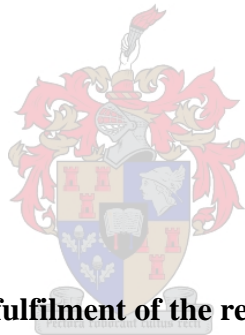


**GENETIC ASSOCIATION ANALYSIS OF
POLYMORPHISMS IN FOUR
CYTOCHROME P450 GENES, THE *MDR1*
GENE AND TREATMENT-OUTCOME IN
XHOSA SCHIZOPHRENIA PATIENTS**

BY

ERIKA TRUTER



**Thesis presented in partial fulfilment of the requirements for the degree of
Masters of Science at the University of Stellenbosch**

Study Leader: Prof L Warnich

Co-study Leader: Prof DJH Niehaus

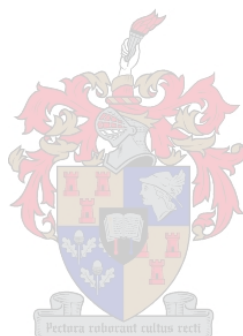
March 2007

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.

Signature:

Date:



ACKNOWLEDGEMENTS

- Patients for participation;
- Prof L Warnich (Department of Genetics, University of Stellenbosch) for being the best supervisor;
- Prof DJH Niehaus (Department of Psychiatry, University of Stellenbosch) for assistance as co-supervisor;
- Dr L Koen (Department of Psychiatry, University of Stellenbosch) for providing clinical samples and patient data;
- Mr W Botes (Department of Genetics, University of Stellenbosch) for statistical guidance and advice;
- Miss H Hitzeroth (Department of Genetics, University of Stellenbosch) for providing the *DRD3* data used in chapter three;
- Mr J Parathyras (Department of Genetics, University of Stellenbosch) for helping with the *MDR1* genotyping in chapter five;
- Miss L Van der Merwe (Department of Genetics, University of Stellenbosch) for advice regarding practical work in the lab;
- Mr C van Heerden and Miss G Agenbag (Central Analytic Facility, University of Stellenbosch) for DNA sequence analysis;
- University of Stellenbosch and the National Research Foundation (NRF) for funding;
- University of Stellenbosch and the NRF for financial support for E Truter
- Renier, my husband, for all the love and motivation throughout the year that kept me going;
- My parents for all their love, support and for always believing in me.

ABSTRACT

Rapidly expanding knowledge of the human genome allows new insight into the interaction between drugs and DNA. The heterogeneous nature of schizophrenia is known to cause different patients to display dissimilar drug responses, reflecting distinct genetic profiles. Resulting adverse side effects include tardive dyskinesia (TD), a movement disorder associated with the long-term use of antipsychotic drugs. The identification of a pharmacogenetic basis of TD may have significant clinical implications in the treatment of schizophrenia, allowing individualised prescription of antipsychotic drugs and eventual elimination of undesirable side effects.

The current study focussed on a number of South African Xhosa schizophrenia patients, some of whom have been diagnosed with TD. The investigation sought to establish whether the underlying mechanism causing the disorder to manifest only in some individuals, might be attributed to differences in DNA sequences, i.e. genomic susceptibility. A number of candidate polymorphisms in the *CYP* and *MDR1* genes were evaluated in three separate analyses. (The same approach was followed in each investigation, and only known polymorphisms were selected.) The incidences of the various variants were compared between TD and non-TD patients. In addition, potential predisposing factors, i.e. tobacco and cannabis smoking and anhedonia, were taken into consideration. These were analysed concurrently with DNA data and TD status.

The first section of the study evaluated a potential association between selected cytochrome P450 (*CYP*) gene polymorphisms and TD. These variants included single nucleotide polymorphisms (SNPs) in the *CYP1A2* and *CYP17 α -hydroxylase* genes, i.e. -3860G>A (*CYP1A2*1C*), -163C>A (*CYP1A2*1F*) and 27T>C (*CYP17 α -hydroxylase*). A statistically significant difference in genotypic and allelic distribution ($P=0.036$ and $P=0.031$ respectively) was observed between patients with and without TD concerning the *CYP17 α -hydroxylase* variant that can be explored by the known influence of this variant on dopamine release. Cannabis smokers who are carriers of this polymorphism had a pronounced decreased risk for TD.

CYP3A4 and CYP3A5 are responsible for the metabolism of several antipsychotic drugs. In the next section of our study the SNPs *CYP3A4*1B*, *CYP3A4*1G*, *CYP3A4* C23081T, *CYP3A5*3* and *CYP3A5*6* were tested as susceptibility loci for TD. The findings suggested that presence of the *CYP3A5*3* polymorphism might contribute to the development of TD ($P=0.038$). A haplotype encompassing the *CYP3A4*1G* and *CYP3A5*3* polymorphisms was more common amongst the TD patients than the non-TD patients ($P=0.049$).

Polymorphisms in the *MDR1* gene have the ability to alter the human drug transporter P-glycoprotein (P-gp) expression which plays an important role in drug secretion and response. The final section of our analysis aimed to determine whether any of four *MDR1* gene SNPs T-129C, C1236T, G2677T/A and C3435T contribute to elevated TD risk. The results indicated that the T-129C and C3435T polymorphisms might be associated with a predisposition to TD. Statistically significant differences ($P=0.0015$ and $P=0.0063$ respectively) were revealed between TD and non-TD regarding Hardy-Weinberg equilibrium (HWE) only.

The current investigation represents the first to study the influence of some of the above mentioned *CYP* and *MDR1* gene polymorphisms in conjunction with the development of TD. Evidence is provided for a possible association between SNPs in the *CYP* (*CYP17 α -Hydroxylase* and *CYP3A5*3*) and the *MDR1* (T-129C and C3435T) genes.

OPSOMMING

Snel toenemende kennis aangaande die mensgenoom bring nuwe insig rakende die wisselwerking tussen DNS en geneesmiddels. Pasiënte reageer verskillend op antipsigotiese medikasie, wat toegeskryf kan word aan verskillende individuele genetiese profiele. Hierdie variasie op DNS vlak, tesame met die komplekse aard van skisofrenie, veroorsaak ongunstige newe-effekte en lei tot tardiewe diskinesie (TD). Sou navorsing 'n farmakogenetiese basis vir TD kon uitlig, sal dit lei tot merkwaardige kliniese implikasies in die behandeling van skisofrenie. Dit sal die weg baan vir geïndividualiseerde medikasie en uiteindelik negatiewe medisinale newe-effekte uitskakel.

Die huidige studie konsentreer op Suid-Afrikaanse Xhosa skisofrenie pasiënte, sommige van wie kliniese simptome van TD toon. Die ondersoek poog om vas te stel waarom sommige pasiënte die toestand ontwikkel en ander nie. Dit het ten doel om vas te stel of die ontstaan van hierdie sindroom verklaar kan word aan die hand van verskille in DNS volgorde-samestelling, of te wel genetiese vatbaarheid. Kandidaat polimorfismes in die Sitochroom P450 (*CYP*) en die *MDR1* gene wat reeds gedokumenteer is, is geëvalueer in drie afsonderlike analyses. Die voorkoms van die variante is vergelyk tussen pasiënte met of sonder TD. Die bydrae wat bykomende faktore, insluitende tabak en dagga rook en anhedonia, mag lewer in verhoogde risiko vir TD, is ook in ag geneem.

Die eerste onderafdeling van die studie toets 'n potensiële verwantskap tussen *CYP* geen-polimorfismes en TD. Die variante sluit in enkel nukleotied polimorfismes in die *CYP1A2* en *CYP17 α -Hidroksilase* gene: -3860G>A (*CYP1A2*1C*), -163C>A (*CYP1A2*1F*) en 27T>C (*CYP17 α -Hidroksilase*). Die resultate het 'n statisties betekenisvolle verskil tussen die genotipe- en alleel-frekwensies ($P=0.036$ en $P=0.031$ onderskeidelik) tussen TD en nie-TD pasiënte opgelewer betreffende die *CYP17 α -Hidroksilase* polimorfisme wat verklaar kan word deur die bekende invloed van hierdie variant op dopamien vrystelling. Individue wat die polimorfisme dra en dagga gerook het, het 'n verlaagde risiko om TD te ontwikkel.

CYP3A4 en CYP3A5 metaboliseer verskillende antipsigotiese middels. Die volgende afdeling behels die evaluasie van die enkel nukleotied polimorfismes *CYP3A4*1B*, *CYP3A4*1G*, *CYP3A4 C23081T*, *CYP3A5*3* en *CYP3A5*6* as moontlike vatbaarheidsloci vir TD. Die bevindinge hier bring na vore dat die *CYP3A5*3* polimorfisme betrokke mag wees by 'n verhoogde TD risiko ($P=0.038$). 'n Haplotipe wat die *CYP3A4*1G* en *CYP3A5*3* polimorfismes behels was meer algemeen onder die TD as onder die nie-TD pasiënte ($P=0.049$).

Polimorfismes in *MDR1* het die vermoë om die uitdrukking van die menslike P-glikoproteïen (P-gp), te verander wat 'n belangrike rol speel in die uitskeiding van medisyne en die reaksie daarop. Die moontlike betrokkenheid van vier *MDR1* geen enkel nukleotied polimorfismes T-129C, C1236T, G2677T/A en C3435T by TD is ondersoek. Die uitslag van die studie vestig die aandag op 'n moontlike verbintenis tussen die T-129C en C3435T polimorfismes en die ontwikkeling van TD. Die TD en nie-TD groepe verskil alleenlik t.o.v. Hardy-Weinberg ewilibrium (HWE) ($P=0.0015$ en $P=0.0063$).

Die onderliggende invloed van bg. polimorfismes in die *CYP* en *MDR1* gene op die oorsprong van TD is nog nie tevore bestudeer nie. Die studie is die eerste om bewys te lewer dat die ontstaan van TD moontlik verband mag hou met sekere polimorfismes in die *CYP* (*CYP17 α -Hydroxylase* and *CYP3A5*3*) en die *MDR1* (T-129C and C3435T) gene.

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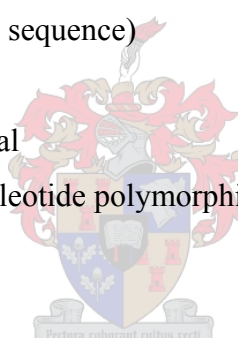
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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations are listed in alphabetical order.

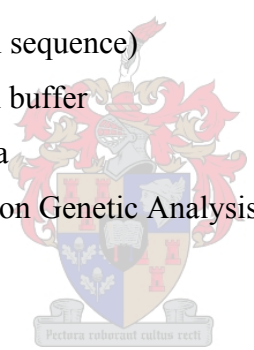
α	Alpha
A	Adenine (in DNA sequence)
ATCB1	ATP-binding cassette, subfamily B gene
ADRs	Adverse drug reactions
AIMS	Abnormal Involuntary Movement Scale
Ala	Alanine
bp	Base pair
ASSP	Alternative splice site predictor
C	Cytosine (in DNA sequence)
$^{\circ}\text{C}$	Degrees Celsius
CI	Confidence interval
cSNP	Coding single nucleotide polymorphism
COOH	Carboxyl group
CYP	Cytochrome P450
DHEA	Dehydroepiandrosterone
DMEs	Drug metabolizing enzymes
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
<i>DRD2</i>	Dopamine receptor D ₂ gene
<i>DRD3</i>	Dopamine receptor D ₃ gene
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
dz	Dizygotic
EDTA	Ethylenediamine tetra-acetic acid (C ₁₀ H ₁₆ N ₂ O ₈)
EMs	Extensive metabolizers



G	Guanine (in DNA sequence)
Gly	Glycine
GST	Glutathione S-transferases
Heter	Heterozygote
Homo mut	Homozygote mutant
Homo wt	Homozygote wild-type
HWE	Hardy-Weinberg equilibrium
IMs	Intermediate metabolizers
iSNP	Intergenic single nucleotide polymorphism
μl	Microlitre
M	Molar (moles per litre)
<i>MDR1</i>	Multidrug resistance gene
MgCl ₂	Magnesium chloride
ml	Millilitre
mRNA	Messenger ribonucleic acid
mz	Monozygotic
n	Sample size
NAT	N-acetyltransferase
NFSE	Nifedipine-specific element
ng	Nanogram
ng/μl	Nanogram per microlitre
NH ₂	Amino group
OR	Odds ratio
%	Percentage
<i>P</i>	Probability
PAA	Polyacrylamide
PCR	Polymerase chain reaction
P-gp	P-glycoprotein



PMs	Poor metabolizers
pSNPs	Perigenic single nucleotide polymorphisms
PXR	Pregnane X receptor
®	Registered trademark
REA	Restriction enzyme analysis
RFLP	Restriction fragment length polymorphism
RSA	Republic of South Africa
SA	South Africa
SAS	Statistical analysis system
Ser	Serine
SNP	Single nucleotide polymorphism
T	Thymine (in DNA sequence)
TBE	Tris borate-EDTA buffer
TD	Tardive dyskinesia
TFPGA	Tools for Population Genetic Analysis
Thr	Threonine
UGT	UDP-glucuronosyltransferases
UMs	Ultra rapid metabolizers
USA	United States of America
UTR	Untranslated region
UV	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume



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CHAPTER ONE

INTRODUCTION

Adverse reaction to medication is a major cause of death and is partly ascribed to inconsistent drug response displayed by different patients. Pharmacogenetics encompasses the study of inter-individual variability in drug response due to polymorphisms in specific genes, i.e. genes of which the encoded protein is involved in drug interaction. Polymorphisms in genes such as Cytochrome P450 (*CYP*) and multi drug resistance (*MDR1*) have the capacity to change the expression of genes, leading to altered drug metabolism and disposition, which in due course may result in adverse drug reactions.

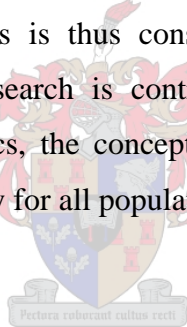
The construction of single nucleotide polymorphism (SNP) profiles constitutes an approach to differentiate between patients who respond favourably to medication and those who experience undesirable side effects. Scientists working in the field of pharmacogenetics aspire to permit the physician to improve medication response by way of individualized prescriptions. This procedure will result in safer, more effective and cost-efficient medicine.

Numerous disorders could greatly benefit from pharmacogenetic research studies. A complex disease that is being subjected to intense scrutiny is schizophrenia and its associated medication induced disorder tardive dyskinesia (TD). Several genes, e.g. *CYP2D6*, *CYP1A2*, *CYP17 α -hydroxylase*, *DRD3* and *MnSOD* have been researched in various other population groups in terms of TD. Genes analysed in this study have been carefully selected based on each gene's importance in the development of TD. However, in our laboratory a study regarding important genes such as *CYP2D6*, *DRD3* and *MnSOD* have already been analysed in relation to TD development in the same Xhosa schizophrenia population used in this study (A Hitzeroth, unpublished). Genes analysed in this study include *CYP1A2* and *CYP17 α -hydroxylase* important in the development of TD and dopamine release respectively (chapter three), *CYP3A4* and *CYP3A5* important in

antipsychotic drug metabolism (chapter four) and *MDR1* important in drug transport (chapter five). To our knowledge genes analysed in this study have not previously been investigated in African population groups such as the Xhosas.

It has to be emphasized that the known high level of variation between the genomes of not only different individuals, but also between different ethnic groups has to be taken into account in molecular studies of drug response. Genetic variants associated with variable sensitivity to medication occur at different frequencies in different population groups. Detailed population based databases have to be drawn up to provide the necessary genomic information to be used in predicting efficient or poor drug metabolism in patients.

This study contributes to provide data for a unique South African population the Xhosas. The discipline of pharmacogenetics is thus considered being applied as a form of predictive testing. While new research is continuously contributing to the rapidly expanding field of pharmacogenetics, the concept of individualized drug therapy is a distant, but certain, achievable reality for all population groups including South Africans.



CHAPTER TWO

LITERATURE REVIEW

2.1 SCHIZOPHRENIA

2.1.1 History

Psychotic disorders constitute a group of severe mental illnesses involving comprehensive impairment in reality testing and are often characterised by delusions and hallucinations (DSM-IV 2000). One of the members of the psychotic disorders group is schizophrenia, a disease marked by disconnection between thoughts, feelings and action. The term is derived from the Greek roots “skhizo” (to split) and “phren” (mind) and was first described by Kraepelin in 1887 as dementia praecox (Kraepelin 1907). It was only in 1908 that Bleuler realised this illness was not a form of dementia, and suggested the name schizophrenia (Stotz-Ingenlath 2000). In the first half of the twentieth century schizophrenia was considered to be a hereditary disease. Individuals diagnosed with schizophrenia were therefore subjected to eugenics in many countries and hundreds of thousands were sterilized or murdered (Allen 1997).

2.1.2 Diagnosis

Schizophrenia is characterised by at least one month of active-phase symptoms (delusions, hallucinations, disorganized speech, behaviour and negative symptoms) and some additional signs and functional impairment persisting for a period of six months (DSM-IV 2000). The diagnostic criteria for schizophrenia are shown in Table 1. Typical symptoms involve a range of perceptual, emotional and cognitive dysfunctions, classified in two categories: positive and negative. Positive symptoms are classified in two distinct dimensions. The psychotic dimension encompasses delusions and hallucinations, while disorganized speech and behavioural abnormalities such as catatonic behaviour distinguish the disorganized dimension. Delusions are defined as the misinterpretation of perceptions or experiences and can include somatic or religious delusions. Bizarre

delusions are very common amongst schizophrenia patients, but are difficult to evaluate across cultures. Auditory hallucinations, i.e. the hearing of voices are extremely common and are usually characterised as third person commentary or conversing types. Catatonic behaviour is typified by decreased reaction to the environment (DSM-IV 2000).

Negative symptoms refer to a loss of normal functions, such as affective flattening, alogia, avolition and anhedonia, also known as limited emotional expression. Affective flattening includes poor eye contact and the lack of responsive facial reactions. Alogia is characterised by limited response to questions, empty replies (also referred to as poverty of speech) and decreased fluency of speech. Avolition refers to a lack of inner drive to participate in work or social activities. Anhedonia is characterised by the inability to experience pleasure and an accompanying loss of interest in pleasurable activities. Anhedonia encompasses physical and social components and has been suggested to increase an individual's risk of developing psychosis (Mathews & Barch 2006). Individuals who have the ability to experience pleasure, have some kind of protection against the negative influences of stress, creating in this manner a buffer against the development of psychotic disorders (Meehl 1962). Anhedonia leads to an "aversive drift" or the tendency to view events as negative or threatening (Meehl 1962). Rating this symptom is based on the individual's rapport of emotional experiences and physiological responses to emotional pictures (Gooding *et al.* 2002), and is measured by the Schedule for the Assessment of Negative Symptoms (SANS) (Andreasen 1982).

Several models have been proposed to explain the development of positive and negative symptoms in schizophrenia, the majority of which highlight deficits in cognitive functioning (Andreasen 1997). Understanding the nature of the cognitive deficits, specifically those related to reduced dopaminergic innervations, may help to elucidate the basic neural mechanisms underlying the overall clinical presentation of schizophrenia. Therefore comparative studies of different individual genetic components may lead to a better understanding of inter-individual variability in cognitive response to certain drugs (Basile *et al.* 2002).

Table 1. Diagnostic criteria for schizophrenia (DSM-IV 2000)

<i>Characteristic symptoms</i>	
<u>Positive symptoms</u>	Psychoses <ul style="list-style-type: none"> Delusions Hallucinations
	Disorganized <ul style="list-style-type: none"> Disorganized speech Grossly disorganized or catatonic behaviour
<u>Negative symptoms</u>	<ul style="list-style-type: none"> Affective flattening Alogia Avolition Anhedonia
<i>Social dysfunction</i>	
When one or more major areas like self-care, interpersonal relation and work are below the level achieved before the onset.	
<i>Duration</i>	
Continues signs must be visible for six months and this must include one month of the active-phase symptoms.	

(Exclude underlying physical conditions or drug use that may explain the symptoms)

2.1.3 Schizophrenia subtypes

Schizophrenia can be divided into five subgroups, namely the paranoid, disorganized, catatonic, undifferentiated and residual types. The paranoid type is characterised by delusions or hallucinations with no prominent disorganized speech or behaviour. In the disorganized type the patient has to meet the criteria for disorganized speech, behaviour and affective flattening. One of the defining features of the disorganized type is the smiling or fatuous facial expression without the appropriate stimulus. The catatonic subtype can be diagnosed on any two of the following five symptoms: motoric immobility, excessive motor activity, extreme negativism, inappropriate postures and echolalia (involuntary echoing of a word or phrase) or echopraxia (involuntary imitation of another person's movement). Symptoms such as disorganized speech, behaviour and

flat or inappropriate affect must be present for the diagnosis of the undifferentiated subtype. The residual type of schizophrenia is characterised by at least one previous episode of schizophrenia without any accompanying symptoms of delusions or hallucinations, but with clear evidence of negative symptoms (DSM-IV 2000).

2.1.4 Incidence

The estimated prevalence rates (calculated in a selected population at a specific point in time), of schizophrenia are similar throughout the world except for some specific areas and range from 0.2% to 2.0% across many studies. The lifetime prevalence of schizophrenia is estimated to be between 0.5% and 1%. The incidence rate (the rate of occurrence in the same set of individuals observed over time), estimated at 1 per 10 000 per year, is much lower than the prevalence rates, due to the chronic characterisation of schizophrenia (DSM-IV 2000). A previous study revealed different schizophrenia incidence rates per 10 000 person-years for Caucasian men and women, as shown in Table 2. The same group determined the cumulative risk for schizophrenia by age 38 as 0.93% for men and 0.35% for women (Bresnahan *et al.* 2000). A study investigating the incidence and prevalence of schizophrenia at latitude reported that the incidence of schizophrenia in men is significantly higher at higher latitudes (Saha *et al.* 2006). Since an assumption has been made that the winter/spring effect (See section 2.2.2) contributes to an increased risk in developing schizophrenia it was of no surprise that latitude could influence the incidence of schizophrenia (Fouskakis *et al.* 2004). Evidence was also found, indicating that the prevalence of schizophrenia as well as the severity of the course is higher in developed countries (Saha *et al.* 2006).

Table 2. The incidence rate per 10 000 person-years for men and women (Bresnahan *et al.* 2000)

Men		Women	
Age	Incidence rates per 10 000 person-years	Age	Incidence rates per 10 000 person-years
15-19	9.4	15-19	1.6
20-24	5.6	20-24	1.3
25-29	3.3	25-29	4.1
30-34	0.9	30-34	-

2.1.5 Course

The age of onset for schizophrenia is in the early to mid 20s in men and in the late 20s in women. The course varies between patients – some may remain chronically ill, whereas others may display exacerbations and remissions. Complete remission, however, is not common amongst schizophrenia patients (DSM-IV 2000). The life expectancy of schizophrenia patients is much shorter than that of the general population and up to 10% of individuals commit suicide (DSM-IV 2000).

2.2 CAUSES OF SCHIZOPHRENIA

It has been suggested that a combination of genetic vulnerability and environmental stressors contribute to the development of schizophrenia. Therefore the disease is one of complex inheritance, where the interaction between several genes is likely to increase the risk of developing schizophrenia (Harrison & Owen 2003).

2.2.1 Hereditary pattern

Research has highlighted the influence of genetic factors in the etiology of schizophrenia. When schizophrenia is present in a family, the first-degree biological relatives have a 10 times greater risk of developing schizophrenia, compared to the rest of the population (DSM-IV 2000). Concordance rates for schizophrenia are higher in monozygotic (mz) twins, suggested at 28%, than in dizygotic (dz) twins. The estimates of heritability of schizophrenia from twin studies revealed rates as low as 11-13.8% amongst mz twins, and 1.8-4.1% amongst dz twins (Koskenvuo *et al.* 1984). The substantial discordance rate in mz twins indicates environmental factors play an important role as well (DSM-IV 2000). However, more recent studies indicated heritability estimates of 71-83% (Cardno *et al.* 2002)

2.2.2 Environmental influences

Abuse or trauma experienced as a child or stressful life events as well as other factors, such as poverty and discrimination, can increase the risk of developing schizophrenia (MacMillan *et al.* 2001). Living in urbanized environments can increase an individual's risk by 68-77% as showed in a Swedish study on 4.4×10^6 individuals (Sundquist *et al.*

2004). Interestingly, individuals born in winter or spring in the northern hemisphere, showed an increased risk of developing schizophrenia (Davies *et al.* 2003).

2.2.3 Predisposing factors

The cannabinoid hypothesis for the pathogenesis of schizophrenia is based on the observation that cannabis (also known as marijuana) abuse can precipitate a clinical psychotic state with hallucinations, delusions and emotional liability resembling schizophrenia (Johns 2001). It has the ability to worsen the positive symptoms of schizophrenia even under regular medication of antipsychotic drugs (Ujike *et al.* 2002). In schizophrenia patients the inhalation of cannabis may lead to poor outcome and liability to relapse (Ujike *et al.* 2002). A Swedish study showed that individuals using cannabis before the age of 18 have a six-fold higher risk of developing schizophrenia (Andreasson *et al.* 1987). It has been suggested that cannabis abuse and schizophrenia could share some neurobiological pathways and therefore putatively some predisposing genes (Leroy *et al.* 2001). Functional interactions have been reported between cannabis and dopamine transmission (Giuffrida *et al.* 1999), which is of great importance since the involvement of dopamine, specifically the dopamine D₃ receptor (*DRD3*), has been suggested in the etiology of schizophrenia (Dubertret *et al.* 1998).



