CYTOCHROME P450 POLYMORPHISMS: RELEVANCE IN TWO SOUTH AFRICAN DISEASE POPULATIONS

BY

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

ABSTRACT

With knowledge of the human genome increasing constantly we are continually faced with new and potentially groundbreaking methods for managing, treating and/or identifying diseases and predisposition to diseases and conditions at a genetic level. The human cytochrome P450 (CYP) super-family of genes code for enzymes that can participate in metabolism of drugs and foreign chemicals and in steroid synthesis and metabolism. Mutations in these genes may contribute to clinically relevant diseases. In this study, the effects of mutations within four CYP genes were evaluated in two South African disease groups - variegate porphyria and breast cancer.

Variegate porphyria (VP) has an unusually high incidence in South Africa due to the R59W founder mutation in the protoporphyrinogen oxidase (*PPOX*) gene that causes a disruption in the haem biosynthetic pathway. VP presents with variable clinical symptoms and has a relatively low penetrance. It is expected that environmental factors and modifier genes play a role in the clinical expression of VP. CYP genes are implicated as candidate modifier genes for the expression of VP due to the function they have in metabolising many drugs contraindicated in porphyria patients, and the necessity of haem binding to the apoprotein to produce a functional CYP enzyme. This is the first study to investigate CYPs as possible modifier genes for VP clinical expression. Six CYP polymorphisms (CYP1A1*m1*, CYP1A1*m2*, CYP1A2 – 734 C>A, CYP1B1 8372 A>C, CYP2D6*3, CYP2D6*4), associated with four CYP loci, were genotyped in a VP population and a suitable control population. The results observed are suggestive of CYP1A1*m1* and CYP1B1 playing a role as modifiers for the clinical expression of VP as they were significantly associated ($P<0.05$) with the presence of VP related symptoms.

Breast cancer is one of the leading causes of death in women due to cancer. It is believed that breast cancer may be the result of co-participation between common DNA alterations and environmental exposures. Polymorphisms in enzymes involved with the metabolism of environmental exposures, such as the cytochrome P450 enzymes, are therefore expected to play an aetiological role in breast cancer. Two groups of South African breast cancer patients (Caucasian and of mixed ancestry) and

population-matched controls were genotyped for four CYP polymorphisms (CYP1A1*m1*, CYP1A1*m2*, CYP1A2 –734 C>A and CYP1B1 8372 A>C). This represents the first investigation of the potential role of CYPs as breast cancer risk modifiers in the two South African populations. Significant differences were observed ($P<0.003$) in genotype and allele frequencies between the breast cancer patients and controls for the CYP1B1 8372 A>C polymorphism in the population of mixed ancestry. Vast differences in allele frequencies were also observed between the two groups of breast cancer populations. These results emphasize the importance of population-based risk assessment when genetic testing and counselling for complex disease susceptibility is offered.

The results of this study provide the first evidence suggesting a role for CYPs in modifying the clinical expression of VP and in acting as risk factors for developing breast cancer in a South African population.

OPSOMMING

Met die konstante toename van kennis oor die mensgenoom kom ons voortdurend te staan voor nuwe metodes vir die beheer, behandeling en/of identifikasie van siektes en vatbaarheid vir siektes op 'n genetiese vlak. Die mens sitochroom P450 geen-superfamilie kodeer vir ensieme betrokke in die metabolisme van medisyne en ander chemiese stowwe en steroïed-sintese en -metabolisme. Mutasies in hierdie gene kan 'n bydrae lewer tot kliniese relevante siektes. In hierdie studie is die effek van mutasies in vier sitochroom gene bestudeer in twee Suid-Afrikaanse siekte groepe, variegate porfirie en borskanker.

Variegate porfirie (VP) het 'n besonderse hoë frekwensie in Suid-Afrika as gevolg van die R59W stigter-mutasie in die protoporfirinogeen oksidase (*PPOX*) geen. Hierdie mutasie lei tot 'n versteuring in die heem biosintese padweg. VP presenteer met variërende kliniese simptome en het 'n betreklike lae penetrasie. Daar word vermoed dat omgewingsfaktore en kandidaat modifiserende gene 'n rol speel in die kliniese beeld van VP. Sitochroom P450 gene is geïdentifiseer as kandidaat modifiserende gene as gevolg van hulle rol in die metabolisme van verbode medikasie vir porfirie pasiënte, asook die binding van heem aan die apoproteïen wat noodsaaklik is vir die produksie van funksionele sitochroom P450 ensiem. Hierdie is die eerste studie wat sitochroom P450 gene as moontlike modifiserende gene vir die kliniese uitdrukking van VP ondersoek. Ses sitochroom P450 polimorfismes (CYP1A1*m1*, CYP1A1*m2*, CYP1A2 –734 C>A, CYP1B1 8372 A>C, CYP2D6*3, CYP2D6*4) is ondersoek in beide 'n VP populasie en 'n geskikte kontrole populasie. Die resultate suggereer 'n rol vir CYP1A1*m1* en CYP1B1 in die modifisering van die kliniese uitdrukking van VP aangesien hulle betekenisvolle assosiasie ($P<0.05$) met die voorkoms van VP-verwante simptome getoon het.

Borskanker is een van die vernaamste oorsake van kankersterftes onder vroue. Borskanker is waarskynlik die resultaat van interaksie tussen algemene DNS-veranderinge en omgewings-blootstelling. Daar word dus verwag dat polimorfismes in ensieme betrokke by die metabolisme van omgewingselemente, soos byvoorbeeld die sitochroom P450 ensieme, 'n etiolgiese rol in borskanker sal speel. Die genotipes

van twee groepe Suid-Afrikaanse borskanker lyers (Kaukasiërs en van gemengde herkoms) en populasie-passende kontroles is bepaal vir vier sitochroom P450 polimorfismes (CYP1A1*m1*, CYP1A1*m2*, CYP1A2 –734 C>A, CYP1B1 8372 A>C). Hierdie studie verteenwordig die eerste ondersoek na die potensiële rol van sitochroom P450s as risiko-modifiserende faktore vir borskanker in die twee populasies. Betekenisvolle verskille ($P<0.003$) in genotipe en allele frekwensies is waargeneem tussen die borskanker lyers en kontroles vir die CYP1B1 8372 A>C polimorfisme in die gemengde herkoms populasie. Beduidende verskille in alleel frekwensies is ook waargeneem tussen die twee borskanker populasies. Hierdie resultate beklemtoon die belangrikheid van populasie gebaseerde risiko-beraming wanneer genetiese toetse en voorligting vir komplekse siekte-vatbaarheid aangebied word.

Die resultate van hierdie studie bied die eerste getuienis dat sitochroom P450s ‘n rol kan speel in die modifisering van die kliniese beeld van VP en ook kan optree as as risiko faktore vir die ontwikkeling van borskanker in ‘n Suid-Afrikaanse populasie.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations are listed in alphabetical order.

α	Alpha
A	Adenine (in DNA sequence)
AA	Acute attacks
AA+AASL	Total acute attacks
AASL	Acute attacks in combination with skin lesions
ABC	Adenosine triphosphate-binding-cassette
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropic hormone
ADR	Adverse drug reaction
$AgNO_3$	Silver nitrate
AIP	Acute intermittent porphyria
ALA	δ-aminolevulinic acid
Asym	Asymptomatic
ATP	Adenosine triphosphate
β	Beta
bp	Base pair
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
C	Cytosine (in DNA sequence)
°C	Degrees Celsius
cAMP	3', 5'-cyclic adenosine monophosphate
CE	Catechol estrogen
$C_{19}H_{10}Br_4O_5S$	Bromophenol blue
CH_3COOH	Acetic acid
$C_{31}H_{28}N_2Na_4O_{13}S$	Xylene cyanol
$(CH_2OH)_3CNH_2$-Cl	Tris(hydroxymethyl)aminomethane
CNS	Central nervous system

cSNP	Coding single nucleotide polymorphism
CYP	Cytochrome P450
Del	Deletion
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPD	Dihydropyrimidine dehydrogenase
EDTA	Ethylenediamine tetra-acetic acid ($C_{10}H_{16}N_2O_8$)
EtBr	Ethidium bromide
EtOH	Ethanol
FMO	Flavin-containing monooxygenase
G	Guanine (in DNA sequence)
GST	Glutathione *S*-transferase
GSTA	Glutathione *S*-transferase-A, class α
GSTM	Glutathione *S*-transferase-M, class μ
GSTM1	Glutathione *S*-transferase M1
GSTP	Glutathione *S*-transferase-P, class π
GSTP1	Glutathione *S*-transferase-P1
GSTT	Glutathione *S*-transferase-T, class θ
H^+	Hydrogen ion
H_3BO_3	Boric acid
HCHO	Formaldehyde
Hex SSCP	Heteroduplex single strand conformation polymorphism
HFE	Haemochromatosis gene
H_2NCHO	Formamide
$HOCH_2CHOHCH_2OH$	Glycerol
Ile	Isoleucine (amino acid)
Inc	Incorporated

iSNP	Intergenic single nucleotide polymorphism
KCl	Potassium chloride
$KHCO_3$	Potassium hydrogen carbonate
KH_2PO_4	Potassium dihyrdrogen orthophosphate
µ	Mu
µg/ml	Micrograms per millilitre
µl	Microlitre
µM	Micromolar
M	Molar (moles per litre)
MDR1	Multidrug resistance P-glycoprotein
$MgCl_2$	Magnesium chloride
mg/ml	Milligrams per millilitre
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance protein
MRP1	Multidrug resistance-associated protein
n	Sample size
Na^+	Sodium ion
NaCl	Sodium chloride
Na_2HPO_4	Di-sodium hydrogen orthophosphate anhydrous
NaOH	Sodium hydroxide
NAT	*N-acetyltransferase*
NAT-1	*N*-acetyltransferase 1
NAT-2	*N*-acetyltransferase 2
NATP	*N*-acetyltransferase pseudogene
ng	Nanogram
ng/µl	Nanograms per microlitre
NH_4Cl	Ammonium chloride
$(NH_2)_2CO$	Urea

OH	Hydroxyl group
OMIM	Online Mendelian Inheritance in Man
%	Percentage
P	Probability
PAA	Polyacrylamide
PAH	Polycyclic aromatic hydrocarbon
PBG	Porphobilinogen
PBS	Phosphate buffered saline solution
PCR	Polymerase chain reaction
PCT	Porphyria cutanea tarda
P-gp	P-glycoprotein
PPAR	Perioxisome proliferator activator receptor
PPOX	Protoporphyrinogen oxidase
pSNP	Perigenic single nucleotide polymorphism
π	Pi
®	Registered trademark
R	Arginine (amino acid)
RFLP	Restriction fragment length polymorphism
rpm	Rotations per minute
RSA	Republic of South Africa
rSNP	Random single nucleotide polymorphism
RXR	Retinoid X receptor
SAMF	South African Medicines Formularly
SDS	Sodium dodecyl sulfate
SISA	Simple interactive statistical analysis
SL	Skin lesions
SL+AASL	Total skin symptoms
SNP	Single nucleotide polymorphism
SSCP	Single strand conformation polymorphism

θ	Theta
T	Thymine (in DNA sequence)
T_A	Annealing temperature (of primers)
TBE	Tris borate-EDTA buffer
TFPGA	Tools for Population Genetic Analysis
™	Trademark
TMA	Tertiary amine trimethylamine
TPMT	Thiopurinemethyl transferase
UDPGT	Uridine diphosphate glucuronosyltransferase
UROD	Uroporpyrinogen decarboxylase
USA	United States of America
UTR	Untranslated region
UV	Ultraviolet
V	Volts
Val	Valine (amino acid)
VP	Variegate porphyria
v/v	Volume per volume
W	Tryptophan (amino acid)
WHO	World Health Organisation
w/v	Weight per volume

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CHAPTER ONE

INTRODUCTION

The multitude of current and envisioned research developments in the fields of genetic information and genetic technologies possess the ability to fundamentally redefine medicine within many of our lifetimes. Major transformations in many aspects of healthcare ranging from diagnostics, disease management and treatment options will be a direct/indirect result of breakthroughs in genetics. Change such as this is inevitable, but this change will not occur over night, rather it will be gradual and will take on multiple forms.

With knowledge of the human genome increasing constantly we are continually faced with new and potentially groundbreaking methods for managing, treating and/or identifying diseases and predisposition to diseases and conditions at a genetic level. Some of these methods have already been realised as seen with genetic tests for predisposition, carrier status determination and diagnosis of a multitude of diseases including, variegate porphyria (protoporphyringen oxidase gene (*PPOX*)) and breast cancer (*BRCA1* and *BRCA2*). Information generated from the human genome about genes and the resulting technologies are moving healthcare into new territory. This forward movement will see demands of the public increasing for access to new tests, drugs and treatment, resulting in growth of public expectations from medicine. This will in turn impact on research efforts in genetics, resulting in the introduction of new interventions, diagnostics and treatments into direct healthcare delivery.

Genetics has played a role in medicine for a long time, and has been acknowledged for the positive contributions it has made. The ability genetic tests hold for early diagnosis, carrier status determination and predisposition of diseases opens the door to lifestyle changes that may possess the ability to alter the course of the disease and reduce its onset hereby improving health. Genetic tests are also advancing treatments and other health interventions. All this said, one must not loose sight of the relative role genetics plays in disease management. A risk associated with progress in

genetics is the tendency for society to attribute an almost mystical power to genetics, and consequently forget the important role environmental factors including housing, nutrition and employment play in shaping health.

The pharmaceutical industry is an example of an area where genetics and environmental factors are encountered together. This industry has evolved from chemically-driven production to genotype-driven analysis of patient subpopulations (Miller, 1998). It makes use of advances in genetic technologies that allow for tailor made treatments that are specific to disease subtypes and individuals. The availability of new drugs, the identification of alternate drugs, and the adjustment of drug dosage all aim to reduce the risk of adverse drug reactions. The identification of further drug targets allows for the development of additional specific drug therapies that may hold more benefits for the patients. Developments such as these allow for better informed, cost-effective disease management practices. Genetic research therefore, continually helps shape the development of assessment tools allowing increasingly more effective treatments that are designed to be genetically compatible with the person that is in need of them.

New techniques, diagnostics and treatments bring hope, but are also burdened with many challenges, some unique to the field of genetics. It is estimated that the birth incidence of infants with trivial or easily correctable congenital disorders is about 25-60/1000. The birth incidence of severe congenital disorders that cause early death or life-long chronic disease ranges from 14/1000 in developed countries to 43/1000 in developing countries (WHO, Human Genetics Programme, 2002). It is estimated that 15% of all cancers have an inherited susceptibility (Schneider, 1994). It is therefore highly likely that a significant proportion of the South African population will experience a disease with some form of genetic component during their lifetime. Genetic research and technology therefore has the potential to improve healthcare in a large fraction of the South African population. The network of genes that govern drug responses in humans will have to be well defined to enable more accurately optimised drug therapy based on each patient's ability to metabolise, transport and respond to medications.

To assist in fulfilling the promises of pharmacogenomics clinical and epidemiological studies are needed to assess how drug response varies among individuals with different genotypes, the prevalence of the genotypes in the population and relevant subpopulations and the degree of interaction between environmental and genetic factors in influencing drug response. The creation of population-based databases providing information necessary for the successful application of pharmacogenomics in the practice of medicine and public health is essential.

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CHAPTER TWO

LITERATURE REVIEW

2.1 PHARMACOGENETICS/PHARMACOGENOMICS

Pharmacogenetics is the study of variability in drug response due to heredity, and includes research of genetic polymorphism in genes encoding drug transporters, drug metabolising enzymes and drug receptors. It encompasses a combination of the fields of genetics, pharmacology and biochemistry. Pharmacogenomics on the other hand is a broader, more recent term that includes the newer sciences of molecular biology, genomics, bioinformatics and their associated technologies (Norton, 2001). Pharmacogenomics focuses on novel drug development, as disease genes become known. The purpose of this field of research is to understand the underlying reasons for differential human response to xenobiotics, to reduce the advent of adverse drug reactions and to provide individualised drug therapy. The terms pharmacogenetics and pharmacogenomics are often used inter-changeably. Pharmacokinetics describes the time course of a drug's actions, i.e. the time to onset and the duration of the drug's effects (Julien, 1996). Molecular pharmacogenetics started with the cloning and characterisation of cytochrome P450 2D6 (CYP2D6). Molecular pharmacogenetics now encompasses the study of many other human genes including genes coding for drug receptors, drug metabolising enzymes and drug transporters.

Early in the 1950s researchers realised that some adverse drug reactions (ADRs) could be caused by genetically determined variations in enzyme activity. The occurrence of ADRs were the clinical events that revealed to researchers genetic variants of drug metabolising enzymes or drug targets (Meyer, 2000). In this way the role of genetic polymorphisms in drug response were accidentally discovered, by observing that some patients and/or volunteers experienced unpleasant reactions when administered standard doses of a drug.

Genetic Polymorphism

A genetic polymorphism exists when an allelic variant (the alteration must be stable within the population) occurs within a human population at a frequency of 1% or more. The number of single nucleotide polymorphisms (SNPs) present in the human genome is estimated to be approximately three million (Roses, 2002). The majority of these however, have no function and are located between genes, so called intergenic or random SNPs (iSNPs/rSNPs). Intergenic SNPs are most likely the result of 4-fold degenerate sites (Nebert, 1999). Single nucleotide polymorphisms present in introns and upstream regulatory regions (non-coding regions) are perigenic SNPs (pSNPs). Perigenic SNPs also include silent codon mutations and changes in the noncoding regions of the mRNA and at least 100 bp 3' of the last exon. Those present in coding regions are so called coding SNPs (cSNPs). Coding SNPs may cause an alteration in the amino acid sequence of the encoded protein that may or may not result in an alteration in the function of the protein. A genetic polymorphism is clinically relevant when it meets with certain drug-related criteria. These criteria include narrow therapeutic range or large inter-individual variation in kinetics or suspicion of overdose (Meyer, 2000).

This brings up the concept of wild-type (normal) allele designation. When considering the obvious ethnic differences in drug metabolising gene polymorphisms, the designation of wild-type seems arbitrary (Nebert, 1999) and the definition of wildtype becomes somewhat flawed when one considers how allelic frequencies differ between different ethnic populations (Yan *et al.,* 2000).

Response to Medication

Mutations found in drug metabolising enzyme genes, drug receptor genes and drug transporter genes and the effect of the mutations on the protein product varies from having no effect, to decreased activity or absence of enzyme. Gene duplications result in increased enzyme/enzyme activity (Ingelman-Sundberg, 2001). Mutations in drug metabolising enzymes allow one to classify patients into one of four groups (patients have to be classified for each gene that is genotyped): extensive metabolisers who are homozygous or heterozygous for the wild type allele, intermediate metabolisers who have two mutations resulting in decreased enzyme

activity, poor metabolisers who carry two loss-of-function mutations and ultra rapid metabolisers who have duplicated or multi-duplicated copies of the gene (Meyer, 2000). The presence of defective alleles results in adverse drug reactions in patients while a lack of drug efficacy is observed due to ultra rapid metabolism. Table 2.01 highlights the differences observed in drug metabolism between poor and ultra rapid metabolisers.

Induction and inhibition of drug transport/metabolism is influenced by environmental factors while aging is known to decrease capacity for drug metabolism and capacity to induce drug metabolising enzymes. It is very difficult to disentangle the contribution of environmental and genetic factors in an individual patient.

Table 2.01: The clinical importance of the different phenotypes with respect to drug metabolism

Poor Metabolisers	Ultra Rapid Metabolisers
• Diminished first pass metabolism, increased bioavailability, exaggerated response	• Lack of therapeutic effect at standard doses
• Diminished drug elimination, side effects or toxic effects	• Too much of the active metabolite
• The therapeutic metabolite is not formed, loss of therapeutic efficacy	• Explanation for suspected bad compliance

(Ingelman-Sundberg, 1998)

Although there are many mutations present within the genes involved with drug transport, drug metabolism and drug receptors, only a few may prove to be common. When analysing an individual's response to medication, one has to take into account that many gene products are involved in drug disposition and drug action. One therefore has to take primary genes, modifier genes and environmental factors into consideration. Differences in allele frequencies and in functional defects are observed for different ethnic groups (Johansson *et al.*, 1994, Ingelman-Sundberg *et al.*, 1999, Evans and Johnson, 2001, Ingelman-Sundberg, 2001). This ethnic diversity (gene geography) suggests that one needs to take ethnic origin into account as well when

performing pharmacogenomic studies and when initiating drug therapy. It has been estimated that 85% of a patients response to a drug is due to genetics (Anonymous, 1998) and that a major cause of ADRs is an individuals genetic predisposition (Spear *et al.*, 2001).

Evans and Relling (1999) have reviewed many of the genes that show association with a specific response to a drug. These include genes encoding proteins that are involved with drug absorption, distribution, metabolism and elimination, genes encoding drug targets and some genes whose relation to drugs are yet unknown. Their findings indicate that these genes demonstrate polymorphism and that the major allele encodes the normal protein while the mutant alleles encode proteins with varied function. The function of the "mutant" protein is usually just reduced or absent however, it has been shown that increased function may also result. Altered protein function is very rare.

2.1.1 Application of Pharmacogenomics

Pharmacogenomics has the potential to revolutionise drug therapy by facilitating both choice and dosage of drug and the development of superior and safer therapies, as summarised in Table 2.02. As population specific data regarding the structure of genes and regulatory pathways that may cause altered response is generated by pharmacogenomic research, improvement of diagnosis and clinical care will be promoted (Weber, 2001). The advantage of pharmacogenomic testing is simply that a single DNA test performed once in a lifetime has the potential of identifying predisposition of patients to at least the extreme phenotypes. The most significant application of pharmacogenomics lies in the development of sensitive, specific diagnostic tests for the identification of specific genetic changes responsible for genetic diversity of human drug response (Norton, 2001, Weber, 2001).

The development of these diagnostic tests has been made possible by the vast array of research into novel molecular genetic techniques. The development of analytical techniques capable of increasing the speed of sequencing, mapping and human gene identification have allowed large amounts of data to be collected from relatively few experiments. These techniques are being used in developmental and physiological

pathway elucidation, and for tracking perturbations in gene activities and patterns of gene expression in cancer and other pathological conditions (Lockhart and Winzeler, 2000, Mir and Southern, 2000). Genetic tests have proved to be formidable forces in areas of disease diagnosis, diagnosis confirmation and genetic disease predisposition identification. Genetic tests are unique in that they are able to confirm the existence of a disease or predisposition to a disease in asymptomatic individuals. Various degrees of accuracy are observed between the different tests. At present there are at least 877 genetic tests available internationally for genetic disorders in adults, children and fetuses (Ontario Provincial Advisory Committee, 2001). The number of genetic tests developed to detect genetic disorders is expected to increase dramatically in the next few years with the vast amount of gene and single nucleotide polymorphisms being identified (Oak Ridge National Laboratory, 2001). Molecular genetic techniques have also found application in screening populations for polymorphisms suspected of playing a role in drug susceptibility and for screening drug candidates for new therapies (Weber, 2001).

Table 2.02: The Scope and Anticipated Benefits of Pharmacogenomics

Scope	Benefits
• Identification and characterisation of candidate genes and polymorphisms	• Advanced screening for diseases • Improvements in drug discovery and approval
• Correlation of polymorphism with therapy, clinical outcomes and drug effects	• Superior, safer drugs • Improved vaccines
• Development of molecular genetic tests for drug selection and for predicting drug response	• More powerful, individualised drugs • Quicker response
• Development of molecular genetic tests for dosing based on genotype or gene expression	• More accurate drug dosing • Minimising adverse drug reactions
OUTCOME: Decreased cost of health care	

(Norton, 2001, Knowledgene, 2001)

Laboratory models have the potential to be very informative regarding pharmacological and toxicological consequences of human conditions that we are unable to study in human models for ethical and/or methodological reasons. Development of inbred mouse strains, as well as recombinant, congenic and recombinant-congenic inbred mouse strains have all aided research of this nature. Mouse models have greatly increased our knowledge and understanding of metabolic, genetic, developmental, toxicological and molecular genetic aspects of human conditions (Goodman, 2002, Botta *et al.*, 2001, Calvisi *et al.*, 2001, Aronson, 1999, Licastro *et al.*, 1999).

Another application of pharmacogenomics is gene therapy which targets specific genetic mutations with the aim of correcting or deleting them. Gene therapy differs from conventional therapy in that it aims to "treat" the DNA underlying the problem rather than the symptoms caused by the disease. It is currently thought that gene therapy has the ability to successfully treat monogenic disorders rather than polygenic disorders. In monogenic disorders identification of targets for treatment is simpler than target identification in diseases caused by the interaction of many genetic factors. Somatic cell line gene therapy is successfully in progress (Mackesen *et al.*, 1997, Ontario Provincial Advisory Committee, 2001). Research into similar therapy for germ cell lines is underway - implications for future generations need to be carefully assessed. Gene therapy holds the promise for treating diseases that cause great pain and suffering for the patients that conventional therapies are unable to treat. Gene therapy may also have the ability to alter the chance that a person will develop a disease (Science Daily, 2001).

The need for high capacity, high throughput tests for enhancing pharmacogenomic research led to the use of and increased demand for the DNA microarray. This technique exploits the sequence complementarity of the two strands forming the DNA duplex (Southern *et al.*, 1999). DNA sampling, genetic research and the field of information technology fused to enhance the development of a DNA chip, which uses microchip technology to accelerate genetics studies. DNA chip technology enables the monitoring of therapy effectiveness and the investigation of complex interactions, dependencies and information that flow between genes (Debouck and Goodfellow,

1999). The number of adverse drug reactions could be greatly reduced and the efficacy of antibiotic prescription greatly increased as DNA chip technology is integrated into pharmacogenetics (Ontario Provincial Advisory Committee, 2001).

By increasing our knowledge of the effects different drugs have on gene expression throughout the entire genome, insufficient and adverse drug reactions can be greatly reduced by pharmacogenomics. If even partial fulfilment of the promise of pharmacogenomics is realised, it will represent progress of a very significant nature.

