DETECTION OF DRUG METABOLIZING ENZYME GENE (DMEs) POLYMORPHISMS AMONG THE ZULU POPULATION OF SOUTH AFRICA

by

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in the

PFIZER MOLECULAR BIOLOGY RESEARCH FACILITY

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Declaration
The experimental work described in this dissertation was conducted in the Pfizer Molecular Biology Research Facility, Nelson R. Mandela School of Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban, under the supervision of Prof. Richard Naidoo. The clinical work described in this dissertation was conducted at the CAPRISA’s Prince Cyril Zulu clinic for communicable diseases, Ethekwini, KwaZulu-Natal under the co-supervision of Dr. K. Naidoo and Dr. P. Chelule.

Ethical approval for the study was obtained from the Ethics and Professional Standards sub-committee (College of Health Sciences), Nelson R. Mandela School of Medicine, University of Kwazulu-Natal.

This study represents original work by the author and has not been submitted in part or whole to any other tertiary institution. Where use was made of the work of others, it has been duly acknowledged in the text.

M. T. Makume

July 2007
Dedication

Mme le Ntate Makume, dissertation ena keya lona. Ke leboha Modimo ho nkabela batswadi ba nkgothaletsang thuto le mamello.

Matseleng Makume ngwaneso, ke o leboha ho menahane ka thuso yohle e omphileng yona. O bile lere laka.

Ana, Dean, Ditebogo-Tsohle, Manthati, Nomaphoso, Ntsoaki, Setsoana, Siyakhona, Thabang, Thato, Refentse le Vuyiswa: Motswalle wa sebele ke a tshepang bokgoni baka, le ha ke sa tshepe.

"Lehlakana la bophelo le tholwa seretseng"
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<td>°C</td>
<td>Degree Celcius</td>
</tr>
<tr>
<td>3TC</td>
<td>Lamivudine</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
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<tr>
<td>ADR</td>
<td>Adverse drug reaction</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency syndrome</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>ARV</td>
<td>Antiretroviral</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutively activated receptor</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>Cys</td>
<td>Cystine</td>
</tr>
<tr>
<td>d4T</td>
<td>Stavudine</td>
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<tr>
<td>DAIDS</td>
<td>Division of AIDS</td>
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ddl  Didanosine
DME  Drug metabolizing enzymes
DNA  Deoxyribonucleic acid
DOTS  Directly Observed Therapy
EM  Extensive metabolizers
FMO  Flavin Monooxygenases
g  Gram
G  Guanine
γ-GT  Gammaglutamyltransferase
Gln  Glutamine
GST  Glutathione transferases
HAART  Highly active antiretroviral therapy
His  Histidine
HIV  Human Immunodeficiency Virus
HNF  Human necrosis factor
hPXR  human Pregnane X Receptor
IA  Intermediate Acetylator
Ile  Isoleucine
IRIS  Immune reconstitution inflammatory syndrome
Leu  Leucine
LFT  Liver function tests
Lys  Lysine
M  Molar (mol/L)
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<tr>
<td>MDR-1</td>
<td>Multi-drug resistant gene</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic acid</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>ml</td>
<td>Millilitre</td>
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<tr>
<td>mm</td>
<td>Milimetre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar (mmol/L)</td>
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<tr>
<td>MT</td>
<td>Mitochondrial toxicity</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate hydrogen</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyltransferases</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymarase chain reaction</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitors</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomole</td>
</tr>
<tr>
<td>P</td>
<td>Probability value</td>
</tr>
<tr>
<td>PM</td>
<td>Poor Metabolizer</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RA</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>SA</td>
<td>Slow Acetylator</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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SULT  Sulfotranferases
T     Thymidine
TB    mycobacterium tuberculosis
UGT   Uridine diphosphate glucoronoslytransferases
UM    Ultrarapid metabolizers
W     Watt
WHO   World Health Organization
μl    Microlitre
μM    Micromolar
ZDV   Zidovudine
ABSTRACT

The ability to metabolise drugs and achieve positive therapeutic outcomes is dependent on both genetic and environmental factors. The focus of this study was to determine the distribution and frequency of clinically relevant DME alleles and to assess the impact of these DME alleles on therapeutic outcomes in a cohort of 50 HIV-TB co-infected Zulu participants.

PCR-RFLP was used to generate a genotypic profile of CYP1A2, 2C9, 2C19, 2E1, 3A4, MDR-1 and NAT-2.

The distributions of the allelic frequencies were as follows. The CYP1A2 (A) - 50.7%, CYP2C9*2 – 100% and *3 – 56.2%, CYP2C19*2 – 35.4%, CYP2E1 (C2) – 28.4%, CYP3A4*1B (G) – 58.2%, MDR-1 (C3435T) - 16% and NAT-2 slow acetylators – 6.5%.

Seventy-three percent of participants had prolonged TB therapy. Within this group, 82.9% of patient displayed wild type and 17.2% variant allele for CYP2E1 gene (p = 0.04) profile. In addition, all the slow acetylators in this study had prolonged TB therapy. In the MDR-1 gene, 87.5% showed wild type allele and 12.5% displayed the variant allele. Unsuccessful TB outcomes were also noted in 22% of this study population. In this group the variant allele was found to be dominant in CYP1A2, CYP3A4 and NAT-2, the opposite was seen in CYP2E1 and MDR-1. It was also interesting to note a similar genetic profile in the group that showed successful TB therapy outcomes. All participants had positive ARV treatment outcomes despite DME genotypic variations. However, 26% of all study participants experienced liver enzyme abnormalities. These findings concur with other studies regarding the ethnic distribution of DME alleles and evidence of an association
between ART and TB therapeutic outcomes and DME genotype variation was inconclusive.
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1. INTRODUCTION

For a clinician to administer therapy, a thorough understanding of how the drugs will interact at a cellular and molecular level is imperative. Knowledge of the pathways a drug will undergo is useful in predicting potential complications, such as drug interactions, and toxicity.

The processes involved in the pharmacokinetics of any drug are categorised into four main components namely absorption, distribution, metabolism and elimination.

1) Absorption is dependent on how the active drug agent is formulated, the route of administration, lipid solubility and gastric acidity,

2) Distribution of the drug from plasma to target tissues,

3) Metabolism, taking place in the liver, where the agent is modified by inherent cytochrome enzymes which eventually render it more soluble for excretion, and finally

4.) Elimination, whereby the kidneys, bile, and to a lesser extent breath and sweat play a role in the elimination of drugs (Kumar and Clark, 2002).

Adverse drug reaction (ADRs) can complicate therapy for patients. They tend to pose a significant challenge to human health. Approximately 10-20% of hospital admissions are a result of ADRs (Kumar and Clark, 2002; Ingelman-Sundberg, 2001, 2004 and Park et al, 2005). The impact of ADRs on the health system in 1994 was recorded to be the cause of over 100 thousand deaths in the US (Ingelman-Sundberg, 2001). In addition to this,
pharmaceutical companies are frequently forced to withdraw a drug from market due to its toxic effects.

There are two types of ADRs:

- Dose-dependent: resulting from known primary or secondary pharmacology of the drug. Symptoms are often dose-related and usually abate following withdrawal of the drug. This type account for 80% of all ADRs and is avoidable by selection of an appropriate dose for the patient.

- Dose-independent: These ADRs are unpredictable and often life-threatening. The factors contributing to these types of ADRs are wide ranging, including variation among individuals' susceptibilities.

Such reactions are mostly attributed to the metabolism of drugs, making the liver a target organ in investigations.

Cellular toxicity (hepatotoxicity) is a known consequence of a majority of ADRs, where drug-induced liver injury is the main reason for more than 50% of cases of acute liver failure. According to Park et al, (2005) more than 600 drugs have been associated with hepatotoxicity.

With regards to drug 'inactivation', drug metabolizing enzymes (DMEs), a family of haem-containing proteins are pivotal in the biotransformation of an active drug agent, additionally, dysfunction within DMEs can contribute to ADRs.

More than 50% of drugs cited in ADR studies undergo phase I metabolism, 86% of which are metabolised primarily by the cytochrome P450 enzymes (Ingelman-Sundberg, 2005).
Thus it is important to elucidate the role of DMEs, in an effort to circumvent the challenges posed by ADRs.

Drug metabolizing enzymes responsible for both phase I and phase II are found in the small intestines, blood-brain barrier and kidneys, however the highest concentration of DMEs is in the liver, specifically in the smooth endoplasmic reticulum of hepatocytes.

Eighty percent of clinically relevant drugs are mainly metabolised by the cytochrome P450 enzymes (Kumar and Johnson, 2002; Ingelman-Sundberg, 2005). The activity of these enzymes can be categorised into induction and inhibition.

- **Induction:** Results from an increased expression of the CYP (cytochrome) enzyme. A drug or xenobiotic substrate binds to the CYP enzyme, causing it to clear the agent.

- **Inhibition:** The drug binds to an enzyme’s active site, preventing its activity, thus delaying the clearance of the drug from the blood (Lin and Lu, 2001).

The development of ADRs could, among other things, be caused by decreased or increased CYP enzyme activity. The challenge is further compounded by drug-drug interactions, where two drugs competing for the same enzyme can cause pre-mature clearance of either or both drugs. The heightened CYP activity often results in reduced efficacy. The opposite trend is observed when one of the co-administered drugs inhibits an enzyme responsible for the metabolism of the other drug. The consequence of such a phenomenon is that the non-metabolized drug remains in the blood-plasma for prolonged periods. Toxicity often arises.
from this effect or its reactive intermediates overwhelm the cellular repair system, causing hepatocellular damage.

The first line of investigation for possible liver stress is the evaluation of liver enzymes, which are known to be elevated in the event of liver stress (Johnson and McFarlane, 1989). Aminotransferases (AST) and (ALT) are enzymes found in hepatocytes. Their elevation in blood would indicate cell damage. High levels of AST are often noted during hepatic necrosis, myocardial infarction and muscle injury. ALT, unlike AST is a liver specific enzyme, found in the cytosol of hepatocytes and high levels of ALT would specifically indicate liver stress (Kumar and Clark, 2002; Johnson and McFarlane, 1989). Alkaline phosphatase (ALP), similar to AST is not exclusive to hepatocytes, however when viewed in conjunction with other liver enzyme levels, its elevation could be linked to cholestasis (lack or reduced bile flow). $\gamma$-glutamyltransferase (GGT) is another liver enzyme whose activity is induced by phenytoin and alcohol. (Kumar and Clark, 2002).

Even with the above-mentioned test, it is still a challenge to predict the occurrence ADRs as these tests play no role in predicting the occurrence of ADRs that will indicate that damage may have occurred (Johnson and McFarlane, 1989). An important element to dose-independent ADRs is the variation among individuals in presentation of liver stress or hepatotoxicity. It has been noted that some individuals have a propensity to develop drug-induced toxicity, whereas others remain asymptomatic. This variability in metabolism is the focus of the present study.
The present study focuses on individuals who are on antiretroviral (ARV) and anti-tuberculosis therapies. In these situations, where concomitant therapy is being administered, clinicians have to be cognisant of potential liver stress, particularly because both therapies are known to be hepatotoxic.

With regards to HIV therapy, the development and implementation of highly active antiretroviral therapy (HAART) has significantly reduced the morbidity and mortality among HIV-infected patients (Pol et al., 2004). However, the liver has come to play an increasingly important role where liver disease is now one of the leading causes of morbidity in HIV-infected patients (Pol et al., 2004). Nunez reported an increase in HIV mortality cases due to discontinuation of ARV therapy from 6% in 1996 to 31.8% in 1998-1999. The main cause of the discontinuation was hepatotoxicity-related (Nunez, 2006).

Genetic studies reveal that variations in the human genome can influence an individuals’ predisposition to illness or even therapeutic failure. It has been revealed that genetic variation within drug-metabolizing genes may be responsible for the dysfunctional enzyme activity that leads to ADRs (Ingelman-Sundberg, 2001; Meyer and Gut, 2002). Variations caused by single nucleotide polymorphism (SNPs), result in functionally variable DMEs. Furthermore the types of SNPs occurring in DME genes are ethnically linked (Meyer and Gut, 2002). This is highlighted by Andersson and co-workers who reported that the presence of CYP2C9*2 allele resulted in poor or reduced metabolism of CYP2C9 substrates and was found in 10% of Caucasians, 2-5% Africans and absent among Asians (Andersson et al, 2005).
The implications of such reports warrant large-scale investigations, which could aid in reducing ADR costs. The determination of an individual’s or an ethnic groups’ propensity for toxicity due to certain therapies by clinicians may be able to reduce morbidity caused by drug-induced liver disease. The present study aimed to address key areas of DME gene polymorphisms and liver stress among a cohort of HIV and TB co-infected Zulu patients. This was done by genotyping the cohort for DMEs known to metabolise anti-TB and ARV drugs. Further an extensive review of clinical charts (LFTs, CD4+ T-cell and viral load counts) was carried out. An attempt at exploring a possible link between therapeutic outcomes and genotypic profile was made by combining the clinical and molecular biology components.
2. LITERATURE REVIEW

2.1 INTRODUCTION

Worldwide, *Mycobacterium tuberculosis* (TB) is known to cause more adult deaths than any other infectious disease, in which one in three infected individuals in the world is thought to be at risk of developing TB (Valadas and Antunes, 2005). According to the World Health Organization (WHO), South East Africa and Sub-Saharan African regions are the regions most heavily burdened by TB infections, with 617 000 and 538 000 deaths, respectively (WHO, 2005).

*Mycobacterium tuberculosis* was once thought to be kept under control, until recently when more cases of TB had been reported. The increased incidences of TB cases can be attributed to several factors such as increasing poverty levels and an increase in Human Immunodeficiency Virus (HIV) infections (Kirschner, 1999; Abdool Karim et al, 2004; Valadas and Antunes, 2005).

Several studies have linked the increase of TB to an increase in HIV infections (Kirchner, 1999; Abdool Karim et al, 2004; Valadas and Antunes, 2005). HIV co-infected TB individuals face increased chances of mortality (500 times more likely than HIV uninfected individuals), as one infection accelerates the progression of the other. Thus TB has been termed “the main opportunistic disease for HIV” (Kirschner, 1999).

It has been shown that HIV infected individuals face an increased risk of activating latent infection due to a decrease in CD4⁺ T-cell count and an increased HI viral load (Kirschner,
Valadas and Antunes (2005) reported that the viral load increased with TB infection and that HIV patients with TB develop AIDS faster than HIV patients without TB (Valadas and Antunes, 2005). Kirschner also showed that TB interferes with the best predictor of AIDS, CD4+ T-cells, by lowering their numbers (Kirschner, 1999).

The South African statistics with regards to HIV and TB have reached epidemic proportions. HIV prevalence rates in 2000 were 24.5% and rose to 29.5% in 2004 (South African HIV and AIDS statistics, 2005). It has been reported that in some Eastern and Southern African regions, 60-70% of new TB cases are also infected with HIV (Abdool-Karim et al, 2004).

2.1.1 Viral Hepatitides

Liver inflammation (hepatitis) is one of the primary disorders, most commonly caused by either hepatitis B or C viruses. Hepatitis B can lead to acute hepatitis, chronic hepatitis, cirrhosis, fulminant hepatitis or a carrier state; hepatitis is the most cause of chronic hepatitis, cirrhosis and hepatocellular (Phillips and Brewer, 2002). HBV or HCV co-infection is frequent in HIV infected patients because of similar routes of infection the resultant liver disorders caused by the viral hepatitides are worsened at it is known that HIV has the ability to significantly modify the natural history of HBV/HCV by increasing the levels of either viremia, especially at the time of HIV seroconversion (Pol et al., 2004). HCV related liver disease is now an important cause of mortality (4.8%) and morbidity (8.6%) in HIV-HCV co-infected patients (Pol et al., 2004).
2.2 THERAPIES

Treatment programs such as Directly Observed Therapies (DOTS) and Highly Active Antiretroviral Therapy (HAART) have been implemented to curb the disease progression of TB and HIV respectively; various medications have been formulated for both agents. Presently, the Department of Health of South Africa utilises the DOTs programme, which involves the usage of drugs such as Isoniazid, Pyrazinamide, Rifampicin and Ethambutol as a first line regimen for a minimum of six months or until the bacteria have been completely cleared. This therapy, if adhered to, would cure TB. On the other hand, the ARV therapy programme makes use of drug groups such as nucleoside reverse transcriptase inhibitors (NRTI’s), Non-nucleoside reverse transcriptase inhibitors (NNRTI’s) and protease inhibitors (PI’s) to arrest the progression of HIV to AIDS (Department of Health of South Africa, 2005).

Regardless of the availability of drugs to treat both diseases, i.e. curing TB and arresting HIV to AIDS progression, it has become a formidable challenge when treating individuals infected with HIV and TB. Various factors may contribute to these often recalcitrant therapies. Resistance to therapy is a growing concern due to the prolonged periods for TB therapy. This often induces non-adherence by the patients to drug therapy thus aiding in the formation of drug resistant strains (Kirchner, 1999). Treatment of multi-drug resistant TB is more costly, problematic, less effective and even more prolonged (Valadas and Antunes, 2005). The emergence and transmission of HIV viruses resistant to one or more HAART classes has been documented and is becoming a challenge (Wainberg, 2004).
2.2.1 Drug-induced toxicity

Besides resistance to therapy, adverse drug reactions (ADRs) have proven to be a significant health problem to patients, contributing to both morbidity and mortality. In addition, ADRs present a major concern to pharmaceuticals. Drug-induced liver injury is the most frequent reason for the withdrawal of an approved drug and it accounts for more than 50% of acute liver failure cases. To date more than 600 drugs have been associated with hepatotoxicity (Park et al, 2005 and Maddrey, 2005).

The metabolic toxicities due to ARV therapy are wide-ranging; from mitochondrial toxicity, glucose intolerance, fat redistribution syndrome and hyperlipideamia. Such toxicities manifest biochemically and morphologically. Mitochondrial toxicity (MT) is the most serious complication associated with the use of NRTIs; Zidovudine (ZDV), Stavudine (d4T), Didanosine (ddl) and Lamivudine (3TC). MT symptoms range from mild to potentially fatal, with each drug causing different complications. Neuropathy is associated with the use of d4T, ddl and 3TC, while hepatic steatosis and lactic acidemia is predominantly associated with ddl, d4T and ZDV (Herman and Easterbrook, 2001; Clark et al, 2002 and Nunez, 2006).

Hence, with the increasing use of ARV therapy, there is an increasing in incidence of myopathy, elevated enzymes, pancreatitis and acute liver failure and lactic acidosis. Nunez (2006) reported an increase in HIV mortality cases due to discontinuation of ARV from 6% in 1996 to 31.8 in 1998-1999. The discontinuation of ARVs was mainly hepatotoxicity related. Generally, a drugs’ propensity to cause liver injury is identified during the large pivotal phase 3 clinical trial. However, some drugs become linked to hepatic problems.
after their release and use in patients in diverse settings (Maddrey, 2005). This problem is further compounded if the patients have an underlying acute or chronic liver disease.

There are limited clinical or laboratory tools which allow for the specific detection of liver injury due to a therapeutic drug. The most important indicator of such injury is often the temporal relationship between initiation of a drug (or drugs) and the appearance of the injury. In an effort to understand and curb drug-induced liver stress, drug metabolism pathways and individual differences affecting such pathways have gained interest (Maddrey, 2005 and Ingelman-Sundberg, 2005).

Recognition and characterization of various enzymes involved in drug metabolism, allow for predictions to be made regarding the likelihood of liver stress, particularly when concomitant therapies are administered (Ingelman-Sundberg, 2001 and Maddrey, 2005).

With respect to HIV therapy, Furin and Johnson (2005) state that South Africa’s use of ARV therapy had decreased the risk of contracting TB from 9.7 to 2.4 cases per 100 people and that ARV therapy has also increased the survival rate of co-infected individuals (Furin and Johnson, 2005). Singingly, the development of ARV therapy and implementation thereof has significantly decreased the morbidity and mortality of HIV-infected patients. Nonetheless, the increased incidences of drug-related hepatotoxicity have highlighted hepatic pathologies when managing HIV infected individuals. Liver enzymes (AST, ALT, etc.) are often elevated in HIV infected patients and the problem is further exacerbated by ARV treatment (Pol et al, 2004).
All classes of antiretroviral drugs have been associated with liver enzyme abnormalities and the mechanisms of such adverse effects appear to differ significantly (Pol et al, 2004 and Clark et al, 2002). Hepatic steatosis and lactic acidosis have been reported in patients treated with zidovudine or didanosine, fatty liver and lactic acidosis is seen in patients following stavudine administration (Clark et al, 2002). Increasing use of ARV therapy was shown to be associated with increased incidence of myopathy, elevated liver enzymes, pancreatitis and acute liver failure and lactic acidosis. Hence liver disease could now be the leading cause of morbidity and mortality (Pol et al, 2004).

Anti-TB drugs such as isoniazid, rifampicin and pyrazinamide are also implicated as the major contributors to hepatotoxicity. Due to this dilemma, determining the time to start anti-retroviral therapy on TB patients remains a concern.

2.3 DRUG METABOLISM

A vast array of chemical compounds can be found circulating in the body at any point in time. These chemicals range from endogenous compounds to xenobiotics. The endogenous component includes bile acids, steroids, prostaglandins and fatty acids. The xenobiotics may range from environmental pollutants such as smoke and pesticides to food and pharmaceutical drugs (Ingelman-Sundberg, 2001; Chelule, 2003).
Although renal excretion may play a role in terminating the biologic activity of some drugs especially ones that have either small molecular weight or polar characteristics, not all drugs have the above mentioned properties. Hence, an alternative mode of removal is required where xenobiotics (generally lipophilic) require alteration of their biological activity (Guengerich, 1995; Chelule, 1998). Drug metabolism or chemical biotransformation of xenobiotics is an alteration process involving various enzyme-catalyzed pathways that ultimately render the compounds inactive and readily excretable. Synthesis of the above-mentioned endogenous substrates may involve the same enzymatic pathways associated with the metabolism of xenobiotics (Guengerich, 1995; Chelule, 1998; Nelson, 1999). However, the main focus will be on xenobiotics, specifically therapeutic drugs, their metabolism and the genetic basis for their variable disposition.

During xenobiotic biotransformation, most of which occurs between absorption of the drug into the circulatory system and its renal elimination, various chemical reactions are involved namely reduction, oxidation, hydrolysis, hydration, conjugation, condensation and isomerisation (Chelule, 1998).

Xenobiotic metabolism occurs in two phases, phase I and II. Phase I metabolism involves the conversion of the parent drug to a more polar metabolite, either by attaching a polar functional group or revealing an inherent one. This reaction often inactivates the compound or modifies its activity, thus if the metabolite is sufficiently polar it will be readily excreted. However, some drugs are still not sufficiently polar and hence proceed to phase II metabolism, during which they become conjugated to another compound which is
usually endogenous and then excreted. In summary, phase I metabolism can be a preparatory reaction for phase II metabolism. Phase I metabolic activity occurs where drugs, administered orally are absorbed into the small intestine and transported first to the liver ultimately undergoing the above-mentioned metabolic processes (Ingelman-Sundberg, 2001; Chelule, 2003 and Park et al, 2005).

Although the liver is the main detoxifying organ, other tissues such as mucosa of the intestine, skin, lung, kidney or brain are also capable of metabolizing drugs (Lin and Lu, 2001).

