17, just like the way metoprolol behaves as will be explained later.

Just like for CPZ, there was great variability in the plasma concentrations among patients which can be due to drug-drug interactions and poor adherence to medication. Excluding two patients who had almost 0 nM thioridazine plasma concentrations, there was almost 16 fold variation in the dose corrected plasma concentrations (0.69-11.1 nM/mg). This is comparable to the 23 fold variation found in another thioridazine study (Berecz *et al.* 2003). These authors carried out a study on in-patients on thioridazine monotherapy and found a relationship between dose-corrected thioridazine plasma concentration, *CYP2D6* genotype and tobacco smoking.

Out of 22 patients on thioridazine, 3 had drug concentrations above the maximum safe concentration of 5391 nM and 5 patients had plasma levels below the MEC of 539 nM. Interestingly, none of these three patients had *CYP2D6*17* or *CYP2D6*29* mutations. This does not mean they did not have *CYP2D6* mutations. It is possible that these patients had other *CYP2D6* mutations which are not common in Africans hence we did not genotype for them e.g. *CYP2D6*4* which results in an inactive enzyme and PM status. One of these patients was also on six other drugs, increasing the likelihood of drug interactions which result in decreased clearance of thioridazine. The two highest dose corrected plasma concentrations were found in two of these patients, showing that there is more to their high drug concentrations than the dosage.

Of the five patients below the MEC, one of them actually had an undetectable level of thioridazine, showing that they were not taking their medication at all. This can be assumed because the number of hours between the time the patient is reported to have taken their last dose and the sampling time (24 hours maximum) is too short for them to have cleared all the drug to reach undetectable levels. Three others had either a *CYP2D6*17* or *CYP2D6*29* mutation, and these may not have been taking their medication due to side effects they may have experienced as a result of elevated drug plasma concentrations. One patient had even been prescribed 300 mg daily and this high dosage may have been an attempt by the clinician to achieve therapeutic effect. Four of these five patients had also been prescribed benzhexol, to counter the extrapyramidal symptoms, usually associated with high drug concentrations and they were also on other antipsychotics. We measured the haloperidol concentration in one of these patients and it was undetectable, further showing their non-compliance. However, in two of these patients, the haloperidol concentration was within the therapeutic range.

5.3.3 HALOPERIDOL

Previous studies in in-patients demonstrated a weak correlation between the number of active *CYP2D6* genes and haloperidol clearance. Poor metabolisers have been shown to be at increased risk of EPS due to high plasma levels, although genetic variation explained only a fraction of the side effects. Interestingly, the same study showed that PMs had higher plasma concentrations of reduced haloperidol than EMs, but not for the parent haloperidol itself (Brockmoller *et al.* 2002). The drug is mainly metabolised by CYP2D6 and CYP3A. The dose corrected plasma concentrations from our study showed lack of effect of *CYP2D6**17 or *CYP2D6**29 on haloperidol levels. This could be due to the presence of CYP3A compensating for CYP2D6 inactivity. For some reason, there was no patient who was on haloperidol monotherapy. The drug was usually combined with chlorpromazine and/or fluphenazine decanoate. Five out of the 22 patients had haloperidol concentrations above the MSC. Two of these patients had one *CYP2D6**17 mutation and another had two *CYP2D6**17 mutations. One patient did not have the *CYP2D6**17 or *CYP2D6**29 mutation and unfortunately, for the fifth patient we were unable to determine the genotype. Three of these five patients were taking 10 mg haloperidol twice daily; one patient was on 15 mg three times daily, whilst one patient was on 5 mg twice daily. Looking at the dose adjusted concentrations, the patient on 10 mg daily had the highest level out of all the 22 patients, showing that the daily dosage did not play a significant role in these patients' high plasma concentrations. The other 4 patients also had high dose corrected concentrations.

Eight patients (36%) had plasma levels below the MEC of 13.3 nM. Four of these patients had been prescribed 5 mg at night only, and another patient was on 2.5 mg at night. From this, it is possible that when 5 mg once daily is prescribed in our population, it is insufficient for achieving any therapeutic benefit. Most of the patients with drug concentrations above the MEC were on a least 10 mg daily, with some going as high as 15 mg three times daily.

During our interview with one patient who was prescribed 10 mg daily, we gathered that the patient was not adhering to their medication, hence their plasma concentration was below the MEC. Looking at the box and whisker plot for *CYP2D6*17, it appears as though the dose corrected plasma concentration was lower in heterozygotes than in wild-type patients. However, a two tailed non-parametric Mann-Whitney test showed that this difference was not significant ($P>0.05$).

5.3.4 FLUPHENAZINE DECANOATE (FD)

In Zimbabwe fluphenazine is usually given intramuscularly as the decanoate, a long acting oily injection with a serum half-life of 7-10 days, which becomes longer with chronic dosing, about 14.3 days (Jann *et al.* 1985). Twenty-three patients in our study were receiving one injection per month. Only 3 of these patients had drug concentrations within the therapeutic window of 0.5-2.5 ng/mL (1.1-5.7 nM) according to the Modecate product information published by Bristol-Myers Squibb. The rest had plasma concentrations below 1.1 nM. Therapeutic concentrations as low as 0.34 nM have also been suggested (Jann *et al*. 1985) in which case 18 of our patients would be within the therapeutic range. Doses that can be given vary from a starting dose of 12.5 mg to a maximum of 100 mg, based on patient response (Bristol-Myers Squibb, Modecate product information, 2004). The 25 mg dosage was prescribed the most with 15 patients receiving it. One of the two patients receiving 12.5 mg per month had a drug concentration below the therapeutic minimum of 0.34 nM. None of the patients had a toxic FD concentration. Drug adherence to FD is not a major concern in our study population since the patients would receive the injection when they present at the clinic for their monthly visits. Most patients would come for their visits and hence receive their dose. FD in our setting is usually given for those patients who have problem with adhering to their oral medication and thus need a long acting antipsychotic. Two of the three patients with concentrations above 1.1 nM had one *CYP2D6*17 mutation. These two patients also

73

belonged to the few patients receiving 50 mg FD per month. We were unable to determine the genotype for the third patient.