2.1.2 Adverse Drug Reactions and Effectiveness of Drug Therapy

Adverse drug reactions have major implications for human health and for healthcare. Patients may suffer adverse drug reactions for various reasons, including misdosing, drug-drug interactions, drug allergies, medication errors, the patient's age and ethnicity, renal and liver function, other disease factors and lifestyle variables such as smoking and alcohol consumption. Hurwitz (1969) found, in a pioneering survey, that the incidence of adverse drug reactions in patients admitted to an acute care hospital, more than tripled in patients older than 70 years of age. Specific drug metaboliser phenotypes have been linked to several adverse drug reactions (Evans and Johnson, 2001). These inherited factors affect the kinetics and dynamics of many drugs as increased or decreased metabolism has the ability to change the concentrations of the drug as well as its active, inactive or toxic metabolites (Meyer, 2000). Genetic variations also have the potential to produce unexpected drug effects or alter the clinical response and frequency of side effects if the polymorphism is located in a gene encoding a drug target (Meyer, 2000). Determining the extent of drug dose alteration for poor or ultra rapid metabolisers is a product of the quantitative role of the effect of the drug metabolising enzyme and the drug uptake mechanism in the overall kinetics of a drug and the agent's therapeutic range (Meyer, 2000). Six different types of ADRs have been described to date (refer to Table 2.03) (University of Cape Town, 2002).

In 1994, over one and a half million hospital admissions in the United States of America were due to adverse drug reactions (Spear *et al.*, 2001). As many as two million hospitalised patients in the United States of America experience adverse drug

reactions per annum (Norton, 2001). The occurrence of fatal ADRs to medications that are beneficial to others results in the death of approximately one hundred thousand inpatients per annum in hospitals around the United States of America (Lazarou, 1998, Meyer, 2000, Spear *et al.*, 2001). This ranks ADRs as the forth to sixth leading cause of death in hospital inpatients in the USA (Nebert, 1999, Meyer, 2000, Spear *et al.*, 2001). Although similar data regarding ADRs in South African hospitals is not currently available, there is nothing to suggest that the situation is any different here.

Table 2.03: The six different types of adverse drug reactions described

Type	Description
A	Augmented (dose related), e.g. hypotension with a beta blocker
B	Bizzare (non-dose related), e.g. anaphylaxis after penicillin injection
C	Continuous (long term), e.g. osteoporosis with oral steroids
D	Delayed (lag time), e.g. teratogenic effect with anti-convulsants
E	Ending of use (withdrawal), e.g. withdrawal syndrome with benzodiazepines
F	Failure of efficacy (no response), e.g. resistance to antibiotics

(University of Cape Town, 2002)

2.2 DRUG TRANSPORTERS, DRUG METABOLISING ENZYMES AND DRUG RECEPTORS

Due to the great significance of drug transporters, drug metabolising enzymes and drug receptors in pharmacological reactions they have been the subjects of many pharmacogenomic research projects. Identifying the genetic variation within the genes encoding these very important proteins is absolutely necessary if successful, optimal drug treatment for all is going to be realised.

2.2.1 Drug Transporters

The role of membrane transporters in drug effects has generated much interest and yet the relevant transporters for most drugs remain unknown (Yan and Sadée, 2000). This is due to the fact that even if a transporter is known to interact with a specific drug it remains unclear if other, yet unknown transporters may too recognise the same drug. A primary goal of drug discovery and development is to understand which

transporters recognise which drugs and which of these transporters can be exploited to target and transport new drugs to the intended tissue within the body (Yan and Sadée, 2000). The intestine, blood brain barrier, central nervous system cells, intestinal cells and the kidney membranes are the locations in which many drug transporters are found. Many of these transporters are members of the adenosine triphosphate (ATP)-binding-cassette (ABC) family and include P-glycoprotein, (*P-gp*, a multidrug efflux pump) and multi-drug resistance proteins (*MRP1-6*). Drug transporters are either Na^+ or H^+-coupled.

Polymorphisms have been found within some of the genes encoding transport proteins, however, the importance of these polymorphisms *in vivo* is yet to be fully understood (Evans and Johnson, 2001). Polymorphisms within these genes may affect drug absorption, distribution, elimination, and tissue targeting. Arranz *et al.* (2000) has identified six genetic polymorphisms within four genes encoding neurotransmitters that are linked to clozapine drug response in schizophrenia sufferers. Hoffmeyer *et al.* (2000) identified 15 polymorphisms in the human *MDR1* (multidrug resistance P-glycoprotein) gene in a Caucasian study population. One of these polymorphisms, a C/T in exon 26 showed a statistically significant correlation with P-glycoprotein expression levels and activity. Resistance of human cells to anticancer drugs is commonly associated with high expression of the multidrug resistance P-glycoprotein (Gottesman *et al.,* 1995) and the multidrug resistance-associated protein (MRP1) (Hipfner *et al.,* 1999).

Results of studies by Borst *et al.* (2000), Hoffmeyer *et al.* (2000), Cascorbi *et al.* (2001) and Glatt *et al.* (2001) indicate that the field of polymorphic drug transporters is a very important field. Ingelman-Sundberg (2001) is of the opinion that the effect of mutations in drug transporters, on pharmacokinetics, will in many cases be more difficult to interpret and will be of a smaller magnitude than that of polymorphic drug metabolising enzymes. Application of microarray gene technology to analyse membrane transporters may help determine tissue expression and allelic distributions on an individual basis (Evans and Relling, 1999). The largest problem facing researchers is, however, the determination of relevant transporters, not only because

of the multitude of genes, but also because of the remarkable structural and functional heterogeneity observed among transporters.

2.2.2 Drug Metabolising Enzymes

Drug metabolising enzymes are generally believed to have evolved as a consequence of the interaction between plants and animals (Schuler, 1996). In order for plants to defend themselves against predation and to enable them to synthesize secondary metabolites for their reproductive cycle, plants require constantly evolving biosynthetic pathways. Plants respond to predation by animals by evolving new genes that synthesize toxic metabolites, forcing the animals to evolve new drug metabolising genes in order to survive (Schuler, 1996, Ingelman-Sundberg *et al.*, 1999).

Xenobiotic metabolism can roughly be divided into two phases. Phase I is characterised by the chemical modification of the compound by addition of a polar functional group (Gonzalez and Nebert, 1990, Iyer and Sinz, 1999) that can be used to attach a conjugate. The compound in this form can then be excreted from the kidneys, in the urine as the addition of the conjugate makes the modified compound more water soluble. A hydroxyl group is frequently added in Phase I of drug metabolism by many cytochrome P450s. This hydroxyl then serves as a site for further modifications during phase II of drug metabolism which is typified by conjugation of the primary metabolites further enhancing their polarity (Iyer and Sinz, 1999). If activation is required to precede toxic response, it will more likely result from phase I metabolism. However, it has recently been shown that phase II conjugating metabolism can play a predominant/highly significant role in determining the response to the toxic xenobiotic administered as they are implicated in the detoxification of such products (Ingleman-Sundberg, 1998). These two sets of enzymes, alone or in combination are responsible for generating metabolites that are readily excretible (Iyer and Sinz, 1999). Multiple xenobiotic-metabolising enzymes with variable and fractionally overlapping catalytic properties are involved with the elimination of lipophilic xenobiotics to avoid their accumulation in cells and tissues (Guengerich, 1992, Nebert *et al.*, 1996).

Molecular mechanisms of inactivation for drug metabolising enzymes include splice site mutations which result in exon skipping (cytochrome P450 19 (CYP19), dihydropyrimidine dehydrogenase *(DPD)*), gene duplication (CYP2D6), enhanced proteolysis (thiopurinemethyl transferase *(TPMT)*), altered promoter functions (cytochrome P450 2A5 (CYP2A5)), critical amino acid substitutions (*N*-acetyltransferase (NAT-2), *CYP2D6*, cytochrome P450 2C19 (CYP2C19), cytochrome P450 2C9 (CYP2C9)), or large gene deletions (glutathione transferase M1 *(GSTM1)*, CYP2D6). Gene duplication (CYP2D6) results in the opposite situation, that of enhanced activity of drug metabolising enzymes (Evans and Johnson, 2001). The majority of gene duplications occurred as animals became terrestrial, due to dietary selection pressure (plant toxins are speculated to have had a major influence) (Ingelman-Sundberg *et al.*, 1999). It is expected that no selection in constitution of cytochrome P450s is expected to occur due to cancer caused by pollution as cancer usually occurs after the human reproductive cycle (Ingelman-Sundberg *et al.*, 1999). Gene duplication can only occur if it offers an advantage to the organism (Clark, 1994).

2.2.2.1 Phase One Metabolising Enzymes

Many phase I enzymes exhibit functional polymorphism that can result in increased pharmacologic effects for medications that are inactivated by these enzymes. These include tricyclic antidepressants (CYP2D6) (Dahl and Bertilsson, 1993), fluoxetine (CYP2D6) (Sallee *et al.*, 2000), warfarin (CYP2C9) (Aithal *et al.*, 1999), 5-fluorouracil and dihydropyrimidine dehydrogenase (Gonzalez and Fernandez-Salguero, 1995). Cytochrome P450 enzyme phase I metabolism converts many compounds to reactive, electrophilic, water-soluble intermediates, some of which can damage DNA (Hande, 1992, Raunio *et al.*, 1995, Smith *et al.*, 1995, Sonnichsen *et al.*, 1995).

2.2.2.1.1 Cytochrome P450 (CYP)

Cytochrome P450 comprise a large super-family of brightly coloured haem thiolate proteins that are of critical importance in the detoxification or activation of a large number of foreign hydrophobic compounds, including therapeutic drugs, chemical carcinogens and environmental pollutants (Guengerich and Shimada, 1991, Gonzalez

and Gelboin, 1994). They are responsible for catabolising compounds to provide a carbon source and for the synthesis of biologically active compounds including steroids, prostaglandins and arachidonate metabolites (Graham and Peterson, 1999). All organisms, protists to mammals, possess cytochrome P450 enzymes (Nelson *et al.*, 1996, Graham and Peterson, 1999).

Cytochrome P450 enzymes are the most important drug-metabolising enzymes in humans and therefore play a major role in many drug-drug and drug-food interactions. Cytochrome P450 enzyme reactions usually function to inactivate a drug but occasionally they may induce activation of the drug (National Community Pharmacists Association, 2001). All the compounds metabolised by CYP enzymes have one characteristic in common, they are all almost insoluble in water, and therefore have the tendency to accumulate in the membranes and other structures within cells and tissues which contain little/no water (American Medical Association, 2001). The extent of drug metabolism by a specific cytochrome P450 isoform is determined by the affinity of the substrate-enzyme complex and the relative abundance of the particular CYP enzyme in relation to the total amount of CYP in the liver (Özdemir *et al.*, 2001). Genetic control of levels of individual CYP expression could influence toxicity of drugs via an increase of CYP (Firozi *et al.,* 1996).

The key to understanding CYPs' importance lies in increasing our knowledge of their location and function. CYP enzymes are found throughout the human body but are most active in tissues that frequently come into contact with external materials e.g. liver, lung, skin, gut and kidneys (American Medical Association, 2001). The location of some of these enzymes may prove to be important in the metabolism of certain medications (National Community Pharmacists Association, 2001). As the liver is the main organ involved in drug and toxin removal, most CYPs are present here. A large amount of CYP is however also present in the small intestine. The multiple forms of cytochrome P450 that exist in liver microsomes have been shown to play important roles in the oxidation of structurally diverse xenobiotic (foreign drugs or chemicals) compounds including drugs, toxic chemicals and carcinogens as well as endobiotic chemicals such as steroids, fatty acids, fat soluble vitamins and prostaglandins (Fuengerich and Shimada, 1991).

As this super-family of genes is responsible for metabolising many toxic chemicals and carcinogens, carrying an allelic variant of any of these genes may affect an individual's capacity to successfully perform theses tasks (Topic *et al.*, 2000). Genetic polymorphism of CYP enzymes has therefore been suggested as a biomarker of susceptibility to carcinogens and toxic compounds (Hong and Yang, 1997).

Many of the CYP enzymes are inducible by the compound they metabolise. Consequently, cytochrome P450s are one of the most extensively studied groups of proteins, being investigated by researchers in fields as diverse as toxicology, pharmacology, environmental biology, biochemistry and molecular biology. Cytochrome P450s are intimately involved with vascular autoregulation in the brain and are vital to the formation of cholesterol, steroids and arachindonic acid metabolites.

Regulatory mechanisms for the CYP enzymes vary greatly. Chemical signals can turn many of these enzymes on or induce their activity. The steroid hormones are under strict endocrine control and their levels are tightly regulated. One example is the induction of steroid biosynthetic CYPs by ACTH (adrenocorticotropic hormone). ACTH stimulates production of 3', 5'-cyclic adenosine monophosphate (cAMP) that presumably activates a protein kinase that phosphorylates some unidentified protein, leading to an increase in gene transcription.

Peroxisome proliferators like clofibrate demonstrate another type of CYP gene regulation. These drugs act through PPAR (perioxisome proliferator activator receptor), a binding protein which when bound to the drug migrates to the nucleus, heterodimerises with retinoid X receptor (RXR) and binds to specific DNA sequences in the regulatory region of genes that are needed for peroxisome generation. In this way, the CYP4A1 gene is turned on. Peroxisomes oxidise fatty acids and CYP4A1 is a known fatty acid hydroxylase.

For data of cytochrome P450 to be accessible and useful, a cytochrome P450 nomenclature system was devised (Nebert *et al.*, 1987). This nomenclature system

relies on evolutionary relationships as depicted in phylogenetic trees of dendrograms derived from the CYP protein sequences. The whole collection of sequences represents the CYP super-family, with families and sub-families being arbitrarily defined as distinct clusters on the tree. Amino acid sequence similarity is the main criteria for categorising cytochrome P450 isozymes. CYPs are classified into families (sharing at least 40% amino acid sequence homology), sub-families (sharing at least 55% amino acid sequence homology) and individual genes. Families are identified by a number, e.g. CYP3, subfamilies by a letter, e.g. CYP3A and individual genes by another number e.g. CYP3A4. The number of human CYP genes has been estimated to 57 and 33 pseudogenes arranged into 18 families and 42 subfamilies (Nebert and Russell, 2002). The majority of these encode enzymes that function to metabolise drugs and xenobiotics. The most recent information regarding all the polymorphic forms of CYPs is found on the Human Cytochrome P450 (CYP) Allele Nomenclature Committee's web page. Information regarding polymorphic cytochrome P450 nomenclature for all enzyme variants can be found here.

Polymorphic CYP nomenclature is complicated, as presently more than eighty allelic variants have been described for CYP2D6 alone. The various allelic CYP forms are found with varying frequencies between various ethnic populations, due to genetic drift and in some cases genetic selection (Ingelman-Sundberg, 2001). Only CYP1A1 and CYP2E1 seem to show signs of conservation. Distribution of the different CYPs is remarkably diverse and the high degree of polymorphism observed within these genes is probably the result of dietary adaptation of the different populations of the world (Ingelman-Sundberg, 2001). Dietary stress in certain regions therefore resulted in the accumulation of specific CYP variants in certain populations. Incidental mutations within the CYP genes are, however, the major cause of the inter-ethnic differences, which were amplified in certain populations as the populations expanded.

Very little homology between human and rodents is observed for the CYP enzymes with the exception of CYP1A1 and CYP2E1. Rodent models can therefore not be effectively used for toxicity testing and analysis of drug metabolism (Ingelman-Sundberg, 1998).

Consequences of Mutations Within Cytochrome P450 genes

Differential cytochrome P450 enzyme activity is observed between individuals and populations due to genetic variation present in the genes. These inter-individual differences in enzyme activity may be responsible for serious adverse drug reactions by a patient when multiple drugs are administered simultaneously. Differential expression of the cytochrome P450 genes in certain organs may also be held responsible for the clinical reaction of a patient to a particular drug. Another factor complicating the prescription of drugs to patients is that cytochrome P450 genes also seem to show variable expression as individuals age. Neonates lack CYP1A2 and are therefore highly sensitive to caffeine as CYP1A2 is responsible for the metabolism of this toxin. The differences in metabolism of drugs due to genetic polymorphism may lead to different levels of effectiveness of certain drugs. Some genes in the CYP super-family show polymorphism with varying occurrence of poor and extensive metabolisers in different populations (Ingelman-Sundberg, 1998). Mutations within the CYPs can cause the resulting enzyme to have eradicated, reduced, altered or increased enzyme activity (Ingelman-Sundberg, 2001).

The involvement of cytochrome P450 enzymes in drug interactions has attracted much attention as knowledge about these enzymes has increased. Certain drugs have been declared illegal due to the severe reactions that they have with cytochrome P450 enzymes. Each CYP enzyme is affected differently by a drug, a fact that must be brought into consideration when one is trying to predict or understand CYP-drug interactions (Evans and Johnson, 2001).

Differences in human cancer susceptibility have been attributed to the occurrence of polymorphism in the enzymes responsible for metabolising the carcinogens (MacLeod *et al.*, 1997, Mucci *et al.*, 2001). CYP1A2 has been implicated as a risk factor in urinary bladder (Landi *et al.*, 1996, MacLeod *et al.*, 1997) and colorectal cancer (MacLeod *et al.*, 1997). MacLeod *et al.* (1997) suggest that when one is evaluating the cancer risk for a specific individual, one must take into consideration the carcinogen exposure and the activities of carcinogen metabolising enzymes (catalytic products of the enzymes may influence the expression or induction of other enzymes which either detoxify or activate potentially carcinogenic compounds) of

that individual. Two genetically linked polymorphisms of CYP1A1, *m1* (6235 T>C, *MspI* polymorphism) and *m2* (4889 A>G, Ile-Val substitution) are associated with increased breast cancer risk (Huang *et al.,* 1999). A single T to C substitution polymorphism within the CYP17 5' promoter-region has been described by Carey *et al.*, 1994. The T-allele has been referred to as the A1 allele and the C-allele as the A2 allele. Data from Feigelson *et al.* (1997) found an increased risk of advanced breast cancer in women who carry a single A2 allele of CYP17 and Spurdle *et al.* (2000) suggest that the CYP17 A2A2 genotype may be implicated in early-onset familial breast cancer. Kristensen and Børresen-Dale (2000) suggest that individuals carrying the long allele of CYP19 are also at an increased risk of developing breast cancer. Imaoka *et al.* (2001) suggests that the possibility exists that gender-specific expression of CYP4B1 in bladder tissue may contribute to male-dominant expression of bladder carcinoma.

Complete inactivation of CYP3A4 is thought to be incompatible with life, which may explain why no completely inactivating mutations have been found within the gene (Evans and Johnson, 2001).

Gene Duplications and Clinical Effects of Gene Duplication

Ultra rapid metaboliser phenotype is the result of duplicated or multi-duplicated copies of a gene encoding a drug metabolising enzyme. Table 2.01 summarises the clinical importance of the ultra rapid metaboliser phenotype in drug metabolism. It is crucial to identify patients with this phenotype before drug treatment commences to allow for optimal treatment.

Multiple copies of a functional CYP2D6 gene have been observed in Ethiopian and Saudi Arabian populations at high frequencies, 29% and 20% respectively (Ingelman-Sundberg, 1998). Duplications of this gene are observed in much lower frequencies in other populations e.g. in Swedes, Zimbabweans and Chinese, multi-duplication occurred at a frequency of only 2% (Ingleman-Sundberg, 1998). The belief therefore exists that dietary selection pressure existed in Saudi Arabia and Ethiopia that favoured the preservation of alleles consisting of duplicated or multi-duplicated genes, and these were introduced to other counties during the Muslim migration. In

support of this hypothesis, it has been observed that very few defective CYP2D6 alleles have been reported in Ethiopian and Saudi Arabian populations.

A consequence of this gene multi-duplication effect is that a drug fails to deliver any therapeutic effect at standard doses in patients carrying more than three functional CYP2D6 genes. Evidence in support of this has been demonstrated by Dalén *et al.* (1998). Dalén and his co-workers showed that plasma levels of nortriptyline are reduced in a proportional manner to the number of gene copies harboured by the individual. The number of primary metabolites are however also proportional to the number of active genes. If this goes undetected in patients, it may result in dire consequences during therapy with drugs whose primary metabolite is toxic or addictive e.g. codeine whose primary metabolite is morphine. Morphine addiction may be induced in patients carrying three or more functional CYP2D6 genes (Ingelman-Sundberg, 1998).

2.2.2.1.2 Other Phase I Enzymes

Other phase I drug metabolising enzymes include the five flavin-containing monooxygenase (FMO) isoforms (FMO1 - FMO5). They comprise a membrane-associated, widely distributed family of enzymes that catalyse the oxidation of nucleophilic nitrogen-, sulfur- and phosphorous-containing xenobiotics, drugs and endogenous substances (Zeigler, 1993). The role of FMOs in human metabolism as suggested by Ziegler (1993), is to metabolise xenobiotics from plant sources to materials that are not pharmacologically or toxicologically threatening to the organism ingesting the chemical. Zhang *et al.* (1995), Cashman *et al.* (1997), Dolphin *et al.* (1997) and Treacy *et al.* (1998) have described mutations of the human *FMO3* gene that cause trimethylaminuria, an inborn error causing a deficiency in *N-oxygenation* of the odorous tertiary amine TMA (tertiary amine trimethylamine) to its non-odorous metabolite TMA *N-oxide*.

2.2.2.2 Phase II Enzymes

Phase II enzymes comprise at least four classes, a, m, p and q (Murata *et al.,* 1998) and are characterised by their diverse substrate specificity and polymorphism (Board *et al.,* 1998).

N-acetyltransferase (NATs) enzymes show genetic polymorphism that influences their capacity to deactivate aromatic amines and other environmental exposures, and activation of heterocyclic amines produced during the cooking of meats. They catalyse generally considered detoxifying reactions (*N*-acetylation) or intermediate reactions leading to DNA reactive intermediates (*O*-acetylation of *N*-hydroxyarylamines stemming from previous CYP metabolism) (Weber and Vatsis, 1993). Two *N-acetyltransferase* genes have been identified, *NAT-1* and *NAT-2*, along with one pseudogene *NATP*. *N*-acetylation polymorphism was identified late in the 1940s where researchers phenotyped individuals as slow or rapid acetylators. *NAT-2* has been identified as the gene principally involved with slow and rapid acetylator phenotype. Three major slow-acetylator alleles have been independently identified in many human populations (Nebert and Roe, 2001). Two of them are common in Caucasians and one in Asians. Slow acetylator phenotype has a high incidence in bladder cancer (Gonzalez and Idle, 1994).

Uridine diphosphate glucuronosyltransferase (UDPGT) enzymes show considerable diversity generated by multiple copies of exon one being alternately spiced with exons two to five (Board *et al.*, 1998). A TA insertion in the *UGT1A1* promoter region reduces the expression of this enzyme and is subsequently the cause of Gilbert's syndrome (Innocenti and Ratain, 2002).

Glutathione *S*-transferases (GSTs) comprise a family of multifunctional dimeric proteins that catalyse glucuronide formation using many endobiotic and xenobiotic compounds as substrates. Their primary function is to detoxify electrophiles capable of binding to DNA (Taningher *et al.*, 1999). Determining and predicting GSTs specificity remains a challenge for researchers. Four classes of human cytosolic GSTs have been distinguished: α (GSTA), μ (GSTM), π (GSTP) and θ (GSTT) (Mannervik *et al.*, 1992). Increased cancer susceptibility due to deficient detoxification has been associated to a deletion of the *GSTM1* gene (Taningher *et al.*, 1999). *GSTP1* polymorphism has also been implicated with other cancer susceptibilities (Harries *et al.*, 1997).

TPMT (thiopurine methyltransferase) is a polymorphic phase II enzyme responsible for catalysing the *S*-methyltransferase of thiopurine medications (e.g. 6-mercaptopurine in chemotherapy), the inactivation pathway in hematopoietic tissues. Patients homozygous for the non-functional *TPMT* allele, develop dose-limiting hemapoietic toxicity that may result in death if the patient receives full doses of thiopurine medications. Treatment with 5-10% of the conventional dose results in successful treatment with only acute toxicity. Individuals heterozygous at the *TPMT* locus have a greater risk of thiopurine toxicity than those homozygous for the normal allele. Dosage has to be moderately reduced for these patients (Evans and Johnson, 2001).

2.2.3 Drug Receptors

Evans and Johnson (2001) define a drug target as "the direct protein target of a drug (e.g. a receptor or an enzyme), proteins involved in the pharmacologic response (e.g., signal transduction proteins or downstream proteins), or proteins associated with disease risk or pathogenesis that is altered by the drug." Generally two types of receptors are recognised based on location and mechanistic features, those present in the cytoplasm and those that insert into and traverse cell membranes. Selectivity and specificity of drug action is a function of the selective localisation of the drug receptors, the specificity of the drugs that bind to the particular receptor, the strength of the drugs' attachment to the receptor and the consequences of interaction between the drugs and the receptors. Receptors display varied levels of sensitivity depending on the individual's age, genetic factors, drug interactions and disease states.

Pharmacogenetics of drug receptors is a very new field of research that only came into being in the 1990's. Many of the researchers in this field have investigated the contribution single polymorphisms or single genes have on the expected response to a medication, and they have been successful in finding associations between certain polymorphisms and the drug response. The results of these studies have, however, been documented very inconsistently. The angiotensin converting enzyme (ACE) gene is a very popular drug target in many of these studies. Studies of an insertion/deletion (I/D) within the gene have yielded contradictory results, in that some studies show no association between response and ACE I/D genotype (Cannella

et al., 1998), others report association of the I/I genotype to greater drug response (Kohno *et al.*, 1999) and others report association of the D/D genotype with greater drug response (Hernandez *et al.*, 2000).

Other genes coding for drug receptors that have received much attention include the β_2-adrenergic receptor gene, dopamine receptor genes and the serotonin (5HT) receptor genes. Many mutations within the androgen receptor have been found associated with prostate cancer (Scher and Kelly, 1993, Schoenberg *et al.*, 1994).

2.2.4 Use of Pharmacogenomics Today and Implications for the Future

The ability to provide every individual patient with an individualised drug efficacy and safety likelihood profile will change the practice and economics of medicine. The test in itself will have great economic significance. Streamlined drug development and the concurrent development of discrete medicines for patients with similar disease phenotypes will result from the ability to rapidly profile patients who seem likely to benefit from a particular medication. Pharmacogenetic profiles will soon be developed which will enable one to determine the likelihood of an individual responding to the medication and/or the likelihood of an individual experiencing adverse side effects from the medication (Roses, 2000).

