

# **Molecular Genetic Analyses of Antipsychotic Pharmacogenes in a South African First Episode Schizophrenia Cohort**

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in Genetics at Stellenbosch University



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**ABSTRACT**

Antipsychotic treatment of schizophrenia is often accompanied by distressing adverse effects and a high relapse rate. Although pharmacogenetic research has identified some promising candidate pharmacogenes, these remain poorly characterized in South African (SA) populations. This study investigated whether polymorphisms in several candidate pharmacogenes (*COMT*, *CYP1A2*, *CYP2D6*, *DRD2*, *DRD3*, *HTR2A* and *SOD2*) were associated with antipsychotic treatment outcome in a SA schizophrenia cohort.

The cohort utilized consisted of 103 first episode schizophrenia (FES) patients from SA Coloured, Caucasian and Xhosa populations, clinically well-characterized and treated with a first generation antipsychotic, flupenthixol decanoate, for at least twelve months. Previously generated exome sequencing data of eleven of the FES patients were used to identify known and novel functional variants within the seven pharmacogenes, and the predicted functional effect of novel variants were assessed with SIFT and PolyPhen-2 algorithms. Based on these analyses and mining of the literature, a total of 33 variants were prioritized for genotyping by PCR-RFLP, long-range and allele-specific PCR, and TaqMan<sup>®</sup> assays. Minor allele frequencies in our cohort were compared to populations from the 1000 Genomes Project or the Human *CYP* Allele Nomenclature databases to distinguish unique genetic variation in SA populations. A mixed model for repeated measures analysis was used to determine whether any of the polymorphisms identified were associated with antipsychotic treatment response, as measured by a change in PANSS scores over time from the twelve-month longitudinal PANSS scores adjusted at baseline. Inheritance models to estimate the effect size (ES) of significant associations were assessed using 95% confidence intervals (CI).

Exome data revealed a total of eighteen functional variants in six of the genes, predominantly present in *CYP2D6*. Novel variants were identified in *COMT* (rs373611092) and *SOD2* (rs372173830), where rs373611092 was predicted to “damage” the *COMT* protein function (SIFT score = 0.015). Of the 33 variants, only variants in *COMT* and *DRD2* were significantly associated with changes in PANSS negative scores after twelve months of treatment. Associations with improved treatment outcomes included *COMT* rs4633 [ $P = 0.0080$  (T allele), ES = -0.17 95% CI (-0.30 to -0.04)], and *DRD2* rs1799732 [ $P = 0.0004$  (-C vs CC), ES = -0.19 95% CI (-0.38 to 0.00)]. *COMT* haplotypes (rs2020917-rs737869-rs6269-rs4633-rs9332377) also showed an improved treatment outcome for negative symptoms. Undesirable treatment outcomes were associated with *COMT* rs9332377 [ $P = 0.0006$  (T allele), ES = 0.25 95% CI (0.11 to 0.39)], and *DRD2* variants rs1799732 [ $P = 0.0004$  (-- vs CC), ES = 0.50 95% CI (0.15 to 0.86)] and rs1079597 [ $P = 0.0020$  (AA vs AG+GG), ES = 0.93 95% CI (0.34 to 1.53)].

This study confirmed the presence of unique variation in a SA FES cohort associated with antipsychotic pharmacogenetic traits, highlighting the value of re-sequencing in SA individuals. Several SNPs in *COMT* and *DRD2* were identified to be involved in treatment response, specifically influencing negative symptoms. These results serve as a platform for future antipsychotic pharmacogenetic studies within the SA context that could aid the optimization of antipsychotic treatment in the uniquely diverse, but understudied populations.

## OPSOMMING

Antipsigotikumbehandeling vir skisofrenie gaan dikwels gepaard met ernstige negatiewe uitwerkings en 'n hoë terugvalkoers. Hoewel farmakogenetiese navorsing op 'n paar belowende kandidaatfarmakogene dui, is dit tot dusver nog swak getipeer onder Suid-Afrikaanse populasies. Hierdie studie het ondersoek ingestel na die verwantskap tussen polimorfismes in verskillende kandidaatfarmakogene (*COMT*, *CYP1A2*, *CYP2D6*, *DRD2*, *DRD3*, *HTR2A* en *SOD2*) en die uitkoms van antipsigotikumbehandeling by 'n kohort Suid-Afrikaanse skisofreniepasiënte.

Die studiekohort het uit 103 skisofreniepasiënte met 'n eerste episode (sogenaamde FES-pasiënte) uit die Suid-Afrikaanse bruin, wit en Xhosa-bevolkingsgroepe bestaan. Die pasiënte is almal klinies goed getipeer en het reeds minstens 12 maande se behandeling met 'n eerstegenerasie-antipsigotikum, flupentiksol-dekanoaat, voltooi. Eksoomvolgorededata wat voorheen vir 11 van die FES-pasiënte verkry is, is gebruik om bekende én nuwe funksionele variante in die sewe farmakogene te identifiseer, en die voorspelde funksionele uitwerking van nuwe variante is met behulp van SIFT- en PolyPhen-2-algoritmes beoordeel. Op grond van hierdie ontledings en literatuurontginning is altesaam 33 variante vir genotipering met PKR-RFLP-, langreeks- en allelspesifieke PKR, en TaqMan<sup>®</sup>-toetse uitgewys. Alleelfrekwensies in die studiekohort is met populasies uit die projek "1000 Genomes" en die databasis van die alleelnomenklatuur vir menslike *CYP* gene vergelyk om unieke genetiese variasie by Suid-Afrikaanse populasies te bepaal. 'n Gemengde model van herhaaldematingsontleding is gebruik om vas te stel of enige van die geïdentifiseerde polimorfismes verband hou met die reaksie op antipsigotikumbehandeling, soos wat geblyk het uit 'n verandering in PANSS-tellings oor tyd vergeleke met die longitudinale PANSS-tellings oor 12 maande wat op die basislyn aangepas is. Oorerwingsmodelle om die uitwerkingsgrootte (UG) van beduidende verwantskappe te bepaal, is met behulp van vertrouensintervalle (VI's) van 95% beoordeel.

Eksoomdata het altesaam 18 funksionele variante in ses van die gene, hoofsaaklik in *CYP2D6*, aan die lig gebring. Nuwe variante is in *COMT* (rs373611092) en *SOD2* (rs372173830) geïdentifiseer, waar rs373611092 na verwagting die *COMT*-proteïenfunksie sal "beskadig" (SIFT-telling = 0.015). Variante in *COMT* en *DRD2* hou sterk verband met veranderinge in negatiewe PANSS-tellings na 12 maande se behandeling. Verbande met beter behandelingsuitkomste sluit in *COMT* rs4633 [ $P = 0.0080$  (T-allele), UG = -0.17 95% VI (-0.30 tot -0.04)], en *DRD2* rs1799732 [ $P = 0.0004$  (-C vs CC), UG = -0.19 95% VI (-0.38 tot 0.00)]. *COMT*-haplotipes (rs2020917-rs737869-rs6269-rs4633-rs9332377) het ook 'n verbeterde behandelingsuitkoms vir negatiewe simptome getoon. Ongewenste behandelingsuitkomste is verbind met *COMT* rs9332377 [ $P = 0.0006$  (T-allele), UG = 0.25 95% VI (0.11 tot 0.39)], en *DRD2*-variante rs1799732 [ $P = 0.0004$  (-- vs CC), UG = 0.50 95% VI (0.15 tot 0.86)] en rs1079597 [ $P = 0.0020$  (AA vs AG+GG), UG = 0.93 95% VI (0.34 tot 1.53)].

Hierdie studie bevestig dus die unieke variasie in die farmakogenetiese kenmerke van antipsigotika by 'n Suid-Afrikaanse FES-kohort, en beklemtoon die waarde van hernude reeksbeplanning by Suid-Afrikaanse individue. Die navorsing toon dat verskeie SNP-verwantskappe vir *COMT* en *DRD2* by behandelingsreaksie betrokke is, wat veral negatiewe simptome beïnvloed. Hierdie resultate dien as 'n platform vir verdere studies oor die farmakogenetika van antipsigotika in Suid-Afrikaanse verband ten einde antipsigotikumbehandeling in eiesoortig diverse dog grootliks onbestudeerde populasies te help optimaliseer.

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## **DEDICATION**

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**LIST OF SYMBOLS AND ABBREVIATIONS**

3'	3-prime end
5'	5-prime end
©	Copyright
°C	Degrees Celsius
°C/hr	Degrees Celsius per hour
=	Equal to
>	Greater than
μM	Micromolar
μl	Microliter
μg/ml	Microgram per millilitre
%	Percentage
®	Registered trademark
<	Smaller than
™	Trademark
&	And
A	Adenine (base)
A	Alanine (amino acid)
ADRs	Adverse drug reactions
AF	African human <i>CYP</i> allele nomenclature database population
AFR	African 1000 Genomes project population
AIMs	Ancestry informative markers
AM	American human <i>CYP</i> allele nomenclature database population
AMR	American 1000 Genomes project population
<i>ANKK1</i>	Ankyrin repeat and kinase domain containing 1 gene

ARC	Activity-regulated cytoskeleton-associated protein
AS	Activity score
AS	Asian human <i>CYP</i> allele nomenclature database population
ASD	Autism spectrum disorder
ASN	Asian 1000 Genomes project population
BD	Bipolar disorder
bp	Base pair
C	Cytosine (base)
C	Cysteine (amino acid)
Cau	Caucasian
CI	Confidence interval
CNS	Central nervous system
CNVs	Copy number variants
<i>COMT</i>	Catechol- <i>O</i> -methyltransferase gene
CYP	Cytochrome P450
<i>CYP1A2</i>	Cytochrome P450, family 1, subfamily A, polypeptide 2 gene
<i>CYP2D6</i>	Cytochrome P450, family 2, subfamily D, polypeptide 6 gene
Cys	Cysteine (amino acid)
D	Deletion (base)
D	Aspartic acid (amino acid)
DALY	Disability-adjusted life years
dbSNP	Single nucleotide polymorphism database
Del	Deletion allele
dH <sub>2</sub> O	Distilled water

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxy-nucleotide-tri-phosphate
<i>DRD2</i>	Dopamine receptor D2 gene
<i>DRD3</i>	Dopamine receptor D3 gene
DSM-IV	Diagnostic and statistical manual of mental disorders version four
DSM-5	Diagnostic and statistical manual of mental disorders version five
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia
EMs	Extensive metabolizers
EPS	Extra-pyramidal side effects
ES	Effect size
<i>et al.</i>	Et alii
EU	European human <i>CYP</i> allele nomenclature database population
EUR	European 1000 Genomes project population
FES	First episode schizophrenia
FGAs	First generation antipsychotics
FMRP	Fragile X mental retardation protein
g	Grams
G	Guanine (base)
G	Glycine (amino acid)
gDNA	Genomic DNA
Gly	Glycine

GWAS	Genome-wide association studies
H	Histidine
HapMap	Haplotype map
HIV	Human immunodeficiency virus
hr	Hours
HREC	Health Research Ethics Committee (Stellenbosch University)
<i>HTR2A</i>	Hydroxytryptamine (serotonin) 2A receptor gene
HTR2A	Hydroxytryptamine (serotonin) 2A receptors
HWE	Hardy Weinberg equilibrium
I	Insertion (base)
I	Isoleucine (amino acid)
ICD-10	International Classification of Diseases - 10
ID	Identification
i.e.	Id est
indel	Insertion/deletion
IMs	Intermediate metabolizers
Ins	Insertion allele
K	Lysine
kb	Kilobase
L	Litres
LD	Linkage disequilibrium
Ltd	Limited

M	Molar
M	Methionine (amino acid)
MAF	Minor allele frequencies
MDD	Major depressive disorder
mg	Milligrams
MgCl <sub>2</sub>	Magnesium chloride
Min	Minutes
ml	Millilitre
mM	Millimolar
N	Asparagine
N	Number of individuals
n	Number of alleles
NA	Not applicable
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
ng	Nanogram
NGS	Next-generation sequencing
nm	Nanometre
<i>NMDA</i>	N-methyl-D-aspartate (glutamate) receptor gene
NMDAR	N-methyl-D-aspartate (glutamate) receptors
OR	Odds ratio
PANSS	Positive and Negative Syndrome Scale
PANSSN	PANSS negative symptoms
PANSSP	PANSS positive symptoms

PANSSG	PANSS general psychopathological symptoms
PANSST	PANSS total score for all symptoms
PCR	Polymerase chain reaction
PMs	Poor metabolizers
PolyPhen-2	Polymorphism phenotyping
Pty	Propriety limited company
PV	Parvalbumin
R	Arginine
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
rs	RefSNP
S	Serine
SA	South Africa
SAC	South African Coloured
Sec	Seconds
Ser	Serine
SGAs	Second generation antipsychotics
SIFT	Sort intolerant from tolerant
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
<i>SOD2</i>	Superoxide dismutase 2 gene
T	Thymine (base)
T	Threonine (amino acid)
Taq	<i>Thermus aquaticus</i>

TB	Tuberculosis
TBE	Tris borate ethylenediaminetetraacetic acid buffer
TD	Tardive dyskinesia
TRIS	Tris (hydroxymethyl) aminomethane
Tyr	Tyrosine
U	Unit (enzyme quantity)
UK	United Kingdom
UMs	Ultra-rapid metabolizers
USA	United States of America
UTR	Untranslated region
V	Valine
V	Volts
v	Version
VNTR	Variable number tandem repeat
vs.	Versus
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
www	World wide web
x	Times
X	Duplication
Xh	Xhosa

## CHAPTER 1: Introduction

**1 CHAPTER 1: INTRODUCTION**

Psychiatric disorders are a great cause of human suffering and contribute significantly to public health and socio-economic burdens. Numerous surveys and assessments, such as the population health metric system, disability-adjusted life years (DALYs), have pointed out the significant role that mental disorders play worldwide, representing about 7.4% of the global disease burden (Becker and Kleinman, 2013). Particularly, the burden of schizophrenia is of great concern as it is ranked one of the top ten disease-related disability causes worldwide (Świtaj *et al.*, 2012). This severe disorder is further complicated by a heterogeneous aetiology (Purcell *et al.*, 2014). While the prevalence is equal in all populations, the burden associated with schizophrenia is much higher in low income countries as compared to developed countries, where countries in Europe receive ~30% mental health care interventions, but Nigeria receives as low as 2% per year due to limited access of mental health resources (Collins *et al.*, 2011; Thornicroft *et al.*, 2012). This highlights the importance of addressing the burdens associated with mental health in African populations. Furthermore, populations with restricted resources are often faced with poor services for mental health and thus countries that are less economically advanced tend to suffer a larger burden in terms of psychiatric diseases when compared to other pressing illnesses like diabetes, cancer, cardiovascular and chronic respiratory diseases (Marrero *et al.*, 2012; Becker and Kleinman, 2013). For example, in most parts of Africa, schizophrenia and related mental diseases have a treatment gap greater than 90%, referring to the percentage of patients that do not receive mental care (Saxena *et al.*, 2007; Patel and Prince, 2010).

In South Africa (SA), a case study performed by Lund and Flisher (2006), revealed that the mental health services are disintegrated, lacking adequate resources, and therefore most schizophrenia patients' first hospitalization experiences were unpleasant and distraught (Zubi and Connolly, 2013). Psychiatric diseases are largely disregarded, especially in comparison with Human Immunodeficiency Virus (HIV) infection, tuberculosis (TB) and malaria (Warnich *et al.*, 2011; Asmal *et al.*, 2013). Further distress is brought about by relapse due to treatment discontinuation and it has been shown that a 50% relapse rate is observed when those suffering from schizophrenia are exposed to criticism or hostility (Emsley *et al.*, 2014). Adverse effects, as well as a poor foundation of social support also have negative effects on treatment adherence (Kritzinger *et al.*, 2011). Studies that will provide evidence for the biological underpinnings of schizophrenia could therefore help in the understanding of the disease, thereby reducing the associated stigma.

Another vital factor to consider with issues pertaining to mental health in SA is the under-representation of its diverse populations in previous research due to the limited resources (Wright *et al.*, 2011; Burns *et al.*, 2014). According to Statistics SA, the SA population is estimated to comprise

## CHAPTER 1: Introduction

of 80.2% Black Africans, 8.8% South African Coloureds (SACs), 8.4% Caucasians and 2.5% Indian and Asian individuals as of mid-2014 (South African Department of Health, 2014). The highest genetic diversity and largest global admixture is observed for the ancient Khoisan and SAC populations, respectively (Tishkoff *et al.*, 2009; Wright *et al.*, 2011). The SAC's interesting admixed genetic background makes it a good candidate for population-genetic-based studies due to genetic contributions from various parts of the world like Europe, South Asia, Indonesia, Sri Lanka, Madagascar, and Western and Southern parts of Africa (Patterson *et al.*, 2010; Alessandrini *et al.*, 2013). Admixture is also displayed between the Khoisan and ancient Bantu tribes, giving rise to the current genetic profiles of the Xhosa population (Tishkoff *et al.*, 2009). The Xhosa population is SA's second largest, comprising about 17.6% of the total population (South African Department of Health, 2014).

By recognizing the complexity in psychiatric disorders such as schizophrenia, and understanding the inter-individual differences present in the diverse and under-studied SA populations, better strategies can be developed to treat mental disorders more efficiently. It is therefore of great importance to identify and understand underlying genetic causes of schizophrenia and treatment outcomes. Studies that will investigate treatment response and promise to take unique genetic profiles into account to improve treatment outcomes will be quite valuable in addressing the current problems associated with treatment. By assessing genetic diversity in relevant genes for SA populations, insight could be provided in improving treatment eventually.

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**2 CHAPTER 2: LITERATURE REVIEW****2.1 Schizophrenia****2.1.1 Description and Epidemiology**

Schizophrenia is a chronic mental disorder, decreasing life expectancy by 12-15 years (van Os and Kapur, 2009). The age of onset usually occurs during the late adolescent or early adulthood years, which is the crucial stage presumed to play a role in the development of neuropathology in the brain (Gogtay *et al.*, 2011). Schizophrenia has a global life-time prevalence of 0.1-1.7%, depending on recovery variation, death and migration rates of suffering individuals (WHO, 2015). A nation-wide population based epidemiological study, exploring incidences between 1971-2010, has shown that the incidence rate of the early onset of schizophrenia has expanded in recent years and revealed a rate of 3.17 in 100 000 person years from ages 0-18 (Okkels *et al.*, 2013).

**2.1.2 Diagnosis and Symptoms**

Schizophrenia is diagnosed according to a standardized classification system described by the recently updated American-based Diagnostic and Statistical Manual of Mental Disorders version five (DSM-5) (Reddy *et al.*, 2014) or by the World Health Organisation's 10th International Classification of Diseases (ICD-10) (van Os and Kapur, 2009). Psychotic symptoms for schizophrenia patients overlap with various mental disorders and therefore schizophrenia needs to be critically evaluated due to its diverse clinical manifestations and the heterogeneity of the disease (Adam, 2013).

Clinical symptoms of schizophrenia are comprehensively assessed based on scales such as the Positive and Negative Syndrome Scale (PANSS) (Ritsner and Susser, 2009) that comprises of 30 different psychopathological symptoms, divided into three main groups, namely positive symptoms, negative symptoms and general pathological symptoms (Emsley *et al.*, 2003; van Os and Kapur, 2009). Positive symptoms refer to general psychotic behaviour that include delusions, hallucinations, hyperactivity and thought disorders, while negative symptoms arise due to deficits in normal social behaviour, such as lack of linguistic skills (alogia), ambition (avolition), pleasure (anhedonia), motivation (abulia), as well as affective flattening, attention deficits and asociality (Xu *et al.*, 2013; Malhotra, 2014). The remaining symptoms fall under the general psychopathology scale for example anxiety, depression, disorientation, etc. and each patient is evaluated through verbal consultation based on the PANSS by which the severity is ranked on a scale from 1 – 7, in order of increasing severity (Yamamoto *et al.*, 2010).

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## 2.1.3 Aetiology of Schizophrenia

## 2.1.3.1 Environmental Risk Factors

Understanding the aetiology of schizophrenia remains challenging due to the complex network of interrelated factors playing a role in its pathophysiology that are attributable to both genetic and non-genetic components (Duan, 2003). Although it is important to investigate genetic factors in psychiatric diseases, non-genetic factors are also fundamental to the development of psychiatric disorders.

The stress-diathesis model has proposed environmental triggers to be responsible for the susceptibility of schizophrenia. This model suggests that factors like substance abuse (e.g. cannabis use), social adversities, traumatic events, infection, paternal age and pregnancy or birth complications all play a role in the early onset and risk of schizophrenia (Hadlich *et al.*, 2010; Piper *et al.*, 2012). Although these environmental factors are important, Figure 1 highlights that none of these contribute as much to the development of schizophrenia as family history, and thus genetics (Sullivan, 2005). However, more recent studies have proposed the neurodevelopmental hypothesis to be involved in schizophrenia pathogenesis, which reveals that environmental triggers could be activated upon genetic predispositions, and epigenetic factors could contribute to defective pre- and postnatal brain development (Schmitt *et al.*, 2014).

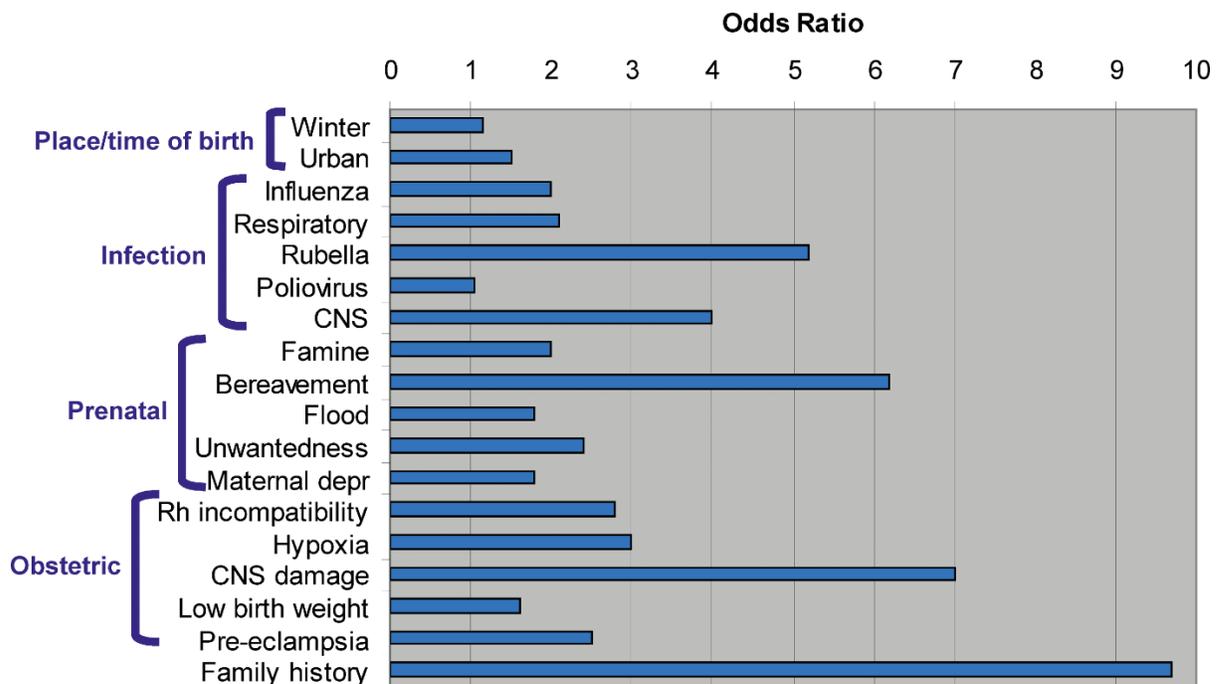
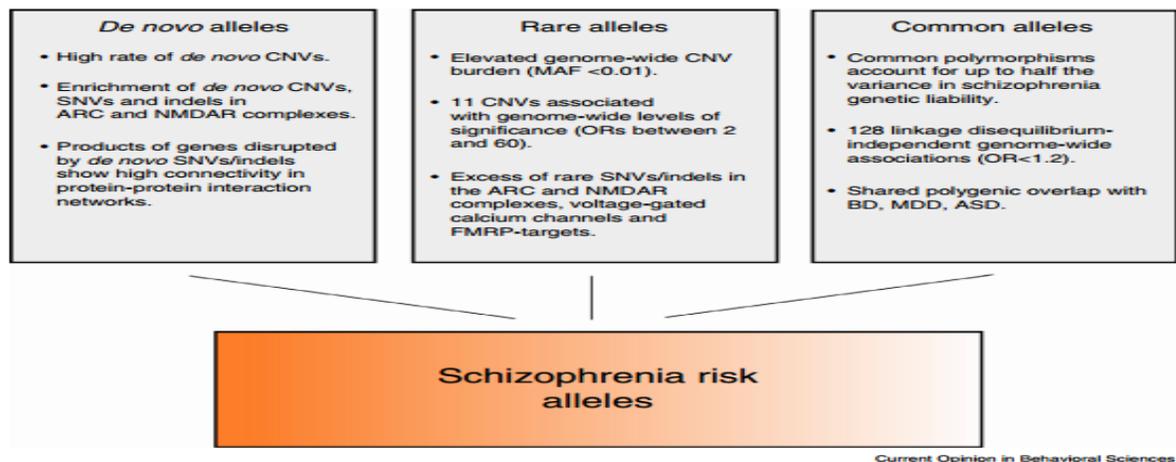


Figure 1. Environmental risk factors involved in the aetiology of schizophrenia (Sullivan, 2005). CNS: central nervous system; depr: depression; Rh: Rhesus. No permission for reuse required from the Public Library of Science

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**2.1.3.2 Genetics of Schizophrenia**

Many studies reveal that genetic factors contribute towards the susceptibility for schizophrenia in patients (Vijayan *et al.*, 2007). Evidence for this is supported by adoption and twin studies which have shown an estimated heritability as high as 80% (Vijayan *et al.*, 2007; van Os and Kapur, 2009). Different models for the genetics of schizophrenia have been proposed, which include the common disease-common variant, and common disease-rare variant models, by which the former describes the collection of a large number of common single nucleotide polymorphisms (SNPs) obtained from genome-wide association studies (GWAS) to contribute to schizophrenia susceptibility, and the latter proposes schizophrenia susceptibility to be caused by rare, highly penetrant copy number variants (CNVs) evident from their higher frequency associated with disease in genome-wide CNV studies (Escudero and Johnstone, 2014). Recent studies have also revealed that the pathogenesis of schizophrenia could be linked to risk alleles like *de novo* mutations which were observed to be associated with higher schizophrenia risk due to late paternal age of conception (Kong *et al.*, 2012; Veltman and Brunner, 2012; McGrath *et al.*, 2014), thus providing evidence for the role of environmental interaction with genetics in the pathogenesis of this disorder. Although schizophrenia has manifested itself as a polygenic, multifactorial disorder, various risk alleles are shared between schizophrenia and other psychiatric diseases like bipolar disorder, major depressive disorder and autism, and these risk alleles are presented in various categories as reported in a recent review (See Figure 2) (Rees *et al.*, 2015). Identification of these risk alleles have been facilitated by the advancement of sequencing technologies that allow the examination of both common and rare variants across multiple genes, which could exhibit significant effects on phenotypes of the disease (Purcell *et al.*, 2014).



**Figure 2: Risk Alleles associated with schizophrenia (Rees *et al.*, 2015). MAF: minor allele frequency; OR: odds ratio; CNV: copy number variation; SNV: single nucleotide variant; indel: insertion/deletion; ARC: activity-regulated cytoskeleton-associated protein; NMDAR: N-methyl-D-aspartate receptor; FMRP: fragile X mental retardation protein; BD: bipolar disorder; MDD: major depressive disorder; ASD: autism spectrum disorder. Reprinted with permission from Elsevier**

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**2.1.4 Neurochemical Influences on Schizophrenia**

Although many factors are speculated to be involved in the pathogenesis of schizophrenia, the most well-documented are the defective neuro-circuitries involving various modulatory neurotransmitters. Chemical imbalances in neurotransmitter systems in the prefrontal cortex of the brain have been hypothesized to be responsible for the pathophysiology of schizophrenia (Orhan *et al.*, 2014). The dopamine hypothesis describes the dysregulation of dopamine neurotransmission resulting in the psychosis of schizophrenia. It is for this reason that antipsychotic drugs are designed to target this system to relieve psychotic symptoms by dopamine receptor antagonism (Cookson, 2013). Currently, this hypothesis is still valid as imbalances in dopamine systems have shown to alter cognitive functioning, where positive symptoms are correlated with dopamine hyperactivity and negative symptoms are displayed by dopamine hypo-activity in the brain (Guillin *et al.*, 2007).

The serotonin hypothesis has also been linked to schizophrenia by the hyperactivity of serotonin, leading to psychotic symptoms and also affecting dopamine regulation as well as other neurotransmitter systems (Eggers, 2013). Animal studies revealed that disruptive serotonin receptors result in psychosis and depression (Richardson-Jones *et al.*, 2011) and dysregulation of serotonin systems in patients is linked to the negative symptomology of schizophrenia (Strauss *et al.*, 2013). Dopamine neurons play a role in the mechanism of drug treatment by releasing serotonin in the midbrain, preventing excessive serotonergic activity in certain brain regions. The interactions between dopamine and serotonin are important and homeostasis is required to prevent psychoses (Seutin, 2005). Therefore, dopamine and serotonergic systems seem to be intertwined and disruptions of the one system might affect the other, which may affect the other neurotransmitter circuitries that contribute to the complexity of schizophrenia pathophysiology (Vyas *et al.*, 2013).

A number of studies have also revealed that oxidative stress, causing cell toxicity and damage as a result of the build-up of free radicals as by-products, may be implicated in the aetiology of schizophrenia (Ng *et al.*, 2008; Bitanihirwe and Woo, 2011; Yao and Reddy, 2011). The brain is vulnerable to oxidative stress due to its high oxygen usage in various metabolic processes like lipid metabolism, neurotransmitter pathways and various enzymatic reactions (Bošković *et al.*, 2011). Superoxide dismutase 2 (SOD2) is an important antioxidant responsible for preventing the damage caused by these free radicals, called reactive oxygen species (ROS). ROS occurs naturally as a by-product of biochemical reactions, causing cell and neural toxicity due to oxidative stress. As a result of the role that this protein plays, aberrant SOD2 enzymes can greatly influence neurodevelopment and therefore it is important to elucidate SOD2 activity for better management of psychotic symptoms (Chowdhury *et al.*, 2011). The importance of oxidative stress is illustrated in Figure 3, which highlights the involvement of many factors in schizophrenia pathogenesis. These aspects

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include both environmental and genetic influences, involving various biological processes like inflammation, neuronal damage, epigenetic factors, mitochondrial dysfunction, N-methyl-D-aspartate (NMDA) or glutamate receptor hypo-activity, etc. (Bitanhirwe and Woo, 2011). These molecules, involving aberrant neurotransmission systems, are all linked to the mechanism of action of antipsychotic drugs. Comprehensive findings of neurotransmission networks will therefore improve our understanding of schizophrenia treatment and enable us to develop effective treatment schemes.

