# Association of genetic variants and the susceptibility to abnormal involuntary movements and tardive dyskinesia (TD) in Xhosa schizophrenia patients

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# Declaration

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

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# Abstract

No obvious explanations exist for the development of abnormal involuntary movements (AIM), but several hypotheses have been proposed for tardive dyskinesia (TD) development. Since TD seems to have a genetic basis, several genetic variants have been investigated in TD development in various populations. Few studies have focused on African populations. This study focused on genetic variants (previously investigated in other populations) and the development and severity of AIM and TD in a Xhosa schizophrenia population. Genotype and allele frequencies determined were compared to those described in the literature for other populations. Following a report of an association between Ala-9Val and schizophrenia in a Turkish population, this study subsequently investigated this association in the Xhosa population.

*MnSOD* Ala-9Val was genotyped using HEX-SSCP analysis and the *DRD3* Ser9Gly variant was genotyped using restriction enzyme digestion by *MscI.* Genotyping was followed by statistical comparisons of the various groups, as well as association analyses between the variant and schizophrenia (only for *MnSOD*), AIM, or TD development and severity. The groups included a Xhosa schizophrenia group, a subgroup of the Xhosa schizophrenia group that had AIM (AIM+) and did not have AIM (AIM-), a subgroup of the AIM+ group that had TD (TD+), and a healthy Xhosa control group. A possible interaction between Ala-9Val and Ser9Gly in the development of AIM and TD was also investigated. Lastly, it was attempted to genotype *CYP2D6*4*, *CYP2D6*10* and *CYP2D6*17* using various PCR methods followed by restriction enzyme analysis.

*MnSOD* Ala-9Val genotype and allele frequencies were similar to those of the Turkish population, but differed to those of the Asian populations. No association between Ala-9Val and the development and severity of schizophrenia was found. However, a relationship between genotype and AIM or TD development was observed, as well as an association between TD severity and Ala-9Val genotype. *DRD3* Ser9Gly genotype and allele frequencies were similar to those of the African American population, but differed from other populations. No significant association between Ser9Gly and the development and severity of AIM or TD was detected, nor was an interactive effect between Ala-9Val and Ser9Gly in AIM or TD development observed. The genotyping of *CYP2D6* proved difficult and these variants could therefore not be analysed. The *CYP2D6*4* genotype and allele frequencies that could be determined from some samples, were similar to the frequencies described previously for African populations.

While we did not find an association between Ser9Gly in TD or AIM development and severity, nor an interaction between Ala-9Val and Ser9Gly, we did observe a relationship between Ala-9Val and

AIM or TD development and TD severity. The effect of this variant is probably small and other variants, specifically those in genes involved in free radical removal should be investigated in combination with Ala-9Val. With regard to *CYP2D6* it is suggested that high-throughput genotyping methods (e.g. microarray technology) should be used in the future. This will enable simultaneous genotyping of several variants and can be used in various populations.

This study is the first of its kind by focusing on the unique South African Xhosa population and TD or AIM development.

# Opsomming

Daar bestaan geen klaarblyklike verklarings vir die ontwikkeling van abnormale nie-vrywillige bewegings (ANB) nie, maar wel ‘n paar hipoteses vir die ontwikkeling van tardiewe diskinesie (TD). Aangesien TD ‘n genetiese basis het, is verskeie genetiese variante ondersoek in die ontwikkeling van die siekte in verskillende populasies. Min studies het egter van populasies uit Afrika gebruik gemaak. Hierdie studie het gefokus op verskeie genetiese variante (alreeds bestudeer in ander populasies) en die ontwikkeling en graad van ANB asook TD in ‘n Xhosa skisofrenie populasie. Genotipe en alleel frekwensies is bepaal en vergelyk met die gedokumenteerde data vir ander populasies. Na aanleiding van ‘n verslag van ‘n assossiasie tussen Ala-9Val en skisofrenie in ‘n Turkse populasie, het ons studie hierdie assosiasie in die Xhosa populasie ondersoek.

*MnSOD* Ala-9Val is gegenotipeer met behulp van HEX-SSCP analise en die *DRD3* Ser9Gly variant is gegenotipeer met die hulp van *MscI* restriksie-ensiem vertering. Na genotipering is statistiese vergelykings vir die verskillende groepe, asook assosiasie analises tussen die variant en skisofrenie (slegs vir *MnSOD*), ANB, of TD ontwikkeling en ernstigheidsgraad gedoen. Die groepe het ‘n Xhosa skisofrenie groep, ‘n subgroep met ANB (AIM+) en daarsonder (AIM-), ‘n subgroep van die AIM+ groep wat TD (TD+) gehad het, en ‘n gesonde Xhosa kontrole groep ingesluit. ‘n Moontlike interaksie tussen Ala-9Val en Ser9Gly in die ontwikkeling van ANB en TD is ook ondersoek. Laastens is ‘n poging aangewend om *CYP2D6*4*, *CYP2D6*10* en *CYP2D6*17* te genotipeer deur verskeie PKR metodes, gevolg deur restriksie-ensiem vertering.

*MnSOD* Ala-9Val genotipe en alleel frekwensies het ooreengestem met dié van die Turkse populasie, maar het verskil van dié van Asiatiese populasies. Geen assosiasie tussen Ala-9Val en skisofrenie ontwikkeling en ernstigheidsgraad is gevind nie. ‘n Verband tussen genotipe en ANB of TD ontwikkeling is egter waargeneem. Verder is ‘n assosiasie tussen die graad van TD en Ala-9Val genotipe opgemerk. *DRD3* Ser9Gly genotipe en alleel frekwensies het ooreengestem met dié van die Afro-Amerikaanse populasie, maar het verskil van ander populasies. Geen betekenisvolle assosiasie tussen Ser9Gly en die ontwikkeling en graad van ANB of TD, nog ‘n interaksie tussen Ala-9Val en Ser9Gly in die ontwikkeling van ANB of TD is waargeneem nie. Die genotipering van *CYP2D6* was problematies en hierdie variante kon dus nie geanaliseer word nie. Die *CYP2D6*4* genotipe en alleel frekwensies, wat wel vir sommige monsters bepaal kon word, het ooreengestem met die frekwensies wat in die literatuur vir ander populasies uit Afrika beskryf is.

Terwyl ons nie ‘n assosiasie tussen Ser9Gly en TD of ANB ontwikkeling en graad, of ‘n interaksie tussen Ala-9Val en Ser9Gly kon vind nie, het ons tog ‘n verband tussen Ala-9Val en ANB of TD

ontwikkeling, en TD ernstigheidsgraad waargeneem. Die effek van hierdie variant is waarskynlik klein en ander variante, spesifiek die in gene wat betrokke is in vrye radikaal verwydering, moet verder saam met Ala-9Val ondersoek word. Die gebruik van toekomstige hoë-deurvloei genotipering metodes (bv. “microarray” gebaseerde tegnologie) vir *CYP2D6* analises word aanbeveel. Dit sal die gelyktydige genotipering van verskeie variante moontlik maak en kan ook in verskillende populasies gebruik word.