The application of pharmacogenomics to the delivery of medicines will maximize the value of each medication. Pharmacogenomics has the potential to impact medical care at many different levels. As medications that treat, cure or prevent common diseases become a more significant part of medical care, the costs of debilitating illnesses will be drastically reduced. A great sum of money will be saved once the practice of treatment and prevention of chronic and common disease improves by simply resulting in a reduction in the length of a hospital stay and long-term care costs. These savings could then be transferred to well-tolerated and effective medicines. Prevention of drug-caused morbidity and mortality should be possible through the successful application of pharmacogenomic research (Nebert, 1999).

Evans and Johnson (2001) suggest that if drug receptor pharmacogenomics is to be used as a reliable tool in the prediction of drug response and for making suitable

therapeutic decisions based on this information, more sophisticated genomic approaches will have to be implemented. This will involve approaches such as the "candidate gene" pharmacogenetic approach, where the polymorphisms in many genes suspected or known to contribute to drug effects or disposition are studied to gain insight of their association with drug response or toxicity. Drysdale *et al.* (2000) have shown that haplotype analysis considerably enhances ones ability to detect important associations between gene polymorphisms and drug response.

The future of pharmacogenomics' acceptance as common practice will rely heavily on its ability to provide results that can be easily understood by persons with a general medical background, and not only molecular biologists. For pharmacogenomics to be widely applied the tests will have to be simplified as much as is allowed, and the tools allowing interpretation of the results will have to be made available in a user friendly manner. Testing technologies and test validation are the two factors that will affect the availability of pharmacogenomic testing as a part of routine drug selection (Spear *et al.*, 2001).

A consequence of pharmacogenomic testing that will have to be addressed is that of genetic counselling, prior to and after a patient has undergone such a test. Topics such as what the test can/can not reveal, the availability of treatment options and the meaning of the test results will have to be discussed at length with the patients. Simultaneous testing for multiple genetic markers of different conditions will complicate issues even further as how does a counsellor prepare an individual for results that indicate that the patient has the genetic markers associated with susceptibility for a number of genetic diseases or disorders? It is valid to therefore say that the volume of information resulting from pharmacogenomic tests may prove to be overwhelming to both the patients and the medical professionals.

2.3 PATIENT GROUPS

Variegate porphyria and breast cancer are two diseases that have great clinical relevance in the South African population due to the high observed prevalence of the diseases. The focus of future studies involving these patients will include determining variation in drug response, but before this can become a reality, a need exists to

identify the relevant polymorphisms in as many South African populations as possible.

2.3.1 Variegate Porphyria (VP)

With the exception of erythrocytes, all tissues exhibit aerobic metabolism and therefore require mitochondrial cytochromes. Functional cytochrome P450 enzyme production requires the incorporation of haem to the apoprotein. All cells should therefore produce enough haem to meet the need for cytochrome production. In the liver fifty percent of synthesised haem is used in the formation of CYP. Synthesis of cytochrome P450 accounts for the high rate of haem synthesis in the liver and changes in the production of CYP would surely cause major changes in the rate of hepatic haem synthesis. The induction of CYPs by a substrate (drug or lipophilic endogenous substance) will lead to the removal or activation of that particular substance, a reaction that requires synthesis of new CYP, which in turn requires the synthesis of new haem. Haem regulates the amount and the activity of CYP proteins in three ways (De Matties and Marks, 1996). These are listed in Table 2.04. In persons suffering from conditions collectively known as porphyrias, this could be problematic as the flow of intermediates within the haem biosynthetic pathway may be partially or totally blocked, limiting the capacity for increased haem biosynthesis.

Table 2.04: Regulatory mechanisms of CYP protein quantity and activity by haem.

• Incorporation of haem to the apoprotein of CYP is essential to ensure the assembly of functional holoprotein
• Haem-exchange allows a haem deficient CYP to require at least part of its original haem compliment
• Inhibition of haem biosynthesis in rats inhibits induction of CYPs

(De Matties and Marks, 1996)

The porphyrias are a heterogeneous group of inherited disorders of haem biosynthesis. All living organisms produce porphyrins, porphyria patients simply accumulate porphyrins in an atypical manner. Haem biosynthesis converts two simple substrates, δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) (porphyrin precursors) into

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more complex porphyrins. Porphyrins are converted from one type to another until protoporphyrin is complexed with iron to eventually form haem. Each step of this pathway is catalysed by a specific enzyme. As free porphyrins are produced only in minute quantities in humans as the accidental side products of haem biosynthesis, the presence of increased porphyrins in urine or faeces is an indication of a disturbance within the haem-biosynthesis pathway.

Variegate porphyria (OMIM #176200) is an autosomal dominant disease with variable clinical expression and a low penetrance, with some carriers being asymptomatic. VP is classified as an acute hepatic porphyria and a toxico-genetic disease (genetically acquired disease showing idiosyncratic reaction to drugs). VP patients and asymptomatic carriers have a 50% reduction in the activity of protoporphyrinogen oxidase which catalyses the conversion of protoporphyrinogen IX to protoporphyrin IX in mitochondria (Deybach *et al.,* 1981 and 1985).

Numerous mutations within the protoporphyrinogen oxidase gene (*PPOX*) have been identified (Whatley *et al.*, 1999, Human Gene Mutation Database, 2002) and support the conclusion that defects within the PPOX gene cause variegate porphyria. Variegate porphyria has an unusually high incidence in South Africa. Genealogical and molecular genetic studies of South African variegate porphyria families have demonstrated that this is the result of a founder gene effect caused by the introduction of a single mutation, R59W, by one of two Dutch immigrants in 1688 (Dean, 1971, Meissner *et al.*, 1996, Warnich *et al.*, 1996, Groenewald *et al.*, 1998). The frequency of this mutation was estimated to be 0.003 in the Caucasian Afrikaans population (Dean, 1971), and is present in approximately 94% of South African VP subjects (Meissner *et al.,* 1996).

Clinical manifestations of the disease include cutaneous photosensitivity and acute neurovisceral crises that may occur separately or in combination (Kirsch *et al.,* 1998). Cutaneous photosensitivity is the result of the circulating porphyrins. Excessive fragility of skin is observed in patients, especially in sun-exposed areas. The skin lesions consist of a bullous eruption leading to ulceration and chronic scarring usually involving the extensor surface of the hands and the face. Damaged areas of the skin

frequently take long to heal properly. The skin of porphyria sufferers often becomes thin, dark and scarred and sometimes rather hairy, particularly on the face. The primary site of skin damage is in the vessel wall (Timonen *et al.*, 1990).

Acute attacks rarely occur before puberty and are most common during the second to forth decades of life (Elder *et al.*, 1997). They occur more frequently among women than men and vary in their clinical presentation. The most common symptom is abdominal pain that may be severe and continuous, and may affect any part of the abdomen, spreading to the back, buttocks and thighs (Elder, 1997). Large amounts of opioids may be required to control the pain. Dehydration is common and inappropriate secretion of vasopressin results in hyponatraemia. Sodium levels frequently drop to levels that result in the occurrence of convulsions. Acute attacks may progress to a neuropathy, affecting mainly the motor nerves but mild disruptions in sensory nerves have been described and include hypoaesthesia. Tachycardia, hypertension, vomiting and constipation may all occur, due possibly to autonomic neuropathy (Gordon, 1999, Medlock *et al.,* 2002). After repeated attacks, wrist and foot dropping may develop. Widespread muscle weakness, dysaesthesia and causalgia may also occur and cranial palsies have been recorded. Other features include abnormal behaviour, confusion, agitation, anxiety and hallucinations. Urinary abnormalities are frequently observed during an attack as an increased excretion of porphyrin precursors ALA and PBG giving rise to red wine/brown coloured urine. In the faeces, increased excretion of protoporphyrinogen and coproporphyrinogen has been observed (Dean, 1971, Mustajoki, 1978, Meyer and Schmid, 1978, Da Silva *et al.*, 1995). Neurological symptoms are also apparent during the attack and range from headaches to paralysis. Abdominal tenderness and diminished bowel sounds are also commonly observed. If death follows an attack it is usually due to respiratory failure and complications due to incorrect management of the respiratory problems.

Variation of genetic characteristics may explain the observed variance in disease manifestation. Commonly used drugs can precipitate an illness, by increasing the synthesis of cytochrome P450 enzymes in the liver that result in an increased synthesis of haem. Known inducers of the symptoms of acute intermittent porphyria (AIP) include sedatives, sulfonamides, erthromycin, griseofulvin, estrogenic

hormones and anti-seizure medications, all of which are metabolised by the enzyme products of CYP genes. Inability, due to CYP polymorphism, to successfully metabolise these drugs may result in a patient developing symptoms of AIP. Barbiturates are absolutely contraindicated in AIP (American Academy of Anaesthesiologist Assistants, 2001). Porphyria cutanea tarda (PCT) is induced in adults due to exposure to environmental toxins including dioxin containing herbicides, cyclic (polychlorinated) hydrocarbons, alcohol and oestrogens. These substances may be inducers of PCT due to the inability of the specific CYP to successfully metabolise these compounds (American Academy of Anaesthesiologist Assistants, 2001). Porphyria patients are strongly recommended to avoid many commonly prescribed medications due to the many negative side effects experienced after their administration. Drugs that can precipitate an acute attack have a common characteristic – the ability to increase the activity of δ-aminolevulinic acid synthase, the rate limiting enzyme of the haem biosynthesis pathway (Gordon, 1999). Other factors precipitating acute attacks include fasting, menstruation, pregnancy, stress, alcohol and infections (Kauppinen and Mustajoki, 1992, Zimmerman *et al.*, 1996, Elder *et al.*, 1997). Menstruation and pregnancy may precipitate acute attacks due to the fact that hormonal changes are experienced and gonadal steroids are metabolised in part by CYPs and may therefore endogeneously increase the rate of haem biosynthesis in women.

Acute attacks may be successfully managed by paying particular attention to the medication the individual is receiving. Many patients in South Africa are however, apparently unaware of their positive porphyria status, and are therefore at an increased risk for experiencing acute attacks (Jenkins, 1996).

2.3.2 Breast Cancer

Cancer is the result of a loss of balanced gene expression caused by a process of mutation in key regulatory genes and epigenetic alterations (Danesi *et al.*, 2001). It results in the excessive proliferation of cells due to the cells' ability to divide without stimulation from other cells and their unresponsiveness to the normal controls of cell proliferation. Cancer cannot be considered purely genetic or purely environmental (Hoover, 2000, Lichtenstein *et al.*, 2000). Research is now focusing on alterations in

DNA that are more common, but that on their own may not significantly impact disease risk. However, together with environmental exposures, these genetic alterations may lead to the development of cancer, so called co-participation (Mucci *et al.*, 2001).

Breast cancer is one of the most common causes of death in women and the most frequently diagnosed cancer in women from all ethnic groups (Greenlee *et al.*, 2001, Jemal *et al.*, 2002). More South African women die annually from breast cancer than any other cancer including cervical cancer which previously was the leading cause of cancer related deaths (SAPA, 10/10/2002). Epidemiological studies indicate that the age of onset is steadily, albeit slowly, decreasing. This is indicative of the role changing environmental factors have on the development of breast cancer.

Last century, *BRCA1*, *BRCA2* and *p53* were identified and declared as the susceptibility genes for breast cancer (Williams and Anderson, 1984, Hall *et al.*, 1990, Wooster *et al.*, 1994). Investigators now conclude that there are as many as four to twelve major genes and many other modifier genes responsible for increased breast cancer risk (Nebert, 1999). It has been postulated that the possibility exists that "low-risk" genetic factors, that are more common than the "high-risk" mutations in *BRCA1* and *BRCA2*, explain the familial aggregation of breast cancer (Spurdle *et al.,* 2000). Modifying factors for increased breast cancer risk or toxicity to drugs and environmental agents clearly include many drug metabolising enzymes, drug receptors, and drug transport genes (Nebert and Roe, 2001). Many of the genes encoding carcinogen metabolising enzymes are polymorphic (Mucci *et al.*, 2001). Some of these variants result in changes in the enzyme, while others increase or decrease the rate at which metabolism of the substrate occurs, and others alter the expression of the enzyme. Susceptibility to cancer may be influenced by altered exposure of cells to the carcinogens, or may be due to the genetic variations within the genes encoding the metabolising enzymes (Mucci *et al.*, 2001). The last 25 years of research have resulted in the identification of many risk and protective factors for breast cancer, all of which culminate in the exposure of breast tissue to oestrogen and perhaps progesterone (Feigelson and Henderson, 1996). The existence of susceptibility genes carrying "low absolute risk" and potentially "high population-

attributable risk", especially when considered in combination, has been hypothesised (Feigelson *et al.*, 1997).

Oxygenated metabolites of oestrogen have newly generated hydroxyl and keto functions, which markedly affect their biological activities (Martucci and Fishman, 1993). Independent studies by Yager *et al.* (1996), Cavalieri *et al.* (1997), and Zhu and Conney (1998) have shown that oestrogen metabolites can bind to DNA, triggering damage. These results have lead to Service (1998) suggesting that oestrogen may be a complete carcinogen that can directly cause genetic alteration and effect tumour initiation. Catechol oestrogen (CE), a metabolite of oestrogen, has been proposed as the initiating mechanism triggering breast cancer (Huang *et al.*, 1999) and has been shown to be carcinogenic in animal models (Shimada *et al.*, 1996). Cytochrome P450 enzymes are involved with CE formation via oestrogen biosynthesis (CYP17) and hydroxylation (CYP1A1). Extrahepatic tissues such as breast tissue rely on CYP1B1 for the homeostasis of oestrogen. Furthermore, CYP1B1 and CYP1A1 have been shown to catalyse the formation of mutagenic intermediates from many polycyclic aromatic hydrocarbons, including many that are potent mammary gland carcinogens in rodents (Bailey *et al.*, 1998). Drugs and foreign chemicals with structural similarity to endogenous ligands that play a role in the regulation of oestrogen metabolism can alter the level of the various oestrogen metabolites. Additional CYPs are responsible for restoring the balance of oestrogen hormone action, hereby neutralising the disturbance (Martucci and Fishman, 1993). Formation of reactive species leading to an increased degree of carcinogenesis has been proposed to be the price paid for tight regulation of steroid hormones (Nebert, 1991).

Interindividual variance in therapeutic drug response and drug toxicities is a cause of great concern for clinicians. This variance results from polymorphism in genes coding for drug metabolising enzymes or drug receptors. Therapeutic agents' pharmacokinetics are altered as a result of polymorphism in drug metabolising enzymes (Iyer and Ratain, 1998). Innocenti *et al.* (2002) is of the opinion that genotypic stratification of patients might identify subgroups with an enhanced prognostic profile to chemotherapy response and survival. Selection of alternative

therapies may be guided by the presence of alleles associated with reduced responsiveness to certain drugs. The unpredictability of cancer treatment will be reduced by the identification of genetic reasons explaining the occurrence of toxicity or lack of tumour response to treatment.

A great challenge in cancer research is to identify, in precise detail, the series of abnormalities that arise in the genomes of all types of cancer, to use the newly identified information to understand malignancy and ultimately to develop more rational and effective strategies for diagnosis and treatment of cancer. The use of chemotherapy as a treatment for cancer is limited due to the fact that the choice of treatment protocol is still empirical and makes no account of tumour biology. Data exists that shows that patients with seemingly identical tumours may respond differently to treatment. Pharmacogenomics now seeks to answer many questions regarding the success of chemotherapy in some cancers and the unsuccessful results in others, as the influence that genetics has on the response to anti-cancer agents is yet to be determined and understood fully. Pharmacogenomics will aid in the determination of the roles played by gene products (enzymes) in either directly or indirectly influencing the activity of chemotherapeutic agents. Decreases or increases in the normal activities of these enzymes are associated with drug resistance, therefore the expression of the target enzyme in the tumour must be taken into consideration in order to maximize the chances of therapeutic success (Danesi, 2001). Creation of individualised chemosensitivity-chemoresistance maps would help in the identification of drug combinations for optimal treatment. Creation of these maps will only be possible once the genes responsible for sensitivity to drugs have been identified and the patients have been screened.

2.4 AIMS OF THE STUDY

The general aim of this study was to investigate whether an association exists between various cytochrome P450 polymorphisms and two disease groups.

Specific Aims of the Study

Variegate Porphyria Study (Addressed in Chapter Three)

i Identification of "unsafe" drugs for porphyria patients and the cytochrome P450s responsible for their metabolism from literature;

ii Selection of polymorphisms within a number of relevant cytochrome P450 genes and subsequent molecular screening of the patient and control groups for the selected polymorphisms;

iii Statistical analysis and evaluation of the results generated to determine whether an association exists between any or all of the polymorphisms and variegate porphyria (disease group and various symptoms).

Breast Cancer Study (Addressed in Chapter Four)

i Selection of polymorphisms within the relevant cytochrome P450 genes and subsequent molecular screening of the patient and control groups for the selected polymorphisms;

ii Statistical analysis and evaluation of the results generated to determine whether an association exists between any or all of the polymorphisms and increased risk of developing breast cancer in two South African population groups (Caucasian and of mixed ancestry).

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CHAPTER THREE

CYP POLYMORPHISMS IN SOUTH AFRICAN VARIEGATE PORPHYRIA PATIENTS: AN ASSOCIATION STUDY

3.1 ABSTRACT

Variegate porphyria (VP), an autosomal dominant disease with low penetrance, is a disorder of haem biosynthesis. The R59W mutation within the PPOX gene (protoporphyrinogen oxidase, the seventh enzyme in the haem biosynthesis pathway) is associated with a founder gene effect among the South African VP population. Clinical expression of the disease varies, but is characterised by neurovisceral attacks and skin lesions, alone or in combination. Some patients are asymptomatic. It is expected that modifier loci and environmental factors are important contributors to the varied clinical expression observed. Neurovisceral attacks may be precipitated by the administration of certain drugs introducing a role for drug metabolising enzymes as possible modifier genes. It is thought that the induction of cytochrome P450 (CYP) enzymes (phase I drug metabolising enzymes) by drugs results in an increased demand for hepatic haem, leading to the induction of haem biosynthesis. Accumulation of excess metabolites in VP patients prior to the enzymatic block may limit the capacity for increased haem synthesis, hereby reducing the expression of functional CYP, leading to incomplete drug metabolism. A clinically characterised VP disease group consisting of four clinical subgroups (asymptomatic, acute attacks, skin lesions, acute attacks and skin lesions) and a healthy control group were included in this study. Six CYP polymorphisms (CYP1A1*m1*, CYP1A1*m2*, CYP1A2 –734 C>A, CYP1B1 8372 A>C, CYP2D6*3 and CYP2D6*4), associated with four CYP loci, were genotyped in these groups. Significant differences in CYP1B1 genotype distributions (P<0.001) and allele frequencies (P<0.0004) were observed after Bonferroni correction for the VP disease group, the skin lesion clinical subgroup, the total acute attacks group and the total skin lesion group versus the control group.

Prior to Bonferroni correction, significant differences in CYP1B1 genotype distributions ($P<0.03$) and allele frequencies ($P<0.025$) were observed for comparisons between the acute attacks clinical subgroup versus the controls and for the total acute attack and the total skin lesion group versus the asymptomatic clinical subgroup. CYP1A1*m1* genotype distributions ($P<0.04$) and allele frequencies ($P<0.05$) yielded significant differences prior to Bonferroni correction for the skin lesion and the acute attack and skin lesion clinical subgroups versus the acute attacks clinical subgroup. The A-allele of CYP1B1 demonstrated an allele frequency of 100.00% for all the clinical subgroups presenting with acute attacks. Although the numbers of patients included in this study are relatively small, the results observed in the CYP1B1 analysis indicate a possible role for CYP1B1 in the modification of the clinical expression of VP in South Africa.

3.2 INTRODUCTION

Variegate porphyria (OMIM #176200), an autosomal dominant disease with low penetrance, is a disorder of haem biosynthesis. Heterozygous individuals have a 50 percent reduction in the activity of the seventh enzyme in the haem biosynthesis pathway, protoporphyrinogen oxidase (*PPOX*). PPOX catalyses the conversion of protoprophyrinogen IX to protoporphyrin IX in mitochondria (Deybach *et al.*, 1981 and 1985). The reduced activity of PPOX in VP patients results in the accumulation of porphyrins and increased porphyrin levels are observed in stool and urine samples of affected individuals, especially during and just after a neurovisceral attack (Dean, 1971, Mustajoki, 1978, Meyer and Schmid, 1978, Da Silva *et al.*, 1995). Clinical expression of the disease varies but is characterised by neurovisceral attacks and skin lesions alone or in combination. Some patients are however asymptomatic. Acute attacks occur more frequently among women than men and vary in their clinical presentation. Menstruation and pregnancy may precipitate neurovisceral attacks due to the fact that hormonal changes are experienced and gonadal steroids are metabolised in part by CYPs and may therefore endogenously increase the rate of haem biosynthesis in women. The mechanism giving rise to the varied symptoms is as yet unknown but it is expected that modifier loci and environmental factors are important in this regard (Elder, 1998, Warnich *et al.*, 2002). The unique founder population of South Africa (Dean, 1971, Meissner *et al.*, 1996, Warnich *et al.*, 1996,

Groenewald *et al.*, 1998) with numerous individuals all having the same mutation (R59W in *PPOX*), provides a unique opportunity to identify genes that may be responsible for modifying the clinical expression of VP.

The administration of certain drugs is known to precipitate neurovisceral attacks therefore great caution should be exercised when prescribing medication to a porphyria patient (SAMF, 2000). Genetic variations in drug metabolising enzymes are therefore implicated as candidate loci responsible for modifying the clinical expression of VP. Fifty percent of the haem produced in the liver is used as a component of cytochrome P450 enzymes, the most important drug metabolising enzymes in humans (Guengerich *et al.*, 1998, Graham and Peterson, 1999, Ingelman-Sundberg, 2001). Drug induced porphyrinogenicity is complex, but it is thought that the induction of CYPs by drugs results in an increased demand for hepatic haem, leading to the induction of haem biosynthesis (Hindmarsh, 1993), and the subsequent accumulation of excess metabolites prior to the enzymatic block. This limits the capacity for increased haem synthesis, which subsequently reduces the expression of functional CYP and leads to the incomplete metabolism of the drug concerned.

Genetic polymorphism of the CYP genes may result in an individual being particularly sensitive to a specific drug. An example of drug sensitivity is shown in known inducers of the symptoms of acute intermittent porphyria (AIP), which include sedatives, sulfonamides, erthromycin, griseofulvin, estrogenic hormones and anti-seizure medications, all of which are metabolised by the enzyme products of CYP genes. Inability due to CYP polymorphism, to successfully metabolise these drugs may result in a patient developing symptoms of AIP. Barbiturates are absolutely contraindicated in AIP (American Academy of Anaesthesiologist Assistants, 2001). Porphyria cutanea tarda (PCT) is induced in adults due to exposure to environmental toxins including dioxin containing herbicides, cyclic (polychlorinated) hydrocarbons, alcohol and estrogens. These substances may be inducers of PCT due to the inability of the specific CYP to successfully metabolise these compounds (American Academy of Anaesthesiologist Assistants, 2001).

Christiansen *et al.* (2000) suggest that the CYP1A2 A/A genotype is associated with predisposition to development of clinically manifest porphyria cutanea tarda (PCT). PCT is characterised by decreased activity of uroporphyrinogen decarboxylase (UROD) (Kappas *et al.*, 1995, Elder, 1998) and the subsequent development of photosensitisation of skin due to the accumulation of uroporphyringen and other substrates of the UROD reaction (Bickers and Pathak, 1993). A 75% decrease in UROD activity is necessary before porphyrin formation is increased to levels that cause photosensitisation (Elder, 1990). The rate of inactivation of hepatic UROD in PCT and experimental uroporphyrias is influenced by three main factors: iron, the CYP1A subfamily and δ-aminolevulinic acid (ALA) (Elder and Roberts, 1994). Approximately 25% of VP patients demonstrate reduced UROD activity (Day *et al.*, 1982, Day 1986). Elder (1990) has suggested a model explaining this: an oxidative reaction catalysed by a CYP (for example CYP1A2) converts uroporphyrinogen to the non-metabolisable uroporphyrin and/or a CYP catalysed oxidative reaction generates a liver specific UROD inhibitor. Smith and Francis (1987) isolated a UROD inhibitor from livers of rodents with uroporphyria. A hypothetical explanation for an association between increased CYP activity and VP therefore lies in the formation of a similar UROD inhibitor for humans. UROD activity seems to be less inhibited without iron loading in a 3-week rat model of PCT (Franklin *et al.*, 1998) while porphyrin accumulation is dramatically reduced under the same conditions.

In this case-control association study, we evaluate the contribution of polymorphisms in CYP1A1, CYP1A2, CYP1B1, and CYP2D6 to a well defined clinical endpoint in South African variegate porphyria (disease group and subgroups) patients and a relevant control group. These four genes were chosen due to the important role they have in metabolising drugs contraindicated in porphyria patients (CYP2D6), the role they have in metabolising environmental pollutants and toxins (CYP1A1 and CYP1B1) and due to demonstrated association with clinical expression of other types of porphyria (CYP1A2).

3.3 MATERIAL AND METHODS

3.3.1 Study Populations

Blood samples from 30 VP patients were collected and were genetically diagnosed as being R59W heterozygotes. Extensive clinical evaluation of the patients at Tygerberg Hospital resulted in the patients being subdivided into four clinical subgroups: acute attacks, skin lesions, acute attacks in combination with skin lesions and asymptomatic (Steyn, 2002). A baseline questionnaire collected information on potential risk factors for VP as well as lifestyle habits. The patients had a mean age of 44 years and ranged in age from 11 years to 71 years. Individuals constituting the healthy control population (n=83) were randomly selected. The same control group was genotyped for each polymorphism. All individuals participating in this study gave their written informed consent. Ethical approval was given by the Ethical Committee of the Faculty of Health Sciences, University of Stellenbosch.

3.3.2 DNA Extraction

Total genomic DNA was extracted from whole blood using a modified protocol of Miller *et al.* (1998) (refer to Appendix A for a detailed protocol) and the extracted DNA was stored at -20°C until genotype analysis.