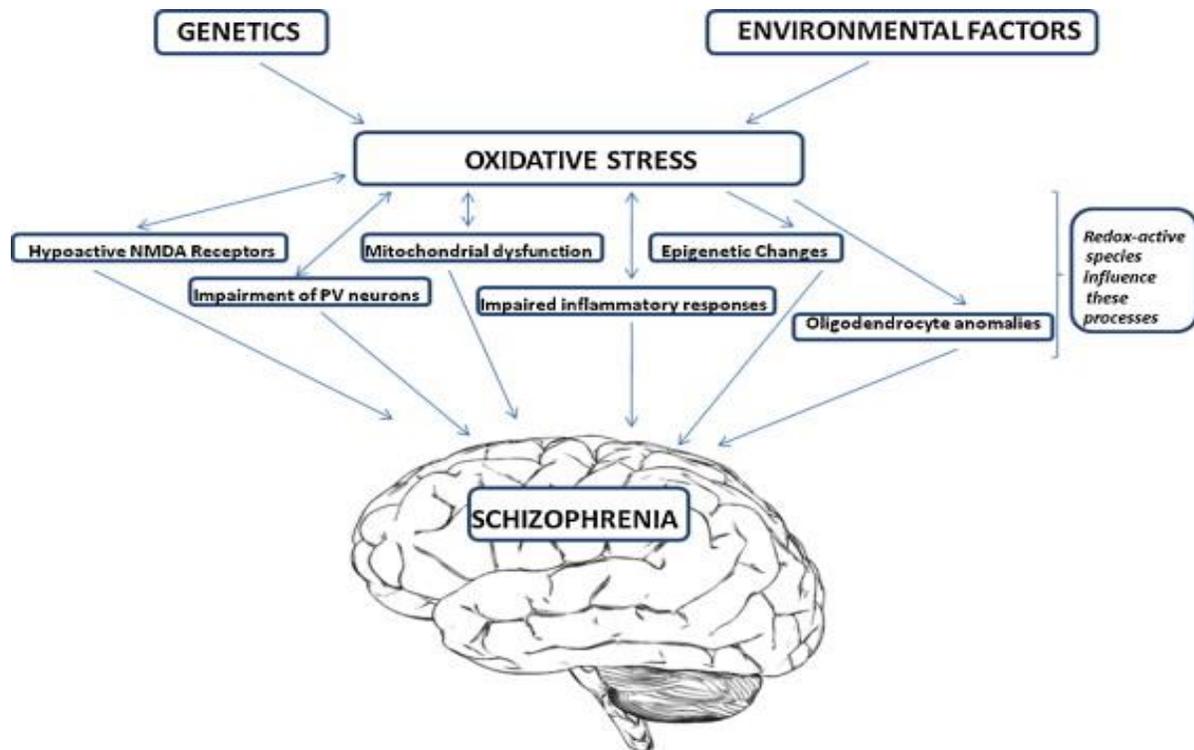


Figure 3: Interrelated factors involved in the oxidative stress in schizophrenia aetiology (Bitanhirwe and Woo, 2011). NMDA: N-methyl-D-aspartate; PV: parvalbumin. Reprinted with permission from Elsevier

### 2.1.5 Treatment

Antipsychotic medication is currently used to treat psychosis in schizophrenia and has been the most effective method of treatment for the disorder in the past fifty years (Peluso *et al.*, 2012). Antipsychotic medications have significantly improved the treatment of schizophrenia and have shown to be more effective than placebo drugs by decreasing relapse rates and readmissions of patients, as well as improving the quality of patients' lives (Leucht *et al.*, 2012). These drugs fall into two categories, the typical or first generation antipsychotics (FGAs) and the more recent, atypical or second generation antipsychotics (SGAs), which display similar efficacies and are therefore both still currently used to treat symptoms of schizophrenia (Hartling *et al.*, 2012). Neurotransmission systems are the main sites of antipsychotic drug therapy as these drugs are designed to act as antagonists by targeting and blocking receptors from binding to neurotransmitters that regulate brain functionality

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(Lohoff and Ferraro, 2010). FGAs generally target dopamine receptors as they have a high affinity for these receptors, ultimately alleviating positive symptoms (Lafuente *et al.*, 2007). Unfortunately, these drugs are not effective in treating other dimensions of schizophrenia symptoms and often cause critical involuntary movements like tardive dyskinesia (TD) and other extra-pyramidal side effects (EPS), and are also associated with a poor quality of life and increased burdens of morbidity and mortality (Correll *et al.*, 2004; Lieberman *et al.*, 2005). FGAs include drugs like chlorpromazine, haloperidol, perphenazine and flupenthixol, which have been used for the past five decades and demonstrate effective treatment of schizophrenia (Shen *et al.*, 2012). SGAs like clozapine, risperidone, olanzapine, ziprasidone, and aripiprazole show less undesirable EPS, but cause acute weight gain and other unwanted side effects that include changes in lipid and glucose metabolism, which in turn can lead to the development of cardiovascular diseases and diabetes mellitus (Kusumi *et al.*, 2011). These atypical drugs not only target dopamine receptors, but also have the ability to target serotonin receptors, distinguishing them from FGAs, and are generally the recommended drugs prescribed to patients as it produces minimal EPS (Meltzer, 2013). However, SGAs are relatively expensive in comparison to FGAs. For this reason typical drugs are often prescribed in circumstances where medical resources are limited (Bukhsh *et al.*, 2014) and are thus used in developing world settings like SA. Consequently, much attention is needed to try and comprehend treatment efficacy in the long-term as both FGAs and SGAs are accompanied by distressing side effects and treatment failures (Lieberman *et al.*, 2005; Lencz and Malhotra, 2009). However, optimizing low cost drugs would be beneficial to resource limited settings in the developing world.

### **2.2 Antipsychotic Pharmacogenetics**

#### **2.2.1 Pharmacogenetics of Schizophrenia**

Pharmacogenetics is a term ascribed to the inherited genetic variation within individuals or across populations, causing changes in the regulation of gene networks, which influences drug pathways and causes individuals to respond differently to therapeutic treatments (Roden *et al.*, 2011). Therefore it is important to characterize the genetic differences observed between individuals and populations. Recent research efforts have greatly contributed to the growing field of pharmacogenetics and the establishment of the International HapMap consortium and 1000 Genomes Project has played an important role in indexing most of the common genetic variation present in the human genome (Deloukas and Bentley, 2004). These projects characterize human variation in global populations, allowing researchers to use these resources to facilitate the use of genetic approaches that could identify networks of polymorphisms from different genes involved in treatment outcomes of patients suffering from various disorders.

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The role of genetics in antipsychotic treatment has been validated by a number of sibling and twin studies, which have demonstrated that improved clozapine and olanzapine outcomes are in strong concordance with monozygotic twins (Reynolds, 2012). As antipsychotic medications do not always exhibit optimal efficacy, pharmacogenetic studies are likely to play an important role in improving these treatments. Not only do antipsychotics fail to reduce schizophrenia symptoms in about 50% of patients (Miyamoto *et al.*, 2005; Lohoff and Ferraro, 2010), but they also cause undesirable side effects or adverse drug reactions (ADRs). FGAs have a narrow therapeutic range since 60% of dopamine receptor blockage is needed for a therapeutic response to occur, but a greater than 80% blockage can lead to the development of ADRs (Lohoff and Ferraro, 2010). It is important to therefore understand the pharmacogenetics of antipsychotic drugs in order to try and improve treatment outcomes by preventing drug toxicity and inefficacy. Therefore, pharmacogenetic studies are urged to identify genetic variants that can help to optimize these important treatments that are plagued by non-optimal outcomes.

Studies that incorporate pharmacogenetics may identify potential genetic predictors of the drug response (Malhotra *et al.*, 2004). The utility of this approach is highlighted by studies such as those performed by Arranz *et al.* (2000), which reported a significant association of six variants across multiple genes with clozapine response, showing a predictive value as high as 77% (Kohlrausch, 2013). Numerous association studies are currently being performed to determine pharmacogenetic traits by comparing responders to non-responders or patients experiencing ADRs, in clinical trials of drugs and other studies (Weiss *et al.*, 2001). However, these approaches are hampered by a number of heterogeneous factors that can influence the statistical outcome of the study, including the multifactorial nature of each individual's response to medication.

Multifactorial components influencing phenotypic response can be difficult to measure and is a huge drawback in understanding the treatment of schizophrenia. Important parameters that should be considered include: i) the type of antipsychotic medication given to each patient, ii) response characterization, iii) the duration of treatment, iv) the population sample size, and v) the ethnic background of the patient (Cichon *et al.*, 2000), as well as inter-individual differences affected by age, gender and lifestyle. Another important parameter is the careful clinical characterization of patients that will improve the ability to detect variants in association with treatment outcome. For instance, the course of illness is quite crucial in detecting a statistical outcome as it has been shown that patients experiencing their first episode of psychosis have a much higher response rate compared to chronically ill patients (Robinson *et al.*, 1999; Chiliza *et al.*, 2015). The importance of considering mental illnesses at early phases can therefore be quite useful to pharmacogenetic research as it avoids issues like concomitant treatment and adherence.

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Treatment adherence is another issue which affects pharmacogenetics studies, as patients suffering from mental diseases are inclined to experience non-adherence with adherence rates for schizophrenia patients reported to be between 20-72% (Julius *et al.*, 2009). This can cause symptomatic relapse in a study focusing on adverse reactions, but the effect of this power has not been enumerated yet (Malhotra *et al.*, 2012). Therefore, improving the adherence rates of medication can allow for small sample sizes to be sufficient, increasing the statistical power. The adherence rates of cohorts can be more accurately measured if the patients receive their medication via injection, thus excluding the inconsistencies in treatment adherence. The use of first episode patients in a pharmacogenetic study is therefore quite beneficial as it addresses many of the issues associated with clinical heterogeneity. Table 1 briefly summarizes some of the challenges that need to be evaluated for antipsychotic treatment response in patients.

**Table 1: Evaluation of challenges concerned with treatment outcome in pharmacogenetics research of schizophrenia patients (Arranz *et al.*, 2011). Reprinted with permission from Elsevier**

Characteristic	Considerations
<b>Patient characteristics</b>	
Diagnosis	Differential diagnosis during an initial episode of psychosis may not be clear. Schizophrenia studies may be confounded by inclusion of diverse disorders (e.g. affective or drug-induced psychoses)
Illness course	Treatment response will be different for patients with treatment-resistant schizophrenia or a first-episode psychosis
Co-morbidities	Drug abuse and depression are common and influence treatment outcome
<b>Treatment episode characteristics</b>	
Antipsychotic drug prescribed	Among different antipsychotics, varying receptor affinities and profiles may result in different gene variants influencing treatment outcome
Treatment adherence	Non-adherence to antipsychotic medication is common (usual)
Concomitant medication	Concomitant mood stabilizers or antidepressants are common. Dose-tailing antipsychotics at switching may also complicate response assessments
Treatment duration	Most improvement in positive symptoms occurs in the initial weeks; however, progressive improvement may occur beyond a year of treatment (e.g. with clozapine)
<b>Outcome phenotype characteristics</b>	
Assessment of efficacy	Antipsychotic efficacy is a complex outcome phenotype. Whilst most studies use standardized outcome measures such as the Positive and Negative Syndrome Scale (PANSS), the association of genetic variants with a multidimensional, composite outcome scale is challenging. 'Real life outcomes' (e.g. level of functioning) may have more relevance for pharmacogenetic tests used in clinical care
Assessment of adverse effects	Some adverse effects may be very robustly measured (e.g. weight gain or lipid levels), while others are more subjective (e.g. tardive dyskinesia)
Confounding factors	Confounding factors may influence outcome (e.g. risk of tardive dyskinesia is increased in female patients, in individuals of African Caribbean origin and with age over 50 years)
Baseline state	Baseline state influences the degree of symptom improvement and adverse effects (e.g. patients with low body mass index have the highest risk of antipsychotic-induced weight gain)

All factors mentioned previously play a huge role in the understanding of the antipsychotic treatment response mechanism. Hence, it is important to develop a study design that will be useful in a clinical setting, allowing patients to be treated more effectively by tailoring their medication according to

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their genetic profiles. A number of candidate genes have been proposed to influence pharmacogenetic traits with regards to antipsychotic exposure. These genetic interactions occur on two dimensions of antipsychotic drug processes, namely pharmacodynamics and pharmacokinetics. Pharmacodynamic processes directly affect neurotransmission networks by targeting receptor sites, altering levels of these neurotransmitters in the brain. This phenomenon explains how drugs interact with these receptors and with other drugs that can create agonistic or antagonistic effects. Pharmacokinetics alternatively focuses on the metabolism of the drug itself and its metabolites and all pathways involved in its mechanism of action (Spina and de Leon, 2007).

### **2.2.2 Pharmacodynamics of Antipsychotics**

Dopamine and serotonin neurotransmitter receptors are the prime targets for antipsychotic drugs, as imbalances in the levels of their relative substrates have been shown to be causal elements of schizophrenia and other psychotic symptoms in psychiatric diseases (Zhang *et al.*, 2010). Various enzymes involved in these neurobiological pathways play an important role in the regulation of these neurotransmitters. Present-day antipsychotic medication operates on a broader scope by targeting other neurotransmitters hypothesized to be associated with negative symptoms of schizophrenia and include epinephrine/norepinephrine, histamine and other acetylcholines (Arranz and de Leon, 2007). However, the roles of dopamine and serotonin in the mechanism of antipsychotic drugs are accentuated since both dopamine (D<sub>2</sub>) and serotonin (HTR2A) receptors have been shown to regulate prefrontal activity in the cortex and antipsychotic treatment response in schizophrenia patients, credited to the genetic variation present in the genes coding for these receptors (Blasi *et al.*, 2015).

#### **2.2.2.1 Dopamine**

Dopamine dysregulation has been implicated in the pathophysiology of schizophrenia and its link to symptom severity is explained by the dopamine hypothesis, stating that psychosis occurs due to dopamine imbalances in the brain (Howes and Kapur, 2009). The first drugs developed were therefore aimed at targeting dopaminergic receptors, hence FGAs' favourable affinity for dopamine receptors (Miyamoto *et al.*, 2005; Arranz and de Leon, 2007). Dopamine receptors are classed into five subtypes, D<sub>1</sub>-D<sub>5</sub>, but most pharmacogenetic studies have focused on receptor types D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub>, mainly because haloperidol, a FGA, shows a stronger affinity for these receptors (Miyamoto *et al.*, 2005; Arranz and de Leon, 2007; Zhang and Malhotra, 2011).

The dopamine receptor D<sub>2</sub> gene (*DRD2*) is involved in controlling motor activity, neuro-endocrine functioning and cognition (Duan, 2003). Several genetic variants that alter regulation and expression levels of *DRD2* have been described. The deletion allele of rs1799732, located in the 5' untranslated region of the gene, has been associated with decreased promoter activity, resulting in a decline in

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expression levels of the downstream coding regions (Arinami *et al.*, 1997; Lafuente *et al.*, 2007; Mi *et al.*, 2011). Patients carrying this allele display poorer response to treatment compared to patients with the Ins/Ins (CC) genotype (Zhang *et al.*, 2010; Zhang and Malhotra, 2011). The presence of the deleterious allele of this SNP was also reported to be associated with escalating positive symptoms in schizophrenia patients (Sáiz *et al.*, 2010). The rs1800497 and rs1079597 SNPs in *DRD2* are also commonly studied polymorphisms that have been reported to result in reduced receptor density in the striatum, indicating increased levels of dopamine in this region, and patients carrying these variants are more likely to respond better to treatment (Vijayan *et al.*, 2007). Patients homozygous for the C or A2 allele of rs1800497 display a significant decrease in PANSS scores after treatment (Mi *et al.*, 2011) whereas for rs1079597, the homozygous G (B1) genotype have shown to be associated with severe hallucinations (Vijayan *et al.*, 2007; Zhang and Malhotra, 2011).

D<sub>3</sub> receptors, encoded by the *DRD3* gene, are located in the limbic regions of the brain (Schwartz *et al.*, 2000), thus controlling impulse and basic emotions and are preferred targets for antipsychotic drugs compared to D<sub>2</sub> receptors (Graff-Guerrero *et al.*, 2009). A non-synonymous variant, rs6280 (Ser9Gly), occurring within this gene, has been investigated in a meta-analysis study and reported the Ser allele to be associated with non-responsiveness to the atypical antipsychotic drug, clozapine (Jönsson *et al.*, 2003). Similarly, further investigation from a larger meta-analysis performed by Hwang *et al.* (2010) revealed a negative trend for the Ser allele to poor response of clozapine treatment. This study showed that this SNP could be a potential candidate involved in treatment response, but highlighted that improved study designs need to be implemented to eliminate inconsistent findings.

Catechol-*O*-methyltransferase (COMT) is a catabolic enzyme responsible for the degradation and removal of dopamine in the prefrontal cortex (Chen *et al.*, 2004). Variation in the *COMT* gene have been implicated in dopamine receptor density, thereby regulating dopaminergic systems (Williams *et al.*, 2007). *COMT* variation have been associated with improved cognition with clozapine treatment and negative symptoms correlated to prefrontal dopaminergic activity (Bosia *et al.*, 2014). A study performed by Wright *et al.* (2012) highlights the important role of the *COMT* P2 promoter region within a SA Xhosa population and negative symptom severity, indicating unique haplotype associations for African populations as they display the highest level of genetic diversity. Genes involved in dopamine neurotransmission are therefore good candidates for investigating antipsychotic treatment outcomes due to the roles of the antipsychotic medication in these networks.

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### 2.2.2.2 Serotonin

Serotonin 2A receptors (HTR2A) are important in maintaining the levels of serotonin and are the principal targets for SGAs, thereby lowering the likelihood of developing ADRs since HTR2A are the favoured targets over dopamine receptors (Casey, 2004). These serotonin receptors are commonly dispersed throughout the cortex of the brain and are more effective in alleviating cognitive defects on which drugs like clozapine, risperidone and olanzapine bind with a high affinity (Mamo *et al.*, 2004; Meltzer, 2013).

A well-studied synonymous variant, rs6313 (T102C) in *HTR2A*, which is in complete linkage disequilibrium (LD) with a functional variant, rs6311 (A-1438G) in the promoter region in Europeans, has shown to be associated with response to clozapine and risperidone with contradictory results. Reduced promoter activity has been observed for the G allele of the promoter polymorphism, indicating less 2A receptors present in the brain, thus altering serotonergic-dopaminergic pathways through antipsychotic mediation of serotonin/dopamine binding affinities (Arranz and de Leon, 2007; Zhang and Malhotra, 2011). However, many studies could not replicate significant findings for rs6313, for both FGA and SGA response, suggesting it is a weak candidate (Masellis *et al.*, 1998; Malhotra, 2014). Various polymorphisms in serotonin genes have been shown to significantly predict treatment response, but findings could not be replicated in later studies (Arranz *et al.*, 2000; Zhang and Malhotra, 2013).

### 2.2.2.3 Other Neurotransmitters

Cognitive abilities are largely affected by several neurotransmitters that include glutamatergic, adrenergic, histamine and muscarinic receptors, to which atypical antipsychotics have a great binding affinity (Chiu *et al.*, 2003; Bishop *et al.*, 2015). Even though adrenergic and histamine receptors are good targets for SGAs, no genetic evidence is available to explain the association to treatment response. Likewise with muscarinic receptors, insignificant associations to clozapine efficacy were detected. However, more research has to be carried out to fully understand the link between these neurotransmitters and antipsychotic efficacy (Arranz and de Leon, 2007).

## 2.2.3 Pharmacokinetics of Antipsychotics

The cytochrome P450 (CYP) system is the chief pharmacokinetic site of most antipsychotic drugs (Nelson *et al.*, 1996; Spina *et al.*, 2003). Two distinct metabolic reactions occur with regards to antipsychotic medication, namely Phase I and Phase II reactions that are involved in the oxidation of antipsychotic metabolism and the inactivation of its metabolites, respectively, where CYP enzymes are primarily involved in Phase I reactions (Roth and Alici, 2014). CYP enzymes are highly polymorphic and have built up several mutations that give rise to variant alleles, resulting in

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differential gene expression and activity (Spina and de Leon, 2007). These enzymes in conjunction with external factors (e.g. smoking, diet, alcohol and substance abuse), influence antipsychotic pharmacokinetics (Arranz *et al.*, 2011). Genetic variations occurring within genes encoding these enzymes result in patients exhibiting disparate phenotypes, such as poor metabolizers (PMs) who lack functional enzymes due to defective or deleted genes, intermediate metabolizers (IMs) that carry one functional and one defective allele, extensive metabolizers (EMs) that carry two functional copies of the gene, and ultra-rapid metabolizers (UMs) that carry more than two active copies of the gene or exhibit increased expression alleles (Spina and de Leon, 2014).

### 2.2.3.1 *CYP1A2*

The *CYP1A2* enzyme plays a significant role in the metabolism of many FGAs and SGAs, specifically clozapine and olanzapine (Tiwari *et al.*, 2005). This enzyme is impeded by caffeine, causing elevated plasma levels of antipsychotic metabolites and can also decrease antipsychotic potency when taken with concomitant drugs that can in turn lead to the development of ADRs (Spina and de Leon, 2007; Arranz *et al.*, 2011). The activity of *CYP1A2* is highly influenced by smoking, showing increased metabolism with *CYP1A2\*1C* and *CYP1A2\*1D* genetic variants (Pavanello *et al.*, 2005), and these patients require dosage adjustments for the antipsychotics they are treated with. A recent study reported that the *CYP1A2\*1F/\*1F* genotype was associated with reduced olanzapine serum concentration in the absence of inducers, indicating a poor treatment outcome (Laika *et al.*, 2010), and included associations with TD development (Fu *et al.*, 2006). However, some studies revealed no association with *CYP1A2* variants and clinical efficacy of olanzapine (Ravyn *et al.*, 2013). Differences in *CYP1A2* frequency variation highlight the importance of investigating these variants in different populations to determine the influence on antipsychotic outcomes (Szalai *et al.*, 2014).

### 2.2.3.2 *CYP2D6*

The *CYP2D6* gene is highly polymorphic, resulting in various phenotypic expressions that occur as a result of diverse variations such as duplications and deletions. Patients with reduced and normal *CYP2D6* activity are classified as PMs and EMs respectively, and when multiple copies of the gene are present they are classed as UMs. Interestingly, not all patients with additional copies of *CYP2D6* are described as UMs because not all copies of the duplicated gene are functional (Ravyn *et al.*, 2013). About 7-10% of Caucasians and 1-2% of Asians are classified as PMs, and require minimal antipsychotic doses for therapeutic responses to occur. PMs may also display ADRs due to high plasma levels of antipsychotic drugs (Arranz and de Leon, 2007; Gaedigk and Coetsee, 2008). Figure 4 briefly describes the genetic mechanisms associated with *CYP2D6* metabolic status and its resulting therapeutic outcome. Ravyn *et al.* (2013) have also reported a number of studies to show a

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significant association of *CYP2D6* PM status with increased plasma concentrations of the typical drug, haloperidol, and its slow clearance as compared to EMs, concluding that the dose be reduced to 50% in these cases. They report similar findings for most FGAs but inconclusive findings when compared to SGAs, where no difference in plasma levels between the various metabolizer phenotypes were observed. Recently, studies have linked *CYP2D6* PM phenotype with an increased risk of Parkinson’s disease (Persson and Ingelman-Sundberg, 2014). However, inferring phenotypic information from *CYP2D6* genotypes is difficult due to the vast number of variants within this gene, by which many have not been characterized, and no set method is available for genotype-phenotype prediction, thus leading to the discrepant results reported. Wright *et al.* (2010) devised a useful genotyping strategy for the SA Xhosa population and demonstrated the unique genetic profiles present within this population, including novel variants detected. The importance of this gene in pharmacogenetic research is quite valuable because of its polymorphic nature, producing inter-individual differences amongst patients in drug response dosages, ultimately affecting drug efficacy (Xu *et al.*, 2013).

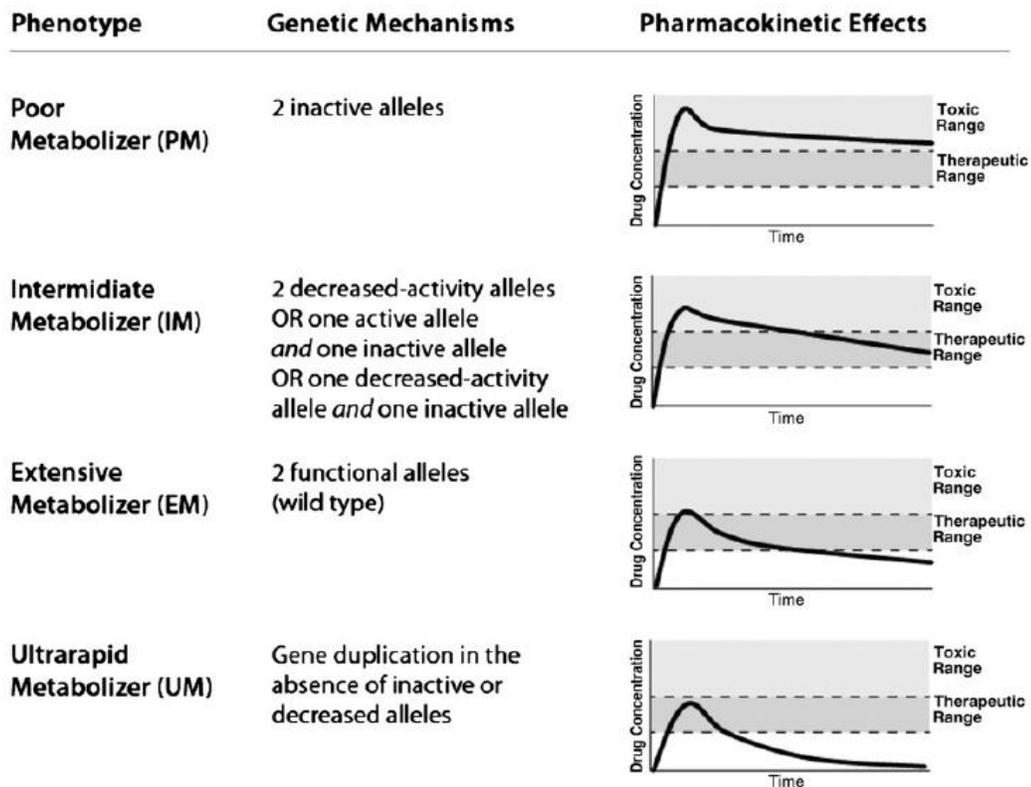


Figure 4. Genetic mechanisms for CYP450 metabolic phenotypes and pharmacokinetic implications (Ravyn *et al.*, 2013). Reprinted with permission from Elsevier

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**2.2.4 Antipsychotic Adverse Drug Reactions**

Although inter-individual differences exist between antipsychotic treatment response profiles in patients, it is important to also consider the differences that occur with the various side effects. ADRs are a huge burden in psychiatric diseases, as they cause severe reactions that in many cases are permanent, leading to high rates of treatment failures and increase the socio-economic burdens placed on patients. The evolution of SGAs has reduced the burden of threatening EPS caused by FGAs, however they are still accompanied with ADRs (Arranz *et al.*, 2011).

TD, a critical involuntary and permanent movement disorder, is amongst one of the most concerning ADRs caused by antipsychotic drugs and about 25% of schizophrenia patients on long-term treatment with typical antipsychotics develop TD. The genetics underlying its mechanism is of great value in pharmacogenetics research as this prior genetic knowledge can assist in treating patients more effectively (Zai *et al.*, 2010). Oxidative stress resulting in neuro-degeneration is an important aspect speculated to be responsible for the pathology of TD. The *SOD2* gene was investigated in a meta-analysis study by (Bakker *et al.*, 2008) and a significant association with TD was discovered, including an association between the rs4880 SNP and TD severity in a study by Kang *et al.* (2008). In a different study, this SNP had relevance to movement disorders because patients that were carriers of the Ala variant developed TD (Chowdhury *et al.*, 2011).

Other ADRs commonly reported in pharmacogenetic studies include weight gain and metabolic syndrome, which have been shown to be associated with most modern antipsychotics, like clozapine and olanzapine, indicating a risk twice as high in schizophrenia patients, and thus causing many health issues that include type 2 diabetes mellitus, coronary heart disease and hypertension (Panariello *et al.*, 2010). Agranulocytosis is a very rare ADR, but quite detrimental and strongly associated with the use of clozapine (Arranz *et al.*, 2011).

Although a considerable number of studies have been performed to understand the antipsychotic pharmacogenetics of schizophrenia, many discrepancies still remain due to the complex mechanism of the disease and its treatment response profiles. Many findings have not been replicated, probably caused by factors like study design, sample size and ethnicity that differed between studies. However, further replicative studies are warranted to elucidate the complexity of treating this disorder. Since antipsychotic response phenotype shows inter-individual variability and prediction of these responses is difficult, pharmacogenetics could play an important role in identifying genetic factors, which could positively influence treatment outcome in psychiatric disorders (Zhang *et al.*, 2010; Zhang and Malhotra, 2011).

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**2.3 Gene Selection and Cohort Used for This Study**

The candidate pharmacogenes selected in this study have been shown to influence treatment outcome based on genetic variants present within these genes. The pathways involved in these genes have been implicated in neurotransmission and drug mechanism in a number of studies. These genes encode important receptors and enzymes involved in dopamine and serotonin pathways, as well as CYP enzymes like CYP1A2 and CYP2D6, primarily involved in antipsychotic metabolism. SOD2 also plays a pivotal role in counteracting the toxicity caused by ROS, preventing neural damage caused by oxidative stress. The following candidate genes were therefore selected for this study due to their implication with antipsychotic pharmacogenetics of schizophrenia: *COMT*, *CYP1A2*, *CYP2D6*, *DRD2*, *DRD3*, *HTR2A* and *SOD2*. Although this study focused on a number of important pharmacogenes for antipsychotics, it is important to note that the study did not include a comprehensive list of all pharmacogenes that are important for antipsychotics.

This study utilized a well-defined first episode schizophrenia (FES) cohort that has been under-represented in pharmacogenetic research. They comprise of SA Coloured (SAC), Caucasian and Xhosa individuals, treated on their first psychotic account without or with limited exposure to previous intervening medication. The value of this cohort is emphasized by the elimination of confounding factors such as inconsistencies in diagnosis and antipsychotic treatment, which could interfere with the phenotypic assessment (Chiliza *et al.*, 2014). This ensures an unbiased study that will enable us to better understand the complexity of genetic interaction and treatment outcome, further relieving disease burden presented on a multifaceted scale. The SAC, Caucasian and Xhosa populations also hold distinctive and unique genetic variation profiles, which could have important implementation in a clinical-diagnostic setting. Furthermore, this study builds on previous work performed in our research group to investigate the effects of genetic variation presented in the under-represented SA populations on treatment outcomes. To our knowledge, this is the first study to investigate these genes in a FES SA cohort.