Metabolism of drugs can be achieved by a wide variety of enzymes, ranging from gastric acids, digestive enzymes or enzymes in the intestinal wall, but the majority are metabolised by specific cellular enzymes, which are found in the liver. These enzymes are located in the endoplasmic reticulum, mitochondria, cytosol, lysozymes or plasma membrane (Lin and Lu, 2001; Chelule, 1998).

2.3.1 Phase I metabolism

Enzymes in this category are also known as “mixed function oxidases” or “monooxygenase”, because their activity requires both molecular oxygen and NADPH to act as a reducing agent (Chelule, 1998; Rettie and Fisher, 1999).

Enzymes belonging to this phase are located in the lipophilic membranes of the endoplasmic reticulum of the liver and other tissues. Isolation of these membranes by homogenization and fractionation yields vesicles called microsomes. The smooth
microsomes tend to be relatively abundant in mixed function oxidases (Chelule, 1998; Rettie and Fisher, 1999; Lin and Lu, 2001).

The overall reaction of these enzymes may be summarised as follows: one molecule of oxygen is reduced per substrate molecule resulting in the production of alcohol and water.

\[ \text{NADPH} + \text{H}^+ + \text{O}_2 + \text{RH} \xrightarrow{\text{CYP Enzymes}} \text{R-OH} + \text{H}_2\text{O} + \text{NADP}^+ \]

Substrate

“Monooxygenase” drug metabolizing enzymes consist of two major groups, Flavin Monooxygenases (FMO’s) and Cytochrome P450 (CYP 450) enzymes.

2.3.1.1 Flavin Monooxygenase (FMO’s)

FMO’s catalyse the oxidation of organic compounds using oxygen and NADPH but unlike CYP P450’s, FMO’s react with oxygen and NADPH in the absence of a substrate to form a flavin enzyme intermediate (4α-hydroxyperoxy flavin). Thus the intermediate can exist in a stable form until coupled with a nucleophilic group to complete the catalytic reaction (Rettie and Fisher, 1999). Five families of FMO’s are identified in humans (FMO 1-5) with FMO3 being the most abundant form in the liver, thus reflecting its dominance in the role of drug metabolism (Rettie and Fisher, 1999; Philips et al, 1995; Cashman, 2005).
2.3.1.2 **Cytochrome P450 enzymes (CYP’s)**

Cytochrome P450 enzymes are a superfamily of haem-containing proteins. These enzymes play a crucial role in drug metabolism since most drugs are metabolised by CYP’s (Ingelman-Sundberg, 2001). These enzymes also play a role in the activation or inactivation of carcinogens and other environmental toxins, as well as biosynthesis and inactivation of many hormones and other endogenous compounds (Oscarson and Ingelman-Sundberg, 2001). The enzymes are so named (P450) because when isolated, they absorb light at 450 nm. Specifically the haemoprotein in its reduced form (Ferrous), binds carbon monoxide to give a ferrocarbonyl adduct that absorbs maximally in the visible region of 450 nm (Estabrook, 2003; Manzi and Shannon, 2005).

In humans, more than 50 CYP P450 enzymes have been identified and further categorised into 17 families and 39 subfamilies (Chelule, 1998; Nelson, 1999; Ingelman-Sundberg, 2005). Classification into families and subfamilies is based on amino acid sequence similarities i.e. enzymes within the same family have more than 40% identity at the amino acid level, while members of the same subfamily are greater than 55% identical (Guengerich, 1995; Nelson, 1999; Oscarson and Ingelman-Sundberg, 2002).

The majority of drug metabolism is carried out by a few isoforms of the CYP 1, 2 and 3 families, all occurring mainly in the liver. Specifically the enzymes CYP1A2, 2E1,2B6, 2C’s, 2D6 and CYP3A’s have been most extensively studied and implicated in metabolizing more than half of pharmaceutically relevant drugs (Ingelman-Sundberg, 2001; Oscarson and Ingelman-Sundberg, 2002).
2.3.2 Phase II metabolism

The main feature of this phase is conjugation. Enzymes belonging to this category are termed “conjugative drug metabolizing enzymes” because they catalyse the coupling of endogenous small molecules to xenobiotics to form a readily excretable soluble “polar” compound (Chelule, 1998; Rettie and Fisher, 1999). These endogenous substrates originate in the diet, thus making nutrition and disease pivotal in the regulation of drug conjugations (Rettie and Fisher, 1999). The conjugative enzyme families include uridine diphosphate glucuronosyltransferases (UGT’s), glutathione transferases (GST’s), sulfotransferases (SULT’s) and N-acetyltransferases (NAT’s) (Coffman et al, 1996). The conjugative reactions involve high-energy intermediates and specific transfer enzymes occurring in the microsomes or in the cytosol (Chelule, 1998).

2.3.2.1 UGT-glucuronosyltransferases (UGTs)

Uridine diphosphate glucuronosyltransferases (UGT’s) are responsible for the addition of UDP-glucoronic acid to xenobiotics. The result is the generation of a more hydrophilic derivative called Glucuronide. The secondary metabolite is then readily excretable in bile or urine (Coffman et al, 1996; Chelule, 1998). Two UGT families, UGT1 and UGT2 are known in humans and both of these families are expressed in the liver (Coffman et al, 1996).

2.3.2.2 Glutathione transferases (GSTs)

The major biological function of GST’s is for protection against electrophilic chemical species (Weber, 1997). They are known to detoxify hydrocarbon epoxides and their
structural impairment has been implicated in carcinogenesis (Chelule, 1998). GST's catalyse the formation of a thioether conjugate by addition or displacements of an electron-withdrawing group. Four families are known namely, GST A1, M1, P1 and T1.

2.3.2.3 Sulfotranferases (SULT's)

Another phase II drug metabolizing enzyme is sulfotranferases. The mode of action of these enzymes is in catalyzing the addition of sulphate groups to xenobiotics with acceptor moieties such as hydroxyl and amine groups (Raftogianis et al, 1997). These enzymes have also been known to bioactivate xenobiotics into highly reactive metabolic intermediates. Ten SULT’s are known in humans of which, only five occur in the liver (Raftogianis et al, 1997).

2.3.2.4 N-acetyltransferases (NAT’s)

This group of phase II enzymes play a significant role in the bioactivation of xenobiotics. NAT’s utilise acetyl-co enzyme A as a donor to transform aromatic amines and hydrazines to amides and hydrazines respectively. Two NAT’s NAT-1 and NAT-2 are known to occur in humans both located in the liver. NAT1 is also known to be expressed in other tissues (Vastis et al, 1995), but NAT-2 is more relevant to drug metabolism.
2.4 CYTOCHROME P450 ACTIVITY

During the course of enzyme-substrate complex formation, certain substrates can either induce or inhibit the activity of a particular CYP enzyme. The induction (increased activity) or inhibition (inactivity) of this enzyme may lead to drug-drug and drug-food interactions (Lin and Lu, 2001; Manzi and Shannon, 2005). The resultant interactions may lead to decreased drug efficacy, toxicity and even mortality. Thus it is imperative that during drug design and prescription by clinicians, that these enzyme interactions be taken into consideration.

2.4.1 Enzyme inhibition

The processes involved in cytochrome inhibition can be categorized as follows:

1.) Reversible inhibition
2.) Quasi-irreversible inhibition
3.) Irreversible inhibition.

Reversible inhibition, which is the most common mechanism responsible for drug interactions, is temporary i.e. inhibition starts after the first dose of the inhibitor and the length of inhibition corresponds to the half-life of the drug (Lin and Lu, 2001; Manzi and Shannon, 2005).

Furthermore, reversible inhibition can be categorised as follows:

1.) Competitive
2.) Non-competitive
3.) Uncompetitive.
4.) Mixed Inhibition

5.) Suicide Inhibition

Competitive inhibition is the type most commonly encountered during drug metabolism, where the binding of the inhibitor prevents binding of the appropriate substrate to the enzyme’s active site (Manzi and Shannon, 2005). In non-competitive inhibition, the inhibitor binds to site other than the active site, producing a non-productive enzyme-substrate complex (Lin and Lu, 2001).

Quasi-irreversible inhibition involves the formation of a reactive metabolic intermediate which leads to enzyme inactivation or destruction. Toxicity is the most common inhibitory effect after drug administration. An enzyme substrate can act as an inhibitor by preventing metabolism of other co-administered drugs which subsequently remain in the bloodstream longer than necessary (Lin and Lu, 2001; Manzi and Shannon, 2005).

2.4.2 Enzyme induction

Induction of a CYP enzyme occurs when a drug substrate increases biosynthesis of that enzyme, further enhancing its metabolic activity (Manzi and Shannon, 2005). Contrary to inhibition, induction is a slow process and usually more complex because other factors are required for transcriptional activation of the gene (Lin and Lu, 2001).

Most of the CYP enzymes are inducible (Lin and Lu, 2001). Regardless of the magnitude of understanding the CYP enzyme activity, it is still a challenge to predict the effect of the drug interactions on the human body. Variability in gene expression and activity of CYPs,
play significant roles in drug metabolism. Furthermore this variability also occurs between individuals (Lin and Lu, 2001; Ingelman-Sundberg, 2001).

2.5 POLYMORPHISMS IN CYTOCHROME P450

Research has revealed that humans are 99.9% identical in their genetic makeup, with only a portion (0.1%) rendering individuality. It is this small portion that has variable clinical implications ranging from genetic predisposition to disease and response to drug therapy. Single nucleotide polymorphisms (SNPs), base deletions and insertions attribute to this variability. SNPs are abundant and are the most frequent type of DNA sequence variation in the human genome, appearing on average at every 300-3000 base pairs. They have frequently been used as genetic markers due to their high abundance and low mutation rates (Meyer and Gut, 2002).

SNPs found within the coding region are of particular interest to biomedical researchers. Coding region SNPs may be:

1.) Non-synonymous, resulting in the alteration of an amino acid which in turn may affect the structure and function of the encoded protein.

2.) Synonymous SNPs may have functional consequences by affecting the stability or folding of mRNA transcripts (Meyer and Gut, 2002).

Mutations within the human genome may be responsible for many diseases. In addition, other factors such as the environment, age and diet can influence disease onset and progression. These factors are often difficult to control within any population group. Thus
the genetic component is becoming important in predicting disease progression or onset as well as potential response to therapy. This is further confirmed by Ingelman-Sundberg (2001), who stated that “knowing the molecular basis of a disease enhances our ability to understand genetic predisposition, onset and progression and this will expedite the development of safer and more effective therapies” (Ingelman-Sundberg, 2001).

There are a wide variety of disciplines in which genetic variation studies can be applied. Our particular concern is genetic variation affecting drug metabolism. Pharmacogenetics addresses the role of genetics and an individual’s response to a particular drug (Ingelman-Sundberg, 2001; Meyer and Gut, 2002). Genetic polymorphisms within drug metabolizing genes are responsible for variation in patient response to therapy. Hence much focus has been channelled, by biomedical researchers and pharmaceuticals alike, into pharmacogenetics and pathways involved in drug metabolism.

Cytochrome P450 enzyme research has provided insight into the genetic basis of DME variability in activity and the differences in individuals’ response to therapy. According to Ingelman-Sundberg, 30-40% of drugs undergoing clinical trials have been withdrawn from further development due to unfavourable therapeutic effects (Ingelman-Sundberg, 2001). In an effort to circumvent developmental and drug design costs as well as reduce potential adverse reactions, research into drug metabolism has gained importance. The cost implications for the United States due to ADRs was reported to be about US $ 100 million and more than 100 000 deaths annually (Ingelman-Sundberg, 2004). The United Kingdom
and Sweden report that 7% and 13% of all hospital admissions respectively are attributed to adverse drug reactions (Ingelman-Sundberg, 2004).

The factors causing adverse reactions are wide ranging, from genetic variability; certain individuals are predisposed to toxic effects, induction or inhibition due to concomitant drug therapies, environmental factors and disease states (Ingelman-Sundberg, 2001). Genetic polymorphisms are of major importance, since it has become apparent that polymorphisms are ethnically linked i.e. the biological background on individuals. Thus during drug design, it is important to take into consideration interracial differences (Ingelman-Sundberg, 2001 and Ozawa et al, 2004).

Besides genetic polymorphisms, other proteins like drug transporters and receptors also play a role in regulating the activity of drug metabolizing enzymes (section 2.9). According to Ozawa et al (2004), polymorphisms may arise as a result of single nucleotide polymorphisms (SNPs), base deletions and insertions which can result in a deficient allele, giving rise to genotypes that predispose individuals to adverse reactions upon exposure to certain medications (Ozawa et al, 2004).

Inactive or abolished enzyme activity is commonly encountered where the entire gene has been deleted, while on the other end of the spectrum, increased activity is seen in subjects with multiple copies of the active CYP gene (Ingelman-Sundberg, 2004; Johnson et al, 2005; Manzi and Shannon, 2005). Mutations at the substrate recognition site have been known to occur and this results in altered substrate specificity.
2.5.1 Phenotype as a consequence of polymorphism

Polymorphisms within various CYP genes can be used to categorise populations into different phenotypes. There are at least 3 phenotypes derived from genotypic mutations, poor (or slow) metabolizers (PM), extensive (or rapid) metabolizers (EM) and ultrarapid metabolizers (UM) (Ingelman-Sundberg, 2001). The PM phenotype has reduced or abolished enzyme activity and can lead to excessive or prolonged therapeutic effect, possibly leading to drug-related toxicity. After a normal drug dose, individuals within this category are then genetically predisposed to drug-induced adverse effects. The UM phenotypes include individuals with multiple copies of the same gene which gives rise to sub-optimal therapeutic levels when the normal drug dose is administered (Ingelman-Sundberg 2001; Scordo et al, 2004). The opposite effect is seen with pro-drugs, which require activation by a specific enzyme before systemic absorption i.e. PM condition may result in decreased response to therapy and UM individuals may experience toxicity (Scordo et al, 2004 and Ingelman-Sundberg, 2004).

Many allelic variants of DME’s have been identified however, only a few have been shown to portray clinical significance. Among the clinically significant variants, their frequency within the Caucasian, Japanese and African populations is different (Ingelman-Sundberg, 2001).
2.6 CYTOCHROME 1 FAMILY

2.6.1 CYP1A2

CYP1A2 is located on chromosome 15 and is comprised of 7 exons and 1 non-coding intron (Ikeya et al, 1989). To date 23 CYP1A2 alleles; including 9 subtypes have been documented (Ikeya et al, 1989; Soyama et al, 2005). Single nucleotide polymorphism (SNP) studies within the transcriptional regulatory regions have shown that the CYP1A2*1C allele (G-3860A) is associated with decreased enzyme inducibility in Japanese smokers (Johnson et al, 2005). In addition, the CYP1A2*1F allele (C163 A) in intron 1 is linked to increased enzyme inducibility in Caucasian smokers. Several other SNPs, including T739G and C729T, in intron 1 were found to be associated with decreased enzyme activity in Ethiopian non-smokers (Soyama et al, 2005).

The CYP1A2 isoform is expressed mainly in the liver, where it accounts for approximately 15% of cytochrome P450 content (Shimada et al, 1994). It plays a major role in drug metabolism. Fifteen percent of pharmaceutical drugs are metabolised by CYP1A2. The drug substrates for CYP1A2 include caffeine and tricyclic antidepressants (Manzi and Shannon, 2005). This isoenzyme is inducible by cigarette smoke, cruciferous vegetables (e.g broccoli and cabbage) and charbroiled foods, while its activity can be inhibited by grapefruit juice, erythromycin and ciprofloxacin. Drugs like phenobarbital and rifampin induce CYP1A2, resulting in clinically significant drug interactions (Manzi and Shannon, 2005). CYP1A2 has been shown to play a key role in chemical carcinogenesis by activating some aromatic amines (Sachse et al, 1999; Soyama et al, 2005).
activity is induced by the binding of aromatic hydrocarbons to the responsive element (Ah-
receptor), 3402-3385 base pairs upstream of the translational initiation site (Sachse et al, 1999; Soyama et al, 2005).

The mRNA expression and enzyme activity levels among individuals have been shown to vary by forty-fold and sixty-fold, respectively. Cigarette smoking and use of oral contraceptives have been shown to modify CYP1A2 activity. Soyama et al, (2005), showed that genetic factors account for 35-75% of variability in gene expression.

2.7 CYTOCHROME 2 FAMILY

The cytochrome 2 family has significantly more isoenzymes involved in drug metabolism than the other two families. CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 are the main isoenzymes belonging to this family. These isoforms metabolize a wide variety of drugs, ranging from antidepressants, antibiotics and antiretrovirals.

2.7.1 CYP2A6

Cytochrome 2A6 plays a significant role in oxidation metabolism and it accounts for 10% of total hepatic CYP’s in human microsomes. Compounds such as nicotine, cotinine and other drugs such as fadrozole, methoxyflurane and letrozole are metabolised by this enzyme (Johnson et al, 2005). CYP2A6 has four different defective alleles i.e Allele that give code for non-functional CYP enzymes. It was found that individuals who smoke and possess more than one defective allele are less likely to be tobacco-dependant than
individuals without the defective allele. The Asian population have a higher proportion of
defective alleles (approximately 23%) compared to approximately 5% in Caucasians
(Ingelman-Sundberg, 2001; Johnson et al, 2005).

2.7.2 CYP2B6

Initially it was thought that CYP2B6 is expressed at low levels of less than 1% but later
reports show this value to be around 6%. The limited research on CYP2B6 was
compounded by the fact that there was a lack of a suitable probe for CYP2B6 investigations
(Lang et al, 2001). Studies, using more sensitive and specific immunochemical and
biochemical detection methods, revealed extensive variation in protein expression among
individuals, ranging from 20- to 250-fold (Lang et al, 2001; Lamba, 2003 and Fauccette et
al, 2004). Expression of CYP2B6 was also detected at lower levels in extrahepatic tissue

The CYP2B6 enzyme, although not as well characterised as other CYPs, has a more
important role in metabolism than previously thought (Ingelman-Sundberg, 2004). This
enzyme is known to be induced by phenobarbital and cyclophosphamide (Ingelman-
Sundberg, 2004). Other reports show CYP2B6 to be involved in the metabolism of
frequently used drugs such as cyclophosphamides, tamoxifen, the dopamine uptake
inhibitor (bupropion), benzodiazepines (diazepam as well as midazolam), nevirapine,
efavirenz and rifampicin. Some recreational drugs such as nicotine, ethylenedioxymeth-
amphetamine (Ecstasy), methylenedioxy-ethylamphetamine (Eve) and procarcinogens
such as aflatoxin B1, 6-aminochrysene and dibenzo[a,h] anthracene are also substrates of CYP2B6 (Lang et al, 2001 and Lamba et al, 2003).

Like CYP1A2’s ar-receptor, the constitutively activated receptor (CAR) was shown to be involved in the induction of CYP2B6 (Lang et al, 2001, Lamba et al, 2003 and Jacob et al, 2004). Lamba et al, (2003) while confirming the study by Lang et al (2001), who also stated that variation in this enzyme’s activity could be linked to variation in the expression of CAR. Thus expression of CYP2B6 is closely linked to, if not dependent on CAR. Lamba and colleagues (2003) revealed significant differences in CYP2B6 and CAR expression between sexes. Liver tissues of females expressed significantly higher amounts of CYP2B6 and CAR mRNA than the males. Furthermore, Jacob et al (2004), studied the metabolism of efavirenz (which is a substrate of CYP2B6) and found that its efficacy could be lower in females than males due to excessive clearance of the drug (Lamba et al, 2003 and Jacob et al, 2004).

2.7.3 CYP2C9

The cytochrome 2C subfamily accounts for 20% total liver CYP content and is responsible for the metabolism of approximately 20% clinically relevant drugs (Johnson et al, 2005). The CYP2C9 enzyme is the most abundant isoform among CYP2C enzymes (Scordo et al, 2004). It accounts for approximately 20% of hepatic CYP2C content (Xie et al, 2002 and Andersson et al, 2005).

The CYP2C9 gene is located on Chromosome 10 at position 10q24 in a cluster of other CYP2C genes. The CYP2C9 gene spans a region of 55 kilobases and consists of 9 exons,
which encode a protein consisting of 490 amino acids (Xie et al., 2002 and Andersson et al., 2005). Fifty SNPs have been shown to occur within the coding and regulatory regions of the gene, yielding 12 allelic variants. Only two coding variants (CYP2C9*2 and 2C9*3) are the most common and the most investigated. They reflect single nucleotide changes C430T and A1075C respectively, which lead to amino acid substitutions Arg144Cys and Ile359Leu. This is implicated in reduced CYP2C9 activity (Kirchheiner and Brockmoller, 2005 and Moridani et al., 2006). Furthermore, CYP2C9*2 substrate affinity is higher than that of the wild type CYP2C9*1 and the catalytic activity of CYP2C9*3 is significantly reduced for most of its CYP2C9 substrates (Kirchheiner and Brockmoller, 2005). In terms of warfarin metabolism, Moridani et al., (2006) found that individuals with either *1/*3 or *X/*X (where X = *2/*3) genotype required 32 to 67% less warfarin dosage than the *1/*1 genotype (Moridani et al., 2006).

Other alleles such as 2C9*5, 2C9*6, 2C9*8 and 2C9*11 have been documented within the African populations, where Allabi and colleagues (2004) found that individuals homozygous for CYP2C9*6/*6 experienced a substantial reduction in phenytoin clearance. Aside from this study, the functional impact of CYP2C9*5, *8 and *11 remains relatively unknown (Allabi et al., 2004 and Kirchheiner and Brockmoller, 2005).

As previously mentioned, the genetic polymorphisms found in CYP2C9 can lead to marked variability in expression and activity of the enzyme among individuals which can result in clinical drug toxicity or reduced drug efficacy in some patients who take standard doses of CYP2C9 substrate drugs (Xie et al., 2002). It is known to metabolize approximately 10%
of clinically relevant drugs, such as tolbutamide, phenytoin, losartan and the anticoagulant warfarin. Rifampin and rifabutin are powerful inducers of CYP2C9 activity, whereas chloramphenicol and sulfonamides are known to inhibit CYP2C9 (Xie et al, 2002; Andersson et al, 2005; Kirchheiner and Brockmoller, 2005; Manzi and Shannon, 2005). Like the CYP2B6’s association with CAR, Xie et al, (2002) report the presence of highly polymorphic hepatic nuclear factor (HNF-1) which could be linked with CYP2C9 expression.

2.7.4 CYP2C19

CYP2C19 is another common isoform within the CYP2C family. The anticonvulsant agent (S)-mephenytoin and several benzodiazepines and anti-depressants are substrates to this enzyme. Like the CYP2C9 enzyme, CYP2C19 is also induced by rifampicin. It is inhibited by drugs such as fluxetine (Manzi and Shannon, 2005; Johnson et al, 2005). CYP2C19 exhibits genetic polymorphism, which can cause variability in drug response. Genetic variation within CYP2C19 can cause the enzyme’s activity to range from high, low or none, therefore populations can be phenotypically categorised according to enzyme activity i.e. poor, intermediate, extensive and ultra-rapid metabolizers (Zand et al, 2005).

Nine variant CYP2C19 alleles have been reported to date, of which two (CYP2C19*2 and CYP2C19*3) are detrimental alleles (Hamdy et al, 2002). The first genetic defect is a single base pair mutation in exon 5, which creates an aberrant splice site, the other more common deficient allele occurs in exon 4 leading to a premature stop codon. Both alleles lead to poor or slow metabolism of CYP2C19 substrates (Hamdy et al, 2002 and Zand et
al, 2005). The frequency of CYP2C19*2 varies between 18 and 23% in Asians, Caucasians and Africans. The allele is inherited as an autosomal recessive trait and accounts for 75% of the defective alleles in Orientals and 93% in Caucasians (Scordo et al, 2004 and Zand et al, 2005).