2.2.4 Treatment

The treatment of schizophrenia comprises a two-fold process. Firstly, medication is administered to control acute psychotic symptoms and secondly to improve the patient's function and quality of life (Kasper 2006). Medication used to treat schizophrenia is called anti-psychotics, and is divided in two categories, namely typical anti-psychotics (older group) and atypical anti-psychotics (newer group). Initial treatment should include one atypical anti-psychotic medication such as Aripiprazole, Olanzapine, Quetiapine, or Risperidone. If no response is visible within four weeks, a second atypical drug should be prescribed where-after a third. The last-line of treatment for schizophrenia is an atypical anti-psychotic called Clozapine that has the potential to cause serious side effects (Murphy *et al.* 2006).

2.2.5 Associated disorders

Due to long term anti-psychotic drug treatment, many patients often develop reversible side effects also known as medication-induced movement disorders, such as Neuroleptic-Induced Parkinsonism, Neuroleptic-Induced Acute Dystonia and Neuroleptic-Induced Tardive Dyskinesia (DSM-IV 2000). The latter is often irreversible.

2.3 SCHIZOPHRENIA AND TARDIVE DYSKINESIA (TD)

2.3.1 The disorder

Tardive dyskinesia (TD) is a heterogeneous disorder, distinguished by involuntary movements that can occur in any muscle group. It develops in many schizophrenia patients following exposure to neuroleptic medication. The risk increases if the drug is used for longer than three months or one month for patients 60 years and older. For a positive diagnosis the movements have to be present for a period longer than four weeks. It is classified in three groups; choreiform (rapid, jerky), athetoid (slow, sinuous) and rhythmic movements (stereotypies). Abnormal orofacial movements occur in 75%, limb involvement in up to 50% and axial dyskinesia of the trunk in 25% of TD individuals. Only 10% of TD individuals are affected in all three of these areas. The dyskinetic movements are assessed by the Abnormal Involuntary Movement Scale (AIMS) that can be divided in two groups, i.e. low count (representing values 1-8) and high count (in the range 8-20). These AIMS scores are used to diagnose TD according to specific criteria (Schooler & Kane 1982).

TD symptoms worsen with stimulants, emotional arousal, stress, distraction and during concentration to move an unaffected body part. The abnormal movements are suppressed temporarily by increasing the medication, are reduced by relaxation and are normally absent during sleep. The incidence of TD is higher among patients that have been exposed to multiple antipsychotic drugs, than those who have used only one drug (Kane 2004). Four major risk factors have been identified for the development of TD, i.e. mood disorders, neurological conditions, cumulative amount of neuroleptic medication and early development of side effects. In addition, the following potential social-

demographic and clinical risk factors have been identified: family history of schizophrenia (with or without TD), affective disorders, diabetes mellitus, alcohol or drug abuse, smoking, menopause, ethnicity (higher in African-Americans) and advanced age (Müller *et al.* 2004).

The onset of TD, often associated with cosmetic problems such as grimacing, tongue protrusion, lip smacking, puckering and pursing of lips and rapid eye blinking, can occur at any age and warning signs are generally very mild. Medical complications, such as ulcers, loss of teeth, depression, weight loss, and difficulty in swallowing and breathing manifest in severe cases of TD (DSM-IV 2000). TD is stable over time in approximately 50%, worsen in 25% and improve in 25% of patients (DSM-IV 2000). TD has been stigmatized and patients experience the disease as very distressing. Finding employment is difficult, as the abnormal involuntary movements are not readily endured (Müller *et al.* 2004).

2.3.2 Incidence

The prevalence rates, (calculated in a selected population at a specific point in time) and the incidence rates, (the rate of occurrence in the same set of individuals observed over time) of neuroleptic-induced TD are shown in Table 3 (Kane 2004). The frequency of TD is the same amongst young males and females but amongst the elderly (>60 year old) it appears to be less frequent in males than in females (DSM-IV 2000). Information regarding the prevalence of TD in Black populations in Africa is limited to three studies conducted in Kenya and South Africa. 11.9% of schizophrenia patients in Nairobi (Gatere *et al.* 2002), 31% of South African schizophrenia Zulu (Holden 1987) and 30% of Xhosa schizophrenia patients in the Western Cape met the criteria for TD according to the AIMS (Fig. 1), (Patterson *et al.* 2005).

The incidence of TD is much higher among smokers than non-smokers. Smoking induces the activity of the hepatic microsomal enzymes and enhances the metabolism of neuroleptic antipsychotic drugs. Therefore smokers require higher dosages of antipsychotic drugs (Ebadi & Srinivasan 1995). It has been put forward that increased

dopaminergic activity of the nicotine leads to dopamine hypersensitivity or neurotoxicity from the free radicals in cigarette smoke, causing damage to the neurons in the basal ganglia (Chong *et al.* 2003).

Table 3. The prevalence and incidence rates of neuroleptic-induced TD among younger and elderly (60 years and older) schizophrenia individuals (DSM-IV 2000).

<i>Prevalence of TD</i>	
Young individuals with long-term neuroleptic treatment	20-30%
Elderly individuals	50%
<i>Incidence rate of TD per year</i>	
Younger individuals	3-5%
Elderly individuals with one year exposure to medication	25-30%

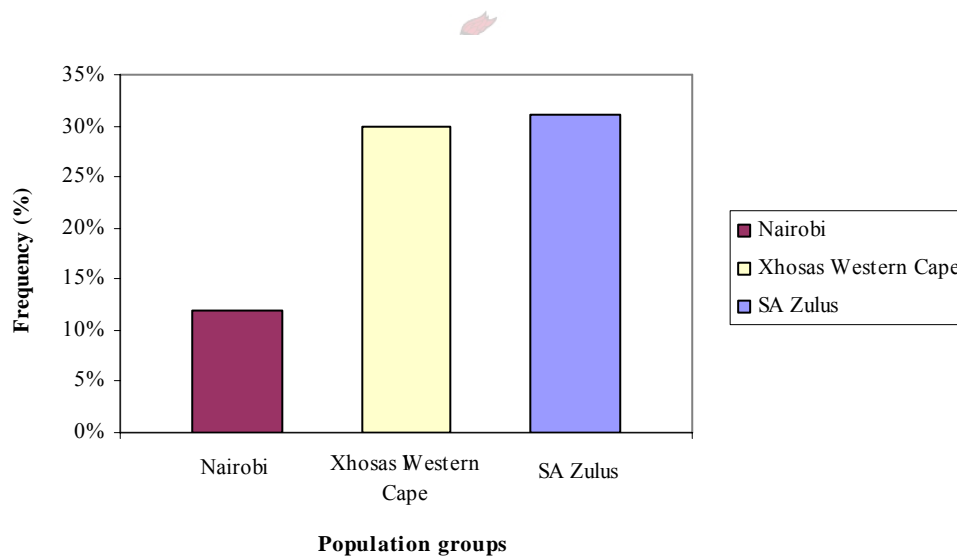


Fig. 1 The prevalence of TD amongst Black African Schizophrenia patients in Nairobi, Xhosas in the Western Cape and SA Zulus (Holden 1987; Gatere *et al.* 2002; Patterson *et al.* 2005).

2.3.3 Hypotheses for the development of TD

A genetic predisposition for the development of TD has been identified, following family and twin studies amongst schizophrenia patients (Muller *et al.* 2001). Several theories have been postulated for the pathophysiology of TD. Of these, the dopamine overactivity hypothesis (Jeste & Wyatt 1981) is the most important and gave rise to a large number of

association studies using polymorphisms in genes involved in the neurotransmitter pathway. These included the dopamine (D₂ & D₃) and serotonin receptor genes *DRD2* and *DRD3* (Tiwari *et al.* 2005a).

The term neuroleptic refers to medication with dopamine-antagonist properties such as haloperidol, chlorpromazine and clozapine (DSM-IV 2000). It has been suggested that neuroleptic-induced movement disorders, e.g. TD, may be caused by chronic blockage of the dopamine receptor sites, resulting in hypersensitivity of dopamine receptors in the brain (Goetz *et al.* 1982; Ebadi & Srinivasan 1995). However, this hypothesis is not universally accepted. Arguments against it are summarized by Ebadi & Srinivasan (1995):

- Noradrenergic over-activity instead of dopamine over-activity may contribute to TD.
- Hyperactivity of the dopaminergic systems in the brain plays a definite role in the etiology of schizophrenia and some studies suggest that the output of dopamine is much lower in schizophrenia patients. No differences between the dopamine receptors (D₁ and D₂) and the occurrence of TD have been found amongst schizophrenia patients.
- Modification in the expression of dopaminergic transmission failed to provide proof of TD.
- TD is sometimes observed only after months or even years of drug treatment, whereas dopamine receptors are noticed within one month of treatment (Goetz *et al.* 1982; Ebadi & Srinivasan 1995).

2.3.4 Future clinical implications in the management of TD

In assessing TD risk profiles for patients receiving antipsychotic drugs, both genetic and environmental factors have to be taken into consideration. The availability of a genetic test to screen for susceptibility to TD will be of great help to the psychiatrist prescribing antipsychotic drugs. It will aid the selection of suitable drugs and appropriate dosage there-of (Müller *et al.* 2004). Since there is no successful treatment for TD at present, onset of the disorder should ideally be prevented. As far as management of TD is

concerned, one study suggests that branched-chain amino acids may reduce the abnormal involuntary movements since it can decrease the synthesis of neurotransmitters such as dopamine (Richardson *et al.* 2003).

2.4 PHARMACOGENOMICS

Pharmacogenomics collects genomic information through genomics, bio-informatics and molecular biology techniques including screening, DNA sequencing and many more (Norton 2001). The functions and interactions of selected genes in the genome are used to identify certain “hot spots” and susceptibility loci in the genome in an attempt to explain inter-individual variation in drug metabolism and medication induced side effects (Basile *et al.* 2002). Individualized drug therapy is then made possible based on knowledge of an individual’s genetic profile (Suarez-Kurtz 2005). Ultimately, it allows the clinician to predict the outcome of medication in areas such as toxicity levels and efficacy of the drugs (Norton 2001). The scope, improved production of new drugs and the potential benefits of pharmacogenomics are indicated in Table 4 (Norton 2001).

Table 4. The scope, improvement in production of new drugs and the potential benefits of clinical pharmacogenomics (Norton 2001).

Scope for clinical pharmacogenomics	Improvement in production of new drugs	Potential benefits
Identification and characterization of candidate genes and polymorphisms	Validating genomic diversity and more suitable drug targets	Reduction in drug-development time
Correlation of DNA polymorphisms with therapy and drug side effects	Elimination of unsuitable drug candidates and targets	Screening for a specific disease, drug response markers
Development of molecular genetic test for prediction of drug response or selection	Faster clinical development by using better trails	Individualised drugs and more accurate dosage thereof
-	Developing drugs with clearly improved medical outcomes	Minimum ADRs

2.5 PHARMACOGENETICS AND ADVERSE DRUG REACTIONS

Pharmacogenetics can be defined as investigating specific polymorphisms in a gene of which the encoded protein is involved in drug interaction (Basile *et al.* 2002). Inter-individual variation in drug response is ascribed to polymorphisms in drug transporters, drug metabolising enzymes (DMEs) and/or drug receptors. Single nucleotide polymorphisms (SNPs) are the most abundant polymorphisms and account for the majority of altered drug responses (See section 2.7). These polymorphisms are capable of altering gene function or protein expression, which potentially gives rise to adverse drug reactions (ADRs) in selected patients (Basile *et al.* 2002).

2.5.1 Adverse drug reactions

10-50% of hospitalized patients respond to drug treatment whereas the remaining (50-90%) are poor or non-responders (Ingelman-Sundberg 2001). ADRs include allergies, hypokalaemia, gastro-intestinal disorders, neurological symptoms and renal failure, some of which can be fatal (Moore *et al.* 1998). It has been estimated that annually $> 2 \times 10^6$ hospitalized patients in the United States show ADRs, 100 000 of whom have been fatal (Norton 2001; Shastry 2006). Three mechanisms have been put forward for the manifestation of ADRs. Firstly, variants in a gene can be associated with an alteration in the metabolism of the drug (pharmacokinetics). Secondly, specific gene variants may be able to induce ADRs. Lastly, gene variants in the drug targets such as enzymes, transporters, receptors and effectors may lead to modulation in the efficacy of the drug or may result in ADRs (pharmacodynamics) (Müller *et al.* 2004). In general medical illnesses it was found that 30-60% of patients were non-responders (Ingelman-Sundberg 2004), whereas approximately 30-40% of patients with mood disorders were non-responders to antipsychotic drugs (Shastry 2006). A diagnostic tool enabling the identification of responders and non-responders would decrease the cost of drug treatment and reduce or prevent ADRs by 70% (Moore *et al.* 1998; Ingelman-Sundberg 2001). The overall benefits to society are improved health care and reduced medical costs (Moore *et al.* 1998). Gene polymorphisms have the ability to alter the metabolism of drugs, resulting in ADRs (de Groot 2006). Thus detailed knowledge of these

polymorphisms are crucial in both drug therapy and drug development (Ingelman-Sundberg 2004). Despite the positive prospects and benefits promised by pharmacogenetics, potential negative implications exist. These include discrimination against certain individuals or population groups for pharmacogenetic testing, high costs and availability, violation of privacy, acceptance to the public and negative psychosocial consequences such as guilt, disbelief, anxiety and fear for passing on the genetic mutations to their offspring (Shastry 2006).

2.6 PHARMACOKINETICS

Pharmacokinetic factors refer to the interaction between the drug metabolizing phase I and phase II enzymes (Müller *et al.* 2004). Phase I enzymes are important in the metabolism of antipsychotic drugs and include cytochrome P450 (CYP) enzymes, flavin-containing monooxygenases, reductases, esterases and alcohol dehydrogenases (Müller *et al.* 2004). Phase II enzymes allow excretion and comprise glutathione S-transferases (GST), N-acetyltransferase (NAT) and UDP-glucuronosyltransferases (UGT) and are able to conjugate drugs using glutathione or acetyl coenzyme A organic donor molecules (Van der Weide & Hinrichs 2006).

Phase I enzymes, the drug metabolizing enzymes (DMEs) contain functional polymorphisms. These polymorphisms give rise to four groups of metabolizers, defined as: poor metabolizers (PMs; deficiencies in or no metabolism of drugs due to mutations), intermediate metabolizers (IMs; reduced enzyme activity), extensive metabolizers (EMs; metabolize the drugs efficiently) and ultra rapid metabolizers (UMs; increased metabolism as a result of gene amplification or over expression (Fig. 2), (Norton 2001; Van der Weide 2006; Ingelman-Sundberg 2004). These categories define the metabolic capacity of an individual. It is known that different individuals display distinct differences in response to the same drug dosages (Ingelman-Sundberg 2004).

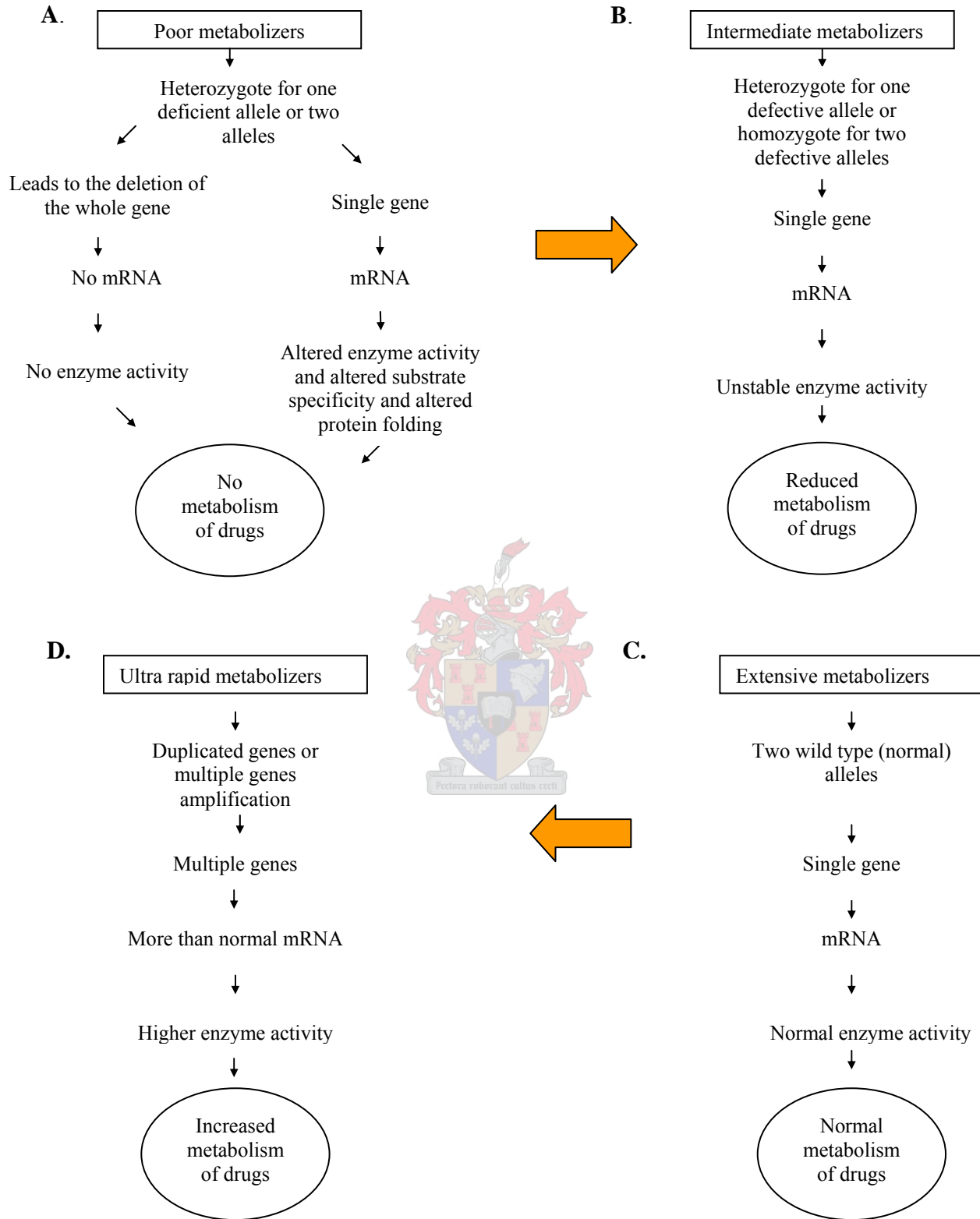
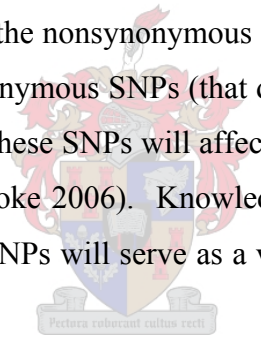


Fig. 2 The four different groups of metabolizers: [A] Poor metabolizers, [B] Intermediate metabolizers, [C] Extensive metabolizers and [D] Ultra rapid metabolizers and their effects on drug metabolism.

2.7 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

There are >19 000 genes in the human genome, many of which contain several different single nucleotide polymorphisms (SNPs). SNPs denote isolated, single nucleotide differences between individuals and include basepair substitutions, nucleotide insertions or basepair deletions (Ingelman-Sundberg 2001). More than 12×10^6 SNPs have been identified in humans, the majority of which are not functional according to the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi). Random SNPs, also called intergenic SNPs (iSNPs), are the most common, found in intergenic DNA regions or in the introns of genes (Marzolini *et al.* 2004). SNPs located in the non-coding regions are called perigenic SNPs (pSNPs) and are found outside the translated regions of the gene, in the promoter area. Coding SNPs (cSNPs) are found in the translated regions (exons) and are the main focus of pharmacogenetics (Ingelman-Sundberg 2001). The cSNPs are divided in two groups, the nonsynonymous SNPs (that lead to alteration of the amino acid complex) and the synonymous SNPs (that do not lead to amino acid changes) (Marzolini *et al.* 2004). Some of these SNPs will affect gene function, protein expression and response to drug therapy (Cooke 2006). Knowledge about the genotype-phenotype correlation and the frequency of SNPs will serve as a valuable aid in individualized drug therapy (Yi *et al.* 2004).

A faint watermark of a university crest is visible in the background of the text. The crest features a shield with various symbols, topped with a crown and supported by two figures. Below the shield is a banner with the Latin motto "Pectora robustant cultus recti".

A single mutation is ideally not viewed on its own, since the resulting enzyme folds for two similar variants can differ tremendously. Mutations located close to each other should be analysed relative to one another, in haplotype studies (Ingelman Sundberg 2001). A haplotype is a specific segment of DNA that is inherited as a unit (Saurez-Kurtz 2005). Utilising biomarkers, a haplotype map is designed to promote the understanding of inter-individual variation in drug response (Shastry 2006). Thus a gene-based haplotype approach takes into account the specific combination of SNPs, rather than single SNPs present in an allele that allow changes in drug response (Fig. 3), (Anglicheau *et al.* 2003). Since schizophrenia is a complex disease that involves many genes, analyses of individual SNPs are unlikely to provide information regarding genome-drug

interactions and gene susceptibility to TD. A better comprehension is expected from haplotypes.

A. SNPs

	SNP		SNP		SNP
	↓		↓		↓
Individual 1	AGTT C AGCC....	GTAC A ACGT....	CGGT T CACT....		
Individual 2	AGTT A AGCC....	GTAC T ACGT....	CGGT G CACT....		
Individual 3	AGTT A AGCC....	GTAC A ACGT....	CGGT T CACT....		
Individual 4	AGTT C AGCC....	GTAC T ACGT....	CGGT G CACT....		



B. Haplotypes

Individual 1	C	A	T
Individual 2	A	T	G
Individual 3	A	A	T
Individual 4	C	T	G

Fig. 3 An illustration of the same short stretch of DNA in four different individuals indicating [A] SNPs at three different positions and [B] the haplotype formed for each individual.

2.8 GENES INVOLVED IN THE METABOLISM AND TRANSPORT OF ANTIPSYCHOTIC DRUGS IN SCHIZOPHRENIA AND TD PATIENTS

2.8.1 Cytochrome P450

Cytochrome P450 is a super family of heme-containing enzymes, responsible for the metabolism of >90% of all drugs. The CYP enzymes are categorized in families and subfamilies according to amino acid sequence. To form part of a family, enzyme members have to be 40% identical and those with over 55% sequence homology form part of the same subfamily (Van der Weide & Hinrichs 2006). Cytochrome P450 plays

an important role in the metabolism of endogenous (steroids and fatty acids) and exogenous (drugs and carcinogens) compounds (de Groot 2006). To date 57 different cytochrome P450s (*CYP*) genes have been identified in humans (Shastry 2006). A specific notation has been devised for the cytochrome P450s. In the example *CYP3A4*, “*CYP*” denotes human cytochrome P-450, “3” the gene family, “A” the subfamily and “4” the gene coding for a specific polypeptide (Keshava *et al.* 2004).

70-80% of *CYP* enzymes are phase I metabolising enzymes responsible for the conversion of lipophilic drugs to polar metabolites and the metabolism of clinical drugs (Ingelman-Sundberg 2004). The *CYP* 1-3 enzymes are responsible for the oxidative metabolism of exogenous compounds such as drugs and xenobiotics (Van der Weide & Hinrichs 2006). The *CYP3A* enzymes, of which *CYP3A4* and *CYP3A5* are most important, are most abundant in the liver and small intestine and metabolise >60% of all drugs (Cascorbi 2006a).

Variability in *CYP* activity is due to endogenous factors (age, gender, morbidity), exogenous factors (foods, smoking, co-medication) and the highly polymorphic characteristics of the *CYP* enzymes (Van der Weide & Hinrichs 2006). 56% drugs causing ADRs have been studied. Of these, 86% are metabolised by polymorphic P450 enzymes (Ingelman-Sundberg 2004; Phillips *et al.* 2001). There are two major reasons why predictive genotyping for P450s is not being performed routinely: Firstly, clinicians are not knowledgeable regarding pharmacogenetics and secondly proven drug efficacy based on favourable genotypic data is still lacking (Ingelman-Sundberg 2004). Four *CYP* genes that have been proven to be of high importance in the metabolic pathway of anti-psychotic drugs and are investigated in this study are *CYP1A2*, *CYP17 α -Hydroxylase*, *CYP3A4* and *CYP3A5*.

2.8.1.1 Cytochrome P450 1A2

CYP1A2, an inducible gene of the cytochrome P450 family of genes, is located at 15q22 alongside two other cytochrome P450 genes (Tiwari *et al.* 2005a) and plays an important role in the metabolism of many commonly used drugs and some neurotoxins (Sachse *et*

al. 1999). CYP1A2 is also one of the major enzymes involved in the metabolism of xenobiotics and the metabolic clearance pathway for typical antipsychotic drugs used by schizophrenia patients (Özdemir *et al.* 2001). It has therefore been proposed that changes in *CYP1A2* could potentially influence risk for the development of TD (Tiwari *et al.* 2005a). It has been shown that the activity of CYP1A2 can be induced by the polycyclic aromatic hydrocarbons in cigarette smoke (Van der Weide & Hinrichs 2006) and by other environmental factors (Tiwari *et al.* 2005a). The wide inter-individual variation of CYP1A2 activity may be due to several polymorphisms in *CYP1A2* causing altered enzyme activity (Sasche *et al.* 1999). Two of the most important SNPs in *CYP1A2* that have been studied in relation to schizophrenia and TD *CYP1A2*1C* (G>A) and *CYP1A2*1F* (C>A) (Tiwari *et al.* 2005a).