Of the 5 patients who had haloperidol concentrations above the MSC, three of them were also on FD, suggesting a drug-drug interaction which resulted in FD increasing the plasma concentration of haloperidol. Fluphenazine is a known inhibitor of CYP2D6, one of the main enzymes which metabolizes haloperidol, thus it may have decreased the clearance of haloperidol in these patients. Thirteen males were on FD, whilst 10 females were on the drug. From our data, it appears *CYP2D6*17 affects fluphenazine metabolism more than *CYP2D6*29. The median dose corrected fluphenazine concentration was higher in patients with one or two *CYP2D6*17 mutations than those without this mutation, whilst for *CYP2D6*29 there was not much of a difference.

5.3.5 AMITRIPTYLINE

The tricyclic antidepressant, amitriptyline (AMT) was not commonly prescribed at the psychiatric clinic, since the most common clinical condition was schizophrenia and not depression. Nine patients were given AMT and of these, two were above the therapeutic range of 288-719 nM. One of these two patients was taking 150 mg per day; a rather high dosage (recommended maximum dose is 200 mg). This patient also was heterozygous for both *CYP2D6*17 and *CYP2D6*29 mutations. In addition, the patient was on CPZ (CYP2D6 inhibitor), haloperidol and benzhexol thus inhibition of AMT CYP2D6 metabolism by CPZ may also have contributed to the high plasma concentration of 1293 nM, almost double the MSC. The metabolism of tricyclic antidepressants is impaired by chlorpromazine, increasing the risk of toxicity (Balant-Gorgia and Balant 1987). The second patient was taking only 50 mg AMT and 5 mg haloperidol but had no *CYP2D6*17 or *CYP2D6*29 mutations. Interestingly, this patient actually had, by far the highest dose adjusted concentration. It is

possible that this patient had other *CYP2D6* mutations which we did not genotype for. Six other patients on AMT had plasma concentrations below the MEC of 288 nM. For one patient, this could be explained by poor adherence since they had not taken their previous night's dose and 37.5 hours had elapsed since they last took a dose and the time blood was drawn from them. Out of these 6 patients, 3 were on a daily dose of 100 mg, 2 were taking 75 mg and one was on 50 mg, so the doses they were receiving were reasonable. Poor adherence could also be an issue for these patients. However, another patient was also on phenobarbitone and carbamazepine, known enzyme inducers and they may have accelerated AMT metabolism by CYP1A2 and other enzymes. As can be seen from our scatter plots, there were just too few patients on AMT for us to be able to observe any difference in the drug levels compared to genotype. For AMT, we were able to measure the plasma concentration of the active metabolite, nortriptyline. This would give us more accurate interpretation of the impact of the mutations, by giving us information with respect to the amount of metabolism that is taking place. With the few samples we had, it can be seen that there was a higher AMT/nortriptyline ratio in *CYP2D6**29 carriers compared to wild type individuals, showing less formation of the metabolite in *CYP2D6**29 carriers. The reverse occurred for the *CYP2D6**17 mutation but the results cannot be useful because of the small numbers involved.

5.4 DRUG CONCENTRATIONS VERSUS GENDER

Figure 16 shows the drug concentrations stratified by gender for CPZ, thioridazine, F.D., and haloperidol. There were not enough patients on AMT to make a meaningful analysis. There was no significant difference in plasma levels between males and females thus it can be concluded that gender does not have a significant effect on CPZ pharmacokinetics.

In our study, plasma thioridazine concentrations were generally higher in females than in males. This could be as a result of cigarette smoking on the part of males. Although, our analysis does not include data on smoking patterns, the frequency of smoking was probably much higher in males than in the females, similar to the general Zimbabwean population. In addition to CYP2D6, thioridazine is metabolised by CYP1A2 as well and lower plasma concentrations have been found in smokers compared to non-smokers due to the inducibility of CYP1A2 by smoking (Berecz *et al.* 2003).

Gender did not have an effect of haloperidol plasma concentrations, with males and females having the roughly the same average concentrations, contrary to other reports (Jann *et al.* 2001).

The median dose corrected FD concentration was higher in females than in males (14.1 compared to 10.2 nM/mg). Although this difference was not statistically significant, it is consistent with our smoking theory, meaning males smoked more than the females, and this resulted in the males having higher FD clearance. This agrees with previous data which says cigarette smoking has been found to be associated with a 2.3 fold increase in the clearance of fluphenazine decanoate (Jann *et al.* 1985).