Hierdie studie is die eerste van sy soort deurdat dit op die unieke Suid Afrikaanse Xhosa populasie en TD of ANB ontwikkeling fokus.

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# List of abbreviations and symbols

| | |
|---|---|
| $(NH_2)_2CO$ | Urea |
| $^{\cdot}OH$ | Hydroxyl radical |
| °C | Degrees Celsius |
| *5-HTT* | 5-@Hydroxytryptamine transporter gene |
| A | Adenine |
| *ACE* | Angiotensin I-converting enzyme gene |
| AD 4 | N-acetyl cysteine amide |
| ADRs | Adverse drug reactions |
| $AgNO_3$ | Silver nitrate |
| AIM | Abnormal involuntary movements |
| AIM- | Patient group without abnormal involuntary movements |
| AIM+ | Patient group with abnormal involuntary movements |
| AIMS | Abnormal involuntary movement scale |
| $Akt_1$ | Protein kinase B gene |
| Ala | Alanine |
| ANCOVA | Analysis of covariance |
| ANOVA | Analysis of variance |
| APS | Ammonium persulfate $(NH_4)_2S_2O_8$ |
| BHPR | Bunney-Hamburg Psychosis Rating |
| bp | Basepairs |
| BPRS | Brief Psychiatric Rating Scale |
| BSA | Bovine serum albumin |
| C | Cytosine |
| $C_{19}H_{10}Br_4O_5S$ | Bromophenol blue |
| $C_{31}H_{28}N_2Na_4O_{13}S$ | Xylene cyanol |
| CAT | Catalase |
| $CH_3COOH$ | Acetic acid |
| $CHRNA_7$ | α7 nicotinic receptor gene |
| cm | Centimetre |
| CNS | Central nervous system |
| *COMT* | Catechol-O-methyltransferase gene |
| CYP | Cytochrome P450 |
| *CYP-17* | Cytochrome P450 17α-hydroxilase gene |

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| | |
|---|---|
| *CYP1A2* | Cytochrome P450, subfamily I, polypeptide 2 gene |
| *CYP2D6* | Cytochrome P450, subfamily IID, Polypeptide 6 gene |
| *CYP2D7P* | Cytochrome P450, subfamily IID, Polypeptide 7 pseudogene |
| *CYP2D8P* | Cytochrome P450, subfamily IID, Polypeptide 8 pseudogene |
| *CYP3A4* | Cytochrome P450, subfamily IIIA, polypeptide 4 gene |
| *CYP3A5* | Cytochrome P450, subfamily IIIA, polypeptide 5 gene |
| Cys | Cysteine |
| d.f. | Degrees of freedom |
| *DAT* | Dopamine transporter gene |
| Del | Deletion |
| DIGS | Diagnostic Interview for Genetic Studies |
| *$DISC_1$* | Disrupted-in-schizophrenia 1 gene |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleoside triphosphate |
| *DRD1* | Dopamine D1 receptor gene |
| *DRD2* | Dopamine D2 receptor gene |
| *DRD3* | Dopamine D3 receptor gene |
| *DRD4* | Dopamine D4 receptor gene |
| DSM-IV | Diagnostic and Statistical Manual of Mental Disorders, fourth edition |
| *$DTNBP_1$* | Dysbindin gene |
| EDTA | Ethylenediaminetetraacetic acid: $C_{10}H_{20}BrN_3$ |
| EM | Extensive metaboliser |
| EPS | Extrapyramidal symptoms |
| EPSE | Extrapyramidal side effects |
| *ESR1* | Estrogen receptor I gene |
| EtBr | Ethidium Bromide |
| EtOH | Ethanol |
| *FokI* | Restriction enzyme with recognition sequence 5'-GGATG -3', and cutting site 5'-GGATGNNNNNNNNN↓NNNN-3' and 5'-CCTACNNNNNNNNNNNNN ↓NNN-3', Source: *E. coli* strain that carries the *FokI* gene from *Flavobacterium okeanokoites* |
| G | Guanine |
| g | Relative centrifugal force |
| Gly | Glycine |

| | |
|---|---|
| *$GRM_3$* | Metabolic glutamate receptor-3 gene |
| GSHPx (GPX1) | Glutathione peroxidase |
| *GSTM1* | Glutathione S-transferase, mu-1 gene |
| *GSTP1* | Glutathione S-transferase, pi gene |
| *GSTT1* | Glutathione S-transferase, theta-1 gene |
| $H_2NCHO$ | Formamide |
| $H_2O_2$ | Hydrogen peroxide |
| HCHO | Formaldehyde |
| HEX-SSCP | Heteroduplex-single strand conformational polymorphism |
| *HhaI* | Restriction enzyme with recognition sequence 5'-GCG↓C-3', Source: *E. coli* strain that carries the *HhaI* gene from *Haemophilus haemolyticus* |
| His | Histamine |
| *HphI* | Restriction enzyme with recognition sequence 5'-GGTGA-3' and cutting site 5'- GGTGANNNNNNNN↓N-3' and 5'-CCACTNNNN NNN↓N-3', Source: *E. coli* strain that carries the *HphI* gene from *Haemophilus parahaemolyticus* |
| *HTR2A* | 5-@Hydroxytryptamine receptor 2A gene |
| *HTR2C* | 5-@Hydroxytryptamine receptor 2C gene |
| *HTR6* | 5-@Hydroxytryptamine receptor 6 gene |
| HWE | Hardy Weinberg equilibrium |
| ICD 10 | International Classification of Diseases version 10 |
| Ile | Isoleucine |
| IM | Intermediate metaboliser |
| Ins | Insertion |
| kb | Kilo basepair |
| KCl | Potassium chloride |
| $KH_2PO_4$ | Potassium dihydrogen orthophosphate |
| $KHCO_3$ | Potassium hydrogen carbonate |
| Leu | Leucine |
| M | Molar |
| *MAOA* | Monoamine oxidase A gene |
| *MAOB* | Monoamine oxidase B gene |
| *MDR1* | Multidrug resistance 1 gene |
| Met | Methionine |
| mg | Milligram |
| $MgCl_2$ | Magnesium chloride |