2.8.1.2 Cytochrome P450 17 α -Hydroxylase

The *CYP17 α -Hydroxylase gene* (*CYP17*), located on chromosome 10q24.3, consists out of eight exons, encodes the steroid 17 α -Hydroxylase and mediates activity (Tofteng *et al.* 2004). It has been suggested that variability in the cytochrome P450 17 α -Hydroxylase could influence susceptibility to TD, because of its effect on neuroprotective capacity and dopamine output (Segman *et al.* 2002). One such variation is the T>C SNP in the 5'-untranslated promoter region of the *CYP17* gene. Its influence on converting pregnanalone to dehydroepiandrosterone (DHEA) and ultimately altering dopamine release is shown in Fig. 4 (Segman *et al.* 2002).

2.8.1.3 Cytochrome P450 3A4

The *CYP3A4* gene located on chromosome 7q21.3-q22.1 comprises 13 exons (Hashimoto *et al.* 1993). CYP3A4 is one of the most important drug-metabolising enzymes and is responsible for the oxidation of drugs, steroids, fatty acids, antipsychotic drugs and xenobiotics (Keshava *et al.* 2004). Almost 40-45% of all drugs are being metabolised by CYP3A4 (Ingelman-Sundberg 2004). It is present in the gut, colon and small intestine, but accounts for 30% of the total CYP in the human liver (Yu *et al.* 2004). At least 78 polymorphisms have been reported in *CYP3A4*, (Keshava *et al.* 2004) which may explain the large inter-individual differences in CYP3A4 activity (Van der Weide & Hinrichs

2006). The most common variant in *CYP3A4* is the *CYP3A4*1B* (-392 A>G), which has a frequency of up to 45% in Black subjects (Lamba *et al.* 2002). *CYP3A4* and *CYP3A5* have similar catalytic specificities (Yu *et al.* 2004) therefore active *CYP3A5* compensates for defective *CYP3A4* metabolism and vice versa (Lee *et al.* 2005).

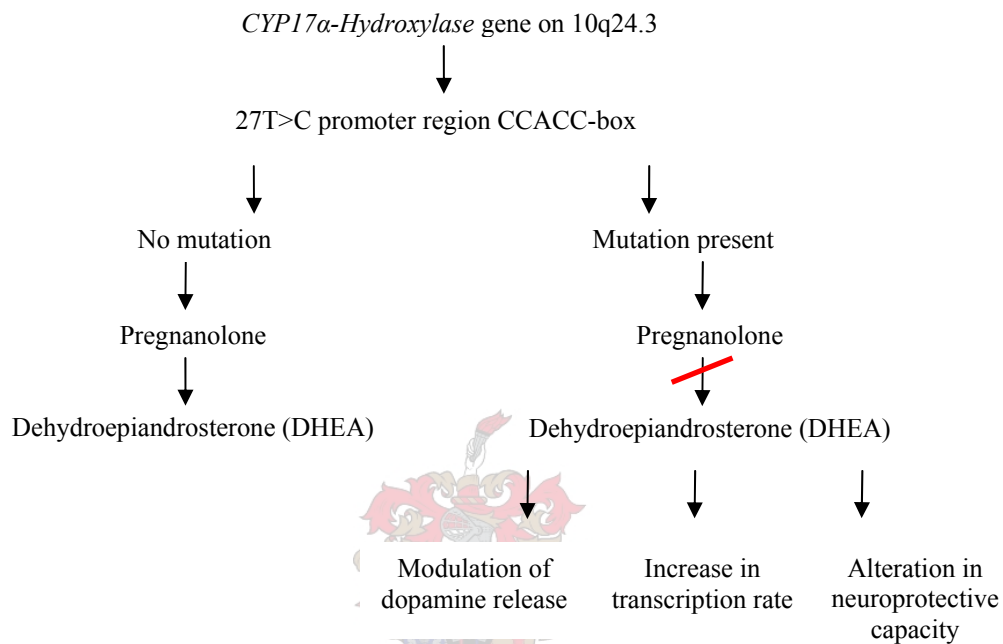


Fig. 4 The 27T>C SNP in the *CYP17α-Hydroxylase* gene alters the conversion of pregnanolone to dehydroepiandrosterone (DHEA), leading to a modulation in dopamine release (Segman *et al.* 2002).

2.8.1.4 Cytochrome P450 3A5

The *CYP3A5* gene, consisting of 13 exons, is situated on chromosome 7q21.3-q22.1. It has been suggested that the presence or absence of expression of active *CYP3A5* depend on functionally correct or incorrect alleles, and in this way accounts for the inter-individual variability in total *CYP3A* activity (Cascorbi 2006a). Several polymorphisms have been reported in *CYP3A5*, two of which, *CYP3A5*3* and *CYP3A5*6*, are the most important.

The *CYP3A5*3* (6986 A>G) polymorphism in intron 3 creates a cryptic splice site that leads to a premature stop codon at amino acid 102 in exon 3, resulting in erroneous

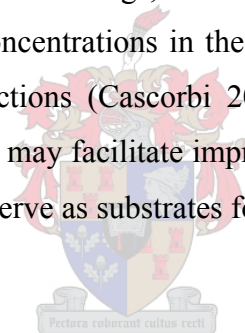
spliced mRNA production and loss of CYP3A5 activity (Yamamoto *et al.* 2005). This polymorphism may be the cause of inter-individual variability in CYP3A activity (Yu *et al.* 2004). It has previously been reported that 66% of Black Africans (Anglicheau *et al.* 2004) and 80% of Caucasians exhibited a low CYP3A5 expression and activity due to this polymorphism (Mathijssen & van Schaik 2006). Interestingly, it was found that carriers of the *CYP3A5**3 allele were more likely to possess the T than the C allele of the polymorphism C3435T in the multi drug resistance gene (*MDR1*) (Anglicheau *et al.* 2004). The *CYP3A5**6 (30570A>G) polymorphism in exon 7 causes absence of CYP3A5 expression in tissues (Kuehl *et al.* 2001). A correlation between this polymorphism and the deletion of exon 7 from *CYP3A5* mRNA, leading to reduced CYP3A5 activity, was observed in African Americans (Kuehl *et al.* 2001; Cascorbi 2006a).

2.8.2 Multidrug resistance gene (*MDR1*)

The human multidrug resistance (*MDR1*) gene contains a core promoter region and 28 exons and is localized to chromosome 7q21.1 (Marzolini *et al.* 2004). P-glycoprotein (P-gp) is a member of the adenosine triphosphate (ATP)-binding cassette family and is encoded by the *MDR1* gene, also known as the ATP-binding cassette, subfamily B (*ABCB1*) gene. The P-gp, ABCB1 is the best characterised human drug transporter (Cascorbi 2006b) and plays an important role in the oral bio-availability, distribution, excretion of and response to drugs. Investigation of genetic heterogeneity regarding SNPs in relation to drug efficacy and response revealed the importance of this transporter (Marzolini *et al.* 2004). P-gp is a phosphorylated and glycosylated transmembrane protein that has a protective function against potentially toxic xenobiotics (Yi *et al.* 2004). It consists of 1280 amino acids and acts as a transmembrane efflux pump responsible for the movement of drugs from the intracellular to the extracellular domain (Marzolini *et al.* 2004).

2.8.2.1 Substrates

Many drugs serve as substrates for MDR1. Therefore expression and functionality of the gene product can directly affect the therapeutic effectiveness of certain drugs, such as antidepressants and neuroleptics, known to be inhibitors or inducers of the P-glycoprotein (Kim *et al.* 2001). High levels of P-gp may lead to the insufficient uptake of certain drugs, whereas low levels of P-gp may cause undesired side effects of drugs (Hoffmeyer *et al.* 2000). Interestingly, the major DME CYP3A4 also metabolizes many drugs that serve as substrates for P-gp. This may be due to the co-ordinated regulation and tissue expression that CYP3A4 and MDR1 share in the liver and intestine and the fact that both genes are located close together on the same chromosome, at 7q22.1 and 7q21.1 respectively (Marzolini *et al.* 2004). Since P-gp is important in drug disposition, it is reasonable to hypothesize that polymorphisms in this transporter may influence the absorption, distribution and excretion of drugs, as well as treatment efficacy (Marzolini *et al.* 2004). Differences in P-gp concentrations in the liver have been reported to have a major effect on drug-drug interactions (Cascorbi 2006b). Predictive testing utilizing polymorphisms in the *MDR1* gene may facilitate improved drug therapy. In this instance the focus should be on drugs that serve as substrates for the *MDR1* gene (Hoffmeyer *et al.* 2000).



2.8.2.2 Polymorphisms

29 SNPs, including 11 non-synonymous, have been identified in the *MDR1* gene (Marzolini *et al.* 2004). The positions of the four most important SNPs are shown in Fig. 5 (Tanabe *et al.* 2001) and their frequencies in different population groups are indicated in Fig. 6 (Marzolini *et al.* 2004).

The noncoding SNP (T-129C) located in exon 1 has been associated with altered P-gp function and expression (Cavaco *et al.* 2003; Marzolini *et al.* 2004). The synonymous SNP in exon 12 (C1236T) has been reported to be in linkage disequilibrium with the G2677T/A and C3435T SNPs in exon 21 and 26 respectively (see below) (Marzolini *et al.* 2004). The SNP in exon 21 at position 2677 results in either of two amino acid changes namely, Ala893Ser (G2677T) or Ala893Thr (G2677A).

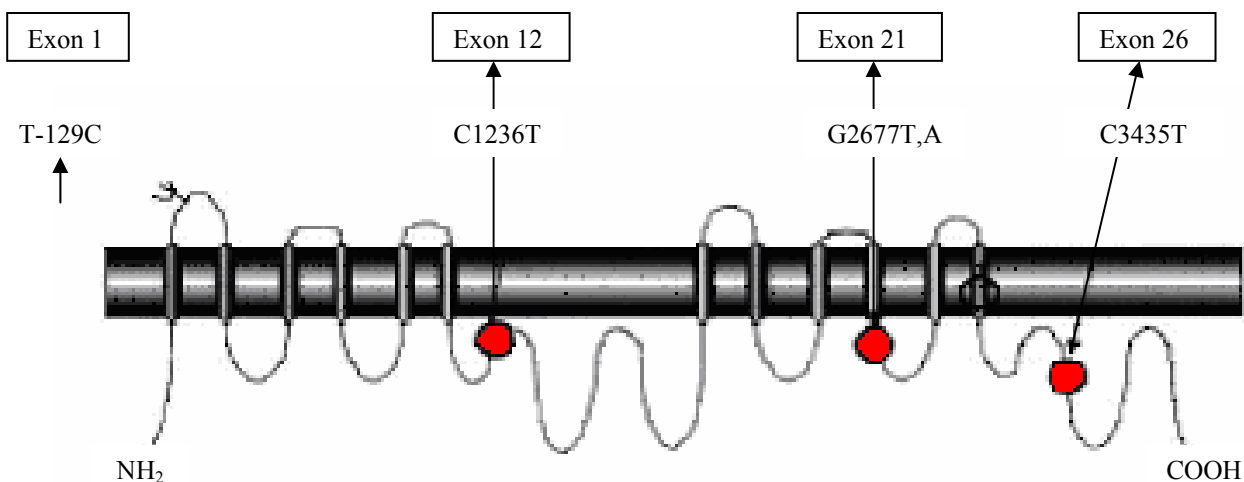


Fig. 5 A schematic representation of the P-gp predicted topology and secondary protein structure. Arrows at the top indicate the relevant positions of four SNPs located within the *MDR1* gene (Tanabe *et al.* 2001).

It was noticed that Caucasian carriers homozygous for the 2677T allele had a higher CYP3A4 expression compared to those who were homozygous for the 2677G allele (Lamba *et al.* 2006). Therefore it has been suggested that the *MDR1* (G2677T/A) SNP may play an important role in DMEs and drug transport in humans (Lamba *et al.* 2006). The synonymous SNP C3435T of exon 26 lies in a wobble position in codon 1145 and gives rise to reduced translation efficiency (Marzolini *et al.* 2004) and lower *MDR1* expression (Yi *et al.* 2004). The CC genotype of this SNP is highest amongst African populations (Fig. 6) and lowest amongst Asian populations (Balram *et al.* 2003). It has been speculated that in Africans the high CC genotype frequency may act as protection against the development of gastrointestinal-tract infections and that the low T allele frequency, may be the reason for an overall lower incidence of renal carcinoma (Schwab *et al.* 2003; Marzolini *et al.* 2004).

Linkage disequilibrium has been reported between G2677T/A and C3435T (Marzolini *et al.* 2004). Furthermore, the 2677 G>T, 3435C>T genotype was found to be an important prognostic indicator of CYP3A4 expression (Lamba *et al.* 2006). The transporter protein P-glycoprotein, encoded by *MDR1*, has a significant influence on brain penetration for some compounds and may influence pharmacodynamics and efficacy as well as distribution of the drugs. There appears to be a definite gene-gene interaction between

MDR1 and *CYP3A4* underlying a pharmacogenetic index of CYP3A4 expression (Williams *et al.* 2006).



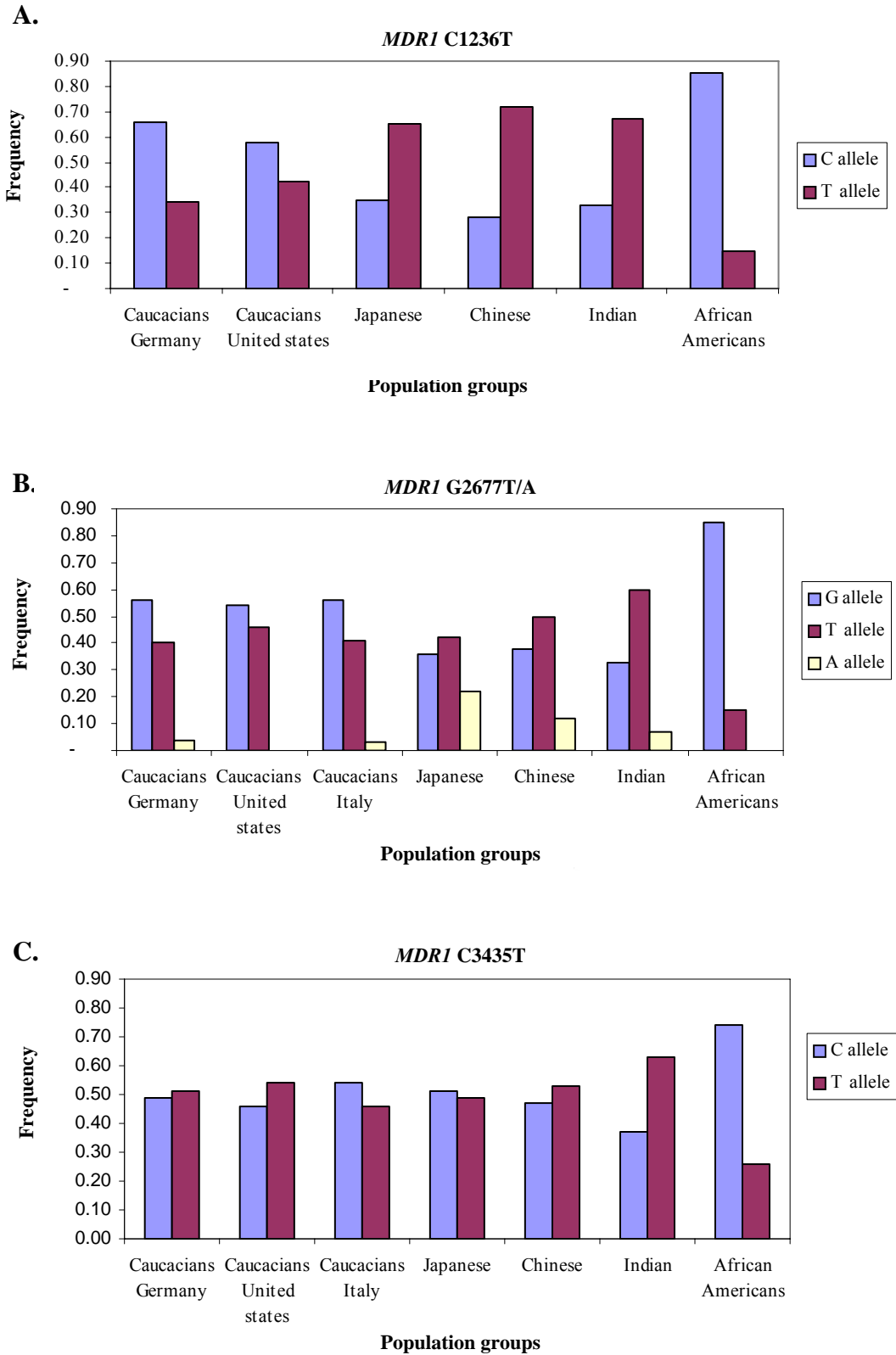


Fig. 6 The allele frequencies of [A] *MDR1* C1236T, [B] *MDR1* G2677T/A and [C] *MDR1* C3435T in various population groups (Marzolini *et al.* 2004).

2.9 INDIVIDUALISED THERAPY

2.9.1 Interethnic differences

Inter-individual variation in drug response poses a serious problem for clinicians prescribing medication. On receiving the same drug dosage, the plasma drug levels of two individuals who are identical in size and weight but from different ethnic groups, can vary more than 1000-fold. This inter-individual, interethnic variation in drug absorption, distribution and excretion is ascribed to disease determinants, genetic and/or environmental factors and the pharmacodynamic response (Ingelman-Sundberg 2001).

To date there is no set of biomarkers available to accurately predict patient's response to medication. A general theory holds that drug-metabolising enzymes evolved as a result of interactions between plants and animals. Food represents a broad range of CYP substrates, which may explain the reason behind interethnic differences (Van der Weide & Hinrichs 2006). It follows that ethnicity needs to be taken into consideration when selecting alleles for an attempted profile. Furthermore, several exogenous factors such as smoking and nutrition should be brought into the equation. Thus inter-ethnic variability in the frequencies of variant gene alleles provides a basis for the observed differences in pharmacokinetics and drug responses between populations (Schwab *et al.* 2003).

2.9.2 Individualised antipsychotic drug therapy aimed at schizophrenia

Schizophrenia is considered to be a heterogeneous disease and therefore different patients display dissimilar drug responses, reflecting distinct genetic profiles. Antipsychotic drug therapies have to progress in the direction of individualised drug therapy to minimise medication induced harmful side effects such as TD (Ohmori *et al.* 2003). Individualised drug therapy will enable psychiatrists to prescribe the right medication and correct dosage for the each patient, accelerating the process of recovery and minimising or eliminating negative side effects. The disciplines of pharmacogenomics and pharmacogenetics are gradually creating an impact in the selection of suitable drug therapies, thereby contributing to improved drug safety and efficacy (Cascorbi *et al.* 2001).

2.10 AIM OF THE STUDY

The aim of this study was to investigate whether an association exists between selected polymorphisms in the cytochrome P450 and *MDR1* genes and the disorder of TD manifesting sporadically in schizophrenia. Schizophrenia patients analysed here comprised individuals from the Xhosa population native to South Africa, some of whom displayed clinical symptoms of TD. The study also aimed to emphasise the importance of ethnic differences and the impact on pharmacogenetics. The ultimate benefits of pharmacogenetic studies such as these are to develop individualised drug therapy and ultimately prevent adverse drug reactions.

Study design

- i Suitable cytochrome P450 (*CYP*) gene polymorphisms were selected for molecular screening of the patients;
- ii Candidate polymorphisms in the *MDR1* gene were chosen to analyse patients ;
- iii Statistical analyses were performed to determine whether the incidences of the above known variants differ between TD and non-TD patients. In addition, tobacco and cannabis smoking and the development of anhedonia were taken into account as risk factors, and analysed in conjunction with DNA data and TD status;
- iv Haplotype analyses were conducted, where appropriate, to establish whether a specific haplotype may be associated with genetic susceptibility to TD in schizophrenia.

CHAPTER THREE

EVALUATION OF *CYP1A2* AND *CYP17 α -HYDROXYLASE* VARIANTS AS MOLECULAR MARKERS OF TARDIVE DYSKINESIA (TD) RISK IN XHOSA SCHIZOPHRENIA PATIENTS

3.1 ABSTRACT

Tardive dyskinesia (TD) is a complication of the long-term use of antipsychotic drugs in schizophrenia patients. The identification of pharmacogenetic basis of TD may have significant clinical implications in the treatment of schizophrenia, allowing individualised prescription of antipsychotic drugs and eventually elimination of adverse side effects. Cytochrome P450 genes are responsible for the metabolism of several antipsychotic drugs and polymorphisms in these genes affect the activity of the drug metabolising enzymes. In this study three polymorphisms were analysed in 117 Xhosa schizophrenia patients (55 of whom were diagnosed with TD) to evaluate a possible association with TD. These variants included single nucleotide polymorphisms (SNPs) in the *CYP1A2* and *CYP17 α -Hydroxylase* genes, i.e. -3860G>A (*CYP1A2*1C*), -163C>A (*CYP1A2*1F*) and 27T>C (*CYP17 α -Hydroxylase*). Both the *CYP1A2*1C* and *CYP1A2*1F* polymorphisms were in Hardy-Weinberg equilibrium (HWE) and did not demonstrate a significant association with TD. These two SNPs were analysed singly and in combination by haplotype analysis. The *CYP17 α -Hydroxylase* polymorphism was not in HWE in either of the two patient groups. Statistically significant difference in genotypic ($P=0.036$) and in allelic frequencies ($P=0.031$) between patients with and without TD, depicted the polymorphism to be more common amongst non-TD patients. This polymorphism also showed a significant difference between TD and non-TD individuals when considering cannabis use ($P=0.013$). It is proven that the *CYP17 α -Hydroxylase* polymorphism modulates the release of dopamine. Furthermore it has been postulated

before that some neurological pathways may be shared by cannabis abuse and schizophrenia. The preliminary results of this study thus warrant additional investigation to elucidate the role of the *CYP17 α -Hydroxylase* variant in the development of schizophrenia and TD.

3.2 INTRODUCTION

Schizophrenia is a psychological disorder and considered a heterogeneous disease. (Diagnostic and Statistical Manual–4th edition (DSM-IV, 2000). Several factors are taken into consideration by the psychiatrist before prescribing antipsychotic drugs and the dosage thereof, because different patients display dissimilar drug responses, reflecting distinct genetic profiles. Since specific genetic variants have been associated with sensitivity to drug response, it has been proposed that genetic tests may allow the design of individualized antipsychotic drug dosage (Ingelman-Sundberg *et al.* 1999; Ohmori *et al.* 2003; Shastry 2006).

Tardive dyskinesia (TD) is a common, adverse and often irreversible side effect associated with long-term antipsychotic drug treatment and does not manifest without the use of medication (Özdemir *et al.* 2001). Use of antipsychotic drugs increases the risk of developing TD by 4-5% per year (Patterson *et al.* 2005). It is an iatrogenic disorder, characterized by involuntary movements in any muscle group. The cause of the disease is unknown, but evidence of genetic components involved in susceptibility to TD has been documented (Özdemir *et al.* 2001; Basile *et al.* 2002).

TD develops in 20% of schizophrenia patients in the Caucasian population (Basile *et al.* 2000), but information regarding the prevalence of TD in black populations in Africa is limited. Only three studies have been documented, indicating that 31% of schizophrenia patients in Johannesburg, South Africa (Holden 1987) and 11.9% of schizophrenia patients in Nairobi met the criteria for TD (Gatere *et al.* 2002). The overall prevalence of TD in the Xhosa population has not yet been evaluated, but 30% of Xhosa schizophrenia patients in the

Western Cape have been diagnosed with the disorder according to the Abnormal Involuntary Movement Scale (AIMS) (Patterson *et al.* 2005).

Cytochrome P450 is responsible for most of the reactions in the metabolism of antipsychotic drugs (Ingelman-Sundberg *et al.* 1999). Differences in the pharmacogenetic responses observed in different individuals have been attributed to three main factors, i.e. absorption, distribution and clearance. Regarding clearance, it has been found that genetic polymorphisms affect the activity of the drug metabolising enzymes (Ohmori *et al.* 2003). The metabolism of antipsychotic drugs is affected by polymorphisms in Cytochrome P450 (*CYP*) genes. Two genes that have been studied in conjunction with drug response are *CYP1A2* on chromosome 15q22 in a Caucasian population (Basile *et al.* 2000) and *CYP17 α -Hydroxylase* on chromosome 10q24.3 in a Jewish population (Segman *et al.* 2002). A single nucleotide polymorphism (SNP) – 3860G>A (*CYP1A2*1C*) (<http://www.imm.ki.se/CYPalleles>) in the 5' upstream region of the human *CYP1A2* gene (Nakajima *et al.* 1999) has been associated with a decrease in *CYP1A2* enzyme activity, but has not been linked to enhanced risk of developing TD (Tiwari *et al.* 2005a). However, Chinese schizophrenia patients who were smokers and carriers of the *CYP1A2*1C* allele showed increased severity of TD (Tiwari *et al.* 2005a). Pharmacogenetic studies demonstrated that elevated *CYP1A2* activity only occurred in smokers. Thus the polymorphism was only of functional importance in smokers, supporting an association between smoking and TD in schizophrenia patients (Chong *et al.* 2003). Another polymorphism -163C>A in intron 1 of the *CYP1A2* gene known as *CYP1A2*1F* (<http://www.imm.ki.se/CYPalleles>) has also been reported in Caucasians (Sachse *et al.* 1999). This SNP was shown to be of clinical importance, because of the association of the A allele with TD in Caucasian schizophrenia patients (Basile *et al.* 2000).