5.5 LIMITATIONS OF PHARMACOGENETICS

At present, there is clearly a gap between the scientific knowledge in pharmacogenetics and its poor development in routine medicine. An increasing number of examples describing differences in drug response as a result of genetic polymorphisms have been published, but most of these reports lack explicit statements on how to translate this information for use in routine therapy. To date, the use of pharmacogenetic testing in routine clinical practice is not

very common. In Australasia, a study showed that pharmacogenetic testing for drug metabolizing enzymes is quite rare (Gardiner and Begg 2005). Phenotypic tests were undertaken more frequently than genotypic tests but both tests were rarely done and were mainly for TMPT and pseudocholinesterase. Genetic tests are used more often for research than for clinical benefit to the patient. The low clinical usage reflects a poor evidence base, unestablished clinical evidence, and in the few cases with strong rationale, a slow translation to the clinical setting (Gardiner and Begg 2005). Bringing pharmacogenetic knowledge to the bedside is not progressing as rapidly as expected when compared to scientific activity in this area (Valdes *et al.* 2003). Most pharmacogenetic studies have been performed to show the effect of genotype on drug plasma concentrations. However, it does not necessarily follow that the effect of a drug (pharmacodynamics) will always be different and this represents one of the major pitfalls of pharmacogenetics. This has clearly been shown for a number of antiretroviral drugs where differences in plasma concentrations due to polymorphism of drug metabolizing enzymes were not translated to differences in immunological or virological response e.g. a study which analyzed nelfinavir + nelfinavir hydroxyl-t-butylamide (M8) plasma concentrations. The authors found a significant difference between wt/wt and wt/*2 for *CYP2C19*2* with respect to the metabolic ratio but indicated that further studies were needed to check for differences in virological responses (Burger *et al.* 2006). Another example is a study which showed only a marginal difference in blood pressure between EMs and UMs when the beta blocker metoprolol was used, yet the clearance and drug plasma concentrations differed markedly between EMs and UMs (Kirchheiner *et al.* 2004). A retrospective study in also showed that *CYP2D6* genotype contributes to clinically relevant variability in plasma concentrations of antidepressants but probably not antipsychotics in daily clinical practice (Mulder *et al.* 2006).

Oftentimes though, the magnitude of the variation in the pharmacokinetics of a drug due to genetic factors far exceeds the range considered as acceptable when comparing the bioavailability of 2 drug formulations. Some authors propose that, in those cases where differences in pharmacokinetic parameters among different genotype groups fall within the acceptable range for stating bioequivalence, the polymorphism can be considered negligible with no genotype-specific dosing being necessary. However, if pharmacokinetic differences cause the drug exposure to fall outside the acceptable range for the corresponding bioequivalence measurement, then adjusting dosage depending on genotype could be beneficial, unless sufficiently powerful studies can show that the pharmacokinetic differences do not affect the clinical outcome. This is necessary because, although there is little doubt that genetic variability causes clinical complications, it is not certain how much variability in drug effects can be alleviated by compensating for these differences (Kirchheiner *et al.* 2005).

As aforesaid, a number of pharmacogenetic studies have shown pharmacokinetic differences between different genotypes but have been lacking evidence of pharmacodynamic differences. Other studies have shown that *CYP2D6* genotype contributes to clinically relevant variability in plasma concentrations of antidepressants but probably not antipsychotics in daily clinical practice, yet without investigating therapeutic response. A flat dose-response relationship can also explain lack of effect of genotype on therapeutic response for some drugs, despite clear differences in plasma levels based on genotype. It is also well appreciated that there are many sources of variability in treatment response to antidepressants including severity of illness, concomitant medication, psychosocial factors etc and genotype alone cannot explain all variability. Further to that, some studies have failed to show a relationship between genotype and treatment response. Apart from the confounding factors mentioned above, the authors attributed this to the fact that most of the drugs did not have

well defined therapeutic ranges (Grasmader *et al.* 2004). However, side effects have been correlated with high drug plasma levels and side effects in several other studies. In some studies, frequencies of genotypes have been similar between patients and healthy volunteers, but in others, frequencies of PMs were higher or lower than in the general population. A lower frequency may indicate that the patients are no longer taking antidepressant medication because they previously encountered side effects to the drugs (Grasmader *et al*. 2004). Alternatively, a higher frequency may indicate that PMs are experiencing more side effects that require additional visits to the clinic.

On a supportive note, thiopurine S-methyltransferase (TPMT) genotyping allows the identification of patients with high risk of severe toxicity if treated with thiopurine drugs. One study reported a higher frequency of haematopoietic toxicity in patients who had TPMT deficiency and those who were heterozygous for non-functional alleles (Schwab *et al.* 2002). In 2001, Evans *et al* found a similar phenomenon in patients who were referred for evaluation of thiopurine haematotoxicity, 70% of whom had one or two non-functional TPMT alleles (Evans *et al.* 2001). TPMT-deficient patients with Acute Lymphoblastic Leukaemia (ALL) could also be successfully treated with 5-15% of the conventional dose of mercaptopurine (Evans *et al.* 1991). TPMT genotyping is now available from some reference labs as a Clinical Laboratory Improvement Act-certified molecular diagnostic that is being used to individualize therapy with thiopurine drugs in the USA. This is driven by the fact that, for the small number of thiopurine drugs metabolized by TPMT, its polymorphisms are a major determinant of severe and potentially fatal haematotoxicity (Evans 2002).

6. CONCLUSION

Our study shows that the frequencies of *CYP2B6*6, CYP2C19*2* and *CYP2D6*17* are similar among the Shona, San and the Psychiatric patients. The frequency of *CYP2D6*29* is however lower in the San compared to the Shona, a finding that might explain the previously reported discordant phenotype results in the San using debrisoquine and metoprolol. The high frequency of the *CYP2B6*6* allele could have clinical implications in the safe use of the CYP2B6 substrate drug, efavirenz in African populations. Association studies of the major *CYP2D6* variants, *CYP2D6*17* and *CYP2D6*29* with plasma concentrations of antipsychotic and antidepressants did not show significant correlations though some genotype-concentration trends were observed for thioridazine and fluphenazine. Carriers of *CYP2D6*29* and *CYP2D6*17* mutations may need lower doses of thioridazine and fluphenazine respectively to avoid side effects related to high drug plasma concentrations.