| | |
|---|---|
| ml | Millilitre |
| *MnSOD* | Manganese superoxide dismutase gene |
| MR | Metabolic ratio |
| mRNA | Messenger ribonucleic acid |
| *MscI* | Restriction enzyme with recognition sequence 5'-TGG↓CCA-3', Source: *E. coli* strain that carries the *MscI* gene from *Micrococcus* species (C. Polisson) |
| MTS | Mitochondrial targeting sequence |
| *MvaI* | Restriction enzyme with recognition sequence 5'-CC↓WGG-3', Source: *E.coli* that carries the cloned *mvaIR* gene from *Micrococcus varians* RFL19 |
| n | Size of group |
| $Na_2HPO_4$ | Di-sodium hydrogen orthophosphate anhydrous |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| ng | Nanogram |
| $NH_4$ | Ammonium |
| $NH_4Cl$ | Ammonium Chloride |
| *NOS1* | Nitric oxide synthase 1 gene |
| $NRG_1$ | Neuregulin gene |
| $O_2^-$ | Superoxide radical |
| OMIM | Online Mendelian Inheritance in Man |
| *OPRM* | Opioid receptor, mu-1 gene |
| *P* | Probability |
| p | Short arm of chromosome |
| PAA | Polyacrylamide |
| *PAH* | Phenylalanine hydroxilase gene |
| PANSS | Positive and Negative Syndrome Scale |
| PBS | Phosphate buffered saline |
| PCP | Phencyclidine |
| PCR | Polymerase chain reaction |
| PM | Poor metaboliser |
| Pro | Proline |
| $PRODH_2$ | Proline dehydrogenase gene |
| PUFAs | Polyunsaturated fatty acids |
| q | Long arm of chromosome |
| $RGS_4$ | Regulator of G-protein signalling gene |

| | |
|---|---|
| ROS | Reactive oxygen species |
| SANS | Scale for the Assessment of Negative Symptoms |
| SAPS | Scale for the Assessment of Positive Symptoms |
| SDS | Sodium dodecyl sulphate: $CH_3(CH_2)_{11}OSO_3Na$ |
| Ser | Serine |
| SNP | Single nucleotide polymorphism |
| SOD | Superoxide dismutase |
| SPECT | Single photon emission computed tomography |
| SPSS | Statistical Package for the Social Sciences |
| SSCP | Single stranded conformational polymorphism |
| T | Thymine |
| TBARS | Thiobarbituric acid reactive substances |
| TBE | Tris-Borate EDTA buffer |
| TD | Tardive dyskinesia |
| TEMED | N', N', N', N', -tetramethylethylenediamine |
| TFPGA | Tools for Population Genetic Analysis |
| *TPH* | Tryptophan hydroxilase 1 |
| Tris | Tris(hydroxymethyl)aminomethan: 2-Amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}NO_3$ |
| Tyr | Tyrosine |
| U | Enzyme activity unit |
| UK | United Kingdom |
| UM | Ultrarapid metaboliser |
| USA | United States of America |
| UV | Ultraviolet |
| V | Volt |
| v/v | Volume per Volume |
| Val | Valine |
| VNTR | Variable number tandem repeat |
| vs. | Versus |
| w/v | Weight per volume |
| µg | Microgram |
| µl | Microlitre |
| µM | Micromolar |
| $\chi^2$ | Chi squared |

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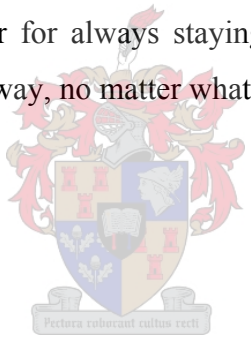


# Chapter One

# Literature review

## 1.1 Pharmacogenetics

The general consensus is that medication is effective in treating diseases. Yet, the efficacy of a drug can lie between 25-80% (Spear *et al*., 2001). In addition, about 6.7% of hospitalised patients develop serious adverse drug reactions (ADRs) in the United States, while in close to 0.32% these ADRs are fatal (Lazarou *et al*., 1998). Clearly this poses a challenge to the medical field and a considerable effort has been made to attempt to elucidate the mechanisms of drug response (efficacy of drug therapy or development of adverse drug effects). Drug response differs between patients (Basile *et al*., 2002; Caraco, 2004; Malhotra *et al*., 2004) as well as populations (Kalow and Bertilsson, 1994; Xie *et al*., 2001), and there seems to be a genetic component to drug response (Spear *et al*., 2001). Hence research has focused on identifying genes and genetic variants determining drug response. This field of study is called pharmacogenetics (Roses 2000; Lindpaintner, 2003) and focuses on individualising therapy (Basile *et al*., 2002). Pharmacogenomics (often used interchangeably with pharmacogenetics), on the other hand, involves the study of the effects different compounds have on gene expression in the entire genome (Lindpaintner, 2003). It is thought that pharmacogenetics will change the practice and economics of medicine, by being able to set up a risk profile for a patient regarding unresponsiveness to medication or development of side effects. In addition, pharmacogenetics can be applied in the pharmaceutical industry, in target and patient group selection (Roses 2000).

Pharmacogenetic studies normally involve the identification of a genetic variant in a specific gene. Candidate genes chosen often code for certain types of proteins, either for drug metabolising enzymes or for proteins the drug interacts with (such as transporters or receptors), (McKinnon and Evans, 2000; Weber, 2001; Bolonna *et al*., 2004; Wilffert *et al*., 2005). Sometimes, variants located in genes not coding for the mentioned type of proteins (such as gene involved in disease self), may indirectly affect drug response and therefore also qualify as a candidate (McKinnon and Evans, 2000; Müller *et al*., 2004). Hence genetic variants in certain genes may affect drug absorption, distribution and metabolism (pharmacokinetics), or the response of the target molecule or a member of its pathway to the medication (e.g. how well the drug binds to its receptor or pharmacodynamics) (Lindpaintner, 2003). The candidate gene selection process is followed by the comparison of gene

polymorphism frequencies between patients responding or not responding to drugs [efficacy (Spear *et al*., 2001)], or between subjects suffering from and those not suffering from side effects [safety (Spear *et al*., 2001)] (Basile *et al*., 2002; Bolonna *et al*., 2004). Significant differences between groups may indicate a role for that polymorphism in drug action or in the development of side effects (Kawanishi *et al*., 2000; Basile *et al*., 2002). Ethnic differences between populations exist regarding treatment response, rate of drug-induced side effects, as well as frequency of certain polymorphisms in the genes relevant to pharmacogenetics (Weber, 2001; Xie *et al*., 2001; Daar and Singer, 2005; Shastry, 2006). This means that research needs to focus not just on interindividual genetic variation but on population genetic differences, if the quest for individualised therapy is to be successful (Weber, 2001; Daar and Singer, 2005; Shastry, 2006). Generally it seems that a combination of several genetic variants are involved in drug response (Kawanishi *et al*., 2000; Bolonna *et al*., 2004; Caraco, 2004; Müller *et al*., 2004). It is, however, important to note that other factors (e.g. disease determinants, age, sex, organ function, ethnicity, and drug interactions) may also play a role in drug response (Johnson and Evans, 2002; Wilkinson, 2005; Shastry, 2006).