CYP17 α -Hydroxylase is responsible for the conversion of pregnanolone to dehydroepiandrosterone (DHEA) (Zwain & Yen 1999). DHEA and its precursor pregnanolone are neuroactive steroids. These steroids have cytoprotective characteristics and provide discerning neuroprotective effects in the brain (Waters *et al.* 1997). A T>C

SNP in the 5'-untranslated promoter region of the *CYP17 α -Hydroxylase* gene creates a potential CCACC-box (Tofteng *et al.* 2004). The polymorphism leads to alterations in the gene's capacity to convert pregnanolone to DHEA, causing modulation of dopamine release and an increase in transcription rate (Segman *et al.* 2002). Schizophrenia patients with the *CYP17 α -Hydroxylase* CC genotype and a serine to glycine polymorphism in exon 1 of the dopamine D3 receptor (*DRD3*) gene were found to be at increased risk of developing TD (Segman *et al.* 2002), however a correlation between TD and the *CYP17 α -Hydroxylase* polymorphism on its own has not been proven. The *D3gly* variant has also independently been associated with TD in chronic schizophrenia patients in other studies (Segman *et al.* 1999; Basile *et al.* 1999; Lovlie *et al.* 2000).

In the current study the *CYP1A2*1C*, *CYP1A2*1F* and *CYP17 α -Hydroxylase* T>C polymorphic sequences were evaluated as potential genetic susceptibility loci for TD in schizophrenia patients belonging to a Black population group native to South Africa, the Xhosa. The identification of a DNA profile associated with the development of TD would provide a simple means of population screening for susceptibility and ultimately prevention of the disorder.

3.3 MATERIAL AND METHODS

3.3.1 Patient samples

Blood samples were obtained from 117 Xhosa individuals, 96 males and 21 females, with clinically diagnosed schizophrenia. AIMS was used to categorize the 55 TD patients. The remaining 62 patients that did not display any TD symptoms included 54 males and 8 females. Information regarding age, tobacco smoking, cannabis smoking and presence of the neurological disorder anhedonia was also obtained. An additional 100 Xhosa healthy control individuals with the same age criteria as the schizophrenia patients were randomly selected. All patients presented at Stikland Hospital, Bellville and provided written informed consent for participation in the study. The Ethics Review Committee of the Faculty of Health Sciences, University of Stellenbosch, Tygerberg approved the

investigation (1907/005:2001/c050). Genomic DNA was extracted according to a protocol of Miller *et al.* (1988).

3.3.2 Polymerase Chain Reaction (PCR) amplification

Genomic DNA from all patients were analysed using the polymerase chain reaction (PCR) to identify the presence of wild type and/or variant alleles in the *CYP1A2* and *CYP17 α -Hydroxylase* genes. A total of three different polymorphisms were studied; the *CYP1A2* locus included two variants.

***CYP1A2*1C* polymorphism:** The primers used to analyse this variant were: F5'-GCT ACA CAT GAT CGA GCT ATA C-3' and R5'-CAG GTC TCT TCA CTG TAA AGT TA-3' (Nakajima *et al.* 1999). PCR was performed in a total reaction volume of 25 μ l containing 100 ng template DNA, 15 pmol of each primer, 100 μ M dNTPs, 0.5 U of *Taq*-polymerase (Bioline, Randolph, MA, USA) and PCR buffer containing 3 mM MgCl₂ (Tiwari *et al.* 2005a). The PCR amplification conditions were as follows: initial denaturing step at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 56°C for 45 s, 72°C for 1 min and a final extension at 72°C for 7 min.

***CYP1A2*1F* polymorphism:** The primer sequences were: F5'-TGG AGT GGT CAC TTG CCT CT-3' and R5'-CTG GCT CAT CCT TGACAG T-3' (Tiwari *et al.* 2005a). The PCR reaction mix was the same as for *CYP1A2*1C*. Amplification conditions were: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 45 s and final extension at 72°C for 5 min.

***CYP17 α -Hydroxylase* polymorphism:** The primers for this variant were: F5'-CAT TCG CAC TCT GGA GTC-3' and R5'-AGG CTC TTG GGG TAC TTG-3' (Feigelson *et al.* 1997). PCR was performed in a total reaction volume of 25 μ l containing 100 ng template DNA, 15 pmol of each primer, 100 μ M dNTPs, 0.5 U *Taq*-polymerase (Bioline, Randolph, MA, USA) and reaction buffer containing 3 mM MgCl₂ (Tofteng *et al.* 2004). Amplification was performed as follows: initial denaturation step at 95°C for 3 min,

followed by 30 cycles of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min and final extension at 72°C for 7 min (Tofteng *et al.* 2004).

3.3.3 Restriction Enzyme Analysis (REA)

All three SNPs analysed in this study are revealed by restriction enzyme digestion following PCR. PCR product (8.8 µl) was used in a total volume of 10 µl containing appropriate buffer and restriction enzymes. Samples were incubated overnight at 37°C.

CYP1A2*1C G>A polymorphism: PCR amplification yielded a fragment size of 596 bp. In the presence of the polymorphic site, digestion with 1 U of *Ddel* I (New England BioLabs Ipswich, MA, USA) resulted in 2 fragments of 464 bp and 132 bp (Tiwari *et al.* 2005a).

CYP1A2*1F C>A polymorphism: The PCR product size of 520 bp was digested with 1 U of *Psp*OMI (SibEnzyme, Novosibirsk, Russia). *Psp*OMI is an isochizomere of *Bsp*120I, described initially for recognition of this particular polymorphic site (Tiwari *et al.* 2005a). Fragment sizes of 373 bp and 147 bp produced upon enzyme digestion represent the C allele. The undigested PCR sample represents the A allele.

CYP17 α -Hydroxylase T>C polymorphism: 1 U of *Msp*AI (New England BioLabs Ipswich, MA, USA.) was used to digest the PCR product. An undigested fragment size of 414 bp represents the wild type, while the digested products of 290 bp and 124 bp represent the C allele (Tofteng *et al.* 2004).

Restriction enzyme digested PCR products were analysed on 1.5% (w/v) agarose gels at 120V for 1hour. 1XTBE (0.089 M Tris, 0.089 M Boric acid and 20 mM EDTA, pH 8.0) was used as electrophoresis buffer and DNA fragments were visualised with UV light following staining in 0.5 µg/ml ethidium bromide.

3.3.4 DNA sequencing

Sequencing was done in two isolated instances upon detection of an uncharacteristic gel banding pattern following restriction enzyme digestion of PCR products. Samples were purified using 5 µl of diluted PCR (6.6 ng/µl) product and 2 µl ExcoSAP-IT (Amersham Biosciences Corporation, Ohio, USA) according to instructions from the manufacturer.

Sequencing was performed in a total reaction volume of 10 µl containing 19.9 ng PCR product, 2 µl sequencing mix (ABI PRISM di-deoxy Terminator Cycle Sequencing kit v3.1 Applied Biosystems) and 13.2 pmol PCR primer (See section 3.3.2) following the manufacturer's instructions. The ABI PRISM 3100 Genetic Analyser (Perkin Elmer, Applied Biosystems, Warrington WA, Great Britain) as well as BioEdit (Tom Hall, version 7.0.1, Isis Pharmaceuticals, Inc) were used to analyze the DNA sequences.

3.3.5 Statistical analysis

Allele and genotype frequencies were estimated and the HWE was tested with the assistance of TFGA (Miller 1997). Statistical difference between the total number of TD and total number of non-TD individuals was determined using an analog of a Fisher's exact test on a contingency table (2 X 2 or 2 X 3 as appropriate) with Microsoft® Excel 2000. A probability (p) value smaller than 0.05 was considered significant.

Differences in the observed frequencies of the three polymorphisms regarding smoking status, cannabis use and prevalence of anhedonia were explored using an analog to Fisher's exact test by means of a 2 X 3 contingency table. In situations where significant genotype differences were observed, allele frequencies were also tested for statistically significant differences by way of an analog to Fisher's exact test (2 X 2 contingency table). Probability (P) values smaller than 0.05 were considered statistically significant. Additional comparisons employing odds ratios (ORs) were performed for variants indicating significant differences in allele frequencies between the TD and non-TD groups (Bland & Altman 2000).

The Pearson Correlation coefficient (r) and standardized linkage disequilibrium coefficient (D') were determined. The arbitrary cut off values for r^2 and D' were considered 0.4 and 0.7 respectively. Thereafter haplotype analysis was conducted using the Haploview 3.31 program (Barrett *et al.* 2005) to determine whether a specific haplotype is associated with TD. Interaction between the *CYP17 α -hydroxylase* and *DRD3* ser9gly polymorphism was calculated according to Zhang *et al.* (2003), and the results were confirmed with the Statistical Analysis System (SAS) Enterprise Guide

(v.9.1 TS level 1M2) program. The *DRD3* ser9gly information used was obtained from unpublished data in our department on the same group of Xhosa schizophrenia patients (A Hitzeroth unpublished).

3.4 RESULTS

In the current study Xhosa schizophrenia patients with and without TD were investigated for the presence of the *CYP1A2*1C*, *CYP1A2*1F* and *CYP17 α -hydroxylase* polymorphisms. All three known SNPs were detected in both patient groups. Examples of the three SNPs are depicted in Fig. 1.

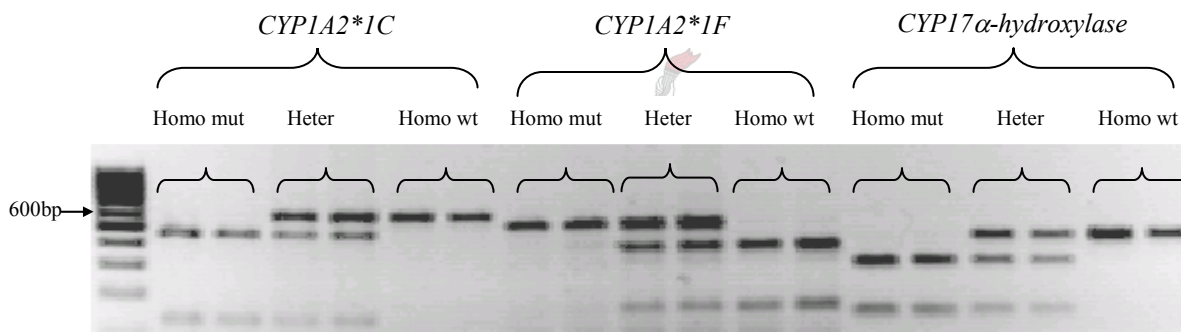


Fig. 1 Examples of the *CYP1A2*1C*, *CYP1A2*1F* and *CYP17 α -hydroxylase* polymorphisms following PCR, digestion with appropriate restriction enzymes and agarose gel electrophoresis (See Material and Methods). Resulting band patterns represent homozygotes for the variant allele (Homo mut), heterozygotes (Heter) and homozygotes for the wild type allele (Homo wt), as indicated. In the first lane a 100bp size marker (Fermentas) was loaded.

In addition, a previously unknown polymorphism was identified in the *CYP1A2* gene in two individuals from the non-TD group. A non-typical banding pattern was observed upon restriction enzyme analysis of the *CYP1A2*1F* PCR product. DNA sequence analysis depicted an identical SNP in both patients, i.e. 2238 T>G. This polymorphism creates an extra cutting site for the restriction enzyme *PspOMI*, along with the existing site, thus producing fragments of 308 bp and 147 bp and 65 bp instead of the usual fragment sizes of 373 bp and 147 bp. Both individuals were heterozygous and thus the configuration seen on the gel consisted of a banding pattern of 373 bp, 308 bp and 147

bp. Electrophoresis conditions utilised did not allow resolution of the 65 bp fragment, but its presence was confirmed by sequence analysis. DNA sequence data identifying the novel SNP are shown in Fig. 2. The two individuals displaying the unique 2238 T>G variant were included in tests evaluating severity of TD according to age and sex, but excluded from HWE and further statistical analyses.

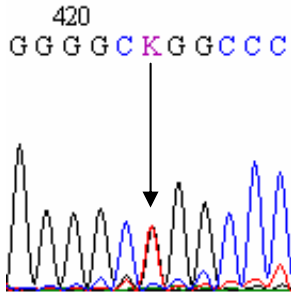


Fig. 2 DNA sequencing results showing the novel 2238T>G SNP observed in two patients.

The mean and standard deviations were calculated for age and AIMS according to gender in the TD and non-TD patient groups. The TD group was also compared to the non-TD group without taking gender into account. The data are presented in Table 1. Individuals in the total TD patient group were much older compared to the non-TD patients. This is not unexpected since TD is associated with the long-term use of antipsychotic drugs.

Table 1. The mean and standard deviation (in brackets) for age and AIMS score comparing the TD with the non-TD patient group for males and females and the total.

	TD		Non-TD		Total	
	Male n=42	Female n=13	Male n=54	Female n=8	TD n=55	Non-TD n=62
Age	38.83 (±11.607)	47.08 (±10.626)	29.12 (±8.454)	33.38(±8.450)	40.78(±11.827)	29.69(±8.508)
AIMS	6.81 (±6.058)	7.83(±4.509)	-	-	7.09(±5.635)	-

The two patient groups, with and without TD, were in HWE for the *CYP1A2*1C* ($P=0.4970$, $P=0.7122$ respectively) and *CYP1A2*1F* polymorphisms ($P=0.5853$, $P=0.9921$ respectively). However, the *CYP17 α -hydroxylase* polymorphism was not in HWE in either the TD or non-TD patient group ($P=0.0172$, $P=0.0349$ respectively). Pooled HWE between the TD and non-TD group were performed in order to assess these

results. The *CYP1A2*1C* polymorphism was in HWE ($P=0.4304$) as well as the *CYP1A2*1F* polymorphism ($P=0.6338$). The *CYP17 α -hydroxylase* polymorphism was not in HWE ($P=0.0026$) as would be expected. Since the *CYP1A2*1F* and *CYP1A2*1C* polymorphisms were in HWE in the pooled analysis an additional 100 normal Xhosa individuals were only genotyped for the *CYP17 α -hydroxylase* polymorphism, and found to be in HWE ($P=0.3558$).

Table 2. Genotypic and allelic distribution of the *CYP1A2*1C*, *CYP1A2*1F* and *CYP17 α -hydroxylase* gene polymorphisms in Xhosa schizophrenia patients with TD compared to patients without TD.

	Genotypic distribution				Allelic distribution		
	No. of patients (%)				G	A	<i>P</i> value
<i>CYP1A2*1C</i>	G/G	G/A	A/A	<i>P</i> value	G	A	<i>P</i> value
TD patients, n=55	26 (47%)	22 (40%)	7 (13%)	0.913	74	36	0.716
Non-TD patients, n=60	26 (43%)	26 (43%)	8 (14%)		78	42	
<i>CYP1A2*1F</i>	A/A	C/A	C/C	<i>P</i> value	A	C	<i>P</i> value
TD patients, n= 55	5 (9%)	26 (47%)	24 (44%)	0.913	36	74	0.863
Non-TD patients, n = 60	6 (10%)	26 (43%)	28 (47%)		38	82	
<i>CYP17α-hydroxylase</i>	T/T	T/C	C/C	<i>P</i> value	T	C	<i>P</i> value
TD patients, n= 55	21(38%)	32 (58%)	2 (4%)	0.036**	74	36	0.031**
Non-TD patients, n = 60	13 (22%)	38 (63%)	9 (15%)		64	56	

** Correlation is significant $P \leq 0.05$

Statistical analyses compared the distribution of the three polymorphisms in all the patients as follows: (a) categorized in two groups, based on presence / absence of TD only, and (b) TD and non-TD patients sub-classified according to tobacco smoking, cannabis smoking and anhedonia status. Genotype and allele frequencies are presented in Table 2. For the *CYP1A2*1C* polymorphism, no significant difference was found between the two groups with respect to genotype ($P=0.913$) or allele frequencies ($P=0.716$). The *CYP1A2*1F* polymorphism also showed no significant difference in genotype ($P=0.913$) or allele frequencies ($P=0.863$). With the *CYP17 α -hydroxylase* polymorphism a significant difference in genotype ($P=0.036$) and allele frequencies ($P=0.031$) was found between the two patient groups.

Genotypic distributions of the three polymorphisms comparing TD and non-TD patients, classified under the categories tobacco smoking, cannabis smoking and anhedonia are shown in Table 3. No significant statistical differences were found between TD and non-TD schizophrenic patients for the *CYP1A2*1C* polymorphism when considering tobacco smoking ($P=0.883$), cannabis use ($P=0.151$) and presence of anhedonia ($P=0.768$). The *CYP1A2*1F* polymorphism also yielded no observed statistical differences when considering the smoking ($P=0.604$), cannabis ($P=0.297$) or anhedonia ($P=0.622$) between the TD and non-TD clinical groups. The *CYP17 α -hydroxylase* polymorphism also did not show significant differences between TD and non-TD individuals when considering smoking ($P=0.120$) or anhedonia ($P=0.113$). Cannabis users, however, showed a significant difference ($P=0.013$) in genotype frequencies between TD and non-TD patients. In addition, a statistical significant difference in allele frequencies between the TD and non-TD group ($P=0.008$) was indicated. The C allele (variant) is more predominant in the non-TD group while the T allele (wt) is more predominant in the TD group when considering cannabis use. The odds ratio (OR) for the subgroup was 3.57 (95% confidence interval (CI), 1.3577 to 9.3939).

Table 3. Genotypic distribution of the *CYP1A2*1C*, *CYP1A2*1F* and *CYP17 α -hydroxylase* polymorphisms in Xhosa schizophrenia patients with TD compared to patients without TD, classified according to tobacco smoking, cannabis smoking and presence of anhedonia.

		Genotypic distribution				Allelic distribution		
		No. of patients (%)						
<i>CYP1A2*1C</i>		G/G	G/A	A/A	<i>P</i> value	G	A	<i>P</i> value
Tobacco Smoking	TD patients, n=37	19 (51%)	13 (35%)	8 (14%)	0.883	51	23	0.794
	Non-TD patients, n=47	22 (47%)	19 (40%)	6 (13%)		63	31	
Cannabis	TD patients, n=16	9 (56%)	3 (19%)	4 (25%)	0.151	21	11	0.684
	Non-TD patients, n=33	16 (49%)	14 (42%)	3 (9%)		46	20	
Anhedonia	TD patients, n=45	20 (44%)	20 (44%)	5 (12%)	0.768	60	30	0.775
	Non-TD patients, n=51	23 (45%)	20 (39%)	8 (16%)		66	36	
<i>CYP1A2*1F</i>		A/A	C/A	C/C	<i>P</i> value	A	C	<i>P</i> value
Tobacco Smoking	TD patients, n=37	19 (51%)	15 (41%)	3 (8%)	0.604	53	21	0.354
	Non-TD patients, n=47	19 (40%)	23 (49%)	5 (11%)		61	33	
Cannabis	TD patients, n=16	9 (56%)	5 (31%)	2 (13%)	0.297	23	9	0.218
	Non-TD patients, n=33	11 (33%)	17 (52%)	5 (15%)		39	27	
Anhedonia	TD patients, n=45	20 (44%)	22 (49%)	3 (7%)	0.622	62	28	0.708
	Non-TD patients, n=51	22 (45%)	21 (43%)	6 (12%)		65	33	

<i>CYP17α-hydroxylase</i>		T/T	T/C	C/C	<i>P</i> value	T	C	<i>P</i> value
Tobacco Smoking	TD patients, n=37	14 (38%)	21 (57%)	2 (5%)	0.120	49	25	0.088
	Non-TD patients, n=47	9 (19%)	32 (68%)	6 (13%)		50	44	
Cannabis	TD patients, n=16	9 (56%)	7 (44%)	0 (0%)	0.013**	25	7	0.008**
	Non-TD patients, n=33	6 (18%)	21 (64%)	6 (18%)		33	33	
Anhedonia	TD patients, n=45	17 (38%)	26 (58%)	2 (4%)	0.113	60	30	0.096
	Non-TD patients, n=51	10 (20%)	36 (70%)	5 (10%)		56	46	

** Correlation is significant $p \leq 0.05$

In a comparison between the total number of TD and non-TD patients, linkage disequilibrium was observed between the *CYP1A2*1C* and *CYP1A2*1F* loci. A search for an association between TD and a specific haplotype failed to provide evidence of a statistically significant difference between TD and non-TD patients (Table 4).

Interaction analysis between the *CYP17α-hydroxylase* and *DRD3* ser9gly polymorphism failed to provide proof of a statistically significant difference between TD and non-TD patients (Table 5).

Table 4. Haplotype analysis between alleles of, *CYP1A2*1C* and *CYP1A2*1F*, in linkage disequilibrium.

Haplotype	Schizophrenia patient ratios		Chi Square	<i>P</i> value
	TD	non-TD		
GA	0.345	0.333	0.038	0.8462
AA	0.327	0.350	0.132	0.7161
GC	0.327	0.317	0.030	0.8634

$D' = 0.999, r^2 = 0.486$

r, Pearson correlation; *D'*, standardized LD coefficient.

Table 5. Combined genotypes of the *DRD3* ser9gly and *CYP17α-hydroxylase* polymorphisms in TD and non-TD Xhosa schizophrenia patients.

Subjects	<i>DRD3</i> T/T		<i>DRD3</i> C/T, C/C	
	<i>CYP17</i> T/C	<i>CYP17</i> C/C	<i>CYP17</i> T/C	<i>CYP17</i> C/C
TD patients (n= 33)	0.70 (23)	0.03 (1)	0.24 (8)	0.03 (1)
non-TD patients (n= 46)	0.43 (20)	0.15 (7)	0.35 (16)	0.07 (3)
Total patients (n= 79)	0.55 (43)	0.10 (8)	0.30 (24)	0.05 (4)

DRD3 T/T; *CYP17* C/C vs *DRD3* C/T, C/C; *CYP17* C/C $P=0.584$

DRD3 C/T, C/C; *CYP17* T/C vs *DRD3* C/T, C/C ; *CYP17* C/C $P=0.741$

3.5 DISCUSSION

Schizophrenia is a distressing illness that exerts a social toll on the community. Paradoxically, medication aimed at alleviating the disturbing symptoms manifesting in schizophrenia has led to the emergence of TD. This disorder develops only in response to treatment and brings about a large measure of discomfort in affected individuals. It has been proposed that genetic predisposition may play an important role in the onset of TD. One approach aimed at improved clinical treatment of schizophrenia and eventually prevention of TD would involve the identification of susceptibility loci in individuals at high risk of contracting TD. The present pharmacogenetic study was undertaken to establish if an association exists between the *CYP1A2*1C*, *CYP1A2*1F* and/or *CYP17 α -hydroxylase* polymorphisms and a tendency to develop TD. The study included 117 Xhosa schizophrenic patients, 55 of whom developed TD while being treated with antipsychotic drugs.

The prevalence of schizophrenia was higher in men than in women which can be expected since the incidence rates per 10 000 person-years is higher in men than in females (Bresnahan *et al.* 2000). In this study 96 of the 117 patients were males and only 21 females. This ratio is reflected in most schizophrenia studies (Hanoeman *et al.* 2004; Veling *et al.* 2006). Findings of different studies regarding gender and the development of TD is very inconsistent. The frequency of TD amongst young males and females is the same, however in elderly patients (>60 years old) it seems to be less frequent in males (DSM-IV 2000). A study performed by Kane and Smith (1982) also reported a higher prevalence of TD amongst females than males. In our study individuals in the TD group were older than those in the non-TD group, probably reflecting longer use of antipsychotic drugs. According to the AIMS no major difference was found between the intensity levels of TD and gender. However a previous study reported a higher severity of TD amongst males than females (Tiwari *et al.* 2005a).

When considering TD and non-TD patients as two major groups, no statistically significant difference was observed regarding genomic distribution of the two

polymorphisms in the *CYP1A2* gene. These SNPs were in HWE in both groups as well as in the pooled group. A sub-classification of the TD and non-TD patients taking into account tobacco smoking, cannabis use and anhedonia also did not show any statistical significant differences for these two variants. Our study thus found no proof of an association between either the *CYP1A2*1C* G>A or the *CYP1A2*1F* C>A polymorphism and TD in the presence or absence of predisposing factors. One previous study in a Caucasian schizophrenia population that had positive AIM scores ($n=85$) reported a correlation between the *CYP1A2*1F* polymorphism and TD. It was reported that individuals harbouring the CC genotype have an increased risk of developing more severe forms of TD (Basile *et al.* 2000). Indian schizophrenia patients ($n=335$), of whom 96 were diagnosed with TD, were studied in relation with the *CYP1A2*1C* and *CYP1A2*1F* polymorphisms. However, no significant difference was observed between either of these two polymorphisms and the development of TD (Tiwari *et al.* 2005a). The lack of association found in this study between *CYP1A2*1C* and *CYP1A2*1F* and TD might be attributed to the small TD sample size ($n=55$) compared to ($n=96$) used in another study (Tiwari *et al.* 2005a) and the different population group used, i.e. the Xhosas. Haplotype analysis in the current study demonstrated, as could be expected, linkage disequilibrium between these two SNPs in the *CYP1A2* gene, located only 3697 base pairs apart, but failed to find a haplotype associated with TD.