7. LIMITATIONS AND SCOPE FOR FURTHER STUDIES

The results of our study suggest that there is good reason to carry out further work in larger populations of at least 100 patients per drug to better determine the significance of pharmacogenetics in a routine outpatient clinic. Most of the differences we saw were quite marked and clear gene-dose relationships existed, particularly for thioridazine versus *CYP2D6*29* as well as fluphenazine versus *CYP2D6*17*, but not statistically significant due to the small sample sizes. For non-parametric t-tests (Mann-Whitney) it may be difficult to prove a difference when the samples are small, no matter how big the difference is. A major reason for the lack of significance is the small number of patients who had homozygous mutations. From a population perspective, these are not very common (2-3%) and most people with mutations are usually heterozygous, thus one would need a large population to get a sufficiently high number of homozygotes for meaningful statistical analysis. Apart from genotype, gender differences need further investigation particularly for thioridazine where females had higher drug concentrations compared to males.

For the genotyping, genotyping for other mutations like *CYP2D6*5*, *CYP2D6*10*, *CYP2D6*41* as well as sequencing the whole *2D6* gene would help explain patients with high plasma concentrations yet they do not have *CYP2D6*17* or *CYP2D6*29* mutations. We chose to genotype for these two mutations as a priority because they occur at the highest frequency in Africans and thus would have greatest significance on a population level. It is our opinion that genotyping for rare mutations is not very useful for routine practice, particularly for resource-limited settings like ours. However, it would make the analysis of our results more accurate. Genotyping for other drug metabolising enzymes e.g. *CYP1A2*, which may affect the pharmacokinetics of the psychotropic drugs, would also be beneficial.

Perhaps the most important work that needs to be carried out is investigating the consequences of the differences in pharmacokinetics between individuals with different genotypes, i.e. pharmacodynamic differences, whether they are differences in therapeutic or toxic responses. A good example would be to measure QTc interval prolongation for the patients on the phenothiazine-based antipsychotics such as thioridazine. We actually made some attempts to bring an electrocardiograph from the University Of Zimbabwe Department Of Physiology to the Parirenyatwa Hospital Psychiatric Unit for use in our patients but failed to obtain the necessary approvals. Several papers have mentioned the possibility of increased risk of extrapyramidal symptoms and other side effects of the drugs but have not actually done studies to show these differences. In our study, we briefly discussed the prescription of benzhexol and limitations of using it in our analysis as mentioned above. Genotyping for mutations of the drug receptors e.g. dopamine receptors would also help explain differences in therapeutic responses with regard to drug plasma levels. In other words, out of the three billion base pairs comprising the human genome, trying to explain different drug responses amongst individuals, cannot be explained to the fullest by analysing one or two positions along the genome. Oftentimes however, one position can explain almost all the variability observed, particularly bimodal tendencies, but outliers would usually need further investigation. Genotyping all genes that affect the drug's absorption, distribution, metabolism, elimination and its mechanism of action would give us the best answers.

Investigating the binding site of CYP2D6 may also explain why we observed differences in plasma concentrations for some, but not all drugs, showing the altered substrate affinity for some mutations of this enzyme, as also described by Wennerholm *et al* in 2002.

Investigating the metabolic ratios would also offer better explanations for some of the differences we observed among genotypes, particularly since some of the patients were on

different drug doses. Unfortunately, we were unable to secure metabolite standards for LC-MS analysis in our study, apart from nortriptyline.

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8. APPENDICES

Appendix 1: Sample Medical Research Council of Zimbabwe (MRCZ) approved consent form.

Page 1 [of 3] MRCZ No.1283

INFORMED CONSENT FORM

PROJECT TITLE: **Genetic polymorphism of drug metabolizing enzymes in major ethnic groups of Zimbabwe and the clinical impact of CYP2D6 polymorphism on efficacy and safety in the use of psychotropics**

Principal Investigator Dr Collen Masimirembwa, (PhD, DPhil)
Phone number: (04)710564

What you should know about this research study:
- We give you this consent so that you may read about the purpose, risks, and benefits of this research study.
- Routine care is based upon the best known treatment and is provided with the main goal of helping the individual patient. The main goal of research studies is to gain knowledge that may help future patients.
- We cannot promise that this research will benefit you. Just like regular care, this research can have side effects that can be serious or minor.
- You have the right to refuse to take part, or agree to take part now and change your mind later.
- Whatever you decide, it will not affect your regular care.
- Please review this consent form carefully. Ask any questions before you make a decision.
- Your participation is voluntary.

PURPOSE

You are being asked to participate in a research study of how the genetic makeup of different types of people in Zimbabwe varies. The purpose of the study is to determine hereditary factors that can affect a person's response to medication. You were selected as a possible participant in this study because of your geographical location as well as your ethnicity. A total of about 600 Zimbabweans shall participate in this study.

PROCEDURES AND DURATION

If you decide to participate, all you will undergo is a needle-prick to take a 5ml blood sample for genetic analysis. We hereby declare that your blood sample shall not be tested for anything else, such as HIV (Human Immunodeficiency Virus).

RISKS AND DISCOMFORTS

You will experience minimal risk as a result of participating in this study.

BENEFITS AND/OR COMPENSATION
This study is mainly for general medical advances but could also benefit you as an individual should your genetic status be associated with drug response and risk of side effects of certain drugs that you may be given in future (not in this study), or may be taking. The use of the DNA (genetic material) extracted from your blood will be the responsibility of the Consortium for the study of Pharmacogenetics in Africans (CoPhA)'s committee and principal investigator. The analysis of your sample may be used for the creation of commercial products from which you may not be entitled to a financial reward. In the event of injury resulting from your participation in this study, treatment can be obtained at any Hospital/Clinic and the costs of such treatment will be our responsibility.