In psychiatry, pharmacogenetics has also become important. Medication prescribed often causes certain side effects and a trial and error approach is used to determine the best type and dosage of medication for the patient (Basile *et al*., 2002; Abidi and Bhaskara, 2003; Malhotra *et al*., 2004). Hence any information gained by pharmacogenetics may aid in predicting a patients drug response and therefore optimise the process of drug and dosage prescription (Basile *et al*., 2002; Shastry, 2006). It has also been suggested to genotype phase II drug trial participants, so that phase III studies will only consist of participants with the genotype that is more likely to result in the desired response (Roses, 2000, 2004), and indeed this is beginning to be applied in the pharmaceutical industry (Roses, 2004). In the future it may be possible to test patients for genetic variants in several genes in the clinical setting, once genotyping methods have become more widely known, gained in accuracy and significance, and become more user friendly and less expensive (Johnson and Evans, 2002). In psychiatry candidate genes for pharmacogenetic studies include the drug metabolising enzymes (Cytochrome P450s) and the neurotransmitter receptors (e.g. dopamine receptors) (Bolonna *et al*., 2004; Wilffert *et al*., 2005). However, other genes such as those involved in free radical metabolism have also been investigated (Akyol *et al*., 2005; Shinkai *et al*., 2006). While we are still not able to perform a genetic test and determine the drug response profile of an individual, progress has been made and several variants have been associated with the development of certain side effects (Johnson and Evans, 2002). For example, Lee *et al.* (2004) found the presence of the T allele of the C825T polymorphism in the G-protein β3 subunit gene to result in severe symptomatology and better antidepressant treatment response.

The importance of a multigenic approach in the application of pharmacogenetics is eloquently demonstrated by clozapine response. A combination of 6 polymorphisms (neurotransmitter-receptor related) was able to give a 76-77% level of prediction for clozapine response in schizophrenia patients (Arranz *et al*., 2000).

## 1.2 Schizophrenia

### 1.2.1 Definition

Schizophrenia (OMIM number: #181500) is a psychiatric disorder affecting about 0.5-1% of the population (APA, 1994). The incidence rate of this disorder is estimated at 1 per 10 000 per year (APA, 1994). Schizophrenia affects several areas of functioning such as interpersonal relations, work, education and self-care (APA, 1994; Mueser and McGurk, 2004). The onset of schizophrenia generally occurs during late adolescence or early adulthood, however, mild social, motor and cognitive problems may be observed during childhood (Sivagnansundaram *et al*., 2003).

### 1.2.2 Symptoms and course

Symptoms of schizophrenia can be divided into negative and positive symptoms (APA, 1994). Negative symptoms are characterised by decreased fluency and productivity of thought and speech (alogia), the reduction of range and intensity of emotional expression (affective blunting), a decrease in initiation of goal-directed behaviour (avolition) and a loss of feeling pleasure (anhedonia) (APA, 1994; Kawanishi *et al*., 2000; Basile *et al*., 2002; Austin, 2005). Positive symptoms can be divided into psychotic and disorganised symptoms (Basile *et al*., 2002). The psychotic category is represented by distortions and exaggerations of inferential thinking (delusions) and perception (hallucinations) (APA, 1994; Basile *et al*., 2002; Austin, 2005), while the disorganised category is characterised by distortions in language and communication (disorganised speech) and behavioural monitoring (grossly disorganised or catatonic behaviour) (APA, 1994; Basile *et al*., 2002). The course of schizophrenia is variable with some patients remaining chronically ill (either stable course, or progressive worsening of symptoms), while others show symptom exacerbations and remissions (APA, 1994). Cognitive impairment, experienced as difficulties in attention and concentration, learning and memory and executive functions, is also observed in schizophrenia (Mueser and McGurk, 2004).

### 1.2.3 Diagnosis

Guidelines for diagnosing schizophrenia is set out in the International Classification of Diseases version 10 (ICD 10) and the Diagnostic and Statistical Manual for mental disorders version 4 (DSM IV) (APA, 1994; Mueser and McGurk, 2004; Austin, 2005). The DSM IV criteria for the diagnosis of schizophrenia are shown in Table 1.

### 1.2.4 Changes in the brain

Brain abnormalities in schizophrenic patients have been reported, although replication of these results has often not been successful (reviewed in Harrison and Weinberger, 2005). Changes observed are generally only small (Sivagnansundaram *et al*., 2003; Harrison and Weinberger, 2005). These include enlargement of the lateral and third ventricles, absence of gliosis, decreased size of certain brain regions, changes in brain blood flow in the subcortical regions, anterior cingulate and the limbic cortex, aberrantly located or clustered neurons (specifically in the entorhinal cortex and the neocortical white matter), smaller pyramidal neuron cell bodies in the hippocampus and neocortex, reduction in interneural density and synaptic projections, decrease in the number of certain neurons (e.g. hippocampal neurons), and lower levels of several presynaptic markers of certain neurons (Harrison, 1999; Harrison and Weinberger, 2005). In addition, morphological, biochemical and molecular evidence has been put forward for the involvement of the mitochondria and metabolism in schizophrenia (Harrison and Weinberger, 2005). Synaptic connectivity (specifically in certain types of connections) seems to be affected in schizophrenia; this only seems to be partly morphological and may for the other part be influenced by more molecular mechanisms (Harrison and Weinberger, 2005). Even though there seem to be some changes in the brain in schizophrenia patients, the exact pathophysiology of this disorder still remains unknown. However, several hypotheses exist.

### 1.2.5 Different hypotheses

#### 1.2.5.1 Dopamine hypothesis

Several hypotheses on the pathophysiology of schizophrenia have been proposed and researched (Sivagnansundaram *et al*., 2003). Of these, the dopamine hypothesis is the oldest and most widely investigated one (Langer *et al.*, 1981; Sivagnansundaram *et al*., 2003). This hypothesis states that schizophrenia may result through an excess of dopamine mediated neuronal activity, potentially

**Table 1:** DSMIV diagnostic criteria for schizophrenia (APA, 1994).

## Diagnostic criteria for Schizophrenia

A. *Characteristic symptoms:* Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated):
   (1) delusions
   (2) hallucinations
   (3) disorganized speech (e.g., frequent derailment or incoherence)
   (4) grossly disorganized or catatonic behavior
   (5) negative symptoms, i.e., affective flattening, alogia, or avolition

   **Note:** Only one Criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behavior or thoughts, or two or more voices conversing with each other.

B. *Social/occupational dysfunction:* For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

C. *Duration:* Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

D. *Schizoaffective and mood disorder exclusion:* Schizoaffective disorder and mood disorder with psychotic features have been ruled out because either (1) no major depressive, manic, or mixed episodes have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.