The novel *CYP1A2* gene 2238 T>G SNP identified in two non-TD patients is located in intron 1, signifying that a resulting phenotypic effect is unlikely. This variant apparently has no effect on splicing as confirmed by the use of two programs, the alternative splice site predictor (ASSP) (Wang & Marín 2006) and the splice site prediction program (Reese *et al.* 1997).

The *CYP17 α -hydroxylase* polymorphism was not in HWE in either of the two patient groups (TD and non-TD) and a significant difference in genotypic and allelic frequencies was found between these two groups ($P=0.036$, $P=0.031$ respectively). The T allele is more prevalent amongst the TD patients while the C allele is more prevalent in the non-TD patients. Our study has thus demonstrated an association between the C allele and

possible protection against the development of TD in the Xhosa population. However, it might be that the C allele is not the functional allele, but is in linkage disequilibrium with another allele. It could be hypothesised that the presence of the T allele leads to a minor conversion of pregnanolone to DHEA resulting in more dopamine release. In turn the presence of the C allele increases the conversion of pregnanolone to DHEA resulting in lowered dopamine output. Therefore individuals carrying the T allele have a higher probability of developing TD than individual's carrying the C allele. It has previously been reported that the presence of the C allele leads to modulated dopamine release and increased transcription rate (Segman *et al.* 2002). Since dopamine overactivity is one of the major, however debatable hypotheses (See section 2.3.3) for the pathophysiology of TD, the impact of this polymorphism was not unexpected. The pooled group of schizophrenia patients was not in HWE either, however, the normal Xhosa individuals ($n=100$) typed for this polymorphism demonstrated HWE. This indicated that the results of this study support an association between the *CYP17 α -hydroxylase* polymorphism and possible protection against TD as well as the development of schizophrenia.

Once the patient group was subdivided to correct for tobacco and cannabis smoking and anhedonia, it was demonstrated that the cannabis users were the only subgroup to show significant differences regarding the genotypic distribution of one variant only, i.e. the *CYP17 α -hydroxylase* polymorphism. Cannabis users with the *CYP17 α -hydroxylase* polymorphism have a significantly decreased risk of developing TD ($P=0.013$). On further investigation the initial findings were confirmed by a statistically significant differences in allele frequencies ($P=0.008$) and OR analyses 3.57 (95% CI), 1.3577 to 9.3939).

The observation of a lowered risk for TD among Xhosa schizophrenia cannabis users who carry the C allele needs to be confirmed in a follow-up study. Additional well-defined case-control association studies including normal healthy control Xhosa individuals who smoke cannabis are advised before a final conclusion can be drawn. Similar study designs on other population groups are also encouraged. This was the first study to conduct an investigation between the *CYP17 α -hydroxylase* polymorphism and

cannabis use amongst Xhosa schizophrenia and TD patients. As yet an association between the *CYP17 α -hydroxylase* polymorphism alone and schizophrenia or TD has not been documented. However, in Caucasian schizophrenia patients ($n=113$) of whom 55 were diagnosed with TD, an association has been established between the homozygous mutant state of *CYP17 α -hydroxylase* and the dopamine *D3gly* allele in either the homozygous or heterozygous configuration and TD (Segman *et al.* 2002). In our study the combinations used to assess the interaction between these two polymorphisms were specifically selected in order to replicate the study design of Segman *et al.* (2002). The outcome of our analysis does not support such an interaction between the *CYP17 α -hydroxylase* and *DRD3* ser9gly polymorphisms in Xhosa TD patients but this may be due to the small number of *D3gly* alleles and *CYP17 α -hydroxylase* CC genotypes detected in this Xhosa population.

Several theories have been postulated for the pathophysiology of TD. Of these, the dopamine overactivity hypothesis is the most important and gave rise to a large number of association studies using polymorphisms in genes involved in the neurotransmitter pathway (Jeste & Wyatt 1981). It has been suggested that neuroleptic-induced movement disorders such as TD may be caused by chronic blockage of the dopamine receptor sites, resulting in hypersensitivity of dopamine receptors in the brain (Goetz *et al.* 1982; Ebadi & Srinivasan 1995). The *CYP17 α -hydroxylase* polymorphism leads to alterations in the gene's capacity to convert pregnanolone to DHEA, causing modulation of dopamine release and an increase in transcription rate. It is thus likely that this polymorphism (C allele) may be associated with schizophrenia and TD (Segman *et al.* 2002). Our study found the exact opposite of the results reported by Segman *et al.* (2002). It has furthermore been suggested that cannabis abuse and schizophrenia may share some neurobiological pathways and therefore possibly some predisposing genes (Leroy *et al.* 2001). The *CYP17 α -hydroxylase* polymorphism associated with cannabis use amongst the Xhosa schizophrenia patients is not unexpected since functional interactions have been reported between cannabis and dopamine transmission (Giuffrida *et al.* 1999). The finding of this study regarding a significant decreased risk of developing TD when smoking cannabis is unexpected since it is known that cannabis smoking increase the risk

of developing schizophrenia (Andreasson *et al.* 1987). Both DHEA and pregnanolone have neuroprotective functions in the brain. Since the presence of the C allele leads to an increase in the conversion of pregnanolone to DHEA, it may be hypothesised that DHEA has a greater neuroprotective capacity than pregnanolone possibly explaining the results found in this study. It has been proven that cannabis holds potential therapeutic applications in the treatment of pain, anxiety and depression (Hampson *et al.* 2000; Robson 2001), therefore the neuroprotective antioxidants of cannabis can be involved in protection against the development of TD.

In conclusion, data presented here suggests an association between the *CYP17 α -hydroxylase* polymorphism, schizophrenia and protection against the development of TD. Taken together, the findings also provide a link between the *CYP17 α -Hydroxylase* polymorphism, cannabis use and TD. The preliminary results warrant additional investigation to elucidate the role of the *CYP17 α -Hydroxylase* variant in the development of schizophrenia and TD.



CHAPTER FOUR

PHARMACOGENETIC ASSOCIATION ANALYSIS BETWEEN CYTOCHROME P450 3A4 AND 3A5 GENE POLYMORPHISMS AND ADVERSE SIDE EFFECTS IN XHOSA SCHIZOPHRENIA PATIENTS CORRELATED WITH ANTIPSYCHOTIC DRUG TREATMENT

4.1 ABSTRACT

Adverse reaction to drugs is an important cause of death and is partly ascribed to inter-individual variability in clinical response. Pharmacogenetics encompasses the study of variability in drug response due to heredity. An important outcome of this expanding discipline is that it will in due course enable the physician to individualize drug treatment, resulting in safer, more effective and cost-efficient healthcare. Tardive dyskinesia (TD) is a known example of an adverse drug reaction. In this study we aimed to assess whether a correlation exists between selected *CYP3A4* and *CYP3A5* gene polymorphisms and the onset of TD. The CYP3A enzymes, including *CYP3A4* and *CYP3A5*, metabolise >60% of all drugs. The patient sample comprised 116 Xhosa schizophrenia individuals from South Africa. Of these, 55 developed TD after being treated with antipsychotic drugs. The four known variants analysed included single nucleotide polymorphisms (SNPs) in the *CYP3A4* and *CYP3A5* genes, i.e. *CYP3A4*1B* (A-392G), *CYP3A4*1G* (G20230A), *CYP3A4* (C23081T), *CYP3A5*3* (A6986G) and *CYP3A5*6* (A30570G). Only *CYP3A4*1B* has formerly been studied in relation to schizophrenia and TD. All the polymorphisms in *CYP3A4* and *CYP3A5* were in Hardy-Weinberg equilibrium (HWE) in the TD and non-TD group. SNPs were analysed singly and in combination by haplotype analysis due to the close proximity of the two genes in conjunction with the development of TD. The *CYP3A5*3* polymorphism showed a statistical significant difference in genotype frequency ($P=0.038$) between the patients with and without TD, suggesting that presence of the variant might contribute to the

development of TD. A statistically significant difference ($P=0.049$) was observed with an AG haplotype comprising *CYP3A4*1G* and *CYP3A5*3* that occurred at a higher frequency in the TD than non-TD individuals. Predisposing factors brought into the equation did not influence the risk of developing TD. In conclusion, the association found between the *CYP3A5*3* polymorphism, AG haplotype and TD warrants further investigation since the P values ($P=0.038$ and $P=0.049$ respectively), were too close to 0.05 and not persuasive enough to act as molecular markers for predicting adverse event occurrence during antipsychotic drug treatment in Xhosa schizophrenia patients.

4.2 INTRODUCTION

Adverse drug reactions constitute one of the ten major causes of death in the United States of America (Nebert 1999). This is partly due to inter-individual variability in drug response. The field of pharmacogenetics explores the inconsistency of drug response in different patients, due to differences in inherited characteristics in these individuals. Genetic variants have been associated with variable sensitivity to drug response (Ingelman-Sundberg *et al.* 1999; Ohmori *et al.* 2003; Shastry 2006). Thus one attempt at reducing the prevalence of adverse drug reactions is the creation of single nucleotide polymorphism (SNP) profiles to distinguish patients who respond clinically to medicine from those who experience undesirable side effects. An important outcome of pharmacogenetics is that it will ultimately permit the physician to prescribe individualised drugs and dosages, resulting in safer, more effective and cost-efficient medicine.

Tardive dyskinesia (TD) is a known example of adverse drug reaction. This disorder is one of complex inheritance, where the interaction between several genes is likely to increase the risk of developing schizophrenia (Harrison & Owen 2003). Drug treatment of schizophrenia aimed at controlling negative symptoms regrettably leads to the emergence of another distressing disorder, TD, characterised by involuntary movements (DSM-IV 2000). It has not been established how TD is brought about but proof has been provided for a genetic component through studies on schizophrenia families and twins

(Muller *et al.* 2001). The incidence of TD is much higher among patients exposed to multiple antipsychotic drugs than those who have been using one drug only (Kane 2004).

This study aimed to determine whether a correlation exists between polymorphisms in two candidate Cytochrome P450 genes and the present of TD symptoms. Cytochrome P450 is a super family of heme-containing enzymes, responsible for the metabolism of >90% of all drugs (Van der Weide & Hinrichs 2006). 57 different cytochrome P450s (*CYP*) genes, of which the CYP1-3 enzymes are most important, have been identified in humans (Shastry 2006). The CYP3A enzymes, including CYP3A4 and CYP3A5, are most abundant in the liver and small intestine and metabolise >60% of all drugs (Cascorbi 2006a), including typical anti-psychotics such as Haloperidol and atypical anti-psychotics such as Risperidone, Olanzapine and Clozapine (de Leon *et al.* 2005; Tiwari *et al.* 2005b). These two genes have been located to 7q21.3-q22.1 and are in close proximity to each other, 136 kb apart (Fig. 1) (Garsa *et al.* 2005). Polymorphisms in these two *CYP* genes bring about altered drug metabolism, modulation in drug efficacy or adverse drug reactions (Müller *et al.* 2004; de Groot 2006).

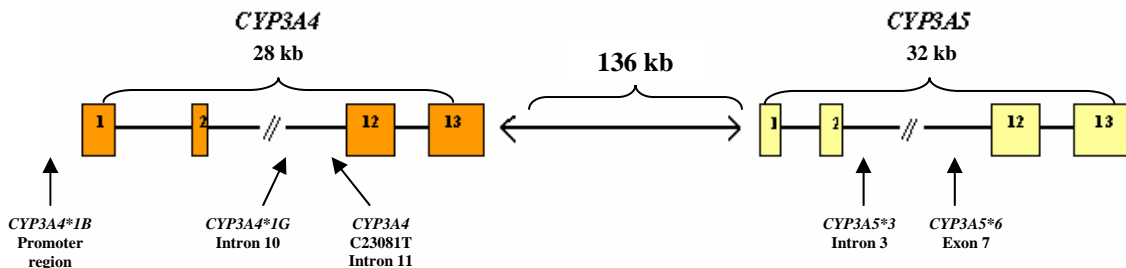


Fig. 1 Illustration of *CYP3A4*, *CYP3A5* and relevant polymorphisms investigated in this study.

The *CYP3A4* gene is 27,592 base pairs long and consists of 13 exons (Hashimoto *et al.* 1993). One polymorphism in *CYP3A4* that has been studied in conjunction with drug response is A-392G (*CYP3A4*1B*) in the nifedipine-specific element (NFSE) of the promoter region (Felix *et al.* 1998; Tiwari *et al.* 2005b). This polymorphism is also known as *CYP3A4-V* (Rebbeck *et al.* 1998) and has been associated with decreased *CYP3A4* expression or decreased enzyme activity. The G20230A (*CYP3A4*1G*) polymorphism in intron 10 is the second most common SNP of the 13 intronic SNPs in

CYP3A4 (Lamba *et al.* 2002) and co-segregates with the *CYP3A4*1B* polymorphism in 62% of African Americans (Lamba *et al.* 2002). The *CYP3A4* C23081T SNP in intron 11 has to our knowledge not yet been used in any association studies. Thus no information is available regarding this SNP, schizophrenia and TD (Agenbag, unpublished results).

The *CYP3A5* gene contains 13 exons and is 31,790 base pairs long. 75% of Whites and 50% of Blacks have an inability to express functional *CYP3A5*, due to genetic polymorphisms (Evans & McLeod 2003). Two polymorphisms in the *CYP3A5* gene, i.e. *CYP3A5*3* (6986 A>G) and *CYP3A5*6* (30570A>G), have been studied in conjunction with drug response. Both affect total *CYP3A* activity (Yamamoto *et al.* 2005) and cause absence of *CYP3A5* in tissues (Kuehl *et al.* 2001). The *CYP3A5*3* polymorphism in intron 3 creates a cryptic splice site that leads to a premature stop codon at amino acid 102 in exon 3, resulting in erroneous spliced mRNA production, loss of *CYP3A5* activity (Yamamoto *et al.* 2005) and decreased expression of *CYP3A5* protein (Kuehl *et al.* 2001). The *CYP3A5*6* (30570A>G) polymorphism in exon 7 causes a total loss of *CYP3A5* expression in tissues (Kuehl *et al.* 2001). A correlation between this polymorphism and the deletion of exon 7 from *CYP3A5* mRNA, leading to reduced *CYP3A5* activity, was observed in African Americans (Kuehl *et al.* 2001; Cascorbi 2006a).

Since many substrates are shared between the two above mentioned genes, drugs metabolized by *CYP3A5* are also being metabolized by *CYP3A4* (Evans & McLeod 2003). *CYP3A4* and *CYP3A5* have similar catalytic specificities (Yu *et al.* 2004), therefore active *CYP3A5* compensates for defective *CYP3A4* metabolism and the other way around (Lee *et al.* 2005). Since these two genes compensate for each other it is evident that a relation must exist between polymorphisms in these genes. Therefore haplotype analysis between SNPs in *CYP3A4* and *CYP3A5* is essential since one SNP in *CYP3A4* may influence the activity of *CYP3A5*. All the SNPs analysed in this study result in a complete loss of activity or decreased activity of *CYP3A4* and *CYP3A5*. In a

study performed on healthy Caucasian and Black people (Evans & McLeod 2003) the combined effect of CYP3A4 and CYP3A5 are noticeable (Fig. 2).

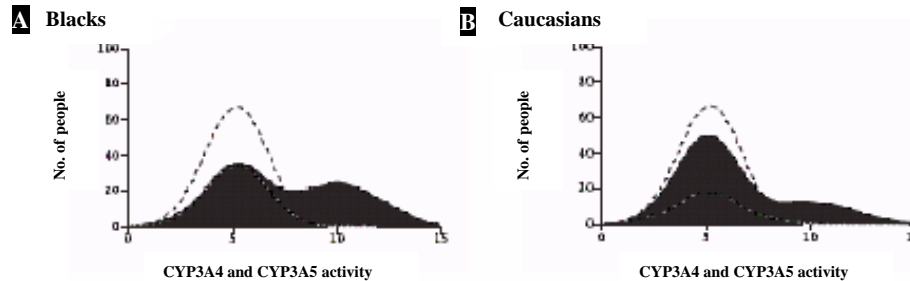
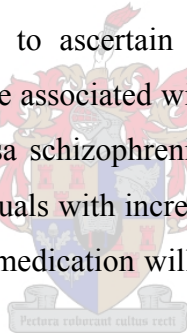


Fig. 2 The combined effects of CYP3A4 and CYP3A5 activities in [A] Blacks and [B] Caucasians. The black dashed lines represent CYP3A4 activity and the white dashed lines represent CYP3A5 activity. Only 25% of Caucasians and 50% of Blacks express functional CYP3A5. The solid area reflects the combined activity of CYP3A4 and CYP3A5. Adapted from Evans & McLeod (2003).

The current study was undertaken to ascertain whether five polymorphisms in the *CYP3A4* and *CYP3A5* genes could be associated with genomic susceptibility to TD. The patient sample comprised 116 Xhosa schizophrenia patients of South Africa. Suitable genomic markers identifying individuals with increased risk of either developing adverse effects or responding favourably to medication will greatly enhance clinical treatment of schizophrenia.



4.3 MATERIAL AND METHODS

4.3.1 Patient samples

The patient group used for this study was the same as for chapter three with exclusion to the normal Xhosa controls (See section 3.3.1).

4.3.2 Polymerase Chain Reaction (PCR) amplification

Genomic DNA from all patients were analysed using the polymerase chain reaction (PCR) to identify the presence of wild type and/or variant alleles in the *CYP3A4* and *CYP3A5* genes. A total of five different polymorphisms were studied; the *CYP3A4* locus included three variants (*CYP3A4*1B*, *CYP3A4*1G* and C23081T) and the *CYP3A5* locus

included two variants (*CYP3A5*3* and *CYP3A5*6*). Primer sequences and PCR product sizes are shown in Table 1. PCR amplifications were performed using a GeneAmp PCR System 2700 (Applied Biosystems, Warrington, WA, USA).

***CYP3A4*1B* polymorphism:** Primers PrF and PrR yielded a fragment of 107 bp. PCR was performed in a total reaction volume of 25 µl containing 30 ng template DNA, 15 pmol of each primer, 100 µM dNTPs, 0.5 U of *Taq*-polymerase (Bioline, Randolph, MA, USA) and PCR buffer containing 3 mM MgCl₂ as supplied by the manufacturer. The two-step PCR amplification conditions were as follows: initial denaturation at 94°C for 4 min, followed by 15 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s and thereafter 20 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 45 s. Final extension was at 72°C for 5 min.

***CYP3A4*1G* polymorphism:** Primer sequences 10F and 10R produced an amplification product of 391 bp. The PCR reaction mix was the same as for *CYP3A4*1B*. Amplification conditions were: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 90 s and final extension at 72°C for 10 min.

***CYP3A4 C23081T* polymorphism:** The primers used to amplify this fragment of 370 bp were 11F and 11R (Table 1). The PCR reaction mix was the same as for *CYP3A4*1B* and *CYP3A4*1G*. Amplification was performed as follows: initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and final extension at 72°C for 10 min.

***CYP3A5*3* polymorphism:** The Int3F and Int3R primers were used to obtain a 166 bp PCR product (Shih and Huang 2002). PCR was performed in a total reaction volume of 25 µl containing 30 ng template DNA, 15 pmol of each primer, 100 µM dNTPs, 0.5 U of *Taq*-polymerase (Bioline, Randolph, MA, USA) and PCR buffer containing 3 mM MgCl₂. Initial denaturation was at 94°C for 5 min. Followed by a two-step amplification, i.e. 15 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, followed by

20 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min.

CYP3A5*6 polymorphism: Primers 7F and 7R yielded a 141 bp PCR product (Shih and Huang 2002). The PCR reaction mix was the same as for CYP3A5*3. The two-step amplification conditions included an initial denaturation step at 94 °C for 5 min, followed by 15 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and subsequently 20 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s. Final extension was at 72 °C for 5 min.

4.3.3 Restriction Enzyme Analysis (REA)

All five SNPs analysed in this study are revealed by restriction enzyme digestion following PCR. PCR product (8.8 µl) was used in a total volume of 10 µl containing appropriate buffer and restriction enzymes (1 U), as outlined below. Samples were incubated overnight at 37°C. The relevant polymorphic sites are shown in Table 1. Gel electrophoresis conditions and resulting digested fragment sizes are indicated in Table 2. Electrophoresis conditions on 15% (w/v) polyacrylamide gels (15% polyacrylamide, 10% (w/v) ammoniumpersulfate and Temed) were at room temperature at 200V for 1-2 hours depending on the fragment sizes. Electrophoresis conditions for agarose gels were 120V for 1 hour. In both instances 1XTBE (0.089 M Tris, 0.089 M Boric acid and 20 mM EDTA, pH 8.0) was used as electrophoresis buffer and DNA fragments were visualised with UV light following staining in 0.5 µg/ml ethidium bromide.

CYP3A4*1B polymorphism: PCR amplification yielded a fragment size of 107 bp. In the presence of the wild type A allele, digestion with *Mbo* II (New England BioLabs Ipswich, MA, USA) resulted in 2 fragments of 73 bp and 34 bp.

CYP3A4*1G polymorphism: The PCR product of 391 bp was digested with *Rsa* I (New England BioLabs Ipswich, MA, USA). Digested fragment sizes of 225 bp and 166 bp represent the wild type G allele. The undigested PCR sample represents the polymorphic A allele.

CYP3A4 C23081T polymorphism: The PCR product was digested with *HpyCH4* IV (New England BioLabs Ipswich, MA, USA). An undigested fragment size of 370 bp

represents the T allele, while the digested products of 293 bp and 77 bp represent the C allele.

***CYP3A5*3* polymorphism:** The PCR product size of 166 bp was digested with 1 U of *Pst* I (New England BioLabs Ipswich, MA, USA). Fragment sizes of 141 bp and 25 bp produced upon enzyme digestion represent the variant G allele. The undigested PCR sample of 166 bp represents the A allele.

***CYP3A5*6* polymorphism:** PCR amplification yielded a fragment size of 141 bp. In the presence of the A allele, digestion with 1 U of *Dra* I also known as *Aha* III (Fermentas, Ontario, Canada, USA) resulted in three fragments of 81 bp, 35 bp and 25 bp whereas the presence of the variant G allele resulted in two fragments of 106 bp and 35 bp.

4.3.4 DNA sequencing

Sequencing was done for the *CYP3A4* C23081T SNP following preliminary indications that the primer set used to amplify this SNP might also be amplifying a pseudogene. Samples were purified using 20 µl of PCR product and 20 µl SureClean (Bioline USA Inc.) according to instructions from the manufacturer. Sequencing was performed in a total reaction volume of 10 µl containing 12 ng diluted PCR product, 4 µl sequencing mix (ABI PRISM di-deoxy Terminator Cycle Sequencing kit v3.1 Applied Biosystems) and 1.1 pmol PCR primer following the manufacturer's instructions. The ABI PRISM 3100 Genetic Analyser (Perkin Elmer, Applied Biosystems, Warrington WA, Great Britain) as well as BioEdit (Tom Hall, version 7.0.1, Isis Pharmaceuticals, Inc) were used to analyze the DNA sequences.

Table 1. Primer sequences used to PCR amplify *CYP3A4* and *CYP3A5* gene variants. The location, bp change giving rise to the variant sequence, and size of the amplified product are also shown.

Variant	Location	Wild- Type	Variant	Primer name	Primer Sequence (5'-3')	PCR Product size
A-392G (<i>CYP3A4*1B</i>)	Promoter region	ggggaAgagag	ggggaGgagag	PrF PrR	GGACAGCCATAGAGACAAGGGgA TACTGGGGAGTCCAAGGGTTCTG	107 bp
G20230A (<i>CYP3A4*1G</i>)	Intron 10	ggatgGtacct	ggatgAtacct	10F 10R	TGATGCCCTACATTGATCTGA CTGCCAGTAGCAACCATTG	391 bp
C23081T (<i>CYP3A4</i>)	Intron 11	accaaCgtgga	accaaTgtgga	11F 11R	GGGTGGCCCCTAAGTAAGAA CTTGGCCCAGAGAACAAAT	370 bp
A6986G (<i>CYP3A5*3</i>)	Intron 3	ctgcaAtatct	ctgcaGtatct	Int3F Int3R	CTTAAAGAGCTCTTTTGTcTgCA ATCCATACCCCTAGTTGTACGACAC	166 bp
A30570G (<i>CYP3A5*6</i>)	Exon 7	tttaaAaagtt	tttaaGaaagtt	7F 7R	ACAAGACCCCTTTGTGGAGAGcTAA TATGTGAATTATATGTCAAGAAAGCAG	141 bp

F, Forward R, Reverse

Table 2. Restriction enzyme assays for the genotypic analysis of RFLPs in the *CYP3A4* and *CYP3A5* genes

Variant	Recognition site	Restriction enzyme	RFLP conditions	Electrophoresis gel	Cutting type
A-392G (<i>CYP3A4*1B</i>)	GAAGA (NNNNNNNN)^	<i>Mbo</i> II	37°C	15% polyacrylamide	wild-type - 2 fragments (73 bp and 34 bp) variant - 1 fragment (107 bp)
G20230A (<i>CYP3A4*1G</i>)	GT^AC	<i>Rsa</i> I	37°C	1.5% agarose	wild-type - 2 fragments (225 bp and 166 bp) variant - 1 fragment (391 bp)
C23081T (<i>CYP3A4</i>)	A^CGT	<i>HpyCH4</i> IV	37°C	1.5% agarose	wild-type - 2 fragments (293 bp and 77 bp) variant - 1 fragment (370 bp)
A6986G (<i>CYP3A5*3</i>)	CTGCA^G	<i>Pst</i> I	37°C	15% polyacrylamide	wild-type - 1 fragment (166 bp) variant - 2 fragments (141 bp and 25 bp)
A30570G (<i>CYP3A5*6</i>)	TTT^AAA	<i>Dra</i> I	37°C	15% polyacrylamide	wild-type - 3 fragments (81 bp, 35 bp and 25 bp) variant- 2 fragments (106 bp and 35 bp)

4.3.5 Statistical analysis

Allele and genotype frequencies were estimated and the Hardy-Weinberg equilibrium (HWE) was tested with the assistance of TFPGA (Miller 1997). Statistical differences between TD and non-TD patients were determined using an analogue of a Fisher's exact

test on a contingency table (2 X 2 or 2 X 3 as appropriate) with Microsoft® Excel 2000. Differences in the observed frequencies of the four polymorphisms when taking into account tobacco and cannabis smoking and anhedonia were explored using an analogue to Fisher's exact test by means of a 2 X 3 contingency table. A probability (*P*) value smaller than 0.05 was considered significant

The Pearson Correlation coefficient (*r*) and standardized linkage disequilibrium coefficient (*D'*) were determined. The arbitrary cut off values for r^2 and *D'* were considered 0.4 and 0.7 respectively. Thereafter haplotype analysis was conducted using the Haploview 3.31 program (Barrett *et al.* 2005) to determine whether a specific haplotype may be associated with TD.