CONFIDENTIALITY
        If you indicate your willingness to participate in this study by signing this document, we plan to disclose the results of the study to health authorities and journals. By results, we mean the number (percentage) of Zimbabweans of a certain genetic makeup. Any information that is obtained in connection with this study that can be identified with you will remain confidential and will be disclosed only with your permission.

ADDITIONAL COSTS
All costs are borne by the study. There are no additional costs to participating in this study.

IN THE EVENT OF INJURY
        In the event of injury as a result of participating in this study, contact Emmanuel Chigutsa on 091921016 or Dr. Collen Masimirembwa, 091 422951.

VOLUNTARY PARTICIPATION
        Participation in this study is voluntary. If you decide not to participate in this study, your decision will not affect your future relations with the University of Zimbabwe, nor the African Institute of Biomedical Science and Technology, its personnel, and associated hospitals. If you decide to participate, you are free to withdraw your consent and to discontinue participation at any time without penalty.

OFFER TO ANSWER QUESTIONS
        Before you sign this form, please ask any questions on any aspect of this study that is unclear to you. You may take as much time as necessary to think it over.

AUTHORIZATION
        You are making a decision whether or not to participate in this study. Your signature indicates that you have read and understood the information provided above, have had all your questions answered, and have decided to participate.

The date you sign this document to enroll in this study, that is, today's date, MUST fall between the dates indicated on the approval stamp affixed to each page. These dates indicate that this form is valid when you enroll in the study but do not reflect how long you may participate in the study. Each page of this Informed Consent Form is stamped to indicate the form's validity as approved by the MRCZ.

_____   _____
Name of Research Participant (please print)   Date

_____   _____   AM
Signature of Participant or legally authorized representative   Time   PM

Relationship to participant: _____

YOU WILL BE GIVEN A COPY OF THIS CONSENT FORM TO KEEP.

If you have any questions concerning this study or consent form beyond those answered by the investigator, including questions about the research, your rights as a research subject or research-related injuries; or if you feel that you have been treated unfairly and would like to talk to someone other than a member of the research team, please feel free to contact the Medical Research Council of Zimbabwe on telephone 791792 or 791193.

Appendix 2: Questionnaire for psychiatric patient details

STUDY ASSIGNED NUMBER: ZWP…….

DATE: ……….

AGE: …….years

GENDER: …..Male or Female

DRUG	DOSING REGIMEN	DATE COMMENCED	DATE AND TIME LAST DOSE TAKEN

TIME OF BLOOD DRAW: …….

Appendix 3: Demographic information, *CYP2D6* genotypes, and drug plasma levels for patients on Chlorpromazine.

Patient number	CPZ plasma conc /nM	dose adjusted CPZ conc nM/mg	*CYP2D6* *17	*CYP2D6* *29	age /years	sex
ZWP21	0.083257	0.000531	CC	GC	46	F
ZWP40	5.3253	0.008493	CC	GC	51	M
ZWP13	4.3841	0.013984	CC	GG		F
ZWP3	21.689	0.034592	CC	GG	20	M
ZWP29	11.344	0.036185	CC	GG	49	F
ZWP42	43.404	0.069225		GG	25	M
ZWP38	22.84	0.072855	CT	GC	56	F
ZWP19	30.48	0.097225	CC	GG	37	F
ZWP1	123.48	0.098469	CT	GG	20	F
ZWP43	33.318	0.106278	CT	GG	42	M
ZWP26	46.735	0.149075	CC	GC	30	M
ZWP2	74.036	0.236159	TT	GG	21	F
ZWP30	79.989	0.255148	CC	GC	32	F
ZWP20	414.93	0.66177		GG	35	M
ZWP7	465.52	0.742456	CC	GC	67	M
ZWP15	176.21	0.74983	CC	GG	50	F
ZWP35	514.52	1.641212	CT	GG	32	M

Appendix 4: Demographic information, *CYP2D6* genotypes, and drug plasma levels for patients on Thioridazine

Patient number	Thioridazine plasma conc nM	dose adjusted thioridazine conc (nM/mg)	*CYP2D6* *17	*CYP2D6* *29	age /years	sex
ZWP27	5.262	0.006508	CC	GG	66	M
ZWP32	8.7912	0.03262	CC	GC	46	M
ZWP16	272.22	2.019436		GC	18	M
ZWP5	372.9	0.691708	CC	GG	37	M
ZWP31	464.17	0.861009	CT	GG	23	M
ZWP1	1184.7	2.197551	CT	GG	20	F
ZWP44	1216.6	2.256724	CC	GG	31	F
ZWP47	1275.9	1.577912	CT	GG	27	M
ZWP17	1482.7	2.750325		GG	28	F
ZWP23	1636.9	6.07384	CC	GC	33	M
ZWP13	1944.5	2.404774	CC	GG		F
ZWP12	2134.9	7.921707	CC	CC		F

			CYP2D6*17	CYP2D6*29		
ZWP14	2392.3	4.437581	CT	GG		M
ZWP3	2655.3	3.283824	CC	GG	20	M
ZWP9	3229.9	5.991282	CT	GC		M
ZWP4	3670.3	4.53908	CC	GG	34	M
ZWP2	3873.9	4.790873	TT	GG	21	F
ZWP28	4228.9	7.84437	CC	GC	24	F
ZWP10	4263.6	5.272817	CT	GG	20	M
ZWP8	5871.5	7.261316	CC	GG		M
ZWP6	5984.3	11.10054	CC	GG	33	F