3.3.3 Selection of CYP polymorphisms

Alphabetical lists of drugs and guidelines for their use by porphyria patients were downloaded from the University of Cape Town's liver clinic (2001) and the University of Queensland's porphyria guide (2001) webpages. Lists of drugs unsafe for use by porphyria patients were compiled using the downloaded lists, SAMF (2000) and MIMS (2001). The CYPs involved in the metabolism of these unsafe drugs were identified using information from Gentest (2001). CYPs important in the metabolism of many of the unsafe drugs were selected and polymorphisms within these genes were identified from literature.

3.3.4 Genotyping of Cytochrome P450 Polymorphisms

PCR-based RFLP assays were used to determine the CYP1A1*m1*, CYP1A1*m2*, CYP1A2 and CYP2D6*4 genotypes (see Appendix B for a detailed protocol). Genotyping of the CYP1B1 polymorphism was performed using the polymerase chain

reaction heteroduplex single strand conformation (PCR-hex SSCP) (Kotze *et al.*, 1995) method of detection. The primers were designed from the CYP1B1 sequence (gi 17446619, NCBI) using the program Primer3 available online (Table 3.01). PCR was employed to amplify a 242 base pair DNA fragment that was subjected to heteroduplex SSCP analysis. The denaturing polyacrylamide gels were stained in 1.5X TBE containing a final concentration of 0.0075% (v/v) EtBr, for 20 minutes and bands were visualised in the gel with UV light and subsequently photographed with the GelDoc system (BioRad, Hercules, CA, USA). Termination cycle sequencing was employed to confirm the presence/absence of the polymorphism. PCR products were purified using a QIAquick® PCR Purification Kit (QIAGEN, GmbH, D-40724, Hilden). Sequencing reactions were performed using a Perkin Elmer BigDye™ Terminator Cycle Sequencing Kit (PE Applied Biosystems, Warrington WA, 4SR, Great Britain) and the forward primer as described in Table 3.01. These reactions were run on an ABI 3100 (PE Applied Biosystems, Warrington WA, 4SR, Great Britain) to determine their sequences. Sequences were analysed using the programs Sequencing Analysis (V3.07) and BioEdit Sequence Alignment Editor (Hall, 1999). Refer to Appendix B for a detailed protocol.

Genotyping of the CYP2D6*3 polymorphism was performed using the polymerase chain reaction single strand conformation (PCR-SSCP) method of detection. In brief, PCR was employed to amplify a 269 base pair DNA fragment that was subjected to SSCP gel electrophoresis on 15% (w/v) polyacrylamide (1% cross linking) gels. The gels were run in 1.5X TBE (Refer to Appendix D) at 4°C for 4 hours at 200V (Refer to Appendix B for a detailed protocol of SSCP electrophoresis). The CYP2D6*3 SSCP gels were silver stained for visualisation of the bands (refer to Appendix C for a detailed protocol for silver staining). Refer to Table 3.01 for primer sequences and annealing temperatures.

Table 3.01: A summary of the CYP polymorphisms genotyped in this study, the sequences of the primers and the annealing temperatures (T_A) of the primers.

Polymorphism	Primer*			Method of Detection	Reference
	Name	Sequence (5'-3')	T_A (°C)		
CYP1A1*m1* 6235 T>C	CYP1A1*mspI*F	TAG GAG TCT TGT CTC ATG CCT	56	C allele creates an *MspI* restriction site	Huang *et al.*, 1999
	CYP1A1*mspI*R	CAG TGA AGA GGT GTA GCC GCT			
CYP1A1*m2* 4889 A>G	CYP1A1valF	GAA CTG CCA CTT CAG CTG TCT	56	G allele creates a *HindII* restriction site	Huang *et al.*, 1999
	CYP1A1valR	GAA AGA CCT CCC AGC GGT CA			
CYP1A2 -734 C>A	CYP1A2F	GGA AGG TAT CAG CAG AAA GCC	66	A allele destroys an *ApaI* restriction site	Christiansen *et al.*, 2000
	CYP1A2R	GGC TCA TCC TTG ACA GTG CC			
CYP1B1 8372 A>C	CYP1B1F	ACT TTG GCA AGC AAA AGA GG	55	Heteroduplex SSCP analysis Termination cycle sequencing	ss# 570246 (NCBI Assay ID) This study
	CYP1B1R	CCC TTT TCA CCT TTT CAG GA			
CYP2D6*3 2637 del A	CYP2D6-3F	GAT GAG CTG CTA ACT GAG CCC	56.5	15% PAA SSCP analysis	Topic *et al.*, 2000
	CYP2D6-3R	CCG AGA GCA TAC TCG GGA C			
CYP2D6*4 1934 G>A	CYP2D6-5F	GGT GTT CCT CGC GCG CTA TG	60	*MvaI/BstNI* digestion	Brown *et al.*, 2000
	CYP2D6-5R	CTC GGT CTC TCG CTC CGC AC			

* The primers were all synthesised by IDT® (Integrated DNA Technologies, Inc, Coralville, IA, USA).

3.3.5 Statistical Analysis

Allele and genotype frequencies were calculated and graphed using Microsoft® Excel 2000. Separate analyses were conducted for each of the polymorphisms. Due to the small sample sizes, Fisher's Exact Tests were favoured over conventional chi-square goodness-of-fit tests. Tools for Population Genetic Analysis, TFPGA (Miller, 1997) was used to perform Fisher Exact Tests on the data to determine whether significant differences in genotype and allele frequencies were found between the various patient groups and clinical subgroups and the controls. TFPGA uses a Markov-Chain method to calculate unbiased estimates of *P* and a Fisher's combined probability test to

calculate multi-loci statistics (Raymond and Rousset, 1994). Sequential Bonferroni corrections (Rice, 1989) were deemed necessary due to the multiple tests performed. Significance prior to sequential Bonferroni corrections was accepted at $P<0.05$, and after application of the sequential Bonferroni correction, significance was accepted at $P<0.00052$. Tests for Hardy-Weinberg Equilibrium were performed using TFPGA (Miller, 1997).

3.4 RESULTS

Lists of drugs considered unsafe for porphyria patients have been compiled but due to the extensive nature of the lists and the fact that they change regularly due to new information that becomes available, they have not been reported on in this study. These lists aided in the selection of CYPs included in this study.

The frequency distributions of the different genotypes and alleles for the CYP1A1*m1*, CYP1A1*m2*, CYP1A2, CYP1B1, CYP2D6*3 and CYP2D6*4 polymorphisms in the VP disease group and clinical subgroups and the controls are shown in Table 3.02 and Table 3.03 respectively. A few specimens were not included in the analyses because of the poor quality of the PCR product after repeated attempts of obtaining suitable PCR products were made. The control population demonstrated Hardy-Weinberg equilibrium for all the polymorphims genotyped in this study, except for the CYP1B1 8372 A>C polymorphism where a deviation was apparent. Differences in the frequencies of the genotypes were observed between the clinical subgroups of VP patients (Appendix I Graph I.01–Graph I.06). Differences in allele frequencies were also observed between the clinical subgroups of VP patients (Appendix I Graph I.07–Graph I.12). Apart from analysing the clinical subgroups, the total patients presenting with acute attacks (AA+AASL) and the total patients presenting with skin lesions (SL+AASL) were also analysed in a further attempt to clarify whether the CYP polymorphisms modify the clinical expression of VP.

Table 3.02: Genotype distributions of CYP1A1*m1*, CYP1A1*m2*, CYP1A2, CYP1B1, CYP2D6*3 and CYP2D6*4 polymorphisms in the variegate porphyria disease group, clinical subgroups and controls.

Genotype frequencies are indicated in (%), n indicates the sample size.

	Variegate Porphyria							Controls
	AA	SL	AASL	Asym	AA+ AASL	SL+ AASL	Total	
n	4	12	6	8	10	18	30	83
CYP1A1*m1*								
T/T	1 (25.00)	11 (91.67)	6 (100.00)	5 (62.50)	7 (70.00)	17 (94.44)	23 (76.67)	66 (79.52)
T/C	3 (75.00)	1 (8.33)	0 (0.0)	3 (37.50)	3 (30.00)	1 (5.56)	7 (23.33)	16 (19.28)
C/C	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.21)
CYP1A1*m2*								
A/A	4 (100.00)	12 (100.00)	6 (100.00)	8 (100.00)	10 (100.00)	18 (100.00)	30 (100.00)	77 (93.90)
A/G	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (6.10)
G/G	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CYP1A2								
C/C	0 (0.0)	1 (8.33)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.56)	1 (3.33)	8 (9.76)
C/A	1 (25.00)	7 (58.33)	2 (33.33)	4 (50.00)	3 (30.00)	9 (50.00)	14 (46.67)	29 (35.37)
A/A	3 (75.00)	4 (33.33)	4 (66.67)	4 (50.00)	7 (70.00)	8 (44.44)	15 (50.00)	45 (54.88)

Key: AA– acute attacks; SL – skin lesions; AASL –acute attacks in combination with skin lesions; Asym – asymptomatic

Table 3.02 continued.

	Variegate Porphyria							Controls
	AA	SL	AASL	Asym	AA+ AASL	SL+ AASL	Total	
n	4	12	6	8	10	18	30	83
CYP1B1								
A/A	4 (100.00)	10 (83.33)	6 (100.00)	3 (37.50)	10 (100.00)	16 (88.89)	23 (76.67)	17 (21.25)
A/C	0 (0.0)	2 (16.67)	0 (0.0)	5 (62.50)	0 (0.0)	2 (11.11)	7 (23.33)	53 (66.25)
C/C	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (12.50)
CYP2D6*3								
A/A	4 (100.00)	12 (100.00)	5 (83.33)	8 (100.00)	9 (90.00)	17 (94.44)	29 (96.67)	73 (90.12)
A/del	0 (0.0)	0 (0.0)	1 (16.67)	0 (0.0)	1 (10.00)	1 (5.56)	1 (3.33)	8 (9.88)
del/del	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CYP2D6*4								
G/G	3 (75.00)	11 (100.00)	5 (83.33)	7 (87.50)	8 (80.00)	16 (94.12)	26 (89.65)	62 (77.50)
G/A	0 (0.0)	0 (0.0)	1 (16.67)	0 (0.0)	1 (10.00)	1 (5.88)	1 (3.45)	16 (20.00)
A/A	1 (25.00)	0 (0.0)	0 (0.0)	1 (12.50)	1 (10.00)	0 (0.0)	2 (6.90)	2 (2.50)

Key: AA– acute attacks; SL – skin lesions; AASL –acute attacks in combination with skin lesions; Asym – asymptomatic

Table 3.03: Allelic distributions of CYP1A1*m1*, CYP1A1*m2*, CYP1A2, CYP1B1, CYP2D6*3 and CYP2D6*4 in the variegate porphyria disease group, clinical subgroups and controls.

Allele frequencies are indicated in (%), n indicates the sample size.

	Variegate Porphyria							Controls
	AA	SL	AASL	Asym	AA+ AASL	SL+ AASL	Total	
n	4	12	6	8	10	18	30	83
CYP1A1*m1*								
T	5 (62.50)	23 (95.83)	12 (100.00)	13 (81.25)	17 (85.00)	35 (97.22)	53 (88.33)	148 (89.16)
C	3 (37.50)	1 (4.17)	0 (0.0)	3 (18.75)	3 (15.00)	1 (2.78)	7 (11.67)	18 (10.84)
CYP1A1*m2*								
A	8 (100.00)	24 (100.00)	12 (100.00)	16 (100.00)	20 (100.00)	36 (100.00)	60 (100.00)	159 (96.95)
G	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (3.05)
CYP1A2								
C	1 (12.50)	9 (37.50)	2 (16.67)	4 (25.00)	3 (15.00)	11 (30.56)	16 (26.67)	45 (27.44)
A	7 (87.50)	15 (62.50)	10 (83.33)	12 (75.00)	17 (85.00)	25 (69.44)	44 (73.33)	119 (72.56)
CYP1B1								
A	8 (100.00)	22 (91.67)	12 (100.00)	11 (68.75)	20 (100.00)	34 (94.44)	53 (88.33)	87 (54.38)
C	0 (0.0)	2 (8.33)	0 (0.0)	5 (31.25)	0 (0.0)	2 (5.56)	7 (11.67)	73 (45.63)
CYP2D6*3								
A	8 (100.00)	24 (100.00)	11 (91.67)	16 (100.00)	19 (95.00)	35 (97.22)	59 (98.33)	154 (95.06)
del	0 (0.0)	0 (0.0)	1 (8.33)	0 (0.0)	1 (5.00)	1 (2.78)	1 (1.67)	8 (4.94)

Key: AA– acute attacks; SL – skin lesions; AASL –acute attacks in combination with skin lesions; Asym – asymptomatic

Table 3.03 continued.

	Variegate Porphyria							Controls
	AA	SL	AASL	Asym	AA+ AASL	SL+ AASL	Total	
n	4	12	6	8	10	18	30	83
CYP2D6*4								
G	6 (75.00)	22 (100.00)	11 (91.67)	14 (87.50)	17 (85.00)	33 (97.06)	53 (91.38)	140 (87.50)
A	2 (25.00)	0 (0.0)	1 (8.33)	2 (12.50)	3 (15.00)	1 (2.94)	5 (8.62)	20 (12.50)

Key: AA– acute attacks; SL – skin lesions; AASL –acute attacks in combination with skin lesions; Asym – asymptomatic

Significant differences in genotype distributions were obtained for the CYP1A1*m1* polymorphism, prior to Bonferroni correction, for the skin lesions group versus the acute attack group of VP and for the acute attack and skin lesions (AASL) group versus the acute attacks group (Table 3.04, indicated in red). Significant *P*-values were observed for the CYP1B1 genotype distributions for the disease group and all the subgroups of VP versus the controls prior to correction (Table 3.04), with the comparison between the acute attacks versus the controls becoming non-significant after correction. The only exception to this was for the asymptomatic group versus the controls. Significant differences were also observed for the CYP1B1 genotype distributions, prior to correction, for the acute attacks and skin lesions (AASL) clinical subgroup, the total acute attacks group (AA+AASL) and for the total skin lesion group (SL+AASL) versus the asymtomatic clinical subgroup (Table 3.04, indicated in red). These results are interesting as neither of the acute attacks or skin lesions clinical subgroup showed significant *P*-values when compared to the asymtomatic group.

Table 3.04: Fishers Exact *P*-values for genotype distributions of CYP1A1*m1*, CYP1A1*m2*, CYP1A2, CYP1B1, CYP2D6*3 and CYP2D6*4 polymorphisms

	P-values					
	CYP1A1*m1*	**CYP1A1*m2***	**CYP1A2**	**CYP1B1**	**CYP2D6*3`**	**CYP2D6*4**
VP vs Controls	1.0000	0.3204	0.3451	0.0000	0.7184	0.0952
AA vs Controls	0.0738	1.0000	1.0000	0.0041	1.0000	0.1613
SL vs Controls	0.7258	1.0000	0.3256	0.0000	0.5915	0.3093
AASL vs Controls	0.6109	1.0000	1.0000	0.0004	0.4905	1.0000
Asym vs Controls	0.4140	1.0000	0.7390	0.5129	1.0000	0.1244
AA+AASL vs Controls	0.4897	1.0000	0.6794	0.0000	1.0000	0.3511
SL+AASL vs Controls	0.4173	0.5833	0.5755	0.0000	1.0000	0.4384
AA vs Asym	0.5442	1.0000	0.5756	0.0830	1.0000	1.0000
SL vs Asym	0.2527	1.0000	0.7980	0.0625	1.0000	0.4191
AASL vs Asym	0.2080	1.0000	0.6266	0.0306	0.4295	0.6871
AA+AASL vs Asym	1.0000	1.0000	0.6335	0.0061	1.0000	1.0000
SL+AASL vs Asym	0.0734	1.0000	1.0000	0.0140	1.0000	0.5442
SL vs AA	0.0267	1.0000	0.4524	1.0000	1.0000	0.2669
AASL vs AA	0.0350	1.0000	1.0000	1.0000	1.0000	0.6643
AASL vs SL	1.0000	1.0000	0.5628	0.5313	0.3313	0.3526
AA+AASL vs SL+AASL	0.1175	1.0000	0.5107	0.5233	1.0000	0.4471

Key: AA– acute attacks; SL – skin lesions; AASL –acute attacks in combination with skin lesions; Asym – asymptomatic; Red value – *P*-value significant prior to sequential Bonferroni correction; Blue value – *P*-value significant after Bonferroni correction

The results of the Fisher's Exact Test for the allele distributions gave similar results to that of the genotype distributions. The same comparisons were significant except for the acute attacks and skin lesion (AASL) clinical subgroup versus the asymptomatic clinical subgroup that was non-significant for CYP1B1 prior to Bonferroni correction. CYP1B1 comparisons between the acute attacks clinical subgroup and the acute attacks and skin lesion (AASL) clinical subgroup versus the controls and the total acute attacks (AA+AASL) clinical subgroup and the total skin lesion (SL+AASL) clinical subgroup versus the asymptomatic clinical subgroup were all non-significant

after the sequential Bonferroni correction was applied (Table 3.05, indicated in red). Comparisons between the skin lesion (SL) clinical subgroup and the acute attacks and skin lesions (AASL) clinical subgroup versus the acute attacks (AA) clinical subgroup for CYP1A1*m1*, became non-significant after the sequential Bonferroni correction was applied. The *P*-values for these analyses are summarized in Table 3.05.

Table 3.05: Fishers Exact *P*-values for allele distributions of CYP1A1*m1*, CYP1A1*m2*, CYP1A2, CYP1B1, CYP2D6*3 and CYP2D6*4 polymorphisms

	***P*-values**					
	CYP1A1*m1*	**CYP1A1*m2***	**CYP1A2**	**CYP1B1**	**CYP2D6*3`**	**CYP2D6*4**
VP vs Controls	1.0000	0.3282	0.7426	0.0000	0.7262	0.4970
AA vs Controls	0.0570	1.0000	0.6828	0.0102	1.0000	0.2763
SL vs Controls	0.4753	1.0000	0.3370	0.0003	0.5980	0.1351
AASL vs Controls	0.6113	1.0000	0.5215	0.0012	0.4822	1.0000
Asym vs Controls	0.4040	1.0000	1.0000	0.3110	1.0000	1.0000
AA+AASL vs Controls	0.4823	1.0000	0.2927	0.0000	1.0000	0.7220
SL+AASL vs Controls	0.2118	0.5901	0.6878	0.0000	1.0000	0.1353
AA vs Asym	0.3579	1.0000	0.6254	0.1286	1.0000	0.5779
SL vs Asym	0.2837	1.0000	0.5028	0.0924	1.0000	0.1667
AASL vs Asym	0.2383	1.0000	0.6724	0.0518	0.4298	1.0000
AA+AASL vs Asym	1.0000	1.0000	0.6733	0.0113	1.0000	1.0000
SL+AASL vs Asym	0.0794	1.0000	0.7489	0.0239	1.0000	0.2346
SL vs AA	0.0398	1.0000	0.3778	1.0000	1.0000	0.0621
AASL vs AA	0.0487	1.0000	1.0000	1.0000	1.0000	0.5367
AASL vs SL	1.0000	1.0000	0.2690	0.5407	0.3301	0.3485
AA+AASL vs SL+AASL	0.1281	1.0000	0.3319	0.5320	1.0000	0.1350

Key: AA– acute attacks; SL – skin lesions; AASL –acute attacks in combination with skin lesions; Asym – asymptomatic; Red value – *P*-value significant prior to sequential Bonferroni correction; Blue value – *P*-value significant after Bonferroni correction

3.5 DISCUSSION

The South African variegate porphyria population, being a founder based population, is a very valuable research tool that can aid the search for genetic factors that may influence the clinical expression of the disease. Warnich *et al.* (2002), failed to demonstrate any correlation between variation in the promoter region of *PPOX* with clinical expression of VP. A need therefore exists to identify other genes that might act as possible modifiers of clinical expression of VP.

The CYP genes are appropriate candidates fitting the role of potential modifiers of clinical expression of VP very well, as they are responsible for the metabolism of many medications and environmental pollutants. Porphyria patients are advised to use medication with extreme caution and in many cases are advised to avoid certain medications altogether due to the risk of the drugs precipitating an acute attack. Administration of the sex hormones oestradiol, oestiol and oestrone is advised to proceed only with extreme caution (SAMF, 2000) in patients suffering from porphyria. CYP1A1, CYP1A2 and CYP1B1 all participate in the metabolism and hydroxylation of these hormones and polymorphism within these genes may lead to altered function and activity of the encoded enzymes resulting in altered metabolism of the hormone. The possibility also exists that these polymorphisms may be in linkage disequilibrium with other regulatory elements elsewhere in the genes, and as such may be responsible for altered expression or activity of the enzyme product. CYP2D6 is responsible for the metabolism of many of the medications contraindicated in VP and was therefore also included as a potential modifier. Christiansen *et al.*, (2000) has shown association with the A/A genotype of CYP1A2 and clinical manifestation of PCT, providing further evidence for the possible modifying role the CYPs may play in the clinical expression of other porphyria types.

In this study we examined the frequency of 5 common CYP polymorphisms (CYP1A1*m1*, CYP1A1*m2*, CYP1A2 -734 C>A, CYP2D6*3 and CYP2D6*4) and a recently described single nucleotide polymorphism (SNP) in the 3'UTR of exon 3 of CYP1B1 in VP patients compared to healthy controls (Table 3.02 and Table 3.03). Uncorrected and corrected *P*-values are shown (Table 3.04 and Table 3.05) as

uncorrected *P*-values may result in Type I errors, while corrected *P*-values may result in Type II errors, due to the stringent, conservative nature of sequential Bonferroni corrections (SISA, 2002). The necessity of Bonferroni corrections in association studies is still uncertain and remains debatable.

CYP1A1*m1* yielded significant differences for comparisons of genotype distribution and allele frequencies involving the skin lesion (SL) clinical subgroup versus the acute attack (AA) clinical subgroup and for the acute attack and skin lesion (AASL) clinical subgroup versus the acute attack subgroup (AA) prior to Bonferroni correction. The T-allele is the predominant allele in patients presenting with skin lesions (Table 3.03) with only one out of twelve patients presenting skin lesions having a single C-allele. This indicates a possible role for the T-allele in increasing the susceptibility of a patient to develop skin lesions, while the C-allele may offer protection against developing skin lesions. The observed frequency of the T-allele is also greater than the frequency of the C-allele observed in the asymptomatic patients. These patients may be protected from developing skin lesions by another allele or, alternatively, environmental factors may influence the development of or protect against the development of skin lesions in these patients.

We show that the T/C genotype of CYP1A1*m1* in the acute attacks subgroup of VP is increased compared to the control group. Although the *P*-value for this comparison is marginally ($P<0.08$), increasing the number of patients included in a follow up study may demonstrate statistical significance between the T/C genotype and the acute attack of VP. The *m1* (C/C) genotype of CYP1A1 represents a phenotype of high inducibility (Peterson *et al.*, 1991, Landi *et al.*, 1994) and has been associated experimentally with higher catalytic activity (Mucci *et al.*, 2001). This increased activity of the enzyme may alter the concentration and state of the relative metabolites of its substrates. These alterations in metabolism may indeed have an effect on the clinical expression of the acute attack of VP. Expression studies in a laboratory model of VP are recommended to ascertain the effect that increased catalytic activity of this enzyme has on the clinical expression of VP, especially the development of the acute attack. A mouse model would be useful in achieving this as CYP1A1 is relatively conserved between humans and mice (Ingelman-Sundberg, 1998). A useful

R59W heterozygous mouse model, for the biochemical phenotype of VP, is described by Medlock *et al.* (2002). Studying this model may give insights into the pathogenic mechanisms operative in the clinical expression of VP.

Our results show that the frequency of the A/A genotype of CYP1B1 in all the clinical subgroups of VP involving acute attacks and skin lesions is greatly increased compared to the control group (Table 3.02). The frequency of the A-allele is increased in all VP clinical subgroups including the total disease group compared to that of the control group. Highly significant *P*-values were observed after Fisher's Exact Tests in comparisons between VP patients (disease group and clinical subgroups) and controls for both genotype and allele distributions (Table 3.04 and Table 3.05). It is of extreme interest to note that significant values were observed for the acute attack and skin lesion clinical subgroups when compared to either the control group or the asymptomatic clinical subgroup. Significant differences were observed for comparisons between the acute attacks and the skin lesions clinical subgroups and the controls. The same is however not true for comparisons between the acute attacks and the skin lesions clinical subgroups and the asymptomatic clinical subgroup. The possibility exists that some of the asymptomatic patients have not yet been exposed to agents known to precipitate the development of acute attacks or skin lesions and therefore these patients may have been incorrectly classified. Some of the significant *P*-values did, however, become non-significant after the sequential Bonferroni correction was applied (*P*-values indicated in red in Table 3.04 and Table 3.05). It is interesting to note that the clinical symptom involved in the majority of the comparisons that lost significance after the application of the Bonferroni correction, was the acute attack. Diagnosis of the acute attack is partial to the subjectivity of the consulting clinician and is further hampered as families that present with the acute attack of VP, may encourage subsequent generations to avoid known precipitating agents of the acute attacks. It is therefore possible that some of the patients that should "genetically" present with acute attacks are missed. Diagnosis of skin lesions on the other hand, is a much simpler, easier process.

Although definite conclusions cannot be made from this study due to the relatively small number of patients included in the study, the difference in genotype and allele

distributions between control and asymptomatic patients and patients presenting with acute attacks, skin lesions or both are suggestive of an association between these symptoms and the A/A genotype or A-allele of CYP1B1. Evidence in support of this conclusion is made even stronger by the observation that in all of the clinical subgroups involving the presentation of acute attacks the frequency of the A-allele is 100.00%. This allele may therefore increase the patients' susceptibility to developing acute attacks or it may be in linkage disequilibrium with other susceptibility loci situated else where in the gene. The structure of the mRNA of this gene may be altered due to this polymorphism, as it has been shown that variations in the 3'UTR of a gene have the potential to alter the stem-loop structure of mRNA and as such affect mRNA processing and stability (Williams *et al.*, 1994, Williams and Marzluff, 1995, Dean *et al.*, 2001 and Grzybowska *et al.*, 2001). The observation that the control population deviated from Hardy-Weinberg equilibrium for the CYP1B1 polymorphism necessitates the expansion of the control population. The reason for this deviation is unknown.