The other deleterious allele CYP2C19*3 is found in 25% of all inactive forms in Orientals but extremely rare in non-Oriental populations (Scordo et al, 2004). A study on mephenytoin metabolism (an anti-anticonvulsant) revealed that 3% of Caucasians are PM’s of this drug and the African population’s PM frequency varies between 4-7% (Scordo et al, 2004 and Johnson et al, 2005).

2.7.5 CYP2D6

CYP2D6 is termed the most polymorphic CYP enzyme. Although the contribution to total CYP activity is approximately 2%, it metabolizes up to 25% of all used drugs (Manzi and Shannon, 2005). The well-known conversion of codeine to morphine is catalysed by CYP2D6 (Manzi and Shannon, 2005).

The activity of CYP2D6 is highly variable and it is the only isoform which is non-inducible by any substrate (Ingelman-Sundberg, 2004). The polymorphisms within CYP2D6, like the CYP2C19, range from complete deficiency to ultrarapid metabolizers. To date, more than 70 allelic variants have been reported and the four major mutated alleles, CYP2D6*3, *4, *5 and *8, are found in approximately 95% in Caucasians who are
poor metabolizers. This gene is also known to yield ultra-rapid metabolizers by duplicating the alleles CYP2D6*1 and *2. This results in increased CYP2D6 activity.

A study by Scordo et al (2004), showed that the incidence of gene duplication ranges from 1% in the Swedish to 10% in the Italian population, and the black Tanzanian population yielded 9% UM's and 36% PM's.

2.7.6 CYP2E1

CYP2E1 enzyme plays a relatively small role in the metabolism of drugs, however in addition to drug metabolism it is reported to deactivate toxins (Manzi and Shannon, 2005). The CYP2E1 gene is located in chromosome 10 at position 10q24.3, close to CYP2C genes, spanning 11 kilobases and contains 9 exons which encode a protein consisting of 493 amino acids (Umeno et al, 1988).

CYP2E1 exhibits polymorphism and several alleles have been reported. The alleles CYP 2E1*1B 5*B and *6 have been identified but as yet, there is no conclusive evidence linking allelic variants to in vivo drug clearance (Ingelman-Sundberg, 2004). Due to inconclusive and contradictory results regarding CYP2E1 genotyping, Ingelman-Sundberg (2004), concluded that this gene is well conserved and this could be attributed to the major role the enzyme plays in the metabolism of endogenous compounds.

Several studies however, have shown CYP2E1 gene to possess the PstI and Rsal polymorphic restriction sites in the 5'-Flanking region of CYP2E1. The resultant alleles were designated C1 (Rsal +, PstI -) and C2 (Rsal-, PstI+) (Hayashi et al, 1991, Watanabe
et al, 1994 and Salama et al, 1999). The C2 allele has been associated with higher transcriptional activity (Hamdy et al, 2002). Among the clinically important substrates for CYP2E1 are enflurane, halothane, the anti-tuberculosis drug isoniazid, paracetamol and alcohol, both of which are potent and common causes of liver injury (Lee, 1997; Manzi and Shannon, 2005).

Generally, most of the substrates for this enzyme are small, organic and hydrophobic in nature (Ingelman-Sundberg, 2004). Acetaminophen, a substrate to CYP2E1 is metabolised to N-acetyl-p-benzoquinoneimine, a hepatotoxin. Chronic ethanol use can induce CYP2E1 activity, thus increasing the risk of hepatotoxicity from acetaminophen (Manzi and Shannon, 2005). Its activity has been reported to cause oxidative stress and the resultant oxy radicals are able to initiate NADPH-dependent lipid peroxidation, ultimately producing cytotoxic aldehydes. These aldehydes have been implicated in ethanol-mediated hepatotoxicity. Hence any variant activity of CYP2E1 could be an important factor in determining the relative risk of alcohol-mediated hepatotoxicity or susceptibility for drug-toxicity (Hu et al, 1997).

The study done by Hayashi et al, (1991) revealed that the C1/C2 polymorphisms affected the CYP2E1’s binding transacting factor and changed its transcriptional regulation. From this, they suggested that this may lead to inter-individual differences in microsomal drug oxidation activity (Hayashi et al, 1991).
The activity of CYP2E1 has been closely linked to two other enzymes, Alcohol dehydrogenase-2 (ADH 2) and Aldehyde dehydrogenase-2 (ALDH 2). Tanaka and colleagues performed a study on Japanese men. They observed among individuals homozygous for the ALDH2 gene, those portraying homozygosity for the C2 allele could consume more ethanol than those homozygous at C1 allele (Tanaka et al, 1997). These findings suggest an interactive effect between ALDH-2 and CYP2E1 on alcohol consumption. This was further confirmed by Sun et al, (1999), whose study revealed that Japanese men with the ADLH2*1 homozygous genotype and the C2 allele of CYP2E1 were at a higher risk at showing excessive alcohol consumption.

Other correlations between CYP2E1 genetic polymorphisms and susceptibility to lung cancer, alcoholic liver disease, hepatocellular carcinoma and nasopharyngeal carcinoma have been reported (Huang et al, 1997).

2.8 CYTOCHROME 3 FAMILY

The CYP3A subfamily is the most prominent family with respect to abundance, oxidation reactions and drug metabolism (Ingelman-Sundberg, 2004 and Johnson et al, 2005).

2.8.1 CYP3A4

The CYP3A4 enzyme is the most abundant isoform in the liver, contributing up to 25% of the total hepatic cytochromes. It is implicated in the metabolism of over 60% of clinically relevant drugs (Lamba et al, 2002, Gorski et al, 2003). This enzyme is expressed, although
to a lesser extent, in the small intestine and the kidney. The presence of CYP3A4 in the intestines has implications in the bioavailability of orally administered drugs.

CYP3A4 is responsible for the metabolism of a wide variety of drugs, such as immunosuppressants, cancer chemotherapeutic agents, antihistamines, sedatives and synthetic estrogens. It also plays a crucial role in the metabolism of endogenous steroids such as cortisol, testosterone and oestradiol (Hsieh et al, 2001 and Eiselt et al, 2001).

A strong feature of this enzyme is its broad substrate specificity and inducibility by different classes of compounds, drugs such as macrolide antibiotics, rifamycins and a few anticonvulsants (Ingelman-Sundberg, 2004). The plant remedy St John’s wort is a potent inducer of CYP3A4. Compounds that inhibit CYP3A4 are HIV protease inhibitors, antidepressants, grapefruit juice as well as some antibiotics like erythromycin and ketoconazole. CYP3A4 has been shown to play a role in the metabolism of efavirenz (anti-HIV drug). This drug is an inducer of the enzyme’s activity (Jacob et al, 2004). In addition to drug metabolism, CYP3A4 is important in the metabolism of dietary and environmental chemicals such as flavanoids, mycotoxins and a number of food additives (Chelule, 2003; Ingelman-Sundberg, 2004).

Variability in CYP3A4 expression seems to be more complex as it is also affected by a multitude of other factors such as diet, disease states and environment (Chelule, 1998; Ingelman-Sundberg, 2001). Studies using liver biopsies, showed that CYP3A4 activity varies up to 40-fold within a population, this variation among individuals may have a
profound effect on systemic exposure, clearance, efficacy and safety of drugs (Eiselt et al, 2001; Hsieh et al, 2001). Variability in expression and activity may also predispose individuals to several common cancers. An example of this is liver cancer in the African and Asian population which is caused by Aflatoxin B1 (Eiselt et al, 2001).

The CYP3A4 gene is located on chromosome 7 at position 7q22.1, spanning 13 exons. Expression varies by up to 40-fold in the liver and the small intestine (Hsieh et al, 2001; Lamba et al, 2002). The most clinically significant variant allele CYP3A4*1B (a point mutation in the 5'-flanking region) has been found to have a critical impact on the enzymes' activity, and ultimately the metabolism of the drug substrates (Hsieh et al, 2001, Lamba et al, 2002). This allele was also found to be associated with higher clinical stages and grade in prostate tumours. Additionally, patients with leukaemia were reported to have an over-expression of CYP3A4*1B (Sata et al, 1999, Hsieh et al, 2001 and Eiselt et al, 2001).

The distribution of the CYP3A4*1B allele is different in different population groups. It was estimated to be 9% in Caucasians, 53% in African Americans and 0% in Asians (Eiselt et al, 2001). The alleles CYP3A4*2 and CYP3A4*12 yield enzymes with a slightly altered substrate specificity. They occur at low frequency, 14% of Caucasians, 10% of Japanese and 15% of Mexicans (Lamba et al, 2002). Other variant alleles have been shown to arise as a result of point mutations. The significance of these genetic changes on the overall drug clearance has not been elucidated (Hsieh et al, 2002 and Lamba et al, 2002). A striking feature about the CYP3A4 gene is that there is no evidence of a "null" or inactive allele.
(Lamba et al, 2002 and Ingelman-Sundberg, 2004), as opposed to the other drug metabolising genes.

Like the CYP2E1 gene, the CYP3A4 gene is extremely well conserved, (Lamba et al, 2002; Ingelman-Sundberg, 2004). The reason behind the conserved status of CYP3A4 could be due to the enzyme’s role in the metabolism of dietary, endogenous and other environmental compounds (Ingelman-Sundberg, 2004).

As with CYP2B6’s CAR and CYP2C9’s HNF-1, the inducibility of CYP3A4 is linked to a transcriptional receptor- human Pregnane X Receptor (hPXR) (Moore and Kliewer, 2000). CYP3A4 drug substrates appear to bind to the hPXR, resulting in transcriptional activation of the CYP3A4 gene (Moore and Kliewer, 2000).

2.9 OTHER DRUG METABOLIZING ENZYMES

In addition to cytochrome P450 enzymes and phase II DMEs, there are other proteins that aid in the completion of the drug metabolism pathway. These proteins are closely linked to CYP enzymes, either chromosomally, transcriptionally or by physiological location. They range from enzymes active in transporting drug compounds to receptors that aid in the activation of DME genes.
2.9.1 P-glycoprotein

P-glycoprotein (P-gp) is a member of the ATP-binding cassette family. It is a gene product of ABCB1 gene, also called a multi-drug resistant gene 1 (MDR-1). This protein was previously studied in relation to tumour cells exhibiting drug resistance to anti-cancer medication (Fromm, 2002; Yan-Hong et al, 2006). Recently, it has been shown to occur in different tissue types, such as the blood-brain barrier, liver, kidney, small intestine, colon, immune system cells and even the haematopoietic stem cells (Fromm, 2002). The presence of P-gp in the above mentioned locations implies another physiological role besides multi-drug resistance in tumour cells (Marzolini et al, 2004 and Fromm 2002). Marzolini and colleagues suggested that the physiological role of P-gp is in the protective barrier, keeping toxic compounds out of the body by excreting them into bile, urine and intestinal lumen (Marzolini et al, 2004).

P-gp is an ATP-dependent transporter protein that is 1280 amino acid long, with six transmembrane domains and an ATP binding site (Marzolini et al, 2004). It acts as an efflux pump, removing its substrate from the lipid bilayer and into the intracellular domain (Fromm, 2005). The ATP-dependent action provides energy for active transport against steep concentration gradients (Marzolini et al, 2004).

Due to its activity in efflux pumping, the transporter’s role in drug disposition and metabolism has received attention. Studies show substrate commonality between P-gp and CYP enzymes. Furthermore, it was also found to be expressed in close proximity to the enzyme CYP3A4, (Marzolini et al, 2004). Whilst some CYP3A4 substrates are not
transported by P-gp, for example midazolam and some P-gp substrates are not metabolised by CYP3A4, for example digoxin. The substrate overlap was found to be significant enough to spur further research into P-glycoprotein (Marzolini et al., 2004).

The drug substrates for P-gp are anti-cancer agents, cardiac drugs, HIV protease inhibitors, immunosuppressant, antibiotics and anti-histamines (Fromm, 2002).

Like most other CYP genes, it was also determined that ABCB1 gene expression is polymorphic (Chelule et al., 2003; Eichelbaum et al., 2004; Fromm, 2002 and Marzolini et al., 2004). Twenty nine SNP’s have been detected in this gene however, only the SNP’s at exons 21 and 26 result in amino acid changes that affect the activity of the P-gp (Marzolini et al., 2004 and Eichelbaum et al., 2004). Exon 26 mutation give rise to C*/T alleles which play an important role in drug transport (Chelule et al., 2003).

Chelule and colleagues report that the wild-type allele *C is associated with increased protein expression which has been linked to reduced drug availability (Fromm 2002; Chelule et al., 2003). The above study was also confirmed by (Eichelbaum et al., 2004) who showed that individuals with the wild type *C/*C had a 2-fold P-gp expression than the *T/*T genotype. However, another showed that Oriental populations had a lower P-gp expression among the *C/*C than the *T/*T counterparts (Fromm, 2002).

Similar to CYP genes, polymorphisms within the MDR-1 gene are ethnically linked. With respect to ethnicity, the *C/*C allele was found to be considerably more frequent in the
African population compared to the Caucasian and non-Asian populations (Fromm, 2002). A study on a South African population revealed that 85.9% of African, 41.7% of Indian and 35.7% of Caucasians are of the *C/*C genotype (Chelule et al, 2003). Schaeffeler et al, (2001) proposed that the high frequency of the C/C genotype compared to the T/T genotype among Africans could be the result of a selective advantage, where this genotype offers protection against gastrointestinal-tract infections. They justified this observation from previous studies, where the glucose-6-phosphate dehydrogenase (G6PD) gene’s associated polymorphism offers increased resistance to Plasmodium Falciparum (malaria) among homozygous individuals in sub-Saharan Africa. Furthermore, P-glycoprotein has been shown to play a role against viral infections. Overexpression of this protein could reduce the CD4 + cell’s susceptibility to infection with HIV-1 (Schaeffeler et al, 2001). The currently available anti-HIV protease inhibitors are substrates to P-gp, the presence of this protein could limit the activity of drugs thus creating a potential sanctuary for viral replication, should the virus be able to pass the protective barrier (Eichelbaum et al, 2004). Like the CYP3A4 gene, there is no evidence as yet of a null mutation within the MDR-1 gene.

2.10 N-Acetyltransferase

The NAT-2 enzyme plays a major role in the metabolism of aromatic amines. It is responsible for mediating the activating steps for some carcinogenic metabolites. Thus any drug that requires inactivation by acetylation would undergo such metabolism. N-acetyltransferases have been linked to the detoxification of several dietary and occupational

The NAT-2 enzyme is expressed in a wide variety of human tissues and has been associated with cancer susceptibility as well as liver injury (Gonzales et al, 1998, Zhang et al, 2005 and Shimizu et al, 2005). The genes (NAT1 and NAT-2) are located on Chromosome 8 at positions 8p23.1-p21.3, however NAT1 appears to encode for a genetically invariant protein, which does not seem to affect the acetylation activity, unlike the NAT-2 gene (Gonzales et al, 1998).

Sequencing studies reveal that 26 SNPs exist within the NAT-2 gene, of which 9 (G191A, C282T, T341C, A434C, C481T, G590A, A803G, A845C and C857A) play a significant role in enzyme activity (Deitz et al, 2000; Shimizu et al, 2005).

The activity of the NAT-2 enzyme varies and can also be used to categorise patients into slow, intermediate and rapid acetylators (Huang et al, 2003; Srivastava et al, 2004 and Kinzig-Schippers et al, 2005) referred to in table 2.1).
Table 2.1: Associations between NAT-2 genotype, nucleotide changes, alleles and phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Nucleotide</th>
<th>NAT-2 allele genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT-2*4</td>
<td>None</td>
<td>NAT-2*4/*4</td>
<td>Rapid Acetylator (RA)</td>
</tr>
<tr>
<td>NAT-2*5B</td>
<td>T341C, C481T, A803G</td>
<td>NAT-2*4/*12A</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>NAT-2*7B</td>
<td>C282T, G857A</td>
<td>NAT-2*4/*7B</td>
<td>Intermediate Acetylator</td>
</tr>
<tr>
<td>NAT-2*12A</td>
<td>A803G</td>
<td>NAT-2*5B/*7B</td>
<td>Slow Acetylator (SA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NAT-2*6A/*6A</td>
<td>Slow Acetylator</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NAT-2*6A/*7B</td>
<td>Slow Acetylator</td>
</tr>
</tbody>
</table>


Due to the role NAT-2 enzyme plays in liver injury and other cancers, research into this protein has revealed several clinically important findings. Shimizu et al, (2005) linked acetylator status to hepatotoxicity predisposition. They showed that slow acetylators had a higher risk of isoniazid induced hepatotoxicity than rapid acetylators (Table 2.2) (Shimizu et al, 2005).
Table 2.2: NAT-2 Genotype linking hepatotoxicity

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Number of Patients</th>
<th>Number of patients with hepatotoxicity</th>
<th>Frequency of hepatotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT-2*4/*4</td>
<td>RA</td>
<td>21</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NAT-2*4/*12A</td>
<td>IA</td>
<td>9</td>
<td>3</td>
<td>RA type 9.1%</td>
</tr>
<tr>
<td>NAT-2*4/*6A</td>
<td>IA</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NAT-2*4/*6C</td>
<td>IA</td>
<td>5</td>
<td>1</td>
<td>IA type 26.7%</td>
</tr>
<tr>
<td>NAT-2*4/*7B</td>
<td>SA</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NAT-2*6A/*7B</td>
<td>SA</td>
<td>3</td>
<td>3</td>
<td>SA type 80%</td>
</tr>
<tr>
<td>NAT-2*6A/*7B</td>
<td>Total</td>
<td>42</td>
<td>10</td>
<td>Total 23.8%</td>
</tr>
</tbody>
</table>


In study using cultured rabbit hepatocytes, a relationship between acetylator phenotype and DNA damage by chemicals that undergo N-acetylation was found. It revealed that markers of DNA damage and DNA repair were expressed by slow acetylators and not by rapid acetylators. The amount of DNA repair measured was dose-dependent. This has lead to
the conclusion that acetylation polymorphism may be a possible factor in susceptibility to toxicity and even perhaps carcinogenicity of substrate chemicals (McQueen et al, 1982).

Another study found that administration of isoniazid with phenytoin (Dilantin) resulted in higher toxic levels of phenytoin, and the effects of this drug interaction were greater in slow acetylators (Kutt et al, 1970 and Timbrell et al, 1977). Further studies also revealed that a significantly higher proportion of slow acetylator diabetics experienced neuropathy than those with rapid acetylator genotypes (McLaren et al, 1977).

PURPOSE OF THE STUDY

Morbidity and mortality due to both HIV and TB infections in South Africa is increasing and 60% of the HIV infected individuals are co-infected with TB (Abdool-Karim, 2004). The treatment for both infections is a challenge as adherence to therapies, resistance to drugs and severe side effects, such as hepatotoxicity, affect therapeutic outcomes. Moreover, drug efficacy and therapeutic responses are influenced by environmental factors, diet, age and genetic predisposition.

The genetic profile together with therapeutic response could provide insight into patient care and management. Genetic variability within drug metabolizing genes influence drug response and these variations have been found to be ethnically linked. In addition concomitant therapies are subject to interactions (drug-drug interactions) that will affect the drug efficacy and ultimately treatment. Concomitant therapy is often the case in an HIV and TB co-infected population.
In light of the above, we undertook a study to investigate the frequency and distribution of drug metabolizing enzymes among a cohort of the Zulu population in South Africa. The study cohort was from the Centre for AIDS Programme of research of South Africa. This included HIV and TB co-infected patient population, who were treated for both infections.

The Polymerase Chain Reaction together with Restriction Fragment Length Polymorphism (PCR-RFLP) was used to assess the genotypic profile of the drug metabolizing Cytochrome P450, N-acetyltransferase and ATP-binding cassette genes. In addition, clinical data from the medical records of the study population was utilised in determining TB and ARV therapeutic outcomes.

Ultimately, this study aimed to determine whether the presence of drug metabolizing variant alleles influences treatment outcomes such as successful TB and ART therapies, duration of TB therapy of patients and the degree of liver derangement of patients on concomitant therapies.
3. MATERIALS AND METHODS

3.1 ETHICAL APPROVAL

This study was approved for sample collection and genotyping, by the University of KwaZulu-Natal Biomedical Research Ethics Committee. Ref: H268/05

3.2 STUDY POPULATION

The study participants comprised 50, randomly chosen Zulu patients within an existing cohort enrolled for a clinical study, intended to determine the best time for the initiation of antiretroviral therapy of patients requiring treatment for tuberculosis, by CAPRISA (Centre for AIDS Programme of research of South Africa). The participants were both HIV and TB co-infected. The rationale for selecting this group was two-fold: firstly, the availability of a well-characterised study group, and secondly, that the success of both antiretroviral and antituberculous therapy might prove to be, in part, influenced by DME genotypes. This led us to use this cohort as the basis of our preliminary study, exploring the feasibility of using drug metabolizing enzymes in determining clinical outcome.

The inclusion and exclusion criteria were as follows:

Inclusion Criteria:

1. HIV infected patients co-infected with TB
2. Receiving any one of the standard anti-TB therapy regimens
3. All patients had to agree to use contraception since they would be on efavirenz.
4. Written, informed consent for both the CAPRISA study and for this present study
Exclusion Criteria:

Patients, who were not unable to maintain a treatment regimen, were excluded.

HCV and HBV testing was not a standard of care in this study setting.

Therapy:

All subjects received standard antituberculous therapy with rifampicin, isoniazid, ethambutol and pyrazinamide.

Patients then received antiretroviral therapy with efavirenz, didanosine and lamivudine. Therapy was introduced at varying times for three cohorts: during the intensive phase of antituberculous therapy (before 8 weeks), the post-intensive phase (before 16 weeks) and during the continuation phase (after 16 weeks). These groups were part of the design of the CAPRISA trial, and were not regarded as relevant for the purposes of the present study.

3.2.3 Definition of TB therapy outcomes

The Sputum and X-rays of study participants were reviewed by a clinician on staff at the CAPRISA Ethekwini clinic. The outcome of TB therapy was defined as follows:

- Treatment success: A negative sputum smear within one month prior to completing therapy, with one previous negative sputum smear and an initially positive sputum smear.

- Treatment failure: A positive sputum smear at any point after five months of therapy.
3.2.4 Definition of ARV Therapy outcomes

The CD4\(^+\) T-cell and the viral load count were reviewed by the attending clinician. An increase of 50 cells per \(\mu l\) as well as a decrease of viral copies, six months after therapy initiation was interpreted as a positive therapeutic outcome.

3.3. SAMPLE COLLECTION FOR GENOTYPING

Blood (10 ml) was collected from patients using a purple top (EDTA) vacutainer, by a trained phlebotomist. The blood was sent to the laboratory for processing. The blood was aliquoted into 500\(\mu l\) volumes in Eppendorf tubes and stored at -70\(^\circ\)C.