**2.3.1 Research Problem and Rationale**

Optimizing antipsychotic treatment of schizophrenia can be challenging due to the complexity of the disorder and treatment outcome. Research efforts are constantly investigating ways to enhance drug efficacy and decrease toxicity and relapse rates, which remains a huge burden. The criteria for the selection of genes were based on the most frequently occurring variants found in literature, which were most consistently reported to be involved in the pharmacogenetics of schizophrenia. By investigating these genes, the relationship between genetic variants and treatment outcome can be established, leading to an improved understanding of treatment outcomes and ultimately facilitating

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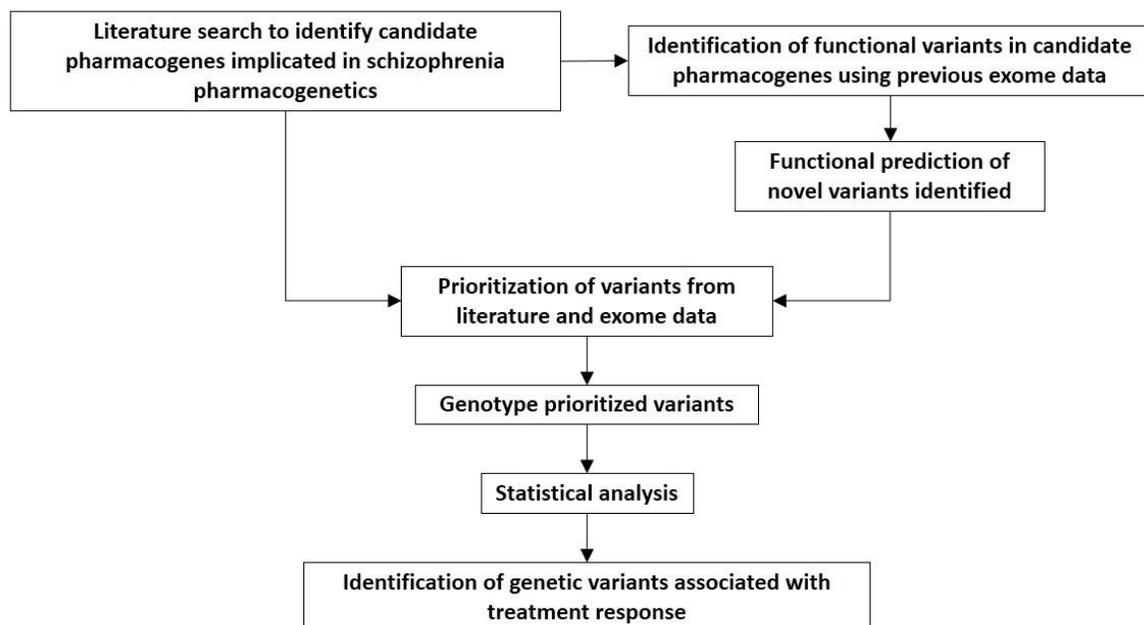
treatment modifications to decrease toxic properties and improve the efficacy of treatment. This will aid in allowing for tailored treatment to be acquired in a clinical setting.

**2.3.1.1 Aim**

This study aims to determine if polymorphisms in candidate pharmacogenes are associated with antipsychotic treatment outcomes in a South African first episode schizophrenia cohort.

**2.3.1.2 Objectives**

- Identify candidate pharmacogenes implicated in pharmacogenetics of schizophrenia from the literature.
- Perform bioinformatic analyses to identify and characterize the genetic variants in the prioritized candidate pharmacogenes using previously generated exome sequencing data.
- Prioritize variants based on the literature and *in silico* analyses of exome data.
- Design genotyping assays and genotype prioritized polymorphisms in *COMT*, *CYP1A2*, *CYP2D6*, *DRD2*, *DRD3* and *SOD2* in a South African first episode schizophrenia cohort.
- Determine the allele, genotype and haplotype frequencies of all prioritized variants.
- Compare minor allele frequencies of variants in our cohort with populations from the 1000 Genomes Project and the Human *CYP* Allele Nomenclature Databases.
- Perform association analyses to establish if there are genetic polymorphisms that are associated with antipsychotic treatment outcome.

**2.3.1.3 Study Design**

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**3 CHAPTER 3: MATERIALS AND METHODS****3.1 Patient Samples**

The patient cohort was selected from a larger cohort obtained for a non-comparative study (Chiliza *et al.*, 2014) that investigated the treatment response profiles to the conventional antipsychotic, flupenthixol decanoate in FES patients, diagnosed using the DSM-IV (American Psychiatric Association, 2000). The drug was administered subsequent to a 7-day wash-out period, by treating each patient with flexible doses of flupenthixol decanoate injections. The initial prescribed dose included 10 mg of flupenthixol decanoate every 2 weeks, with 10 mg increments every 6 weeks allowed, not exceeding 30 mg every 2 weeks. Concomitant psychotropic treatment was allowed if necessary, which included benzodiazepines, antidepressants or anticholinergics. Other antipsychotics, mood stabilizers or psychostimulants were not permitted (Chiliza *et al.*, 2015). From the 207 patients that were eligible in the study, 126 resided in and around Cape Town and for 104 of these, genomic DNA (gDNA) samples were available. However, since only unrelated individuals were considered in this study, one patient was excluded leaving a total of 103 patients for inclusion in this study. These individuals' identity was anonymized for the purpose of this study and the patients were recruited from Stikland Hospital in the Western Cape, where they have been clinically evaluated for at least 12 months. Extensive clinical and demographic data were collected for all the patients (Table 2) at baseline, week 2, week 4, week 6 and every 3 months thereafter. The data included PANSS scores assessed accurately by medical doctors trained to use assessment instrumentation and underwent inter-rater reliability testing of a >75% for all scales prior to the study. These PANSS scores were used to measure treatment response in this study. gDNA was previously extracted from whole blood using the Miller *et al.* (1988) protocol. Genotype data for ancestry informative markers (AIMs) were also available for the cohort (Daya *et al.*, 2013; Drögemöller *et al.*, 2013). Prior to the study, written or verbal informed consent was obtained from the patients themselves or their caregivers. Ethical approval was obtained from the Health Research Ethics Committee (HREC), Faculty of Health Sciences, Stellenbosch University, in terms of the Health Act No 61.2003 (ethics review numbers for clinical and genetic aspects: N06/08/148 and 1907/005, respectively).

**Table 2. Patient demographic data (Chiliza *et al.*, 2014; Drögemöller *et al.*, 2014a). Reprinted with permission from *Future Medicine***

<b>Patient demographic descriptor</b>	<b>Count, n (%)</b>
Mean age (years)	24 ± 7
<b>Gender</b>	
Male	76 (74)
Female	27 (26)
<b>Ethnicity</b>	

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African descent (Xhosa)	13 (13)
Mixed ancestry (SAC)	82 (80)
European descent (Caucasian)	8 (8)
<b>Highest level of education</b>	
Elementary school	7 (7)
Secondary school	65 (63)
Matriculation	21 (20)
Tertiary education	8 (8)
Technical college	2 (2)
<b>Marital status</b>	
Single	87 (84)
Married	8 (8)
Divorced	5 (5)
Widowed	2 (2)
Cohabiting	1 (1)
<b>Urban vs rural living</b>	
Urban	101 (98)
Rural	2 (2)
<b>Previous antipsychotic medication</b>	
No	57 (55)
Yes	46 (45)
<b>Family history of psychosis</b>	
None	41 (41)
Schizophrenia	38 (38)
Bipolar	1 (1)
Depression	5 (5)
Substance abuse	7 (7)
Other	9 (9)

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### 3.2 Prioritization of Variants

Genes selected in this study (*COMT*, *CYP1A2*, *CYP2D6*, *DRD2*, *DRD3*, *HTR2A* and *SOD2*) have been chosen based on pathways implicated in the pharmacogenetics of schizophrenia, i.e. dopamine and serotonin neurotransmission, the cytochrome P450 system and oxidative stress (Zhang *et al.*, 2011). A literature search was conducted on Google Scholar (<https://scholar.google.co.za/>) using the keywords “schizophrenia pharmacogenetics” and “antipsychotic pharmacogenetics” in the search tool (accessed 16 January 2012). The search criteria was filtered according to a publication date of at least five years prior to the study and only “full-text” articles were considered, available via the Stellenbosch University Library and Information Service portal (<http://library.sun.ac.za/English/Pages/default.aspx>) (accessed 16 January 2012). References of identified articles were examined for additional relevant citations. The remaining articles were mined to identify the most commonly cited candidate genes implicated in the pharmacogenetics of schizophrenia and the chosen variants have all demonstrated evidence for playing a role in the dopamine, serotonin and drug metabolism pathways, which included intronic, missense, synonymous and frameshift mutations.

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The candidate genes selected were further investigated for novel and known functional variants within previously generated exome data for eleven of the FES patients from SAC ethnicity, of which five were responders and six non-responders to antipsychotic medication. These data were available in variant call format (vcf) and were generated as described by Drogemöller *et al.* (2014b). Data for the *COMT*, *CYP1A2*, *CYP2D6*, *DRD2*, *DRD3*, *HTR2A* and *SOD2* genes were extracted from the vcf file using the *awk* utility (`awk '$21 ~ /gene_name/' vcf_file_name.txt > gene_name.txt`). These data were subsequently submitted to SeattleSeq Annotation 141 (Ng *et al.*, 2010) using default settings in order to annotate variants within the candidate pharmacogenes. Variants were classified as functional if they were predicted *in silico* to affect gene products (loss-of-function, missense or splice-site mutations). Variants predicted by SeattleSeq Annotation 141 to affect the gene function were further analyzed by means of the Sorting Intolerant from tolerant (SIFT) (Kumar *et al.*, 2009) and Polymorphism Phenotyping (PolyPhen-2) (Adzhubei *et al.*, 2010) algorithms and the severity of the variant was considered likely to affect the protein function if the variant was classified as “damaging” by at least one of the algorithms. Novel variants identified were submitted to the National Center for Biotechnology Information Single Nucleotide Polymorphism database (NCBI dbSNP) (<http://www.ncbi.nlm.nih.gov/snp/>) to obtain rs IDs for these variants. Relevant genotyping assays were developed for novel functional variants for *COMT* and *SOD2* and subsequently validated by Sanger sequencing as described in section 3.3.5 if the variants were predicted to be damaging by either one of the algorithms. The novel variants identified in the *CYP* genes from the exome data were not investigated further due to the genes’ complex polymorphic nature and high sequence similarity which hampers next-generation sequencing (NGS) capabilities (Drögemöller *et al.*, 2013).

### 3.3 Experimental Procedures for SNP Genotyping and Sanger DNA Sequencing

#### 3.3.1 Amplification of SNP Regions in Pharmacogenes

##### 3.3.1.1 Primers for Polymerase Chain Reaction (PCR)

For the majority of SNPs, primer sequences were available from previous studies. The primer sequences are shown in Table 3 and allowed for the amplification of the regions surrounding the variants of interest, under conditions described in Table 4. In order to design genotyping assays for the novel variants identified in *COMT* and *SOD2*, the reference sequences for the relevant genomic regions were obtained from the Ensembl Genome Browser (Cunningham *et al.*, 2014) (Ensembl Gene ID ENSG00000093010 and ENSG00000112096, respectively). The novel variant present in *COMT* was located in close proximity to both rs6269 and rs4633 and was thus amplified with primers used for the region including these SNPs, and subsequently genotyped using restriction enzymes as described in section 3.3.3. For the novel variant present in *SOD2* primers were designed in a web-

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based primer design application by Integrated DNA Technologies (IDT - PrimerQuest Input), using the OligoAnalyzer 3.1 and PrimerQuest computational tools and a genotyping assay was subsequently designed for the amplification of the region containing the novel variant for *SOD2* by means of the allele-specific PCR method as described in section 3.3.1.5.

**3.3.1.2 PCR Conditions**

PCR was used to amplify regions of interest within pharmacogenes from patient gDNA previously extracted from whole blood. All reaction mixtures comprised of 20 ng DNA, 1x reaction buffer, and 0.5 U BIOTAQ™ Polymerase (Bioline™, London, UK). The remaining reagents were optimized specifically for each SNP as shown in Table 4. PCR amplifications were performed using the GeneAmp® PCR Systems 2700 (Applied Biosystems™, California, USA) and the cycle conditions are displayed in Table 5.

**Table 3. Primer sequences used in experimental procedures, including expected amplicon sizes and genetic variation**

SNPs	Amplicon Size (bp)	Primer Sequence (5'-3')	Polymorphism	Reference
<i>COMT</i>				
<sup>1</sup> rs9617850	413	F – TGCATCACGTGGGTTAGCAGAGAA R – TTCATGGCTGAACTGCCTTCCTCA	G>A	(Wright <i>et al.</i> , 2012)
rs9606186	486	F – TCACTGCACATAGTGCCACTCTCA R – TCATGGCAGACGCTAGGCTCTACTG	G>C	(Wright <i>et al.</i> , 2012)
rs2075507	681	F – CTCTGGCGAAAGGAAT R – CCCACAGGCAGTGAGTGTA	A>G	(Wright <i>et al.</i> , 2012)
rs45551837	774	F – GACTGCCTCCCAGAACTCTGTT R – CATAACGGCCTCTTGGTCTA	G>A	(Wright <i>et al.</i> , 2012)
rs45536341	678	F – TCAGCACAGCAGGACCTTAGACAA R – ACCTCAGCTTCCACAAGGAGTCT	C>T	(Wright <i>et al.</i> , 2012)
<sup>1</sup> rs737865	350	F – CTGCCCTGCTAACAGACCTGC R – GTGGCAAACCCAGATCAGTTGG	A>G	(Wright <i>et al.</i> , 2012)
rs6269, rs4633 and rs373611092	499	F – TTCTGAACCTTGCCCTCTgC R – ATGGGGTGATAACAGCTTCTC	A>G, C>T <sup>3</sup> (C186T) and A>G <sup>2</sup> (M90V), respectively	(Wright <i>et al.</i> , 2012)

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<sup>1</sup> rs4818 and <sup>1</sup> rs4680	295	F – GGGATCCAAGTTCCCTCTC R – CCCTTTTCCAGGTCTGACAAC	C>G <sup>3</sup> (G408C) and G>A <sup>2</sup> (V158M), respectively	(Wright <i>et al.</i> , 2012)
rs9332377	332	F – AGCATCCTATGAGGTTTCTCC R – AGTGCCAGGAACTCAGCCTTA	C>T	(Wright <i>et al.</i> , 2012)
<sup>1</sup> rs165599	414	F – ACCTCTCTGAACTGCAACACTGGA R – TGTGAATGCTGGCTGACTCCTCTTC	G>A	(Wright <i>et al.</i> , 2012)
<b>DRD2</b>				
<sup>1</sup> rs1799732	303	F – ACTGGCGAGCAGACGGTGAGGACCC R – TGCGCGCGTGAGGCTGCCGGTTCCGG	Del>C	(Arinami <i>et al.</i> , 1997)
<sup>1</sup> rs1079597	459	F – GATACCCACTTCAGGAAGTC R – GATGTGTAGGAATTAGCCAGG	C>T	(Wu <i>et al.</i> , 2000)
<sup>1</sup> rs1800497	310	F – CCGTCGACCCCTTCTGAGTGTCATCA R – CCGTCGACGGCTGGCCAAGTTGTCTA	G>A <sup>2</sup> (E713K)	(Wu <i>et al.</i> , 2000)
rs6275	494	F – AGGAGCTGGAGATGGAGATGCT R – TGCCCATCTGTAAAGTGAGCACAA	T>C <sup>3</sup> (C939T)	(Röhrich, 2009)
<b>DRD3</b>				
rs6280	463	F – GCTCTATCTCCAACCTCTCACA R – AAGTCTACTCACCTCCAGGTA	G>A <sup>2</sup> (G9S)	(Hitzeroth <i>et al.</i> , 2007)
<b>HTR2A</b>				
rs6311	469	F – AACCAACTTATTTCTACCAC R – AAGCTGCAAGGTAGCAACAGC	G>A <sup>3</sup> (A- 1438G)	(Röhrich, 2009)
rs6313	345	F – TCTGCTACAAGTTCTGGCTT R – CTGCAGCTTTTCTCTAGGG	C>T <sup>3</sup> (C102T)	(Röhrich, 2009)
<b>SOD2</b>				
rs4880	91	F – CCAGCAGGCAGCTGGCACCG R – TCCAGGGCGCCGTAGTCGTAGG	T>C <sup>2</sup> (V16A)	(Chistyakov <i>et al.</i> , 2001)
rs372173830	575	First amplification F – CCAGGTGTCGCATTCTGATGTT R – CAACAGTGAGATCTGTCTCA	C>G <sup>2</sup> (E133D)	novel

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	406	Second amplification (nested)		
		F1 – GTTCCTTTGACAAGTTTAAGGAG		
		F2 – GTTCCTTTGACAAGTTTAAGGAC		
		R – CAACAGTGAGATCTGTCTCA		
<i>CYP1A2</i>				
rs2069514 (*1C)	596	F – GCTACACATGATCGAGCTATAC	G>A	(Nakajima <i>et al.</i> , 1999)
		R – CAGGTCTCTTCACTGTAAAGTTA		
rs762551 (*1F)	519	F – TGGAGTGGTCACTTGCCCTCT	C>A	(Tiwari <i>et al.</i> , 2005)
		R – CTGGCTCATCCTTGACAGT		
<i>CYP2D6</i>				
<i>CYP2D6*1</i>	6.6 kb	F – ATGGCAGCTGCCATACAATCCACCTG	Reference sequence	(Wright <i>et al.</i> , 2010)
		R – CGACTGAGCCCTGGGAGGTAGGTAG		
<i>CYP2D6*1xN</i>	3.5 kb	F – CCATGGAAGCCCAGGACTGAGC	Duplication	(Wright <i>et al.</i> , 2010)
		R – CGGCAGTGGTCAGCTAATGAC		
<i>Duplication test</i>	8.6 kb	F – CGGCAGTGGTCAGCTAATGAC	5' duplication	(Gaedigk <i>et al.</i> , 2007)
		R – CCAGAAGGCTTTGCAGGCTTCAG		
<i>CYP2D6*5</i>	3.5 kb	F – ACCAGGCACCTGTACTCTCTCA	Deletion	(Wright <i>et al.</i> , 2010)
		R – GCATGAGGAAGGCACCCAGAC		
rs3892097/ rs72549356 (*4/*40)	184/202	F – GTGGGTGATGGGCAGAAG	G>A and 18bp insertion, respectively	(Wright <i>et al.</i> , 2010)
		R – GAGGGTCGTCGTACTIONGAA		
rs5030655 (*6)	600	F – TGTGCTGTAAGCTCAGTGTGGGT	T>Del	(Wright <i>et al.</i> , 2010)
		R – GAGGGTCGTCGTACTIONGAA		
<i>CYP2D6*13</i>	5kb	F – TCCGACCAGGCCTTTCTACCAC	2D7/2D6 Hybrid	(Wright <i>et al.</i> , 2010)
		R – ACTGAGCCCTGGGAGGTAGGTAG		

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<sup>1</sup>Polymorphisms that have been genotyped previously (Higgins, 2011) but still tested for association analyses in this study

<sup>2</sup>Amino acid substitutions

<sup>3</sup>Synonymous changes

### 3.3.1.3 Long-range PCR for the Detection of *CYP2D6* Alleles

Analysis of the duplication and deletion of the 6.6 kb *CYP2D6* gene was performed using long-range PCR as described by Wright *et al.* (2010). This gene was amplified using ~20 ng DNA, 0.4 U KAPA Long Range DNA Polymerase (KAPABiosystems™, Cape Town, South Africa), 1x KAPA Long Range 5x buffer, 0.4 μM *CYP2D6* primers for the 6.6 kb fragment and 0.3 μM primers for the

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fragment tested specifically for either the duplication or deletion, 2 mM KAPA Long Range MgCl<sub>2</sub> and 0.4 mM dNTPs, and 1 M Betaine (Sigma-Aldrich (Pty) Ltd, Aston Manor, South Africa) with a final reaction volume of 8 µl. Samples were amplified by conditions described in Table 5 after pre-setting the thermal cycler programme to heat block at 85°C using the GeneAmp® PCR Systems 2700 (Applied Biosystems™, California, USA). The nature of gene duplications were further investigated by amplifying the duplicated gene under the same conditions using 2.5 mM KAPA Long Range MgCl<sub>2</sub>, and 0.4 µM primers, and genotyping these fragments by restriction fragment length polymorphism (RFLP) PCR for the allele-defining SNPs for *CYP2D6\*4* and *CYP2D6\*6* (Table 6), and by PCR for *CYP2D6\*40* (See Table 4 and Table 5 for reagent concentrations and conditions, respectively). Specific exons containing SNPs of interest were amplified by nested PCR using ~1000x dilutions of long-range products and subsequently genotyped using RFLP-PCR as described in Table 6 and visualized as described in section 3.3.2.

**3.3.1.4 Hybrid PCR for the Detection of CYP2D6 Hybrid Gene**

*CYP2D6* is known to result in hybrid pseudogenes (*CYP2D7/CYP2D6*) from recombination activities (Black *et al.*, 2012). Patient samples that tested homozygous for all SNPs genotyped were therefore selected for hybrid testing. The 1000x diluted product obtained from the long-range PCR described in section 3.3.1.3 was used as a template for this assay that was performed in two separate reactions. The first reaction was performed as a control to amplify the 6.6 kb *CYP2D6* gene as well as the 5 kb hybrid, *CYP2D7/CYP2D6*, and the second reaction was specific for the hybrid only to confirm no spurious amplification took place in the first run, using the specified primers indicated in Table 3. Concentrations of reaction mixtures were the same as described for the long range PCR, using 1.5 mM KAPA Long Range MgCl<sub>2</sub>. Products were visualized as described in section 3.3.2.

**3.3.1.5 Allele-specific PCR for the Detection of rs372173830 in SOD2**

The allele-specific PCR method was employed for investigating the novel SNP in *SOD2*, rs372173830, identified from the exome sequencing data. Two sets of primers (Table 3) were designed, of which the first set was used to amplify the region of interest containing the SNP, and the second set was used for amplification of either the wild-type or variant allele by means of a nested PCR, narrowing down the region of interest and using a 1 in 50 µl dilution of the previously amplified product as a template. All samples therefore underwent three successive rounds of PCRs to successfully genotype this SNP using reagent concentrations described in Table 4 and PCR conditions described in Table 5.

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**Table 4. Concentration of Reagents used in PCR analysis**

Genes	SNPs	MgCl <sub>2</sub> (mM)	dNTPs (mM)	Primers ( $\mu$ M)	Additives	Final Volume ( $\mu$ l)
<i>COMT</i>	rs9617850	1.5	0.4	0.4	-	25
	rs9606186	2.5	0.4	0.4	5% v/v DMSO	25
	rs2075507	1.5	0.4	0.4	-	25
	rs45551837	3	0.4	0.4	5% v/v DMSO	25
	rs45536341	1.5	0.4	0.4	5% v/v DMSO	25
	rs737865	1.5	0.4	0.4	5% v/v DMSO	25
	rs6269, rs4633, rs373611092	1	0.4	0.4	-	30
	rs4818, rs4680	2.5	0.4	0.4	-	25
	rs9332377	1.5	0.4	0.4	-	25
	rs165599	2	0.4	0.2	-	15
<i>DRD2</i>	rs1799732	1.5	0.4	0.2	1M Betaine	20
	rs1079597	1.5	0.4	0.3	5% v/v DMSO	15
	rs1800497	1.5	0.4	0.3	5% v/v DMSO	15
	rs6275	1.5	0.4	0.5	-	15
<i>DRD3</i>	rs6280	3	1	0.3	-	15
<i>HTR2A</i>	rs6311	1.5	0.4	0.5	-	15
	rs6313	1.5	0.4	0.5	-	15
<i>SOD2</i>	rs4880	1	0.8	0.5	5% v/v DMSO	15
	rs372173830 – First and second amplification	1.5	0.4	0.5	-	15
<i>CYP1A2</i>	rs2069514 (*1C), rs762551 (*1F)	1.5	0.4	0.4	-	25
<i>CYP2D6</i>	rs3892097/ rs72549356 (*4/*40)	1.5	0.4	0.4	-	25
	rs5030655 (*6)	2	0.4	0.2	-	25

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**3.3.2 Visualization of PCR products**

Each fragment was visualized by electrophoresis on 1, 1.5 or 3% (w/v), agarose gels (Appendix A) depending on fragment sizes and stained with 0.5 µg/ml ethidium bromide, in 1x TBE buffer (Appendix A), using molecular weight markers (Hyperladder I, IV or V, Bioline™, London, UK) relevant to the expected size of the fragment. The fragments were subsequently loaded onto the gel using 5 µl CRESOL Loading Buffer (Appendix A). Fragments were allowed to migrate through the gel for 30 minutes at 120 V, followed by visualization under ultra-violet light at 260 nm with a MultiGenius Bio Imaging Capture System (Syngene, Cambridge, UK). Long-range and hybrid products were visualized on 0.6% (w/v) agarose gel using a molecular weight marker of 200 bp (Hyperladder I, Bioline™, London, UK).

**3.3.3 Restriction Fragment Length Polymorphism (RFLP) Analysis**

Amplified fragments were subsequently used for PCR-RFLP analysis using the restriction enzymes and conditions as shown in Table 6. Relevant restriction enzymes were identified from an online application tool, RestrictionMapper version 3 (<http://www.restrictionmapper.org/>). Digests were all performed on the GeneAmp® PCR Systems 2700 (Applied Biosystems™, California, USA). Fragments were separated on agarose gels depending on the size of the respective fragments and stained with 0.5 µg/ml ethidium bromide for visualization under ultra-violet light with a MultiGenius Bio Imaging Capture System (Syngene, Cambridge, UK). Positive controls, if available, were used to act as representatives of the different genotypes, ensuring that the digests were successful.

**3.3.4 TaqMan® Assays**

TaqMan® probe-based assays (Applied Biosystems™, California, USA) were performed for the SNPs in *COMT* rs5748489 and rs2020917 (Higgins, 2011), and *DRD2* rs6277 (Welham, 2015), since these SNPs do not create or abolish recognition sequences of known restriction enzymes. The reactions for *COMT* rs5748489 and rs2020917 were carried out using 15 ng gDNA, 1x TaqMan® probes and 1x TaqMan® Genotyping Master Mix. For *DRD2* rs6277, the reaction was performed using 30 ng gDNA, 1x ROX High Reference Dye, 20x TaqMan® SNP assay (Applied Biosystems™, California, USA) and 1x KAPA Probe Fast qPCR Master Mix (KAPABiosystems™, Cape Town, South Africa). All reactions were performed using a final volume of 10 µl in 96-well plates. These reactions were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems™, California, USA) using the conditions described by the manufacturer. The genotyping was performed using the Sequence Detection System (SDS) Software (Applied Biosystems™, California, USA) with an autocaller confidence level of 95%.

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**3.3.5 Bi-directional Sanger Sequencing**

Due to its functional consequence, the novel variant in *COMT* (rs373611092) was selected for sequencing. PCR products were bi-directionally sequenced using Sanger sequencing to confirm the presence of this variant as called by the exome sequencing. PCR products, representing the different genotype classes, where available, were purified using the SureClean Protocol (Bioline<sup>TM</sup>, London, UK) and sample concentrations were measured on the Nanodrop spectrophotometer (NanoDrop<sup>®</sup> ND-100, Nanodrop Technologies Inc., Wilmington, Delaware, USA) at a wavelength of 260 nm followed by preparation of the sequencing reaction as described in the BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit Manual (Applied Biosystems<sup>TM</sup>, California, USA). The samples were analyzed by capillary electrophoresis on a 3130*xl* Genetic Analyser (Applied Biosystems<sup>TM</sup>, California, USA) at the Central Analytical Facility, Stellenbosch University and results were aligned to the relevant reference sequences with the sequence alignment programme, BioEdit (Ibis Biosciences, Carlsbad, California, USA).

**Table 5. PCR cycle conditions used**

SNPs	PCR Cycle conditions	Reference
<b><i>COMT</i></b>		
rs9617850, rs45551837, rs737865, rs6269, rs4633, rs373611092 and rs9332377	94°C 3 min; 40 cycles of 94°C 15 sec, 60°C 15 sec and 72°C 30 sec; 72°C 5 min	(Wright <i>et al.</i> , 2012)
rs9606186	94°C 3 min; 10 cycles of 94°C 15 sec, 60°C 15 sec and 72°C 30 sec; 30 cycles of 94°C 15 sec, 55°C 15 sec and 72°C 30 sec; 72°C 5 min	(Wright <i>et al.</i> , 2012)
rs2075507 and rs45536341	94°C 3 min; 40 cycles of 94°C 15 sec, 55°C 15 sec and 72°C 30 sec; 72°C 5 min	(Wright <i>et al.</i> , 2012)
rs4818 and rs4680	94°C 3 min; 40 cycles of 94°C 15 sec, 66°C 15 sec and 72°C 30 sec; 72°C 5 min	(Wright <i>et al.</i> , 2012)
rs165599	94°C 3 min; 10 cycles of 94°C 15 sec, 68°C 15 sec and 72°C 30 sec; 30 cycles of 94°C 15 sec, 62°C 15 sec and 72°C 30 sec; 72°C 5 min	(Wright <i>et al.</i> , 2012)
<b><i>DRD2</i></b>		
rs1799732	95°C 4min; 35 cycles of 95°C 1 min, 55°C 1min and 72°C 1,5min; 72°C 5min	(Breen <i>et al.</i> , 1999)
rs1079597	94°C 4 min; 10 cycles of 94°C 30 sec, 65°C 30 sec and 72°C 30 sec; 25 cycles of 94°C 30 sec, 55°C 30 sec and 72°C 30 sec; 72°C 4 min	(Röhrich, 2009)

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rs1800497	94°C 4 min; 10 cycles of 94°C 30 sec, 65°C 30 sec and 72°C 30 sec; 25 cycles of 94°C 30 sec, 55°C 30 sec and 72°C 30 sec; 72°C 4 min	(Röhrich, 2009)
rs6275	94°C 3 min; 40 cycles of 94°C 15 sec, 60°C 15 sec and 72°C 30 sec; 72°C 5 min	(Röhrich, 2009)
<b>DRD3</b>		
rs6280	94°C 5 min; 35 cycles of 94°C 30 sec, 57°C 30 sec and 72°C 45 sec; 72°C 7 min	(Hitzeroth <i>et al.</i> , 2007)
<b>HTR2A</b>		
rs6311	94°C 5 min; 30 cycles of 94°C 20 sec, 62°C 20 sec and 72°C 20 sec; 72°C 10 min	(Röhrich, 2009)
rs6313	94°C 5 min; 30 cycles of 94°C 20 sec, 60°C 20 sec and 72°C 20 sec; 72°C 10 min	(Röhrich, 2009)
<b>SOD2</b>		
rs4880	94°C 3 min; 35 cycles of 94°C 1 min, 60°C 1 min and 72°C 1 min; 72°C 5 min	(Chistyakov <i>et al.</i> , 2001)
rs372173830	First amplification: 94°C 3 min; 35 cycles of 94°C 20 sec, 62°C 20 sec and 72°C 30 sec; 72°C 5 min  Second amplification: 94°C 3 min; 19 cycles of 94°C 15 sec, 60°C 15 sec and 72°C 30 sec; 72°C 5 min	*novel
<b>CYP1A2</b>		
rs2069514 (*1C)	95°C 5 min; 35 cycles of 95°C 45 sec, 56°C 45 sec and 72°C 1 min; 72°C 7 min	(Truter, 2007)
rs762551 (*1F)	95°C 5 min; 35 cycles of 95°C 30 sec, 58°C 30 sec and 72°C 45 sec; 72°C 5 min	(Truter, 2007)
<b>CYP2D6</b>		
<i>CYP2D6*1xN</i> and <i>CYP2D6*5</i>	95°C 5 min; 35 cycles of 95°C 15 sec and 68°C 10 sec; 68°C 7 min	(Wright <i>et al.</i> , 2010)
Duplication test	95°C 5 min; 35 cycles of 95°C 15 sec and 68°C 10 sec; 68°C 9 min	(Gaedigk <i>et al.</i> , 2007)
rs3892097/ rs72549356 (*4/*40)	94°C 3 min; 35 cycles of 94°C 15 sec, 55°C 15 sec and 72°C 30 sec; 72°C 5 min	(Wright <i>et al.</i> , 2010)
rs5030655 (*6)	94°C 3 min; 40 cycles of 94°C 15 sec, 55°C 15 sec and 72°C 30 sec; 72°C 5 min	(Wright <i>et al.</i> , 2010)
<i>CYP2D6*13</i>	95°C 2 min; 35 cycles of 95°C 15 sec, 67°C 10 sec; 68°C 7 min	(Wright <i>et al.</i> , 2010)

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\*Conditions for reactions were optimized.