Expression studies involving CYP1B1 carrying these two alleles will have to be performed to identify if one of the alleles (wild type or variant) carries higher activity of the enzyme. Using a mouse model of VP is not recommended in this case, as there is no notable homology between the rodent ortologue and the human CYP1B1 enzyme (Ingelman-Sundberg, 1998). The *P*-value observed for the comparison involving the asymptomatic clinical subgroup and the controls for CYP1B1, was so insignificant that it suggests further evidence that this SNP or a locus linked to it may indeed participate in the modification of the clinical expression of VP. Our results therefore "strongly" suggest that CYP1B1 may be a major modifier gene of the clinical expression of VP, as even with these small sample sizes extremely significant *P*-values were observed.

CYP1B1 is in a key position to be a modifier gene of the clinical expression of VP due to its location in extrahepatic organs (Shimada *et al.*, 1996, Hakkola *et al.*, 1997) including the skin (Sutter *et al.*, 1994), where its expression is induced by UV light (Katiyar *et al.*, 2000), and the central nervous system (CNS), where its localisation at the interface of the blood and brain suggests that this enzyme may act as an enzymatic

barrier preventing the entry of chemicals into adjacent brain parenchyma (Rieder *et al.*, 2000). As the clinical expression of VP involves the CNS and the skin, CYP1B1 may be responsible for the modification of the symptoms.

The results observed for the other CYP polymorphisms genotyped in this study all rendered non-significant *P*-values. We show that the frequency of the A/A genotype of CYP1A2 in the acute attack (AA), acute attack and skin lesion (AASL) and the acute attack and acute attack + skin lesion (AA+AASL) clinical subgroups of VP are increased when compared to the control group (*P*-values all non-significant). The A/A genotype of this polymorphism is associated with the skin lesions observed in patients with PCT (Christiansen *et al.*, 2001). We would therefore have expected a similar association with the skin lesions of VP. The C/A polymorphism located in the non-coding region of CYP1A2 may be responsible for a differential binding of regulatory proteins to the surrounding sequence leading to variations in the expression level of CYP1A2 or it may be in linkage disequilibrium with regulatory loci elsewhere in the gene. The A/A genotype represents a highly inducible genotype that is associated with increased activity of CYP1A2 upon exposure to inducing agents including smoking (MacLeod *et al.*, 1998, Sachse *et al.*, 1999). The highly inducible genotype (A/A) of CYP1A2 may be a susceptibility factor for the development of symptoms and may be induced by environmental factors that these patients are exposed to. Increasing the number of patients included in this study may allow this proposed effect to be observed.

The G-allele of CYP1A1*m2* is very rare in our study populations with only 5 of 83 individuals of the control group having one copy of this allele. The substitution of a G for an A in exon 7 of CYP1A1 causes an amino acid change (Ile to Val) in the haem-binding region of the protein. The Val variant has almost two fold higher catalytic enzyme activity than the Ile form (Landi *et al.*, 1994, Autrup, 2000). It seems that this polymorphism has no relevance in the South African VP population. Mucci *et al.* (2001) reports that homozygotes of this allele are more common in Japanese than Caucasian populations.

CYP2D6 plays an important role in the metabolism of many of the medications contraindicated in porphyria patients and in the metabolism of opioids, used to treat an acute attack. We therefore screened our VP patients and healthy controls for two of the poor metaboliser genotypes (CYP2D6*3 and CYP2D6*4) of this gene. Our data show that the frequency of the wild type alleles are far increased compared to the number of variant alleles in our populations (Table 3.03). Although not statistically significant, there were slightly higher wildtype frequencies in the VP disease group compared to the controls. The frequency of the CYP2D6*4 A-allele in our healthy controls was increased compared to the frequency observed by Topic *et al.*, (2000) in their healthy control group. This is indicative of the expected inter-ethnic difference in allele frequencies. The number of patients included in this study was however limited, therefore it is suggested that more patients be included in a follow up study to provide a clearer picture of the extent of correlation between VP (and its clinical expression) and CYP2D6.

Although the numbers of patients included in this study are relatively small, the results observed suggest that CYP1B1 plays a more important role in the modification of the clinical expression of variegate porphyria than the other CYPs studied. It may be that CYP1A1, CYP1A2 and CYP2D6 are less significant modifiers of the clinical expression of VP and that increasing the number of patients may give a truer reflection of the role they play. It would be very interesting to study the effects of these polymorphisms on the clinical expression of VP in other populations (Japanese, Finnish, Turkish etc.) to ascertain the relevance of our results compared to results in these populations. It may be possible that these polymorphisms exercise subtle effects, increasing the susceptibility for certain clinical features that will only be apparent under specific environmental conditions. Furthermore, the results we obtained may be population specific or may be the result of an error due to the small sample size.

Although cytochrome P450s have been implicated as possible modifier genes of the clinical expression of VP (Von und zu Fraunberg *et al.*, 2002), this is, to the best of our knowledge, the first study to investigate the association between the CYPs and VP. We have shown that the cyotochrome P450 genes, especially CYP1B1 due to the

highly significant associations it displayed, are good candidates for modifying the clinical expression of VP. Clinical expression of VP is complex and the possibility exists that more than one gene plays a role in modifying it. Future studies using DNA chip technology would enable the simultaneous identification of different alleles from genes suspected of playing a role in the modification of the clinical expression of VP. DNA chips have the potential to exert an enormous impact on the elucidation of the possible multi-factorial modification of the clinical expression of VP. Looking simultaneously at all of the pieces of the VP puzzle could, in future studies, greatly increase our understanding of the relative roles played by genetics and the environment in modifying clinical expression of variegate porphyria.

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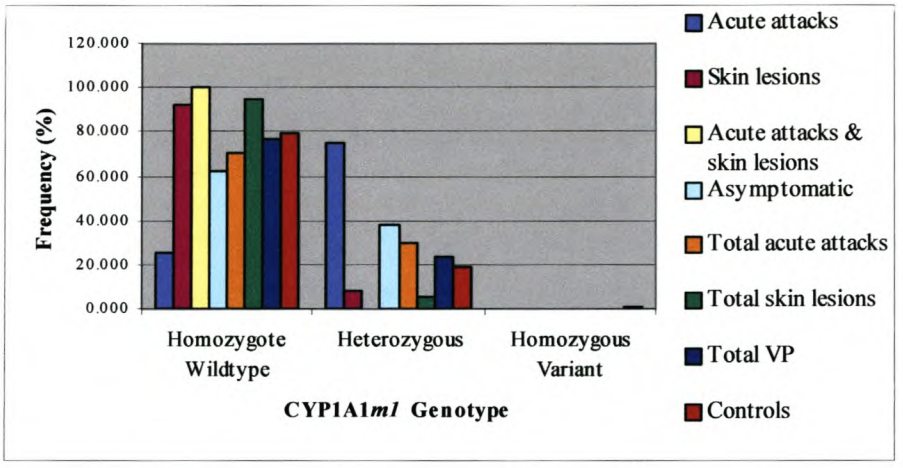
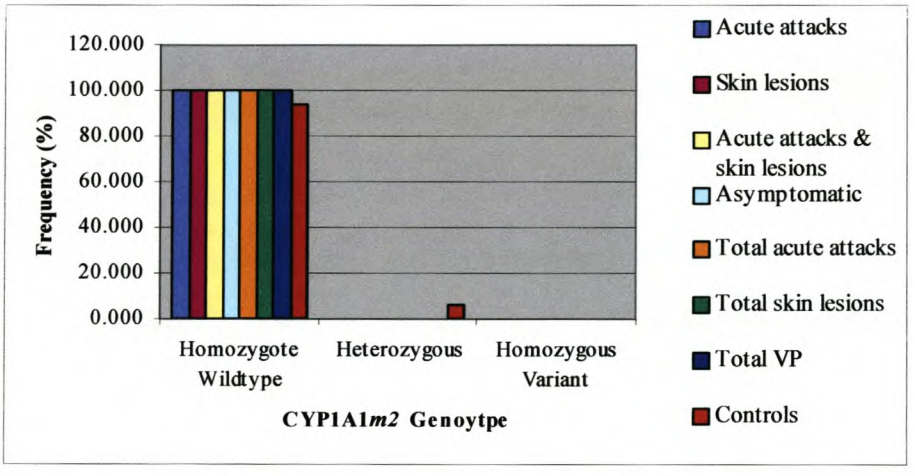
    - Determining the CYPs involved with metabolism of the unsafe drugs
    - Literature search for CYP polymorphisms

- Selection of CYP polymorphisms to include in the study (with assistance from Prof Warnich)
- DNA extractions of individuals making up the control population
- R59W screening of the control individuals
- Primer design of CYP1B1 primers
- Screening and genotyping of CYP polymorphisms
    - PCR amplification
    - Restriction enzyme digests
    - Agarose gel electrophoresis
    - SSCP gel electrophoresis
    - Heteroduplex SSCP gel electrophoresis
    - PCR purification
    - DNA sequencing reactions
- Data analysis
- Writing of manuscript

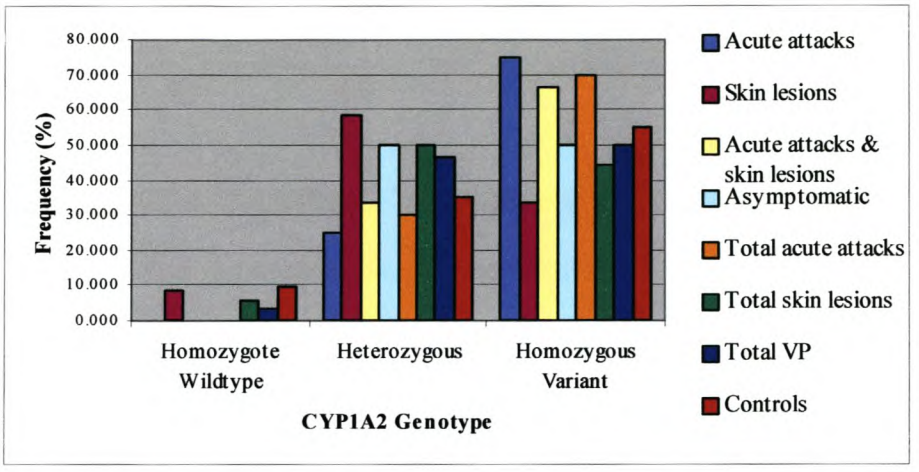
APPENDIX I



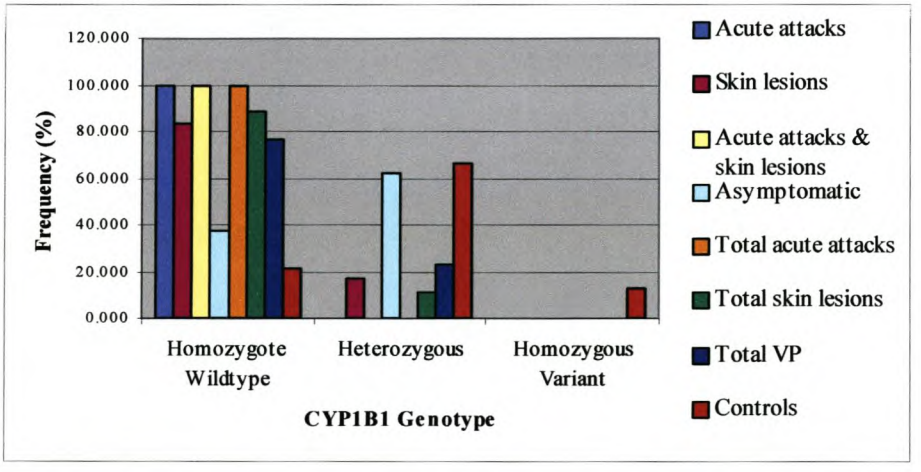
Graph I.01: Comparison of CYP1A1*m1* genotype frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



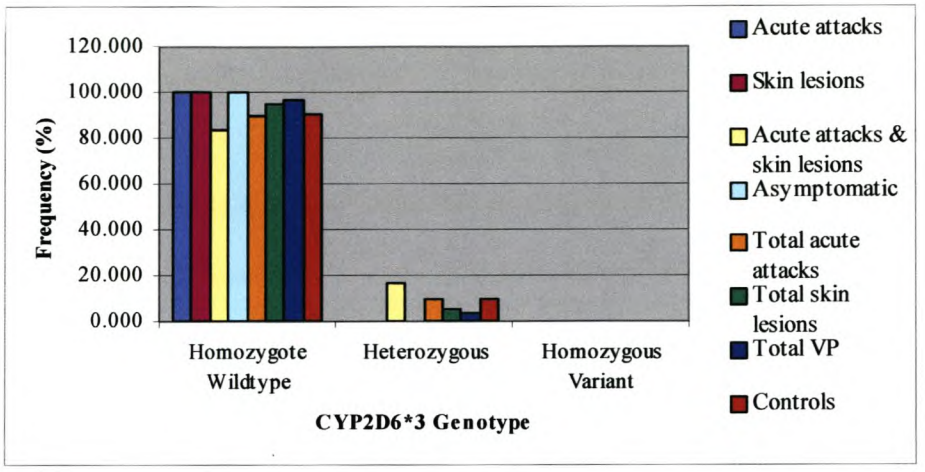
Graph I.02: Comparison of CYP1A1*m2* genotype frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



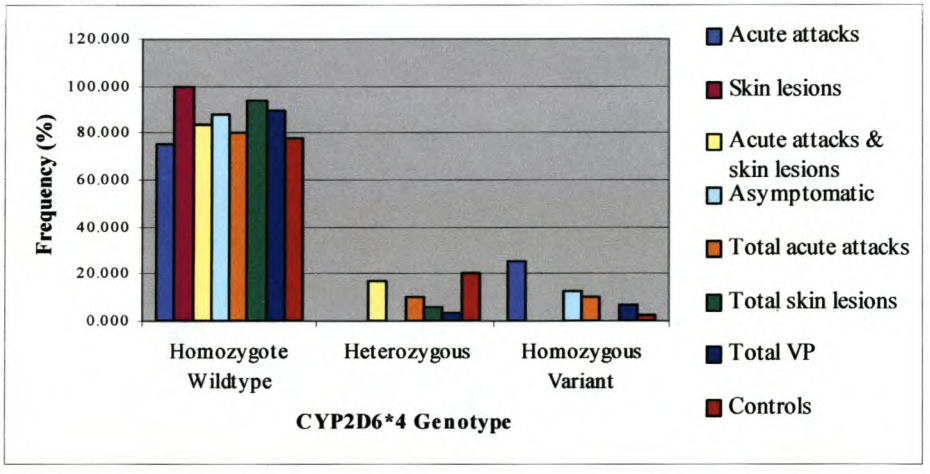
Graph I.03: Comparison of CYP1A2 genotype frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



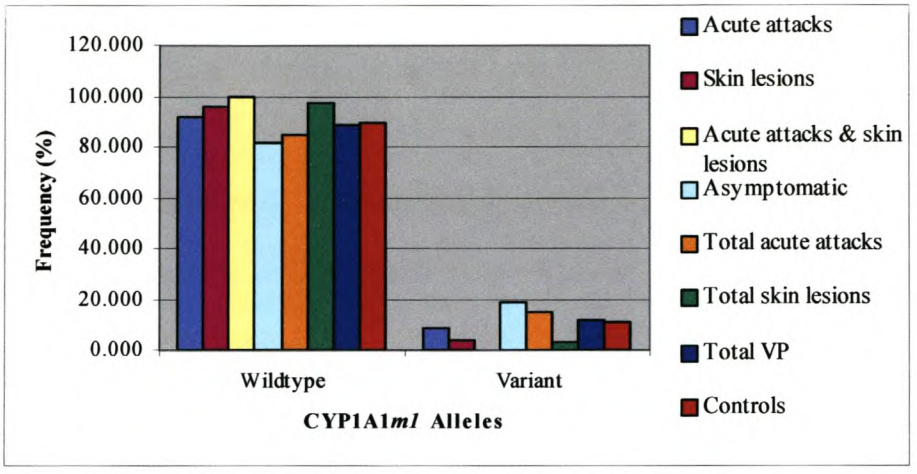
Graph I.04: Comparison of CYP1B1 genotype frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



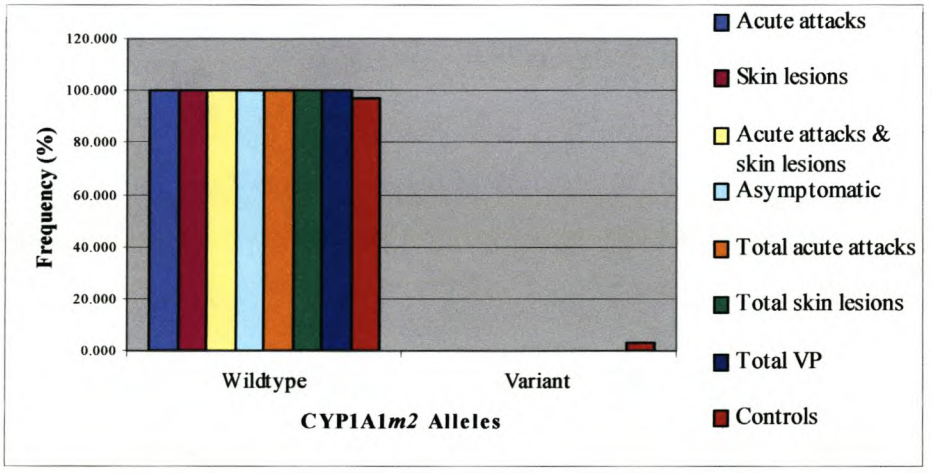
Graph I.05: Comparison of CYP2D6*3 genotype frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



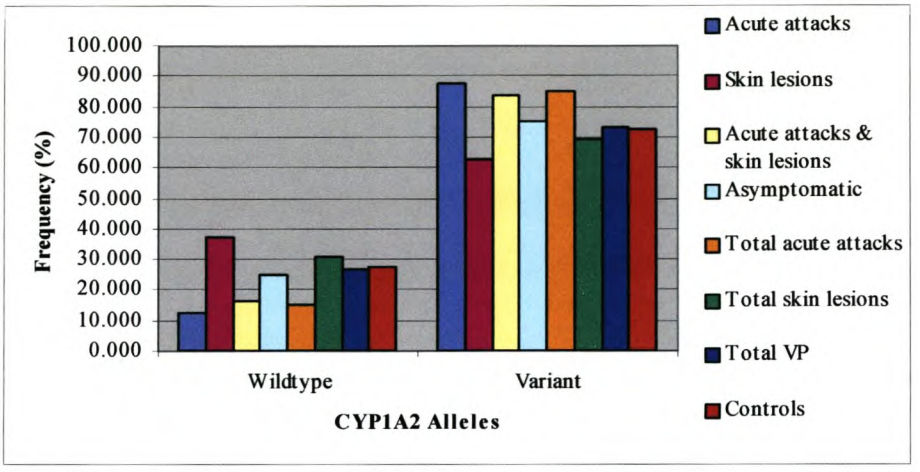
Graph I.06: Comparison of CYP2D6*4 genotype frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



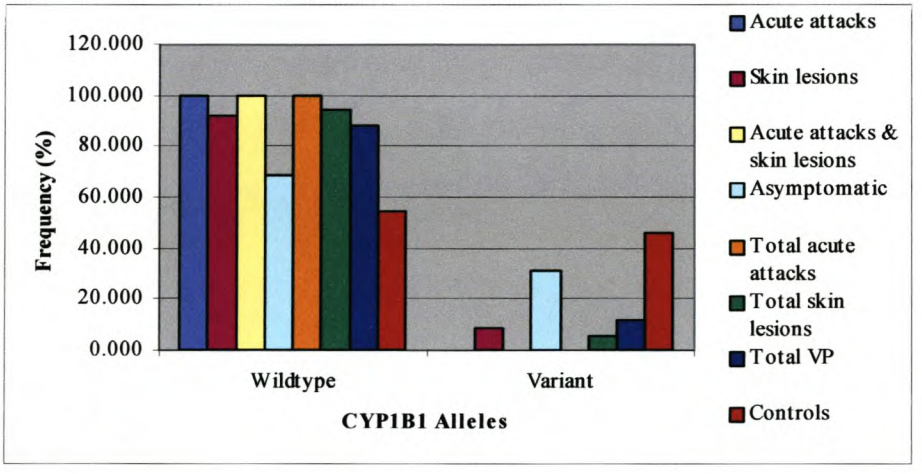
Graph I.07: Comparison of CYP1A1*m1* allele frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



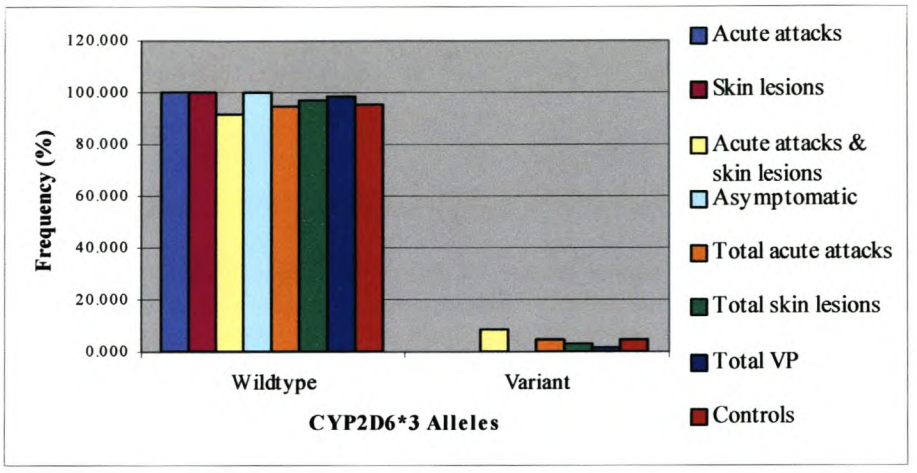
Graph I.08: Comparison of CYP1A1*m2* allele frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



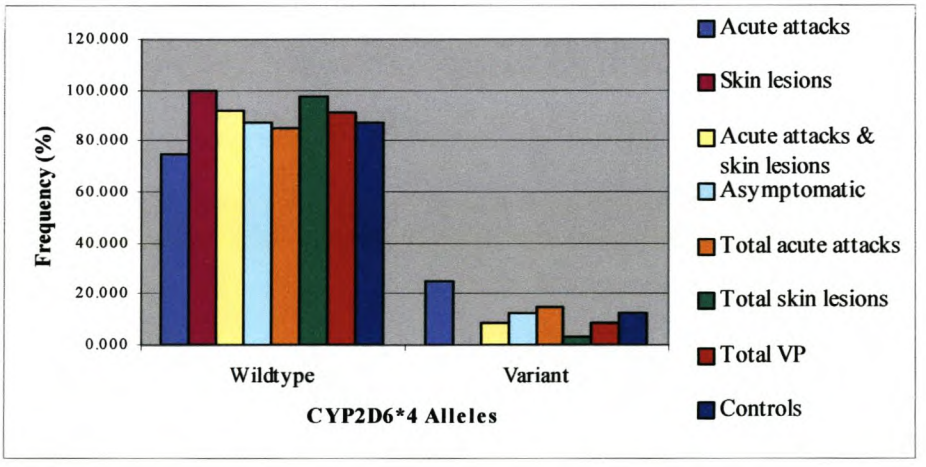
Graph I.09: Comparison of CYP1A2 allele frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



Graph I.10: Comparison of CYP1B1 allele frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



Graph I.11: Comparison of CYP2D6*3 allele frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls



Graph I.12: Comparison of CYP2D6*4 allele frequencies for variegate porphyria patients (disease group and clinical subgroups) and controls

CHAPTER FOUR

CYP POLYMORPHISMS IN TWO SOUTH AFRICAN BREAST CANCER POPULATIONS: AN ASSOCIATION STUDY

4.1 ABSTRACT

Breast cancer is one of the leading causes of death in women due to cancer. It is a multifactorial disease and is neither purely genetic nor purely environmental. At 50 years of age, risk of developing breast cancer is approximately 1 in 50, at 80 years of age, risk increases to 1 in 10. Mutations in major cancer susceptibility genes identified to date, such as *BRCA1* and *BRCA2* are responsible for only 5-10% of all observed breast cancer cases. It is believed that breast cancer may be the result of co-participation between common DNA alterations and environmental exposures, including hormone replacement therapy (oestrogen), smoking, absence of breast-feeding and contraception use. Polymorphisms in enzymes involved with the metabolism of these environmental exposures, such as the cytochrome P450 enzymes (CYP), are therefore expected to play an etiological role in breast cancer. Four CYP polymorphisms (CYP1A1*m1*, CYP1A1*m2*, CYP1A2 –734 C>A and CYP1B1 8372 A>C) were genotyped in two groups of breast cancer patients (29 Caucasians and 67 of mixed ancestry) and healthy population-matched controls. Significant differences in genotype distributions ($P<0.0001$) and allele frequencies ($P<0.03$) were observed between the mixed ancestry breast cancer patients and the mixed ancestry controls for the CYP1B1 8372 A>C polymorphism. Significant differences in allele frequencies were also observed between the two control populations for the CYP1A1*m1* ($P<0.02$) and CYP1B1 8372 A>C ($P<0.03$) polymorphisms. Differences in allele frequencies for the variant alleles were observed between the two groups of breast cancer patients. These results emphasize the importance of population-based risk assessment when genetic testing and counselling for complex disease susceptibility is offered.

4.2 INTRODUCTION

Breast cancer is the most prevalent cause of death due to malignancy in South African women (SAPA, 10/10/2002). Breast cancer is a complex disease, as it is neither exclusively genetically, nor exclusively environmentally developed. The role of changing environmental factors in the development of breast cancer is indicated by a decrease in age of onset of the disease (Kristensen and Børresen-Dale, 2000). The genetic basis of breast cancer does not only result from the inheritance of specific rare mutations, but may be the result of inheriting various relatively common mutations, with many genes each making a small contribution to overall susceptibility (Campbell *et al.,* 2000). Research is currently focused on the identification of susceptibility genes i.e. genes that encode a product with which an environmental agent may interact, causing an alteration of normal cellular functions and critical life processes of the cell, leading ultimately to toxicity or malignancy (Nebert and Roe, 2001). Allelic variants of genes encoding for drug metabolising enzymes, drug receptor molecules and drug transporter genes are expected to be factors affecting enhanced or reduced risk of cancer susceptibility and toxicity to drugs and environmental carcinogens (Nebert, 1999).