E. *Substance/general medical condition exclusion:* The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.

F. *Relationship to a pervasive developmental disorder:* If there is a history of autistic disorder or another pervasive developmental disorder, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

*Classification of longitudinal course* (can be applied only after at least 1 year has elapsed since the initial onset of active-phase symptoms)*:*

**Episodic With Interepisode Residual Symptoms** (episodes are defined by the reemergence of prominent psychotic symptoms); *also specify if:*
**With Prominent Negative Symptoms**
**Episodic With No Interepisode Residual Symptoms**
**Continuous** (prominent psychotic symptoms are present throughout the period of observation); *also specify if:* **With Prominent Negative Symptoms**
**Single Episode In Partial Remission;** *also specify if:* **With Prominent Negative Symptoms**
**Single Episode In Full Remission**
**Other or Unspecified Pattern**

through dopamine receptor supersensitivity (Langer *et al*., 1981). It is based on the antagonistic action of antipsychotic medication at the D2 receptors, as well as the psychotogenic properties of drugs that increase dopamine activity (Sivagnansundaram *et al*., 2003). Some evidence for this hypothesis has come from single photon emission computed tomography (SPECT) measurements of baseline D2 receptor availability, and D2 receptor availability during acute dopamine depletion. Comparison between the baseline and dopamine depletion values showed an increase in D2 receptor stimulation in schizophrenic patients compared to controls (Abi-Dargham *et al*., 2000). However, this hypothesis cannot account for the late onset of the disease. Furthermore, research has as yet not been able to associate schizophrenia development with any of the dopamine receptors (except DRD3 (Jönsson *et al*., 2003; Crocq *et al*., 1992)) or any components in dopamine effect-mediating pathways. Hence this hypothesis cannot be singly responsible for the development of schizophrenia (Sivagnansundaram *et al*., 2003).

#### 1.2.5.2 Free radical hypothesis

It has also been suggested that free radical damage, as a result of ineffective antioxidant defence or increased free radical production, may cause (reviewed in Yao *et al.*, 2001) or at least worsen the course of schizophrenia (Mahadik *et al.*, 2001; Arvindakshan *et al.*, 2003a). Sources of free radicals or reactive oxygen species (ROS) include autooxidation, electron leakage in the mitochondrial respiratory chain reaction (Fridovich, 1983; Halliwell, 1997) and phagocytes as defence (Curnutte and Babior, 1987; Halliwell, 1997). They can also be produced in the presence of certain toxicants (Davies, 2000). If left unchecked reactive oxygen species (ROS) can interact with lipids, proteins and nucleic acids, which may ultimately lead to cell death (Halliwell, 1991; Mahadik and Mukherjee, 1996). An antioxidant defence system exists to scavenge these harmful free radicals, namely enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT), as well as several antioxidant compounds such as α-tocopherol (vitamin E) and ascorbic acid (vitamin C). Dietary or genetic factors affecting the antioxidant defence system may therefore result in an excess of free radicals and hence damage to the cells. Neurons are considered to be very susceptible to free radical damage, due to their high polyunsaturated fatty acid contents (reacts with free radicals), high oxygen consumption (contributes to free radical production), as well as low levels of antioxidant enzymes in the brain. In addition, due to the neuronal inability of DNA replication, no DNA repair occurs and may therefore compromise neural cells. The presence of iron in certain areas of the brain may also predispose the brain to free radical damage (Mahadik *et al*., 2001; Rao and Balachandran, 2002). Free radical damage in the brain may eventually lead to

neurodegeneration, abnormal neurodevelopment, as well as membrane impairment of the neurons (Mahadik *et al*., 2001).

Evidence that supports the free radical hypothesis in schizophrenia is, for example, the finding of decreased levels of plasma antioxidants (albumin, bilirubin, uric acid, ascorbic acid) (Yao *et al*., 1998b; Yao *et al*., 2000; Reddy *et al*., 2003; Dakhale *et al*., 2004; Pae *et al*., 2004), and aberrant plasma or central nervous system antioxidant enzyme (superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase (CAT)) levels and activity (Ravikumar *et al*., 2000; Kuloglu *et al*., 2002; Ranjekar *et al*., 2003; Michel *et al*., 2004). Findings, however, have been ambiguous (Abdalla *et al*., 1986; Yao *et al*., 1999). Other evidence for the free radical hypothesis is the higher level of lipid peroxidation products (Mahadik *et al*., 1998; Akyol *et al*., 2002; Dakhale *et al*., 2004), and lower levels of membrane phospholipid polyunsaturated fatty acids (target for free radicals) (Khan *et al*., 2002; Arvindakshan *et al*., 2003a). Schizophrenic patients also showed improvement in outcome after supplementing their diet with antioxidants such as vitamins E and C, as well as omega-3 fatty acids (Arvindakshan *et al*., 2003b) in schizophrenic patients compared to controls. While some neuroleptics have pro-oxidant qualities (Parikh *et al*., 2003; Polydoro *et al*., 2004) and may therefore confound findings of increased oxidative damage in schizophrenic patients, studies on drug-naïve patients have also shown aberrant levels of antioxidant enzymes (Mukherjee *et al*., 1996), lipid peroxidation products (Mahadik *et al*., 1998) as well as antioxidant compounds (Dakhale *et al*., 2004; Pae *et al*., 2004). In addition to the above evidence, schizophrenic patients are also known to have an unhealthy lifestyle with a high fat diet, little exercise and a high percentage of patients smoking, which may also exacerbate oxidative stress (Hughes *et al*., 1986; Brown *et al*., 1999). It must be noted, however, that free radical damage is not thought to be the primary event causing schizophrenia, but rather affect deterioration and poor outcome of the disorder (Mahadik *et al*., 2001; Arvindakshan *et al*., 2003a). An example of this would be the positive correlation of free radical excess or low total antioxidant status with negative symptoms in schizophrenic patients (Yao *et al*., 1998a; Sirota *et al*., 2003).

#### 1.2.5.3 Neurodevelopmental hypothesis

The neurodevelopmental hypothesis has also been proposed as a model to explain schizophrenia development. It suggests that due to genetic and/or environmental factors abnormalities in neurodevelopmental processes may occur, which then ultimately result in clinical symptoms of schizophrenia (Mueser and McGurk, 2004; Rapoport *et al*., 2005). Some minor signs of abnormality may already be visible early on, before the development of signs of the disease. This

hypothesis has become very popular, since it is able to account for several factors important in the pathogenesis of schizophrenia, such as age of onset, brain abnormalities, the pre- and perinatal risk factors, and the cognitive defects that stay static (Sivagnansundaram *et al.*, 2003; Rapoport *et al*., 2005).

#### 1.2.5.4 Glutamate hypothesis

A dysfunction in the glutamate system has also been suggested to play a role in the pathophysiology of schizophrenia (Moghaddam, 2003; Sivagnansundaram *et al.*, 2003). This is supported by the finding that some genes that have been linked to schizophrenia are related to the glutamate system (Collier and Li, 2003), as well as other evidence from post-mortem brain studies and the psychosis-inducing drug phencyclidine (PCP), which was found to be an antagonist of glutamate receptors (Moghaddam, 2003).