3.4 MOLECULAR BIOLOGY

3.4.1 Drug Metabolising Enzyme Genes for genotyping

Genes for the cytochrome P450 families 1, 2 and 3 as well as N-acetyltransferase 2 and MDR-1 (a drug transporter) were chosen because of their role in drug metabolism (Table 3.1). Limited information was available, specifically on the genotypic status of the Zulu Southern African population. Due to the prevalence of TB and HIV, particularly in KwaZulu-Natal, it was imperative that some of the enzymes implicated be investigated in terms of their effects on the metabolism of anti-TB and HIV drugs. Not only were the chosen genes involved in anti-TB and HIV drug metabolism, but also influence the metabolism of other more commonly used drugs.
Table 3.1: Genes genotyped for this particular study and their substrates

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>Variant Investigated</th>
<th>Drug substrate</th>
<th>Inducer</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>*1F</td>
<td>C-163A</td>
<td>Fluoroquinolones, Ciprofloxacin, Insulin, Tobacco</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>*2</td>
<td>C430T, C1075T</td>
<td>Ibuprofen, Tamoxifen, S-warfarin, Fluconazole, Isoniazid, Rifampin</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>*3</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>*2</td>
<td>*m1</td>
<td>Rifampin</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>*5A</td>
<td>C-1053</td>
<td>Ethanol, Benzene, Isoniazid</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>*1B</td>
<td>A290G</td>
<td>Erythromycin, Midazolam, Tacrolimus, Efavirenz, Nevirapine, Rifampin, Rifabutin</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>MDR-1</td>
<td>C/T</td>
<td>C3435T</td>
<td>anti-cancer agents, cardiac drugs, HIV protease inhibitors, immunosuppressant, antibiotics and antihistamines</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>NAT 2</td>
<td>*5</td>
<td>C481T, G590A, G857A, G191A</td>
<td>Isoniazid</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

3.4.2 DNA EXTRACTION

DNA was extracted from whole blood using the PUREGENE DNA Purification System, Gentra Systems, Minnesota, USA kit, according to the manufacturer’s instructions, with minor modifications to improve DNA yield, briefly:
300 μl of whole blood was added to a 2 ml microfuge tube containing 900 μl Red Blood Lysis Solution and incubated for 10 minutes at room temperature, to ensure efficient lysis of the cells. 100 μl Protein Precipitation Solution was added to the cell lysate and vortexed vigorously, ensuring uniform mixing of the sample. The solution was centrifuged for 3 minutes at 14 000 rpm. The supernatant was removed from the dark-red protein pellet. The protein-free supernatant was added to a clean 1.5 ml microfuge tube, containing 300 μl of (100%) isopropanol. The sample was inverted gently, where DNA strands became visible and 300 μl of (70%) ethanol was added to wash the DNA pellet. DNA was washed twice using ethanol and by centrifugation for 1 minute at 14 000 rpm. 100 μl DNA Hydration Solution was added to the dry DNA pellet and vortexed for 5 seconds at 5 000 rpm. The re-suspended DNA was left to stand at room temperature overnight.

3.4.3 DNA QUANTIFICATION

3.4.3.1 Agarose Gel Electrophoresis

Principle

Nucleic acids (DNA or RNA) is loaded into a gel and subjected to an electric current. The positively charged nucleic acid will migrate through the gel, from the positive to the negative electrode. The speed of nucleic acid migration is inversely related to its size, therefore different size fragments would be discernible, meaning that bigger fragments would not migrate as fast or as far as the smaller fragments. For visualisation, Ethidium Bromide- a chemical that intercalates between the nucleotides-in mixed within the gel mixture and will fluoresce under Ultra violet light.
Procedure to make Agarose Gel

For 1% Gel: 0.7 g of Agarose was mixed in 70 ml (1X TBE buffer)

For 2% Gel: 1.4 g of Agarose was mixed in 70 ml (1X TBE buffer)

The Agarose was measured into a flask and mixed with the TBE buffer. The mixture was placed into a microwave oven for approximately 1 minute or until the agarose had dissolved. The mixture was left to cool to 40-50°C and 3.5 μl of Ethidium Bromide was added. The warm agarose gel was poured into a set casting tray and comb and left to set for approximately 30 minutes.

Procedure to run the Gel

1X TBE buffer was poured into the gel tank, to cover both the gel tray and electrodes. The combs were removed from the gel mould and the gel immersed into the buffer-filled tank. 2-3 μl gel-loading buffer per sample was mixed with 5 μl of DNA into microtitre plate well. The buffer-DNA mixture was loaded into the pre-set gel and subjected to 60 Volts for 60 to 90 minutes. The gel was removed from the tank and viewed under UV light.

3.4.3.2 Gel Photography

The DNA, PCR products and restriction digests were separated by gel electrophoresis and visualised by ethidium bromide staining under UV radiation at a wavelength of 300 nm using the ChemiDox UV transilluminator. The gel was scanned into the computer using a video camera and the Quantity One version 4.4.1 software was used to adjust the light and contrast of the picture before printing.
3.4.3.3 Nanodrop Spectrophotometer

The quantity and the purity of the DNA was assessed by spectrophotometry using a Nanodrop ND-1000 spectrophotometer according the manufactures instructions. Briefly, the DNA sample (1.5μl) was placed onto the Nanodrop stand in order to create a single path between the two electrodes. In-built software was used to calculate the amount and purity of the sample.

3.5 STORAGE OF DNA

The stock DNA samples, with a concentration of 600 ng/μl was diluted to 300 ng/μl using TE buffer (Hydration Solution), were then aliquoted into 50μl volumes per sample, and stored at -70°C until use.

3.6 POLYMERASE CHAIN REACTION- RESTRICTION FRAGMENT LENGTH POLYMORPHISM

The PCR-RFLP technique of genotyping was used for genotyping assays.

3.6.1 Polymerase Chain Reaction

Principle

PCR is a necessary application when making a large number of copies of a particular gene. The purpose for this amplified gene product is for further downstream reactions such as restrictions and sequencing. The technique occurs in several steps, requiring
reagents such as a buffer, MgCl₂ (Salt), nucleotides, a polymerase enzyme capable of copying gene fragments, primers and the DNA serving as a template for amplification. The steps include:

- **Denaturation**: The double strand DNA melt into single strand, this occurs usually at 94-95 °C.
- **Annealing**: The primers attach to complementary nucleotides on the original single strand to form stable hydrogen bonds. This step occurs at 54-65 °C, depending on the gene being amplified.
- **Extension**: The attached primers form a basis for the addition and the elongation of the copy strand. This step occurs at 72 °C, an ideal working temperature for the polymerase enzyme.

**Procedure for amplifying a DNA fragment**

The amounts and concentration for making a 50 µl PCR reaction volume were as follows: 1X *Taq* Buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 15-35 pmol Forward and Reverse Primers, 10 U/µl *Taq* DNA Polymerase. A master mix, containing all the above reagents was prepared and 44µl was added into fresh 200 µl thin-walled PCR tubes. 6µl (300ng) of DNA was added to each tube as a template. The solution was vortexed and centrifuged. The PCR tubes were then slotted into the allocated holes on the in the PCR –GeneAmp 9700 (Applied Biosystem)-machine.

**PCR Cycling Conditions:**

The various PCR cycling conditions were performed according to table 3.4 using the primers set out in table 3.3
3.6.2 Purification of PCR product from Agarose Gel

Despite numerous attempts at optimising PCR, there were occasions where the undesired product amplified and appeared on Agarose gel. The desired band was then excised to the agarose gel using a commercially available kit (Appendix B).

3.6.3 Restriction Fragment Length Polymorphism

Principle

This technique uses the presence or absence of particular nucleotide sequence within a DNA fragment to its advantage. Detection of particular mutation is achieved by using endonucleases enzymes to cleave at specific recognition sites. Should a fragment contain a recognition site, the endonucleases enzyme will cleave and produce fragments of different base pairs. Organisms could then be compared on the basis of the number and lengths of DNA fragments produced.

Procedure for RFLP

This procedure was performed on ice. A master mix, containing Nuclease-Free water, 1 Restriction enzyme buffer, 10 U/µl Restriction enzyme and 1µg DNA PCR Product (Template) was added to a microfuge tube and incubated. The period and temperature for incubation varied depending on the type of restriction enzyme (Table 3.5). Verification of restriction was performed by subjecting the restriction products to Agarose gel electrophoresis. The genes that were investigated in the study were genotyped according to references in table 3.2
<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>Nucleotide change Investigated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>C/A</td>
<td>C to A substitution at position 734 downstream of the first transcribed nucleotide of CYP1A2.</td>
<td>Sachse et al, 1999</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>*2</td>
<td>C430T mutation (Arg144Cys)</td>
<td>Moridani et al, 2006 &amp; Yasar et al, 1999</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>*3</td>
<td>C1075T mutation (Ile359Leu)</td>
<td>Moridani et al., 2006 &amp; Yasar et al, 1999</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>*2</td>
<td>m₁</td>
<td>Zand et al, 2005</td>
</tr>
<tr>
<td>CYP2E1</td>
<td></td>
<td>C₁/C₂ mutation</td>
<td>Huang et al., 2003 &amp; Salama et al, 1999,</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>*1B</td>
<td>A-290G</td>
<td>Van Schaik et al, 2000</td>
</tr>
<tr>
<td>MDR-1</td>
<td></td>
<td>C3435T in exon 26</td>
<td>Cascorbi et al, 2001</td>
</tr>
<tr>
<td>NAT2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*4</td>
<td>T341C, C481T, A803G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*5</td>
<td>T341C, C481T, A803G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*6</td>
<td>A590G, A803G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*7</td>
<td>C282T, G857A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*14</td>
<td>A803G</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Primer sequences</td>
<td>Amplicon size (bp)</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>--------------------</td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>1) 5'-CAA CCC TGC CAA TCT CAA GCA C-3' 2) 5'-AGA AGC TCT GTG GCC GAG AAG G-3'</td>
<td>920</td>
<td></td>
</tr>
<tr>
<td>CYP2C9*2</td>
<td>1) 5'-GGA GGA TGG AAA ACA GAG ACT TA-3' 2) 5'-TGA GCT AAC AAC CAG GAC TCA T-3'</td>
<td>396</td>
<td></td>
</tr>
<tr>
<td>CYP2C9*3</td>
<td>1) 5'-GCT GTG GTG CAC GAC GTC AGA TGC -3' 2) 5'-ACA CAC ACT GCC AGA CAC TAG G-3'</td>
<td>298</td>
<td></td>
</tr>
<tr>
<td>CYP2C19*2</td>
<td>1) 5'-AAT TAC AAC CAG AGC TTG GC-3' 2) 5'-TAT CAC TTT CCA TAA AAG CAA G-3'</td>
<td>196</td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>1) 5'-TTC ATT CTG TCT TCT AAC TGG-3' 2) 5'-CCA GTC GAG TCT ACA TTG TCA-3'</td>
<td>410</td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>1) 5'-GGA CAG CCA TAG ACA CAA CTG CA -3' 2) 5'-CTT TCC TGC CCT GCA CAG -3'</td>
<td>334</td>
<td></td>
</tr>
<tr>
<td>MDR-1</td>
<td>1) 5'-TGT TTT CAG CTG CTT GAT GG -3' 2) 5'-AAG GCA TGT ATG TTG GCC TC -3'</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>NAT2</td>
<td>1) 5'-TCT AGC ATG AT CAC TCT GC-3' 2) 5'-GGA ACA AAT TGG ACT TGG-3'</td>
<td>1093</td>
<td></td>
</tr>
</tbody>
</table>

1) Forward primer; 2) Reverse primer

The primer sequences used in this study were obtained from references in table 3.2 and synthesized by Inqaba Biotech™.
<table>
<thead>
<tr>
<th>Gene</th>
<th>1A2</th>
<th>2C9*2</th>
<th>2C9*3</th>
<th>2C19*2</th>
<th>2E1</th>
<th>3A4</th>
<th>MDR1</th>
<th>NAT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer concentration</td>
<td>20 pmol</td>
<td>20 pmol</td>
<td>20 pmol</td>
<td>30 pmol</td>
<td>20 pmol</td>
<td>20 pmol</td>
<td>20 pmol</td>
<td>35 pmol</td>
</tr>
<tr>
<td>Total reaction mix (μl)</td>
<td>25</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Initial Denaturation</td>
<td>95°C for 5 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C for 30 sec</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C for 30 sec</td>
<td>58°C for 30 sec</td>
<td>58°C for 30 sec</td>
<td>60°C for 30 sec</td>
<td>58°C for 30 sec</td>
<td>55°C for 30 sec</td>
<td>58°C for 30 sec</td>
<td>55°C for 30 sec</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C for 30 sec</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C for 10 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4 shows the PCR conditions of the 8 drug metabolizing enzyme genes. The initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, elongation at 72°C for 30 sec and final extension at 72°C for 10 min steps of the PCR conditions were constant throughout all the genes. The number of cycles for all PCR procedures was 35.
Table 3.5: RFLP conditions for DME gene SNP detection

<table>
<thead>
<tr>
<th>Gene</th>
<th>1A2</th>
<th>2C9*2</th>
<th>2C9*3</th>
<th>2C19*2</th>
<th>2F1</th>
<th>3A4</th>
<th>MDR1</th>
<th>NAT2*12</th>
<th>NAT2*7</th>
<th>NAT2*5</th>
<th>NAT2*7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction Enzyme</td>
<td>Bsp120I</td>
<td>AvaiI &amp; NsiI</td>
<td>AvaiI &amp; NsiI</td>
<td>SmaI</td>
<td>PstI</td>
<td>PstI</td>
<td>Sau3AI</td>
<td>MspI</td>
<td>BamHI</td>
<td>TaqI</td>
<td>KpnI</td>
</tr>
<tr>
<td>Incubation Temp</td>
<td>37°C</td>
<td>37°C</td>
<td>37°C</td>
<td>25°C</td>
<td>37°C</td>
<td>37°C</td>
<td>37°C</td>
<td>37°C</td>
<td>37°C</td>
<td>56°C</td>
<td>37°C</td>
</tr>
<tr>
<td>Incubation Time</td>
<td>O/N</td>
<td>90 minutes</td>
<td>90 minutes</td>
<td>60 minutes</td>
<td>O/N</td>
<td>90 minutes</td>
<td>90 minutes</td>
<td>O/N</td>
<td>O/N</td>
<td>O/N</td>
<td>O/N</td>
</tr>
</tbody>
</table>

O/N: overnight

Table 3.5 shows the restriction conditions for the 11 alleles. This was performed as per Appendix F. The use of four restriction enzymes on the NAT2 PCR product was to differentiate the wild type NAT2*4 allele and from the above-stated allele.
3.7 LABORATORY PARAMETERS TO MONITOR PATIENT PROGRESS

3.7.1 Liver function tests

Total bilirubin, lactate dehydrogenase, alkaline phosphatase, alanine aminotransferase, gamma- glutamyltransferase and aspartate aminotransferase levels were monitored as a proxy for possible hepatic stress or injury. Levels were determined three-monthly, or more frequently where clinically indicated. All biochemical tests were performed by qualified technicians at the Lancet Laboratories. (Appendix D).

The liver enzyme levels for normal and abnormal were graded according to the clinical trials protocol (Appendix G).

3.7.2 Viral Load Count

The tests were performed by qualified technicians at the CAPRISA Laboratory at the University of KwaZulu-Natal. (Appendix E)

3.7.3 CD4⁺ T-Cell Count

The tests were performed by qualified technicians at the CAPRISA Laboratory at the University of KwaZulu-Natal. (Appendix F).
3.8 STATISTICAL ANALYSES

After completion of the data collection and data entry, a descriptive analysis was conducted. Frequency distributions of categorical variables (gender, genotype, liver function test results) and means, standard deviation and ranges of continuous variables (age, CD4+ counts and viral load counts) were calculated.

All data was captured on computer using Microsoft Excel Software (Seattle, USA) and the analyses were carried out using the SPSS and SAS statistical packages.

3.8.1 Descriptive statistics

The following observations were sought and reported as study outcomes:

- Difference between expected and observed allele frequencies, assessed by Chi² Test for significance

- Significance of variance of baseline, 6 months and 1 year CD4+ T-cell and viral load counts, using the Chi² Test

- Genotype profiles of patients with positive and negative ARV therapeutic outcomes to assess associations between ARV therapeutic outcomes and genotype, using the Chi² Test

- Genotype profiles of patients on TB therapy to assess associations between TB therapeutic outcomes and genotype, using the Chi² Test
• DME gene profile of individuals who had successful TB therapy. Association between liver enzyme levels and genotype assessed for significance by Wilcoxon test.
4. RESULTS

4.1 GENOTYPING OF THE DRUG METABOLIZING ENZYME GENES

4.1.1 Polymerase Chain Reaction (PCR) Amplification of Drug Metabolizing Enzyme genes

Genomic DNA samples extracted from 50 individuals were removed from storage and used as a template for PCR reactions targeting an array of DME, according to section 3.6 of materials and methods. The concentrations of the samples were determined by Nanodrop ND-1000 readings (section 3.4.3.3) to be 600 ng/µl. The DNA samples were then diluted with TE buffer to a final concentration of 300 ng/µl and stored until use. 6 µl of the DNA was used for the amplification of each gene according to the methodologies outlined in section 3.6 and tables 3.2-3.4. A human DNA sample, previously used successfully as a template for multiple PCR reactions, and of a known concentration was used in all the PCR reaction as a positive control and a blank PCR tube (without DNA) was used as a negative control. Despite repeated attempts of amplification, some of the patient DNA did not produce any PCR product (Table 4.1, successful amplifications). 5 µl of PCR product was evaluated using Agarose Gel Electrophoresis. The expected product size for all the gene fragments are shown in Table 4.1 and representative pictures are shown below.
Table 4.1: Expected fragments sizes of the PCR product for each gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele(s)</th>
<th>Expected Fragment size (base pairs)</th>
<th>Number of successful amplifications</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>C/A</td>
<td>920</td>
<td>50</td>
<td>4.1a</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>*2</td>
<td>396</td>
<td>50</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>*3</td>
<td>298</td>
<td>48</td>
<td>4.3</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>*2</td>
<td>168</td>
<td>48</td>
<td>4.4</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>C_{l}</td>
<td>410</td>
<td>49</td>
<td>4.5</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>*1B</td>
<td>334</td>
<td>48</td>
<td>4.1b</td>
</tr>
<tr>
<td>MDR-1</td>
<td>C3435T</td>
<td>197</td>
<td>46</td>
<td>4.6</td>
</tr>
<tr>
<td>NAT-2</td>
<td>NAT2*4</td>
<td></td>
<td>47</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>NAT2*5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAT2*6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAT2*7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAT2*14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1093</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In the case of the CYP3A4 gene, multiple bands were obtained in addition to the desired band of 334 bps (see figure 4.1b). The 334 bp band was excised and purified using a Qiagen PCR product purification band Appendix E.

Figure 4.1a): Agarose gel electrophoresis (2%) of CYP3A4 PCR product before Product extraction from Gel. Lane 1, shows 3 μl of 100 bp DNA ladder (Fermentas™).
Figure 4.1 b): Agarose gel electrophoresis (2\%) of the correct 334 bp CYP3A4 PCR product after clean-up of the PCR product.

Lanes 3 to 15 show successful amplification of a 334 bp product from 13 patients. Lane 2, positive control and lane 1, shows 3 µl of 100 bp DNA ladder (Fermentas™).

Figure 4.2: Agarose gel electrophoresis (2\%) of CYP1A2 PCR product

Lanes 3 to 10 showing successful amplification of a 920 bp product from 7 patients, lane 2 positive control, and lane 1, shows 3 µl of 100 bp DNA ladder (Fermentas™).
Figure 4.3: Agarose gel electrophoresis (2%) of CYP2C9*2 PCR product

Lanes 3 to 11 showing successful amplification of a 396 bp product from 9 patients, lane 2 positive control, lane 12 negative control and lane 1, shows 3 μl of 100 bp DNA ladder (Fermentas™).
Figure 4.4: Agarose gel electrophoresis (2%) of CYP2C9*3 PCR product

Lanes 3 to 16 showing successful amplification of a 298 bp product from 14 patients, lane 2 positive control, lane 17 negative control and lane 1, shows 3 μl of 100 bp DNA ladder (Fermentas™).
Figure 4.5: Agarose gel electrophoresis (2%) of CYP2C19*2 PCR product

Lanes 3 to 16 showing successful amplification of a 168 bp product from 14 patients, lane 2 positive control and lane 1, shows 3 µl of Molecular weight marker IIIX -Lambda DNA ladder (Roche™).

Figure 4.6: Agarose gel electrophoresis (2%) of CYP2E1 PCR product

Lanes 3 to 8 show successful amplification of a 410 bp product from 8 patients. Lane 2, negative control and lane 1, shows 3 µl of 100 bp DNA ladder (Fermentas™).
Figure 4.7: Agarose gel electrophoresis (2%) of MDR1 PCR product

Lanes 4 to 10 show successful amplification of a 197 bp product from 7 patients. Lane 2 is negative control, lane 3 shows the positive control and lane 1, shows (3 μl) of Phi X DNA Ladder (Roche™).

Figure 4.8: Agarose gel electrophoresis (2%) of NAT2 PCR product

Lanes 2 to 7 & 9 show successful amplification of a 1093 bp product from 6 patients. Lane 8, negative control, lane 9 is the positive control and lane 1, shows 3 μl of 100 bp DNA ladder (Fermentas™).
4.1.2 Restriction Fragment Length Polymorphism analysis of Drug Metabolizing Enzyme Genes

The PCR products generated from patient DNA samples were restricted according to previously described methodology (Tables 3.2 and 3.5). 10 µl of PCR product was used in the analysis of each gene.

Table 4.2 shows a summary of all the restrictions performed for all genes, as well as the expected band sizes for the possible genotypes. Restriction digests containing a combination of wild type and homozygous bands were denoted as heterozygous (see table 4.2).
Table 4.2: Expected Band size for possible genotypes in each PCR fragment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele/ Mutation</th>
<th>PCR product Size (bp)</th>
<th>Restriction enzyme</th>
<th>Expected Band size per genotype (bp)</th>
<th>(%) Successful Restriction</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wild type (Wt)</td>
<td>Heterozygous (Ht)</td>
<td>Homozygous (Hm)</td>
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<tr>
<td>CYP1A2</td>
<td>CA</td>
<td>920</td>
<td>Bsp1201</td>
<td>709, 211</td>
<td>920, 709 and 211</td>
<td>920</td>
</tr>
<tr>
<td></td>
<td>*2</td>
<td>396</td>
<td>Avall and Nsil</td>
<td>223, 173</td>
<td>396, 223, 173</td>
<td>396</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>*3</td>
<td>298</td>
<td>Avall and Nsil</td>
<td>246, 28 and 24</td>
<td>274, 246, 28 and 24</td>
<td>274 and 24</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>*2</td>
<td>168</td>
<td>Smal</td>
<td>120 and 48</td>
<td>168, 120 and 48</td>
<td>168</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>C1</td>
<td>410</td>
<td>PstI</td>
<td>410</td>
<td>410, 290 and 120</td>
<td>290 and 120</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>*1B</td>
<td>334</td>
<td>PstI</td>
<td>220, 81, 33</td>
<td>220, 199, 81, 33 and 21</td>
<td>199,81,33 and 21</td>
</tr>
<tr>
<td>MDR-1</td>
<td>C3435T</td>
<td>197</td>
<td>Sau3AI</td>
<td>197</td>
<td>197, 158 and 39</td>
<td>158 and 39</td>
</tr>
</tbody>
</table>

Table 4.3: Expected Band size for possible genotypes in NAT2 PCR fragment

<table>
<thead>
<tr>
<th>Allele</th>
<th>Bp</th>
<th>Kpn I</th>
<th>Taq I</th>
<th>Bam HI</th>
<th>Msp I</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT2*4</td>
<td>1093</td>
<td>Restricted</td>
<td>Restricted</td>
<td>Restricted</td>
<td>Restricted</td>
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<td>NAT2*5</td>
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<td>Unrestricted</td>
<td>Unrestricted</td>
<td>Unrestricted</td>
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<td></td>
<td></td>
<td>4.19</td>
</tr>
</tbody>
</table>
4.1.2.1  Restriction analysis of CYP1A2 gene

The C to A substitution at position 734 of CYP1A2 gene was investigated. The 920 bp PCR product was restricted with the Bsp 120I restriction enzyme. The restriction digest yielded 1 band for the homozygous genotype, 2 and 3 bands for the wild type and heterozygous genotypes respectively (see table 4.2). All 50 individuals were genotyped, of which 24.0% (n=12) of individuals possessed restriction recognition sites on both alleles, thus designated the wild type CC genotype (lanes 4-6, 8 & 12 of figure 4.10). Half of the study population (n=25) had a C to A substitution on one allele, thus making them heterozygous CA for the CYP1A2 gene (lanes 3, 9, 11, 13 & 15 of figure 4.10). Thirteen (26.0%) individuals were homozygous for the AA genotype (lane 7, 10, 14 of figure 4.10).