Early age at menarche, late menopause, high serum or urine oestrogen levels and low levels of sex hormone binding protein (that leads to a high availability of unbound oestradiol) have been identified as risk factors for the development of breast cancer, suggesting a role for endogenous and exogenous oestrogens in breast cancer development (Hankinson *et al.,* 1998). Oestrogens provide mitotic stimulation and promote tumour growth in breast cancer (Huber *et al.,* 2002). Hsu *et al.* (1991) has shown that upregulation of oncogenes by oestrogen results in the stimulation of breast cancer cell proliferation while Tesarik *et al.* (1999) attributes modulation of breast cancer cell apoptosis to oestradiol. Oestradiol synthesis from cholesterol occurs in a number of enzymatic steps many of which are catalysed by cytochrome P450 (CYP) enzymes.

Of all CYPs, CYP1B1 is expressed at the highest levels in breast cancer tissue (Kristensen and Børresen-Dale, 2000) and has the strongest oestradiol activity (Mucci *et al.,* 2001). CYP1B1 variants are associated with altered oestrogen metabolism (Mucci *et al.,* 2001). CYP1B1 is therefore placed in a key position for mammary carcinogenesis due to the

endogenous production of potentially carcinogenic catechol oestrogens and its ability to metabolically activate exogenous procarcinogens (Bailey *et al.,* 1998a). CYP1B1 is also important for the homeostasis of oestrogen in extrahepatic tissues such as the breast (Bailey *et al.,* 1998a), for transformation of xenobiotics (Huber *et al.,* 2002) and is capable of activating environmental procarcinogens and anticancer agents (Huber *et al.,* 2002).

CYP1A1, a highly inducible enzyme (Coughlin and Piper, 1999), is said to be the principle enzyme in the activation of cigarette-smoke and other environmental pollutants including, but not limited to, polycyclic aromatic hydrocarbons (PAH), dioxin, aromatic amines, polychlorinated biphenyls and nitrosamines (reviewed in Kristensen and Gut, 1994). Heterocyclic aromatic amines (Morris and Seifter, 1992) and PAHs (Nagao *et al.,* 1994) are, in experimental models, potent mammary carcinogens. CYP1A1 is a participant in the first step of procarcinogen conversion to DNA-binding, carcinogenic forms (Kawajiri, 1999) and is involved in the metabolism of oestrogens (Mucci *et al.,* 2001) and in oestrogen hydroxylation (Huang *et al.,* 1999b). CYP1A1 is also involved in xenobiotic metabolism (Huber *et al.,* 2002). Polymorphism within CYP1A1 may affect the distribution of the relative metabolites of PAHs and oestrogen, and as such determine susceptibility to cancer (Yager, 1996, Zhu and Conney, 1998). Exposure to cigarette smoke from a young age combined with the *m1* or *m2* polymorphism of CYP1A1 (refer to Table 4.01) has been suggested to increase risk of developing breast cancer (Kristensen and Børresen-Dale, 2000). It is however unclear whether increased risk due to CYP1A1 genotype is dependant on exposure to environmental factors (Huber *et al.,* 2002). As these two polymorphisms have been associated with increased catalytic activity of the enzyme, it is suggested that polymorphism present within this gene may have an effect on the rate of PAH activation or may increase the concentration of oestrogen agonists and may therefore play a role in hormone or tobacco-dependant cancers (Mucci *et al.,* 2001). Great ethnic variation exists in the genotype distributions of CYP1A1 polymorphisms (Huber *et al.,* 2002).

CYP1A2 metabolises aromatic amine procarcinogens and is involved in hepatic and extrahepatic oestrogen hydroxylation to a lesser extent than CYP1A1 and CYP1B1 (Hanna *et al.,* 2000). Differences in CYP1A2 mRNA levels were observed in a study by Nebert *et al.* (1996) and the suggestion is made that these interindividual differences in expression may

play a role in toxicity and cancer. CYP1A2 is one of the enzymes primarily responsible for oestrogen hydroxylation in human liver (Martucci and Fishman, 1993).

In this association study, we evaluate the contribution of four SNPs (CYP1A1*m1*, CYP1A1*m2*, CYP1A2 –734 C>A and CYP1B1 8372 A>C) to a well defined clinical endpoint in two well characterised South African breast cancer populations and relevant control groups.

4.3 MATERIAL AND METHODS

4.3.1 Study Populations

Blood samples, from 96 breast cancer patients (29 Caucasian and 67 of mixed ancestry (Nurse *et al.*, 1985)) were collected at Tygerberg Hospital after clinical and pathological evaluation and diagnosis of breast cancer was obtained. A baseline questionnaire collected information on potential risk factors for breast cancer as well as lifestyle habits (e.g. smoking status, alcohol consumption, cooking methods (oil, gas, electricity, fire)). Individuals constituting the healthy control populations were randomly selected and consisted of 76 Caucasians and 80 individuals of mixed ancestry. All individuals participating in this study gave their written informed consent. Ethical approval was obtained from the Ethics Committee, Faculty of Health Sciences, University of Stellenbosch.

4.3.2 DNA Extraction

Total genomic DNA was extracted using a modified protocol of Miller *et al.* (1998) (refer to Appendix A for a detailed protocol) and the extracted DNA was stored at -20°C until genotype analysis.

4.3.3 Genotyping of Cytochrome P450 Polymorphisms

PCR-based RFLP assays were used to determine the CYP1A1*m1*, CYP1A1*m2* and CYP1A2 genotypes (refer to Appendix B for a detailed protocol). Genotyping of the CYP1B1 polymorphism was performed using the polymerase chain reaction heteroduplex single strand conformation (PCR-hex SSCP) (Kotze *et al.*, 1995) method of detection. The primers were designed from the CYP1B1 sequence (gi 17446619, NCBI) using the program Primer3 available online (Table 4.01). In brief, PCR was employed to amplify a 242 base pair DNA

fragment that was subjected to heteroduplex SSCP analysis. The denaturing polyacrylamide gels were stained in 1.5X TBE containing a final concentration of 0.0075% (v/v) EtBr, for 20 minutes and bands were visualised in the gel with UV light and subsequently photographed with the GelDoc system (BioRad, Hercules, CA, USA). Termination cycle sequencing was employed to confirm the presence/absence of the polymorphism. PCR products were purified using a QIAquick® PCR Purification Kit (QIAGEN, GmbH, D-40724, Hilden). Sequencing reactions were performed using a Perkin Elmer BigDye™ Terminator Cycle Sequencing Kit (PE Applied Biosystems, Warrington WA, 4SR, Great Britain) and the forward primer as described in Table 4.01. These reactions were run on an ABI 3100 (PE Applied Biosystems, Warrington WA, 4SR, Great Britain) to determine their sequences. Sequences were analysed using the programs Sequencing Analysis (V3.07) and BioEdit Sequence Alignment Editor (Hall, 1999). Refer to Appendix B for a detailed protocol.

Table 4.01: A summary of the CYP polymorphisms genotyped in this study and the sequences and annealing temperatures (T_A) of the primers.

Polymorphism	Primer*			Method of Detection	Reference
	Name	Sequence (5'-3')	T_A (°C)		
CYP1A1*m1* 6235 T>C	CYP1A1*mspI*F	TAG GAG TCT TGT CTC ATG CCT	56	C allele creates an *MspI* restriction site	Huang *et al.*, 1999b
	CYP1A1*mspI*R	CAG TGA AGA GGT GTA GCC GCT			
CYP1A1*m2* 4889 A>G	CYP1A1valF	GAA CTG CCA CTT CAG CTG TCT	56	G allele creates a *HindII* restriction site	Huang *et al.*, 1999b
	CYP1A1valR	GAA AGA CCT CCC AGC GGT CA			
CYP1A2 -734 C>A	CYP1A2F	GGA AGG TAT CAG CAG AAA GCC	66	A allele destroys an *ApaI* restriction site	Christiansen *et al.*, 2000
	CYP1A2R	GGC TCA TCC TTG ACA GTG CC			
CYP1B1 8372 A>C	CYP1B1F	ACT TTG GCA AGC AAA AGA GG	55	Heteroduplex SSCP analysis Termination cycle sequencing	ss# 570246 (NCBI Assay ID) This study
	CYP1B1R	CCC TTT TCA CCT TTT CAG GA			

* The primers were all synthesised by IDT® (Integrated DNA Technologies, Inc, Coralville, IA, USA).

4.3.4 Statistical Analysis

Allele and genotype frequencies were calculated and graphed using Microsoft® Excel 2000. Separate analyses were conducted for each of the polymorphisms. Due to the small sample sizes, Fisher's Exact was favoured over conventional chi-square goodness-of-fit tests. Tools

for Population Genetic Analysis, TFPGA (Miller, 1997) was used to perform Fisher Exact Tests to determine whether significant differences in genotype and allele frequencies were found between the various patient groups and the controls. TFPGA uses a Markov-Chain method to calculate unbiased estimates of *P* and a Fisher's combined probability test to calculate multi-loci statistics (Raymond and Rousset, 1994). Due to the conservative nature of exact tests and the lack of multiple tests (Rice, 1989), Bonferroni corrections were not deemed necessary (Louis and Dempster, 1987). Significance was accepted at $P < 0.05$. Tests for Hardy-Weinberg Equilibrium were performed using TFPGA (Miller, 1997).

4.4 RESULTS

The frequency distributions of the different genotypes and alleles for the CYP1A1*m1*, CYP1A1*m2*, CYP1A2 and CYP1B1 polymorphisms are shown in Table 4.02 and Table 4.03 respectively. A few specimens were not included in the analyses because of the poor quality of the PCR product after numerous attempts were made to achieve suitable product. Differences were observed in the frequencies of genotypes (Graph II.01 - Graph II.04) and alleles (Graph II.05 - Graph II.08) between the two ethnic groups (Caucasian and mixed ancestry). The Caucasian and mixed ancestry control populations demonstrated Hardy-Weinberg equilibrium for all the polymorphims genotyped in this study, except for the CYP1B1 8372 A>C polymorphism where a deviation was apparent.

Table 4.02: Genotype distributions of CYP1A1*m1*, CYP1A1*m2*, CYP1A2 and CYP1B1 polymorphisms for Caucasian and mixed ancestry breast cancer cases and controls.

	Caucasian		Mixed Ancestry	
Genotype	**Cases (%)**	**Control (%)**	**Cases (%)**	**Control (%)**
CYP1A1*m1*				
T/T	24 (82.76)	60 (78.94)	42 (64.62)	49 (62.03)
T/C	5 (17.24)	15 (19.74)	21 (31.31)	26 (32.91)
C/C	0 (0.0)	1 (1.32)	2 (3.08)	4 (5.06)
	n=29	n=76	n=65	n=79
CYP1A1*m2*				
A/A	28 (96.55)	70 (93.33)	60 (92.31)	73 (92.41)
A/G	1 (3.45)	5 (6.67)	5 (7.69)	6 (7.6)
G/G	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	n=29	n=75	n=65	n=79
CYP1A2				
C/C	2 (6.9)	8 (10.67)	8 (12.31)	3 (3.9)
C/A	9 (31.03)	27 (36.0)	25 (38.46)	31 (40.26)
A/A	18 (62.07)	40 (53.33)	32 (49.23)	43 (55.84)
	n=29	n=75	n=65	n=77
CYP1B1				
A/A	6 (21.43)	16 (21.91)	14 (22.22)	23 (29.11)
A/C	18 (64.29)	51 (69.86)	45 (71.43)	24 (30.38)
C/C	4 (14.29)	6 (8.23)	4 (6.35)	32 (40.51)
	n=28	n=73	n=63	n=79

Table 4.03: Allele distributions of CYP1A1*m1*, CYP1A1*m2*, CYP1A2 and CYP1B1 polymorphisms for Caucasian and mixed ancestry breast cancer cases and controls.

	Caucasian		Mixed Ancestry	
Allele	**Cases (%)**	**Control (%)**	**Cases (%)**	**Control (%)**
CYP1A1*m1*				
T	53 (91.38)	135 (88.80)	158 (84.04)	124 (78.48)
C	5 (8.62)	17 (11.18)	30 (15.96)	34 (21.52)
CYP1A1*m2*				
A	57 (98.28)	145 (96.67)	182 (96.81)	152 (96.20)
G	1 (1.72)	5 (3.33)	6 (3.19)	6 (3.8)
CYP1A2				
C	13 (22.41)	43 (28.67)	54 (28.72)	37 (24.03)
A	45 (77.59)	107 (71.33)	134 (71.28)	117 (75.97)
CYP1B1				
A	30 (53.57)	83 (56.85)	103 (56.59)	70(44.30)
C	26 (46.43)	63 (43.15)	79 (43.41)	88 (55.70)

Significant differences in genotype distributions (P<0.0001, Table 4.04) and allele frequencies (P<0.03, Table 4.05) were observed between the mixed ancestry breast cancer patients and the mixed ancestry controls for the CYP1B1 8372 A>C polymorphism. Significant results were obtained for the two control populations for CYP1A1*m1* (P<0.02, Table 4.05) and CYP1B1 (P<0.03, Table 4.05).

Table 4.04: Fishers Exact *P*-values for genotype distributions of CYP1A1*m1*, CYP1A1*m2*, CYP1A2 and CYP1B1 polymorphisms.

			Controls	
Polymorphism	**Breast Cancer Patients**	**Caucasian Breast Cancer Patients**	**Caucasians**	**Mixed Ancestry**
CYP1A1*m1*	Caucasian		1.0000	
	Mixed Ancestry	0.2240		0.8749
	Caucasian Controls			0.0680
CYP1A1*m2*	Caucasian		1.0000	
	Mixed Ancestry	0.6593		1.0000
	Caucasian Controls			1.0000
CYP1A2	Caucasian		0.7675	
	Mixed Ancestry	0.5789		0.1829
	Caucasian Controls			0.2760
CYP1B1	Caucasian		0.6776	
	Mixed Ancestry	0.4457		0.0000
	Caucasian Controls			0.0000

Table 4.05: Fishers Exact *P*-values for allele distributions of CYP1A1*m1*, CYP1A1*m2*, CYP1A2 and CYP1B1 polymorphisms.

			Controls	
Polymorphism	**Breast Cancer Patients**	**Caucasian Breast Cancer Patients**	**Caucasians**	**Mixed Ancestry**
CYP1A1*m1*	Caucasian		0.801	
	Mixed Ancestry	0.0871		0.766
	Caucasian Controls			0.0195
CYP1A1*m2*	Caucasian		1.000	
	Mixed Ancestry	0.6663		1.000
	Caucasian Controls			1.0000
CYP1A2	Caucasian		0.388	
	Mixed Ancestry	0.2327		0.1475
	Caucasian Controls			0.3476
CYP1B1	Caucasian		0.758	
	Mixed Ancestry	0.6269		0.0231
	Caucasian Controls			0.0253

A multivariate logistic regression analysis of the populations based on confounding factors (history of breast feeding, use of contraception, hormone replacement therapy, number of pregnancies and smoking) was not feasible due to the unavailability of suitably defined control populations. However, Fisher exact tests were performed on the genotype and allele frequencies of the breast cancer patients (across all populations) based on their history of confounding factors, to determine if there was any significant association between a genotype/allele and risk of breast cancer. These results are summarised in Table 4.06 and Table 4.07. Some of the patients were excluded from this analysis due to an incomplete history.

Table 4.06: Fisher Exact *P*-values for breast cancer patients' genotype frequencies

	***P*-values**			
	CYP1A1*m1*	**CYP1A1*m2***	**CYP1A2**	**CYP1B1**
Breast feeding (no vs yes)	0.587	0.5799	0.292	0.183
Contraception use (no vs yes)	1.000	1.000	0.221	0.079
Hormone replacement therapy (no vs yes)	1.000	1.000	0.340	0.118
Number of pregnancies (<2 vs >=2)	0.635	0.111	0.866	0.545
Smoking (no vs yes)	0.527	0.428	0.392	1.000

Table 4.07: Fisher Exact P-values for breast cancer patients' allele frequencies

	***P*-values**			
	CYP1A1*m1*	**CYP1A1*m2***	**CYP1A2**	**CYP1B1**
Breast feeding (no vs yes)	0.595	0.587	0.2697	0.170
Contraception use (no vs yes)	1.000	1.000	0.496	0.212
Hormone replacement therapy (no vs yes)	1.000	0.579	0.103	0.142
Number of pregnancies (<2 vs >=2)	0.804	0.116	0.691	0.467
Smoking (no vs yes)	0.688	0.434	0.196	1.000

4.5 DISCUSSION

The importance of low-penetrance susceptibility genes in the aetiology of cancer is now emerging. Genes involved in carcinogen metabolism and DNA repair modestly increase the risk for cancer in exposed individuals at perhaps low doses of carcinogens (Bartsch *et al.*, 2000). Population based studies are required to determine the frequency of polymorphisms in the low-penetrating susceptibility genes as different ethnic groups often show different

polymorphism characteristics (Huang *et al.*, 1999a, Nebert, 1999, Evans and Johnson, 2001, Ingelman-Sundberg, 2001, Weber, 2001). Epidemiological and cell biology studies have highlighted the contribution of oestrogen to the development of breast cancer. Oestrogen can bind to DNA triggering damage (Yager *et al.*, 1996, Cavalieri *et al.*, 1997, Zhu and Conney, 1998) suggesting that oestrogen might be a complete carcinogen that can directly cause genetic alteration and effect tumour initiation. In this study we examined the frequency of three common CYP polymorphisms (CYP1A1*m1*, CYP1A1*m2*, CYP1A2 –734 C/A) and a recently described single nucleotide polymorphism (in the 3'UTR of exon 3 of CYP1B1 8372 A>C) present in CYPs involved with oestrogen metabolism in breast cancer patients from two different South African populations. Differences in genotype (CYP1A1*m1*) and allele (CYP1A1*m1* and CYP1A2) frequencies were observed between the two population groups, indicating the ethnic variability of these polymorphisms (Table 4.02 and Table 4.03). To the best of our knowledge this is the first study to investigate the frequency of cytochrome P450 polymorphisms in mixed ancestry breast cancer patients and South African breast cancer patients of European descent.

The genotypic distributions for CYP1A1*m1*, CYP1A1*m2*, CYP1A2 and CYP1B1 for the Caucasian population were all very similar between the breast cancer patients and the controls. The same is true for the allele frequencies. Although the numbers are small, this may be an indication that no significant increase in risk of developing breast cancer is associated with any of these polymorphisms in the Caucasian population. The mixed ancestry population showed similar results for CYP1A1*m1*, CYP1A1*m2* and CYP1A2 genotypic and allele distributions. Significant differences were, however, observed for comparisons between mixed ancestry breast cancer patients and mixed ancestry controls for genotypic and allele distributions of the CYP1B1 polymorphism.

We show that significant *P*-values were obtained for both genotype and allele distributions for CYP1B1 between mixed ancestry breast cancer patients and mixed ancestry controls (Table 4.04 and Table 4.05). The C/C genotype frequency was notably higher for the mixed ancestry controls than for the mixed ancestry breast cancer patients (Table 4.02). It is therefore possible that possessing a double dose of the C allele may therefore decrease ones risk of developing breast cancer. Although the numbers of patients included in this study are too low to allow definite conclusions, the results obtained for the CYP1B1 polymorphism in

the mixed ancestry population are suggestive of an association between CYP1B1 polymorphism and increased risk of developing breast cancer in this population. The possibility exists that this association may be spurious due to population substructures that arise as a consequence of admixture linkage disequilibrium (the control population was found to deviate from Hardy-Weinberg equilibrium for this polymorphism). By estimating individual admixture it is possible to control for admixture as a confounder in association studies of genetic factors with disease risk (McKeigue *et al.,* 2000). Exclusion or control of admixture as a possible confounding factor will be achieved by genotyping other population-specific polymorphisms.

CYP1B1 is over expressed in a wide variety of cancers (McKay *et al.*, 1995, Murray *et al.*, 1997, McFadyen *et al.*, 1999, Murray *et al.*, 2001) including breast cancer tissue (Kristensen and Børresen-Dale, 2000). This raises questions about the functional role of CYP1B1 in tumours. An increase in CYP1B1 expression might favour oestrogen catabolism (Dasmahapatra *et al.*, 2002) due to the fact that CYP1B1 is responsible for the hydroxylation of oestradiaol-17β (E_2) (Spink *et al.*, 2000). Further work is required to investigate the mechanism by which CYP1B1 modifies breast cancer risk. Williams *et al.* (1994), Williams and Marzluff (1995), Dean *et al.* (2001) and Grzybowska *et al.* (2001) have reported that variations in the 3'UTR of genes can alter the stem-loop structure of mRNA and can affect mRNA processing and stability. Functional studies would be advantageous to investigate the effect of this polymorphism on the mRNA and to determine the mechanisms by which altered mRNA may influence risk of developing breast cancer. This polymorphism may, however, be in linkage disequilibrium with functional polymorphisms elsewhere in this gene or in a gene present on the same chromosome.

McFadyen *et al.* (2001) have demonstrated that CYP1B1 may be a mechanism of anticancer drug resistance, and conclude that the presence of CYP1B1 in tumour cells may have an important role in drug resistance. More research into the functions of CYP1B1 is however necessary to completely understand the role it plays in breast cancer risk, and how to successfully manage it to reduce risk. Insignificant *P*-values were obtained for the genotype and allele distributions for the Caucasian breast cancer patients and controls when analysing CYP1B1.

The results for the CYP1A1*m1* polymorphism in the Caucasian groups were in accordance with results of previous studies (Rebbeck *et al.*, 1994, Ambrosone *et al.*, 1995, Taioli *et al.*, 1995, Bailey *et al.*, 1998b) all of which failed to show a significant association overall between CYP1A1*m1* polymorphism and Caucasian breast cancer risk. Taioli *et al.* (1995) also reports a lack of association of the CYP1A1*m2* polymorphism and breast cancer risk in patients of Caucasian origin. An increased risk of breast cancer was however reported by them for women of African-American descent. It would therefore be interesting to screen the African populations present in South Africa for the CYP1A1*m1* and CYP1A1*m2* polymorphisms. These results may prove to be of great merit in the fight against breast cancer.

The variant allele of CYP1A2, i.e. the A-allele was by far the most predominant allele observed in our study in both the breast cancer group and the control group of both populations. In a case-control study involving South African variegate porphyria patients and controls, the A allele of CYP1A2 was also more frequently observed than the wildtype C allele (This study, Chapter Three). This finding is indicative of the ethnic variation observed for the cytochrome P450 genes. The A/A genotype presents a highly inducible genotype of this enzyme, and it has been shown that smoking further induces activity of CYP1A2 (Sachse *et al.*, 1999). We showed a non-significant *P*-value for the comparison between breast cancer patients with a history of smoking and those without a history of smoking for CYP1A2. However it would be of more value to investigate the effect of smoking on breast cancer risk by comparing breast cancer smokers to healthy control smokers. This was however not possible in this study due to the limited information available about the control samples.

Studying the effects of confounders (absence of breast feeding, contraception use, hormone replacement therapy, number of pregnancies and smoking status) on the risk of breast cancer was hampered by the limited information available regarding the individuals included in the control population. It is suggested that in future studies, control populations included in the studies need to be as well defined as the individuals making up the disease population. Analysis of the genotype distribution for contraception use among the breast cancer patients for CYP1B1 rendered an insignificant, yet encouraging *P*-value (Table 4.06). A follow up study will investigate the effect of different contraceptives (oral versus injection and

oestrogen versus progesterone) on the risk of breast cancer and these results should prove to be very informative.

Our data did not support a substantial interaction effect between either of the CYP polymorphisms and the risk of breast cancer overall. However it seems that the CYP1B1 C/C genotype may be a protective factor for decreased risk of breast cancer in the mixed ancestry population. These results will be confirmed in a resampled mixed ancestry breast cancer group.

It should be emphasized that it is absolutely necessary to genotype individuals from each population represented in South Africa for CYP polymorphisms, as each population is unique. Data such as this would be of great value to clinicians and pharmacists, as it will aid in the elucidation of the differential response to medication among the South African population.

A complete understanding of the aetiological role of oestrogen in breast cancer will, however, require studies evaluating the roles of genes involved in oestrogen metabolism and the extent to which oestrogen exposure modifies the associations of these genes with breast cancer risk. Genetic polymorphisms have been found in phase I and phase II drug metabolising enzymes, drug transporters and drug receptors, therefore, it is likely that risk assessments would become more sensitive and reasonable if polymorphisms in all categories of enzymes were taken into consideration as biomarkers for breast cancer risk.