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Table 6: PCR-RFLP reactions

Gene	SNP	Restriction Enzyme	Enzyme Concentration (U)	Additives	Incubation Temperature, Incubation Time (°C, hr)	Genotype	Restriction Fragment Length (bp)	Electrophoresis for fragment separation (Gel % (w/v) and V and time indicated)	Reference
<i>COMT</i>	rs9617850	<i>Bsa</i> AI (NEB)	3	-	37°C, 16 hr	GG	275, 130, 8	2% Agarose Gel	(Wright <i>et al.</i> , 2012)
						GA	405, 275, 130, 8	80 V for 1 hr	
						AA	405, 8		
	rs9606186	<i>Bsr</i> I (NEB)	3	-	65°C, 2 hr (inactivation step of 80°C for 20 min)	GG	320, 76, 54, 36	3% Agarose Gel	(Wright <i>et al.</i> , 2012)
						GC	320, 295, 76, 54, 36, 25	80 V for 2 hr and 20 min	
						CC	295, 76, 54, 36, 25		
	rs2075507	<i>Hind</i> III (NEB)	5	-	37°C, 16 hr	AA	407, 274	2% Agarose Gel	(Wright <i>et al.</i> , 2012)
						AG	407, 274, 230, 177	80 V for 1 hr	
						GG	274, 230, 177		
rs45551837	<i>Taq</i> AI (NEB)	4	BSA (100µg/ml)	65°C, 2 hr	GG	364, 251, 159	2% Agarose Gel	(Wright <i>et al.</i> , 2012)	
					GA	410, 364, 251, 159	80 V for 1 hr		
					AA	410, 364			
rs45536341	<i>Bsa</i> HI (NEB)	2	BSA (100µg/ml)	37°C, 16 hr	CC	457, 221	2% Agarose Gel	(Wright <i>et al.</i> , 2012)	
					CT	678, 457, 221	80 V for 1 hr and 45 min		
					TT	678			
rs737865	<i>Bs</i> II (NEB)	2	-	55°C, 16 hr	AA	300, 50	2% Agarose Gel	(Wright <i>et al.</i> , 2012)	
					AG	300, 259, 50, 41	80 V for 1 hr and 20 min		
					GG	259, 50, 41			

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rs6269	<i>TaqI</i> (NEB)	2	BSA (100µg/ml)	65°C, 2 hr	AA	499	4% Agarose Gel	(Wright <i>et al.</i> , 2012)
					AG	499, 479, 20		
					GG	479, 20		
rs4633	<i>BsaAI</i> (NEB)	2	-	37°C, 2 hr	CC	305, 194	2% Agarose Gel	(Wright <i>et al.</i> , 2012)
					CT	499, 305, 194		
					TT	499		
rs373611092	<i>BstXI</i> (NEB)	2	-	37°C, 16 hr	AA	392, 107	2% Agarose Gel	*NA
					AG	499, 392, 107		
					GG	499		
rs4818	<i>MboI</i> (NEB)	5	-	37°C, 16 hr	CC	126, 103, 64, 2	15% Polyacrylamide Gel	(Wright <i>et al.</i> , 2012)
					CG	126, 114, 103, 64, 12, 2		
					GG	114, 103, 64, 12, 2		
rs4680	<i>NlaIII</i> (NEB)	3	BSA (100µg/ml)	37°C, 16 hr	GG	151, 114, 30	15% Polyacrylamide Gel	(Wright <i>et al.</i> , 2012)
					GA	151, 114, 96, 30, 18		
					AA	151, 96, 30, 18		
rs9332377	<i>EcoNI</i> (NEB)	1	-	37°C, 16 hr	CC	332	4% Agarose Gel	(Wright <i>et al.</i> , 2012)
					CT	332, 310, 22		
					TT	310, 22		
rs165599	<i>MspI</i> (NEB)	5	-	37°C, 16 hr	GG	376, 38	2.5% Agarose Gel	(Wright <i>et al.</i> , 2012)
					GA	414, 376, 38		
					AA	414		

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	rs1799732	<i>Bst</i> NI (NEB)	5	BSA (100µg/ml)	65°C, 16 hr	CC C- --	160, 144 160, 144, 303 303	2.5% Agarose Gel 80 V for 1 hr	(Arinami <i>et al.</i> , 1997)
<b>DRD2</b>	rs1079597	<i>Taq</i> 1α (NEB)	2	BSA (100µg/ml)	65°C, 2 hr	GG GA AA	267, 192, 459, 267, 192 459	2% Agarose Gel 80 V for 1 hr	(Wu <i>et al.</i> , 2000)
	rs1800497	<i>Taq</i> 1α (NEB)	2	BSA (100µg/ml)	65°C, 2 hr	GG GA AA	180, 130 310, 180, 130 310	2% Agarose Gel 80 V for 1 hr	(Wu <i>et al.</i> , 2000)
	rs6275	<i>Nco</i> I (NEB)	2	-	37°C, 16 hr	TT TC CC	274, 113, 107 381, 274, 113, 107 381, 113	2% Agarose Gel 80 V for 1 hr	(Röhrich, 2009)
<b>DRD3</b>	rs6280	<i>Bst</i> XI (NEB)	2	-	37°C, 16 hr	GG GA AA	260, 203 463, 260, 203 463	15% Polyacrylamide Gel 200 V for 1 hr	(Hitzeroth <i>et al.</i> , 2007)
<b>HTR2A</b>	rs6311	<i>Msp</i> I (NEB)	5	-	37°C, 16 hr	GG GA AA	245, 224 469, 245, 224 469	2% Agarose Gel 80 V for 1 hr	(Röhrich, 2009)
	rs6313	<i>Msp</i> I (NEB)	5	-	37°C, 16 hr	CC CT TT	217, 125 342, 217, 125 342	2% Agarose Gel 80 V for 1 hr	(Röhrich, 2009)

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<b>SOD2</b>	rs4880	<i>AgeI</i> (NEB)	3	-	37°C, 16 hr (inactivation at 65°C for 20min)	TT	74, 17	4% Agarose Gel 80 V for 1 hr	(Chistyakov <i>et al.</i> , 2001)
						TC	91, 74, 17		
						CC	91		
<b>CYP1A2</b>	rs2069514 (*IC)	<i>DdeI</i> (NEB)	1	-	37°C, 16 hr	GG	596	1.5% Agarose Gel 80 V for 1 hr and 20 min	(Truter, 2007)
						GA	596, 464, 132		
						AA	464, 132		
	rs762551 (*IF)	<i>ApaI</i> (NEB)	3	-	30°C, 16 hr	CC	372, 147	1.5% Agarose Gel 80 V for 1 hr and 20 min	*NA
						CA	519, 372, 147		
						AA	519		
<b>CYP2D6</b>	rs3892097 (*4)	<i>BstNI</i> (NEB)	5	BSA (100µg/ml)-	65°C, 16 hr	GG	98, 86	2% Agarose Gel 80 V for 1 hr	(Wright <i>et al.</i> , 2010)
						GA	184, 98, 86		
						AA	184		
	rs5030655 (*6)	<i>BsaXI</i> (NEB)	2	-	37°C, 16 hr	TT	600	2% Agarose Gel 80 V for 1 hr	*NA
						T-	600, 350, 219, 30		
					--	350, 219, 30			

NA: not applicable

\*Conditions for reactions were optimized.

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## 3.4 Statistical Analysis

3.4.1 Genotype and Allele Distributions and *CYP2D6* Activity Scores

Differences in allele and genotype frequencies were determined for all variants, including analysis for deviations from Hardy-Weinberg Equilibrium (HWE) using SNPStats (Solé *et al.*, 2006). *P* values  $\leq 0.01$  were regarded as significant for HWE. Minor allele frequencies (MAF) for the different FES population groups were compared to MAF of the African, American, East Asian and European populations retrieved from the 1000 Genomes Project (Abecasis *et al.*, 2012). *CYP* minor allele frequencies for the FES populations were compared to frequencies retrieved from The Human Cytochrome P450 (*CYP*) Allele Nomenclature Database (Sim and Ingelman-Sundberg, 2010) of African, African American, East Asian and European populations. Haplotypes and inferred frequencies were calculated in R (Ihaka and Gentleman, 1996). LD plots for significant haplotypes were constructed in Haploview 3.31 (Barret *et al.*, 2005). Activity scores for *CYP2D6* were calculated to deduce phenotypic information of metabolizer groups as described by Gaedigk *et al.* (2008) using Model A in Table 7.

**Table 7. Values assigned to alleles in AS-Model A (Gaedigk *et al.*, 2008). Reprinted with permission from Wiley**

Value assigned to allele	Alleles - AS-Model A
0	*3, *4, *4xN, *5, *6, *7, *16, *36, *40, *42, *56B
0.5	*9, *10, *17, *29, *41, *45, *46
1	*1, *2, *35, *43, *45xN
2	*1xN, *2xN, *35xN

AS: activity score. The AS of a genotype is the sum of the values assigned to each allele (e.g., *CYP2D6*\*1/\*1 and *CYP2D6*\*2/\*5 genotypes have AS of 2 and 1, respectively).

## 3.4.2 Association Testing

Statistical analyses were performed in R and various R packages, including genetics v3.0.3 (Ihaka and Gentleman, 1996) by a qualified statistician. Mixed model repeated measures analysis was used to determine associations between genotyped SNPs, haplotypes, and activity scores across changes in longitudinal PANSS scores over twelve months. Analyses were performed by correcting for age, gender, proportion ancestry and baseline PANSS scores. Proportion ancestry was calculated using the ancestry informative markers (AIMs) data for the entire study cohort and 1000 Genomes populations using ADMIXTURE (Alexander *et al.*, 2009). These data were previously obtained in our study group to correct for population stratification (Drögemöller *et al.*, 2013). Significant associations with genotyped SNPs were tested with additive, dominant and recessive inheritance models by which the strength of the associations was assessed and effect sizes (ES) with 95% confidence intervals (CI) are reported. Unadjusted patterns of the associations with a change in PANSS scores and each SNP were determined and presented in graphs. *P* values  $\leq 0.01$  were regarded as significant.

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**4 CHAPTER 4: RESULTS****4.1 Selection of Variants**

After literature mining and quality control, a total of 31 variants in seven genes that met the criteria for candidate pharmacogenes were prioritized for inclusion in this study (Table 8).

Evaluation of the exome data revealed a total of eighteen missense variants in six of the seven genes, predominantly present in *CYP2D6* (Table 9). As a result of information that became available after the initial characterization of the variants identified from the exome data for the *CYP* genes, including the novel variants reported, it was decided not to include these in further analyses due to the unreliability of NGS data for these genes (Drögemöller *et al.*, 2013). Exclusion of all *CYP* variants left a total of eight missense variants in *COMT*, *DRD3*, *HTR2A* and *SOD2* of which six have previously been reported and two are novel. Novel variants submitted to NCBI dbSNP were assigned the rs IDs, *COMT* rs373611092 and *SOD2* rs372173830, using the submitter handle “WARNICH\_LAB”. PolyPhen-2 and SIFT algorithms predicted *COMT* rs373611092 to damage the resulting gene product (SIFT score = 0.015) and *SOD2* rs372173830 to be benign or tolerating (SIFT score = 0.48). Bi-directional sequencing was performed to confirm the single novel variants that predicted functional damage (Appendix B1).

Finally 33 variants were selected for genotyping in the FES cohort. These variants consisted of 31 variants prioritized from the literature and the two novel SNPs identified from the exome data in *COMT* (rs373611092) and *SOD2* (rs372173830). Examples of the PCR-RFLP and TaqMan<sup>®</sup> analyses for each of the SNPs are depicted in Appendix B2 and Appendix B3, respectively.

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**Table 8. Candidate pharmacogenes associated with antipsychotic treatment outcome (variants listed in order from 5'-3')**

Gene	Prioritized Variants	Class of Variation	Antipsychotic Associated Pharmacogenetic Trait	References
COMT	rs9617850	upstream	Treatment response, Movement disorder	Di Giorgio <i>et al.</i> , 2009; Gerretsen <i>et al.</i> , 2009; Lencz and Malhotra, 2009; Blanc <i>et al.</i> , 2010; Foster <i>et al.</i> , 2010; Gesteira <i>et al.</i> , 2010; Šagud <i>et al.</i> , 2010; Arranz and Munro, 2011; Arranz <i>et al.</i> , 2011; Burdick <i>et al.</i> , 2011; Cacabelos <i>et al.</i> , 2011; Chowdhury <i>et al.</i> , 2011; Lee and Kang, 2011; Zhang and Malhotra, 2011; Reynolds, 2012; Vyas <i>et al.</i> , 2012; Wright <i>et al.</i> , 2012
	rs9606186	upstream		
	rs5748489	upstream		
	rs2075507	upstream		
	rs45551837	upstream		
	rs2020917	upstream		
	rs45536341	upstream		
	rs737865	intron		
	rs6269	intron		
	rs4633	synonymous codon (C186T)		
	rs4818	synonymous codon (G408C)		
	rs4680	missense (V158M)		
rs9332377	intron			
rs165599	3' UTR			
CYP1A2	rs2069514 (*1C)	upstream	Treatment response, Movement disorder	Gerretsen <i>et al.</i> , 2009; Foster <i>et al.</i> , 2010; Gesteira <i>et al.</i> , 2010; Kores Plesničar, 2010; Lohoff and Ferraro, 2010; Tandon <i>et al.</i> , 2010; Arranz and Munro, 2011; Arranz <i>et al.</i> , 2011; Lee and Kang, 2011; Zhang and Malhotra, 2011
	rs762551 (*1F)	intron		
CYP2D6	CYP2D6*1xN	gene duplication	Treatment response, Movement disorder, Weight gain, QT prolongation	Gerretsen <i>et al.</i> , 2009; Foster <i>et al.</i> , 2010; Gesteira <i>et al.</i> , 2010; Kores Plesničar, 2010; Wright <i>et al.</i> , 2010; Arranz and Munro, 2011; Arranz <i>et al.</i> , 2011; Balt <i>et al.</i> , 2011; Chowdhury <i>et al.</i> , 2011; Lee and Kang, 2011; Risselada <i>et al.</i> , 2011; Zhang and Malhotra, 2011; Lett <i>et al.</i> , 2012
	CYP2D6*5	gene deletion		
	rs3892097 (*4)	splice acceptor		
	rs72549356 (*40)	18 bp insertion		
	rs5030655 (*6)	frameshift		
CYP2D6*13 (2D7/2D6)	gene hybrid structures			
DRD2	rs1799732	upstream	Treatment response, Movement disorder, Weight gain, Hyperprolactinemia, Neuroleptic malignant syndrome	Gerretsen <i>et al.</i> , 2009; Lencz and Malhotra, 2009; Blanc <i>et al.</i> , 2010; Foster <i>et al.</i> , 2010; Gesteira <i>et al.</i> , 2010; Kores Plesničar, 2010; Shiroma <i>et al.</i> , 2010; Steimer, 2010; Arranz and Munro, 2011; Arranz <i>et al.</i> , 2011; Balt <i>et al.</i> , 2011; Burdick <i>et al.</i> , 2011; Chowdhury <i>et al.</i> , 2011; Lee and Kang, 2011; Risselada <i>et al.</i> , 2011; Zhang and Malhotra, 2011; Lett <i>et al.</i> , 2012
	rs1079597	intron		
	rs1800497 (ANKK1)	missense (E713K)		
	rs6275	synonymous codon (C939T)		
rs6277	synonymous codon (C957T)			
DRD3	rs6280	missense (G9S)	Treatment response, Movement disorder	Di Giorgio <i>et al.</i> , 2009; Gerretsen <i>et al.</i> , 2009; Lencz and Malhotra, 2009; Blanc <i>et al.</i> , 2010; Foster <i>et al.</i> , 2010; Gesteira <i>et al.</i> , 2010; Kores Plesničar, 2010; Lohoff and Ferraro, 2010; Steimer, 2010; Tandon <i>et al.</i> , 2010; Arranz and Munro, 2011; Arranz <i>et al.</i> , 2011; Cacabelos <i>et al.</i> , 2011; Chowdhury <i>et al.</i> , 2011; Lee and Kang, 2011; Zhang and Malhotra, 2011; Reynolds, 2012
HTR2A	rs6311	upstream (A-1438G)	Treatment response, Movement disorder, Weight gain	Gerretsen <i>et al.</i> , 2009; Lencz and Malhotra, 2009; Blanc <i>et al.</i> , 2010; Foster <i>et al.</i> , 2010; Gesteira <i>et al.</i> , 2010; Kores Plesničar, 2010; Lohoff and Ferraro, 2010; Steimer, 2010; Tandon <i>et al.</i> , 2010; Arif and Mitchell, 2011; Arranz <i>et al.</i> , 2011; Balt <i>et al.</i> , 2011; Cacabelos <i>et al.</i> , 2011; Chowdhury <i>et al.</i> , 2011; Risselada <i>et al.</i> , 2011; Zhang and Malhotra, 2011; Lett <i>et al.</i> , 2012; Reynolds, 2012
	rs6313	synonymous codon (C102T)		
SOD2	rs4880	missense (V16A)	Movement disorder	Hitzerth <i>et al.</i> , 2007; Arranz <i>et al.</i> , 2011; Chowdhury <i>et al.</i> , 2011; Lee and Kang, 2011

UTR: untranslated region

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**Table 9: Known and novel functional variants identified from previously generated exome data (variants listed in order from 5'-3')**

<b><u>Known Variants</u></b>				
<b>Gene</b>	<b>Locus</b>	<b>Effects</b>	<b>PolyPhen-2</b>	<b>SIFT</b>
<i>COMT</i>	rs4680	V158M	Benign	Damaging
<i>CYP1A2</i>	rs17861157	S298R	Benign	Damaging
	rs144148965	D436N	Benign	No score
<i>CYP2D6</i>	rs1135840	T486S	Benign	Tolerated
	rs59421388	V338M	Probably damaging	Damaging
	rs16947	C296R	Benign	Tolerated
	rs28371717	A237S	Benign	Tolerated
	rs61736512	V136I	Benign	Tolerated
	rs28371696	R26H	Benign	Tolerated
<i>DRD3</i>	rs6280	G9S	Benign	Tolerated
<i>HTR2A</i>	rs6314	H425Y	Benign	Tolerated
	rs6304	I197V	Benign	Tolerated
	rs6312	D49N	No score	No score
<i>SOD2</i>	rs4880	V16A	Benign	Tolerated
<b><u>Novel Variants</u></b>				
<b>Gene</b>	<b>Locus</b>	<b>Effects</b>	<b>PolyPhen-2</b>	<b>SIFT</b>
<i>COMT</i>	rs373611092	M90V	Probably damaging	Damaging
<i>CYP2D6</i>	<sup>1</sup> 22:42522641	H477N	Benign	Tolerated
	<sup>2</sup> rs769157652	E410K	Benign	Tolerated
<i>SOD2</i>	rs372173830	E133D	Benign	Tolerated

<sup>1</sup>Novel variant chromosome position. No rs ID is available for this variant yet

<sup>2</sup>Variant was detected as novel at the start of this study, but recently reported in the dbSNP database by another research group. *CYP* variants were excluded from further analyses due to unreliable data obtained from NGS

## 4.2 Frequency Data for SNPs

Allele and genotype frequencies were calculated in the FES cohort for each of the subpopulations in this cohort. Genotype frequencies are displayed in Appendix C for each of the SNPs evaluated. MAF for each SNP across different populations in the FES cohort were calculated and compared to the MAF of the African (AFR), American (AMR), Asian (ASN) and European (EUR) populations from the 1000 Genomes Project database (Table 10). None of the SNPs deviated from HWE in any of the population groups (Appendix D).

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**Table 10: Minor allele frequency comparisons for SA FES populations and 1000 Genomes Project populations**

SNPs	Populations from SA FES cohort			Populations from 1000 Genomes Project			
	Cau	SAC	Xh	AFR	AMR	ASN	EUR
<b><i>COMT</i></b>							
rs9617850	0.19	0.26	0.46	0.43	0.19	0.10	0.20
rs9606186	0.31	0.32	0.31	0.39	0.34	0.28	0.41
rs5748489	0.31	0.28	0.15	0.24	0.32	0.28	0.41
rs2075507	0.31	0.31	0.23	0.36	0.34	0.28	0.41
rs45551837	0.00	0.03	0.15	0.11	0.02	0.06	0.00
rs2020917	0.38	0.23	0.13	0.08	0.18	0.30	0.28
rs45536341	0.00	0.01	0.00	0.01	0.00	0.00	0.00
rs737865	0.38	0.26	0.12	0.13	0.18	0.29	0.28
rs6269	0.19	0.34	0.31	0.37	0.31	0.34	0.41
rs4633	0.50	0.41	0.27	0.29	0.38	0.27	0.50
rs373611092	0.00	0.01	0.00	NA	NA	NA	NA
rs4818	0.31	0.28	0.19	0.17	0.30	0.34	0.40
rs4680	0.50	0.38	0.23	0.28	0.38	0.28	0.50
rs9332377	0.13	0.19	0.35	0.36	0.13	0.00	0.15
rs165599	0.21	0.42	0.50	0.75	0.50	0.47	0.31
<b><i>DRD2</i></b>							
rs1799732	0.06	0.29	0.54	0.57	0.16	0.14	0.08
rs1079597	0.19	0.22	0.12	0.17	0.27	0.41	0.15
rs1800497	0.21	0.33	0.35	0.39	0.31	0.41	0.19
rs6275	0.75	0.40	0.19	0.34	0.61	0.47	0.69
rs6277	0.56	0.16	0.00	0.06	0.33	0.06	0.54
<b><i>DRD3</i></b>							
rs6280	0.63	0.42	0.12	0.18	0.57	0.69	0.66
<b><i>HTR2A</i></b>							
rs6311	0.31	0.30	0.54	0.41	0.36	0.59	0.44
rs6313	0.31	0.27	0.50	0.39	0.35	0.59	0.44
<b><i>SOD2</i></b>							
rs4880	0.44	0.36	0.50	0.42	0.58	0.13	0.47
rs372173830	0.00	0.05	0.04	NA	NA	NA	NA
<b><i>CYP1A2</i></b>							
rs2069514 (* <i>IC</i> )	0.00	0.19	0.31	0.31	0.36	0.28	0.02
rs762551 (* <i>IF</i> )	0.25	0.35	0.50	0.44	0.24	0.33	0.32

NA: not applicable as no data is available for novel SNPs; SA: South African; FES: First episode schizophrenia cohort; Cau: FES Caucasian; SAC: FES South African Coloured; Xh: FES Xhosa; AFR: African; AMR: American; ASN: Asian; EUR: European

Frequencies for the *CYP2D6* alleles in this study were compared to frequencies in the African (AF), American (AM), Asian (AS) and European (EU) populations from the Human *CYP* Allele Nomenclature database (Table 11), since the frequency data for some *CYP* alleles were not available on the 1000 Genomes Project database.

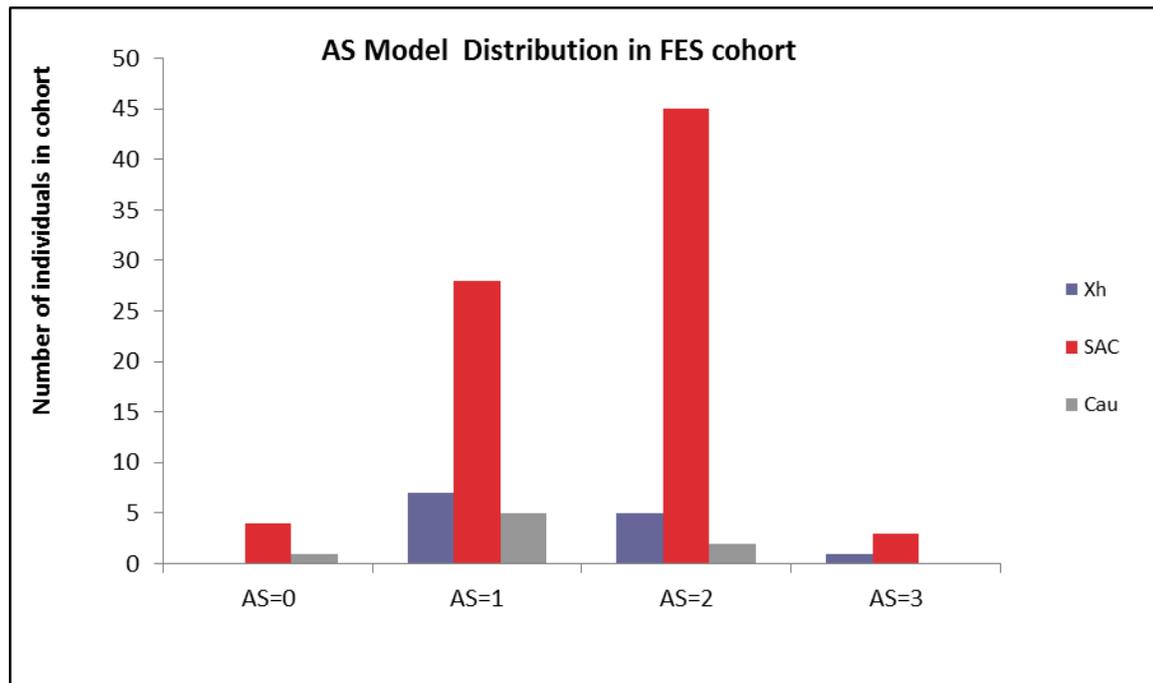
## CHAPTER 4: Results

**Table 11. Frequency comparisons for *CYP2D6* alleles in the SA FES populations and the Human *CYP* Allele Nomenclature populations**

Alleles	Populations from SA FES cohort			Populations from the Human <i>CYP</i> Allele Database			
	Cau	SAC	Xh	AF	AM	AS	EU
rs3892097 (*4)	0.25	0.09	0.08	0.04	0.11	0.00	0.19
deletion (*5)	0.13	0.13	0.19	0.06	0.02	0.06	0.03
rs5030655 (*6)	0.06	0.00	0.00	0.00	0.00	0.00	0.01
rs72549356 (*40)	0.00	0.01	0.00	0.01	0.00	0.00	0.00
duplication (*1xN)	0.00	0.03	0.08	0.01	0.01	0.00	0.01
<i>CYP2D6</i> *4xN	0.00	0.01	0.04	0.01	0.01	0.00	0.00

SA: South African; *CYP*: Cytochrome P450; FES: first episode schizophrenia cohort; Cau: FES Caucasian; SAC: FES South African Coloured; Xh: FES Xhosa; AF: African; AM: American; AS: Asian; EU: European

The *CYP2D6* activity score distribution for each population in the FES cohort is depicted in Figure 5, where most individuals for all three FES populations were assigned the value 1 and 2, denoting IM/EM phenotypes. Only a small number of individuals (less than 10) were classed as PMs (AS = 0) and UMs (AS = 3). None of the Xhosa individuals displayed PM phenotypes and none of the Caucasian individuals exhibited UM phenotypes.



**Figure 5: Individuals in SA FES cohort classed into metabolizer groups based on Activity Score. AS: Activity score; FES: first episode schizophrenia; Xh: Xhosa; SAC: South African Coloured; Cau: Caucasian**

## CHAPTER 4: Results

**4.3 Association Tests****4.3.1 SNP associations with Treatment Outcome**

Changes in PANSS scores across twelve months, measured at regular intervals throughout this time period, were used to assess treatment outcome. Tests were performed for genotyped SNPs, calculated *CYP2D6* activity scores, and additive allelic models of the SNPs and the changes in PANSS scores over twelve months, adjusting for age, gender, proportion ancestry and baseline PANSS scores, where  $P \leq 0.01$  was regarded as significant. Table 12 shows the significant  $P$  values in the highlighted cells obtained from the mixed model repeated measures analyses for the tests of association between individual SNPs and a change in PANSS scores over time. Apart from the significant associations observed between variants in *COMT* and *DRD2* and change in PANSS negative scores, additional variants surpassing the significance threshold were considered for nominal associations ( $P \leq 0.05$ ) (Table 12). The effect estimates of these SNP associations were further investigated (Table 13) to determine the mode of inheritance that best fitted these significantly associated variants. The results from the complete data set can be found in Appendix E1 and Appendix E2. None of the significant associations survived Bonferroni corrections for multiple testing.

From the fifteen SNPs investigated in the *COMT* gene, statistical data revealed the significant associations for two of the SNPs with changes in PANSS negative scores after twelve months of treatment (Table 12), a synonymous variant (rs4633) and intronic variant (rs9332377). Both these variants acted in an additive manner. In the case of rs4633, each copy of the T allele was associated with an improved treatment outcome for PANSS negative scores [ $P = 0.0080$ , ES = -0.17 95% CI (-0.30 to -0.04)], while statistical analysis of rs9332377 revealed that each copy of the T allele of this variant was associated with worse treatment outcome [ $P = 0.0006$ , ES = 0.25 95% CI (0.11 to 0.39)] (Table 13).

Two of the five SNPs present in the upstream region (rs1799732) and intron 1 (rs1079597) of *DRD2* were also significantly associated with PANSS negative scores when the genotypic model was used. These associations were not observed when allelic models were investigated (Table 12). Interestingly, further analysis of the “best fit” inheritance model revealed that the genotypic model remained the “best fit” for rs1799732. In the case of rs1799732, when compared to C/C homozygous individuals, heterozygous individuals (C- vs CC) exhibited improved treatment of PANSS negative scores [ $P = 0.0004$ , ES = -0.19 95% CI (-0.38 to 0.00)], while homozygous del/del individuals (-- vs CC) showed worsening of PANSS negative scores each week after treatment [ $P = 0.0004$ , ES = 0.50 95% CI (0.15 to 0.86)] (Table 13). Further examination of

## CHAPTER 4: Results

rs1079597 revealed that this variant acted in a recessive manner and was associated with a poor treatment outcome for PANSS negative scores [ $P = 0.0020$ , ES = 0.93 95% CI (0.34 to 1.53)] (Table 13).