Figure 4.9: Agarose gel electrophoresis (2%) of CYP1A2 PCR products digested with the Bsp120I restriction endonuclease enzyme.

Lane 1 shows a 100 bp DNA ladder (O’GeneRuler™, Fermentas). Lane 2 is the unrestricted control. The restriction digests were run on a 2% Agarose gel at 80V for 120 minutes.
4.1.2.2  Restriction analysis of the CYP2C9*2 gene

The C430T mutation, corresponding to the allele CYP2C9*2 was investigated. The 398 bp PCR product was restricted with \textit{Avall} and \textit{Nsil} restriction enzymes as per section 3.6. After restriction, all the 50 individuals possessed the restriction sites on both alleles, thus wild type for the C430T substitution.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure410.png}
\caption{Agarose gel electrophoresis (2\%) of CYP2C9*2 PCR product digested with \textit{Avall} and \textit{Nsil}}
\end{figure}

Lane 1 shows a 100 bp DNA ladder (O’GeneRuler™, Fermentas) and lane 2 shows an undigested control.
4.1.2.3 Restriction analysis of the CYP2C9*3 gene

The C1075T substitution was investigated. The 298 bp PCR product was digested with \textit{Avall} and \textit{Nsil} restriction enzymes. Forty two (87.5\%) individuals possessed a recognition site corresponding to C1075T, making them homozygous (T/T) *3/*3 genotype (figure 4.12). Six (12.5\%) individuals yielded a 246 bp band, depicting the wild type (C/C) (TT) *1/*1 genotype for the CYP2C9*3 gene (not shown on gel).

![Figure 4.11: Agarose gel electrophoresis (2\%) of \textit{Avall} and \textit{Nsil} restriction of the CYP2C9*3 PCR product](image)

Lane 1 shows a 100 bp DNA ladder (Fermentas\textsuperscript{TM}), lane 2 shows an undigested CYP2C9*3 PCR product, serving a positive control. Lanes 3 – 16 are the digestion products from patient samples and lane 17 is a negative control, without DNA template. \textbf{Note:} The 274 bp band is accompanied by a 28 and 24 bp bands, not visible on the gel.

4.1.2.4 Restriction analysis of the CYP2C19*2 gene

The *2 allele was investigated for the CYP2C19 gene. A total of 48 individuals were genotyped, the other two were unable to produce a PCR fragment. Fourteen (29.2\%)
individuals, did not possess *Sma*I restriction recognition site on both alleles and hence designated homozygous for the CYP2C19*2 mutation (lanes 5 and 8 of figure 4.13). Thirty four (70.8%) individuals possessed recognition sites for *Sma*I (lanes 3,4,6 & 7 of figure 4.13) hence wild type for CYP2C19*3 gene.

**Figure 4.12: Agarose gel electrophoresis (2%) of *Sma*I restriction of the CYP2C19*2 PCR products**

Lane 1 shows a 100 bp DNA ladder (Fermentas™) and lane 2 shows an undigested PCR product serving as a control. **Note:** The 120 bp band is accompanied by a 49 bp band, not visible on the gel. There were instances where there were faint, additional bands as seen in the above picture; this was probably due to partial restriction. We chose to assign the more visible band to particular genotypes.
4.1.2.5 Restriction analysis of the CYP2E1 gene

The C1/C2 mutation, which results in a PstI restriction site was investigated for 49 individuals. The PCR products for 75.5% (n=37) of individuals did not possess a PstI recognition site on either allele, depicting their wild type (C1/C1) genotype (lanes 3-6, 8-10 of figure 4.14). Of the remaining 24.5% (n=12) individuals who possessed the PstI recognition site, 5 individuals yielded two fragments of 290 and 120 bp, hence homozygous (C2/C2). The other 7 individuals were found to have an additional pattern, containing bands sizes of 410, 120 and 290. These were classified as heterozygous, although it was not possible to rule out partial restriction or additional genotypes.

![Agarose gel electrophoresis](image)

**Figure 4.13: Agarose gel electrophoresis (2%) of PstI restriction of CYP2E1 PCR product**

Lane 1, shows (3 µl) of Phi X DNA Ladder (Roche™) and lane 2 in the undigested CYP2E1 PCR fragment, serving as a control.
4.1.2.6 **Restriction analysis of the CYP3A4 gene**

The CYP3A4*1B allele with an A290G substitution was investigated. Forty eight individuals were able to be genotyped. The previous study using the same methodology described the wild type as a band size of 220bp, with the homozygous genotype a further restriction site, giving 2 bands of 199 and 21 bp (Van Schaik, et al, 2000). However, in this study, although the band patterns appeared the same, the sizes of the bands were smaller than 220 and 199bp. It was not possible to sequence these fragments to determine if they corresponded to the previously described patterns. Therefore a single band as in lane 3 was taken to be the wild type. A double bands as in lane 4, 5 and 6 was taken to be heterozygous. Where there was a single band corresponding to the same size as the smaller band in the heterozygote pattern, this was taken as homozygote. 10 individuals showed one fragment designated equivalent to the 220 bp band, denoting the wild type (A/A) genotype (lane 3 of figure 4.15). Twenty two (45.8%) individuals were found to be homozygous (G/G). The remaining 33.0% (n=16) were heterozygous (A/G), showing 2 fragments after restriction by *PstI* (lanes 4-6 of figure 4.15).
Figure 4.14: Agarose gel electrophoresis (2%) of *PstI* restriction of the CYP3A4 PCR Product

Lane 1 shows the 100 bp DNA ladder (Fermentas™) and lane 2 the undigested PCR product serving as a control. Lane 3 shows a fragment denoting the wild type (A/A) genotype and lanes 4-6 shows two fragments denoting the heterozygous AG genotype. Lane 7 is the negative control, without the digestion template.

### 4.1.2.7 Restriction analysis of the MDR-1 gene

The C3435T mutation, sensitive to *Sau3AI* restriction was investigated. PCR product was obtained for 46 of the 50 participants. Forty (86.9%) individuals did not possess a recognition site for the enzyme and hence were positive for the CC wild type genotype. Six
(12.5%) individuals possessed a restriction or partial restriction site; they were classified as variant according to the previous study (Cascorbi et al., 2001).

Figure 4.15: Agarose gel electrophoresis (2%) of Sau3AI restriction of MDR1 PCR product

Lane 1: 100 bp DNA ladder (Fermentas™) and lane 2 shows the undigested PCR product serving as a control. Lanes 3-10, 12-13 & 15 are the PCR samples restricted to yield a 158 bp fragment and lanes 11, 14 & 16 show partial restrictions of PCR fragments. Lane 17 shows the negative control. Note: The 158 bp band is accompanied by a 39 bp band, not visible on gel.
Table 4.4: Allele distribution and frequencies of DME genes among the Zulu population of South Africa.

<table>
<thead>
<tr>
<th>Gene (SNP investigated)</th>
<th>Alleles</th>
<th>n</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>C</td>
<td>74</td>
<td>49.3</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>76</td>
<td>50.7</td>
</tr>
<tr>
<td>CYP2C9*2</td>
<td>*1/*2</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>CYP2C9*3</td>
<td>*1</td>
<td>42</td>
<td>43.8</td>
</tr>
<tr>
<td></td>
<td>*3</td>
<td>54</td>
<td>56.2</td>
</tr>
<tr>
<td>CYP2C19*2</td>
<td>*1</td>
<td>62</td>
<td>64.6</td>
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<tr>
<td></td>
<td>*2</td>
<td>34</td>
<td>35.4</td>
</tr>
<tr>
<td>CYP2E1</td>
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<td>106</td>
<td>71.6</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>42</td>
<td>28.4</td>
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<tr>
<td>CYP3A4</td>
<td>A</td>
<td>61</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>85</td>
<td>58.2</td>
</tr>
<tr>
<td>MDR-1</td>
<td>CC</td>
<td>126</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>CT/TT</td>
<td>24</td>
<td>16</td>
</tr>
</tbody>
</table>
### Table 4.5: Genotype distribution and frequencies of DME genes among the Zulu population of South Africa.

<table>
<thead>
<tr>
<th>Gene (SNP investigated)</th>
<th>Genotype</th>
<th>(n)</th>
<th>Frequency (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>CC</td>
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<td>24.3</td>
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<tr>
<td></td>
<td>CA</td>
<td>25</td>
<td>50.0</td>
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<td></td>
<td>AA</td>
<td>13</td>
<td>25.7</td>
</tr>
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<td>CYP2C9*3</td>
<td>*1/*1</td>
<td>42</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td>*3/*3</td>
<td>8</td>
<td>31.6</td>
</tr>
<tr>
<td>CYP2C19*2</td>
<td>*1/*1</td>
<td>14</td>
<td>41.7</td>
</tr>
<tr>
<td></td>
<td>*1/*2</td>
<td>34</td>
<td>12.5</td>
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<td>CYP2E1</td>
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<td>C2/C2</td>
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<tr>
<td></td>
<td>CT/TT</td>
<td>6</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Tables 4.4 and 4.5 show a summary of the frequency of the different alleles and genotypes.

#### 4.1.2.8 Restriction analysis of the NAT-2 gene

Restriction analysis for the NAT-2 gene was based on the previously mentioned methodologies (Table 3.2). Both Huang <i>et al</i>, (2003) and Srivastava <i>et al</i>, (2004), did not sequence for the various genotypes of the NAT-2 gene. Their studies used the presence of restriction enzyme recognition sites, within the 1093 bp NAT-2 fragment, to define genotypes and defined the wild type genotype as possessing restriction sites for the four
restriction enzymes used on the fragment. Four restriction enzymes were used to discern between the alleles (*5, *6, *7 and *14), thus should an enzyme not restrict the fragment, the individual was deemed positive for that particular allele. If there was a mixed pattern showing bands corresponding to both the digested and undigested fragment then the result was classified as heterozygous. In addition a recent study (published after completion of this thesis) (Sabbagh et al, 2008) showed by sequencing the NAT2 gene there were multiple possible genotypes for some of the restriction patterns. This could explain why for example with the allele NAT2*5, in addition to the three predicted patterns of wild-type, heterozygous, and homozygous there were samples that gave an additional band with **KpnI** restriction. However in this thesis the older less discriminatory method was used and the alleles were defined as either wild-type, heterozygous or homozygous.

![Agarose gel electrophoresis (2%) of KpnI restriction of NAT2 PCR product to detect NAT2*5 allele](image)

**Figure 4.16: Agarose gel electrophoresis (2%) of KpnI restriction of NAT2 PCR product to detect NAT2*5 allele**

Lane 1: 100 bp DNA ladder (Fermentas™) and lane 2 shows the undigested PCR product serving as control. The PCR product of lane 3 was undetected and lane 4 did not restrict denoting homozygosity (M1/M1), whereas lanes 5, 6, 7, 10, 11, 12, 13 show complete
restriction, denoting the wild type. Lanes 8, 9, 14, 15, 16 displayed a combination of the previous two patterns indicating heterozygosity. The results of the 47 individuals who were able to produce viable PCR product are shown in table 4.4. In lanes 7 and 15 there was an additional band that may have been due to the presence of an additional restriction site corresponding to another mutation.

Figure 4.17: Agarose gel electrophoresis (2%) of TaqI restriction of NAT2 PCR product to detect NAT2*6 allele

Lane 1 shows the 100 bp DNA ladder (Fermentas™) and lane 2 the undigested PCR product serving as control. Digestion of the 1093 bp PCR product by TaqI was done to identify the NAT2*6. Multiple TaqI restriction sites existed in the fragment giving a complex pattern. It was therefore difficult to distinguish between the various genotypes. However all the individuals gave a pattern that corresponded most closely with the heterozygous genotype, as defined in previous studies and therefore the 48 individuals successfully genotyped with the TaqI digestion were designated as such.
Figure 4.18: Agarose gel electrophoresis (2%) of *BamHI* restriction of NAT2 PCR product to detect NAT2*7 allele

Digestion of the 1093 bp PCR product by *BamHI* was done to differentiate between NAT2*7 allele and wild type NAT2*4 allele. Lane 1 shows the 100 bp DNA ladder (Fermentas™) and lane 2, the undigested PCR product serving as control. Lanes 3 & 7: Show partial loss of restriction of the PCR product corresponding to heterozygity for the NAT2*7 (M3) allele. Lanes 4-6 & 8-20 show PCR product possessing a restriction site for *BamHI*, compatible with the NAT2*4 wild type allele. Results for patients are shown in table 4.4.
Figure 4.19: Agarose gel electrophoresis (2%) of *MspI* restriction of NAT2 PCR product to detect NAT2*14 allele

Digestion of the 1093 bp PCR product by *MspI* was done to differentiate between NAT2*14 and wild type NAT2*4 allele. Lane 1 shows the 100 bp DNA ladder (Fermentas™) and lane 2, the undigested PCR product serving as control. Lanes 3, 5-10 & 12-18 show complete restriction, denoting wild type for the NAT2*14 (M4) allele and lanes 4, 11 & 19-20 show partial restriction indicating heterozygosity. Lane 21 served as a negative control. The results of the 47 individuals who were able to produce viable PCR are shown in table 4.4.
Table 4.5: Summary of NAT2 restriction profiles of the study population

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Kpn (M1)</th>
<th>Taq (M2)</th>
<th>BamH1 (M3)</th>
<th>Msp (M4)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>121001</td>
<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/M2</td>
<td>wt/M2</td>
<td>Rapid Acetylator</td>
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<tr>
<td>121002</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121003</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121004</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
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<tr>
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<td>wt/M1</td>
<td>wt/M2</td>
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</tr>
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<td>M1/M1</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
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<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/M4</td>
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<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
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<td>wt/M2</td>
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<td>wt/M4</td>
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<tr>
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<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121068</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121070</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121074</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121076</td>
<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121078</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121082</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/M3</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121083</td>
<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121091</td>
<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121092</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121093</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121100</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121104</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121105</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121110</td>
<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>121131</td>
<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122058</td>
<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/M2</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122065</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/M2</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122071</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122079</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122082</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122093</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122099</td>
<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122102</td>
<td>wt/wt</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122104</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/wt</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122115</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
</tr>
<tr>
<td>122118</td>
<td>wt/M1</td>
<td>wt/M2</td>
<td>wt/wt</td>
<td>wt/M4</td>
<td>Rapid Acetylator</td>
</tr>
</tbody>
</table>
Table 4.5 shows the restriction patterns of the study population. We were unable to produce clear restriction images of 8 of the patients. They were excluded from subsequent analyses.

The genotypes were assigned according to Huang et al, 2003. Each restriction enzyme was able to discern between M/M (unrestricted), WT/M (partial restriction) and Wt/Wt (complete restriction). The presence of any two (M/M) - highlighted in red- for an individual, is indicative of a slow acetylator genotype.

Table 4.5 shows that 46.5% of the study population are slow acetylators.
4.2 CLINICAL REVIEW OF THE STUDY POPULATION

4.2.1 Study Population demographic information

Table 4.6 presents the demographic data of the study populations.

Table 4.6: Patient demographics

<table>
<thead>
<tr>
<th></th>
<th>N (%)</th>
<th>Mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (yr)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>50</td>
<td>33.50 (20-52)</td>
</tr>
<tr>
<td>Male</td>
<td>25 (50)</td>
<td>36.36 (24-52)</td>
</tr>
<tr>
<td>Female</td>
<td>25 (50)</td>
<td>30.88 (20-51)</td>
</tr>
<tr>
<td><strong>Age group categories (yrs)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-30</td>
<td>22 (44)</td>
<td></td>
</tr>
<tr>
<td>31-40</td>
<td>20 (40)</td>
<td></td>
</tr>
<tr>
<td>41-60</td>
<td>8 (16)</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>37 (74)</td>
<td></td>
</tr>
<tr>
<td>Occasional</td>
<td>6 (12)</td>
<td></td>
</tr>
<tr>
<td>Frequent</td>
<td>6 (12)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td><strong>Alcohol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>35 (70)</td>
<td></td>
</tr>
<tr>
<td>Occasional</td>
<td>7 (14)</td>
<td></td>
</tr>
<tr>
<td>Frequent</td>
<td>7 (14)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td><strong>History of Traditional Medicine use</strong></td>
<td>8 (16)</td>
<td></td>
</tr>
</tbody>
</table>

The sex distribution was equal with 25 males and 25 females, with a median age of 35 and 29 years respectively. 84.0% of individuals are under 40 years, a profile typical of the HIV epidemic. 70.0% of individuals were reported not to consume alcohol.
4.2.2 Immune status of study participants

Table 4.7: Baseline, 6 months and 1 year CD4\(^+\) T-cell and viral load counts

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>6 months</th>
<th>12 months</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4(^+) (Cells/(\upmu)l)</td>
<td>167 (112)</td>
<td>238 (121)</td>
<td>353 (254)</td>
<td>P&lt;0.001 (ANOVA)</td>
</tr>
<tr>
<td>Viral load (copies per ml)</td>
<td>201073 (236492)</td>
<td>289 (169)</td>
<td></td>
<td>P=0.0001 (Paired t test)</td>
</tr>
</tbody>
</table>

All time points are defined from the time of recruitment into the study. Note therefore that the duration of exposure to ARV therapy was not uniform for all participants in view of the study-determined variable points of initiation of such therapy.
### 4.2.3 Anti-retroviral therapy clinical outcomes

**Table 4.8: ARV therapeutic outcomes among study population**

| CD4<sup>+</sup> T-cell count improvement<sup>a</sup> | 29 (58) |
| Viral load drop<sup>b</sup> | 48 (96) |
| Number of Days on ARV therapy<sup>c</sup> | |
| Less than 90 days | 2 (4) |
| 90-180 days | 3 (6.3) |
| 181-270 days | 6 (12.5) |
| 270-360 | 12 (25) |
| More than 360 days | 25 (52.1) |
| Unknown | 2 (4) |

---

<sup>a</sup>: Improvement defined as an increase of 50 or more T-cells per ml at baseline months to 6 months post ARV therapy.

<sup>b</sup>: Virological response was defined as a drop in viral load to undetectable levels from baseline to 12 months post randomization.

<sup>c</sup>: Calculated from ART initiation to end of study (30 April 2007).
Table 4.9: Genotype profile of individuals (n=44) classified by ARV therapeutic success or failure (Success defined as a CD4\(^+\) count increase from baseline to 6 months post-recruitment of a least 50 cells/\(\mu\)l)

<table>
<thead>
<tr>
<th>THERAPEUTIC SUCCESS</th>
<th>CYP1A2</th>
<th>CYP2E1</th>
<th>CYP3A4</th>
<th>CYP2C19*2</th>
<th>MDR-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6 (13.6%)</td>
<td>23 (46%)</td>
<td>6 (13.6%)</td>
<td>19 (43.2%)</td>
<td>25 (56.8%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>10 (22.7%)</td>
<td>3 (6.8%)</td>
<td>9 (20.5%)</td>
<td>10 (22.7%)</td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>13 (29.5%)</td>
<td>3 (6.8%)</td>
<td>14 (31.8%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Hetero/homozygous</td>
<td>4 (9.1%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THERAPEUTIC FAILURE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>7 (15.9%)</td>
<td>11 (25%)</td>
<td>3 (6.8%)</td>
<td>9 (20.5%)</td>
<td>12 (27.3%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>5 (11.4%)</td>
<td>2 (4.5%)</td>
<td>4 (9.1%)</td>
<td>6 (13.6%)</td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>3 (6.8%)</td>
<td>2 (4.5%)</td>
<td>8 (18.2%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Hetero/homozygous</td>
<td></td>
<td></td>
<td></td>
<td>1 (2.3%)</td>
<td></td>
</tr>
<tr>
<td>Significance</td>
<td>0.048</td>
<td>0.68</td>
<td>0.8181</td>
<td>0.98</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Table 4.9 describes the frequency of genotypes of individuals who had positive and negative ARV therapeutic outcomes. Significance assessed by \(\chi^2\) (\(\chi^2\) for trend for 2x3 tabulations).
4.2.4 TB therapy clinical outcomes

Figure 4.20: Summary of TB therapeutic outcomes of the study population

Figure 4.20 summarises the outcome of treatment for tuberculosis. 60% of patients recorded successful therapy in that the final sputum result was negative. 8% recorded a treatment failure, whereas 32% either failed to complete therapy or to produce a sputum sample.
Table 4.10: Genotype profile of individuals (n=34) classified by TB therapeutic success (n=30) or failure (n=4). Significance assessed by $\chi^2$ ($\chi^2$ for trend for 2x3 tabulations).