4.4 RESULTS

In the current study Xhosa schizophrenia patients with and without TD were investigated for the presence of five *CYP3A* gene polymorphisms, three of which are in *CYP3A4* (*CYP3A4*1B*, *CYP3A4*1G* and C23081T) and two in *CYP3A5* (*CYP3A5*3* and *CYP3A5*6*). All five known SNPs were detected, and these were in both patient groups. Examples of the five SNPs detected are depicted in Fig. 3. Extra fragments were observed while detecting the *CYP3A4*1B* SNP in Fig. 3A. The origin of these bands is unknown, but is probably due to binding of the primers to additional sites, in spite of the fact that primers were designed to prevent this. Expected fragments can be seen at the bottom part of the gel. The secondary lighter band patterns noticeable at the top of the gel served as internal controls since the pattern was different for each genotype. These constant fragments detected throughout all of the samples did not affect genotyping.

DNA sequencing analysis of *CYP3A4* C23081T SNP (depicted in Fig. 3C) revealed that primers used to amplify this polymorphism may also have amplified a pseudogene, not only the area of interest. In this case the secondary amplification products could not be distinguished from the desired amplification products. Therefore, this set of data was excluded from statistical analysis.

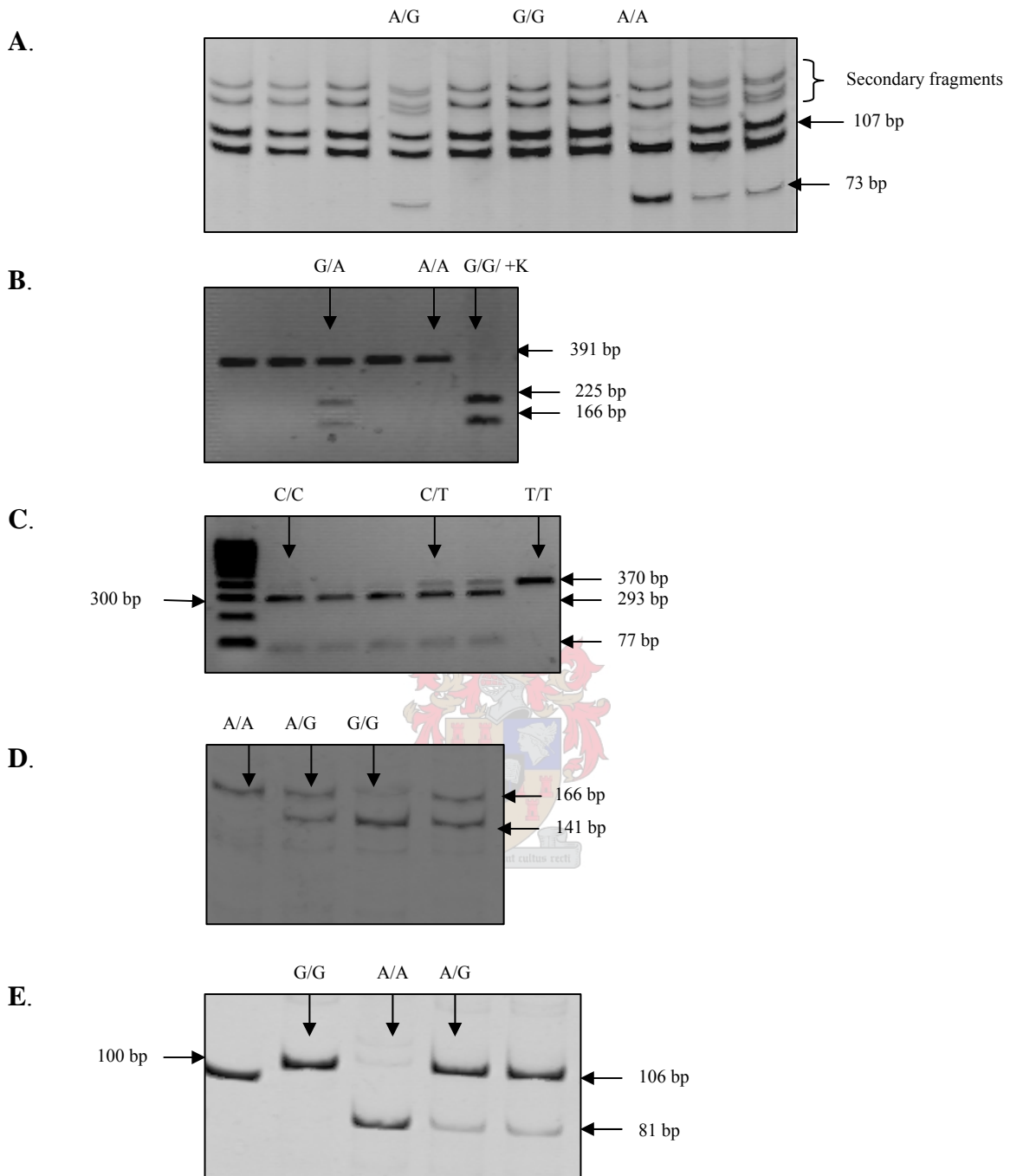


Fig. 3 Restriction enzyme analysis of five known SNPs located in the *CYP3A4* and *CYP3A5* genes, observed in our sample of schizophrenia patients: [A] *CYP3A4**1B, [B] *CYP3A4**1G, [C] *CYP3A4* C23081T, [D] *CYP3A5**3, [E] *CYP3A5**6. The +K in [B] represents a positive control. Indicated fragment sizes left of certain gel photos refer to the ladder while sizes on the right hand side of the gel refer to the actual fragments obtained. For appropriate restriction enzymes revealing polymorphisms and description of wild type and variant alleles see Table 2.

The two patient groups, with and without TD, were in HWE for all four polymorphisms analysed, i.e. *CYP3A4*1B* ($P=0.7415$, $P=0.7638$ respectively), *CYP3A4*1G* ($P=0.4945$, $P=0.4856$ respectively) *CYP3A5*3* ($P=0.0685$, $P=0.6123$ respectively) and *CYP3A5*6* ($P=0.1215$, $P=0.9887$ respectively).

Table 3. Genotypic and allelic distribution of the *CYP3A4*1B*, *CYP3A4*1G*, *CYP3A5*3*, *CYP3A5*6* polymorphisms in Xhosa schizophrenia patients with TD compared to patients without TD.

	Genotypic distribution				Allelic distribution		
	No. of patients (%)				A	G	P value
<i>CYP3A4*1B</i>	A/A	A/G	G/G	P value	A	G	P value
TD patients, n=55	2(4%)	19(34%)	34(62%)	0.538	23	87	0.275
Non-TD patients, n=61	4(7%)	25(41%)	32(52%)		33	89	
<i>CYP3A4*1G</i>	G/G	G/A	A/A	P value	G	A	P value
TD patients, n= 52	0(0%)	9(17%)	43(83%)	0.897	9	95	0.902
Non-TD patients, n = 61	0(0%)	10(16%)	51(84%)		10	112	
<i>CYP3A5*3</i>	A/A	A/G	G/G	P value	A	G	P value
TD patients, n= 55	28(51%)	26(47%)	1(2%)	0.038**	82	28	0.062
Non-TD patients, n = 61	44(72%)	15(25%)	2(3%)		103	19	
<i>CYP3A5*6</i>	G/G	G/A	A/A	P value	G	A	P value
TD patients, n=55	36(65%)	19(35%)	0(0%)	0.359	91	19	0.880
Non-TD patients, n=61	41(67%)	18(30%)	2(3%)		100	22	

** Correlation is significant $p \leq 0.05$

Statistical analyses subsequently compared the distribution of the four polymorphisms in the following manner: (a) schizophrenia patients were categorized in two groups, based on the presence / absence of TD only, (b) TD and non-TD patients were sub-classified according to three major groups, i.e. tobacco smoking, cannabis smoking and anhedonia status, and combinations thereof. The three major groups as well as the combined groups such as smoking / cannabis and cannabis / anhedonia were analysed separately.

For the *CYP3A4*1B*, *CYP3A4*1G* and *CYP3A5*6* polymorphisms, no significant differences were found between TD and non-TD individuals with respect to genotypic or

allelic frequencies (Table 3). The *CYP3A5*3* polymorphism showed a significant difference between the two groups with respect to genotype ($P=0.038$), but not with respect to allele frequency ($P=0.062$). Furthermore, there were no significant statistical differences between TD and non-TD patients in the distributions of any of the four polymorphisms once clinical information on tobacco smoking, cannabis smoking, presence of anhedonia, and combinations of these predisposing factors were taken into account (Table 4).

Table 4. Genotypic distribution of *CYP3A4*1B*, *CYP3A4*1G*, *CYP3A5*3* and *CYP3A5*6* polymorphisms in Xhosa schizophrenia patients with TD compared to patients without TD, classified according to tobacco smoking, cannabis smoking and/or presence of anhedonia.

		Genotypic distribution				Allelic distribution		
		No. of patients (%)				A	G	P value
<i>CYP3A4*1B</i>		A/A	A/G	G/G	P value	A	G	P value
Tobacco Smoking	TD patients, n=37	2(5%)	13(35%)	22(60%)	0.927	17	57	0.701
	Non-TD patients, n=47	3(6%)	18(38%)	26(56%)		24	70	
Cannabis	TD patients, n=16	1(6%)	5(31%)	10(63%)	0.931	7	25	0.735
	Non-TD patients, n=32	3(9%)	10(31%)	19(60%)		16	48	
Anhedonia	TD patients, n=45	2(4%)	13(29%)	30(67%)	0.385	17	73	0.161
	Non-TD patients, n=49	4(8%)	19(39%)	26(53%)		27	71	
Smoking/Cannabis	TD patients, n=15	1(7%)	5(33%)	9(60%)	0.876	7	23	0.933
	Non-TD patients, n=29	3(10%)	8(28%)	18(62%)		14	44	
Cannabis/Anhedonia	TD patients, n=14	1(7%)	5(36%)	8(57%)	0.877	7	21	0.927
	Non-TD patients, n=27	3(11%)	8(30%)	16(59%)		14	40	
<i>CYP3A4*1G</i>		G/G	G/A	A/A	P value	G	A	P value
Tobacco Smoking	TD patients, n=36	0(0%)	7(19%)	29(81%)	0.776	7	65	0.787
	Non-TD patients, n=47	0(0%)	8(17%)	39(83%)		8	86	
Cannabis	TD patients, n=15	0(0%)	2(13%)	13(87%)	0.645	2	28	0.661
	Non-TD patients, n=32	0(0%)	6(19%)	26(81%)		6	58	
Anhedonia	TD patients, n=42	0(0%)	7(17%)	35(83%)	0.965	7	77	0.967
	Non-TD patients, n=49	0(0%)	8(16%)	41(84%)		8	90	
Smoking/ Cannabis	TD patients, n=14	0(0%)	2(14%)	12(86%)	0.806	2	26	0.814
	Non-TD patients, n=29	0(0%)	5(17%)	24(83%)		5	53	
Cannabis/Anhedonia	TD patients, n=13	0(0%)	2(15%)	11(85%)	0.613	2	24	0.633
	Non-TD patients, n=27	0(0%)	6(22%)	21(78%)		6	48	

<i>CYP3A5*3</i>		A/A	A/G	G/G	<i>P</i> value	A	G	<i>P</i> value
Tobacco Smoking	TD patients, n=37	21(57%)	15(40%)	1(3%)	0.149	57	17	0.082
	Non-TD patients, n=47	36(77%)	10(21%)	1(2%)		82	12	
Cannabis	TD patients, n=16	11(69%)	5(31%)	0(0%)	0.499	27	5	0.846
	Non-TD patients, n=32	23(72%)	7(22%)	2(6%)		53	11	
Anhedonia	TD patients, n=45	25(56%)	19(42%)	1(2%)	0.117	69	21	0.162
	Non-TD patients, n=49	36(73%)	11(23%)	2(4%)		83	15	
Smoking/ Cannabis	TD patients, n=15	10(67%)	5(33%)	0(0%)	0.531	25	5	0.719
	Non-TD patients, n=29	22(76%)	6(21%)	1(3%)		50	8	
Cannabis/Anhedonia	TD patients, n=14	11(79%)	3(21%)	0(0%)	0.523	25	3	0.270
	Non-TD patients, n=27	18(67%)	7(26%)	2(7%)		43	11	

<i>CYP3A5*6</i>		G/G	G/A	A/A	<i>P</i> value	G	A	<i>P</i> value
Tobacco Smoking	TD patients, n=37	28(76%)	9(24%)	0(0%)	0.354	65	9	0.221
	Non-TD patients, n=47	31(66%)	14(30%)	2(4%)		76	18	
Cannabis	TD patients, n=16	11(69%)	5(31%)	0(0%)	0.765	27	5	0.846
	Non-TD patients, n=32	22(69%)	9(28%)	1(3%)		53	11	
Anhedonia	TD patients, n=45	29(64%)	16(36%)	0(0%)	0.365	74	16	0.777
	Non-TD patients, n=49	32(65%)	15(31%)	2(4%)		79	19	
Smoking/Cannabis	TD patients, n=15	11(73%)	4(27%)	0(0%)	0.761	26	4	0.635
	Non-TD patients, n=29	20(69%)	8(28%)	1(3%)		48	10	
Cannabis/Anhedonia	TD patients, n=14	9(64%)	5(36%)	0(0%)	0.765	23	5	0.785
	Non-TD patients, n=27	17(63%)	9(33%)	1(4%)		43	11	

Haplotype analysis of the four *CYP3A* SNPs in the total number of patients revealed eight different haplotypes, all with incidences greater than 1%. The linkage disequilibrium (LD) plot generated by Haploview while considering all four SNPs, indicated a standardized linkage disequilibrium coefficient of $D'=0.736$ between *CYP3A4*1G* and *CYP3A5*3*. Subsequent analysis of these two selected SNPs (*CYP3A4*1G* and *CYP3A5*3*) revealed four different haplotypes, each occurring at a frequency higher than 1%. A search for an association between TD and a specific haplotype between the four loci failed to provide evidence of a statistically significant difference between TD and non-TD patients (Table 5). However, a statistical significant difference was observed with a specific haplotype (AG) found between *CYP3A4*1G* and *CYP3A5*3* ($P=0.0496$). This haplotype was more common amongst TD than non-TD individuals. The frequency of the AA haplotype was very high in both the TD and non-TD Xhosa schizophrenia group (0.779) (Table 6).

Table 5. Haplotype analysis for the four loci, *CYP3A4*1B*, *CYP3A4*1G*, *CYP3A5*3* and *CYP3A5*6*

Haplotype	Frequency	Schizophrenia patient ratios		Chi Square	P value
		TD	non-TD		
GAAG	0.463	0.456	0.470	0.047	0.8276
AAAG	0.159	0.128	0.187	1.514	0.2185
GAAA	0.137	0.134	0.140	0.022	0.8833
GAGG	0.112	0.153	0.075	3.529	0.0603
AGGG	0.036	0.035	0.037	0.005	0.9426
GGGG	0.030	0.027	0.032	0.044	0.8343
AAAA	0.021	0.014	0.027	0.499	0.4800
AAGG	0.015	0.024	0.008	0.973	0.3240

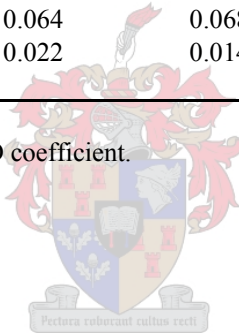
Table 6. Haplotype analysis for *CYP3A4*1G* and *CYP3A5*3*

Haplotype	Frequency	Schizophrenia patient ratios		Chi Square	P value
		TD	non-TD		
AA	0.779	0.730	0.823	2.884	0.0895
AG	0.137	0.184	0.095	3.854	0.0496**
GG	0.066	0.064	0.068	0.012	0.9128
GA	0.018	0.022	0.014	0.181	0.6706

$D' = 0.736$, $r^2 = 0.181$

r, Pearson correlation; D', standardized LD coefficient.

** Correlation is significant $p \leq 0.05$



4.5 DISCUSSION

The identification of DNA susceptibility loci would lead to improved clinical treatment of schizophrenia and minimise adverse drug reactions such as TD. Tobacco and cannabis smoking have been suggested to act as predisposing factors in the development of schizophrenia and/or TD. Eradication of TD will bring major benefits to the individual, including improved well being and enhanced capacity to engage in occupational activities.

The current investigation aimed to determine whether any of four selected *CYP3A* gene polymorphisms, *CYP3A4*1B*, *CYP3A4*1G*, *CYP3A5*3* and *CYP3A5*6* constitute DNA susceptibility loci for TD. Additional criteria, i.e. tobacco and cannabis smoking and anhedonia were also taken into account. The study included 116 South African Xhosa

schizophrenia patients, 55 of whom developed TD while being treated with antipsychotic drugs.

The *CYP3A4* and *CYP3A5* genes play a major role in the metabolism of drugs, including antipsychotic drugs, and the response thereof. Since these two genes are so closely situated to each other on chromosome 7q21.3, polymorphisms in the one gene can affect the expression of the other. It is anticipated that enhanced knowledge of polymorphisms in these two genes will stimulate progress in the discipline of individualized drug therapy.

No statistically significant differences were detected between TD or non-TD individuals regarding the genomic distribution of three of the polymorphisms studied, i.e. *CYP3A4*1B*, *CYP3A4*1G* and *CYP3A5*6*. *CYP3A5*3* demonstrated a statistically significant difference in genotypic distribution between TD and non-TD ($P=0.038$), but not in terms of allelic distribution. This observation may be due to the higher, almost doubled, frequency of heterozygotic individuals in the TD group compared to the non-TD group as well as the high frequency of the AA (wt) genotype amongst non-TD patients. The variant G allele has a higher frequency amongst the TD than non-TD patients. *CYP3A5*3* is known as the most important *CYP3A5* polymorphism since it affects total CYP3A activity (Yamamoto *et al.* 2005). It has previously been reported that a loss of CYP3A5 activity and decreased expression of CYP3A5 protein were results of this polymorphism (Kuehl *et al.* 2001). It has been noted that CYP3A5, together with CYP3A4 are responsible for the metabolism of antipsychotic drugs (Cascorbi 2006a). Thus, it is not unexpected that the variant G allele has a higher frequency in TD than non-TD patients since it results in the absence of CYP3A5 activity causing insufficient metabolism of antipsychotic drugs and thereby promoting the development of TD. Therefore, the high frequency of the AA genotype amongst the non-TD patients could possibly act as protection against the development of TD, however this warrants further investigation. A previous study reported a high frequency of the *CYP3A5*3* A allele and a low frequency of the G allele in a randomly selected African American population. These frequencies in comparison with other population groups are depicted in Fig. 4 (Hustert *et al.* 2001). These results mirror the findings in our Xhosa schizophrenia

population. Sub-classification of the TD and non-TD patients based on tobacco and cannabis smoking and anhedonia did not show any statistical significant differences for any of these four variants. Knowledge about *CYP3A4*, *CYP3A5*, TD and sub-classifications such as tobacco and cannabis smoking and anhedonia are limited. However, it is well known that smoking affects the overall metabolism of humans, therefore it is most likely to affect the metabolism of antipsychotic drugs. Smoking has only been associated with the development of TD in one cytochrome P450 gene, i.e. *CYP1A2* (Tiwari *et al.* 2005). To our knowledge no association studies have been performed regarding *CYP3A4* polymorphisms and smoking. A lower expression of *CYP3A5* amongst smokers than non-smokers was previously reported in two studies on healthy Caucasians (Piipare *et al.* 2000; Hukkanen *et al.* 2003).

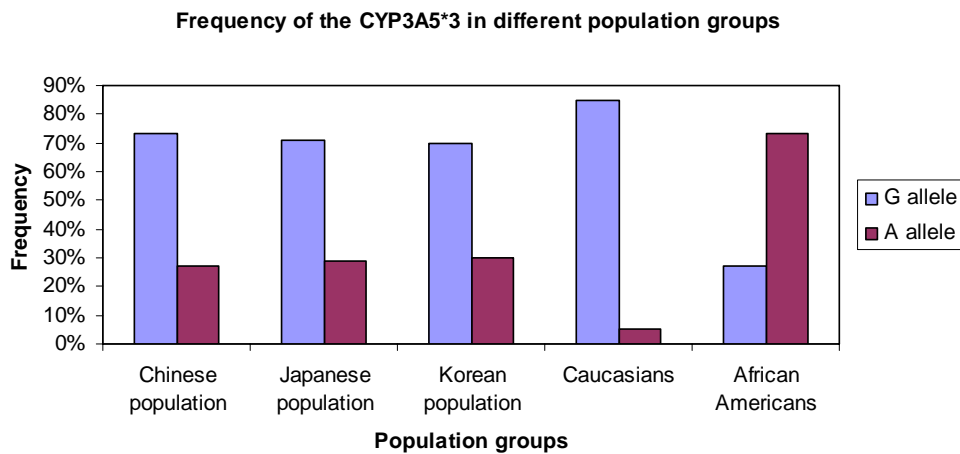


Fig. 4 The frequency of the *CYP3A5**3 variant in different ethnic groups. (Hustert *et al.* 2001)

The *CYP3A4**1B polymorphism has been studied extensively in different population groups and different diseases (Felix *et al.* 1998; Rebbeck *et al.* 1998; Lamba *et al.* 2002, Tran *et al.* 2006) and has thus far, only been significantly associated with the development of prostate cancer (Rebbeck *et al.* 1998) and treatment-related leukaemia (Felix *et al.* 1998). Not much research has, however, been done regarding this polymorphism and the development of TD.

One previous study on Indian schizophrenia patients failed to provide proof of an association between the *CYP3A4*1B* polymorphism and the development of TD (Tiwari *et al.* 2005b). Other studies documented the occurrence of this variant (*CYP3A4*1B*) at high frequencies in African Americans and low frequencies in Caucasians (Fig. 5) (Sata *et al.* 2000; Lamba *et al.* 2002). These results are in agreement with the observation in our study. High frequencies of the A allele of the *CYP3A4*1G* SNP has previously been reported in African Americans (Fig. 5), (Lamba *et al.* 2002). With not one GG genotype found for the *CYP3A4*1G* SNP and only a few heterozygotes these results are mirrored in our Xhosa schizophrenia population.

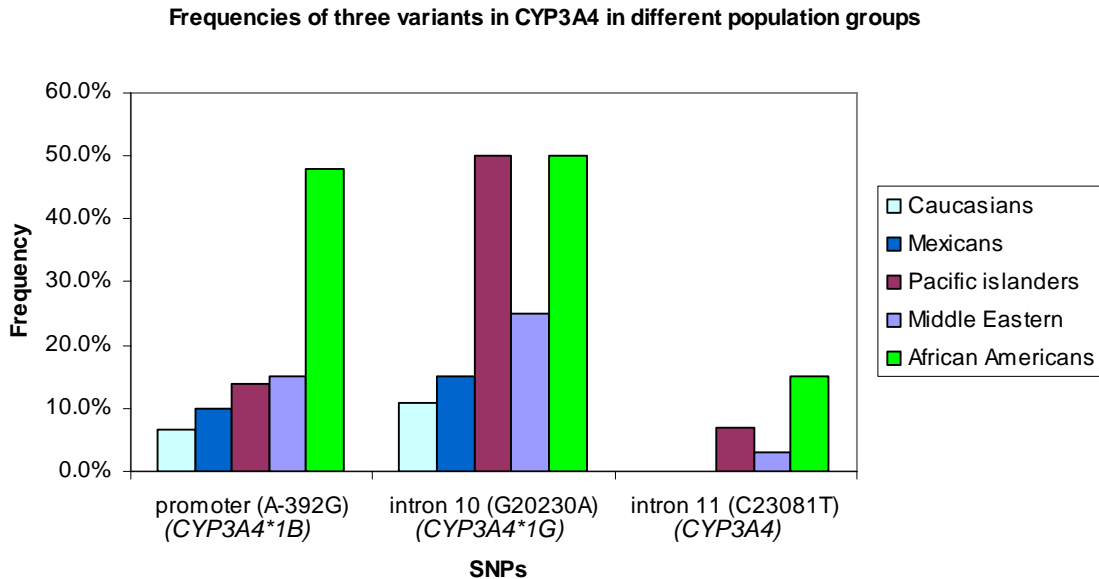


Fig. 5 Reported frequencies of the three polymorphisms in *CYP3A4* (Lamba *et al.* 2002).