Unadjusted patterns for the significantly associated SNPs with changes in PANSS scores were determined and are illustrated with graphs in Appendix F.

**Table 12: Association tests with  $P$  values from mixed model repeated measures analyses for tests of association between genotyped SNPs and additive allelic model for the same SNPs and change in PANSS scores**

SNPs	PANSSN	PANSSP	PANSSG	PANSST
<b><u>Significant Associations (<math>P &lt; 0.01</math>)</u></b>				
Genotypic Model				
<b><u>COMT</u></b>				
rs9332377	0.0026	0.0861	0.1187	0.0674
<b><u>DRD2</u></b>				
rs1799732	0.0004	0.7079	0.0759	0.0282
rs1079597	0.0082	0.6399	0.5887	0.1965
Additive Allelic Model				
<b><u>COMT</u></b>				
rs4633 (T)	0.0080	0.9516	0.3482	0.1769
rs9332377 (T)	0.0006	0.9638	0.3841	0.1174
<b><u>Nominal Associations (<math>P &lt; 0.05</math>)</u></b>				
Genotypic Model				
<b><u>COMT</u></b>				
rs737865	0.8019	0.0201	0.0423	0.0955
rs6269	0.0569	0.0433	0.1517	0.0356
<b><u>HTR2A</u></b>				
rs6311	0.0298	0.2801	0.0842	0.0442
<b><u>CYP1A2</u></b>				
rs762551 (*1F)	0.0101	0.7254	0.1983	0.2226
Additive Allelic Model				
<b><u>CYP2D6</u></b>				
<i>CYP2D6</i> *1xN (duplication)	0.5831	0.0148	0.0233	0.0617
rs3892097 (A)	0.6003	0.0281	0.9670	0.5438

PANSSP: Positive symptoms total; PANSSN: Negative symptoms total; PANSSG: General psychopathology total; PANSST: Total score for all questions

## CHAPTER 4: Results

Table 13: Significant *P* values from inheritance models estimating percentage change in PANSS scores after adjusting for covariates

SNP	Outcome	<i>P</i> -value			Mode of inheritance	Comparison	% Change per week (95% CI)
		Genotype	Allelic	"Best"			
<b><u>Significant Associations (<math>P \leq 0.01</math>)</u></b>							
<b><u>COMT</u></b>							
rs4633	PANSSN	0.0293	0.0080	0.0080	Additive T	Each T allele	-0.17 (-0.30 to -0.04)
rs9332377	PANSSN	0.0026	0.0006	0.0006	Additive T	Each T allele	0.25 (0.11 to 0.39)
<b><u>DRD2</u></b>							
rs1799732	PANSSN	0.0004	0.3669	0.0004	Genotype	-C vs CC -- vs CC	-0.19 (-0.38 to 0.00) 0.50 (0.15 to 0.86)
rs1079597	PANSSN	0.0082	0.1131	0.0020	Recessive	AA vs AG+GG	0.93 (0.34 to 1.53)
<b><u>Nominal Associations (<math>P \leq 0.05</math>)</u></b>							
<b><u>COMT</u></b>							
rs737865	PANSSP	0.0201	0.0495	0.0092	Dominant	AG+GG vs AA	0.32 (0.08 to 0.56)
<b><u>HTR2A</u></b>							
rs6311	PANSSN	0.0298	0.0975	0.0170	Dominant	AA+AG vs GG	-0.22 (-0.40 to -0.04)
	PANSST	0.0442	0.1268	0.0258	Dominant	AA+AG vs GG	-0.19 (-0.37 to -0.02)
<b><u>CYP1A2</u></b>							
rs762551	PANSSN	0.0101	0.0125	0.0027	Dominant	AC+CC vs AA	-0.28 (-0.46 to -0.10)
<b><u>CYP2D6</u></b>							
<i>CYP2D6*1xN</i>	PANSSP	0.0302	0.0148	0.0091	Dominant	XX+IX vs II	-0.60 (-1.05 to -0.15)
	PANSSG	0.0374	0.0233	0.0118	Dominant	XX+IX vs II	-0.44 (-0.77 to -0.10)
rs3892097 (*4)	PANSSP	0.0806	0.0281	0.0281	Additive A	Each A allele	0.26 (0.03 to 0.49)

PANSSP: Positive symptoms total; PANSSN: Negative symptoms total; PANSSG: General psychopathology total; PANSST: Total score for all questions; X: gene considered as duplicated; I: gene considered as wild-type

Haplotype analyses were also performed for *COMT* and *DRD2*, as well as for the genes showing nominal associations ( $P \leq 0.05$ ) in Table 12 for *HTR2A*, *CYP1A2* and *CYP2D6*. LD plots for *COMT* and *DRD2* are presented in Appendix G. Due to the large number of SNPs present in *COMT*, haplotype analyses including all the variants genotyped in this gene could not be performed for association testing. For this reason, a smaller region was therefore selected for haplotype analysis. This region included the SNPs that showed significant associations from the mixed model repeated measures analyses performed previously (rs2020917-rs737865-rs6269-rs4633-rs9332377). The haplotypes displaying significant associations with changes in PANSS scores and their corresponding effect sizes are shown in Table 14. The results of the full analyses are shown in Appendix E3. Three main haplotypes were examined for the five investigated SNPs, two of which were associated with a poor treatment outcome for PANSS negative scores (AAACT and CAGCT haplotypes). One of these haplotypes (CAGCT) was also associated with a poor treatment outcome for PANSS total scores. The third haplotype was associated with an improvement in PANSS positive scores (GAATC haplotype) after the twelve months. Further analyses for haplotypes demonstrating nominal associations from Table 12 ( $P \leq 0.05$ ) are shown in Table 15.

**Table 14. *COMT* haplotype associations with percentage change in PANSS scores, estimating effect sizes**

rs2020917	rs737865	rs6269	rs4633	rs9332377	Frequency	Phenotype	P-value	% Change per week (95% CI)
A	A	A	C	T	0.03	PANSSN	0.003	0.2 (0.6 to 1.0)
C	A	G	C	T	0.07	PANSSN	<0.000	0.3 (0.5 to 0.8)
						PANSSST	0.006	0.1 (0.3 to 0.6)
G	A	A	T	C	0.02	PANSSP	0.003	-6.4 (-3.9 to -1.4)

*DRD2* haplotypes, using the significant associations of regions identified previously, did not reveal any associations with changes in PANSS scores that were significant ( $P \leq 0.01$ ). However, one haplotype, *HTR2A.CA* (rs6311-rs6313) was shown to be significantly associated with PANSS general scores and improved treatment outcome. Effect sizes for haplotypes that met the  $P \leq 0.01$  cut-off are shown in Table 15.

**Table 15. Haplotype associations with percentage change in PANSS scores, estimating effect sizes of nominal associations**

Haplotype	Phenotype	P-value	% Change per week (95% CI)
<i>HTR2A.CA</i>	PANSSG	0.0030	-0.67 (-1.10 to -0.23)
	PANSSST	0.0104	-0.55 (-0.98 to -0.13)
<i>CYP1A2.GC</i>	PANSSN	0.0125	-0.18 (-0.31 to -0.04)
<i>CYP2D6.IIADI</i>	PANSSP	0.0131	0.35 (0.07 to 0.62)

## 5 CHAPTER 5: DISCUSSION

Schizophrenia poses a great socio-economic burden in SA and SA populations are understudied with regards to pharmacogenetic research of psychiatry (Dodgen *et al.*, 2013; de Wet *et al.*, 2015). This study was aimed at investigating polymorphisms in several pharmacogenes identified from the literature (*COMT*, *CYP1A2*, *CYP2D6*, *DRD2*, *DRD3*, *HTR2A* and *SOD2*) in a SA FES cohort. In addition, the variation present in these genes was further examined by identifying novel functionally relevant variants within these pharmacogenes by using exome data from a previous study performed by Drogemöller *et al.* (2014a). Variants selected for the study were then genotyped in the FES cohort and minor allele frequencies (MAF) were compared to other worldwide populations. Lastly, variants were evaluated for association with treatment outcomes by looking at changes in PANSS scores across twelve months (adjusting for age, gender, ethnicity and baseline PANSS scores). Thus, this study played an important role in both the characterization of the variation present in antipsychotic pharmacogenes in a South African population, while also determining the relevance of these variants for antipsychotic pharmacogenetics applications.

### 5.1 Study Design

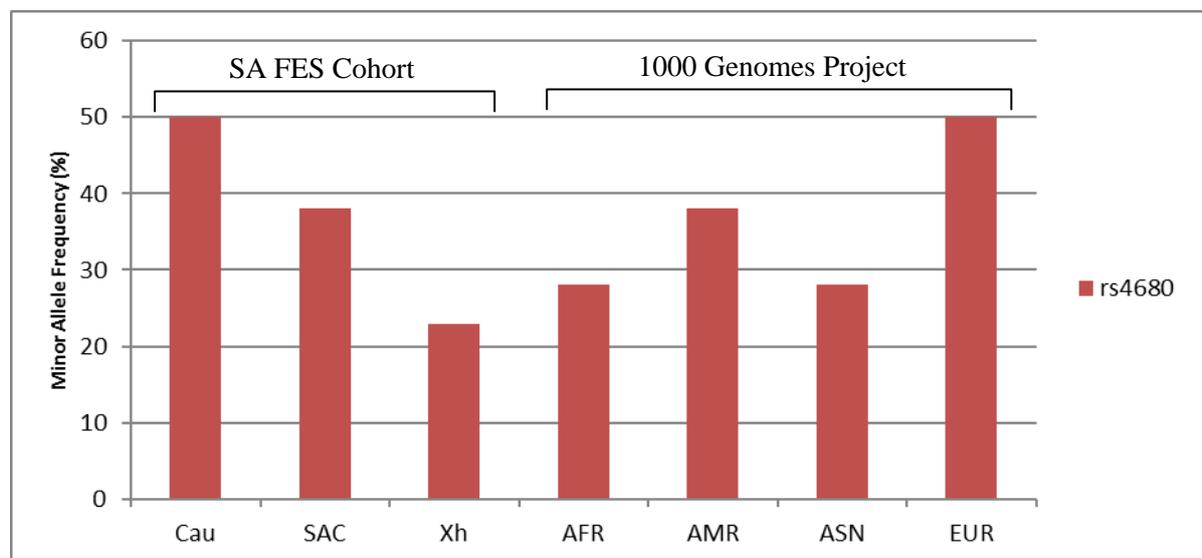
A number of drawbacks have been reported with regards to the implementation of pharmacogenetic studies of antipsychotic medication to identify genetic variants that are of relevance to the clinical context (refer to Table 1). These include poor replication of findings, lack of significant associations, clinical heterogeneity and low contribution of single-marker genetic variants (Zhang and Malhotra, 2013). Hence, comprehensive study designs need to be implemented to advance the field of antipsychotic pharmacogenetics in order to overcome these issues. Taking all these considerations into account, this study utilized a well-defined FES cohort and although the cohort was relatively small, comprising of only 103 individuals, it has some important advantages for pharmacogenetic research. The value of this cohort in terms of pharmacogenetic research is emphasized by the lack of confounding factors, such as inconsistencies in diagnosis and course of illness, which are eliminated by the fact that the individuals in the cohort are experiencing their first episode of psychosis. In fact it has been reported that a 50% greater effect size was observed for the association of the *DRD2* polymorphism, rs17997323, with antipsychotic treatment response in patients experiencing their first episode of schizophrenia when compared to chronic patients (Zhang *et al.*, 2010). Additional benefits of the use of this cohort include the elimination of the effects of prior antipsychotic drug exposure for majority of the patients, and due to the fact that these patients receive their medication via injection, reliable and accurate results regarding adherence can be obtained for this study.

## 5.2 Genetic Variation Contributing to Schizophrenia Treatment in SA

This study investigated variants within seven pharmacogenes identified from literature that have previously been shown to be implicated in schizophrenia pharmacogenetics within the past five years. Novel functional variants in the candidate pharmacogenes were identified from previously generated exome data and were included in the study. Prioritized variants were genotyped and assessed to distinguish unique genetic profiles in SA populations. To our knowledge, this is the first study to provide frequency data for these variants in a unique South African FES population, including many patients from the SAC population. Thus, these data have provided valuable information pertaining to schizophrenia pharmacogenetic research in the context of South Africa.

### Genetic Variation between SA and Worldwide Populations

When the MAFs of the investigated variants in the SA populations were compared to other populations, most of the MAF showed a similar pattern across populations. Populations with similar ancestry had approximately similar frequencies while admixed populations displayed intermediate frequencies between populations (Table 10). For instance, the FES Caucasian and European populations showed similar frequencies, as well as the FES Xhosa and African populations, as would be expected. This trend is illustrated in Figure 6 using *COMT* rs4680 as an example.



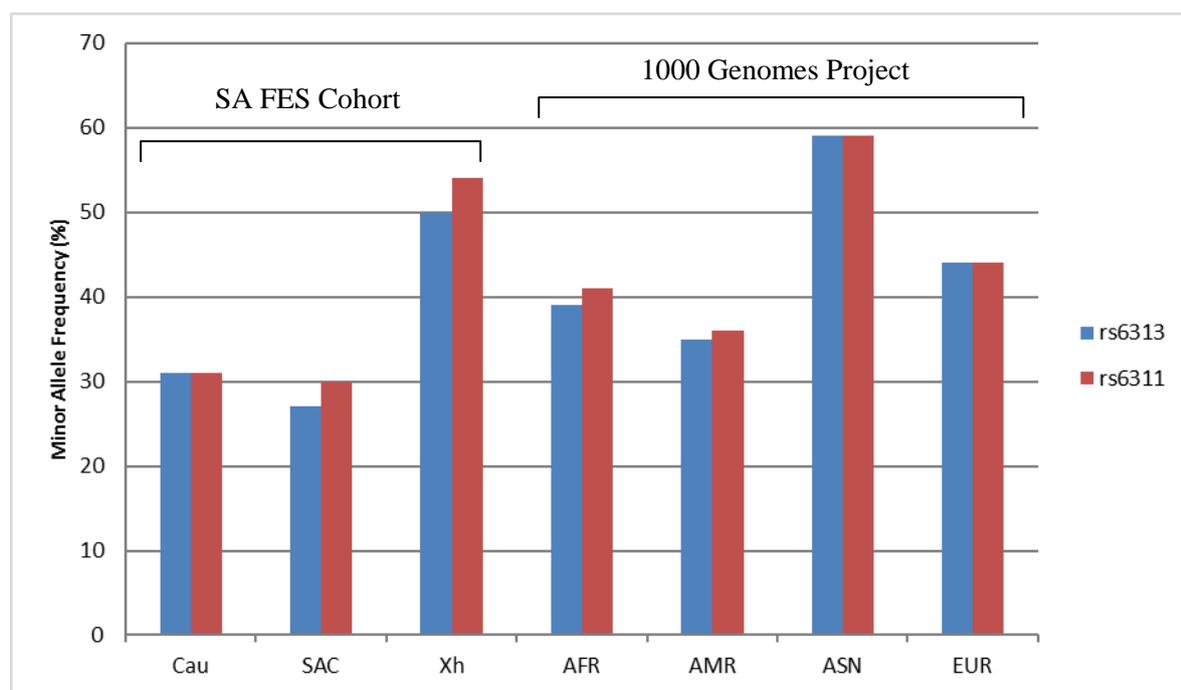
**Figure 6. Minor allele frequency comparisons of *COMT* rs4680, between different populations in the SA cohort and populations from the 1000 Genomes Project**

As seen in the case of *COMT* rs4680, SA Caucasians and Europeans displayed similar frequencies for the variant (A) allele (50%). This allele was shown to be less prevalent in populations with African ancestry (20-30%). The SAC displayed intermediate frequencies between the other two SA populations (~40%), as did the admixed Americans between Europeans and Africans. Investigating the frequencies of the *COMT* rs4680 SNP in previous studies revealed that this low activity A allele (Met) have been shown to occur from as low as 1% in South Americans to as high as 62% in

European and South-western Asians, where the high activity Val allele was associated with dysfunctional frontal cognition due to high levels of dopamine (Gupta *et al.*, 2009). The high variability of this functionally relevant variant in global populations further supports the evidence that different ethnic groups may exhibit distinctive genetic variation patterns in genes of relevance to pharmacogenetics. This once again highlights the value of performing studies which examine the diverse populations residing within South Africa.

Although the abovementioned pattern of variation was observed for the majority of SNPs investigated in this study, the admixed populations did not exhibit intermediate allele frequencies for all variants examined. With regards to *DRD2* rs1079597, the SAC and AMR exhibited a much higher MAF when compared to the other populations, most likely due to Asian contributions as the Asian population had the highest MAF of 41%. These frequencies obtained for the FES SAC seem to be plausible due to the Asian ancestry components that they carry, which have been described by Daya *et al.* (2013). However, FES Xhosa and Caucasian frequencies are derived from very small sample sizes and are therefore not the most accurate representations in all cases.

An additional important consideration in terms of differences between populations groups is differences in the patterns of LD. The commonly studied *HTR2A* SNPs in pharmacogenetic research for schizophrenia, rs6313 and rs6311 have been reported to be in complete LD (Ghadirivasfi *et al.*, 2011; Reynolds *et al.*, 2014). However, our results reported differences in MAF between these two SNPs in the FES SAC and Xhosa populations, as well as the 1000 Genomes African and American populations (Figure 7). The rest of the populations showed identical MAFs for both of these SNPs. This suggests that these SNPs are only in perfect LD in European and Asian populations, but are not in perfect LD in populations of African descent. This is not unexpected, as individuals with African ancestry have lower LD compared to the other worldwide populations due to the fact that these populations are more ancient and have thus had more time for recombination to occur and LD to diminish over time (McEvoy *et al.*, 2011). Careful consideration therefore needs to be given when investigating these SNPs in populations of African descent because assuming LD can result in inaccurate allele assignment, and tag SNPs for Caucasian studies may not be applicable in African studies.



**Figure 7. Minor allele frequency comparisons of *HTR2A* rs6313 and rs6311, between different populations in the SA cohort and populations from the 1000 Genomes Project**

### Rare and Unique Genetic Variation Profiles in SA

Novel functional exonic variants were identified in *COMT* and *SOD2* in our cohort (Table 9). The novel SNP in *COMT* (rs373611092) occurred in the SAC population. Only one individual carried this mutation and was heterozygous for this SNP, hence limiting its implementation for current pharmacogenetic applications. The SNP thus had a frequency of 1% in the SAC population and was predicted by PolyPhen-2 to have probable damaging effects on the translated product as it results in a missense mutation, Met90Val, being situated approximately 1000 bp downstream from rs4680. The novel missense mutation in *SOD2*, rs372173830, results in the Glu133Asp substitution, and was predicted *in silico* as benign or tolerated to the resulting protein by Polyphen-2 and SIFT algorithms, respectively. This variant was detected in heterozygous samples in both our Xhosa (N = 1) and SAC (N = 8) populations with frequencies of 4% and 5% respectively.

Possible new *CYP* alleles may also point to unique patterns of genetic variation that exist in SA. However, due to the lack of the reliability of NGS technologies for these genes with high sequence similarities, *CYP* variants were not investigated further (Drögemöller *et al.*, 2013). Previous studies by our group, which have utilized specially designed genotyping assays to investigate the variation in the *CYP* genes, have consistently identified novel variants. However, these variants occur at very low frequencies and are thus not likely to contribute substantially to antipsychotic treatment response outcomes (Gaedigk *et al.*, 2007; Drögemöller *et al.*, 2010; Wright *et al.*, 2010; Drögemöller *et al.*, 2013). Nonetheless, the presence of novel variants in SA populations highlights the importance of re-sequencing these under-studied populations as these novel variants could play a potential role in pharmacogenetic applications. As more and more rare variation is detected we will improve our

capabilities to account for the effects of these variants and prediction algorithms will improve (Drögemöller *et al.*, 2014a).

The *CYP2D6* variants that were chosen for genotyping had a higher frequency in our cohort (Table 11) when compared to the frequencies of populations worldwide retrieved from the Human *CYP* Allele Nomenclature Database (Table 11), which consisted of much larger sample sizes. The presence of the duplication (\*1xN) and deletion (\*5) of *CYP2D6* was quite prevalent in our Xhosa sample. Conversely, the loss-of-function alleles (\*4 and \*6) that have been reported to be mostly prevalent in Europeans (Sistonen *et al.*, 2009), demonstrated higher frequencies in our small Caucasian cohort when compared to the European populations.

Similar trends are reported on indigenous African populations in a previous study performed by Sistonen *et al.* (2007), by which the South-eastern and South-western Bantu and San populations showed a frequency of 18.8% and 14.3% respectively for *CYP2D6*\*5, corresponding with our results. These results are also consistent with the results obtained from a later study from the same research group that reported a frequency of 12.5% for *CYP2D6*\*5 in a SA Bantu population and frequencies ranging between 3.3-6.6% for populations in the rest of the African continent (Sistonen *et al.*, 2009). Although this allele is rare in European and Asian populations, it is quite evident that this allele is more prevalent in African populations, specifically Southern African populations.

Other *CYP2D6* alleles in our cohort were also compared with the frequencies observed in other studies in SA populations, i.e. Xhosas by Wright *et al.* (2010) and SACs and Caucasians by Gaedigk and Coetsee (2008) (Figure 8). These frequencies demonstrate *CYP2D6*\*5 to be quite prevalent in populations with a large African component and *CYP2D6*\*4 with European components. Frequencies for *CYP2D6*\*1xN also seem to be more prevalent in African populations but Gaedigk and Coetsee (2008) did report a small proportion presented (0.3%) in their Caucasian cohort. Although the same trend is seen with variants in the same population group, some proportions differ slightly, most likely due to the size of the cohorts and therefore this still needs further investigation in larger cohorts.

Discrepancies between the frequencies observed in SA cohorts and those of the 1000 Genomes Project which has indexed the human variome by the utilization of next-generation sequencing, could be a result of *CYP2D6*'s inability to sequence well. Drögemöller *et al.* (2013) has demonstrated that variation in *CYP* genes cannot be well captured by these NGS technologies due to genomic complexities of GC rich regions, high sequence similarities and presence of pseudogenes. Although NGS technologies have remarkably advanced the field of pharmacogenetics and pharmacogenomics, alternative genotyping strategies are still required for capturing unique variation in complex pharmacogenes, such as *CYP2D6*. Improvement in these NGS technologies will also be required to address the current shortcomings.

### **Inferring CYP2D6 Metabolizer Phenotypes using the AS Model**

It is important to consider the overall CYP2D6 activity by comparing all the alleles in conjunction to establish metabolizer phenotype profiles. Hence, the AS is an important parameter to consider when investigating the combined effect of all allele-defining variants. An AS of 0 is denoted by individuals characterized as PMs and an assigned value greater than 2 is an indication of UM since the individual would carry a functional allele together with a functional duplication of the gene (Gaedigk *et al.*, 2007). Population sizes for our Caucasian and Xhosa samples were too small to accurately infer metabolizer phenotypes using the AS. Examination of the SAC population revealed that 5% of this cohort were PMs (Figure 5). Interestingly, Dandara *et al.* (2001) also found PMs to occur at low frequencies in the SA Venda population (2.6%) due to the presence of the non-functional *CYP2D6\*4* allele, and a PM frequency of 2.3% in psychiatric Tanzanian patients accounted by the prevalence of the reduced function *CYP2D6\*17* allele, which was not genotyped in our cohort. This study reported that populations closely related to the Bantu tribes in SA were likely to exhibit similar frequencies of PMs, which was confirmed by the current study. Frequencies for UMs in the SACs were shown to occur at 4%, which is consistent with frequencies of 5% for this same population in the study by Gaedigk and Coetsee (2008).

The majority of individuals from our cohort belonged to the IM/EM category (35-56%) (Figure 5). The AS association study should however be replicated in a larger cohort by comparing interethnic frequencies for the *CYP2D6* alleles and improving classification of these variants in order to document the different frequencies observed between populations groups. Treatment dosages can therefore be optimized once the AS is calculated and patients will be better identified by their relative metabolizing phenotypes and be treated accordingly. By adapting treatment dosages, a more desirable treatment outcome can be achieved for PMs and UMs since deficient treatment regimens can be eliminated. For instance, PMs will need dose reductions of about 50-70% for FGAs and antidepressants, where UMs will need adjustments of up to 150% due to its rapid clearance, usually making these patients non-responsive to the medication and also resulting in toxic adverse effects (Swen *et al.*, 2011). However, no association with the AS and treatment response was observed in this study, most likely due to the fact that not all alleles were examined. Additionally, CYP2D6 may be more involved in toxicity (Teh and Bertilsson, 2012), which was not examined in this study, than efficacy. Borges *et al.* (2010) implemented the AS model in a study to predict CYP2D6 metabolizer phenotypes to tamoxifen response. They reported the AS to be twice as effective in predicting the exposure to drugs, but also showed that concomitant treatment seems to confound the prediction using this system. Implementation of the AS system to predict phenotype outcome will be of a great advantage within the clinical setting if *CYP2D6* variation is well elucidated, but it is also important to consider factors that are involved in drug exposure, which will influence the predicted phenotype.

CHAPTER 5: Discussion

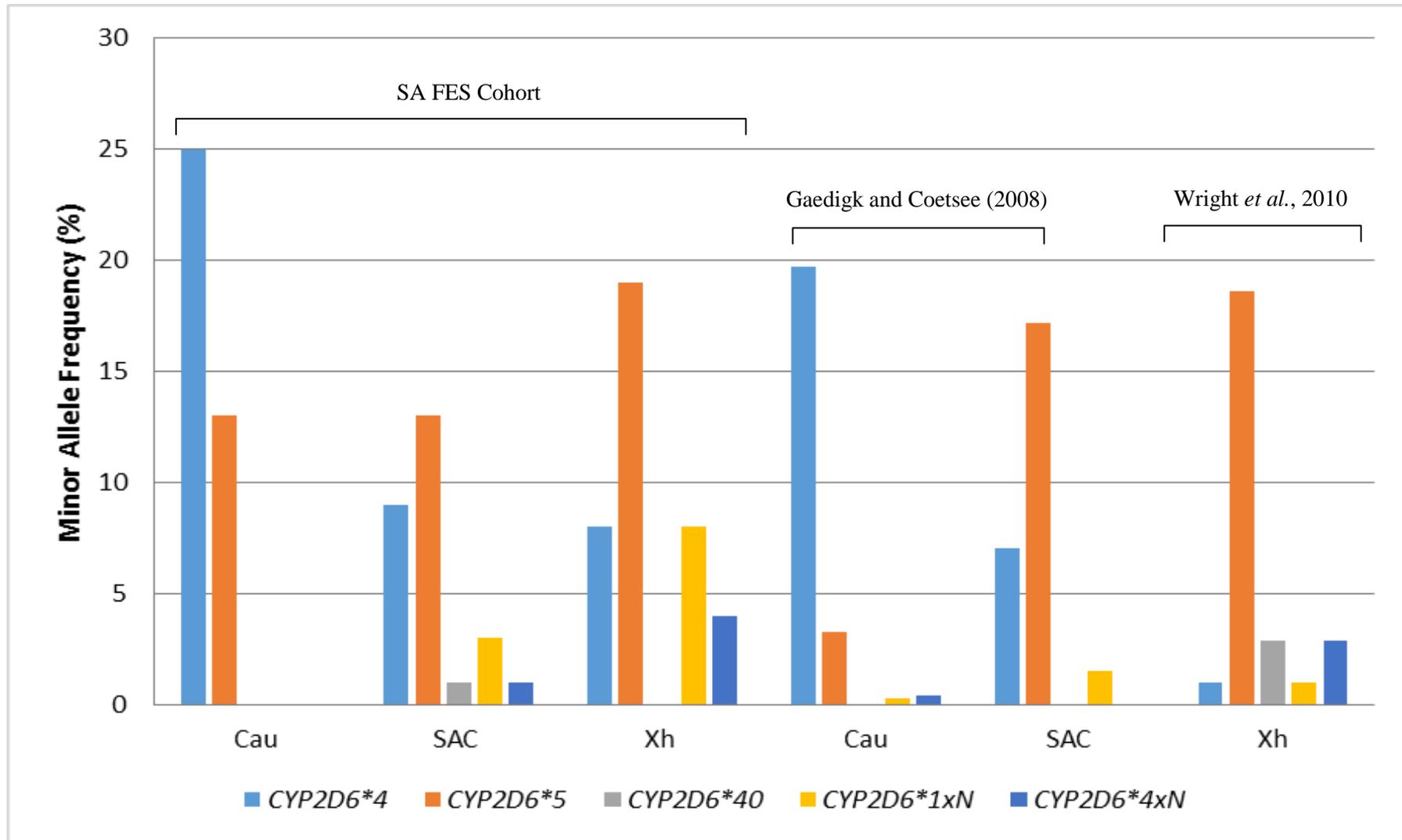


Figure 8. Minor allele frequency (%) comparisons for *CYP2D6* variants between the SA FES cohort and different Southern African populations obtained from literature

## CHAPTER 5: Discussion

**5.3 Significant Associations of Pharmacogene Variation and Antipsychotic Treatment Response**

A huge concern with antipsychotic treatment of schizophrenia is that the medication is only effective in ~50% of the patients (Lohoff and Ferraro, 2010). Trial and error methods have been used to treat schizophrenia patients, which have resulted in poor treatment administrations due to the inter-individual variation presented in treatment response profiles (Gardner *et al.*, 2014). Understanding these variations and studying symptom categories separately can allow patients to be grouped in a more homogenous manner than the conventional DSM-5 method, whilst recognizing that the polymorphisms associated with treatment outcome can direct the establishment of improved treatment and management. As a result this could eventually lessen the communal burdens associated with mental health systems that SA is currently faced with.

Aberrant neurotransmission circuitries have shown evidence for being involved in the pathophysiology of schizophrenia (Lisman *et al.*, 2008), highlighting the importance in understanding the complex networks affecting brain function on which antipsychotic medication target to alleviate psychotic symptoms. This study investigated a large amount of variation using several genotyping strategies to examine the contribution of genetics in the context of a well-characterized cohort of SA FES patients. This study therefore demonstrated the important roles of *COMT* and *DRD2* with regards to PANSS negative scores and treatment outcome. These findings are quite interesting as it fits in well with the dopamine hypothesis and FGAs that act on dopamine receptors (Changasi *et al.*, 2014), providing further substantiation with the recognized biological mechanism of action in schizophrenia. The most significant associations with polymorphisms in these genes varied with regards to (i) direction of response and (ii) effect sizes. This once again highlights the complexity of antipsychotic treatment response outcomes. Figure 9 gives an indication of the genetic variants playing a role in the treatment of negative symptoms each week, using flupenthixol decanoate after twelve months, with *HTR2A* and *CYP1A2* playing more minor roles in the treatment of negative symptoms.