<table>
<thead>
<tr>
<th>THERAPEUTIC SUCCESS (n=30)</th>
<th>CYPIA2</th>
<th>CYP2E1</th>
<th>CYP3A4</th>
<th>CYP2C19*2</th>
<th>MDR-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>7 (23.3%)</td>
<td>22 (73.3%)</td>
<td>4 (13.3%)</td>
<td>22 (73.3%)</td>
<td>22 (73.3%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>15 (50%)</td>
<td>4 (13.3%)</td>
<td>10 (33.3%)</td>
<td>6 (20%)</td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>8 (26.7%)</td>
<td>4 (13.3%)</td>
<td>16 (53.3%)</td>
<td>2 (6.7)</td>
<td></td>
</tr>
<tr>
<td>Hetero/homozygous</td>
<td>8 (26.7%)</td>
<td>4 (13.3%)</td>
<td>16 (53.3%)</td>
<td>2 (6.7)</td>
<td></td>
</tr>
<tr>
<td>THERAPEUTIC FAILURE (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 (26.7%)</td>
</tr>
<tr>
<td>Wild type</td>
<td>3 (75%)</td>
<td>3 (75%)</td>
<td>0 (0%)</td>
<td>3 (75%)</td>
<td>4 (100%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>1 (25%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (25%)</td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>0 (0%)</td>
<td>1 (25%)</td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Hetero/homozygous</td>
<td>0 (0%)</td>
<td>1 (25%)</td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Significance</td>
<td><strong>0.04</strong></td>
<td><strong>0.80</strong></td>
<td><strong>0.10</strong></td>
<td><strong>0.79</strong></td>
<td><strong>0.58</strong></td>
</tr>
</tbody>
</table>
4.2.5 Severe adverse events of study population

![Organization chart](image)

Figure 4.21: Organization chart of liver disorders noted in the study population

The reported adverse events (AE's) presented above are all grade 4 laboratory events and grade 3 and 4 clinical events, as defined by the Regulatory Compliance Centre (see Appendix G for description and references) (figure 4.21). All grades of liver enzyme abnormalities are further described in table 4.13 below. Of note is that only 1 out of 12 reported events were reported as due to drug induced toxicity. These results are unsurprising as a small proportion of patients were slow acetylators, which are the group most at risk of drug-induced hepatotoxicity (Huang et al, 2003).
4.2.6 Liver Enzyme Functions

Table 4.11: Liver enzyme levels at baseline, 6 and 12 months post-recruitment

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Normal Range</th>
<th>Baseline (range)</th>
<th>6 months (range)</th>
<th>12 months (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>2-26 umol/L</td>
<td>11.08 (2-31)</td>
<td>6.96* (3-31)</td>
<td>4.68* (2-9)</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP)</td>
<td>53-128 IU/L</td>
<td>100.37 (26-295)</td>
<td>102.11 (43-387)</td>
<td>106.57 (26-517)</td>
</tr>
<tr>
<td>Gamma glutamyltransferase (γGT)</td>
<td>0-44 IU/L</td>
<td>64.84 (16-259)</td>
<td>82.85 (18-428)</td>
<td>85.60 (14-715)</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT)</td>
<td>&lt;35 IU/L</td>
<td>21.27 (5-104)</td>
<td>23.10 (7-77)</td>
<td>27.83 (7-129)</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST)</td>
<td>13-35 IU/L</td>
<td>31.88 (12-110)</td>
<td>31.77 (14-60)</td>
<td>35.04 (17-157)</td>
</tr>
<tr>
<td>Lactate dehydrogenase (LDH)</td>
<td>120-230 IU/L</td>
<td>316.43* (146-986)</td>
<td>221.15 (146-366)</td>
<td>226.15* (143-394)</td>
</tr>
</tbody>
</table>

* p<0.005 with respect to baseline

Table 4.11 reflects the liver enzyme profile of the study participants at baseline; 6 months and 12 months post recruitment (see 3.2.2). Biochemical liver tests show a statistically but not clinically significant decrease in bilirubin and LDH levels from baseline to 6 and 12 months. Changes in other parameters were not significant.

4.2.6.1 Bilirubin

All patients had normal range bilirubin for all the time points. However, the levels are shown to drop significantly from baseline to 12 months TB and ART therapy initiation. This is not clinically relevant.
4.2.6.2 Alkaline Phosphatase

All the study participants had normal ALP levels for all time points. One patient had grade 2 (2.6-5 X ULN) ALP toxicity at 6 months after TB therapy initiation. Another patient had grade 2 ALP toxicity at 6 months after ART initiation.

4.2.6.3 Gamma-glutamyltransferase

Elevated γ-GT levels were common throughout the study. At 6 months, there was no recorded significant difference among patients. 5 patients were reported to have elevated Grade 2 baseline levels and 1 patient had grade 3 (5-10 X ULN) at baseline. 9 individuals were found to have elevated γ-GT at 6 months post therapy initiation, 6 were reported to have Grade 2 elevation and 3 had grade 3. 8 patients were reported to have elevated Grade 2 elevations, 12 months after therapy initiation, 1 patient had grade 3 and 1 patient had a grade 4 (>10 X ULN) elevation.

However, γ-GT abnormalities are usually ignored in the clinical management of HIV, as it is a non-specific marker of liver enzyme induction (Nunez, 2006). Thus the significance of this elevation in this context is uncertain.

4.2.6.4 Alanine aminotransferase

A single patient, who was reported to have grade 3 elevated γ-GT at baseline, was also found to have grade 2 ALT toxicity. All other patients had normal ALT levels throughout the study. None of the patients were reported to have enzyme elevations above grade 1 at 6 months post therapy initiation.
4.2.6.5 Aspartate Aminotransferase

A majority of the patients did not experience elevated AST; however, there was a gradual though non-significant increase in AST levels from baseline to 12 months post therapy initiation. The patient who was reported to have grade 3 elevated γ-GT and Grade 2 ALT toxicity at baseline was also found to have grade 2 AST toxicity.

4.2.6.6 Lactate Dehydrogenase

Baseline levels of LDH for all the patients were elevated to grade 1 toxicity. Thirty nine of the patients recorded grade 1 toxicity and 2 recorded grade 2 toxicity at baseline. All the LDH levels subsequently dropped to the normal range and maintained the levels at 6 months but started to increase at 12 months post recruitment (table 4.11).

4.2.7 Association between liver enzyme levels and genotype

Table 4.12 shows the association between genotype and liver enzyme levels of the study population. The Kruskal-Wallis test was used to assess significance for the association.
### Table 4.12: Table of Association between genotype and liver enzyme levels: median (IQR)

<table>
<thead>
<tr>
<th></th>
<th>CYP1A2</th>
<th>CYP2E1</th>
<th>CYP3A4</th>
<th>CYP2C19*2a</th>
<th>MDR-1a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>6 Months</td>
<td>Baseline</td>
<td>6 Months</td>
<td>Baseline</td>
</tr>
<tr>
<td>Bilirubin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>5 (4-6)</td>
<td>4 (3-6)</td>
<td>6 (5-9)</td>
<td>5 (4-7)</td>
<td>9 (5-12)</td>
</tr>
<tr>
<td>Ht</td>
<td>7 (5-11)</td>
<td>5 (4-6)</td>
<td>10 (5-14)</td>
<td>5 (4-6)</td>
<td>5 (4-10)</td>
</tr>
<tr>
<td>Hm</td>
<td>6 (4-12)</td>
<td>6 (4-7)</td>
<td>6 (4-7)</td>
<td>5</td>
<td>6 (5-8)</td>
</tr>
<tr>
<td>AST</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>27 (23-36)</td>
<td>21 (20-24)*</td>
<td>27 (22-38)</td>
<td>28 (22-37)</td>
<td>29 (21-48)</td>
</tr>
<tr>
<td>Ht</td>
<td>28 (20-38)</td>
<td>28 (23-37)*</td>
<td>29 (24-33)</td>
<td>24 (22-32)</td>
<td>24 (19-31)</td>
</tr>
<tr>
<td>Hm</td>
<td>27 (22-38)</td>
<td>23 (21-39)*</td>
<td>24 (19-36)</td>
<td>22</td>
<td>28 (22-38)</td>
</tr>
<tr>
<td>ALT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>18 (15-28)</td>
<td>16 (14-22)</td>
<td>19 (13-28)</td>
<td>22 (15-28)</td>
<td>28 (11-45)</td>
</tr>
<tr>
<td>Ht</td>
<td>23 (13-28)</td>
<td>22 (17-29)</td>
<td>24 (18-26)</td>
<td>27 (16-33)</td>
<td>18 (12-25)</td>
</tr>
<tr>
<td></td>
<td>CYP1A2</td>
<td></td>
<td>CYP2E1</td>
<td></td>
<td>CYP3A4</td>
</tr>
<tr>
<td>-----</td>
<td>--------</td>
<td>-----</td>
<td>--------</td>
<td>-----</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>6 Months</td>
<td>Baseline</td>
<td>6 Months</td>
<td>Baseline</td>
</tr>
<tr>
<td>ALP</td>
<td>Wt</td>
<td>67 (60-102)</td>
<td>98 (59-123)</td>
<td>76 (66-108)</td>
<td>99 (86-117)</td>
</tr>
<tr>
<td></td>
<td>Ht</td>
<td>80 (68-113)</td>
<td>90 (78-101)</td>
<td>105 (69-139)</td>
<td>85 (66-112)</td>
</tr>
<tr>
<td></td>
<td>Hm</td>
<td>87 (73-106)</td>
<td>101 (88-119)</td>
<td>73 (62-107)</td>
<td>99</td>
</tr>
<tr>
<td>GGT</td>
<td>Wt</td>
<td>40 (37-50)</td>
<td>51 (34-70)</td>
<td>49 (31-94)</td>
<td>54 (43-110) *</td>
</tr>
<tr>
<td></td>
<td>Ht</td>
<td>43 (25-146)</td>
<td>55 (41-114)</td>
<td>110 (50-172)</td>
<td>77 (56-97) *</td>
</tr>
<tr>
<td></td>
<td>Hm</td>
<td>77 (53-115)</td>
<td>57 (42-88)</td>
<td>24 (18-51)</td>
<td>33 *</td>
</tr>
<tr>
<td></td>
<td>Ht</td>
<td>242 (200-291)</td>
<td>205 (173-237)</td>
<td>234 (207-361)</td>
<td>199 (177-261)</td>
</tr>
<tr>
<td></td>
<td>Hm</td>
<td>240 (208-337)</td>
<td>214 (210-254)</td>
<td>230 (185-287)</td>
<td>217</td>
</tr>
</tbody>
</table>
a: Heterozygous categories include both heterozygous and homozygous

*: A near significant association between AST levels and CYP1A2 gene was seen, between baseline and 6 months time points (p= 0.0502) as well as between GGT levels and CYP2E1 gene was seen, between baseline and 6 months time points (p= 0.0513).

**: A significant association between AST levels and CYP3A4 gene was seen, between baseline and 6 months time points (p= 0.0413)

4.2.8 Clinical and genotypic description of patients with elevated liver enzymes

13 of 50 subjects (26%) experienced elevated liver enzymes at some point after initiation of therapy (Table 4.13). The greatest elevations were found for the GGT, where one patient experienced grade 4 (>10 X ULN) toxicity at the 12 month time point.

The group consisted of 6 females and 7 males. 4 of the patients were in the intensive phase of TB therapy (4 TB drugs with or without ARVs) and 7 were in the post intensive phase (2 TB drugs and 3 ARVs) when they experienced enzyme elevations. A majority of the individuals (11) were exposed to prolonged TB therapy (191-> 222 days). One individual was diagnosed with hepatitis B and two patients reported a heavy exposure to alcohol.

In most cases enzyme abnormalities were restricted to elevations in GGT or LDH, both of which are non-specific markers of enzyme induction or injury. Only three patients revealed aminotransferase elevations: a mild ALT elevation in one, a moderate elevation in AST in the second and a mild elevation of AST and LDH in the third. These are the only patients in whom a drug-induced hepatitis might be queried, and the biochemical severity of this was mild to moderate.
Table 4.13: LFT derangement with relatedness to TB or ARV therapy or an unrelated cause as suggested by attending clinician

<table>
<thead>
<tr>
<th>No</th>
<th>Parameter</th>
<th>Grade</th>
<th>Definitely</th>
<th>Probably</th>
<th>Possibly</th>
<th>Unrelated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ARV</td>
<td>TB</td>
<td>ARV</td>
<td>TB</td>
</tr>
<tr>
<td>A. γ-GT elevated alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>γ-GT</td>
<td>1</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>γ-GT</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>γ-GT</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>γ-GT</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>γ-GT</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>γ-GT</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>γ-GT</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>γ-GT</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>γ</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. Other parameters elevated

| 4  | AST, ALT  | 2     |     |     |       |     |     | x   |       |         |
|    | γ-GT      | 3     |     |     |       |     |     |     |       |         |
| 9  | LDH, γ-GT | 2     |     |     |       |     | x   |     |       |         |
| 12 | AST, LDH  | 1     |     |     |       |     | x   | x   |       |         |
| 13 | LDH       | 2     |     |     |       |     | x   | x   |       |         |

Patient records were drawn for all subjects who demonstrated elevated liver tests at any stage. These records were independently reviewed by the principal (clinical) investigator for the study from which these patients were drawn, and categorised as most likely to ARV therapy, TB therapy or unknown on an interpretation of the timing and circumstances of the elevation. These results are shown in Table 4.13.
The genotype profile of the above 13 individuals was found to be as follows.

**Table 4.14: Deranged liver enzymes and possible toxicity**

<table>
<thead>
<tr>
<th>n (%)</th>
<th>Genotype (Gene)</th>
<th>Possible consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 (92.0)</td>
<td>Ht/Hm (CYP 1A2)</td>
<td>Decreased enzyme inducibility</td>
</tr>
<tr>
<td>10 (77.3)</td>
<td>Wt (CYP2E1)</td>
<td>Increased risk for INH-induced hepatotoxicity</td>
</tr>
<tr>
<td>8 (62.0)</td>
<td>SA (NAT-2)</td>
<td>Metabolize some TB drugs such as isoniazid and ethanol-based compounds at a slower rate than the rapid acetylators, leading to increased risk of hepatotoxicity</td>
</tr>
<tr>
<td>9 (69.2)</td>
<td>Ht/Hm (CYP3A4)</td>
<td>Decreased enzyme inducibility</td>
</tr>
<tr>
<td>10 (76.9)</td>
<td>Wt (MDR-1)</td>
<td>Increased drug clearance resulting in sub-optimal efficacy</td>
</tr>
</tbody>
</table>

A majority of the above patient group exhibit the variant genotypes, known to contribute to liver stress.
5. DISCUSSION

This study assessed the frequency of DME alleles among the Zulu population, using a cohort of HIV and TB co-infected Zulu patients recruited into a clinical study as the sample population. As a secondary objective, retrospective clinical data from this cohort was used to search for correlations between DME alleles and therapeutic outcomes, including treatment success or failure, and documented liver stress.

Table 3.1 shows the important drug substrates for which each DME gene is responsible.

5.1 CYTOCHROME P450 1A2

Table 5.1: Comparison of CYP1A2 genotype frequency among different ethnic groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C/C</td>
</tr>
<tr>
<td>Zulu (present study)</td>
<td>24.3</td>
</tr>
<tr>
<td>Caucasian (Sachse et al, 1999)</td>
<td>10.0</td>
</tr>
<tr>
<td>Japanese (Soyama et al, 2005)</td>
<td>62.8</td>
</tr>
</tbody>
</table>

75% of the Zulu population carry the A allele which is associated with reduced enzyme activity. This might potentially alter the rate of metabolism of drug substrates such as ciprofloxacin, leading to a prolonged plasma half-life. This frequency is shared with the Caucasian population, in contrast to the Japanese population in whom the C allele is more common. Some important antimicrobial agents, including rifampicin and erythromycin, are powerful inducers of CYP1A2. It is unknown whether this inducing effect is influenced by the genotype: were this the case, concomitant administration of an agent such as rifampicin might be expected to have variable effects on the plasma levels of the CYP1A2 drug.
substrate depending on genotype, an effect which might be clinically important. This is worthy of further investigation. With regard to ARV therapy, the role of CYP1A2 is as yet unknown.

As shown in Tables 4.9 and 4.10, we have shown a consistent association between CYP1A2 genotype and clinical outcomes for both response to therapy and potential liver toxicity. The significance or otherwise of this is discussed in a later section. It does however appear that the potential importance of CYP1A2 genotype for clinical practice is worthy of further, more focused investigation.

5.2 CYTOCHROME P450 2C9

Table 5.2: Comparison of CYP2C9 genotype frequency among different ethnic groups

<table>
<thead>
<tr>
<th>Genotype Frequency %</th>
<th>*1/*3</th>
<th>*3/*3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zulu (present study)</td>
<td>87.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Caucasian (Moridani et al (2006))</td>
<td>78</td>
<td>22</td>
</tr>
<tr>
<td>Asian (Kirchheiner &amp; Brockmoller 2005)</td>
<td>100</td>
<td>0.0</td>
</tr>
</tbody>
</table>

A similar frequency between Caucasian and Zulu population was observed and the *1 allele was found to be the predominant allele among all three ethnic groups. The Asian population exhibited little variation in CYP2C9 alleles.

The clinical significance of CYP2C9 metabolism has been investigated using warfarin which is a known substrate for the CYP2C9 enzyme. Moridani et al (2006) showed that *3/*3 genotype individuals displayed a reduced clearance rate of warfarin compared to the *1/*3 genotype. These individuals were shown to require a reduced warfarin dosage as compared to the *1/*1 wild type and thus were genetically predisposed to warfarin toxicity.
Rifampicin and isoniazid have also been shown to be important substrates for the CYP2C9 enzyme, thus a reduced clearance of rifampicin as a result of carriage of one of these alleles could contribute to an individuals’ risk of drug-induced toxicity (Xie et al, 2002; Andersson et al, 2005; Kirchheiner and Brockmoller, 2005; Manzi and Shannon, 2005). Our findings show a universal occurrence of variant *3 allele among the study population, with 12.5% being homozygous. However, only a very small proportion (Table 4.14) of the study cohort showed probable hepatotoxicity due to TB therapy. Larger, population-based studies need to be conducted to examine the whether the frequency of significant hepatotoxicity in patients taking TB therapy is indeed influenced by genotype, to inform dosing guidelines for rifampicin and isoniazid at a programmatic level.

5.3 CYTOCHROME P450 2C19

Table 5.3: CYP2C19 Genotype frequency comparison among different ethnic groups

<table>
<thead>
<tr>
<th>Genotype Frequency %</th>
<th>*1</th>
<th>*2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zulu (present study)</td>
<td>70.8</td>
<td>29.2</td>
</tr>
<tr>
<td>Tanzanian (Bathum et al, 1999)</td>
<td>82.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Egyptians (Hamdy et al, 2004)</td>
<td>78.0</td>
<td>22.0</td>
</tr>
<tr>
<td>Italians (Scordo et al, 2004)</td>
<td>62.0</td>
<td>32.0</td>
</tr>
<tr>
<td>Iranians (Zand et al, 1999)</td>
<td>86.0</td>
<td>14.0</td>
</tr>
</tbody>
</table>

The distribution of both the *1 and *2 allele appears to similar among the above groups. When administering CYP2C19 drug substrates, ethnic significance need not be considered as a possible influencing factor. Of note, none of the samples from our study demonstrated a CYP2C19*3 fragment. These results accord with those of Bathum, Hamdy and Scordo who did not detect the CYP2C19*3 among Tanzanian, Egyptian and Italian populations.
respectively. It would appear that this allele is predominantly an Asian mutation (Hamdy et al, 2002; Scordo et al, 2004).

Rifampicin has also been shown to be an inducer of CYP2C19 (Manzi and Shannon, 2005; Johnson et al, 2005). As with to CYP2C9, the presence of the *3 allele confers slower metabolism of CYP2C19 substrates, thereby potentially influencing the therapeutic outcomes of patients taking CYP2C19 substrates such as rifampicin.

5.4 CYTOCHROME P450 2E1

Table 5.4: CYP2E1 Genotype Frequency comparison among different ethnic groups

<table>
<thead>
<tr>
<th></th>
<th>Genotype Frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1/C1</td>
</tr>
<tr>
<td>Zulu (present study)</td>
<td>75.5</td>
</tr>
<tr>
<td>African-American Liu et al (2001)</td>
<td>89</td>
</tr>
<tr>
<td>Chinese (Cai L et al, 2001)</td>
<td>63.7</td>
</tr>
<tr>
<td>Caucasians (Liu et al, 2001)</td>
<td>93</td>
</tr>
</tbody>
</table>

The differences in CYP2E1 frequencies among the different populations may have a significant clinical impact. The wild type (C1/C1) genotype is the predominant genotype among the listed ethnic groups. The activity of the CYP2E1 enzyme was highlighted in a study by Huang et al, (2003) who investigated the role of CYP2E1 in individuals’ susceptibility to anti-tuberculous drug-induced hepatitis. Patients homozygous for the C1 (wild type) allele were found to have a higher risk of hepatotoxicity than those with the C2 variant allele. Although Huang and co-workers reported that the CYP2E1 gene could be an independent risk factor in hepatotoxicity, they postulated an interaction with NAT-2
acetylator status and its role in isoniazid metabolism. Their findings indicate that subjects with the CYP2E1 (C1/C1) genotype who were NAT-2 slow acetylators had a higher enzyme activity and increased risk of hepatotoxicity than the C1/C2 or C2/C2 genotypes when administered isoniazid (Huang et al, 2003). It would seem that the C1/C1 genotype had a higher CYP2E1 activity and hence could potentially produce a greater volume of secondary metabolites, which have a hepatotoxic effect. The higher activity of CYP2E1 enzyme combined with reduced acetylation by the NAT-2 enzyme could overwhelm the hepatocytes and result in cellular damage (Huang et al, 2003).

5.5 CYTOCHROME P450 3A4

Table 5.5: Comparison of CYP3A4 genotype frequency among different ethnic groups

<table>
<thead>
<tr>
<th>Genotype Frequency %</th>
<th>A/A</th>
<th>A/G</th>
<th>G/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zulu (present study)</td>
<td>21.0</td>
<td>16.0</td>
<td>46.0</td>
</tr>
<tr>
<td>Zulu (Chelule et al, 2003)</td>
<td>7.5</td>
<td>17.8</td>
<td>74.8</td>
</tr>
<tr>
<td>Caucasian (Chelule et al, 2003)</td>
<td>16</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Taiwanese (Sata et al, 1999)</td>
<td>94</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

The observed frequency of the variant (G/G) genotype in the present study of 46.0% differed significantly from the frequency calculated by Chelule et al (2003) (p=0.015, Fischer’s exact test), where 110 Africans from the Kwa-Zulu Natal region of South Africa were genotyped and 74.8% of them were homozygous (G/G) genotype. We are unable to account for this. However, the data from our analysis with respect to the CYP3A4 frequency is similar to other studies by Wandel et al, 2000 (66.0%); Hsieh et al, 2001 (53.0%); Lamba et al, 2002 (35-67.0%).
It has been speculated that the CYP3A4*1B is associated with reduced activity due to altered gene expression (Lamba et al, 2002). However Wandel et al, (2000) postulated that this variation could lead to reduced activity, and recommended that more studies should be done to clarify this further. Furthermore, Lamba et al, (2002) suggested an association between the CYP3A4*1B and CYP3A5 activity and its possible role in substrate metabolism.

5.6 P-GLYCOPROTEIN (MDR-1)

Table 5.6: Comparison of MDR-1 genotype frequency among different ethnic groups

<table>
<thead>
<tr>
<th>Genotype Frequency %</th>
<th>C/C</th>
<th>C/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zulu (present study)</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>Zulu (Chelule et al, 2003)</td>
<td>75.5</td>
<td>20.9</td>
</tr>
<tr>
<td>Caucasian (Cascorbi et al, 2001)</td>
<td>20.8</td>
<td>79.2</td>
</tr>
<tr>
<td>West Africans (Schaeffeler et al, 2001)</td>
<td>83</td>
<td>17</td>
</tr>
</tbody>
</table>

The wild-type allele is associated with increased intestinal expression of P-glycoprotein and thus reduced drug availability (Chelule et al, 2003; Marzolini et al, 2004). A review on the clinical relevance of MDR-1 gene polymorphism stated that individuals homozygous for the C3435T (T/T) allele had a 2-fold reduction in intestinal P-glycoprotein expression (Eichelbaum et al, 2004). Hence, they would be more likely to have a slower clearance of drug substrates than the C/T or C/C counterparts. Furthermore, the consequence of a higher P-gp expression could lead to a lower plasma drug concentration among Africans compared to Caucasians. Eichelbaum and colleagues found that with a higher drug concentration in plasma, individuals with the T/T genotype are more likely to have a
significantly improved CD4$^+$ cell count and viral load suppression compared to the C/T (heterozygous) or C/C (homozygous) genotype, six months after anti-retroviral therapy initiation (Eichelbaum et al, 2004). This observation was in relation the context of protease inhibitor treatment, warranting further study for other antiretroviral drug groups and MDR-1 expression.

Genotyping may have a clinical relevance in that the higher prevalence of the C/C genotype among Africans might result in higher P-pg expression and lower drug concentrations. However, given that 30% of the African population does in fact carry the alternative C/T genotype, race alone would not be a suitable proxy for formal genotype profiling.