The dual pathway shared between the *CYP3A4* and *CYP3A5* genes complicates a separate assessment of the clinical effects of their polymorphisms (Evans & McLeod 2003). Therefore an individual evaluation of each SNP and haplotype analysis is advisable. In a study performed on healthy African volunteers the haplotype analysis revealed that a haplotype containing the *CYP3A4*1B* G allele, *CYP3A5*3* A allele and *CYP3A5*6* G allele has a high frequency of 57% (Garsa *et al.* 2005). In our study the haplotype analysis was performed between all four SNPs and a haplotype containing the *CYP3A4*1B* G allele, *CYP3A4*1G* A allele, *CYP3A5*3* A allele and *CYP3A5*6* G allele

were obtained at a frequency of 46%. This frequency is slightly less than that obtained in healthy African volunteers Garsa *et al.* (2005). This might be ascribed to the fact that the African volunteers used in the above-mentioned study were mixed Africans and healthy individuals whereas our study was conducted only on Xhosa schizophrenia patients.

Haplotype analysis in the current study demonstrated linkage disequilibrium between two of the four *CYP3A* gene SNPs (*CYP3A4*1G* and *CYP3A5*3*), and found a statistical significant difference between TD and non-TD patients ($P=0.0496$). Previous studies on healthy Japanese individuals reported strong linkage disequilibrium between SNPs in *CYP3A4* and *CYP3A5* (Saeki *et al.* 2003; Fukushima-Uesaka *et al.* 2004). The AG haplotype (*CYP3A4*1G* and *CYP3A5*3*) was more frequently observed, almost twice as high, in TD individuals. Our results may indicate an association between this specific haplotype and the development of TD, however, this result needs to be treated with caution since the border line level of significance. The *CYP3A4*1G* polymorphism has not yet been studied in conjunction with drug response, therefore this haplotype has not previously been described. This finding warrants further investigation in schizophrenia patients of other population groups. Information regarding interethnic differences in *CYP3A4* and *CYP3A5* variants will enhance our understanding of antipsychotic drug response and TD at the molecular level and will provide more insight into gene-drug interactions amongst different ethnic groups.

Of the four polymorphisms included in our analysis, only *CYP3A4*1B* has previously been studied in schizophrenia and TD. To our knowledge *CYP3A4*1G*, *CYP3A5*3* and *CYP3A5*6* have not formerly been studied in conjunction with drug response, schizophrenia or TD. In conclusion, data presented here suggests an association between the *CYP3A5*3* polymorphism, the AG haplotype comprising (*CYP3A4*1G* and *CYP3A5*3*), and the development of TD. Further studies are encouraged to elucidate the role of these polymorphisms in the development of TD.

CHAPTER FIVE

ASSOCIATION STUDY BETWEEN MULTIDRUG RESISTANCE GENE (*MDR1*) VARIANTS IN XHOSA SCHIZOPHRENIA PATIENTS AND THE DEVELOPMENT OF INVOLUNTARY MOVEMENTS

5.1 ABSTRACT

Tardive dyskinesia (TD), distinguished by involuntary movements in the tongue, jaw, or trunk, is an irreversible side effect in some schizophrenia patients, associated with long-term antipsychotic drug treatment. The heterogenic nature of schizophrenia is known to cause different patients to display dissimilar drug responses, reflecting distinct genetic profiles. Should the underlying cause of TD be explained by a pharmacogenetic foundation, major clinical implications in the treatment of schizophrenia will follow. Individualised drug prescriptions and ultimately elimination of medication induced adverse side effects will become possible. The human multidrug resistance (*MDR1*) gene encodes the human drug transporter P-glycoprotein (P-gp), which plays an important role in amongst others, drug excretion and response. Polymorphisms in the *MDR1* gene have the ability to alter P-gp expression, resulting in altered drug disposition and response. Four DNA polymorphisms in this gene were studied in 116 South African Xhosa schizophrenia patients, 55 of whom displayed clinical symptoms of TD. This study sought to determine whether any of these variants contribute to genomic vulnerability for TD. The variants, detected by polymerase chain reaction (PCR) analysis, included the known single nucleotide polymorphisms (SNPs) T-129C, C1236T, G2677T/A and C3435T. Additional potential predisposing factors, i.e. tobacco and cannabis (also known as marijuana) smoking and anhedonia were also evaluated. Two of the four polymorphisms studied, T-129C and C3435T, were out of Hardy-Weinberg equilibrium (HWE) only in the patients with TD ($P=0.0015$ and $P=0.0063$ respectively). This may indicate a possible association between either or both of these SNPs and a predisposition in the development of TD. No statistically significant

differences were observed regarding genotypic distribution of any of the four *MDR1* gene polymorphisms when comparing TD and non-TD. Sub-classifications of the TD and non-TD patients based on tobacco and cannabis smoking and anhedonia also did not show any statistical significant difference for any of these four variants. Haplotype analysis identified linkage disequilibrium between the C1236T, G2677T/A and C3435T polymorphisms, but failed to find a haplotype associated with TD. We conclude that the *MDR1* gene polymorphisms employed in this study do not all constitute molecular markers of TD risk in Xhosa schizophrenia patients however, the two SNPs not in HWE warrant further investigation. For the present time the probability of a genetic component underlying TD remains unclear.

5.2 INTRODUCTION

The heterogeneity of schizophrenia gives rise to dissimilar drug responses displayed by different patients, reflecting distinct genetic profiles. Specific genetic variants have been associated with variation in sensitivity to drug response. Therefore antipsychotic drug medication has to progress in the direction of individualised drug therapy, in order to minimize or eliminate discomfort and side effects to the patient (Ohmori *et al.* 2003).

Tardive dyskinesia (TD) is a side effect distinguished by involuntary movements in any muscle group. The following potential social-demographic and clinical risk factors have been identified: family history of schizophrenia (with or without TD), drug abuse, smoking, ethnicity (higher in African-Americans) and advanced age (Müller *et al.* 2004). Genetic susceptibility to TD has been documented (Özdemir *et al.* 2001; Basile *et al.* 2002; Ohmori *et al.* 2003). The availability of a genetic test to screen for susceptibility to TD will be of great help to the psychiatrist prescribing antipsychotic drugs – the selection of suitable medication and its appropriate dosage will become possible (Müller *et al.* 2004).

The human multidrug resistance (*MDR1*) gene contains a core promoter region and 28 exons and is localized to chromosome 7q21.1 (Marzolini *et al.* 2004). P-glycoprotein (P-gp) encoded by the *MDR1* gene is the best characterised human drug transporter and plays an important role in the oral bio-availability, distribution, excretion of and response to drugs (Cascorbi 2006b). High levels of P-gp may lead to the insufficient

uptake of certain drugs, whereas low levels of P-gp may cause undesired side effects of drugs (Hoffmeyer *et al.* 2000).

Typical antipsychotic medication was prescribed to 78% of the schizophrenia patients included in the study presented here. Many drugs, including certain typical antipsychotic drugs, serve as substrates for MDR1. Several single nucleotide polymorphisms (SNPs) located within this gene have been studied in conjunction with drug response, four of which have proved to be important. The noncoding SNP (T-129C) located in the promoter region has been associated with altered P-gp function and expression (Cavaco *et al.* 2003; Marzolini *et al.* 2004). In healthy Portuguese the -129C allele was present in 5% of individuals (Cavaco *et al.* 2003). The SNP (C1236T) in exon 12 is synonymous and the C allele occurs in a much higher frequency in Africans Americans than in other population groups (Kim *et al.* 2001). The SNP in exon 21 at position 2677 results in either of two amino acid changes, i.e. Ala893Ser (G2677T) or Ala893Thr (G2677A) and has been associated with altered P-gp function (Marzolini *et al.* 2004). Caucasian individuals, homozygous for the 2677T allele associated with lower P-gp function, showed a higher expression of CYP3A4 than individuals carrying the 2677G allele (Fig. 1) (Lamba *et al.* 2006). The frequency of the 2677A allele is very low amongst all population groups, ranging from 0.5% in Black subjects (Yi *et al.* 2004) to 2% in Germans (Cascorbi *et al.* 2001) and 6% in Chinese subjects (Tang *et al.* 2002).

The synonymous SNP C3435T of exon 26 is the only SNP in the *MDR1* gene that alters transport function of P-gp (Schwab *et al.* 2003). A study performed on unrelated individuals from Berlin revealed a significant difference of expression levels of intestine P-gp between the two alleles of the SNP. Individuals homozygous for the T allele had a significant decrease in P-gp expression (Cascorbi *et al.* 2001). The C allele, associated with an increased expression of P-gp and reduced drug availability, was found to be predominant amongst Africans, who had a CC frequency of 75.5% (Chedule *et al.* 2003).

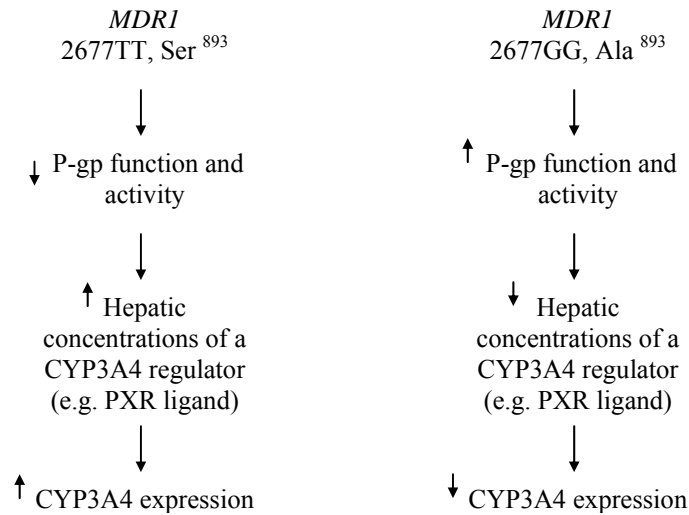


Fig. 1 The influence of *MDR1* genotypes on the expression of CYP3A4 (Lamba *et al.* 2006).

Linkage studies showed a significant association between the promoter SNP and the SNPs located in exon 21 ($P=0.006$) and exon 26 ($P=0.001$) (Anglicheau *et al.* 2004). Furthermore, the exon 12 SNP has been linked to the SNPs of exons 21 and 26 respectively in healthy European Americans and African Americans (Kim *et al.* 2001). Strong linkage disequilibrium has also been reported between the SNPs in exon 21 and 26 in different population groups (Marzolini *et al.* 2004) and both have been associated with induced activity of CYP3A4 in men (Lamba *et al.* 2006).

The current study on South African Xhosa schizophrenia patients investigated evidence for a probable association between any of the four above-mentioned variants of the *MDR1* gene and a tendency to develop TD. The effects of cannabis (also known as marijuana) and tobacco smoking, as well as the presence of the neurological disorder anhedonia were also taken into account as potential contributing factors in the onset of TD.

5.3 MATERIAL AND METHODS

5.3.1 Patient samples

The patient group used for this study was the same as for chapter three with exclusion to the normal Xhosa controls (See section 3.3.1).

5.3.2 Polymerase Chain Reaction (PCR) amplification

Genomic DNA from all patients were analysed using the polymerase chain reaction (PCR) to identify the presence of wild type and/or variant alleles in the *MDR1* gene. A total of four different SNPs were studied (Table 1).

T-129C polymorphism in the promoter region: Primers Fpr and Rpr were used to amplify a 197 bp PCR product. PCR was performed in a total reaction volume of 25 μ l containing 30 ng template DNA, 15 pmol of each primer, 100 μ M dNTPs, 0.5 U GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA), 5X Green GoTaq Flexi buffer and 4 mM MgCl₂. The two-step PCR amplification conditions were as follows: initial denaturing step at 94 °C for 4 min, followed by 15 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s and then 20 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 45 s. Final extension was at 72 °C for 5 min.

C1236T polymorphism in exon 12: Primers F12 and R12 yielded a 345 bp PCR product. The PCR reaction mix as well as the amplification conditions were the same as for the T-129C variant described above.

G2677T/A polymorphism in exon 21: Primers F21 and R21 were used to obtain a 108 bp PCR product. The PCR reaction mix was the same as for the T-129C variant, but the amplification conditions differed. The two-step amplification comprised an initial denaturation step at 94 °C for 4 min, followed by 15 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s. Subsequently 20 cycles were performed at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 45 s and the final extension was at 72 °C for 5 min.

C3435T polymorphism in exon 26: Primers F26 and R26 used to detect this variant yielded a 581 bp PCR product. The PCR reaction mix as well as the amplification conditions were the same as for the T-129C and C1236T variants.

5.3.3 RFLP Restriction Enzyme Analysis (REA)

All four SNPs analysed were revealed by restriction enzyme digestion following PCR amplification. Restriction enzyme analysis (REA) and electrophoresis conditions are shown in Table 2. PCR product (15 μ l) was used in a total volume of 20 μ l containing appropriate buffer and restriction enzymes. Samples were incubated overnight at the respective temperatures.

T-129C polymorphism: After PCR amplification, the DNA fragments were digested with 1 U of *MspA1* I (New England BioLabs Ipswich, MA, USA). In the presence of the polymorphic site, digestion resulted in 3 fragments of 148 bp, 32 bp and 17 bp.

C1236T polymorphism: The PCR product was digested with 1 U of *Eco*0109 I (Fermentas, Ontario, Canada, USA). Fragment sizes of 256 bp and 89 bp produced upon enzyme digestion represent the C allele. The undigested PCR sample represents the T allele.

G2677T/A polymorphism: 1 U of *Alw*21 I (Fermentas, Ontario, Canada, USA) was used to digest the PCR product. An undigested fragment size of 108 bp depicts the variant T/A while the digested products of 87 bp and 21 bp denote the G allele. To distinguish between the T and A allele, the *Alw*21 I digested sample was subsequently incubated with 1 U of *Rsa* I (New England BioLabs Ipswich, MA, USA) Fragment sizes of 106 bp and 2 bp produced upon *Rsa* I digestion represent the G or T allele whereas the 82 bp, 24 bp and 2 bp represent the A allele.

C3435T polymorphism: The PCR product was digested with 1 U of *Mbo* I (New England BioLabs Ipswich, MA, USA). Fragment sizes of 211, 198 and 172 bp produced upon enzyme digestion comprise the C allele. The 370 and 211 bp fragments represent the T allele.

Restriction enzyme digested PCR products were analysed on either polyacrylamide or agarose gels (Table 2). Electrophoresis through 15% (w/v) polyacrylamide gels (15% polyacrylamide, 10% (w/v) ammoniumpersulphate and Temed) was at room temperature at 200V for 1-2 hours depending on the fragment sizes. Electrophoresis conditions for agarose gels were 120V for 1 hour. In both instances 1XTBE (0.089 M Tris, 0.089 M Boric acid and 20 mM EDTA, pH 8.0) was used as electrophoresis buffer and DNA fragments were visualised under UV light following staining in 0.5 µg/ml ethidium bromide.

5.3.4 Statistical analysis

Allele and genotype frequencies were estimated and the Hardy-Weinberg equilibrium (HWE) was tested with the assistance of TFGA (Miller 1997). Statistical differences between TD and non-TD patients were determined using an analog of a Fisher's exact test on a contingency table (2 X 2 or 2 X 3 as appropriate) with Microsoft[®] Excel 2000. Differences in the observed frequencies of the four polymorphisms regarding tobacco and cannabis smoking, and anhedonia were explored by using an analog to Fisher's exact test by means of a 2 X 3 contingency table. A probability (*P*) value smaller than 0.05 was considered significant.

The Pearson correlation coefficient (r) and standardized linkage disequilibrium coefficient (D') were subsequently determined. The arbitrary cut off values for r^2 and D' were considered 0.4 and 0.7 respectively. Haplotype analysis was conducted using the Haploview 3.31 program (Barrett *et al.* 2005) to determine whether a specific haplotype may be associated with TD.

Table 1. Primer sequences used to PCR amplify *MDR1* gene variants

Variant	Location	Wild- Type	Variant	Primer name	Primer Sequence (5'-3')	PCR Product size	Reference
T-129C	Promoter region	cgagTagecg	cgagCagecg	Fpr Rpr	TCTCGAGGAATCAGCATTCAATCC CTAAAGGAAACGAAGAGCGGCTCTG	197 bp	(Cavaco <i>et al.</i> 2003)
C1236T	exon 12	agggCctga	agggTctga	F12 R12	TTTAAACCTAGTGAACAGTCAGTTCC ATATCCTGTCCATCAACTGACC	345 bp	(Hoffmeyer <i>et al.</i> 2000)
*G2677T/A	exon 21	aggtGctgg	aggt(T/A)ctgg	F21 R21	GTACCCATCATTGCAATAGCA TTTAGTTTGACTCACCTTCCgAG	108 bp	(Cavaco <i>et al.</i> 2003)
C3435T	exon 26	agatCgtga	agatTgtga	F26	CTACCACATGCATACATCAGAAAC	581 bp	(Hoffmeyer <i>et al.</i> 2000)

F, Forward R, Reverse

* Two sequential restriction enzyme digestions required to reveal polymorphism. See text.

Table 2. Restriction enzyme assays for the genotypic analysis of four RFLPs in the *MDR1* gene

<i>MDR1</i> Variant	Recognition site	Restriction enzyme	RE Incubation temp	Electrophoresis gel	Gel banding pattern
T-129C	C(C/A)G^C(T/G)G	<i>MspA11</i> (Cavaco <i>et al.</i> 2003)	37°C	2.0% agarose	wild-type - 2 fragments (180 bp and 17 bp) variant - 3 fragments (148 bp, 32 bp and 17 bp)
C1236T	(A/G)G^G(N)CC(C/T)	<i>Eco 0109I</i>	37°C	1.6% agarose	wild-type - 2 fragments (256 bp and 89 bp) variant - 1 fragment (345 bp)
G2677T/A	G(T/A)GC(T/A)^C	<i>Alw211</i> (Cavaco <i>et al.</i> 2003)	37°C	15% polyacrylamide	wild-type - 2 fragments (87 bp and 21 bp) variant - 1 fragment (108 bp)
G2677A	GT^AC	<i>Rsa I</i>	37°C	15% polyacrylamide	"G" or "T" - 2 fragments (106 bp and 2 bp) "A" - 3 fragments (82 bp, 24 bp and 2 bp)
C3435T	^GATC	<i>Mbo I</i> (Balram <i>et al.</i> 2003)	37°C	2.0% agarose	wild-type - 3 fragments (211 bp, 198 bp and 172 bp) variant - 2 fragments (370 bp and 211 bp)

RE restriction enzyme

5.4 RESULTS

In the current study Xhosa schizophrenia patients with and without TD were investigated for the presence of four polymorphisms in the *MDR1* gene namely: T-129C in the promoter region, C1236T in exon 12, G2677T/A in exon 21 and C3435T in exon 26. All four known SNPs were detected, and these were seen in both patient groups. Examples of the four SNPs detected are depicted in Fig. 2.

The two patient groups, with and without TD, were in HWE for the C1236T ($P=0.4099$ and $P=0.7386$ respectively) and G2677T/A polymorphisms ($P=0.8908$ and $P=0.8964$ respectively). The two remaining polymorphisms (T-129C and C3435T) were not in HWE in the TD group, but in HWE in the non-TD group. The P values for TD and non-TD respectively were as follows: T-129C polymorphism, $P=0.0015$ and $P=0.9887$; C3435T polymorphism, $P=0.0063$, $P=0.5104$.

Statistical analyses subsequently compared the distribution of the four polymorphisms in the following manner: (a) schizophrenia patients were categorized in two groups, based on the presence / absence of TD only (Table 3), and (b) TD and non-TD patients were sub-classified according to tobacco smoking, cannabis smoking and anhedonia status, and combinations thereof (Table 4). All of these groups were analysed separately. No significant differences were found between TD and non-TD patients with respect to genotype or allele frequencies for any of the four polymorphisms studied (Table 3).

No significant statistical differences were found between TD and non-TD schizophrenia patients of the *MDR1* T-129C polymorphism when considering smoking status ($P=0.412$), cannabis smoking ($P=0.073$), presence of anhedonia ($P=0.306$), smoking/cannabis ($P=0.983$) and cannabis/anhedonia ($P=0.997$). The *MDR1* C1236T polymorphism also yielded no observed statistical differences when considering the smoking ($P=0.619$), cannabis ($P=0.774$), anhedonia ($P=0.439$), smoking/cannabis ($P=0.822$) or cannabis/anhedonia ($P=0.576$) between the TD and non-TD clinical groups. The *MDR1* G2677T/A polymorphism also did not show significant differences between the TD and non-TD individuals when considering tobacco smoking ($P=0.372$), cannabis smoking ($P=0.593$), anhedonia ($P=0.608$),

smoking/cannabis ($P=0.767$) and cannabis/anhedonia ($P=0.296$). Furthermore, the *MDR1* C3435T polymorphism yielded no observed statistical differences when considering smoking ($P=0.807$), cannabis ($P=0.550$), anhedonia ($P=0.479$), smoking/cannabis ($P=0.716$) or cannabis/anhedonia ($P=0.436$).

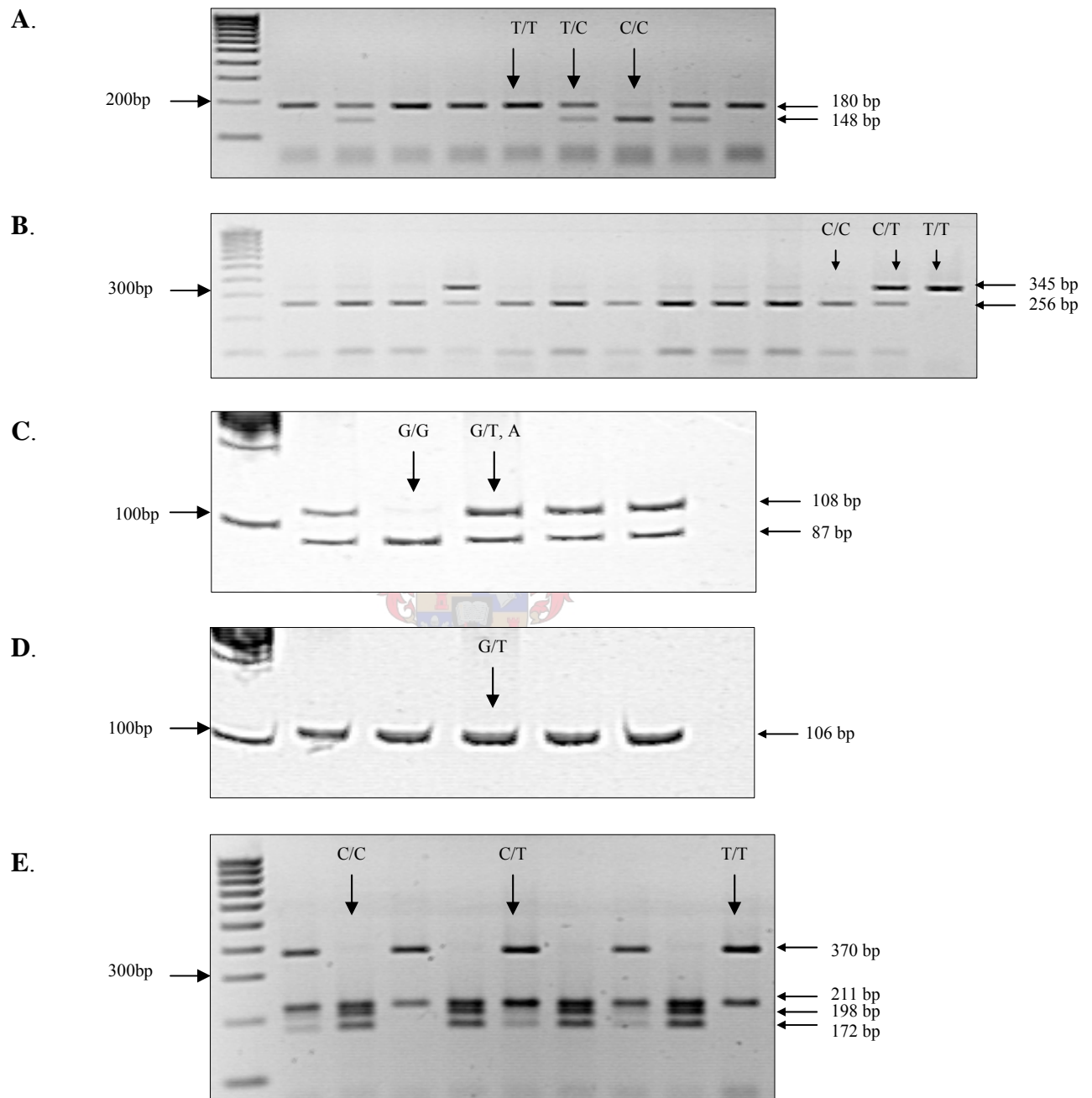


Fig. 2 Restriction enzyme analysis of four known SNPs located in the *MDR1* gene: [A] T-129C, [B] C1236T, [C] G2677T/A, [D] 2677T/A, [E] C3435T. For appropriate restriction enzymes revealing polymorphisms and relevant fragment sizes see Table 2. In the first lane a 100bp size marker (Hyperladder IV, Bioline) was loaded.