### 1.2.6 Risk factors

While the exact pathophysiology of schizophrenia is unknown, several risk factors have been identified in the development of schizophrenia. Family history of the disorder and social class have been strongly associated with schizophrenia development. Two theories may explain the association with social class, namely that an unfavourable environment may lead to schizophrenia development, or social drift (Bromet and Fennig, 1999). Age, gender, rheumatoid arthritis, season of birth, as well as obstetric, birth and early childhood complications are considered potentially strong risk factors for the development of schizophrenia (Bromet and Fennig, 1999; Mueser and McGurk, 2004). Other possible risk factors include substance abuse, stress, and geographic location (Bromet and Fennig, 1999). Since family history is considered a strong risk factor in the development of schizophrenia, a genetic component to this disorder is to be expected.

### 1.2.7 Genetics and schizophrenia

Family, twin and adoption studies indicate that schizophrenia has a strong genetic component, however, other factors, such as epigenetic (Abdolmaleky *et al*., 2005; Sullivan, 2005), developmental and environmental factors (Kawanishi *et al*., 2000; Sullivan *et al*., 2003; Mueser and McGurk, 2004), may interact in a complex manner with genetic factors in the development of the disorder. Genetic analyses further suggest that schizophrenia may result through the epistatic or multiplicative interaction of several loci (Mueser and McGurk, 2004; Austin 2005). Some studies have been able to show genetic linkage with schizophrenia to a certain genomic region, but results

could often not be replicated (Harrison and Weinberger, 2005). At the moment, regions with the most evidence for genetic linkage with schizophrenia are situated on chromosome: 1q21-q22, 1q32-q42, 5q21-q34, 6p24-p21, 6q13-26, 8p22-21, 10p11-15, 13q14-32, 15q14 and 22q11-q13 (Sivagnansundaram *et al.*, 2003). Two meta-analyses found 8p, 13q and 22q (Badner and Gershon, 2002) and 1q, 3p, 5q, 6p, 8p, 11q, 14p, 20q, and 22q (Lewis *et al*., 2003) to be linked with schizophrenia. Only 8p and 22q were common to both meta-analyses. Blood-based gene expression profiles, making use of 8 biomarker genes, were also recently shown to differentiate between schizophrenia, bipolar disorder and the control group with 95-97% accuracy (Tsuang *et al*., 2005). Some specific genes that may be implicated in the pathophysiology of schizophrenia are catechol-O-methyl transferase (*COMT*), dysbindin ($DTNBP_1$), neuregulin ($NRG_1$), regulator of G-protein signalling ($RGS_4$), disrupted-in-schizophrenia 1 ($DISC_1$) and metabolic glutamate receptor-3 ($GRM_3$) (Austin, 2005; Harrison and Weinberger, 2005). In addition genes such as calcineurin, α7 nicotinic receptor gene ($CHRNA_7$), proline dehydrogenase ($PRODH_2$) and $Akt_1$ (protein kinase B) have also been suggested to play a role in schizophrenia development (Harrison and Weinberger, 2005). Several dopaminergic genes, including the dopamine D3 receptor (*DRD3*) have also been investigated (based on the dopamine hypothesis) for their role in schizophrenia development; however, results have varied (Crocq *et al*., 1992; Dubertret *et al*., 1998; Jönsson *et al*., 2003; Ambrósio *et al*., 2004; Baritaki *et al*., 2004; Hoogendoorn *et al*., 2005). Based on the free radical hypothesis genes involved in the antioxidant pathway, such as *GSHPx* and specifically *SOD*, have also been investigated for their possible role in schizophrenia (Shinkai *et al*., 2004; Akyol *et al*., 2005). One of these candidate genes, *MnSOD*, will be discussed in more detail below.

#### 1.2.7.1 *MnSOD*

SOD plays a role in neurodevelopment, specifically growth termination and differentiation initiation (Mahadik and Mukherjee, 1996). Three types of SODs exist, namely copper-zinc SOD in the cytosol (McCord and Fridovich, 1969), extracellular SOD (Marklund, 1982), and manganese SOD (MnSOD) (Weisiger and Fridovich, 1973). The latter is localized in the mitochondria (Weisiger and Fridovich, 1973). MnSOD (OMIM number: *147460) converts $O_2^-$ to $H_2O_2$ (Fridovich, 1974; Chance *et al*., 1979). SOD works in combination with GSHPx and CAT to render harmless free radicals (Chance *et al.*, 1979) (Figure 1), produced by several mechanisms such as auto-oxidation, the immune system, the respiratory chain reaction and toxicants (Fridovich, 1983; Curnutte and Babior, 1987; Halliwell, 1997; Davies, 2000). Since MnSOD scavenges free radicals, any change in activity of the enzyme may result in damage to the cell, and if this occurs in the brain, to neurodegeneration (Akyol *et al*., 2005). The genetic variant Ala-9Val is located in the