Appendix 5: Demographic information, *CYP2D6* genotypes, and drug plasma levels for patients on Haloperidol

Patient number	Haloperidol plasma conc /nM	dose adjusted haloperidol conc (nM/mg)	*CYP2D6*17	*CYP2D6*29	age /years	sex
ZWP39	2.5317	0.380707	CT	GG	73	M
ZWP38	3.5258	0.132549	CT	GC	56	F
ZWP37	6.2199	0.467662	CT	GG	38	F
ZWP22	6.8048	0.511639	CC	GG	23	F
ZWP18	10.071	0.757218		GG	69	F
ZWP42	11.4	0.428571	CC	GG	25	M
ZWP43	12.098	0.909624	CT	GG	42	M
ZWP33	12.814	0.481729	CT	GG	27	M
ZWP31	14.881	0.279718	CT	GG	23	M
ZWP25	16.337	0.614173	CC	GG	39	F
ZWP24	18.215	0.684774	CC	GC	43	F
ZWP50	19.291	0.362613	CC	GG	28	M
ZWP11	19.683	1.479925	CC	GG	46	M
ZWP19	20.086	1.510226		GG	37	F
ZWP32	21.196	1.593684	CC	GC	46	M
ZWP26	21.609	0.812368	CC	GC	30	M
ZWP28	25.109	0.943947	CC	GC	24	F
ZWP49	27.27	1.370352	CC	GG	36	F
ZWP6	41.959	1.577406	CC	GG	33	F
ZWP14	45	0.56391	CT	GG		M
ZWP2	49.717	0.93453	TT	GG	21	F
ZWP20	53.126	1.997218		GG	35	M
ZWP4	62.666	1.177932	CC	GG	34	M
ZWP47	80.668	1.516316	CT	GG	27	M
ZWP10	96.722	0.808037	CT	GG	20	M

Appendix 6: Demographic information, *CYP2D6* genotypes, and drug plasma levels for patients on Fluphenazine Decanoate (FD)

Patient number	Fluphenazine plasma conc /nM	Fluphenazine dose adjusted conc X1000 (nM/mg)	age /years	sex	*CYP2D6**17	*CYP2D6**29
ZWP28	0.13075	2.289842	24	F	CC	GC
ZWP31	0.20427	2.386332	23	M	CT	GG
ZWP24	0.22002	3.85324	43	F	CC	GC
ZWP32	0.22193	7.787018	46	M	CC	GC
ZWP36	0.2293	4.015762	64	F	CC	GG
ZWP42	0.33079	5.79317	25	M	CC	GG
ZWP45	0.34075	5.967601	44	M	CT	GG
ZWP49	0.57499	20.17509	36	F	CC	GG
ZWP4	0.57756	6.747196	34	M	CC	GG
ZWP27	0.58309	10.21173	66	M	CC	GG
ZWP18	0.59849	10.48144	69	F		GG
ZWP43	0.6826	11.95447	42	M	CT	GG
ZWP34	0.78822	13.8042	21	M	TT	GG
ZWP29	0.7888	13.81436	49	F	CT	GG
ZWP37	0.82285	14.41068	38	F	CT	GG
ZWP17	0.83077	14.54939	28	F		GG
ZWP21	0.83251	14.57986	46	F	CC	GC
ZWP30	0.90598	15.86655	32	F	CC	GC
ZWP47	0.91469	8.023596	27	M	CT	GG
ZWP40	0.94151	16.48879	51	M	CC	GC
ZWP20	2.0428	23.86449	35	M		GG
ZWP10	3.3284	29.19649	20	M	CT	GG
ZWP14	3.6979	32.43772		M	CT	GG

Appendix 7: Demographic information, *CYP2D6* genotypes, and drug plasma levels for patients on Amitriptyline

Patient number	Amitriptyline plasma conc /nM	Dose adjusted AMT conc (nM/mg)	age /years	sex	*17	*29	Nortriptyline plasma conc /nM
ZWP46	1.9605	0.007261	45	M	CT	GG	< 0
ZWP48	21.716	0.060322	66	F	CC	GC	13.344
ZWP39	63.97	0.355389	73	M	CT	GG	58.727
ZWP30	158.37	0.439917	32	F	CC	GC	197.18
ZWP41	159.53	0.590852	48	F	CC	GG	41.438

ZWP24	196.26	0.726889	43	F	CC	GC	144.6
ZWP25	666.5	2.468519	39	F	CC	GG	205.04
ZWP11	892.47	4.958167	46	M	CC	GG	518.24
ZWP38	1292.6	2.393704	56	F	CT	GC	867.77

Appendix 8: Drug regimens of psychiatric patients

SAMPLE NUMBER (ZWP)	DRUGS	TIME FROM LAST DOSE/hrs	Steady state Y/N
01	THIORIDAZINE 200mg N CHLORPROMAZINE 200mg bd	22 8	Y N
02	THIORIDAZINE 100am, 200mg N CHLORPROMAZINE 100 im stat (29/03/06) HALOPERIDOL 10mg b.d.	9 9	Y N N
03	THIORIDAZINE 100mg am, 200mg N CHLORPROMAZINE 200mg im (9/04 and 18/04/06) HALOPERIDOL 10mg bd CARBAMAZEPINE 200mg tds BENZHEXOL 5mg bd	9 21 6	Y N Y
04	THIORIDAZINE 100am, 200mg N HALOPERIDOL 10 mg bd F.D. 37.5mg im BENZHEXOL5mg od	6 6 123	Y Y N
05	THIORIDAZINE 200mg N SULPIRIDE 200mg tds LITHIUM CARBONATE 750mg N	19	Y
06	THIORIDAZINE100mg bd HALOPERIDOL 5mg bd AMOXYCILLIN, STEROLINS, STALANEV 40	6(smpl A)2(B) 6(smpl A)2(B)	Yes for B sample
07	CHLORPROMAZINE 200mg N	15	Y
08 (A) B C	THIORIDAZINE 200mg N THIORIDAZINE 100mg am, 200mg N THIORIDAZINE 100mg am, 200mg N	15 12 12	N N Y
09			
10	THIORIDAZINE 100mg bd 200mg N HALOPERIDOL 15mg tds F.D. 50mg im stat CARBAMAZEPINE 200mg tds BENZHEXOL 5mg od	1 1 72	Y Y N
11	HALOPERIDOL 5mg N AMITRIPTYLINE 50mg N	14 14	Y Y
12	SODIUM VALPROATE 200mg tds COTRIMOXAZOLE, STALANEV		