Table 3. Genotypic and allelic distribution of the *MDR1* gene T-129C, C1236T, G2677T/A and C3435T polymorphisms in Xhosa schizophrenia patients with TD compared to patients without TD.

	Genotypic distribution				Allelic distribution		
	No. of patients (%)				T	C	P value
<i>MDR1 T-129C</i>							
	T/T	T/C	C/C	P value	T	C	P value
TD patients, n=55	41(75%)	9(16%)	5(9%)	0.136	91	19	0.880
Non-TD patients, n=61	41(67%)	18(30%)	2(3%)		100	22	
<i>MDR1 C1236T</i>							
	C/C	C/T	T/T	P value	C	T	P value
TD patients, n= 55	44(80%)	11(20%)	0 (0%)	0.445	99	11	0.274
Non-TD patients, n = 61	44(72%)	16(26%)	1(2%)		104	18	
<i>MDR1 G2677T/A</i>							
	G/G	G/T	T/T	P value	G	T	P value
TD patients, n= 55	53(96%)	2(4%)	0(0%)	0.916	108	2	0.917
Non-TD patients, n = 61	59(97%)	2(3%)	0(0%)		120	2	
<i>MDR1 C3435T</i>							
	C/C	C/T	T/T	P value	C	T	P value
TD patients, n=51	41(80%)	7(14%)	3(6%)	0.382	89	13	0.629
Non-TD patients, n=60	44(73%)	14(23%)	2(4%)		102	18	

Table 4. Genotypic distribution of the *MDR1* T-129C, C1236T, G2677T/A and C3435T polymorphisms in Xhosa schizophrenia patients with TD compared to patients without TD, classified according to tobacco smoking, cannabis smoking, presence of anhedonia and combinations of these factors.

		Genotypic distribution				Allelic distribution		
		No. of patients (%)				T	C	P value
<i>MDR1 T-129C</i>								
Tobacco Smoking	TD patients, n=37	27(73%)	6(16%)	4(11%)	0.412	60	14	0.614
	Non-TD patients, n=47	34(72%)	11(24%)	2(4%)				
Cannabis	TD patients, n=16	11(69%)	4(25%)	1(6%)	0.973	26	6	0.856
	Non-TD patients, n=32	21(66%)	9(28%)	2(6%)				
Anhedonia	TD patients, n=45	32(71%)	8(18%)	5(11%)	0.306	72	18	0.641
	Non-TD patients, n=49	34(69%)	13(27%)	2(4%)				
Smoking/Cannabis	TD patients, n=15	10(67%)	4(27%)	1(6%)	0.983	24	6	0.907
	Non-TD patients, n=29	20(69%)	7(24%)	2(7%)				
Cannabis/Anhedonia	TD patients, n=14	9(64%)	4(29%)	1(7%)	0.997	22	6	0.934
	Non-TD patients, n=27	17(63%)	8(30%)	2(7%)				

<i>MDR1 C1236T</i>		C/C	C/T	T/T	<i>P</i> value	C	T	<i>P</i> value
Tobacco Smoking	TD patients, n=37	30(81%)	7(19%)	0(0%)	0.619	67	7	0.641
	Non-TD patients, n=47	36(77%)	11(23%)	0(0%)		83	11	
Cannabis	TD patients, n=16	13(81%)	3(19%)	0(0%)	0.774	29	3	0.650
	Non-TD patients, n=32	25(78%)	6(19%)	1(3%)		56	8	
Anhedonia	TD patients, n=45	37(82%)	8(18%)	0(0%)	0.439	82	8	0.250
	Non-TD patients, n=49	36(73%)	12(25%)	1(2%)		84	14	
Smoking/ Cannabis	TD patients, n=15	12(80%)	3(20%)	0(0%)	0.822	27	3	0.831
	Non-TD patients, n=29	24(83%)	5(17%)	0(0%)		53	5	
Cannabis/Anhedonia	TD patients, n=14	13(93%)	1(7%)	0(0%)	0.576	27	1	0.247
	Non-TD patients, n=27	22(81%)	4(15%)	1(4%)		48	6	
<i>MDR1 G2677T/A</i>		G/G	G/T	T/T	<i>P</i> value	G	T	<i>P</i> value
Tobacco Smoking	TD patients, n=37	37(100%)	0(0%)	0(0%)	0.372	74	0	0.374
	Non-TD patients, n=47	46(98%)	1(2%)	0(0%)		93	1	
Cannabis	TD patients, n=16	16(100%)	0(0%)	0(0%)	0.593	32	0	0.312
	Non-TD patients, n=32	30(94%)	2(6%)	0(0%)		62	2	
Anhedonia	TD patients, n=45	44(98%)	1(2%)	0(0%)	0.608	89	1	0.611
	Non-TD patients, n=49	47(96%)	2(4%)	0(0%)		96	2	
Smoking/ Cannabis	TD patients, n=15	15(100%)	0(0%)	0(0%)	0.767	30	0	0.469
	Non-TD patients, n=29	28(97%)	1(3%)	0(0%)		57	1	
Cannabis/Anhedonia	TD patients, n=14	14(100%)	0(0%)	0(0%)	0.296	28	0	0.303
	Non-TD patients, n=27	25(93%)	2(7%)	0(0%)		52	2	
<i>MDR1 C3435T</i>		C/C	C/T	T/T	<i>P</i> value	C	T	<i>P</i> value
Tobacco Smoking	TD patients, n=35	28((80%)	6(17%)	1(3%)	0.807	62	8	0.486
	Non-TD patients, n=46	34(74%)	10(22%)	2(4%)		78	14	
Cannabis	TD patients, n=14	12(86%)	2(14%)	0(0%)	0.550	26	2	0.266
	Non-TD patients, n=32	23(72%)	8(25%)	1(3%)		54	10	
Anhedonia	TD patients, n=41	33(80%)	6(15%)	2(5%)	0.479	72	10	0.400
	Non-TD patients, n=48	34(71%)	12(25%)	2(4%)		80	16	
Smoking/Cannabis	TD patients, n=13	11(85%)	2(15%)	0(0%)	0.716	24	2	0.425
	Non-TD patients, n=29	22(76%)	6(21%)	1(3%)		50	8	
Cannabis/Anhedonia	TD patients, n=12	11(92%)	1(8%)	0(0%)	0.436	23	1	0.174
	Non-TD patients, n=27	20(74%)	6(22%)	1(4%)		46	8	

Haplotype analysis of the four *MDR1* SNPs (Fig. 3A) revealed five different haplotypes, all with incidences greater than 1%. Subsequent analysis (Fig. 3B) of three selected SNPs (C1236T, G2677T/A and C3435T) revealed four different haplotypes, each occurring at a frequency higher than 1%. No association was found between TD and a specific haplotype (Tables 5 and 6). Interestingly, the CGC haplotype has a very high frequency (0.819) in the Xhosa schizophrenia population.

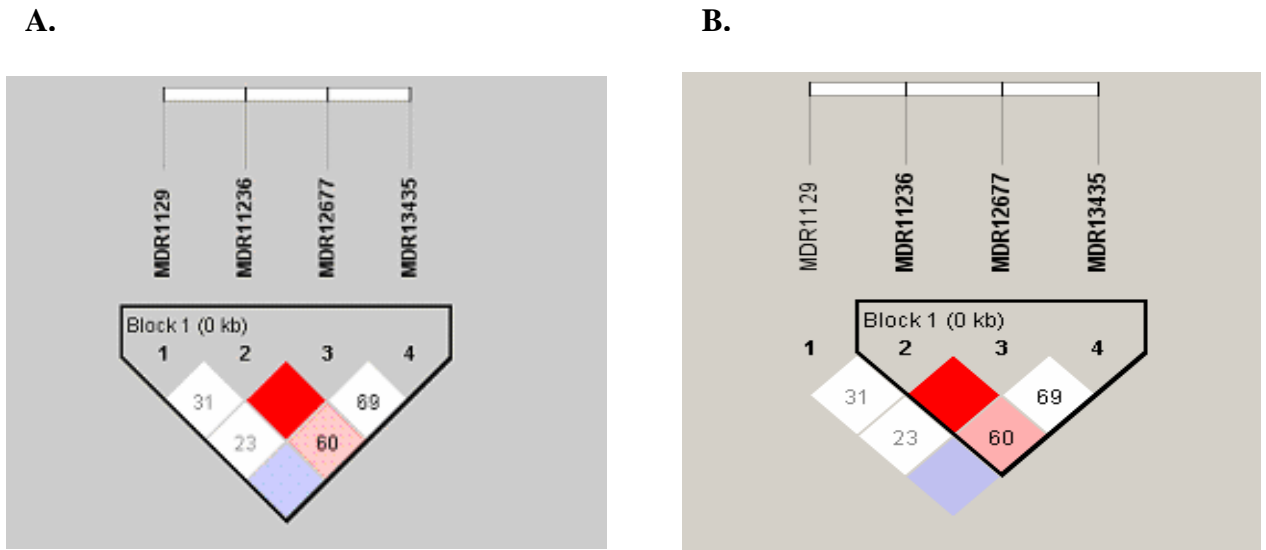


Fig. 3 LD plot results from haplotype analysis between [A] all four *MDR1* gene SNPs studied and [B] between three selected SNPs. The colour scheme is as follows: white = LOD < 2, D' < 1; blue = LOD < 2, D' = 1; shades of pink = LOD ≥ 2, D' < 1; red = LOD ≥ 2, D' = 1.

Table 5. Haplotype analysis between alleles of the *MDR1* gene: T-129C, C1236T, G2677T/A and C3435T (Fig. 3A).

Haplotype	Frequency	Schizophrenia patient ratios		Chi Square	P value
		TD	non-TD		
TCGC	0.661	0.683	0.641	0.440	0.5072
CCGC	0.158	0.158	0.159	0.000	0.9913
TTGT	0.078	0.058	0.096	1.168	0.2798
TCGT	0.049	0.046	0.053	0.050	0.8223
TTGC	0.026	0.023	0.030	0.111	0.7390

Table 6. Haplotype analysis between alleles of the *MDR1* gene: C1236T, G2677T/A and C3435T (Fig 3B)

Haplotype	Frequency	Schizophrenia patient ratios		Chi Square	P value
		TD	non-TD		
CGC	0.819	0.840	0.800	0.644	0.4223
TGT	0.074	0.054	0.093	1.267	0.2603
CGT	0.054	0.056	0.053	0.013	0.9076
TGC	0.035	0.031	0.038	0.085	0.7713

$D' = 0.607, r^2 = 0.327$

r, Pearson correlation; D', standardized LD coefficient.

5.5 DISCUSSION

Schizophrenia is a severe mental illness. The goal of medication is two-fold: to control acute psychotic symptoms and to improve patients' function and quality of life. Unfortunately many patients develop medication-induced movement disorders, including neuroleptic induced Parkinsonism, acute dystonia and TD. TD is often irreversible and causes severe discomfort in affected individuals.

It has been suggested that genetic predisposition may represent the underlying cause of TD. The identification of DNA susceptibility loci in individuals at high risk of contracting TD will bring about key innovations in schizophrenia research. Other factors that have been proposed to contribute to the development of TD include tobacco and cannabis smoking and the diagnosis of anhedonia. The present pharmacogenetic study aimed to evaluate whether any of four selected *MDR1* gene polymorphisms (T-129C, C1236T, G2677T/A and C3435T) contributed to enhanced risk for TD. Supplementary factors, i.e. tobacco and cannabis smoking and anhedonia were also considered. 116 South African Xhosa schizophrenia patients were studied, of whom 55 developed TD.

The *MDR1* gene plays an important role in drug disposition and response and has been studied in conjunction with other disorders such as Parkinson's disease (Tan *et al.* 2004a) and HIV infection (Schwab *et al.* 2003). The current study is to our knowledge the first study to investigate a possible association between polymorphisms in the *MDR1* gene and the development of TD. Pharmacogenetic studies regarding the *MDR1* gene, encoding the P-gp glycoprotein, are highly significant due to its important role in drug disposition. Polymorphisms in the *MDR1* gene have previously been implemented in studies regarding drug-resistant epilepsy that results from multiple drug use (Kim *et al.* 2006). Since TD can also develop due to multiple drug combinations used over a long period of time, it highlights the importance of the *MDR1* gene as a perfect candidate gene to investigate. Contradictory results exist regarding the effect of cannabinoids on the MDR1 P-gp transport function. It has previously been reported that plant derived cannabinoids inhibit the transport function of P-gp (Zhu *et al.* 2006) and modulate P-gp activity (Niere *et al.* 2006). However, on the contrary Holland *et al.* (2006) reported that

cannabinoids do not inhibit the function of P-gp. Thus the importance of the *MDR1* gene and cannabis use remains unclear and the investigation into the role of this *MDR1* gene in TD is warranted.

None of the four *MDR1* gene polymorphisms studied displayed differences in genotypic distribution between TD and non-TD patient groups. Sub-classifications of the TD and non-TD patients based on tobacco smoking, cannabis abuse and anhedonia also did not reveal any major differences for any of these four variants.

The C1236T and G2677T/A SNPs were in HWE in both the TD and non-TD groups. The T-129C and C3435T polymorphisms were in HWE in the non-TD group but not in the TD group. Several reasons, i.e. selection, genetic drift, gene flow and mutation have been used to explain statistically significant HWE deviations, but it is unclear which of them could have caused the deviation in this case. The strong statistical differences observed in the TD group regarding the T-129C and C3435T polymorphisms ($P=0.0015$ and $P=0.0063$ respectively), may be ascribed to a deficiency of heterozygotes or an increase of wild-type and variant homozygotes between the TD and non-TD group compared with that expected under Hardy-Weinberg. These results might indicate a possible association between either or both of these SNPs and a predisposition to the development of TD.

A previous study on Korean epileptic individuals indicated that the *MDR1* C3435T SNP CC genotype was associated with drug-resistant epilepsy (Siddiqui *et al.* 2003). However, two other studies failed to replicate this finding (Tan *et al.* 2004b; Sills *et al.* 2005). In our study the CC genotype was slightly more prevalent amongst TD than non-TD individuals. Since drug-resistant epilepsy and possibly TD are the result of multiple drugs used during treatment, it could be expected that there would be some similarities between the present study and that of Siddiqui *et al.* (2003) regarding the C3435T polymorphism. A high frequency of the C3435T SNP C allele was previously reported in a randomly selected Ghanian African population (Schaeffeler *et al.* 2001). The C allele was also the predominant allele in the Xhosa schizophrenia population

The incidences of three SNPs (C1236T, G2677T/A and C3435T) were compared between African Americans (Marzolini *et al.* 2004) and the Xhosa schizophrenia patients (with and without TD) of the current study (Fig. 4).

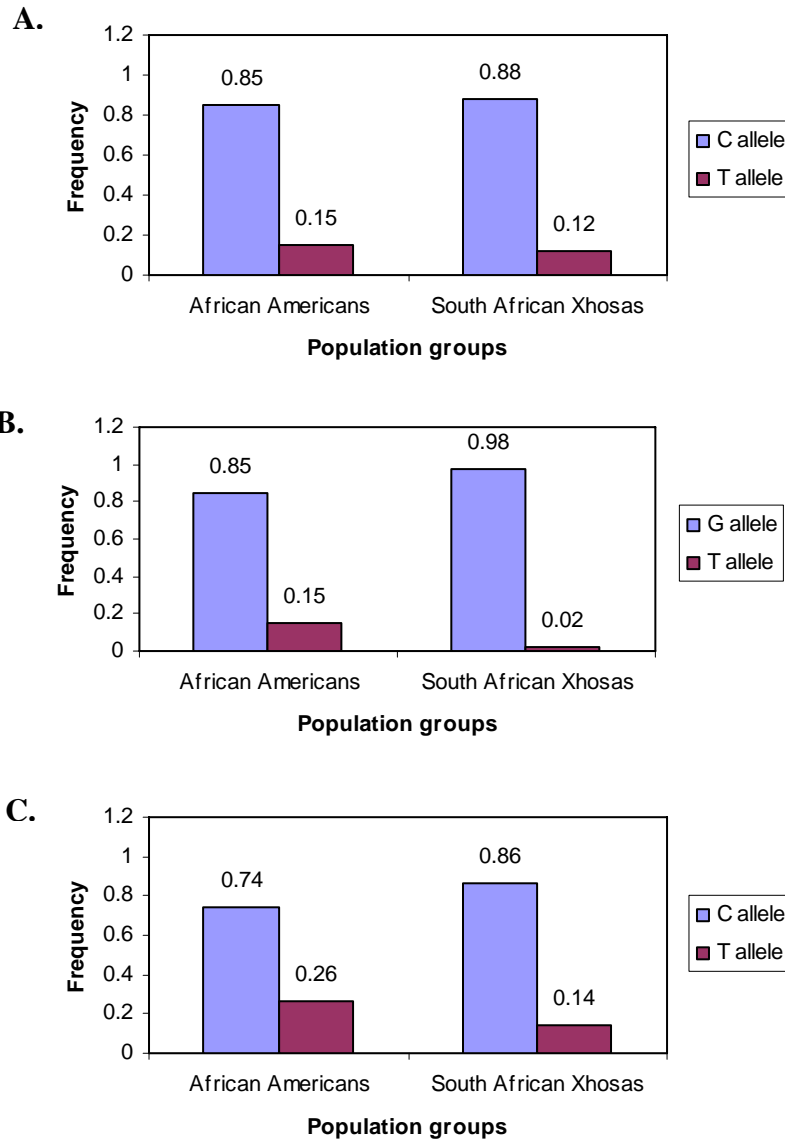


Fig. 4 The allele frequencies of [A] *MDR1* C1236T, [B] *MDR1* G2677T/A and [C] *MDR1* C3435T in the African American (Marzolini *et al.* 2004) and South African Xhosas diagnosed with schizophrenia (data obtained from the study presented here).

In all instances the frequencies were comparable; however the frequencies of the G2677T and C3435T polymorphisms deviated slightly between the two population groups. These deviations may be ascribed to two reasons. Xhosa schizophrenia individuals were compared to normal healthy African Americans and secondly, a great deal of integration has taken place between African Americans and Caucasians

resulting in admixture. Interestingly enough it has previously been reported that the alleles most common amongst African Americans are usually infrequent in Caucasians (Kim *et al.* 2001). These results stress the importance of different allele frequencies in different population groups. Therefore, frequencies obtained from studies on African Americans cannot be blindly applied to other African population groups such as the Xhosas.

Haplotype analysis in the current study demonstrated linkage disequilibrium between three of the four *MDR1* gene SNPs (C1236T, G2677T/A, C3435T), but did not identify a haplotype associated with TD. The CGC haplotype occurred at a very high frequency, i.e. 81.9%, compared to other haplotypes. The second most common was a TGT at a frequency of only 7.4%. Since the CGC haplotype occurred at high frequencies in both the TD and non-TD patients, the configuration has no relevance regarding resistance or susceptibility to TD.

In conclusion, data presented here found proof of a possible association between the *MDR1* T-129C and C3435T polymorphisms and a predisposition in the development of TD. This association may be ascribed to the TD group being out of HWE for both SNPs, whereas the non-TD group was in HWE. However, future studies will cast light on the influence of *MDR1* gene polymorphisms on MDR1 activity and drug metabolism, particularly antipsychotic drugs.

CHAPTER SIX

CONCLUSIONS

The applications of pharmacogenomics and pharmacogenetics are vast in the drug developing industry, but several challenges remain to be met. The major scopes of clinical pharmacogenomics include the identification of interactions between gene variants and drugs and the development of molecular tests for the prediction of drug response. Other potential exciting benefits that will evolve from this information are individualised drug prescriptions which in itself will herald a significant reduction in ADRs.

Schizophrenia is a complex, multifactorial disease, influenced by genetic and environmental factors. Long-term exposure of these patients to antipsychotic drugs often, but not always, leads to the development of TD. TD probably indicates the inability of an individual to fully metabolise the drugs, which in turn is thought to be accounted for by genetic variability amongst different individuals. Even though none of the DNA polymorphisms included in the present study could unequivocally prove a genetic basis for TD, the information generated is considered to contribute to the gradual step-wise accumulation of data casting light on the interaction between drugs and the genome. Of all the gene variants analysed here, only four, i.e. *CYP1A2*1C*, *CYP1A2*1F*, *CYP17 α -hydroxylase* and *CYP3A4*1B*, have previously been studied in conjunction with schizophrenia, TD and drug metabolism and response. Our study represents the first to conduct an inquiry into the pharmacogenetic nature of schizophrenia in Xhosa patients, utilizing this particular series of 12 gene polymorphisms.

6.1 CYTOCHROME P450 (CYP) GENE POLYMORPHISMS AND TD

The study detailed in Chapter three did not find proof of an association between TD and either of two polymorphisms, *CYP1A2*1C* and *CYP1A2*1F*, in the *CYP1A2* gene.

Evidence of a relationship between the *CYP17 α -hydroxylase* polymorphism and TD was provided in the following manner: presence of the polymorphism protects against development of the negative medication side effects. Cannabis smokers who carry the variant are at even lower risk of developing TD.

Both *CYP3A4* and *CYP3A5* play a very important role in drug metabolism however, no association was observed between either of the *CYP3A4* SNPs (*CYP3A4*1B* and *CYP3A4*1G*) and the development of TD in the investigation outlined in Chapter four. Analysis of the DNA results, in association with tobacco and cannabis smoking and anhedonia, did not succeed in identifying additional risk factors causing TD. Heterozygotes of the *CYP3A5*3* polymorphism were detected more frequently amongst TD patients. The other *CYP3A5* SNP analysed, *CYP3A5*6*, did not appear to play a role in conveying increased or decreased susceptibility for TD. It was revealed that a haplotype encompassing *CYP3A4*1G* and *CYP3A5*3* accompanied heightened risk for TD. From this study it is clear that the influences of the *CYP* genes on schizophrenia, TD or in a broader sense, on drug metabolism are indispensable.

6.2 MULTIDRUG RESISTANCE GENE (*MDR1*) POLYMORPHISMS AND TD

Chapter five documented an investigation into the role of four *MDR1* gene SNPs (T-129C, C1236T, G2677T/A and C3435T) in predisposition to TD. No association was found between TD and the C1236T and G2677T/A SNPs. Both the T-129C and C3435T SNPs were out of HWE in TD, and in HWE in non-TD patients, indicating a possible association between either or both of these SNPs and a predisposition to TD. In terms of genotypic and allelic distributions, no differences were observed between the two patient groups.

6.3 LIMITATIONS OF THIS STUDY

As is the case in many other projects of this nature, one of the biggest limitations of our study was the sample size. The study was performed on 116 individuals, that for some geneticist might be enough and for others not nearly enough. Some of the rare polymorphisms, i.e. *MDR1* 2677 AA and *CYP3A4*1G* GG were not observed and the scope of statistical analyses were restricted due to the small numbers of patients available. Incomplete clinical data such as period of treatment, dosage and drug cocktail mix prevented any additional conclusions.

6.4 CHALLENGES AND FUTURE PROSPECTS

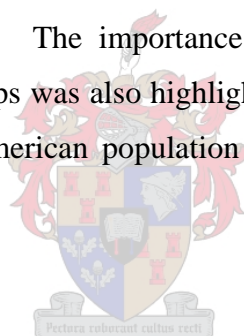
The potential clinical applications of pharmacogenomics are promising and exciting, and may yield hitherto unexpected results. Prospective studies are encouraged to assess how an individual's genotypic profile can be utilized to predict his / her metabolic capacity. The fact that each individual has a unique genetic makeup and responds differently to different drugs, raises a few interesting questions. Will genotyping identify only a fraction of schizophrenia patients that will benefit from individualised drug therapy? Will it be possible to make adjustments in the treatment of schizophrenia patients with more severe metabolic polymorphisms? How exactly will this genotype based approach differ from the current status quo when prescribing drugs? How successful will this genotype based methodology be in eliminating all ADRs such as TD? No doubt the pharmacogenomic treatment approach promises to be highly complex and sufficient guidelines and training should be made available to the clinician.

Clinical utility of CYP450 genotyping is expected to be proven in the near future. Ideally an individual will be genotyped only once for all the relevant CYP450 enzymes. P-gp, encoded by the *MDR1* gene, plays an important role in the oral bio-availability, distribution, excretion of and response to drugs and future studies will illuminate the

importance of the gene and its variants in relation to schizophrenia, TD and drug metabolism. Clearly, the issue of interethnic differences warrants a lot of attention and should be addressed in great depth. A vast amount of research still lies ahead before the goal is realised and successful application of the discipline of pharmacogenomics becomes a daily, feasible reality, one epitomized by individually designed drug therapies and minimum ADRs.

6.5 CONTRIBUTION OF THIS STUDY

The aim of this study was met and has provided evidence of associations between certain polymorphisms in the cytochrome P450 and *MDR1* genes and the disorder of TD manifesting sporadically in schizophrenia patients. This was the first study to investigate the possible involvement of these polymorphisms in the development of TD amongst the South African Xhosa population. The importance of genotypic differences existing between different population groups was also highlighted by this study since frequencies obtained amongst the African American population was not precisely mirrored in the Xhosa population.



CHAPTER SEVEN

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Home page of the human cytochrome P450 (*CYP*) allele nomenclature committee:
<http://www.imm.ki.se/CYPalleles>