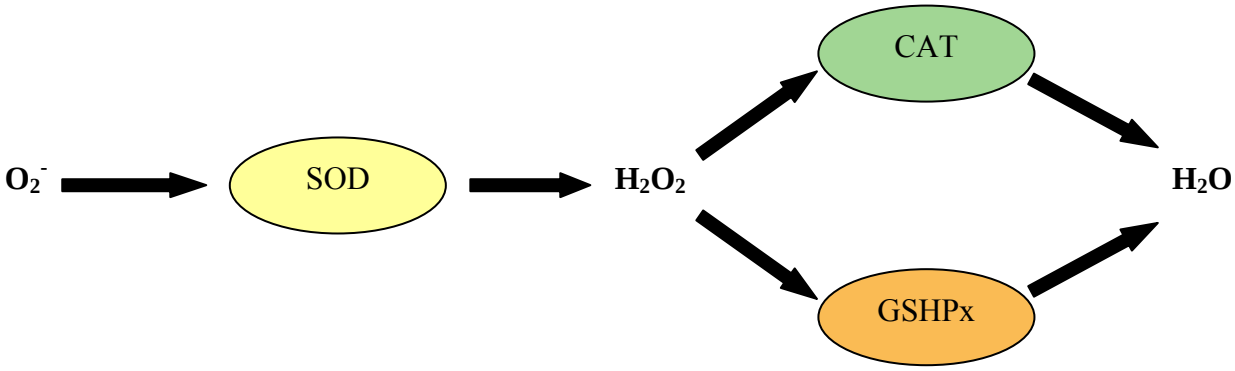



**Figure 1:** The enzymatic antioxidant pathway.

mitochondrial targeting sequence of MnSOD (see Figure 2). A change at residue 16 of the protein results in an amino acid change from alanine (GCT) to valine (GTT) (Ala16Val) (Rosenblum *et al*., 1996; Sutton *et al*., 2003). This amino acid is located at position -9 in the signal peptide, and hence this polymorphism is also referred to as Ala-9Val (Shimoda-Matsubayashi *et al*., 1996). It was discovered that Ala-9Val may influence mitochondrial transport (Rosenblum *et al*., 1996; Shimoda-Matsubayashi *et al*., 1996; Sutton *et al*., 2003; Sutton *et al*., 2005). The Val allele resulted in less efficient transport of MnSOD into the mitochondrion compared to the Ala allele in rat liver cells and HuH7 human hepatoma cells (Sutton *et al*., 2003; Sutton *et al*., 2005). This may mean that the –9Ala allele will result in higher MnSOD activity and hence protect against free radical damage. Recently Akyol *et al*. (2005) reported an association between the Ala-9Val polymorphism of the MnSOD gene and schizophrenia in a Turkish population. The heterozygous Ala/Val genotype was found to be higher in schizophrenics compared to healthy controls. They also reported a lower Ala/Ala genotype in the schizophrenic group compared to the controls. The Ala/Ala genotype, as postulated by the above group, may have a protective effect against schizophrenia, while the Ala/Val genotype may predispose to the development of the disorder. This finding supports the hypothesis that reactive oxygen species (ROS) may play a role in the development of neuropsychiatric disorders (Akyol *et al*., 2005). Yet there have been contradictory results with Zhang *et al.* (2002a) for example not finding an association between schizophrenia development and the Ala-9Val variant. Ala-9Val has also been associated with other disorders such as age-related macular degeneration (Kimura *et al*., 2000), where the development of the disorder was more likely when the Ala allele was present. It was therefore proposed that very efficient MnSOD could result in more production of $H_2O_2$, which may then result in free radical-mediated damage and the appropriate disorder.

#### 1.2.7.2 Complexity of schizophrenia

It is clear that schizophrenia is transmitted in a complex non-mendelian manner. The possibility that several gene loci may interact with each other to cause schizophrenia, as well as clinical heterogeneity complicates molecular studies of the disorder (OMIM number: #181500). The latter may result from factors such as different ethnicity, inadequate sample size, inaccurate assessment of schizophrenia symptoms by clinicians, overlapping symptoms with other disorders, lack of clear disease pathology, uncertain symptom boundaries, phenocopies and incomplete penetrance of the disease, (Sivagnansundaram *et al.*, 2003). Possible ways of solving this problem have been the study of endophenotypes or of atypical and typical antipsychotic action (Rybakowski *et al.*, 2001; Sivagnansundaram *et al.*, 2003).

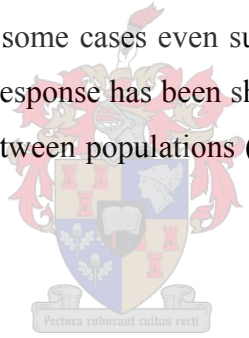
### 1.2.8 Treatment of schizophrenia

Management of schizophrenia is based on a bio-psycho-social approach (Mueser and McGurk, 2004). Schizophrenia itself is treated by either typical (conventional, first generation) antipsychotics, or with the newer atypical (second generation) antipsychotics (Kawanishi *et al*., 2000; Abidi and Bhaskara, 2003). Typical antipsychotics, which mainly target dopaminergic receptors, seem to alleviate positive symptoms and patient disability (Kawanishi *et al*., 2000). However, they seem to have little impact on the negative symptoms and carry a higher risk of side effects, such as tardive dyskinesia (TD) and extrapyramidal side effects (EPSE) (Kawanishi *et al*., 2000; Abidi and Bhaskara, 2003). Atypical antipsychotics, which mostly target serotonergic receptors, have been shown to be effective in the treatment of both positive and negative symptoms, with a lower risk of TD and EPSE (Kawanishi *et al*., 2000; Abidi and Bhaskara, 2003; Casey, 2004; Pierre, 2005; Tenback *et al*., 2005). Nonetheless, recent research suggests that these drugs, especially clozapine, do show side effects such as weight gain, hyperprolactinemia, obesity, dyslipidemias, diabetes and cardiac adverse effects (Basile *et al*., 2001a; Basile *et al*., 2002; Abidi and Bhaskara, 2003). Atypical antipsychotics also do not totally alleviate the risk of TD, and cases where TD development occurred after atypical antipsychotic treatment have been reported (Miller, 2003; Yeh *et al*., 2003; Ananth *et al*., 2004; Jeste, 2004; Pierre, 2005). Typical antipsychotics are generally prescribed in third world countries, since they are less expensive than atypical antipsychotics (Müller *et al*., 2004). The drug-induced side effects observed with typical antipsychotics, together with a generally unhealthy lifestyle (smoking and weight gain), as well as a high suicide rate found to exist in the schizophrenic population, increases this groups mortality risk

(Hughes *et al*., 1986; Yao *et al*., 2001; Abidi and Bhaskara, 2003). The smoking incidence has been shown to be extremely high among schizophrenics (Hughes *et al*., 1986). This may be due to a temporary decrease in negative symptoms through smoking. It is, however, not known whether this decrease is due to nicotine, the act of smoking, or other substances in cigarettes (Smith *et al*., 2002).

### 1.2.9 Side effects

As seen above, antipsychotics may induce certain side effects. This puts psychiatrists in a dilemma when prescribing drug treatment for their patients (Basile *et al*., 2002; Malhotra *et al*., 2004). Deciding what dosage to administer is a difficult task, since the efficacy of a drug at a certain dosage has to be balanced with the risk of developing side effects (Basile *et al*., 2002; Abidi and Bhaskara, 2003; Malhotra *et al*., 2004). This is complicated even more with some patients not responding to treatment, with for example only 60% of patients with schizophrenia responding to a specific drug (Spear *et al*., 2001). Psychiatric symptoms may persist, or side effects may develop, resulting in some degree of discomfort for the patient, which can lead to employment loss, social dysfunction, medical morbidity, and in some cases even suicide (Basile *et al*., 2002; Chouinard *et al*., 2002; Malhotra *et al*., 2004). Drug response has been shown to vary between patients (Basile *et al*., 2002; Malhotra *et al*., 2004) and between populations (Kalow and Bertilsson, 1994; Xie *et al*., 2001)




### 1.2.10 Genetics of psychiatric drug response and development of side effects

Variability in drug response seems to have a genetic component (Spear *et al*., 2001); although there is little heritability data available on this, due to various different drugs being prescribed over generations (Malhotra *et al*., 2004). Interethnic variability in drug response has been noted and this is explained by genetic and/or environmental factors (Kalow and Bertilsson, 1994). Based on the partly hereditary nature of drug response, as well as the development in the pharmacogenetic field and better molecular genetic techniques, research has focused on the analysis of several candidate genes, their variants and their possible role in drug response (Malhotra *et al*., 2004). In psychiatry, pharmacogenetic studies have focused mainly on the clinical efficacy of antipsychotics, efficacy of antidepressant medications and the development of adverse effects associated with psychotropic drugs (Basile *et al*., 2002; Malhotra *et al*., 2004). Studies on the efficacy of antipsychotic medication have mainly focused on the genetic basis of clozapine response, where the serotonergic and dopaminergic receptors have been extensively investigated (Basile *et al*., 2002). Tardive dyskinesia (TD), weight gain, sedation, extrapyramidal symptoms (EPS), long QT syndrome,

sexual dysfunction, blood lipid abnormalities and diabetes are some of the adverse effects that have been reported for the use of antipsychotics (Basile *et al*., 2002; Malhotra *et al*., 2004). TD, which seems to have a genetic component (Weinhold *et al*., 1981; Yassa and Ananth, 1981; Waddington and Youssef, 1988), has been one of the main side effects studied in conjunction with several genetic variants.