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	SIGNIFICANT ASSOCIATIONS ( $P \leq 0.01$ )		NOMINAL ASSOCIATIONS ( $P \leq 0.05$ )	
Improved Treatment Outcome	rs4633 (synonymous)	rs1799732 (intronic): -C vs CC	rs6311 (upstream variant)	rs762551 (intronic)
	<i>COMT</i>	<i>DRD2</i>	<i>HTR2A</i>	<i>CYP1A2</i>
Worse Treatment Outcome	rs9332377 (intronic)	rs1799732 (intronic): -- vs CC rs1079597 (intronic)		

Figure 9. Associations with genetic polymorphisms and treatment outcome of negative symptoms after twelve months of treatment with flupenthixol decanoate

The results in this study showed that the *COMT* gene was significantly associated with the treatment of negative symptoms. Our findings suggest the T allele of *COMT* rs4633 contributes to the improvement of negative symptoms [ $P = 0.0080$ , ES = -0.17 95% CI (-0.30 to -0.04)]. Although the role of the rs4633 SNP is unclear in pharmacogenetics, recent studies have commonly investigated this SNP with regards to haplotypes of rs4680 and rs4818 which have been associated with *COMT* activity for mental disorders in Europeans and Asians (Htun *et al.*, 2014; Li *et al.*, 2014). Wang *et al.* (2009) reported the T allele for rs4633 to be significantly associated with paranoia ( $P = 0.004$ ) and hallucinations ( $P = 5.141 \times 10^{-5}$ ) in paranoid schizophrenic patients, possibly induced by the increased *COMT* expression. In a later study, this same research group reported the rs4633-rs4680 haplotype to be associated with an increase in severity of total negative symptom scores as well as subcategories of negative symptoms, which included affective flattening ( $P = 0.003$ ), asociality ( $P = 0.011$ ), apathy ( $P = 0.007$ ) and emotional withdrawal ( $P = 0.003$ ) (Wang *et al.*, 2010). The rs4633 SNP is a synonymous variant that have previously been reported to be in complete LD with rs4680 in Europeans but not Chinese patients (Voisey *et al.*, 2012). It has been shown to influence *COMT* expression that could lead to increased *COMT* activity and hence, decreased dopamine levels (Bray *et al.*, 2003; Nackley *et al.*, 2006; Wang *et al.*, 2009). This phenomenon could explain the improvement of negative symptoms observed in our study due to the hypo-frontality and decreased dopamine neurotransmission (Potkin *et al.*, 2002; Shi *et al.*, 2014). In another study, Gupta *et al.* (2009)

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found rs4633 to be significantly associated with a good response to risperidone in schizophrenia patients using allelic models [ $P = 0.036$ , ES = 1.80 95% CI (1.03-3.15)].

The *COMT* rs4680 SNP is very well-studied and often found in literature with regards to genetic studies performed on schizophrenia. Our study did not replicate the significant findings that most recent pharmacogenetics studies have found regarding its association with treatment outcome in schizophrenia even though this SNP was quite prevalent in our cohort and its occurrence corresponded with populations worldwide (Figure 6). These previous studies reported significant associations with the low activity Met (A) allele and worse treatment outcome of negative symptoms for schizophrenia (Pelka-wysiecka *et al.*, 2013; Green *et al.*, 2014; Bosia *et al.*, 2015). Wright *et al.* (2012) also reported the lack of significant associations of *COMT* rs4680 with schizophrenia pathophysiology in a Xhosa cohort highlighting the need to investigate African populations independently in pharmacogenetic research in order to develop unique genotyping strategies on these populations. The lack of association found for rs4680 by Wright *et al.* (2012) and our study could indicate that the variation in LD in African populations have helped to show that rs4633 is rather the causal SNP for African populations ( $D' = 1$ , See Appendix G).

In contrast to the findings for rs4633, rs9332377 showed allelic associations with worsening of negative symptoms after treatment [ $P = 0.0006$ , ES = 0.25 95% CI (0.11 to 0.39)] with the T allele. Although only limited association studies for schizophrenia pharmacogenetics research are available for this SNP, it has previously been reported to be associated with other negative effects of treatment outcome, namely cisplatin-induced ototoxicity, presenting more common in Africans than Europeans (Drögemöller *et al.*, 2011; Aminkeng *et al.*, 2014), thus highlighting the role of this SNP in developing ADRs with treatment outcome. Nieratschker *et al.* (2010) also investigated rs9332377 in Germans for association with negative symptoms of schizophrenia and recruited 631 schizophrenia patients and 207 nuclear families. However, no significant associations were detected for this large cohort after correcting for multiple testing. This SNP have also been associated with anger in a study investigating suicidal behaviour in Germans and Italians ( $P = 0.03$ ) (Calati *et al.*, 2011), suggesting it may be involved in certain psychopathological symptom domains in mental disorders. This SNP also showed an involvement with its C allele and worsening of positive symptoms in the *COMT*.GAATC (rs2020917-rs737865-rs6269-rs4633-rs9332377) block; and therefore may be of particular relevance to African populations. Further *COMT* haplotype analyses also revealed improvement of PANSS negative scores (Table 14) for two of the haplotype blocks, AAAC and CAGCT by

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which the latter showed strong associations [ $P < 0.00$ , ES = 0.30 95% CI (0.50 to 0.80)]. This haplotype block was also associated with improved PANSS total scores.

Additionally, our study revealed nominal associations with worsening of treatment outcome for rs737865 and rs6269 (Table 12), where negative symptoms showed associations with worse treatment for the latter ( $P = 0.0206$ ). Although it is clear that COMT is responsible for the degradation of dopamine in the prefrontal cortex (Chen *et al.*, 2004), these results indicate the important role that *COMT* plays in the pathophysiology of schizophrenia, which needs to be well elucidated in order to improve treatment outcome. *COMT* also demonstrates cardinal influence on negative symptomology within South African schizophrenia populations. Evidence for this can be supported by significant findings from a previous study that utilized a South African Xhosa population and reported significant associations of *COMT* variation (rs45536341, rs6269 and rs4633) with negative symptom severity and suggests that variants in LD within this gene, specifically in the P2 promoter region, play a role in negative symptom severity in African populations (Wright *et al.*, 2012). This relates to our findings which show the involvement of *COMT* variation with negative symptoms in SA populations.

Dopamine receptors play a fundamental role in the regulation of neural circuitries responsible for the pathological symptoms of schizophrenia, which is important to understand in order to develop future drug discovery attempts (Howes *et al.*, 2012), especially due to its relevance of being targeted by FGAs. Not only were variants in *COMT*, which is involved in the metabolism of dopamine, significantly associated with PANSS negative scores, but our results also pointed towards *DRD2*'s considerable involvement with treatment outcomes for negative symptoms. However, the directions of effects for these associations are ambiguous. The commonly studied rs1799732 SNP involved in translational activity of the gene (Mi *et al.*, 2011), was shown to exhibit both improved and worsening of negative symptoms with genotypic models. The -/C vs C/C genotypes showed an improvement to treatment outcome after twelve months [ $P = 0.0004$ , ES = -0.19 95% CI (-0.38 to 0.00)], where -/- vs C/C genotypes were more strongly associated with a worse treatment outcome [ $P = 0.0004$ , ES = 0.50 95% CI (0.15 to 0.86)]. A meta-analysis study conducted by Zhang *et al.* (2010) reported six studies (N = 698) to show a worse antipsychotic treatment outcome for del carriers compared to C/C ( $P = 0.03$ ), concluding that del carriers contributed to a worse outcome, which is in line with what was observed for homozygous del individuals, but not heterozygous individuals. Additionally, the fact that the del allele has been reported in the literature to be associated with a poorer treatment response (Arranz *et al.*, 2013) further supports the need to investigate this variant in more SA populations to determine

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the exact role that this variant plays in antipsychotic treatment outcomes and larger cohorts are needed to tease out the intricacies of the detected association.

A further significant association was identified for the intronic variant, *DRD2* rs1079597, involved in decreased D<sub>2</sub> receptor density that display irregular dopamine levels in the striatum (Vijayan *et al.*, 2007). Our results showed association for rs1079597 with worsening of negative symptoms each week after twelve months of treatment [ $P = 0.0020$ , ES = 0.93 95% CI (0.34 to 1.53)]. Recessive models indicated AA vs AG+GG genotypes to contribute to an undesirable treatment outcome. In line with these results, unadjusted models also evidently illustrate the decline in PANSS negative scores (Figure 10) with the AG+GG genotypes, confirming that the A (B2) allele contributes to a poor treatment outcome. A recent study showed that the G (B1) allele demonstrated improved treatment outcome of negative symptoms after six weeks of treatment in Korean schizophrenia patients using both genotypic ( $P = 0.017$ ) and haplotype analysis (rs1079597-rs1800497: T-C associated with poor response to negative symptoms,  $P = 4.11 \times 10^{-7}$ , permuted  $P = 0.014$ ) (Kang *et al.*, 2015). In an earlier study, the variant allele A (B2) was associated with better clozapine response in African Americans but the association was absent in Caucasians (Hwang *et al.*, 2005). Discrepancies in these findings could be a result of frequency and LD variation between different ethnicities, which accounts for inter-individual variation for treatment response. The opposing direction of the effects observed in our study may also be explained by the different drugs used, since clozapine has a very different mechanism of action as compared to flupenthixol decanoate (Zhang and Malhotra, 2013).

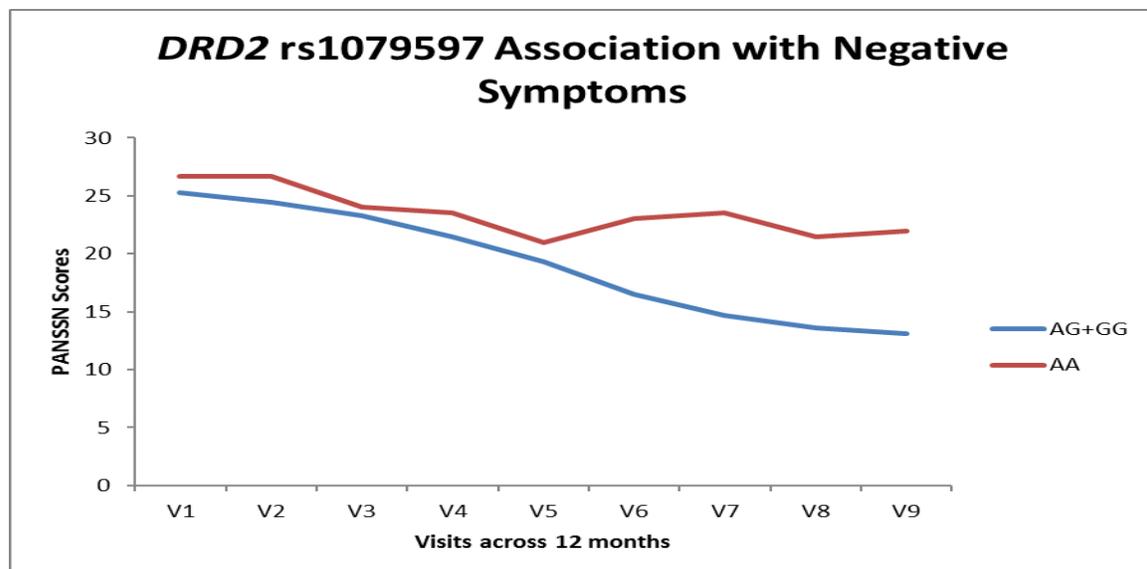


Figure 10: *DRD2* rs1079597's association with negative symptoms after twelve months of treatment with flupenthixol decanoate

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Additionally, nominal associations have been illustrated with improved treatment outcome for negative symptoms in *HTR2A* (rs6311) and *CYP1A2* (rs762551) ( $P \leq 0.05$ ) where further haplotype investigations unveiled more findings for these genes (Table 15). Variation in the *HTR2A* gene is important in pharmacogenetic research of schizophrenia and plays a role in treatment response since many antipsychotics target these receptors that are involved in dopamine circuitries (Blasi *et al.*, 2015). In this study, although rs6311 was associated with improved negative symptoms after treatment, haplotype analyses of the two SNPs in *HTR2A* (rs6313-rs6311) revealed that the CA block showed significant associations to PANSS general ( $P = 0.0030$ ) and total ( $P = 0.0104$ ) scores, also showing an improvement to treatment. This shows how *HTR2A* may be involved in various symptom domains. This also further supports the notion that haplotype analyses are necessary for these SNPs because they are not in complete LD in SA populations as demonstrated from the MAF results. Chen *et al.* (2009) performed a study on a Han Chinese population suffering from schizophrenia and found that the rs6313-rs6311 haplotype with CC/GG genotypes predicted the influence of negative symptoms on treatment outcome with aripiprazole. In another study performed on a Caucasian population from Moscow, the CC genotype for rs6313 showed to be more prevalent with positive, negative and general symptoms in patients suffering from major psychoses (Golimbet *et al.*, 2002). This highlights the role that *HTR2A* plays in regulating drug plasma levels of SGAs, which in turn reduces negative symptoms and corresponds with our results depicting how these SNPs affect symptoms in our cohort by its improvement after treatment. However, the minor associations found for this gene could be related to the smaller role of *HTR2A* in FGA response (Reynolds *et al.*, 2014).

*CYP1A2* is involved in the metabolism of many antipsychotic drugs and demonstrates a wide range of inter-individual variability (Perera *et al.*, 2012). The *CYP1A2* rs762551 variant is readily inducible and leads to an increased *CYP1A2\*1F* activity which would ultimately lead to decreased plasma levels of antipsychotic drugs (Changasi *et al.*, 2014). In this study, rs762551 (*CYP1A2\*1F*) was associated with changes in negative symptoms where patients carrying the C allele were more inclined towards showing treatment improvement of negative symptoms per week after twelve months [ $P = 0.0027$ , ES = -0.28 95% CI (-0.46 to -0.10)]. The haplotype block containing the C allele, *CYP1A2.GC*, also showed an improvement with negative symptoms after twelve months of treatment [ $P = 0.0125$ , ES = -0.18 95% CI (-0.31 to -0.04)], providing further evidence of rs762551's C allele playing a role in the treatment of negative symptoms. Similar to

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our results, Balibey *et al.* (2011) reported that the A allele was associated with poor clozapine response and that AA genotypes were associated with treatment resistance of clozapine.

This study revealed no associations with treatment outcome for *DRD3* and *SOD2*, and only marginal associations with *CYP2D6* and changes in positive scores after treatment (Table 12). However, further replicative studies are warranted, especially for the nominal associations found in this study. Although many of these results did not survive correction for multiple testing, these associations are replications of robust findings from previous studies and thus the corroboratory evidence provided by this study shows that these variants are likely to be true positive findings, especially since this study used a well-characterized cohort. The key finding for this study suggests that *COMT* and *DRD2* play vital roles in the treatment of negative symptoms, as these genes are crucial in regulating dopamine neurotransmission and further supports the dopamine hypothesis, indicating its ideal candidacy in studying and understanding schizophrenia pathogenesis and treatment. These findings for *COMT* and *DRD2* are very valuable since negative symptoms are generally not well treated by antipsychotic medication (Abou-Setta *et al.*, 2012). By identifying variants that will improve our understanding of the negative symptom domain, we can develop better strategies to treat these symptoms and alleviate ineffective treatment regimens. Since SA and many third-world countries still use FGAs to treat patients, these countries will strongly benefit from studies like ours that will aid in improving treatment with FGAs.

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This study provided important pharmacogenetic data for under-represented populations in South Africa, where health care institutes are faced with restricted or inadequate mental health resources and services. This study therefore met its aim and objectives by genotyping a large number of polymorphisms in several pharmacogenes associated with antipsychotic treatment outcomes in a SA FES cohort. Polymorphisms were successfully genotyped for *COMT*, *CYP1A2*, *CYP2D6*, *DRD2*, *DRD3*, *HTR2A* and *SOD2* in the entire cohort of 103 FES individuals using several different genotyping strategies. The allele and genotype frequencies enabled us to distinguish the differences between SA populations, particularly the SAC population, and those from African, American, East Asian and European populations from the 1000 Genomes Project and the Human *CYP* Allele Nomenclature Database, indicating unique genetic diversity present in SA populations. Association analyses were successfully performed to establish if the polymorphisms investigated in this study are associated with treatment outcomes by looking at the changes in PANSS scores across twelve months of treatment.

The exome data obtained from a previous study by Drögemöller *et al.* (2014a) enabled us to successfully identify novel variants that were unique to our cohort. These novel functional variants in *COMT* and *SOD2* were successfully genotyped in the entire cohort. This highlights the unique genetic profile that SA populations have which distinguishes them from populations around the world. Successful genotyping strategies were performed for these novel variants and they can therefore be further investigated in future studies of SA populations. However, these variants occurred at a very low frequency and therefore a lack of significant findings was associated with these variants due to reduced statistical power. Although rare variation demonstrates evidence for playing a role in treatment response, next-generation sequencing technologies should further be advanced to capture these variants as it might demonstrate important inferences in future pharmacogenetic research (Drögemöller *et al.*, 2014b).

Furthermore, the minor allele frequencies for most of the SNPs investigated from our populations showed correlation with frequencies in populations from the 1000 Genomes project, where the FES Caucasian group was similar to Europeans, and Xhosas similar to Africans, indicating evidence of common ancestry. The SAC population showed intermediate frequencies between the Caucasians, Africans and Asians, illustrating the contribution from various ancestries and making this a unique admixed group that needs careful attention when evaluating their treatment response profiles. These frequencies suggest that we need to take into account that studies performed on

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SA populations should be done independently from other populations because of our unique diversity.

Our data revealed promising evidence for the *COMT* and *DRD2* variants with negative symptoms of schizophrenia after antipsychotic treatment. This study successfully identified associations of dopaminergic polymorphisms involved in the treatment of negative symptoms of schizophrenia symptoms after twelve months of treatment with flupenthixol decanoate. Variation in *COMT* (rs4633) and *DRD2* (rs1799732) displayed positive treatment outcomes. It is quite difficult to treat negative symptoms and therefore this study provided important data that could unravel clues underlying the treatment of negative symptoms. Lack of significant findings for *COMT* rs4680 in both this study and by Wright *et al.* (2012) further supports unique profiles for SA populations in pharmacogenetic research, even though this SNP was observed at similar frequencies to the worldwide populations from the 1000 Genomes Project database. This suggests that variation in SA populations compared to worldwide populations should be treated differently as they might result in differential drug response phenotypes.

This study has enabled a guideline for researchers to discover clues underlying the intricate nature of schizophrenia aetiology in SA populations by enhancing treatment efficacy, which will ultimately lead to the improvement of mental health systems. Pharmacogenetic research has been advancing in recent years, but further studies need to be performed on these under-studied cohorts to aid the facilitation of developing tailored treatment in the future which will show a significant improvement of drug efficacy between 10-15% and decrease toxicity between 15-20% (Arranz *et al.*, 2013).

### **6.1 Limitations and Future Directions**

It is important to incorporate environmental factors into a study since these largely affect the outcome of schizophrenia pathophysiology as well (Duan, 2003). Environmental factors can include factors like smoking status, alcohol or drug abuse, past traumatic experiences, living environment, caffeine consumption, etc. and many of these factors have been implicated in the variability of drug response and metabolism of schizophrenia in past studies (Perera *et al.*, 2012; Schmitt, 2014). By incorporating these factors into a study, it might be possible to understand the mechanism involving the disease progression, aetiology, and inter-individual variability of treatment response, and thus finding the link between genetics and the environment will enable us to predict treatment outcome more efficiently. Therefore, limitations to this study include the lack of investigation of environmental factors as this may help explain the complexity of some of the findings, e.g. those associated only with specific genotype classes.

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This study only focused on a number of pharmacogenes important for antipsychotic medication and a more comprehensive list is reported by Arranz and de Leon (2007), Foster *et al.* (2010) and Kohlrausch (2013). Drögemöller *et al.* (2013) investigated pharmacogenetic variation in South African populations, and also provides a list of important genes involved in antipsychotic medication. All variants contributing to antipsychotic efficacy is, however, not yet known and future prospective studies may identify additional genes that could be screened and investigated.

As South African populations are both unique and understudied, this study has played an important role in providing information to aid in understanding the sequence diversity of SA populations in order to overcome the mismanagement of antipsychotic treatment in SA health sectors. However, due to the highly polymorphic nature of *CYP2D6* alleles, consisting of over 100 alleles (Sim and Ingelman-Sundberg, 2010), this study only examined a few selected clinically relevant alleles. In order to accurately infer the CYP status of patients as it is important to take into account all alleles influencing metabolizer phenotypes that will affect the statistical significance (Almoguera *et al.*, 2013). As mentioned previously, better genotyping strategies for *CYP* alleles or advancement in sequencing technologies would aid in the development of pharmacogenetic applications due to the unique genetic profiles for SA. Due to this unique variation, it may be required to sequence SA individuals who do not respond to treatment. A good option would be to utilize Sanger sequencing as the data obtained for the 11 FES individuals demonstrated the presence of rare novel variants in the SAC population. This option would not only be cost-effective in a resource-limited setting as compared to whole genome sequencing, but also have the potential to identify rare variants unique to SA individuals (Drögemöller *et al.*, 2013).

Other limitations include small cohort sizes, especially for Caucasian and Xhosa patients and to date, there is no replicative cohort available for this study. Although this study is in itself a replication cohort as the variants investigated in this study have already been previously associated with treatment outcomes, further replicative studies are necessary in future to provide corroborating evidence for the exact phenotypes investigated in this study. Larger SA cohorts are needed, especially for the different ethnicities in order to tease out some of the complexities observed with regards to the direction and mode of inheritance of the significant associations. Populations should be evaluated separately, such as the various Bantu tribes in SA, Caucasians from European ancestry, as well as the highly admixed Coloured population. It is therefore recommended to repeat these studies in more SA cohorts.

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Our study only included a small region for *COMT* haplotype analyses due to the inability of the statistical model to compute a quantitative variable of PANSS scores changes with a large set of polymorphisms in this gene and only examined the effects of single genes. Development of complex statistical modelling may therefore provide further insight into associations with variation in the human genome and phenotypes being assessed. This will also be useful for identifying associations with variations across multiple genes and complex biological networks, as well as its interaction with environmental factors, that all contribute to the aetiology of complex diseases like schizophrenia and subsequent treatment response.

Pharmacogenetics studies like this one would benefit from investigating a large number of variants with treatment response. Again this emphasizes the need for alternative advanced genotyping technologies that can generate high-throughput data from genetic sequences. Not only will this allow for effective genotyping of complex regions within the genome, but it will also be able to give accurate results in a short period of time, making genotyping applications more feasible. Ultimately results from these studies could be developed to such a level that a pharmacogene SNP array or similar test could be designed that would be unique to the populations from SA, having a great advantage for clinicians to treat patients effectively based on unique genetic profiles. This research promises to further enhance the field of pharmacogenetics by bringing about the revolution for personalized medication for SA individuals and improved mental healthcare. Although big international studies like the Psychiatric Genomics Consortium (<http://www.med.unc.edu/pgc>), the International Schizophrenia Consortium (<http://pngu.mgh.harvard.edu/isc/>) and various pharmacogenetic studies are underway in aiming to elucidate schizophrenia aetiology and improve treatment, results from this study can provide information to these large-scale studies on African populations. This study has played an important role in providing much needed information for these under-represented cohorts, however, larger studies using high-throughput genotyping strategies will continue to add to this knowledge base.

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## 8 APPENDIX A

*Supplementary Table*

Table S 1: Components of all reagents in PCRs

Preparation of Reagents used for PCR			
<b>TBE Buffer</b>	<b>Cresol Loading Buffer</b>	<b>DMSO</b>	<b>Agarose Gel</b>
10x Buffer:	Stock:	15% DMSO:	1.5% - 4% (w/v) gels:
➤ 108g TRIS <sup>a</sup>	➤ 10mg Cresol powder <sup>a</sup>	➤ 85µl dH <sub>2</sub> O	➤ 1.5g – 4g Agarose powder <sup>b</sup>
➤ 55g Boric Acid <sup>a</sup>	➤ 1ml dH <sub>2</sub> O	➤ 15µl 100% DMSO <sup>a</sup>	➤ 100ml 1x TBE Buffer <sup>a</sup>
➤ 7.44g EDTA <sup>a</sup>	Loading Buffer:		➤ 5µl Ethidium Bromide <sup>a</sup>
➤ 1L dH <sub>2</sub> O	➤ 3.4g sucrose <sup>c</sup>		
1x Buffer:	➤ 9.5ml dH <sub>2</sub> O		
➤ 100ml 10x TBE Buffer <sup>a</sup>	➤ 200µl Cresol stock <sup>a</sup>		
➤ 900ml dH <sub>2</sub> O			

<sup>a</sup>Reagents supplied by Sigma-Aldrich (Pty) Ltd.<sup>b</sup>Reagent supplied by SeaKem®<sup>c</sup>Reagent supplied by Merck Chemicals (Pty) Ltd.

9 APPENDIX B1



Figure S 1: Bi-directional Sanger sequencing for *COMT* rs37361102

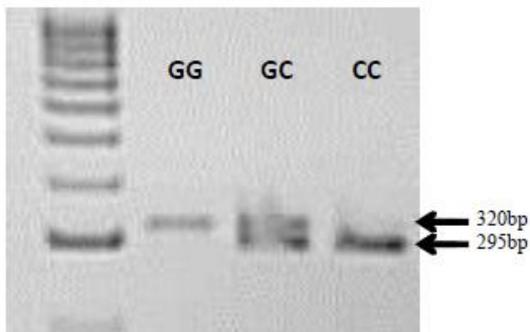
10 APPENDIX B2

*Electrophoresis: Genotyping*

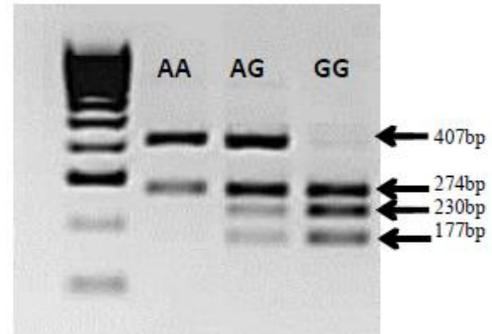
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**COMT**

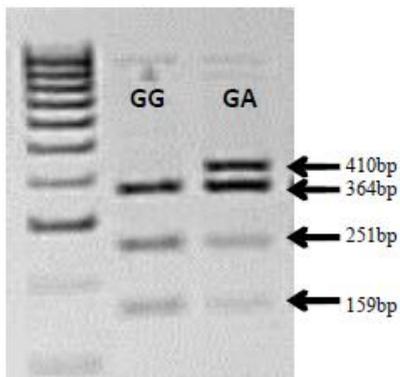
rs9606186



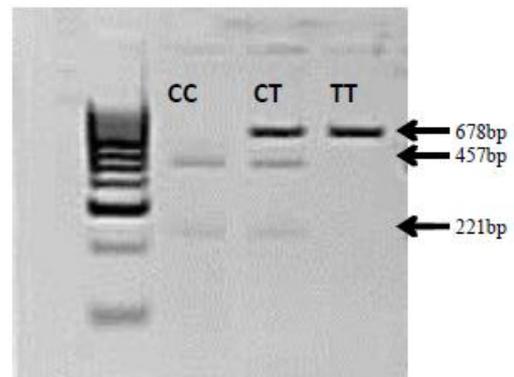
rs2075507



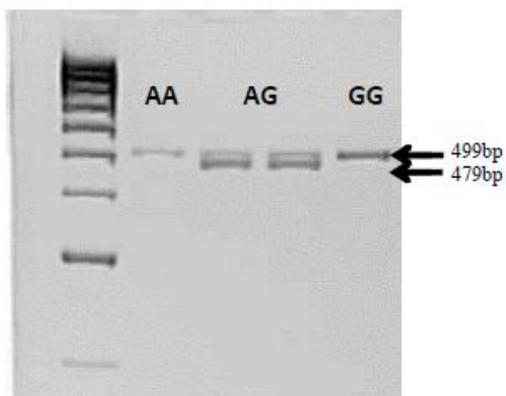
rs45551837



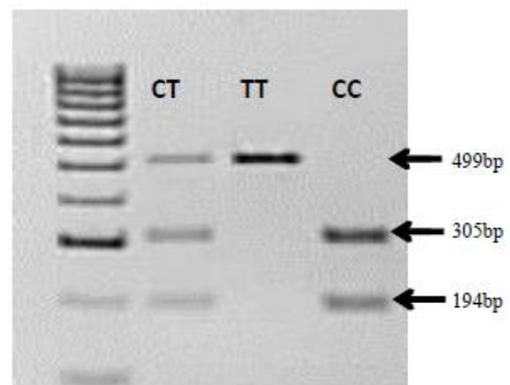
rs45536341



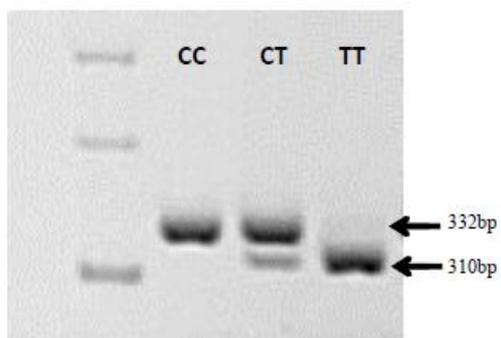
rs6269



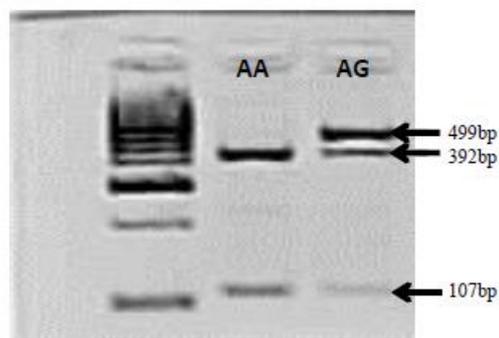
rs4633



rs9332377

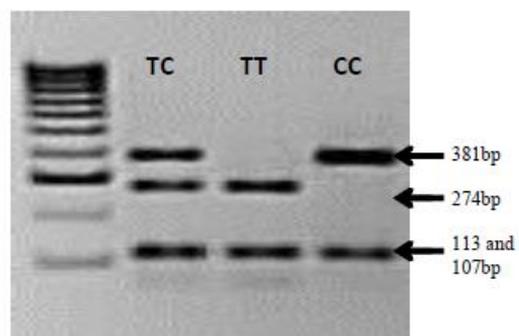


rs373611092

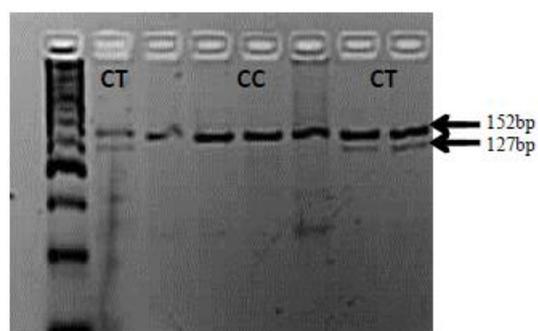


DRD2

rs6275

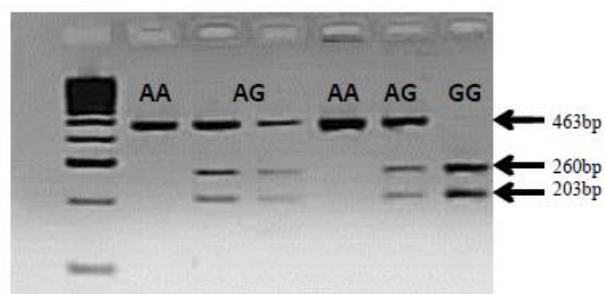


rs6277



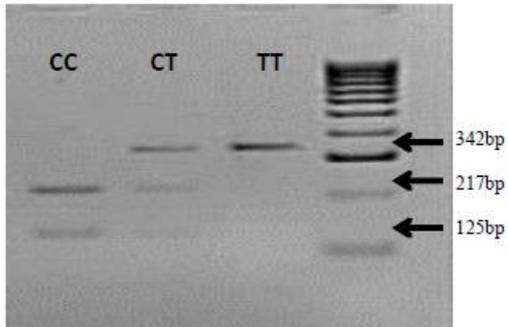
DRD3

rs6280

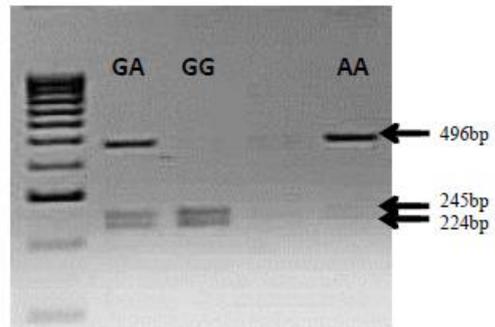


**HTR2A**

rs6313

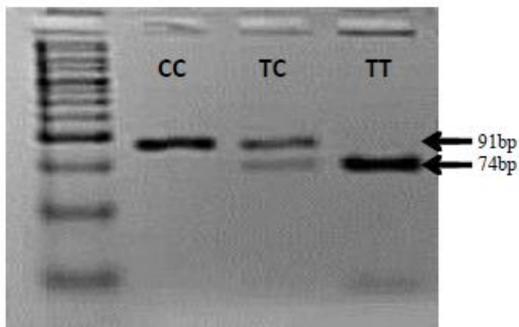


rs6311

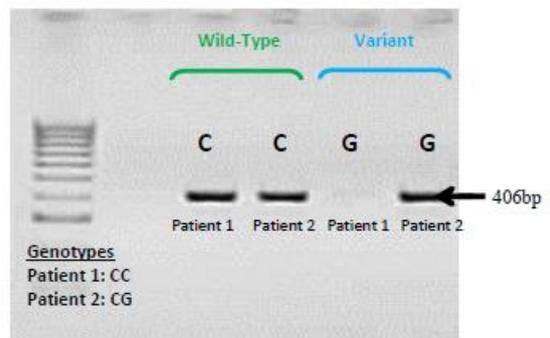


**SOD2**

rs4880

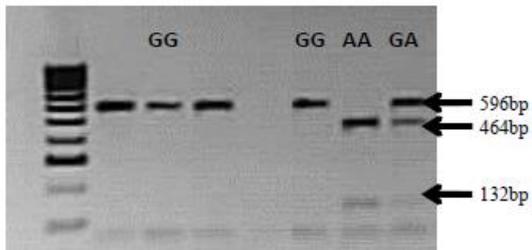


rs372173830 (Allele-specific PCR)