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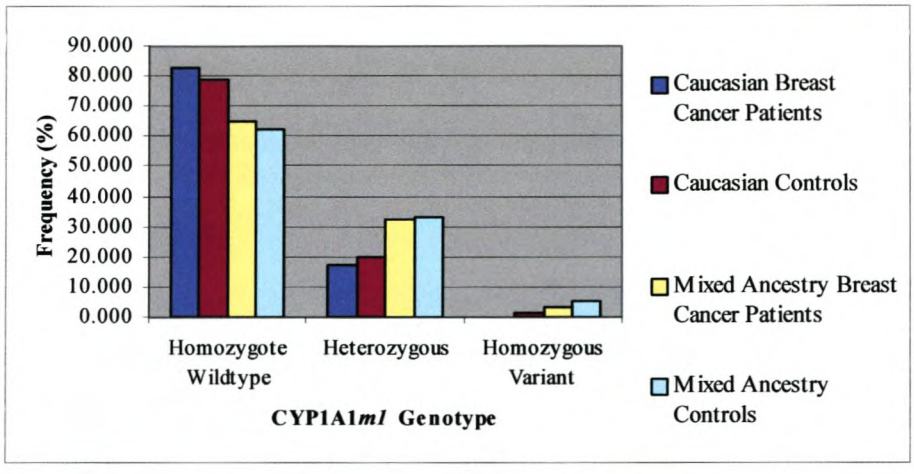
- Tasks performed by the author:
    - Selection of CYP polymorphisms to include in the study (with assistance from Prof Warnich)
    - DNA extractions of individuals making up the Caucasian control population
    - Primer design of CYP1B1 primers
    - Screening and genotyping of CYP polymorphisms
        - PCR amplification
        - Restriction enzyme digests
        - Agarose gel electrophoresis
        - SSCP gel electrophoresis

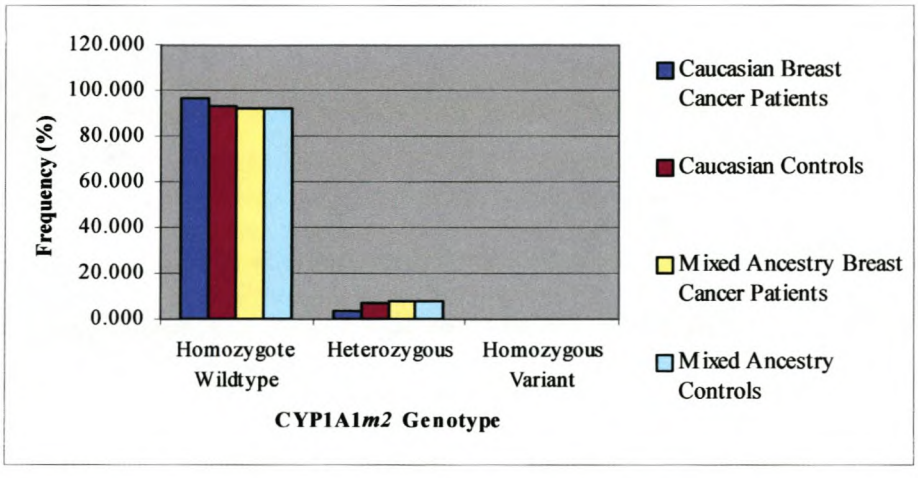
- Heteroduplex SSCP gel electrophoresis
- PCR purification
- DNA sequencing reactions

- Data analysis
- Writing of manuscript

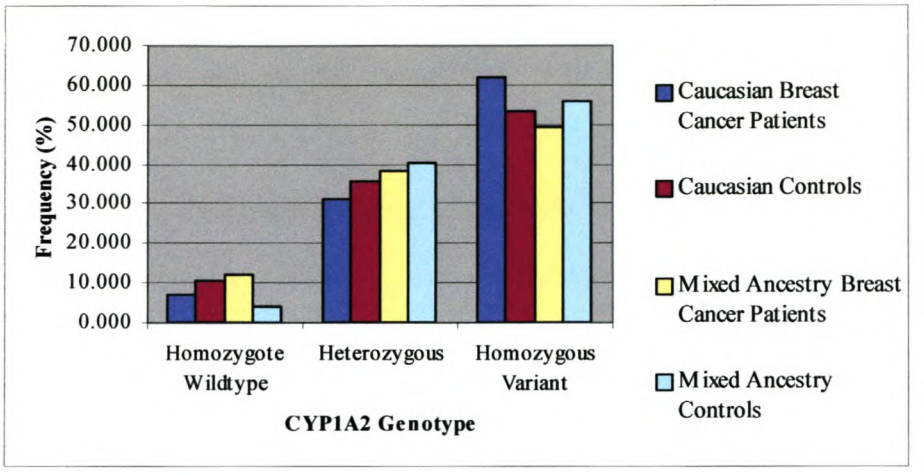
APPENDIX II



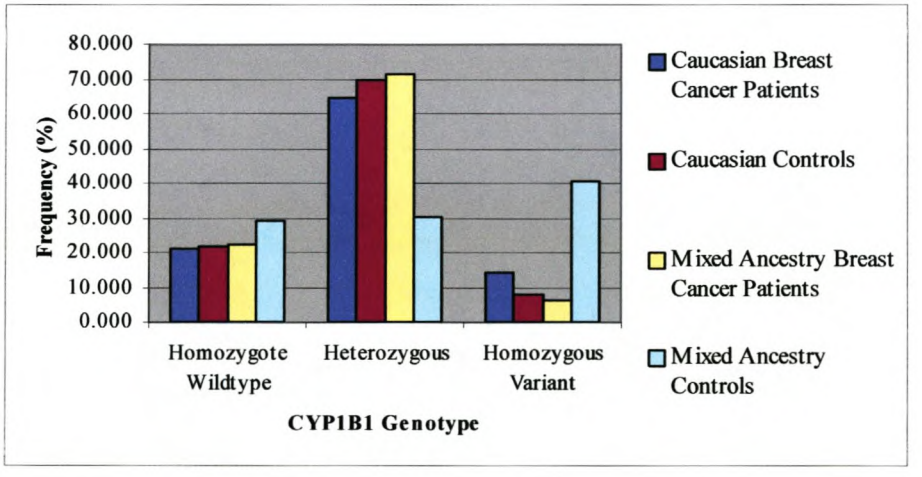
Graph II.01: Comparison of CYP1A1*m1* genotype frequencies for breast cancer patients and controls (Caucasian and mixed ancestry)



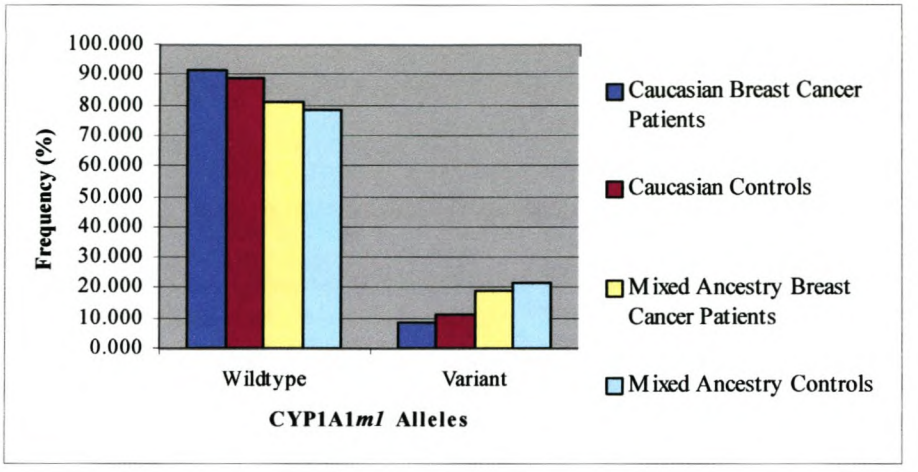
Graph II.02: Comparison of CYP1A1*m2* genotype frequencies for breast cancer patients and controls (Caucasian and mixed ancestry)



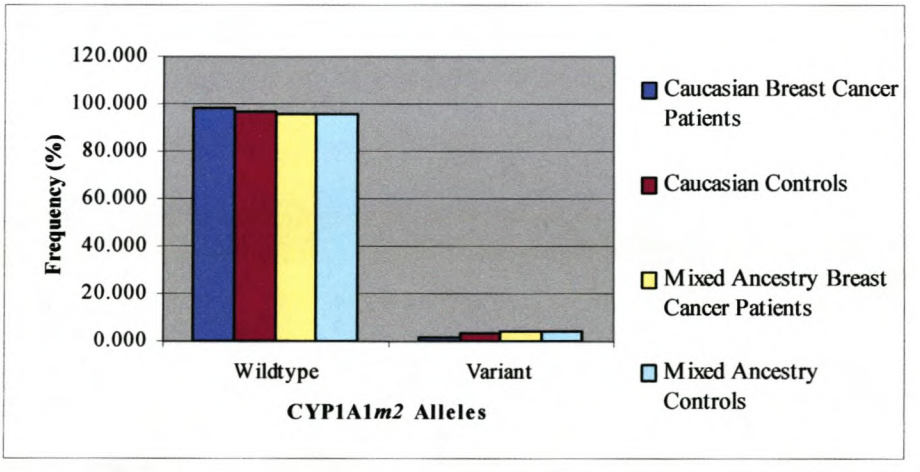
Graph II.03: Comparison of CYP1A2 genotype frequencies for breast cancer patients and controls (Caucasian and mixed ancestry)



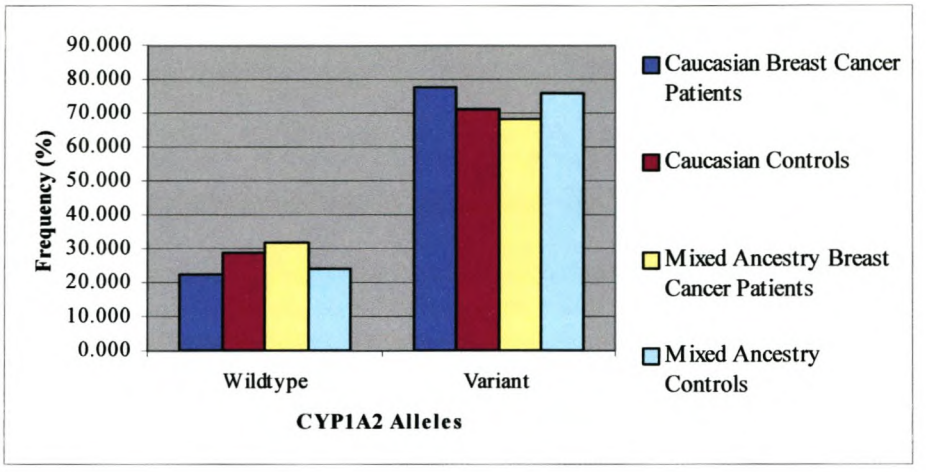
Graph II.04: Comparison of CYP1B1 genotype frequencies for breast cancer patients and controls (Caucasian and mixed ancestry)



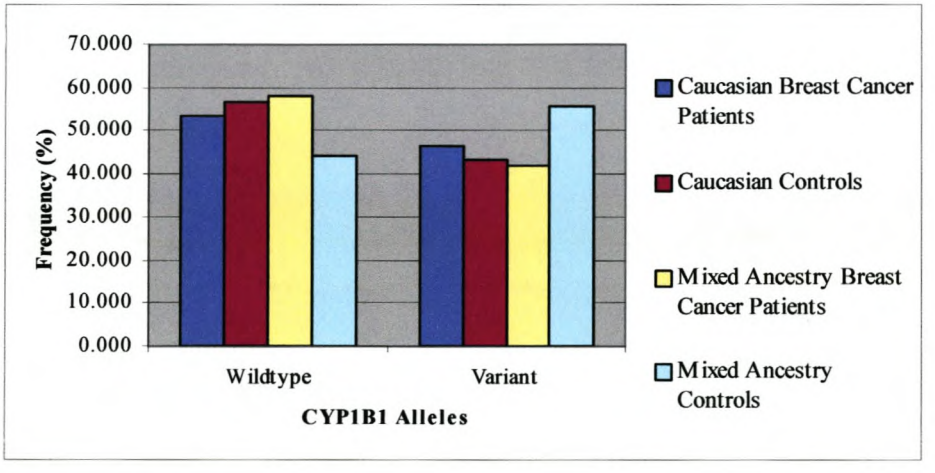
Graph II.05: Comparison of CYP1A1*m1* allele frequencies for breast cancer patients and controls (Caucasian and mixed ancestry)



Graph II.06: Comparison of CYP1A1*m2* allele frequencies for breast cancer patients and controls (Caucasian and mixed ancestry)



Graph II.07: Comparison of CYP1A2 allele frequencies for breast cancer patients and controls (Caucasian and mixed ancestry)



Graph II.08: Comparison of CYP1B1 allele frequencies for breast cancer patients and controls (Caucasian and mixed ancestry)

CHAPTER 5

CONCLUSIONS

The applications of pharmacogenomics in research and drug development are vast, but not straight forward. In order to have the most useful outcome, pharmacogenomic studies need to be optimally designed. This includes sufficient characterisation of the patient and control groups, and sufficient numbers of participants that are all thoroughly phenotyped. It is recommended that different ethnic groups are genotyped as ethnic specificity may be useful to improve diagnosis and clinical care of patients by providing information about the structure of genes and regulatory pathways that may cause an altered response in that specific population (Nebert, 1999, Evans and Johnson, 2001, Weber, 2001).

Benefits of pharmacogenomics for industry include the reduction of clinical trial sizes, which may be limited to only genetically defined subpopulations. Screening the drugs for possible interactions with polymorphic enzymes early in the development process has the potential to reduce problematic dosing of future drugs due to the fact that fewer drugs that are substrates for polymorphic enzymes will be released to the commercial market.

5.1 NEW TECHNOLOGY

With the development of newer methods of molecular analysis to detect DNA polymorphisms, such as DNA chip technology, genotyping screening approaches are increasingly becoming feasible. DNA chips are becoming increasingly important in pharmocogenetics as they are used as research tools to screen drug candidates for the development of new therapies and to score individuals and populations for polymorphisms suspected of playing a role in drug susceptibility. They will however not reach their full potential in pharmacogenomic testing until their cost has been reduced, they have greater robustness and reliability and have improved production (Weber, 2001).

Once a polymorphism has been defined in a gene, functional significance of the polymorphism requires demonstration. This introduces the field of functional genomics (McKusick, 1997) in which genetically manipulated animals will be phenotyed to determine

the biological function of the particular polymorphic gene. This experimental approach in itself involves many new technologies (Ohlstein *et al.*, 2000) including *in vivo* imaging, mass spectrometry and microarray hybridization.

Single strand conformation polymorphism and restriction enzyme digests were the methods of choice for polymorphism detection in these studies. These techniques are not without limitations, including their laborious and tedious nature and the relatively small amount of data that one is able to collect from many hours of work. DNA chip technology would have greatly increased the amount and speed of data collection from this study, as it would have enabled the screening of further polymorphisms in additional CYP genes. Technology such as the DNA chip is currently not freely available on the local market and is very expensive.

5.2 VARIEGATE PORPHYRIA

Von und zu Fraunberg *et al.* (2002) suggest that cytochrome P450s are good candidates for modifying porphyrin metabolism and variegate porphyria phenotype. The function of CYPs may be decreased by an insufficient supply of haem in porphyria patients. To the best of our knowledge, this is the first study to investigate the association of CYP polymorphisms with the clinical expression of VP. Christiansen *et al.* (2000) has, however, performed a similar study in porphyria cutanea tarda patients investigating the association of a CYP1A2 polymorphism with clinical expression.

In Chapter 3 of this study we demonstrate a lack of association between 4 (CYP1A1*m2*, CYP1A2, CYP2D6*3 and CYP2D6*4) of the polymorphisms genotyped in this study and clinical expression of VP. This may be the result of a sample size with too low numbers to allow for the effect of the polymorphisms to be successfully observed, or may indicate that the polymorphisms are simply not involved with modifying the clinical expression of VP. Future haplotype studies on the VP families may be useful in elucidating the role of these polymorphisms on the clinical expression of VP.

Two of the polymorphisms (CYP1A1*m1* and CYP1B1 8372 A>C) genotyped in this study did, however, demonstrate association with clinical expression of VP. Although some of the *P*-values for the comparisons became non-significant after the Bonferroni correction was applied, sufficient evidence is provided to suggest a possible role for these CYPs in

modifying the clinical expression of VP. We have shown significant association of the CYP1B1 8372 A>C polymorphism with all the clinical subgroups of VP expressing symptoms. The frequency of the A-allele in the subgroups expressing acute attacks is 100%, a clear indication that this allele may indeed increase a patients' susceptibility to developing an acute attack. Strong evidence is provided to suggest that CYP1B1 may be a modifier of the clinical expression of VP even with the low numbers of patients included in the study. Further we observed significant differences in genotype and allele frequencies, prior to Bonferroni correction, between the skin lesion (SL) and the acute attack (AA) clinical subgroups, and the acute attack and skin lesion (AASL) and the acute attack clinical subgroups (AA) for CYP1A1*m1*, further implicating CYPs as candidate modifier genes of VP clinical expression. It seems that the T-allele of this polymorphism may increase a patients' susceptibility to developing skin lesions or that the C-allele may offer protection to developing skin lesions.

This study has provided evidence in support of the suggestion that CYPs are candidate genes for modifying VP clinical expression. Effective use of a mouse model of VP will enhance studies investigating the effect of increased catalytic activity of CYP1A1 on the clinical expression of VP. Future studies would also include functional studies to determine whether the CYP1B1 polymorphism alters the expression of the gene due to it possibly having an effect on the mRNA structure. One would also have to investigate whether linkage disequilibrium is present between this polymorphism and other functional polymorphisms in CYP1B1 or in another gene on the same chromosome. Haplotype studies on the variegate porphyria families will also increase our knowledge about the effect of CYP polymorphisms on the clinical expression of VP. Studies in other populations are required to confirm our findings and to determine the molecular mechanisms involved in clinical expression of VP in those populations.

This study has, using a rather small study population, shown that CYPs may be genes responsible for modifying the clinical expression of VP to varying degrees. A Pandora's box of questions regarding the possible effects that these genes have on the clinical expression and the mechanisms involved with modifying expression of this disease has been opened – questions that will hopefully be successfully answered in future studies. Future studies investigating the role of CYPs in the development of symptoms, may find reasons explaining

the lack of uniformity in the severity of the symptoms observed. Polymorphisms within these CYP genes may potentially be used as markers for clinical expression of symptoms and susceptibility to developing various clinical symptoms of VP.

5.3 BREAST CANCER

Bartsch *et al.* (2000), suggest that the future challenge of molecular epidemiological studies involving cancer patients will be to analyse individuals that are exposed to carcinogens for a combination of genotypes associated with susceptibility to cancer due to the fact that cancer is a largely preventable disease. Establishment of risk profiles for individuals and subgroups in known exposure situations will be aided by studies that integrate biomarkers for carcinogen exposure, early biological effects and susceptibility. This study (Chapter 4) is significant in that it is to the best of our knowledge, the first study to determine the frequencies of CYP polymorphisms in the mixed ancestry population of South Africa and the breast cancer population of South Africa as a whole. The results that were obtained will be of great importance to the development of breast cancer risk profiles for this population. Our results have emphasized the large inter-ethnic differences in allele frequencies in CYP genes observed throughout the world.

In Chapter 4 of this study, we demonstrate non-significant results for three of the polymorphisms (CYP1A1*m1*, CYP1A1*m2* and CYP1A2) genotyped in this study. This may be the result of a sample size with too low numbers to allow for the effect of the polymorphisms to be successfully observed, or may indicate that the polymorphisms are simply not involved with modifying the risk of breast cancer in the South African populations.

One of the polymorphisms (CYP1B1 8372 A>C) genotyped in this study did, however, demonstrate association with increased risk of breast cancer in the mixed ancestry population. This polymorphism, situated in the 3'UTR of exon 3 of the CYP1B1 gene, may have an effect on the mRNA structure and as such alter the expression of the gene. The possibility also exists that this polymorphism may be in linkage disequilibrium with another functional polymorphism in CYP1B1 or a functional polymorphism in another gene situated nearby on the same chromosome. Studies in other populations are required to confirm our findings. Since the mixed ancestry control population deviated from Hardy-Weinberg equilibrium, it is

suggested that an alternative control population be genotyped and included in this study. Controling for admixture as a confounder is also suggested.

It is unfortunate that all the relevant lifestyle information was not available for the control individuals included in this study, as this would have added more depth to the study. Stratification of the participants would have been possible, and as such, more knowledge would have been gained regarding the various confounding factors of breast cancer in the two South African populations studied. The results were however encouraging, and future studies will investigate the involvement of additional CYPs on the risk of developing breast cancer, in these and other populations unique to South Africa, in an attempt to develop risk profiles for the South African populations. The resources that are required to conduct such studies successfully necessitate large collaborations between epidemiologists, molecular biologists and clinicians. These collaborations have been realised between the academics of the University of Stellenbosch and scientists from the private sector. Participation like this will help foster a deeper understanding into the complexity of breast cancer in the unique populations of South Africa.

5.4 CHALLENGES AND FUTURE PROSPECTS OF PHARMACOGENOMICS

Incorporation of genetic technologies into mainstream medicine will raise many issues including financial, ethical, legal, social and operational questions. Many of these questions will touch on the core values, principles and ethical beliefs of the population. Bioethics is therefore becoming increasingly important in the regulation and future of human genetic technologies. If we are to benefit from these technologies, it is essential that public support for the technologies is gained and that social and ethical implications and concerns are addressed thoroughly.

Focused genome wide scans for polymorphisms associated with disease risk or drug response will be aided by technological advances. Data generated from these scans, will aid clinicians in making preventative health and prospective treatment decisions. The end result will be the optimal selection of medications and their dosages based on the individual patient and will not rely on average experiences from the global population of patients with a similar diagnosis.

5.5 REFERENCES

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APPENDIX A

TOTAL GENOMIC DNA ISOLATION FROM WHOLE BLOOD

A modified protocol of Miller *et al.* (1998) was followed to extract total genomic DNA from whole blood.

Whole blood was collected in glass tubes containing ethylenediamine tetra-acetic acid (EDTA, $C_{10}H_{16}N_2O_8$) as the preservative. The blood was stored at 4°C until the DNA extraction was performed the following day. If the extraction was unable to proceed the day following the collection of the blood sample, the blood was stored at -20°C until genomic DNA was extracted.

The tube containing the thawed blood was shaken and the contents poured into a 40ml centrifugation tube (Greiner Labortechnik, Kremsmuenster, Austria). The EDTA tube was rinsed out with 30ml cold lysis buffer (8.3mg/ml ammonium chloride (NH_4Cl), 1.1mg/ml potassium hydrogen carbonate ($KHCO_3$), 25µg/ml EDTA, pH 7.4) and this was added to the blood in the 40ml tube. This mixture was incubated on ice for 15 minutes with gentle inversion every 5 minutes. The mixture was centrifuged at 1500 rpm, at 4°C for 20 minutes (Beckman Avanti 30™, Beckman Coulter, Inc). The supernatant was discarded and the pellet was resuspended in 10ml phosphate buffered saline solution (PBS) (6mg/ml potassium chloride (KCl), 24mg/ml sodium chloride (NaCl). 3.42mg/ml di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4), 0.6mg/ml potassium dihyrdrogen orthophosphate (KH_2PO_4)). This mixture was centrifuged at 1500 rpm, at 4°C for 10 minutes and the supernatant was once again discarded. The pellet was resuspended in 3ml nuclear lysis buffer (12.11mg/ml tris(hydroxymethyl)aminomethane ($(CH_2OH)_3CNH_2$-Cl), 23.4mg/ml NaCl, 0.6mg/ml EDTA, pH 8.2), 30µl proteinase K (10mg/ml) (Roche Diagnostics Corporation, Indianapolis, IN, USA), and 150µl 20% (w/v) sodium dodecyl sulfate (SDS). This mixture was shaken well and incubated in a 50°C water bath overnight.

One milliliter 6M NaCl was added to the overnight incubation. This was followed by a vigorous one minute shake, after which the mixture was centrifuged at 2500 rpm, at

21°C for 15 minutes. The supernatant was transferred to a new 40ml tube (Greiner Labortechnik, Kremsmuenster, Austria) taking care to leave the foam and pellet behind. After a 15 second shake, this was centrifuged at 2500 rpm, at 21°C for 15 minutes. The supernatant was subsequently transferred to a 30ml glass Corex tube (USA) taking note of the volume. Extreme caution must be exercised when transferring the supernatant to avoid any contamination by the foam or the pellet. Two 2 volumes of cold 100% (v/v) ethanol (EtOH) was added to the aqueous phase and the tube was covered with Parafilm® (American National Can™, Menasha, WI, USA). This was allowed to stand until a white spool formed and rose to the top of the solution. The spool was transferred to a 1.5ml Safe Lock Eppendorf® tube (Eppendorf, Hamburg, Germany) with an innoculation needle (Looplast®, LP Italiana SPA, Milan, Italy). The DNA was washed with 300µl 70% (v/v) EtOH and this mixture centrifuged at 14 000 rpm, at 4°C for 5 minutes (Beckman GS-15R, Beckman Coulter, Inc). The 70% EtOH was poured off and the pellet was allowed to air dry. The pelleted DNA was resuspended in 250µl sterile SABAX water (Adcock Ingram, Johannesburg, RSA) overnight. DNA concentration and purity was determined by running aliquots of the extracts on a 1% (w/v) agarose gel (stained with 0.0075% (v/v) EtBr, 1X TBE (refer to Appendix D) running buffer) with Lamda DNA (Promega Corporation, Madison, WI, USA) standards. Lamda standards of 10ng/µl, 20 ng/µl, 40 ng/µl, 60 ng/µl and 80ng/µl were loaded. The gel was run at 80V for thirty minutes. Bands were visualised in the gel with UV light and were subsequently photographed with the GelDoc system (BioRad, Hercules, CA, USA). The concentration of the DNA was determined and it was subsequently diluted to 20-30ng/µl, aliquoted and stored at -20°C.

APPENDIX B

GENOTYPING TECHNIQUES

CYP1A1*m1*, CYP1A1*m2*, CYP1A2 and CYP2D6*4

Genotyping of the CYP1A1*m1* (6235 T>C, *MspI* polymorphism), CYP1A1*m2* (4889 A>G, Ile/Val substitution), CYP1A2 (-734 C>A) and CYP2D6*4 (1934 G>A) polymorphisms was successfully performed using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) (Kristensen *et al.*, 2001) method of detection. In brief, DNA fragments were amplified using PCR and the primers described in Table 3.01 and Table 4.01. The reaction mixtures contained approximately 20ng of genomic DNA, 20.0 µM of the forward primer, 20.0 µM of the reverse primer, 200 µM of each dNTP (Promega Corporation, Madison, WI, USA), 0.5 units of *Taq* polymerase (BIOTAQ^{TM} DNA Polymerase, Bioline, Springfield, NJ, USA) and a final concentration of 1.0mM (CYP1A1*m1* and CYP1A1*m2*) or 1.5mM (CYP1A2 and CYP2D6*4) $MgCl_2$ in a total volume of 25.0µl. PCR was performed at 95°C for five minutes for the initial denaturation, followed by thirty-five cycles of denaturation at 95°C for thirty seconds, annealing at T_A (refer to Table 3.01) for thirty seconds, and extension at 72°C for one minute. This was followed by a ten minute extension at 72°C after which the samples were held indefinitely at 4°C. All PCR reactions were performed in a Perkin Elmer GeneAmp PCR system 9600 (Applied Biosystems, Warrington, WA, USA).