5.7 N-ACETYLTRANSFERASE 2

Table 5.7: NAT-2 Genotype Frequency comparison among different ethnic groups

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Slow Acetylator</th>
<th>Rapid Acetylator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zulu (present study)</td>
<td>45.6</td>
<td>55.4</td>
</tr>
<tr>
<td>Caucasian (Schippers et al, 2005)</td>
<td>83.3</td>
<td>16.7</td>
</tr>
<tr>
<td>Indian (Mittal et al, 2004)</td>
<td>53.6</td>
<td>45.4</td>
</tr>
</tbody>
</table>

The NAT-2 acetylator status has clinical relevance in terms of drug metabolism. This was shown in a study on 318 Taiwanese patients which found that slow acetylator individuals were 2.3 times more likely to experience hepatotoxicity than their rapid acetylator counterparts (Huang et al, 2003). These researchers also studied the interactive effect of CYP2E1 and NAT-2, and found that patients who were homozygous for the wild type C1/C1 genotype had a higher risk of hepatotoxicity than the C1/C2 or C2/C2 counterparts.
In addition, when the C1/C2 or C2/C2 genotypes combined with rapid acetylator status, this provided a basis for comparison, the odds ratio for hepatotoxicity among slow acetylators increased from 3.94 to 7.43 for C1/C1-slow acetylator individuals.

These findings were confirmed in another study where 80% of slow acetylators experienced hepatotoxicity, compared to 9.1% of rapid acetylators (Shimizu et al, 2005) (Table 2.2). This would indicate that determining the acetylator status of patients prior to treatment of tuberculosis may alleviate liver injury (Shimizu et al, 2005 and Kinzig-Schippers et al, 2005).

Our study revealed that the genotypes previously associated with slow acetylator phenotype were less common that in other studies, and this may have contributed to the low incidence of hepatotoxicity in the study population.

**Conclusion**

In summary, some variation in allelic frequencies of the DME genes was shown between the Zulu subjects and those reported for other populations, indicating racial differences in terms of DME allele and genotype distribution. Drug substrates such as rifampicin, isoniazid and some ARVs may be negatively influenced by the predominant genotypes found in the Zulu population for example; those with a NAT-2 (slow acetylator) status (45.6%); CYP3A4 homozygotes (46%) and MDR-1 wild types (70%). However, intra-ethnic variability is common and race is a poor predictor of actual genotype in the Zulu population. In view of this, we conclude that consideration of the individual genotype is necessary when administering therapy, as race is too imprecise a predictor of genotype.
5.8 CLINICAL STUDY AND PATIENT DEMOGRAPHICS

Our clinical study attempted to:

1. Assess therapeutic outcomes of TB and ARV treatment relative to genotype
2. Correlate abnormal liver enzyme elevations (indicative of liver stress) with DME genotype variability.

To our knowledge, this is the first study to relate the genotypic profile of drug metabolizing enzymes to ARV and TB clinical outcomes in South Africa, though, owing to methodological limitations, it is unable to attribute these outcomes solely to DME genotype. As described in 2.2, the study was nested in a larger study, in which an immunocompromised study population received multiple drugs for HIV, TB and other conditions, including Pneumocystis prophylaxis. This constitutes a very heterogeneous population in which multiple confounding variables may operate. The administration of TB and ARV therapies in an already immunocompromised individual poses health risks, mainly due to additive toxicities, drug-drug interactions and potential worsening of clinical symptoms due to immune reconstitution inflammatory syndrome (IRIS) (Nagy, 2006). Furthermore, both treatment success and failure, and adverse events, are influenced by multiple factors beyond genetically controlled drug metabolism, including compliance, microbial resistance patterns, comorbidities and others. This study was unable to separate out these disparate variables, and might therefore be expected able, at best, to identify gross associations for further, focused studies.
In view of this, we conducted our study as a pilot study in which we attempted to generate a genotypic profile of DME in an ill population and link this profile to clinical outcomes and to liver injury following exposure to potentially hepatotoxic drugs.

5.9 CD4+ AND VIRAL LOAD PROFILE IN STUDY POPULATION

The median pre-ARV therapy CD4+ cell count of the study population was 166.8 cells/μl (range 11-426) with a median increase to 238.7 cells/μl (range 198-375). This is similar to a study by Hoffmann et al (2007), assessing the extent of liver toxicities in a cohort of HIV positive individuals on ARV therapy in individuals from Johannesburg South Africa, where the median count of 136 cells/μl (range 73-208) at baseline increased to 300 (range 150-422). 46.0% of our patients were significantly immunocompromised with a CD4 cell count of less than 200, a figure similar to the study by Hoffmann et al, (2001), where 72.0% of the patients had less than 200 cells per μl at baseline.

The viral load for the present study population was 232 000 Copies/ ml at baseline. 58.0% of the cohort registered an overall increase in CD4+ cell count. All patients suppressed their viral load to less than 400 viral copies per μl, indicating successful ARV therapy.

5.10 GENOTYPE AND ARV THERAPY OUTCOMES

The relationship of drug metabolism to ARV therapy has mainly been investigated in pharmacokinetic studies (Li and Chan, 1999; Bean, 2000; Schinazi et al, 2006), with limited data on the genetic contribution to metabolism.
We were unable to show any relationship between genotype and ARV therapeutic outcomes, where therapeutic success was defined as an increase in CD4 count of more than 50 cells/µl in 6 months (Table 4.9) except for CYP1A2. Here subjects AA homozygous appeared more likely to demonstrate treatment success (p=0.048). However application of the Bonferroni principle would set the required level of significance at 0.05/6 (0.008), since six independent hypotheses-genotypes-were being tested. Thus, though we are unable to demonstrate an unequivocal relationship between genotype and outcome, this does suggest an association which might be studied in more detail, with a larger patient sample, in order to determine whether the association is real or not. Furthermore, it must be noted that all patients successfully suppressed their viral loads indicating that the ARV drugs were effective: the implications of a failure to improve CD4 count for an understanding of drug metabolism and its genotypic control is therefore quite unclear.

The enzyme CYP3A4 has been shown to play a role in the metabolism of NNRTI's, NRTIs and most anti-TB drugs (Table 3.1), hence activity of the CYPs could potentially influence the success of ARV therapy. A majority of the individuals (52.3%) who showed ARV therapy success (Table 4.9) were found to have at least one variant (G) which confers a reduced CYP3A4 enzyme activity. Justesen et al, (2004) performed a study on the pharmacokinetics of ARV therapy which revealed that HIV infected individuals displayed a reduced CYP3A4 activity as compared to non-HIV infected control patients. Therefore, they are more likely to show negative therapeutic outcomes. Our study revealed that more patients with the variant G allele were among the group who experienced successful ARV therapy. However, Justesen et al, (2004) found a negative outcome; our results are contrary
as the majority of the patients with the allele conferring reduced activity had positive therapeutic outcomes. In the context of the current understating of drug metabolism, our results are plausible as the variant CYP3A4 gene confers reduced enzyme activity and therefore increased bioavailability of drugs. Our difficulty in relating viral suppression to CD4 count improvement does however remain, and complicates our analysis. Genotyping and pharmacokinetic studies investigating ARV therapy metabolism in a HIV-TB co-infected cohort would further contribute to our understanding of this field.

Thus far, research into the role of MDR-1 and ARV therapy metabolism has been centred on protease inhibitors (PI), where it was shown that individuals with the wild type (C/C) genotype are at an increased risk of ARV therapeutic failure (Eichelbaum, 2004). The present study population did not receive PI therapy. 56.8.0% of individuals with ARV therapeutic success in this study possess the wild type MDR1 (C/C) genotype. In view of this, the MDR-1's role in the metabolism of other ARV therapy groups should be investigated especially as in the majority of African settings, treatment regimens initially include NNRTI's and NRTI's.

**Conclusion**

In conclusion, we have been unable to demonstrate a significant effect of DME polymorphisms on ARV therapeutic outcomes in this limited pilot study. We recognise however that our study was underpowered, and also subject to multiple confounding factors which may have masked such an effect.
5.11 TB THERAPY OUTCOMES

Figure 4.21 highlights a drawback of the study, where 32% (9, sputum not tested and 7, defaulted treatment) of the study population’s data were inapplicable for any statistical interpretation. The remaining 68% of the study cohort presented uneven categories (30, treatment success and 4, treatment failure). Categorizing the 4 unsuccessful patients into the various genotypes would not be expected to yield significant findings, given the very low numbers. However, despite this, Table 4.10 shows a significant association between CYP1A2 and therapeutic outcomes (p= 0.04). In a manner similar to CYP1A2 and ARV therapeutic outcomes (section 5.10), this value may not be a true reflection of CYP1A2’s role in TB therapy outcomes, given Bonferroni’s theorem. However, we note that our results are congruent in that CYP1A2 genotype appears to be significant for both ARV and TB therapy. This may be an indication that such an association does indeed exist, and this appears to may warrant further investigation.

In retrospect, duration of therapy very was a poor proxy for treatment outcome, insufficient characterisation of the patients, as well as inefficient microbial sensitivity testing in determining success or failure may have contributed to the insignificant results seen in figure 4.21 and Table 4.10.

It should be noted however, that duration of TB therapy is multi-factorial. Possible resistance of the bacterium to therapy as well as drug intolerance by the patient may have been, among others, major factors influencing therapeutic outcome. The average period of susceptible-TB therapy is six months; hence any deviation from this time period should be thoroughly investigated in an effort to identify toxicity and potential drug-resistance.
5.12 LIVER ENZYME LEVELS

The enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are contained in high concentration within hepatocytes. Elevated blood levels of ALT and AST result when there is a loss of hepatocyte membrane integrity due to the toxic effect of oxy radicals arising from secondary drug metabolites (Johnson and McFarlane, 1989). Liver function tests may be used as markers of liver cell injury, though their utility is limited, and should where necessary be supplemented by histological assessment. Serum elevations of ALT and AST approximately three to five times the upper limit of normal are conventionally regarded as suggestive of toxin-induced injury (Johnson and McFarlane, 1989; Nunez, 2006).

Bilirubin, ALP, γGT, ALT, AST and LDH were used as markers of liver stress during therapy in this study. Contrary to studies by Lawn et al, (2005); Nunez (2006) and Hoffmann et al, (2007) who evaluated only the AST and ALT, this study together with Martinez et al, (2001) incorporated all of the above enzymes for a comprehensive overview of liver injury patterns. It has been shown that both ARVs and anti-TB medications are associated with increased risk of toxicity, clinically manifested by the elevation of liver enzymes (Pol et al, 2004 and Clark et al, 2002). Elevated enzymes are not exclusive to ARV and TB therapy. Patients infected with HIV are more likely to also experience liver enzyme abnormalities due to other co-infections such as HBV or HCV as well as from other drug therapies used for HIV-associated conditions (Martinez et al, 2001; Sulkowski et al, 2002; Nunez, 2006). In this study, we were unable to demonstrate any significant rise in median enzyme levels during the study (Table 4.11) for any parameter other than for GGT,
for which the levels rose from 64.84 IU (range 16-259) to 82.85 IU (range 18-428) 
(p=0.05). This is in contrast to the study by Martinez et al, (2001), where the estimated 
incidence of hepatotoxicity, evidenced by elevated ALT, γ-GT, ALP and AST levels, 
increased from 3.7% at 3 months to 20.1% 12 months post ARV therapy initiation.

With respect to GGT, Nunez (2006) points out that this is not a reliable marker for liver 
stress and its elevation is merely an indication of enzyme induction. This γ-GT elevation 
was also seen in the study by Martinez et al, (2001) who reported a 45.0% increase. We are 
uncertain of the role of γ-GT and the significance of its elevation in patients with HIV and 
TB receiving multiple drug therapy.

Hepatitis B and C co-infections are common in HIV-infected subjects in the developed 
world, probably because of shared routes of transmission such as intravenous drug abuse. 
In this study 10.0% of the cohort (n= 5) were hepatitis B surface antigen positive (section 
4.2.5). HCV testing was not a standard of care in this study setting. We found no 
significant association between HBV infections and elevated liver enzymes.

The genotype profile and clinical data of 13 individuals, who demonstrated deranged liver 
enzyme levels during the treatment period, were evaluated. Table 4.13 shows that enzyme 
elevations among the sub-group were attributed to either ARV or TB drugs by the 
evaluating clinician. It must be borne in mind however that attribution of disturbed liver 
function to a specific cause can be highly problematical owing to the non-specificity of 
disturbed liver enzymes for any particular type of liver injury, particularly where full data
including dechallenge and rechallenge experience and liver biopsy are not available, and these results should be regarded as subjective. When the clinical observations were correlated with the genotypic profile, the findings reveal that a majority of these individuals had variant DME alleles which confer reduced enzyme activity, highlighting the relevance of pharmacogenetics within clinical trials. However, most of these abnormalities were limited to elevations in GGT alone, which, as stated above, is very non-specific. Only three subjects showed mild elevations in other enzymes, which would constitute better evidence for actual liver injury. The lack of careful characterisation of these patients as well as the very low number makes it impossible to prove any associations with DME alleles.

Biochemical liver tests (Table 4.13) show a statistically but not clinically significant decrease in bilirubin and LDH levels from baseline to 6 and 12 months. This may indicate some TB- or HIV-related disturbance of liver function which improved with treatment of these conditions. In the case of bilirubin, a fall in levels may also reflect the enzyme-inducing effect of rifampicin.

5.12.1 Liver derangement and genotype associations

12 out of the 13 patients with abnormal enzyme levels were shown to possess either 1 or 2 CYP1A2 variant alleles. The presence of these variant alleles, resulting in reduced enzyme activity, could have contributed to the deranged liver enzyme levels. A combination of ARV (Table 4.9), TB therapy (Table 4.10) outcomes, as well as the above liver enzyme profile (Table 4.14), are suggestive of the role CYP1A2 plays in this population. Additionally, the CYP2E1 profile demonstrated a majority (73.3%) with wild type (C1/C1)
genotype, thereby increasing the risk of hepatotoxicity when metabolising isoniazid or ethanol-based drug. This is in keeping with another study by Huang et al (2003). The patient diagnosed with drug-induced hepatitis, also included in this subgroup, (figure 4.21) has a wild type CYP2E1 genotype. 62% of this subgroup were found to possess the SA (slow acetylator) genotype, which has previously been described to clear drug substrates at a slower rate than rapid acetylators, thus increasing their risk of toxicity due to the delayed clearance of drug substrates (Shimizu et al, 2006). Among the patients with deranged liver enzymes, 69.2% of them were found to have the variant (G/G) CYP3A4*1B genotype. Clinically, this highlights the importance of generating genotypic profiles of patients prior to therapy initiation, as this could contribute to understanding ADRs, underscoring the need for further investigation in this area.

Conclusion

These findings suggest the need for further studies assessing the influence of DME genotypes on therapeutic outcomes and toxicity profiles in the South African population, particularly with respect to the treatment of HIV and TB. When attempting to link such genetic data to therapeutic outcomes, a clear definition of ARV and TB therapeutic outcome is imperative. As previously discussed, a larger sample size is required in order to provide an adequately powered study for appropriate subgroup analysis. Lack of liver biopsies to support laboratory reports, was a draw back because liver enzyme levels alone could not accurately provide evidence for drug-induced hepatitis, and patients with possible toxicity require very careful and expert evaluation, backed up by a full range of ancillary investigations, including liver biopsy where necessary. Table 4.13 reflects 13 of the 50
patients who exhibited deranged liver enzyme levels at some point during treatment, however only 3 patients show a significant change, and then only mild. A genotypic profile of these patients indicates a high prevalence of detrimental alleles, which may influence therapeutic outcomes or risk of toxicity. Clearly much larger numbers of patient will be required in order to capture sufficient patients with true drug-induced hepatitis for meaningful analysis.
6. CONCLUSION AND FUTURE RECOMMENDATIONS

Our study generated a genotypic profile of drug metabolising enzyme genes in a cohort of the Zulu population of South Africa. Differences in the distribution and frequencies of the investigated DMEs between the Zulu population and other ethnic groups were observed, confirming other studies in that the occurrence of DME variant alleles is ethnically linked (Ingelman-Sundberg, 2001).

The PCR-RFLP method was used in our study, it was chosen because it was the most frequently used method as well as for its sensitivity and specificity in detecting SNPs. The technique is relatively inexpensive as it does not require specialized laboratory equipment. The techniques was efficient for generating reliable data, however it have its drawback in that it is laborious and time-consuming. The method is cost effective, especially in poor-resource settings.

Other methods for genotyping, such as single-stranded conformation polymorphism (SSCP), DNA sequencing, Fluorescence Resonance Energy Transfer (FRET) and Oligonucleotide Microarray have been utilised to genotype for DMEs. These methods may be relatively faster than the PCR-RFLP in that they can be used to generate multiple gene profiles on numerous samples simultaneously however, they are not cost effective and require to specialized equipment, accompanied by high reagent costs. Further highly skilled individuals are required to carry out the techniques. The prohibitive costs of performing microarray experiments however, can be overcome by incorporating more DMEs and their
accompanying transcriptional receptors as well as utilizing this technology for both research and diagnostic purposes. The Roche AmpliChip CYP450 Pharmacogenomic Microarray for Clinical Applications is one of these typical applications.

It should be noted however, that the sample population was not representative in that, the patient were selected based on their ill-health. The choice of such a population was based on the premise that genotype is constant, regardless of health status. Interrogation of this cohort for DME correlations were confounded by the heterogeneous nature of the patient population, the varying times of initiation of ARV therapy, the exposure to multiple drugs, the small sample size (including a high dropout rate) and shortcomings in the documentation of liver-related adverse events.

Our study however, highlighted a small number of DMEs within a much larger range, whose activity could potentially impact therapeutic outcomes of individuals on concomitant therapies, namely anti-TB and HAART. The findings failed to yield conclusive evidence of these DME's role in influencing therapeutic outcomes. This was to be expected, as this was a preliminary pilot study, exploring the possibility of the influence of DMEs on clinical outcomes. The study does however provide a basis for future studies which will provide evidence for the role of DMEs in the mediation of treatment-related toxicities. In this instance, it is the recommendation of the study that a larger cohort, longer observational period and more stringent clinical parameters be considered; especially as treatment toxicity caused by ADRs may contribute to the morbidity and mortality of patients.
HIV infections coupled with TB co-infection, reiterates the need to monitor individuals in terms of genetic predisposition to develop hepatotoxicity. Furthermore, focused attention to genetic mediation of hepatotoxicity in future studies would aid in developing strategies to reduce the drug burden of HIV-TB co-infected individuals and the morbidity due to ADRs.

A shortcoming of this study is evident in the lack of pharmacokinetic studies to support the genetic contribution in therapeutic outcomes. It is thus recommended that more comprehensive studies, which would include:

- A larger cohort of South Africans, by comparing the genotypic profile of all ethnic groups, for example: Indian, Zulu and Caucasian populations. This cohort should ideally be comprised of healthy individuals, thus removing potentially confounding factors stated in the present study.
- Inclusion of other DME genes and the accompanying DME transcriptional regulatory factors such as hPXR, CAR and HNF-1.
- Pharmacokinetic studies, evaluating HAART and anti-TB drug absorption levels among the African, Indian and Caucasian populations
- Ultimately combining the broader genotypic findings and pharmacokinetic studies to gain a larger insight to the role of DMEs on clinical outcomes.
- Some of the Anti-HIV drugs (NNRTIs) do not undergo CYP metabolism; hence their impact should also be evaluated.

The field of drug metabolism is complex and multi-faceted and response to therapy is dependent on various factors such as genetic predisposition, age, diet environment and co-morbidities (Meyer and Gut, 2002). Clinical outcomes of any one individual are subject to
a myriad of variable factors, of which drug metabolism is a minor component. Therefore, a comprehensive and stringent study, including elements of pharmacodynamics, pharmacogenomics and pharmacogenetics is essential in alleviating the cost of therapy related to HIV and TB.
References


Estabrook, R.W. 2003 ‘A Passion for P450s (Remembrances of the Early history of research on Cytochrome P450)’, *Drug Metabolism and Disposition*, vol. 31, pp. 1461-1473.


CYP2D6, CYP2C19, CYP3A4s and MDR1/ABCB1', Drug Metabolism and pharmacokinetics, vol. 19, pp. 83-95.


APPENDICES

Appendix A: DNA Extraction Protocol

A) Cell Lysis

- Add 300 µl whole blood to a 2 ml microfuge tube containing 900 µl Red Blood Lysis Solution and incubate for 1 min at room temperature.
- Centrifuge the solution for 30 seconds at 14 000 rotations per min (rpm). Remove supernatant. Leaving a white pellet with 10-20 µl residual liquid.
- Vortex the white pellet vigorously for 20 seconds, ensuring complete resuspension of pellet.
- Add 300 µl Cell Lysis Solution to the re-suspended white cells and vortex briefly.

B) Protein Precipitation

- Place sample on ice for 1 min to cool the sample
- Add 100 µl Protein Precipitation solution to the cell lysate.
- Vortex vigorously at high speed for 20 seconds to mix the sample uniformly.
- Centrifuge solution for 1 min at 14 000 rpm. The protein should form a tight dark brown pellet

C) DNA Precipitation

- To a clean 1.5 ml microfuge tubes, containing 300 µl (100%) isopropanol, add the supernatant.
- Mix the sample by inverting gently 50 times, strands of DNA should be visible.
• Centrifuge the solution for 1 min at 14 000 where the DNA will form a white pellet.
• Pour off the supernatant and drain the tube briefly onto a clean absorbent paper. Add 300 μl (70%) ethanol and invert the tube to wash the DNA pellet.
• Centrifuge the solution for 1 min at 14 000 rpm and pour off the supernatant.
• Drain the tube by inverting it and allowing it to air-dry for 5 seconds.

D) DNA Hydration

• Add 100 DNA hydration solution and vortex for 5 seconds at medium speed.
• Incubate sample overnight at room temperature.

E) Modifications to DNA Extraction protocol

• The incubation time was increased from 1 min to 10 min in step 1 of the cell lysis phase to ensure sufficient lysis of red blood cells.
• Step 1 of the protein precipitation phase (RNA degradation) was excluded.
• During the protein precipitation phase in step 4, centrifugation was increased from 1 to 3 min.
• During the DNA hydration phase, centrifugation of the solution was increased from 1 min to 2 min at steps 3 and 5. Step 6, instead of air-drying the DNA pellet for 5 seconds, the pellet was allowed to air-dry for 10 min. The hydration solution added to the pellet varied (50μl -150 μl) depending on the size of the pellet.
Appendix B: Preparation of Solutions and Buffers

10X TBE (Tris-Borate-EDTA) Buffer

For 1L solution

Tris- 108g
Boric Acid- 55g
EDTA- 9.3g

- Measure out the above reagents into a flask.
- Add 700 ml distilled water and dissolve at low heat.
- Adjust volume to 1L with distilled water.
- Filter and store at room temperature in a Schott bottle.

Sample Loading Buffer

For 100 ml solution

Tris-HCl- 5 ml
EDTA (500 mM)- 1 ml
Bromophenol Blue- 0.05g
Rnase (10 mg/ml) - 300µl
Distilled Water- to 100 ml

- Measure out the above reagents into a Schott bottle,
- Boil for 15 min at 100°C and cool overnight.
Appendix C: Agarose Gel Electrophoresis

Principle

Nucleic acids (DNA or RNA) is loaded into a gel and subjected to an electric current. The positively charged nucleic acid will migrate through the gel, from the positive to the negative electrode. The speed of nucleic acid migration is inversely related to its size, therefore different size fragments would be discernible, meaning that bigger fragments would not migrate as fast or as far as the smaller fragments.