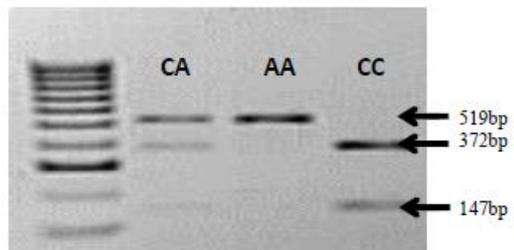


**CYP1A2**

*CYP1A2*\*1C (rs2069514)

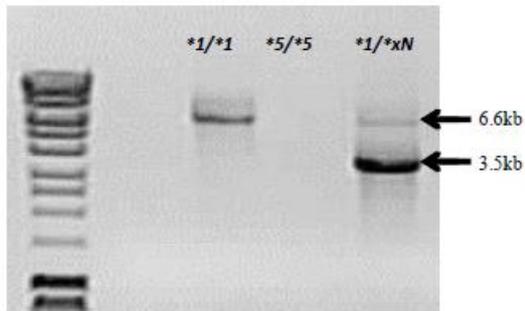


*CYP1A2*\*1F (rs762551)

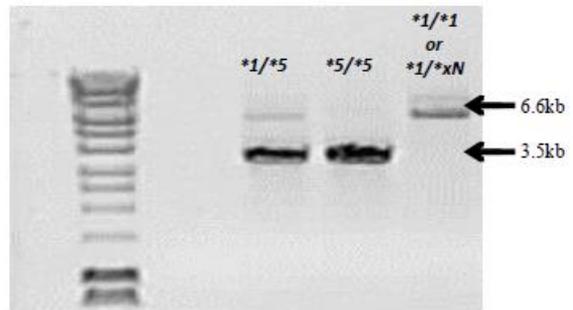


***CYP2D6* (Long-range PCR)**

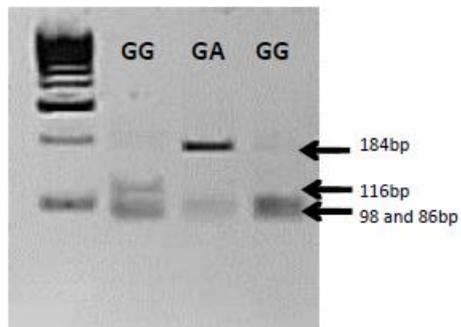
*CYP2D6*<sup>\*xN</sup> (Duplication)



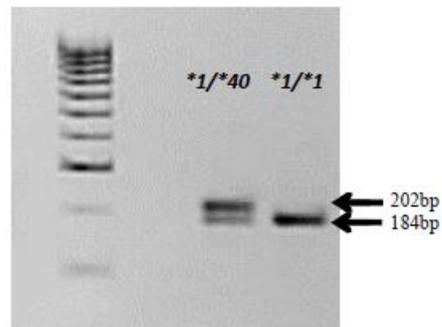
*CYP2D6*<sup>\*5</sup> (Deletion)



*CYP2D6*<sup>\*4</sup>



*CYP2D6*<sup>\*40</sup> (PCR)



*CYP2D6*<sup>\*6</sup>

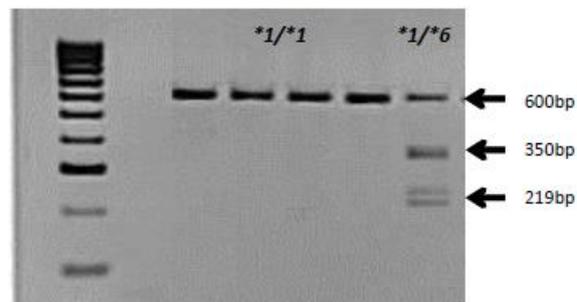


Figure S 2: Electrophoresis results for the genotyping of SNPs in pharmacogenes

11 APPENDIX B3

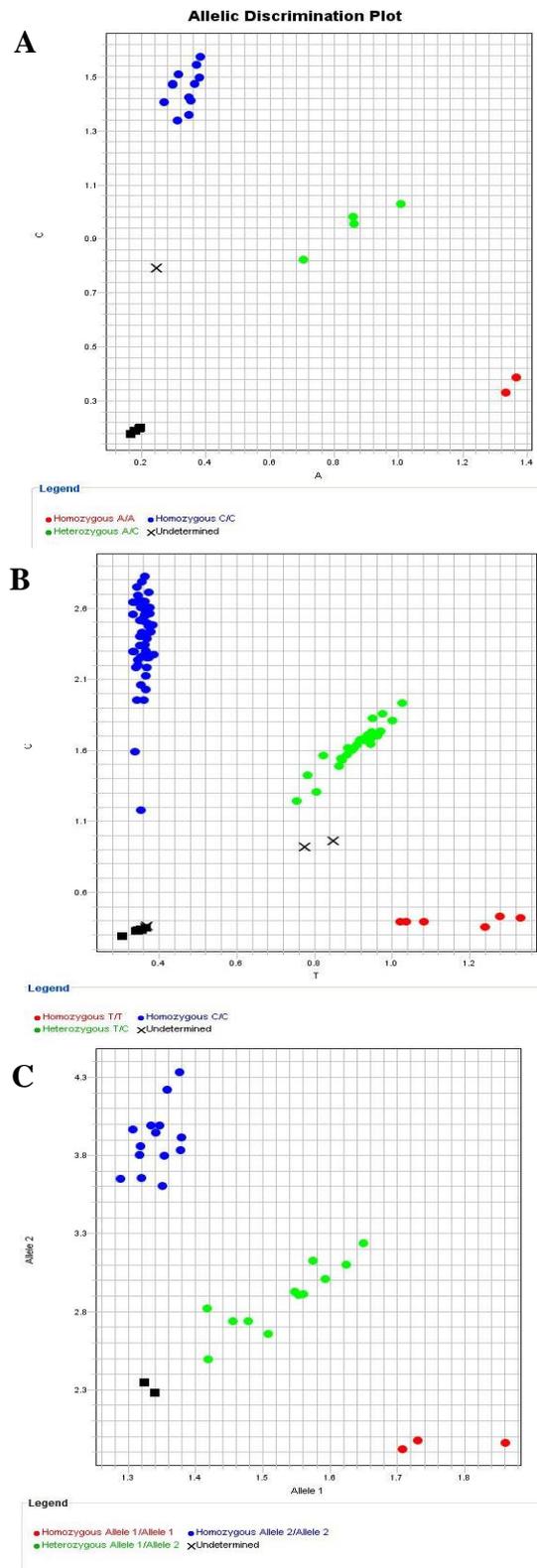


Figure S 3: TaqMan SNP genotyping results for (A) *COMT* rs5748489; (B) *COMT* rs2020917 and (C) *DRD2* rs6277

12 APPENDIX C

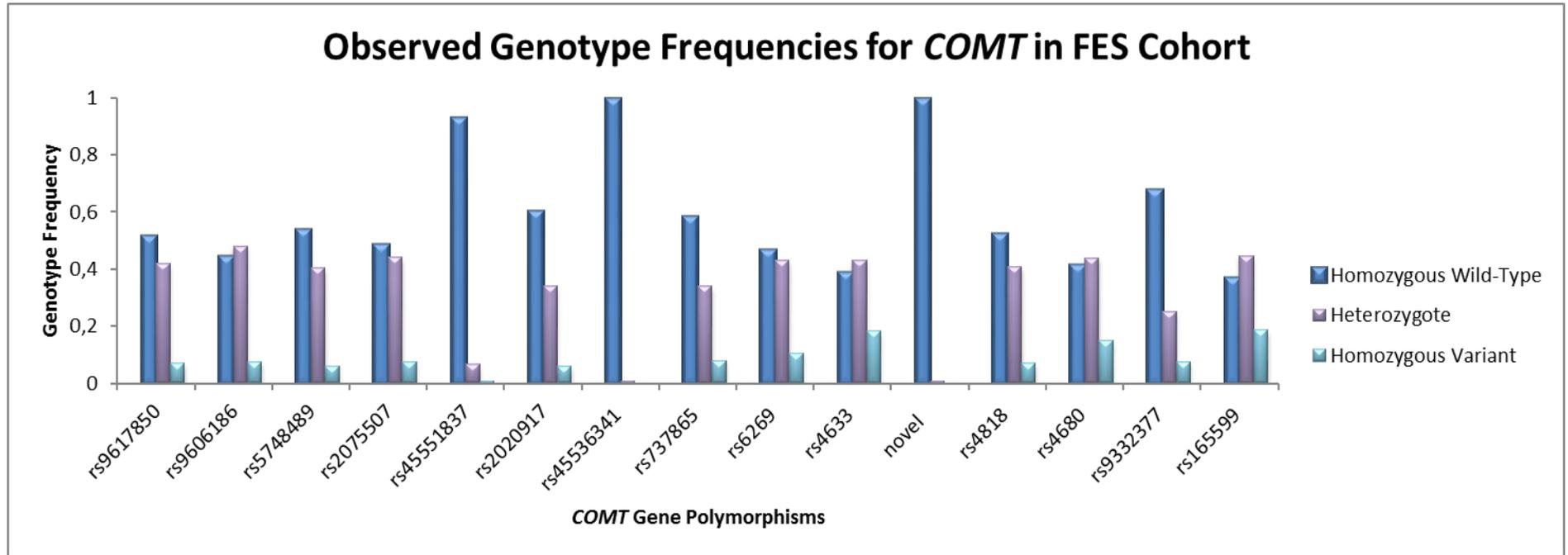


Figure S 4: Genotype frequencies of cohort

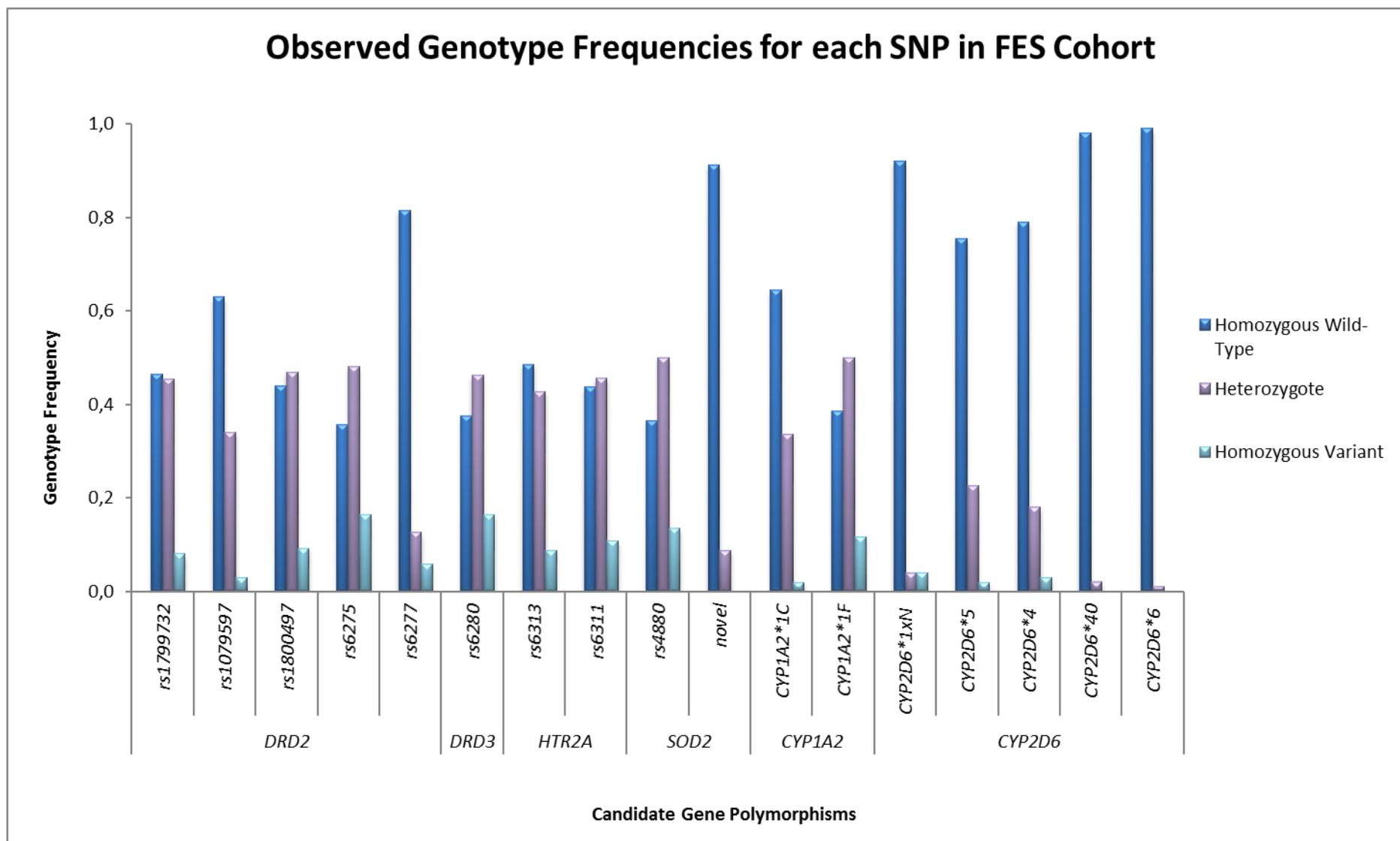


Figure S 5: Genotype frequencies of cohort

## 13 APPENDIX D

Table S 2: Probability testing for Hardy Weinberg Equilibrium distribution across different populations in the FES cohort

GENE	POLYMORPHISM	POPULATION	P-value
<i>COMT</i>	rs9617850	Cau	1.00
		SAC	0.56
		Xh	1.00
	rs9606186	Cau	0.49
		SAC	0.32
		Xh	0.51
	rs5748489	Cau	0.49
		SAC	1.00
		Xh	1.00
	rs2075507	Cau	0.49
		SAC	1.00
		Xh	0.52
	rs45551837	Cau	NA
		SAC	1.00
		Xh	0.23
	rs2020917	Cau	1.00
		SAC	1.00
		Xh	0.13
	rs45536341	Cau	NA
		SAC	1.00
		Xh	NA
	rs737865	Cau	1.00
		SAC	0.77
		Xh	0.12
	rs6269	Cau	0.20
		SAC	0.62
		Xh	0.51
	rs4633	Cau	0.20
SAC		1.00	
Xh		0.17	
rs373611092	Cau	NA	
	SAC	1.00	
	Xh	NA	
rs4818	Cau	0.08	
	SAC	0.27	
	Xh	0.37	
rs4680	Cau	0.20	
	SAC	1.00	
	Xh	0.52	
rs9332377	Cau	1.00	
	SAC	0.03	
	Xh	0.58	
rs165599	Cau	1.00	
	SAC	0.82	

		Xh	0.28
		Cau	1.00
	rs1799732	SAC	0.58
		Xh	0.60
		Cau	1.00
	rs1079597	SAC	1.00
		Xh	1.00
DRD2		Cau	1.00
	rs1800497	SAC	0.45
		Xh	0.58
		Cau	0.38
	rs6275	SAC	0.65
		Xh	1.00
		Cau	0.53
	rs6277	SAC	0.38
		Xh	NA
DRD3		Cau	1.00
	rs6280	SAC	1.00
		Xh	1.00
HTR2A		Cau	0.49
	rs6311	SAC	1.00
		Xh	1.00
		Cau	0.49
	rs6313	SAC	0.78
		Xh	0.28
SOD2		Cau	1.00
	rs4880	SAC	0.81
		Xh	1.00
		Cau	NA
	rs372173830	SAC	1.00
		Xh	1.00
CYP1A2		Cau	NA
	<i>CYP1A2*1C</i>	SAC	0.06
		Xh	0.51
		Cau	1.00
	<i>CYP1A2*1F</i>	SAC	0.63
		Xh	1.00
CYP2D6		Cau	NA
	<i>CYP2D6*1xN</i>	SAC	0.00
		Xh	0.23
		Cau	1.00
	<i>CYP2D6*5</i>	SAC	0.34
		Xh	1.00
		Cau	0.38
	<i>CYP2D6*4</i>	SAC	0.21
		Xh	1.00
		Cau	NA
	<i>CYP2D6*40</i>	SAC	1.00
		Xh	NA
	Cau	1.00	
<i>CYP2D6*6</i>	SAC	NA	
	Xh	NA	

## 14 APPENDIX E1

Table S 3: Association tests of *P* values from mixed model repeated measures analyses between genotyped SNPs

	PANSSN	PANSSP	PANSSG	PANSST
<b>Mixed Model Repeated Measures Analysis</b>				
<b>Genotypic Model</b>				
<i>COMT</i>				
rs9617850	0.9240	0.8454	0.6948	0.7585
rs9606186	0.2171	0.7185	0.5979	0.6065
rs5748489	0.1238	0.9078	0.3064	0.4049
rs2075507	0.2831	0.3326	0.1772	0.1931
rs45551837	0.2662	0.8988	0.5547	0.9112
rs2020917	0.2011	0.0820	0.1418	0.2362
rs45536341	0.5113	0.0777	0.8085	0.6735
rs737865	0.8019	0.0201	0.0423	0.0955
rs6269	0.0569	0.0433	0.1517	0.0356
rs4633	0.0293	0.9953	0.2191	0.2984
rs373611092	0.3912	0.8940	0.9355	0.7659
rs4818	0.8284	0.0824	0.2029	0.3322
rs4680	0.1260	0.8180	0.4094	0.6703
rs9332377	0.0026	0.0861	0.1187	0.0674
rs165599	0.2318	0.1068	0.8361	0.5637
<i>DRD2</i>				
rs1799732	0.0004	0.7079	0.0759	0.0282
rs1079597	0.0082	0.6399	0.5887	0.1965
rs1800497	0.0897	0.4111	0.3465	0.6038
rs6275	0.2332	0.5723	0.9001	0.8242
rs6277	0.0580	0.3350	0.2420	0.1140
<i>DRD3</i>				
rs6280	0.5276	0.4389	0.2030	0.2688
<i>HTR2A</i>				
rs6311	0.0298	0.2801	0.0842	0.0442
rs6313	0.5634	0.4290	0.4571	0.3965
<i>SOD2</i>				
rs4880	0.9680	0.6026	0.7806	0.7983
rs372173830	0.3553	0.0643	0.3806	0.8746
<i>CYP1A2</i>				
<i>CYP1A2*1C</i>	0.3200	0.2882	0.2578	0.3223

	PANSSN	PANSSP	PANSSG	PANSST
<i>CYP1A2*1F</i>	0.0101	0.7254	0.1983	0.2226
<b><i>CYP2D6</i></b>				
<i>CYP2D6*1xN</i>	0.1698	0.0302	0.0374	0.0689
<i>CYP2D6*5</i>	0.9191	0.8825	0.7779	0.9284
<i>CYP2D6*4</i>	0.8657	0.0806	0.8841	0.7872
<i>CYP2D6*40</i>	0.0992	0.6766	0.3042	0.3541
<i>CYP2D6*6</i>	0.5481	0.1283	0.8671	0.7293
<b><i>CYP2D6_AS</i></b>	<b>0.0993</b>	<b>0.0539</b>	<b>0.6683</b>	<b>0.7177</b>
<b>Additive Allelic Model</b>				
<b><i>COMT</i></b>				
rs9617850 (A)	0.8801	0.5680	0.5391	0.6438
rs9606186 (C)	0.1158	0.8455	0.5841	0.4908
rs5748489 (A)	0.0611	0.6647	0.1830	0.2479
rs2075507 (G)	0.1550	0.7239	0.3336	0.2397
rs45551837 (A)	0.9687	0.8846	0.4437	0.6983
rs2020917 (T)	0.0767	0.2063	0.5413	0.6283
rs45536341 (T)	0.5113	0.0777	0.8085	0.6735
rs737865 (G)	0.6390	0.0495	0.7369	0.4873
rs6269 (G)	0.0383	0.1124	0.0572	0.0247
rs4633 (T)	0.0080	0.9516	0.3482	0.1769
rs373611092 (G)	0.3912	0.8940	0.9355	0.7659
rs4818 (G)	0.5889	0.2814	0.4538	0.5582
rs4680 (A)	0.1275	0.9884	0.4457	0.3750
rs9332377 (T)	0.0006	0.9638	0.3841	0.1174
rs165599 (G)	0.3669	0.0921	0.5502	0.2836
<b><i>DRD2</i></b>				
rs1799732 (del)	0.4642	0.5848	0.4816	0.5140
rs1079597 (A)	0.1131	0.3637	0.4140	0.1935
rs1800497 (A)	0.3472	0.6946	0.4274	0.3419
rs6275 (C)	0.9496	0.5810	0.8181	0.7294
rs6277 (T)	0.1110	0.7450	0.2620	0.2190
<b><i>DRD3</i></b>				
rs6280 (A)	0.2978	0.3778	0.0752	0.1076
<b><i>HTR2A</i></b>				
rs6311 (A)	0.0975	0.3204	0.1032	0.1268
rs6313 (T)	0.3021	0.6792	0.5391	0.5182
<b><i>SOD2</i></b>				
rs4880 (C)	0.8038	0.4620	0.6876	0.6712

	PANSSN	PANSSP	PANSSG	PANSST
rs372173830 (G)	0.3553	0.0643	0.3806	0.8746
<b><i>CYP1A2</i></b>				
<i>CYP1A2*1C</i> (A)	0.6200	0.1251	0.9685	0.7695
<i>CYP1A2*1F</i> (C)	0.0125	0.5648	0.0952	0.1107
<b><i>CYP2D6</i></b>				
<i>CYP2D6*1xN</i> (duplication)	0.5831	0.0148	0.0233	0.0617
<i>CYP2D6*5</i> (deletion)	0.6859	0.6392	0.9946	0.8149
<i>CYP2D6*4</i> (A)	0.6003	0.0281	0.9670	0.5438
<i>CYP2D6*40</i> (ins)	0.0992	0.6766	0.3042	0.3541
<i>CYP2D6*6</i> (del)	0.5481	0.1283	0.8671	0.7293

## 15 APPENDIX E2

Table S 4: Associations of the significantly associated variants examining the “best fit” inheritance models estimating percentage change in PANSS scores after adjusting for covariates

SNP	Outcome	P-value			Comparison	% Change per week (95% CI)	
		Genotype	Allelic	"Best"			
<b><u>COMT</u></b>							
rs737865	PANSSP	0.0201	0.0495	0.0092	Dominant	AG+GG vs AA	0.32 (0.08 to 0.56)
	PANSSG	0.0423	0.7369	0.0423	Genotype	AG vs AA	0.19 (0.00 to 0.39)
						GG vs AA	-0.18 (-0.50 to 0.15)
rs6269	PANSSN	0.0569	0.0383	0.0206	Recessive	GG vs AA+AG	0.34 (0.05 to 0.63)
	PANNSP	0.0433	0.1124	0.0126	Recessive	GG vs AA+AG	0.48 (0.10 to 0.86)
	PANNST	0.0356	0.0247	0.0126	Recessive	GG vs AA+AG	0.35 (0.07 to 0.62)
rs4633	PANSSN	0.0293	0.0080	0.0080	Additive T	Each T allele	-0.17 (-0.30 to -0.04)
rs9332377	PANSSN	0.0026	0.0006	0.0006	Additive T	Each T allele	0.25 (0.11 to 0.39)
<b><u>DRD2</u></b>							
rs1799732	PANSSN	0.0004	0.3669	0.0004	Genotype	DI vs II	-0.19 (-0.38 to 0.00)
						DD vs II	0.50 (0.15 to 0.86)
rs1079597	PANNST	0.0282	0.4642	0.0160	Recessive	DD vs DI+II	0.39 (0.07 to 0.72)
	PANSSN	0.0082	0.1131	0.0020	Recessive	AA vs AG+GG	0.93 (0.34 to 1.53)
<b><u>HTR2A</u></b>							
rs6311	PANNSN	0.0298	0.0975	0.0170	Dominant	AA+AG vs GG	-0.22 (-0.40 to -0.04)
	PANNST	0.0442	0.1268	0.0258	Dominant	AA+AG vs GG	-0.19 (-0.37 to -0.02)
<b><u>CYP1A2</u></b>							
rs762551	PANNSN	0.0101	0.0125	0.0027	Dominant	AC+CC vs AA	-0.28 (-0.46 to -0.10)
<b><u>CYP2D6</u></b>							
CYP2D6*1xN	PANNSP	0.0302	0.0148	0.0091	Dominant	XX+IX vs II	-0.60 (-1.05 to -0.15)
	PANNSG	0.0374	0.0233	0.0118	Dominant	XX+IX vs II	-0.44 (-0.77 to -0.10)
CYP2D6*4	PANNSP	0.0806	0.0281	0.0281	Additive A	Each A allele	0.26 (0.03 to 0.49)

## 16 APPENDIX E3

Table S 5: Association tests of *P* values from mixed model repeated measures analyses between haplotypes

Haplotype	PANSSN	PANSSP	PANSSG	PANSST
<i>DRD2</i> .DAACC	0.6326	0.0418	0.3978	0.1850
<i>DRD2</i> .DGACC	0.3800	0.4153	0.2799	0.3094
<i>DRD2</i> .DGATC	0.9438	0.7990	0.7775	0.8267
<i>DRD2</i> .DGGCC	0.5138	0.9089	0.8416	0.6929
<i>DRD2</i> .DGGCT	0.0007	0.9027	0.2150	0.0803
<i>DRD2</i> .DGGTC	0.4171	0.8750	0.3014	0.5264
<i>DRD2</i> .IAACC	0.1374	0.9410	0.5268	0.3665
<i>DRD2</i> .IGACC	0.9416	0.3373	0.0513	0.1748
<i>DRD2</i> .IGACT	0.1828	0.0514	0.5474	0.1926
<i>DRD2</i> .IGATC	0.9903	0.9947	0.6981	0.7969
<i>DRD2</i> .IGGCC	0.5106	0.3274	0.5565	0.6588
<i>DRD2</i> .IGGCT	0.1983	0.6462	0.6902	0.6153
<i>DRD2</i> .IGGTC	0.4999	0.4995	0.2205	0.3206
<i>HTR2A</i> .CA	0.0583	0.1047	0.0030	0.0104
<i>HTR2A</i> .CG	0.0910	0.3653	0.1124	0.1359
<i>HTR2A</i> .TA	0.2839	0.6299	0.4842	0.4659
<i>HTR2A</i> .TG	0.3607	0.6484	0.9096	0.8476
<i>SOD2</i> .CC	0.6673	0.8080	0.6002	0.6978
<i>SOD2</i> .CG	0.6150	0.1274	0.7614	0.8795
<i>SOD2</i> .TC	0.9945	0.2829	0.9274	0.7664
<i>SOD2</i> .TG	0.2992	0.1824	0.2329	0.6220
<i>CYP1A2</i> .AA	0.6200	0.1251	0.9685	0.7695
<i>CYP1A2</i> .AC	0.1331	0.5738	0.8503	0.4034
<i>CYP1A2</i> .GA	0.0354	0.5053	0.0876	0.0660
<i>CYP1A2</i> .GC	0.0125	0.5648	0.0952	0.1107
<i>CYP2D6</i> .IDADI	0.2333	0.0922	0.7380	0.2267
<i>CYP2D6</i> .IDGDI	0.4144	0.2878	0.9321	0.5241
<i>CYP2D6</i> .IIADI	0.5070	0.0131	0.6548	0.3979
<i>CYP2D6</i> .IIGDD	0.5539	0.1241	0.8439	0.7137
<i>CYP2D6</i> .IIGDI	0.3537	0.8465	0.2282	0.4001
<i>CYP2D6</i> .IIGII	0.0969	0.6788	0.3080	0.3544
<i>CYP2D6</i> .XDGDD	0.4360	0.8887	0.8356	0.8329
<i>CYP2D6</i> .XDGDI	0.4360	0.8887	0.8356	0.8329
<i>CYP2D6</i> .XIADI	0.0768	0.0204	0.0169	0.0093
<i>CYP2D6</i> .XIGDI	0.2522	0.0373	0.0647	0.1788