An aliquot of 15µl of PCR product was digested with 1µl of the relevant restriction enzyme: CYP1A1*m1* with *MspI* (Roche Diagnostics Corporation, Indianapolis, IN, USA) (recognition sequence: 5'- C↓CGG -3'), CYP1A1*m2* with *HindII* (Roche Diagnostics Corporation, Indianapolis, IN, USA) (recognition sequence: 5'- GT(T/C)↓(A/G)AC –3'),CYP1A2 with *ApaI* (Promega Corporation, Madison, WI, USA) (recognition sequence: 5' - GGGCC↓C - 3') and CYP2D6*4 with *MvaI* (*BstNI*) (Promega Corporation, Madison, WI, USA) (recognition sequence: 5'- CC↓AGG – 3') in a reaction with 2.5µl reaction buffer in a total volume of 20µl. The PCR

products were digested at 37°C overnight according to the conditions on the package insert provided with the restriction enzyme and enzyme buffer.

On completion of the digest, the products of the restriction enzyme digest were run on a 3% (w/v), 1X TBE (refer to Appendix D) agarose gel (CYP1A1*m1*, CYP1A1*m2* and CYP2D6*4) at 60V for 2.5 hours or a 1% (w/v), 1X TBE (refer to Appendix D) agarose gel (CYP1A2) at 60V for 1.5 hours. Five microlitres of 6x loading dye (refer to Appendix D) were added to each digest and 15.0µl of this mixture was loaded to the gel. The bands were stained with ethidium bromide (EtBr) added to the agarose gel mix (final concentration 0.0075% v/v). A 100 bp ladder (Promega Corporation, Madison, WI, USA) served as the molecular size marker (3.5 µl ladder + 3.0 µl 6x loading buffer). Bands were visualised in the gel with UV light and subsequently photographed with the GelDoc system (BioRad, Hercules, CA, USA, Hercules, CA, USA, Hercules, CA, USA). Three different genotypes were defined for each of the polymorphism, being the homozygous wild-type, the heterozygous and the homozygous variant genotypes. Refer to Table B.01 for the respective genotypes for each of the polymorphisms.

Table B.01: Definition of the genotypes of the CYP1A1*m1*, CYP1A1*m2*, CYP1A2 and CYP1B1 polymorphisms.

Polymorphism	Genotype		
	Homozygous Wildtype	**Heterozygous**	**Homozygous Variant**
CYP1A1*m1*	T/T	T/C	C/C
CYP1A1*m2*	A/A	A/G	G/G
CYP1A2	C/C	C/A	A/A
CYP1B1	A/A	A/C	C/C
CYP2D6*3	A/A	A/del	del/del
CYP2D6*4	G/G	G/A	A/A

CYP1B1

Genotyping of the A/C polymorphism present in the 3' UTR of exon 3 of CYP1B1 (8372 A>C) was successfully performed using the polymerase chain reaction single

strand conformation polymorphism (PCR-SSCP) (Kristensen *et al.*, 2001) method of detection. In brief, primers were designed from the CYP1B1 sequence (gi 17446619, NCBI) using the program Primer3 available online. PCR was employed to amplify a 242 base pair DNA fragment. The reaction mixture contained approximately 20ng of genomic DNA, 0.8 μM of the forward primer, CYP1B1F, 0.8 μM of the reverse primer, CYP1B1R, 200 μM of each dNTP (Promega Corporation, Madison, WI, USA), 1.0 units of *Taq* polymerase (BIOTAQ^{TM} DNA Polymerase, Bioline, Springfield, NJ, USA) and a final concentration of 1.5mM $MgCl_2$ in a total volume of 25.0μl. PCR was performed at 95°C for five minutes for the initial denaturation, followed by thirty-five cycles of denaturation at 95°C for thirty seconds, annealing at 55°C for thirty seconds, and extension at 72°C for thirty seconds. This was followed by a ten minute extension at 72°C after which the samples were held indefinitely at 4°C. All PCR reactions were performed in a Perkin Elmer GeneAmp PCR system 9600 (Applied Biosystems, Warrington, WA, USA). Refer to Table 3.01 for the primer sequences and annealing terperatures.

SSCP

Five microlitres of formamide denaturing loading dye (refer to Appendix D) was added to each PCR product and this mixture was subsequently denatured at 98°C for 8 minutes, before snap cooling on ice. Fifteen microlitres of this mixture were loaded on a 15% (w/v) polyacrylamide (PAA) (1% cross-linking) 103 X 98mm (Mighty Small, Hoefer Pharmacia Biotech Inc, BioRad, Hercules, CA, USA, Hercules, CA, USA) SSCP gel. The gels were run at 200V, at 16°C, for 4 hours. The gels were stained in 1.5X TBE buffer (refer to Appendix D) containing a final concentration of 0.0075% (v/v) EtBr for 20 minutes and bands were visualised in the gel with UV light and subsequently photographed with the GelDoc system (BioRad, Hercules, CA, USA).

SSCP-Heteroduplex

Samples that showed band differences were subjected to another round of PCR (conditions as above) and were then run on 37cm X 16.5cm denaturing heteroduplex

SSCP gels (Kotze *et al.*, 1995). Five microlitres of formamide denaturing loading dye were added to each PCR product and this mixture was subsequently denatured at 98°C for 5 minutes, before snap cooling on ice. Fifteen microlitres of this mixture were loaded to a 15% (w/v) PAA, 1% (w/v) urea ($(NH_2)_2CO$), 1% cross-linking heteroduplex SSCP gel. The gels were run at 300V, at 4°C overnight. The gels were removed from the electrophoresis apparatus and were stained in 1.5X TBE buffer (refer to Appendix D) containing a final concentration of 0.0075% (v/v) EtBr for 20 minutes and bands were visualised in the gel with UV light and subsequently photographed with the GelDoc system (BioRad, Hercules, CA, USA).

Samples with identical band patterns (SSCP and heteroduplex) were identified and representatives from each band pattern were sequenced to determine the absence or presence of the polymorphism. PCR products were purified using a QIAquick® PCR Purification Kit (QIAGEN, GmbH, D-40724, Hilden). Sequencing reactions were performed using a Perkin Elmer BigDye™ Terminator Cycle Sequencing Kit (PE Applied Biosystems, Warrington WA, 4SR, Great Britain) and forward primer as described in Table 3.01. These reactions were run on an ABI 3100 (PE Applied Biosystems, Warrington WA, 4SR, Great Britain) for sequencing. Sequences were analysed using the programs Sequencing Analysis (V3.07) and BioEdit Sequence Alignment Editor (Hall, 1999).

CYP2D6*3

Genotyping of CYP2D6*3, the single base deletion (A_{2637}) in exon 5 of CYP2D6 was successfully performed using the polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) (Kristensen *et al.*, 2001) method of detection. In brief, PCR amplification of a 269 base pair DNA fragment was performed using the primers described in Topic *et al.*, (2000) (Refer to Table 3.01 for the primer sequences). The reaction mixture contained approximately 20ng of genomic DNA, 0.8 µM of the forward primer, CYP2D6-3F, 0.8 µM of the reverse primer, CYP2D6-3R, 200 µM of each dNTP (Promega Corporation, Madison, WI, USA), 1.25 units of *Taq* polymerase (BIOTAQ™ DNA Polymerase, Bioline, Springfield, NJ, USA) and a final concentration of 0.8mM $MgCl_2$ in a total volume of

25.0µl. PCR was performed at 92°C for ten minutes for the initial denaturation, followed by thirty-eight cycles of denaturation at 92°C for forty-five seconds, annealing at 56.5°C for thirty seconds, and extension at 72°C for thirty seconds. This was followed by a ten minute extension at 72°C after which the samples were held indefinitely at 4°C. All PCR reactions were performed in a Perkin Elmer GeneAmp PCR system 9600 (Applied Biosystems, Warrington, WA, USA).

Five microliters of formamide denaturing loading dye (refer to Appendix D) was added to each PCR product and this mixture was subsequently denatured at 98°C for 8 minutes, before snap cooling on ice. Six microliters of this mixture was loaded on a 15% (w/v) PAA (1% cross linking) Mighty Small (103 X 98mm) (Hoefer Pharmacia Biotech Inc) SSCP gel. The gels were run in 1.5X TBE (refer to Appendix D), at 200V, at 4°C, for 4 hours. The gels were silver stained for visualisation of bands (refer to Appendix C for a detailed silver staining protocol).

APPENDIX C

SILVER STAINING OF POLYACRYLAMIDE GELS

After removing the polyacrylamide gels from the plates, they were placed in a plastic container and were covered with fixing solution (10% (v/v) EtOH, 0.5% (v/v) acetic acid (CH_3COOH), stored at room temperature). They were shaken on a Belly Dancer (Stovall Life Science, Inc) for 10 minutes to allow optimal covering of the gels by the fixing solution. The fixing solution was discarded and the gels were rinsed twice (one minute per rinse) with distilled water. All the water was discarded and the gels were covered with freshly made staining solution (0.1% (w/v) silver nitrate ($AgNO_3$)). They were shaken on the Belly Dancer (Stovall Life Science, Inc) for a minimum of ten minutes. The staining solution was discarded and the gels were rinsed once for five seconds with distilled water. The water was discarded and the gels were covered with cold developing solution (1.5% (w/v) sodium hydroxide (NaOH), 0.4% (v/v) formaldehyde (HCHO) added just before use) and shaken on the Belly Dancer (Stovall Life Science, Inc), allowing developing to continue for ten minutes or until the gel turned yellow and red-brown bands of DNA were visible. The developing solution was discarded and the gels were rinsed twice with distilled water. The gels are stored in the dark in a plastic covering. Caution must be exercised not to touch the gel with ones hands after the gel has been developed. All solutions after and including the staining solution were discarded into well marked bottles for disposal.

APPENDIX D

GENERAL SOLUTIONS

1X TBE

10.8mg/ml tris(hydroxymethyl)aminomethane ($(CH_2OH)_3CNH_2$-Cl)

5.5mg/ml boric acid (H_3BO_3)

0.7445mg/ml EDTA ($C_{10}H_{16}N_2O_8$)

1.5X TBE

16.2mg/ml tris(hydroxymethyl)aminomethane ($(CH_2OH)_3CNH_2$-Cl)

8.25mg/ml boric acid (H_3BO_3)

1.116mg/ml EDTA ($C_{10}H_{16}N_2O_8$)

6X Loading Dye

0.25% (w/v) bromophenol blue ($C_{19}H_{10}Br_4O_5S$)

0.25% (w/v) xylene cyanol FF ($C_{31}H_{28}N_2Na_4O_{13}S$)

30.0% (w/v) glycerol ($HOCH_2CHOHCH_2OH$)

Formamide Loading Dye

98% (v/v) formamide (H_2NCHO)

10mM EDTA ($C_{10}H_{16}N_2O_8$)

0.05% (w/v) bromophenol blue ($C_{19}H_{10}Br_4O_5S$)

0.05% (w/v) xylene cyanol FF ($C_{31}H_{28}N_2Na_4O_{13}S$)

APPENDIX E

CYTOCHROME P450 POLYMORPHISM ANALYSIS

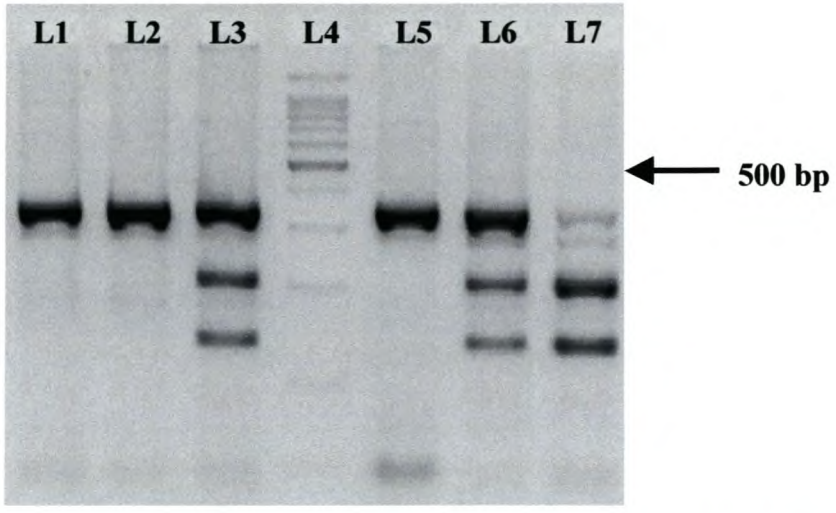


Figure E.01: **A 3 % (w/v) agarose gel of the CYP1A1*m1* polymorphism (*MspI* restriction enzyme digest) in six of the Caucasian breast cancer patients (L1-L3, L5-L7). Lanes 1, 2, and 5 are individuals homozygous for the T-allele, lanes 3 and 6 are individuals heterozygous for the polymorphism (T/C genotype), while lane 7 is an individual homozygous for the C-allele. Lane 4 contains a 100 bp ladder loaded as a molecular size marker. The gel was stained in 0.0075% (v/v) EtBr.**

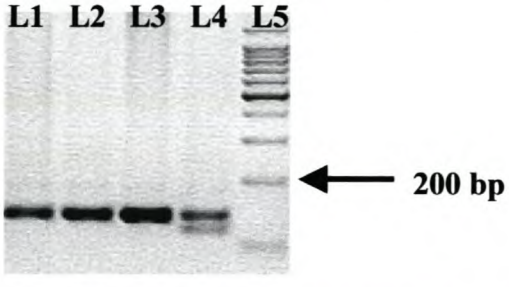


Figure E.02: **A 3 % (w/v) agarose gel of the CYP1A1*m2* polymorphism (*HindII* restriction enzyme digest) in four of the mixed ancestry controls (L1-L4). Lanes 1, 2, and 3 are individuals homozygous for the A-allele, while lane 4 contains an individual heterozygous for the polymorphism (A/G genotype). Lane 5 contains a 100 bp ladder loaded as a molecular size marker. The gel was stained in 0.0075% (v/v) EtBr.**

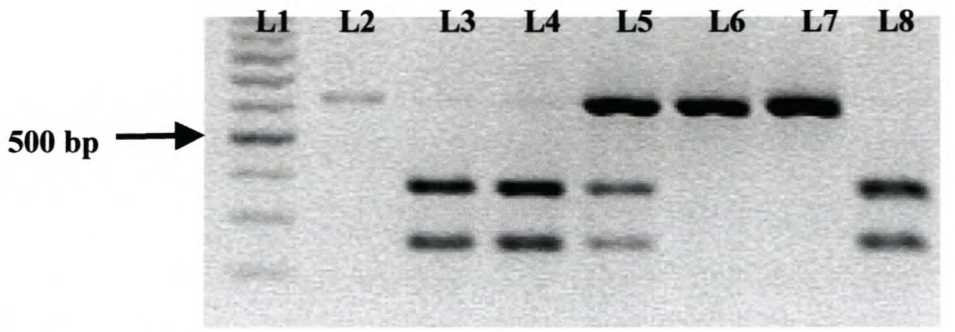


Figure E.03: A 1 % agarose gel of the CYP1A2 polymorphism (*ApaI* restriction enzyme digest) in 7 of the mixed ancestry controls (L2-L8). Lanes 2, 6, and 7 are individuals homozygous for the A-allele, lanes 3, 4 and 8 are individuals homozygous for the C-allele and lane 5 is an individual that is heterozygous for the polymorphism (C/A genotype). Lane 1 contains a 100bp ladder loaded as a molecular size marker. The gel was stained in 0.0075% (v/v) EtBr.

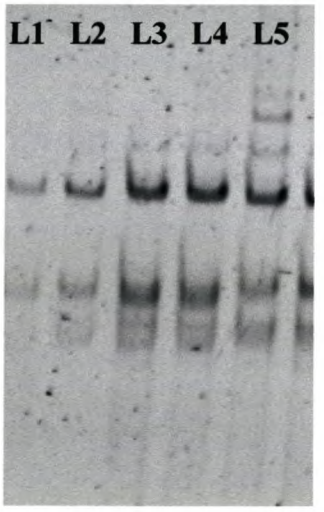


Figure E.04: A 10% (v/v) urea SSCP polyacrylamide gel of the CYP1B1 polymorphism in five of the Caucasian controls (L1-L5). Lanes 1, 2, 3 and 4 are individuals heterozygous for the polymorphism (C/A genotype), while lane 5 contains an individual homozygous for the A-allele. The gel was stained in 0.0075% (v/v) EtBr for visualisation of the bands.

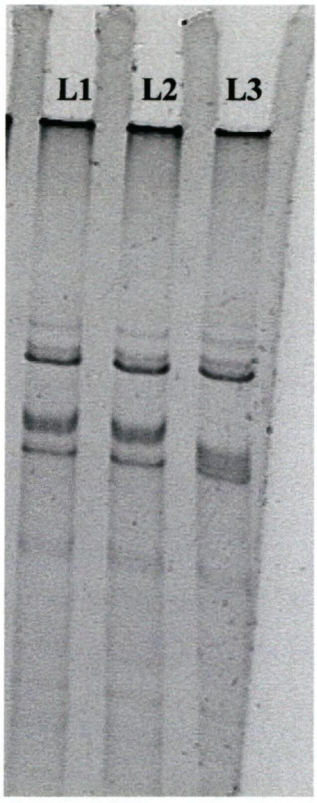
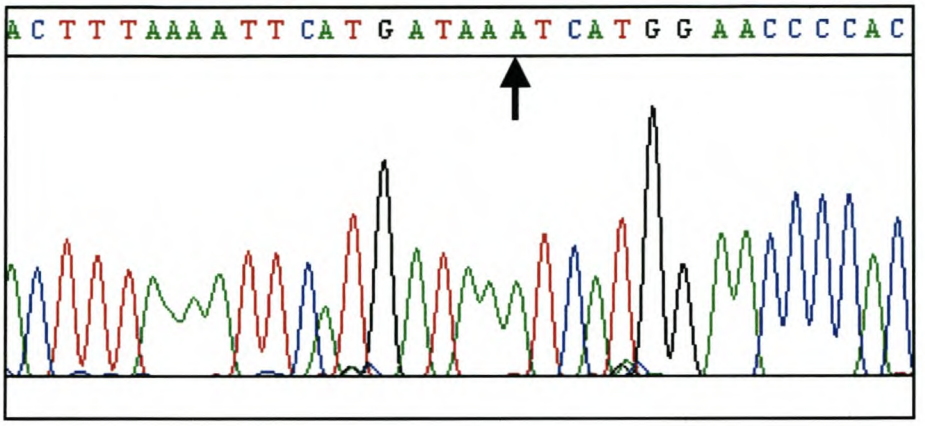
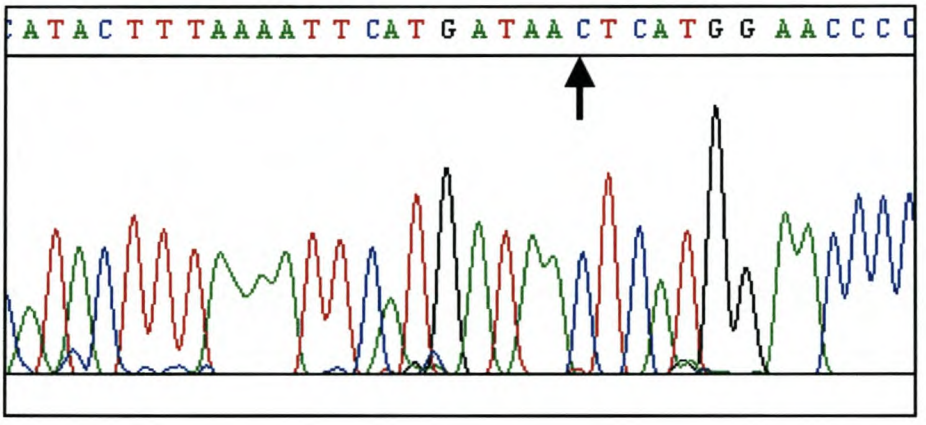


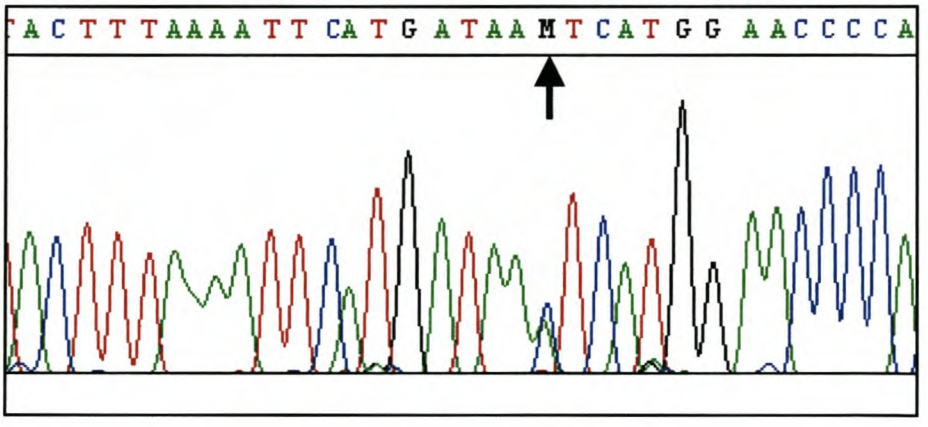
Figure E.05: A 15% (v/v) SSCP polyacrylamide gel of the CYP1B1 polymorphism in three of the Caucasian controls (L1-L3). Lanes 1 and 2 are individuals heterozygous for the polymorphism (C/A genotype), while lane 3 contains an individual homozygous for the A-allele. The gel was stained in 0.0075% (v/v) EtBr for visualisation of the bands.



a



b



c

Figure E.06: **Sequence outputs for the three possible genotypes for the CYP1B1 polymorphism. Figure E.06a indicates the homozygous A-allele state, figure E.06b indicates the homozygous C-allele state and figure E.06c indicates the heterozygous C/A state. PCR products were sequenced using the CYP1B1F primer as described in Table 3.01 and Table 4.01.**

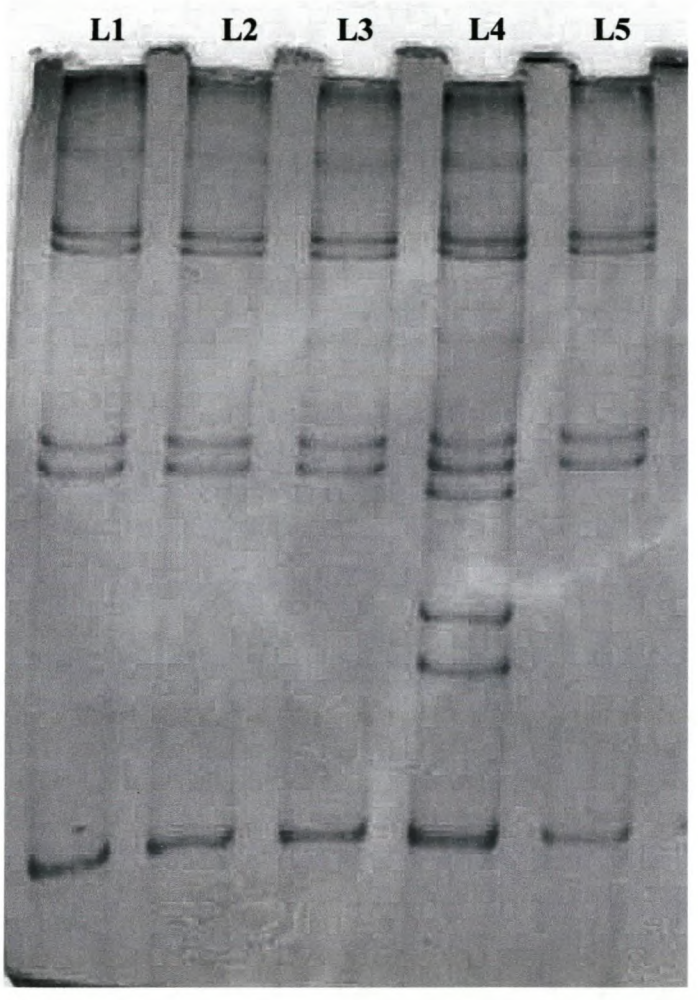


Figure E.07: A 15% (v/v) SSCP polyacrylamide gel of the CYP2D6*3 polymorphism in five of the variegate porphyria patients (L1-L5). Lanes 1, 2, 3 and 5 are individuals homozygous for the A-allele, while lane 4 contains an individual heterozygous for the polymorphism (A/del genotype). The gel was silver stained for visualisation of the bands.

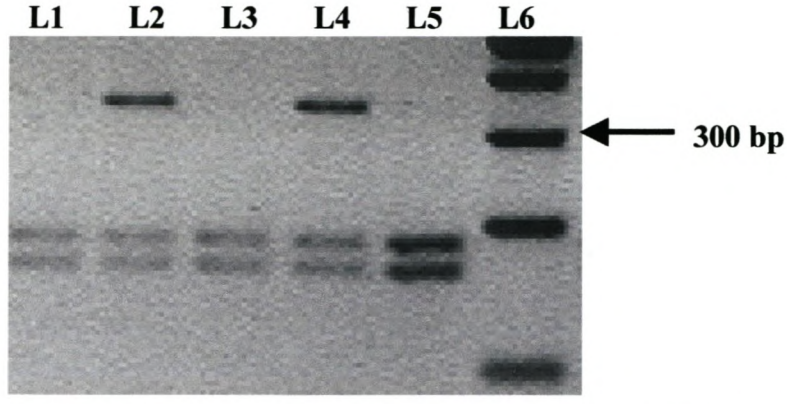


Figure E.08: **A 3% (w/v) agarose gel of the CYP2D6*4 polymorphism (*MvaI/BstNI* restriction enzyme digest) in five of the variegate porphyria patients (L1-L5). Lanes 1, 3 and 5 are individuals homozygous for the G-allele, while lanes 2 and 4 contain individuals heterozygous for the polymorphism (G/A genotype). Lane 6 contains a 100 bp ladder which served as a molecular size marker. The gel was stained in 0.0075% (v/v) EtBr for visualisation of the bands.**

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