For visualisation, Ethidium Bromide- a chemical that intercalates between the nucleotides- in mixed within the gel mixture and will fluoresce under Ultra violet light.

Procedure to make Agarose Gel

For 1 % Gel

Agarose – 0.7 g

1X TBE buffer (Electrophoresis buffer) – 70 ml

For 2% Gel

Agarose- 1.4g

1XTBE (Electrophoresis buffer) – 70 ml

- Measure out the above reagents into a flask and swirl.
- Place into a microwave oven for approximately 1 min or until the agarose has dissolved.
- Cool to 40-50 °C and add 3.5 μl Ethidium Bromide

(Gloves must be worn when handling Ethidium bromide).
• Set the gel casting tray and comb as required.
• Pour the warm agarose and allow approximately 30 min to set, at room temperature while taking care that bubbles do not form inside the gel.

Procedure to run the Gel

• Add 1X TBE buffer into the gel tank, enough to cover both the gel tray and electrodes
• Remove combs from the gel mould
• Immerse gel into the buffer-filled tank
• Add 2-3 µl gel loading buffer per sample into a microtitre plate wells. Bromophenol blue is used for larger sized nucleic acid and Orange G is used for smaller DNA products.
• Add appropriate amount (5-7 µl) of sample to the loading gel, mix and add into the gel wells.
• Place the lid of the gel tank into position and ensure the electrodes a correctly connected, switch on the power supply.
• Leave the gel to run at 100 Volts for approximately 60 to 90 min.
• Remove gel from tank.
• View gel under the Chemidox UV transillumination system.
Appendix D: Polymerase Chain reaction

**Principle**

PCR is a necessary application when making a large number of copies of a particular gene. The purpose for this amplified gene product is for further downstream reactions such as restrictions and sequencing. The technique occurs in several steps, requiring reagents such as a buffer, MgCl$_2$ (Salt), nucleotides, a polymerase enzyme capable of copying gene fragments, primers and the DNA serving as a template for amplification.

The steps include:

**Denaturation-**

The double strand DNA melt into single strand, this occurs usually at 94- 95 °C.

**Annealing-**

The primers attach to complementary nucleotides on the original single strand to form stable hydrogen bonds.

This step occurs at 54-65 °C, depending no the gene being amplified.

**Extension-**

The attached primers form a basis for the addition and the elongation of the copy strand. This step occurs at 72 °C, an ideal working temperature for the polymerase enzyme.
Procedure for amplifying a DNA fragment

For a 50 μl reaction volume:

- Into a clean 2 ml microfuge tube, add:

<table>
<thead>
<tr>
<th>Amount</th>
<th>Concentration</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.1 μl</td>
<td>10X</td>
<td>Taq Buffer</td>
</tr>
<tr>
<td>5 μl</td>
<td>1.5 mM</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>3 μl</td>
<td>0.2 mM of each dNTP</td>
<td>Nucleotide mix</td>
</tr>
<tr>
<td>0.6 μl</td>
<td>15-35 pmol*</td>
<td>Forward Primer</td>
</tr>
<tr>
<td>2 μl</td>
<td>15-35 pmol*</td>
<td>Reverse Primer</td>
</tr>
<tr>
<td>0.3 μl</td>
<td>10 U/ μl</td>
<td>Taq DNA Polymerase</td>
</tr>
</tbody>
</table>

*Note: Variable, depending to the gene and size of fragment being amplified.

- Vortex the master mix thoroughly and aliquot into 200μl thin-walled PCR tubes.
- Add 6μl DNA into PCR tubes
- Vortex the solution and spin down.
- Put the PCR tube into the allocated slots in the PCR –GeneAmp 9700 (Applied Biosystem-machine

For a 25 μl reaction volume:

- Into a clean 2 ml microfuge, add:

<table>
<thead>
<tr>
<th>Amount</th>
<th>Concentration</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.6 μl</td>
<td>10X</td>
<td>Taq Buffer</td>
</tr>
<tr>
<td>2.5 μl</td>
<td>1.5 mM</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>0.3 μl</td>
<td>0.2 mM of each dNTP</td>
<td>Nucleotide mix</td>
</tr>
<tr>
<td>1 μl</td>
<td>15-35 pmol*</td>
<td>Forward Primer</td>
</tr>
<tr>
<td>1 μl</td>
<td>15-35 pmol*</td>
<td>Reverse Primer</td>
</tr>
<tr>
<td>0.15 μl</td>
<td>10 U/ μl</td>
<td>Taq DNA Polymerase</td>
</tr>
</tbody>
</table>
PCR Cycling Conditions:

- Set the following conditions on the PCR machine:

  Initial Denaturation 94-95°C for 5 min

  20-35 cycles of:

  - Denaturation 94-95°C* for 30 seconds
  - Annealing 54-65°C* for 30-40* seconds
  - Elongation 72°C for 30-40* seconds
  - Final Elongation 72°C for 10 min
  - Storage 4°C till use

- *Note: the conditions are variable depending on the gene fragment being amplified.

- Verify amplification by subjecting PCR products to agarose gel electrophoresis for 90 at 100 Volts.
Appendix E: Purification of PCR Product from Agarose Gel

Principle
Despite numerous attempts at optimising PCR, there are occasions where undesired product will amplify and appear on Agarose gel. Should the desired band be visible, it can be excised from the Agarose gel and purified using a kit.

Procedure
- Excise the DNA fragment from the agarose gel with a scalpel.
- Weight the gel slice and add 3 volumes of Buffer QG to 1 volume of gel.
- Incubate at 50°C for 10 min and vortex the tube every 2-3 min during incubation
- Add 1 gel volume of isopropanol to the sample mix
- Place the spin column in a provided 2 ml collection tube
- Apply the solution to the QIAquick column and spin for 1 min, this will bind the DNA
- Discard the flow-through and add 0.5 ml Bugger QG to the QIAquick column and spin for 1 min.
- Discard the flow-through again and add 0.75 ml Buffer PF, to the QIAquick column and centrifuge for 1 min.
- Discard the flow-through and centrifuge at maximum speed for 1 min.
- Place the QIAquick column into a clean microfuge tube and add 50 μl Buffer EB to elute the DNA.
- Run 5 μl DNA on Agarose Gel Electrophoresis to verify DNA product.
Appendix F: Restriction Fragment Length Polymorphism

Principle

This technique uses the presence or absence of particular nucleotide sequence within a DNA fragment to its advantage. Detection of particular mutation is achieved by using endonucleases enzymes to cleave at specific recognition sites. Should a fragment contain a recognition site, the endonucleases enzyme will cleave and produce different length fragments. Organisms could then be compared on the basis of the number and lengths of DNA fragments produced.

Procedure for RFLP

- Perform the procedure on ice
- Into a clean 2 ml microfuge tube, add:

<table>
<thead>
<tr>
<th>Amount</th>
<th>Concentration</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-15µl</td>
<td></td>
<td>Nuclease-Free water</td>
</tr>
<tr>
<td>3µl</td>
<td>10X</td>
<td>Restriction enzyme buffer</td>
</tr>
<tr>
<td>2µl*</td>
<td>10 U/µl</td>
<td>Restriction enzyme</td>
</tr>
<tr>
<td>10-12µl</td>
<td>≈ 1µg DNA</td>
<td>PCR Product (Template)</td>
</tr>
</tbody>
</table>

- Mix gently and spin down for a few seconds
- Incubate sample at 37-65°C* for 1-16 hours*
• * Note: variable, depending on the restriction enzyme and the PCR product being restricted.

• Verify restriction by subjecting restriction sample to agarose gel electrophoresis for 120 min at 80V.

**Restriction Enzymes**

**Principle**

Restriction enzymes originate from bacteria which possess these types of enzyme to protect the bacterial cell from foreign invading DNA. Any DNA not recognised as ‘self’, is digested into smaller pieces by the restriction enzymes.

The functioning of any restriction is dependent on a specific DNA nucleotide sequence. Some enzymes recognize sequences 4 base pairs long, some 6 and still other 8 or more. The common feature of most enzyme recognition sites is that they are palindromic.

The restriction endonucleases functions by ‘scanning’ the length of a DNA molecule. Once it encounters a specific recognition sequence it will bond to the DNA and cut at each of the two sugar phosphate backbone of the helix, weakening the hydrogen bond and eventually breaking the DNA strands.

Cleavage of the DNA by the enzymes can produce two types of ends:

Sticky ends- the cut produced by the enzymes is staggered, and thus producing a single stranded ends. This end is termed sticky because it can bind to another complementary strand, even to another strand originating from another organism.

Blunt ends- this type of end has no single strand, thus requiring no complementarity.
Restriction enzyme nomenclature is based on its bacterial source, i.e. the first letter of the name come from the genus, the next two are from the species name for example *Bam HI* comes from *Bacillus Amylophilus* and *Sau 3AI* comes from *Staphylococcus Aureus*.

**Restriction Enzymes used for Genotyping**

- **Bsp120I**
  - Source: *Bacillus subtilis RFL120*
  - Recognition site: 5'...GGGCC C C...3'
    3'...C C C G G G...5'

- **PstI**
  - Source: *Providencia stuARVi*
  - Recognition site: 5'...CTGCAG...3'
    3'...GACGTC...S'

- **AvaII**
  - Source: *E. coli* that carries the cloned *eco471R* gene from *E.coli RFL47*
  - Recognition site: 5'...G G A C C C...3'
    3'...C C T G G...5'

- **NsiI**
  - Source: *Neisseria sicca*
  - Recognition site: 5'...ATGCA T...3'
    3'...T A C G T A...5'

- **SmaI**
  - Source: *Serratia marcescens*
  - Recognition site: 5'...C C C G G G...3'
    3'...G G G C C C...5'

- **BamHI**
  - Source: *Bacillus amyloliquefaciens H*
  - Recognition site: 5'...GGATCC...3'
    3'...CC TAGG...5'

- **Sau3AI**
  - Source: *Staphylococcus aureus 3A*
  - Recognition site: 5'...G A T C...3'
    3'...C T A G...5'

- **TaqI**
  - Source: *Thermus aquaticus YT-1*
  - Recognition site: 5'...TCG A...3'
    3'...AGCT...5'
MT
Source: *E. coli* strain that carries the *MspI* gene from *moraxella* species (TCC49670)
Recognition site: 5’… C C G G …3’
: 3’… G G C C .5’

*KpnI*
Source: *Klebsiella pneumoniae* OK8
Recognition site: 5’… G G T A C C …3’
: 3’… C C A T G G…5’

*BsrI*
Source: *Bacillus* species N
Recognition site: 5’… A C T G G N…3’
: 3’… T G A C C N…5’

*Bsp143I*
Source: *Bacillus* species RFL143
Recognition site: 5’… G A T C …3’
: 3’… C T A G…5’

*StyI*
Source: *Eschericia coli* RFL130
Recognition site: 5’… C C T T G G…3’
: 3’… G G A A C C…5’

T T
Appendix G: Liver Function Tests

The Kinetic UV test for the quantitative determination of AST is performed on the OLYMPUS analysers according to the manufacturer’s protocol.

A) Liver Function Test for Aspartate aminotransferase

Test Principle

The biochemical method is based on the recommendations of the “International Federation for Clinical Chemistry” (IFCC). The aspartate aminotransferase (AST) catalyses the transamination of aspartate and 2-oxoglutarate, Forming L-glutamate and oxalacetate. The addition of pyridoxal phosphate to the reaction mixture ensures maximum catalytic activity of AST. The oxalacetate is reduced to L-malate by malate dehydrogenase (MDH), while NADH is simultaneously converted to NAD\(^+\). The decrease in absorbance due to consumption of NADH is measured at 340 nm and is proportional to the AST activity in the sample.

Reaction Principle:

\[
\begin{align*}
\text{(AST)} & \\
2\text{-oxoglutarate} + \text{L-aspartate} & \rightarrow \text{L-glutamate} + \text{Oxalacetate} \\
\text{(MDH)} & \\
\text{Oxalacetate} + \text{NADH} + \text{H}^+ & \rightarrow \text{L-Malate} + \text{NAD}^+ 
\end{align*}
\]
B) Liver Function test for Lactate dehydrogenase

Test Principle

The biochemical method is based on the recommendations of the “International Federation for Clinical Chemistry” (IFCC). LDH catalyses the oxidation of lactate to pyruvate coupled with the reduction of NAD$^+$ to NADH. The increase of NADH is measured at 340nm and is directly proportional to the enzyme activity in the sample.

Reaction principle:

\[
(LDH) \quad \text{Lactate} + \text{NAD}^+ \rightarrow \text{Pyruvate} + \text{NADH} + \text{H}^+
\]

C) Liver Function test for Alanine aminotransferase

Test Principle

ALT transfers the amino group from alanine to 2-oxoglutarate to form pyruvate and glutarate. The addition of pyridoxal phosphate to the reaction mixture ensures maximum catalytic activity of ATL. The pyruvate enters a lactate dehydrogenase (LDH) catalysed reaction with NADH to produce Lactate and NAD$^+$. The decrease in absorbance due to consumption of NADH is measured at 340 nm and is proportional to the ALT activity in the sample. Endogenous pyruvate is removed during the incubation period.

Reaction Principle:

\[
(ALT) \quad 2\text{-oxoglutarate} + \text{L-Alanine} \rightarrow \text{L-Glutamate} + \text{Pyruvate}
\]

\[
(LDH) \quad \text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{Lactate} + \text{NAD}^+
\]
Pyruvate + NADH + H⁺ → L-lactate + NAD⁺

D) Liver Function Test for Alkaline Phosphatase

Test Principle

The biochemical method is based on the recommendations of the “International Federation for Clinical Chemistry” (IFCC). Alkaline phosphatases (ALP) activity is determined by measuring the rate to conversion of p-nitro-phenylphosphate (pNPP) to p-nitrophenol (pNP) in the presence of magnesium and zinc ions and of 2-amino-2-methyl-1-propanol (AMP) as phosphate acceptor at pH 10.4.

The rate of change in absorbance due to formation of pNP is measured bichromatically at 410/480 nm and is directly proportional to the ALP activity in the sample.

Reaction Principle:

\[
(ALP)
\]

\[
pNPP + AMP → pNP + AMP-PO_4^{3-}
\]

E) Liver Function Test for Total Bilirubin

Test Principle

The biochemical method is based on the recommendations of the “International Federation for Clinical Chemistry” (IFCC). A stabilised diazonium salt, 3,5-dichlorophenyldiazonium tetrafluoroborate (DPD), reacts with conjugated bilirubin and directly with unconjugated bilirubin in the presence of an accelerator to form azobilirubin. The absorbance at 540nm is proportional to the total bilirubin concentration. A separate sample blank is performed to reduce endogenous serum interference.
Reaction Principle:

\[
\text{(Caffeine)} \quad \text{Bilirubin} + \text{DPD} \rightarrow \text{Azobilirubin} \quad \text{Surfactant}
\]

F) Liver Function Tests for Gamma-glutamyltransferase

Test Principle

The biochemical method is based on the recommendations of the “International Federation for Clinical Chemistry” (IFCC). GGT catalyses the transfer to the gamma-glutamyl group from the substrate, gamma-glutamyl-3-carboxy-4-nitroanilide, to glycylglycine, yeiling 5-amino-2-nitrobenzoate. The change in absorbance at 410/480nm is due to the formation of 5-amino-2-benzoate and is directly proportional to the GGT activity in the sample.

Reaction Principle:

\[
\text{(γ-GT)} \quad \text{L-γ-Glutamyl-3-carboxy-4-nitroanilide} + \text{Glycylglycine} \rightarrow \text{L-γ-Glutamylglycylglycine} + 5\text{-amino-2-nitrobenzoate}
\]
## Division of AIDS Table for Grading Severity of Adult and Paediatric Adverse Events

<table>
<thead>
<tr>
<th>Liver enzyme</th>
<th>Normal range</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Bilirubin (Total)</td>
<td>2-26 umol/L</td>
<td>32.5 - 65</td>
<td>67.6 - 130</td>
<td>132.6 - 260</td>
<td>&gt;260</td>
</tr>
<tr>
<td>S-Bilirubin conjugated</td>
<td>1-7 umol/L</td>
<td>8.75 - 17.5</td>
<td>18.2 - 35</td>
<td>35.7 - 70</td>
<td>&gt;70</td>
</tr>
<tr>
<td>S-g-Glutamyl transferase (GGT)</td>
<td>0-44 IU/L</td>
<td>55 - 110</td>
<td>114.4 - 220</td>
<td>224.4 - 440</td>
<td>&gt;440</td>
</tr>
<tr>
<td>Alkaline Phosphatase (ALP)</td>
<td>53-128 IU/L</td>
<td>160 - 320</td>
<td>332.8 - 640</td>
<td>652.8 - 1280</td>
<td>&gt;1280</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT)</td>
<td>&lt;35 IU/L</td>
<td>43.75 - 87.5</td>
<td>91 - 175</td>
<td>178.5 - 350</td>
<td>&gt;350</td>
</tr>
<tr>
<td>AST</td>
<td>13-35 IU/L</td>
<td>43.75 - 87.5</td>
<td>91 - 175</td>
<td>178.5 - 350</td>
<td>&gt;350</td>
</tr>
<tr>
<td>S-Lactate Dehydrogenase (LDH)</td>
<td>120-230 IU/L</td>
<td>287.5 - 575</td>
<td>598 - 1150</td>
<td>1173 - 2300</td>
<td>2300</td>
</tr>
</tbody>
</table>

Table adapted from: http://rcc.techres.com/DAIDS%20RCC%20Forms/ToxicityTables_DAIDS_AE_GradingTable_FinalDec2004.pdf
Division of AIDS Estimation for Grading Severity of Adult Adverse Events

<table>
<thead>
<tr>
<th>Grade</th>
<th>Mild</th>
<th>Transient or mild discomfort; no limitation in activity; no medical intervention/ therapy required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 2</td>
<td>Moderate</td>
<td>Mild to moderate limitation in activity- some assistance may be needed; no or minimal medical intervention/ therapy</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Severe</td>
<td>Marked limitation in activity, some assistance usually required; medical intervention/ therapy required, hospitalization possible</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Life-threatening</td>
<td>Extreme limitation in activity, significant assistance required; significant medical intervention/ therapy required, hospitalization or hospice care probable</td>
</tr>
</tbody>
</table>

http://rcc.tech-res.com/DAIDS%20RCC%20Forms/ToxicityTables_Adult_TRP_v01a.pdf
Appendix H: Determination of Viral Load

Purpose

Use of the Roche Amplicor HIV 1 Monitor test, version 1.5 for quantitative determination of HIV.

References:

a.) Package insert
b.) Amplicor Operator’s Manual
c.) Ampliprep Operator’s Manual

Suitable Specimens:

- Plasma collected in ACD or EDTA tubes only.
- Other body fluids are suitable, but their viral load is generally low compared to plasma.
- Blood should be stored for no longer than 6 hours before plasma is separated and stored at −70°C.
- If a specimen is delayed before the plasma can be separated it may be stored between 2 and 8°C for no more than 18 hours before separation.
- ACD specimens will result in viral load measurements approximately 15% lower than EDTA specimens refer to XI. Method Limitations.

Unsuitable Specimens:

- Specimens older than 24 hours
- Grossly haemolysed specimens
- Test requires
  - 200 μL of plasma for the Standard test
  - 500μL of plasma for the Ultra-sensitive test.
Procedure

- Refer to the package insert.

- Two specimen preparation procedures are illustrated.
  a. In the Standard specimen preparation procedure, HIV-1 RNA is isolated directly from plasma by lysis of virus particles with a chaotropic agent, followed by precipitation of RNA with alcohol.
    i. The reportable range is 400 to 750,000 copies/mL
  b. With the UltraSensitive specimen preparation procedure, HIV-1 viral particles in body fluids are concentrated by a high speed centrifugation, followed by lysis of the virus particles with a chaotropic agent and precipitation of the HIV-1 RNA with alcohol.
    i. The reportable range is 50 to 100,000 copies/mL

- Use the standard method for patients not on treatment (especially in the acute phase of HIV infection) where results are expected to be very high

- Use the UltraSensitive procedure for body fluids with low viral loads and for samples from patients on treatment when the viral load is expected to be undetectable. This ultrasensitive preparation utilises a high speed centrifugation of the plasma to concentrate the virus before extraction.
Appendix I: Determination of CD4⁺ T-cell count

Principle

MultiTest reagents employ fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population to reduce contamination of unlysed or nucleated red blood cells in the gate.

When whole blood is added to the reagent, the fluorochrome-labelled antibodies in the reagent bind specifically to the leucocyte surface antigens. During acquisition cells pass in front of the laser beam and scatter the light, with resultant fluorescence of stained cells. The scatter- and fluorescence-signals indicate cell size, internal complexity and fluorescence intensity.

A known volume of sample is stained directly in the TruCOUNT tube. The lyophilised bead pellet in the tube dissolves, releasing a known number of fluorescence beads. During analysis, the absolute number (cells/µl) of positive cells in the sample can be determined by comparing cellular events to bead events. Events are acquired and analysed using MultiSet™ software and absolute counts are determined automatically.

Purpose

CD4 and CD8 determinations using the MultiTest reagents and TruCOUNT tubes on the Becton Dickinson FACSCalibur Flow cytometer

Procedure

Specimen Collection, Transport and Handling

- Peripheral blood in EDTA anticoagulated vacutainer tubes.
• Must be maintained at room temperature (20 - 25°C) during transportation and storage (i.e. Do not freeze or expose to very high temperatures).

• The blood must be stained within 48 hours of draw and analysed within 6 hours of staining. If samples are stained within 24 hours of collection, they can be analysed up to 24 hours. Samples stained after these time-points may result in inaccurate counts.

• Refer to Table 1 for rejection criteria

Table 1.

<table>
<thead>
<tr>
<th>ACCEPTABLE SPECIMENS</th>
<th>UNACCEPTABLE SPECIMENS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh specimen</td>
<td>Specimen older than 48 hours</td>
</tr>
<tr>
<td>Full draw specimen in EDTA</td>
<td>Low volume specimen (&lt; 2.5mL)</td>
</tr>
<tr>
<td>Labelled specimen</td>
<td>Clotted or exhibiting fibrin clots</td>
</tr>
<tr>
<td></td>
<td>Haemolysed specimens ((gross)</td>
</tr>
<tr>
<td></td>
<td>Specimen exposed to temperature extremes</td>
</tr>
<tr>
<td></td>
<td>Unlabelled specimen</td>
</tr>
</tbody>
</table>

Specimen and Reagent Storage

• Maintain the specimens at an ambient temperature of approximately 23°C (Air-conditioning) until testing.

• Store the TruCOUNT tubes in their original pouch at 2-25°C and the monoclonal antibodies at 2-8°C.
• Do not use after expiration date shown on the label. Open the pouch only after it has reached room temperature and carefully reseal the pouch immediately after removing the tube.

• If the desiccant in the pouch has turned from blue to lavender, discard the remaining tubes.

Precautions

• Do not use a reagent if you observe any change in appearance.

• Precipitation or discolouration indicates instability or deterioration.

• The antibody reagent contains sodium azide, handle with care refer to SOPs SHAZ006 and SGEN008.

• Bead count varies according to the lot of TruCOUNT tubes. It is critical to use the bead count shown on the current Lot/Batch of TruCount tubes when entering this value in the software.

• Do not mix multiple lots of tubes in the same assay.