	SULPIRIDE THIORIDAZINE 100mg N BENZHEXOL 5mg od	14	Y
13	THIORIDAZINE 100am 200mg N CHLORPROMAZINE 100mg am HALOPERIDOL 10mg bd	2 2 2	N Y N
14	F.D. 50mg im stat HALOPERIDOL 10mg tds THIORIDAZINE 200mg N	288 2 14	N Y Y
15	CHLORPROMAZINE 75mg N HYDROCHLOROTHIAZIDE 25mg od GLIBENCLAMIDE 10mg bd	13.5	Y
16	PHENOBARBITONE 90mg N CARBAMAZEPINE200mg bd THIORIDAZINE 50mg N	13.5	Y
17	LITHIUM CARBONATE 500mg N THIORIDAZINE 200mg N F.D. 25mg im BENZHEXOL 5mg od	15.5 28 days	Y Y
18	HALOPERIDOL 5mg N BENZHEXOL 2.5mg bd SODIUM VALPROATE 200mg N F.D. 25mg im PROPRANOLOL 40mg od	39 25days	Y Y
19	CHLORPROMAZINE 100mg N HALOPERIDOL 5mg N BENZHEXOL5mg N	14 14	Y Y
20	CHLORPROMAZINE 200mg N HALOPERIDOL 5mg bd F.D. 37.5mg im BENZHEXOL 5mg od	16 2 28days	Y Y Y
21	CHLORPROMAZINE 50mg N F.D. 25mg im	14 30days (and 90min earlier)	Y Y
22	CARBAMAZEPINE 400mg N HALOPERIDOL 5mg N BENZHEXOL5mg N	16.5	Y
23	THIORIDAZINE 100mg N BENZHEXOL 5mg N	14.5	Y
24	HALOPERIDOL 10mg N CARBAMAZEPINE 200mg bd BENZHEXOL 5mg od F.D. 25mg im AMITRIPTYLINE 75mg N	14.5 15 days 14.5	Y Y Y
25	AMITRIPTYLINE 75mg N TRIFLUOPERAZINE 10mg N	14	Y Y
26	CHLORPROMAZINE 100mg N HALOPERIDOL 10mg N BENZHEXOL 5mg od	14.5 14.5	Y Y
27	TRIFLUOPERAZINE 5mg bd CARBAMAZEPINE 200mg bd		Y Y

	THIORIDAZINE 300mg N F.D. 25mg im BENZHEXOL 5mg od SALBUTAMOL, HYDROCHLOROTHIAZIDE	15.75 28 days	Y N
28	HALOPERIDOL 5mg bd CARBAMAZEPINE 200mg N F.D. 25mg im THIORIDAZINE 200mg N	3.5 28days 14	Y Y Y Y
29	CHLORPROMAZINE 100mg N F.D. 25mg i.m. monthly	13.3 35 days	Y Y
30	CHLOPROMAZINE 100mg N AMITRIPTYLINE 100mg N F.D. 25mg im	13.5 14 days ago ?	Y Y Y
31	F.D. 37.5mg im HALOPERIDOL 10mg bd THIORIDAZINE 200mg N CARBAMAZEPINE 200mg am 400mg N BENZHEXOL 5mg od	28 days 2.5 14	Y Y Y Y
32	F.D. 12.5mg im HALOPERIDOL 5mg N THIORIDAZINE 100mg N BENZHEXOL	28 days 17 17	Y Y Y
33	CHLORPROMAZINE200mg N HALOPERIDOL 10mg N CARBAMAZEPINE 200mg bd BENZHEXOL 5mg N	12 12	Y Y
34	CARBAMAZEPINE 400mg N TRIFLUOPERAZINE 15mg N BENZHEXOL 5mg od F.D. 25mg im	 24 days	 Y
35	CHLORPROMAZINE 100mg N HALOPERIDOL 10mg N CARBAMAZEPINE 200mg bd LITHIUM CARBONATE 1000mg N BENZHEXOL 5mg od	13.5 13.5	Y Y
36	THIORIDAZINE 100mg N HALOPERIDOL 5mg am, 5mg afternoon F.D. 25mg im BENZHEXOL 5mg od	14.5 20.5 28 days	Y Y Y
37	F.D. 25mg im HALOPERIDOL 5mg N BENZHEXOL 5mg od PHENOBARBITONE 120mg N PROPRANOLOL 20mg bd	28 days 12.75	Y Y
38	CHLORPROMAZINE 100mg N HALOPERIDOL 10mg N AMITRIPTYLINE 150mg N BENZHEXOL 5mg od	14 14 14	Y Y Y
39	CARBAMAZEPINE 200mg N PHENOBARBITONE 90mg N		