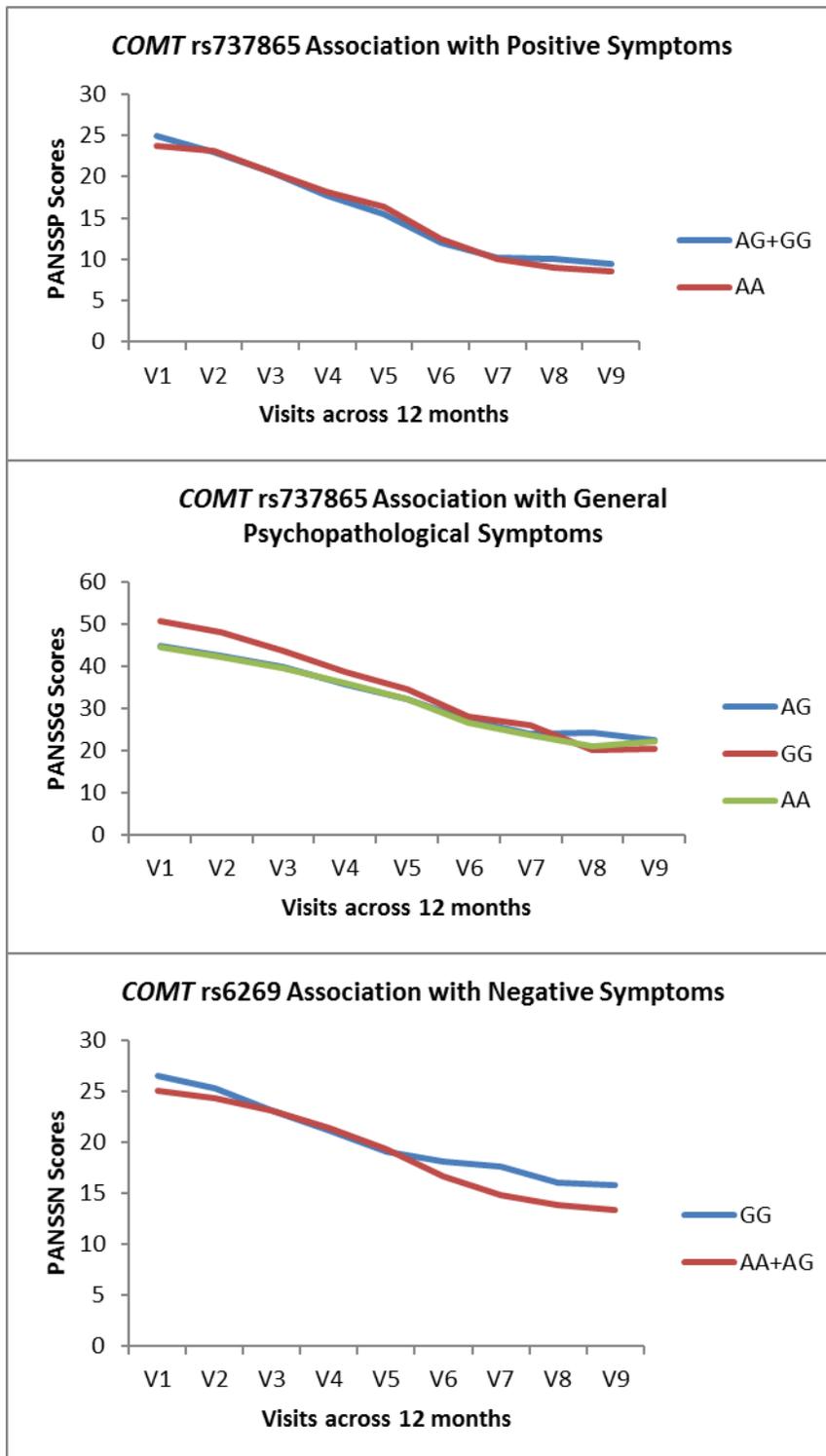
Table S 6. Association tests of *P* values from mixed model repeated measures analyses for *COMT* haplotypes

rs2020917	rs737865	rs6269	rs4633	rs9332377	Frequency	PANSSP	PANSSN	PANSST	PANSSG
A	A	A	C	C	0.04	0.507	0.483	0.247	0.181
A	A	A	C	T	0.03	0.277	0.003	0.036	0.083
A	A	A	T	C	0.09	0.606	0.372	0.509	0.567
A	A	G	C	C	0.02	0.451	0.275	0.188	0.145
A	A	G	C	T	0	0.679	0.007	0.02	0.01
A	G	A	C	C	0	0.848	0.191	0.345	0.343
A	G	A	T	C	0.01	0.081	0	0.002	0.016
A	G	A	T	T	0.01	0.556	0.337	0.791	0.673
A	G	G	C	C	0.05	0.279	0.753	0.341	0.282
A	G	G	C	T	0.01	0.352	0.051	0.209	0.51
C	A	A	C	C	0.09	0.275	0.544	0.713	0.705
C	A	A	C	T	0.01	0.314	0.539	0.352	0.47
C	A	A	T	C	0.25	0.449	0.017	0.547	0.908
C	A	G	C	C	0.02	0.677	0.973	0.819	0.809
C	A	G	C	T	0.07	0.438	0	0.006	0.015
C	G	A	C	C	0.01	0.404	0.679	0.952	0.868
G	A	A	C	C	0.04	0.077	0.261	0.742	0.935
G	A	A	C	T	0.01	0.056	0.537	0.094	0.088
G	A	A	T	C	0.02	0.003	0.685	0.053	0.291
G	A	A	T	T	0.01	0.018	0.458	0.006	0
G	A	G	C	C	0.03	0.144	0.063	0.116	0.253
G	A	G	C	T	0.01	0.002	0.346	0.013	0.026
G	G	A	C	C	0.01	0.481	0.233	0.966	0.711
G	G	A	C	T	0.01	0.224	0.832	0.375	0.367
G	G	A	T	C	0.02	0.025	0.078	0.225	0.777
G	G	G	C	C	0.08	0.077	0.178	0.484	0.378
G	G	G	C	T	0.01	0.946	0.087	0.527	0.911
T	G	A	C	C	0.02	0.608	0.15	0.31	0.506
T	G	A	C	T	0	0.136	0.053	0.005	0
T	G	G	C	T	0.01	0.93	0.236	0.84	0.187

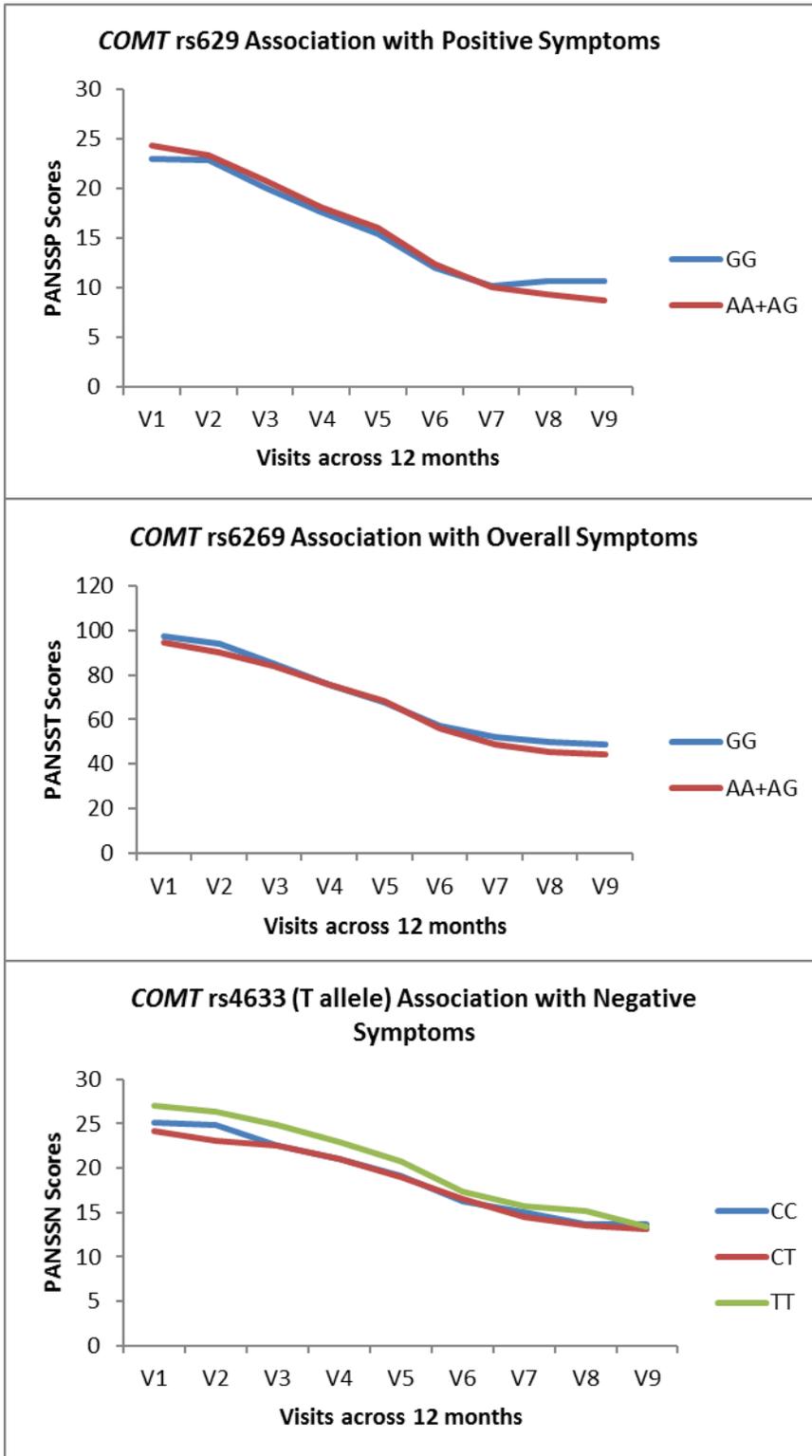
All haplotypes with a frequency  $\leq 1\%$  were excluded from further analyses.

Appendices

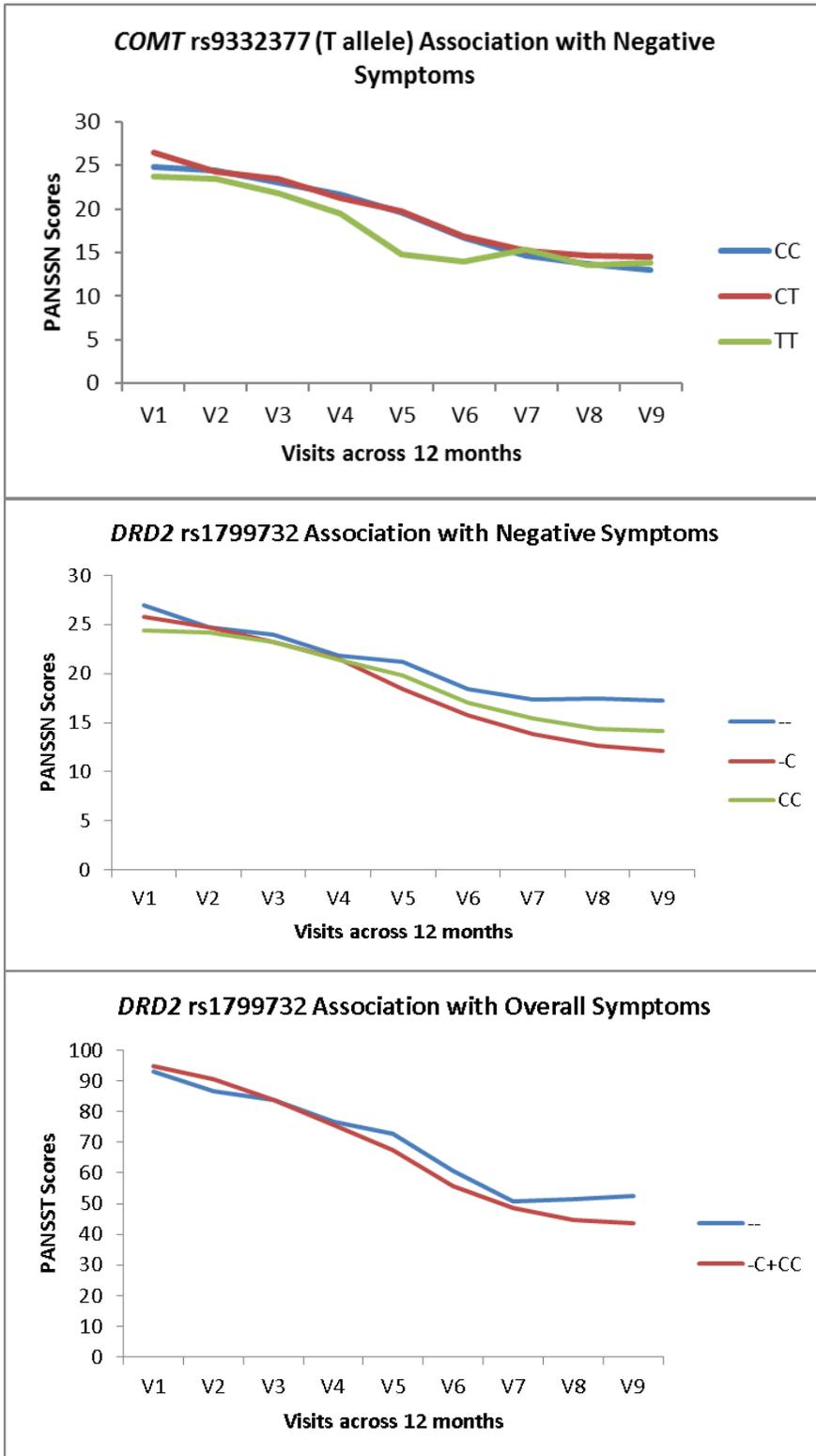
17 APPENDIX F



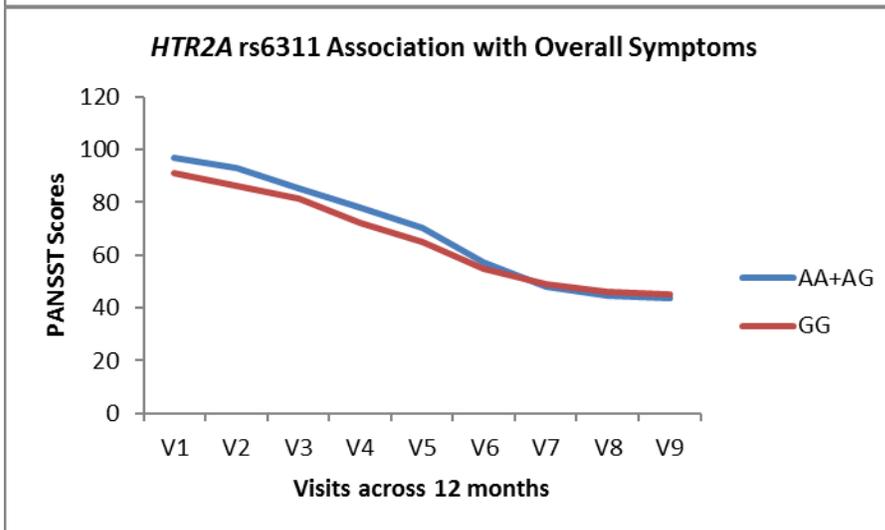
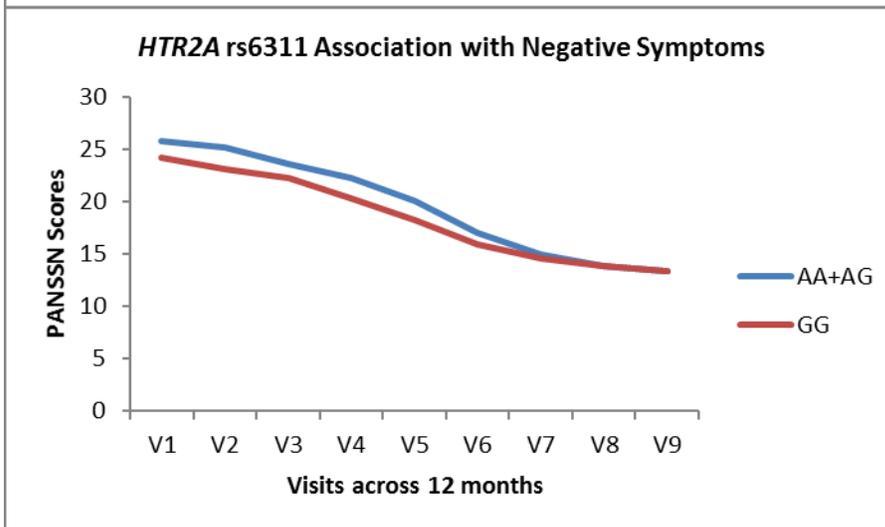
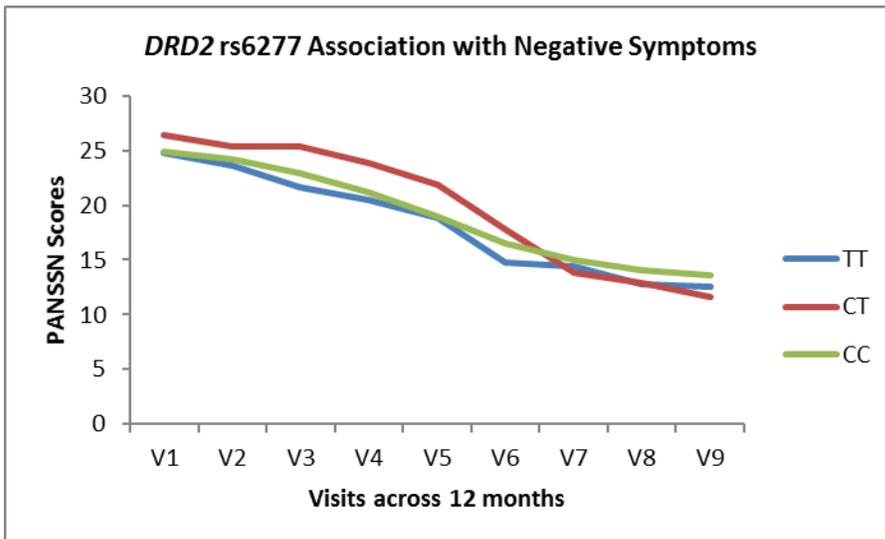
Appendices



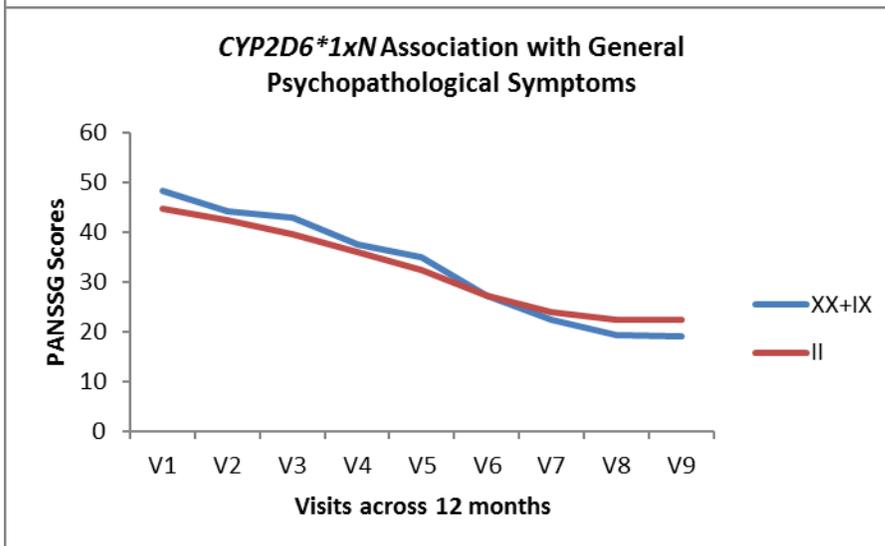
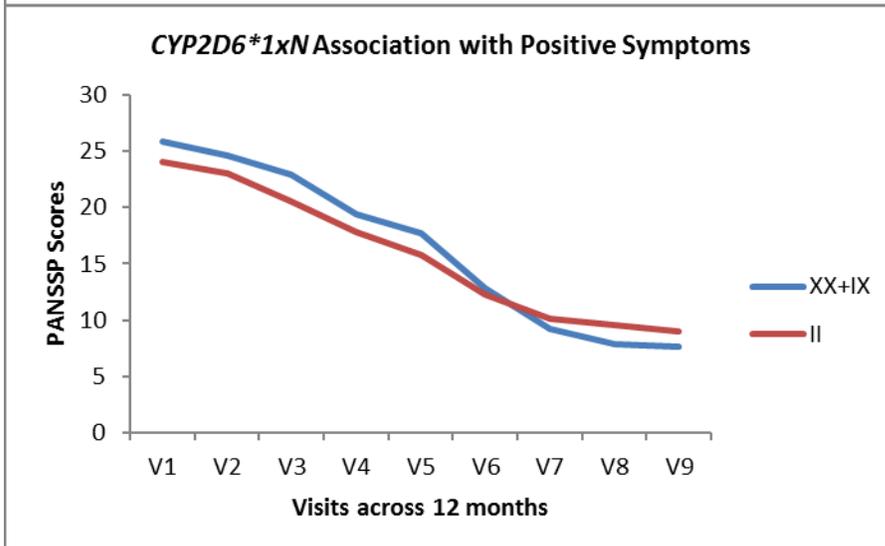
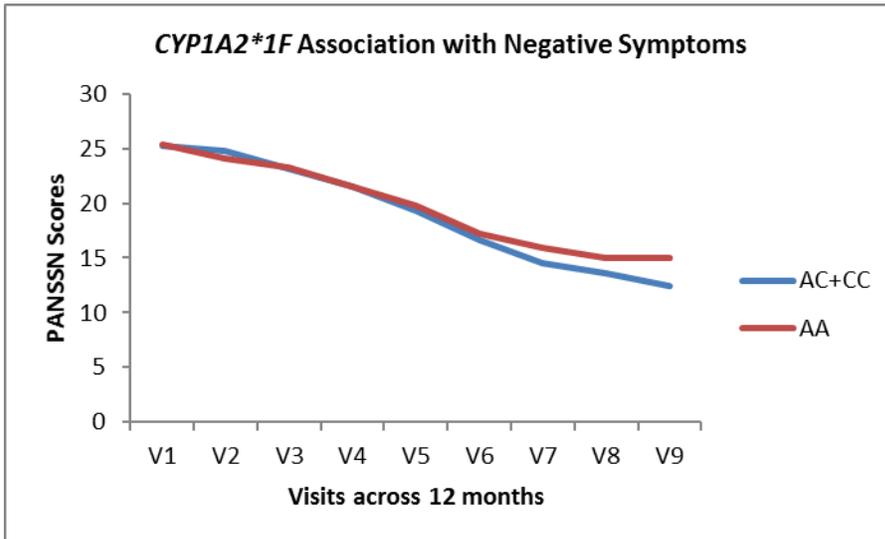
Appendices



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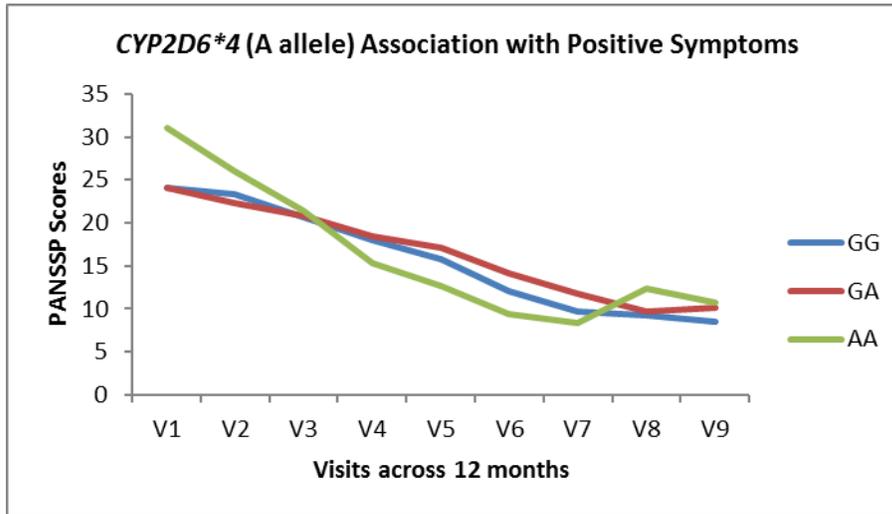


Figure S 6: Significant associations for percentage change in PANSS scores for each SNP after twelve months of treatment

Appendices

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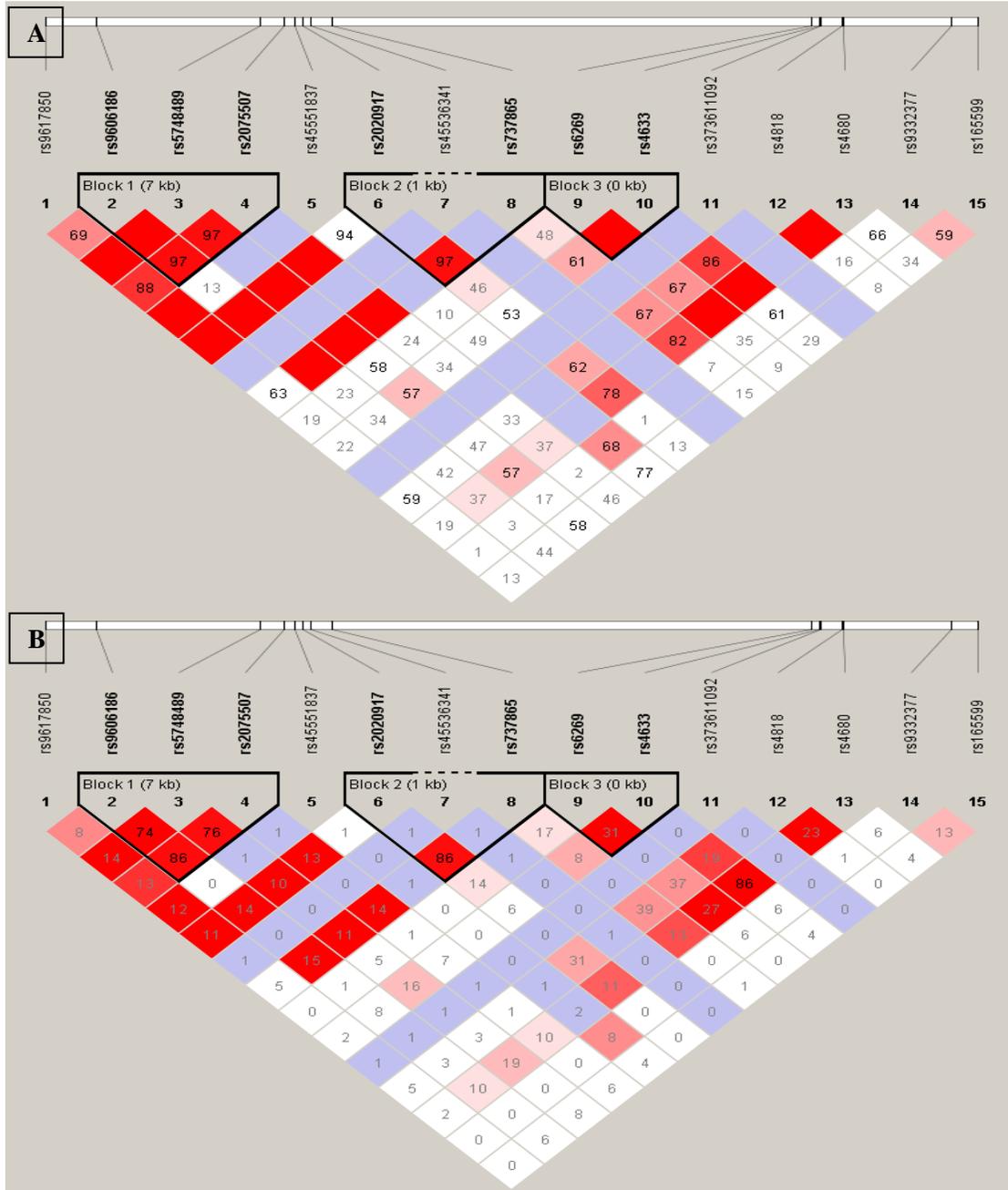


Figure S 7: *COMT* LD plots for the entire FES cohort presenting (A)  $D'$  and (B)  $r^2$  values.

Appendices

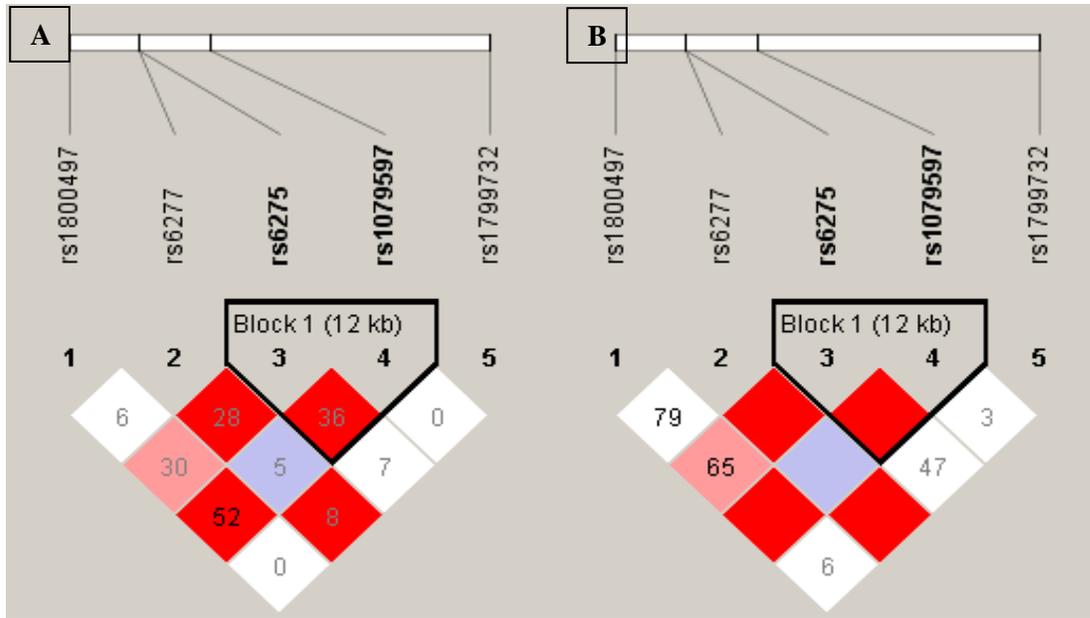


Figure S 8: DRD2 LD plots for the entire FES cohort presenting (A) D' and (B) r<sup>2</sup> values.