	HALOPERIDOL 2.5mg N	13.3	Y
	BENZHEXOL 5mg N		
	ATENOLOL 50mg N		
	AMITRIPTYLINE 50mg N	13.3	Y
40	F.D. 25mg im	28 days	Y
	CHLORPROMAZINE 200mg N	13.5	Y
	BENZHEXOL 5mg N		
41	AMITRIPTYLINE 75mg N	15.5	Y
	CHLORPROMAZINE 100mg N	15.5	Y
	HYDROCHLOROTHIAZIDE 25mg od		
	NIFEDIPINE 10mg bd		
	INDOMETHACIN 25mg tds		
42	CHLORPROMAZINE 200mg N	13.75	Y
	HALOPERIDOL 10mg N	13.75	Y
	F.D. 25mg im	21 days	Y
	PHENOBARBITONE 120mg N		
43	CHLORPROMAZINE 100mg N	14	Y
	HALOPERIDOL 5mg N	14	Y
	F.D. 25mg im	28 days	Y
	BENZHEXOL 5mg od		
44	THIORIDAZINE 200mg N	15	Y
45	F.D. 25mg im	60 days	Y
46	AMITRIPTYLINE 75mg N	37.5	Y
47	F.D. 50mg im	32 days	Y
	HALOPERIDOL 10mg bd	4	Y
	THIORIDAZINE 300mg N	38	Y
	BENZHEXOL 5mg od		
48	HALOPERIDOL 10mg N	15	Y
	AMITRIPTYLINE 100mg N	15	Y
49	F.D.12.5mg im	28 days	N
	HALOPERIDOL 7.5mg N	15	Y
	BENZHEXOL 5mg od		
50	CHLORPROMAZINE 200mg N	15.5	Y
	HALOPERIDOL 10mg bd	4.5	Y
	BENZHEXOL 5mg od		

Appendix 9: MRCZ ethical approval

Telephone: 791792/791193/792747
Telefax: (263) - 4 - 790715
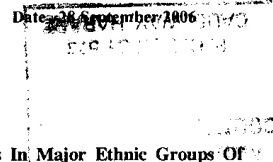E-mail: mrcz@mrczimshared.co.zw

Medical Research Council of Zimbabwe
Josiah Tongogara / Mazoe Street
P. O. Box CY 573
Causeway
Harare

MRCZ APPROVAL LETTER

Ref: MRCZ/A/1283
XRef: MRCZ/B/5

Date: 28 September 2006

Prof. T. E. Chigwedera
Box 2294
Harare

RE: **"Genetic Polymorphism On Drug Metabolizing Enzymes In Major Ethnic Groups Of
        Zimbabwe And Clinical Impact Of CYP2D6 Polymorphism On Efficacy And Safety In
        The Use Of Psychotropics"**

Thank you for the above titled proposal that you submitted to the Medical Research Council of
Zimbabwe (MRCZ) for review. Please be advised that the Medical Research Council of Zimbabwe has
reviewed and **approved** your application to conduct the above titled study.

> The above details should be used on all correspondence, consent forms and documents as
> appropriate.

- **APPROVAL NUMBER**   **:MRCZ/A/1283**
- **APPROVAL DATE**   **: 28 Setember 2006**
- **EXPIRATION DATE**   :This approval expires on **27 September 2007**
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress
  report on a standard form obtainable from the MRCZ Offices, should be submitted one month
  before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING** :All serious problems having to do with subject
  safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ
  within 10 working days using standard forms obtainable from the MRCZ Offices.
- **MODIFICATIONS:** Prior MRCZ and IERC approval using standard forms obtainable from the
  MRCZ Offices is required before implementing any changes in the Protocol (including changes in
  the consent documents).
- **TERMINATION OF STUDY:** On termination of a study, a report has to be submitted to the
  MRCZ using standard forms obtainable from the MRCZ Offices.
- **QUESTIONS:** Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on
  mrcz@mrczimshared.co.zw.
- **Other:**
- Please be reminded to send in copies of your final research results for our records as well as for
  Health Research Database
- You're also encouraged to submit electronic copies of your publications in peer reviewed journals
  that may emanate from this study.

Kind regards from the MRCZ Secretariat.

Buesera
....

PROMOTING THE ETHICAL CONDUCT OF RESEARCH
Executive Committee: *Ms G.N. Mahlangu (Chairperson), Prof E. Gomo (Vice Chairperson), Mrs S. Munyati
(Secretary), Dr T. Chipato (Executive Member)*
Registered with the USA Office for Human Research Protections (OHRP) as an International IRB
(IRB Number IRB00002409 IORG0001913)

109

Appendix 10: Approval from Provincial Medical Director for Matebeleland South

Tel: 263-9-6834
: 263-9-62914

Fax: 263-9-77915

ZIMBABWE

MINISTRY OF HEALTH
AND CHILD WELFARE
MATABELELAND
SOUTH P Bag A 5225
BULAWAYO

31 October 2006

School of Pharmacy
P O Box MP 167
Mount Pleasant
Harare

Attention Emmanuel Chigutsa

REQUEST TO CARRY OUT STUDY IN PLUMTREE DISTRICT

I am in receipt of your request to carry out a study in Plumtree District on Drug Metabolizing Enzymes in major ethnic groups of Zimbabwe and clinical impact of CYP2D6 Polymorphism on efficacy and safety in the use of Psychotropics.

I am also aware that your proposal was passed through University of Zimbabwe College of Health sciences Ethics Committee as well as Medical Research of Zimbabwe (BDCZ)

I have no objection in you carrying out the study.

Dr J Ndlovu
PROVINCIAL MEDICAL DIRECTOR MATABELELAND SOUTH

/gd