## 1.3 Tardive dyskinesia (TD)

Very little is known about the pathophysiology of abnormal involuntary movements (AIM) and no hypotheses exist that explain the development of AIM. Hence, in the following section the focus will be on TD specifically. TD (OMIM number: 272620) is a neuroleptic-induced involuntary movement disorder, with abnormal movements occurring in the tongue, jaw, trunk or extremities. Patients with TD are affected mainly on a social level, but in severe cases it may lead to problems such as having trouble eating or with dentures (orofacial dyskinesia) resulting in weight loss or cachexia development; problems during ambulation, respiratory dysfunction (diaphragmatic involuntary movements) and speech problems (APA, 1994; Sachdev, 2000).

### 1.3.1 Symptoms

The involuntary movements can take different forms, such as choreiform, athetoid, dystonic or stereotypic, or a combination of these forms. Choreiform movements are characterised by rapid, jerky, non-repetitive movements especially in the proximal muscles, whereas athetoid movements involve the distal muscles with slow, sinuous or writhing motions. Dystonic movements are slow and sustained muscle contractions, while stereotypic movements are rhythmic and repetitive (Sachdev, 2000). Generally the involuntary movements are more common in the orobuccal, lingual and facial muscles. However the limbs and trunk may also be affected (APA, 1994; Ebadi and Srinivasan, 1995; Sachdev, 2000). Involuntary movements in the facial, trunkal and limb area can also occur in combination (APA, 1994; Sachdev, 2000). TD symptoms may be worsened by stimulants, neuroleptic withdrawal, and anticholinergic medications and can be temporarily worsened by stress, emotional arousal, as well as distraction by voluntary movements in unaffected areas of the body. Abnormal movements are generally absent during sleep, while relaxation and voluntary movements in the affected area may also transiently reduce symptoms. Higher neuroleptic dosages may temporarily suppress TD (APA, 1994). As mentioned, neuroleptic-induced TD can show various types of involuntary movements, and this has prompted some to distinguish between TD and other involuntary movement disorders such as tardive dystonia, tardive akathisia, tardive tics and Tourette's syndrome. Other features that have been associated with TD are of a

neurological, behavioural and cognitive nature, and include saccadic eye movement abnormalities, neurological soft signs and ventricular enlargement (Sachdev, 2000).

### 1.3.2 Onset and diagnoses

TD generally develops after neuroleptic treatment and may occur at any age. Dyskinesias may occur as an acute effect of neuroleptic treatment, but also due to other drugs such as anticonvulsants, secondary to other neurological and systemic disorders as well as spontaneously (see sections 1.3.3 and 1.3.4) (Sachdev, 2000). According to the DSM IV, diagnosis of neuroleptic–induced TD occurs according to the criteria in Table 2. Diagnosis of TD can be considered accurate in the presence of moderately severe involuntary movements in at least one body part or mild movements in two or more body parts. Reassessment of patients with mild symptoms should occur within one week, to confirm the diagnoses (Schooler and Kane, 1982). To evaluate the severity of the symptoms, several scales can be used of which the Abnormal Involuntary Movements Scale (AIMS) is the most commonly used. Seven body regions are rated on a five-point scale for dyskinetic movements. Furthermore, a global severity rating of movements, the extent to which

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**Table 2:** DSMIV diagnostic criteria for tardive dyskinesia (APA, 1994).

**Research criteria for 333.82 Neuroleptic-Induced Tardive Dyskinesia**

A. Involuntary movements of the tongue, jaw, trunk, or extremities have developed in association with the use of neuroleptic medication.

B. The involuntary movements are present over a period of at least 4 weeks and occur in any of the following patterns:
   (1) choreiform movements (i.e., rapid, jerky, nonrepetitive)
   (2) athetoid movements (i.e., slow, sinuous, continual)
   (3) rhythmic movements (i.e., stereotypies)

C. The signs of symptoms in Criteria A and B develop during exposure to a neuroleptic medication or within 4 weeks of withdrawal from an oral (or within 8 weeks of withdrawal from a depot) neuroleptic medication.

D. There has been exposure to neuroleptic medication for at least 3 months (1 month if age 60 years or older).

E. The symptoms are not due to a neurological or general medical condition (e.g. Huntington's disease, Sydenham's chorea, spontaneous dyskinesia, hyperthyroidism, Wilson's disease), ill-fitting dentures, or exposure to other medications that cause acute reversible dyskinesia (e.g., L-dopa, bronocriptine). Evidence that the symptoms are due to one of these etiologies might include the following: the symptoms precede the exposure to the neuroleptic medication or unexplained focal neurological signs are present.

F. The symptoms are not better accounted for by a neuroleptic-induced acute movement disorder (e.g., Neuroleptic-Induced Acute Dystonia, Neuroleptic-Induced Acute Akathisia).

they incapacitate the patient and the patient's awareness of the movements is performed (Guy, 1976).

The course of TD is variable in terms of symptom severity, location, and duration (Ebadi and Srinivasan, 1995; Sachdev, 2000). Onset of TD is mainly gradual, with mild symptoms, but in some cases symptoms may be quite severe. TD symptoms remain stabile in half of all patients, while they may also worsen or improve in the rest (APA, 1994). In addition, remission of symptoms is also not uncommon (APA, 1994; Sachdev, 2000).

### 1.3.3 Dyskinesia as a feature of schizophrenia

Neuroleptically-naive schizophrenic patients have been reported to show dyskinesias, with the risk of developing TD symptoms and the severity of these symptoms increasing with age. The possibility exists that these dyskinesias are part of the pathophysiology of schizophrenia and symptoms are only exacerbated by neuroleptic treatment (Sachdev, 2000). A further finding implicates TD as a feature of schizophrenia, where negative symptoms severity as measured on SANS was also correlated with TD status, independent of risk factors such as age and antipsychotic exposure (Van Os *et al.*, 2000).

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### 1.3.4 Prevalence/incidence

About 20-30% of schizophrenics treated with antipsychotic medication develop tardive dyskinesia (Kane and Smith, 1982; Kane *et al*., 1985; Holden, 1987; APA, 1994). The prevalence of TD seems to vary between different populations, with for example lower prevalence rates (9.3%) in the Chinese population (Chiu *et al*., 1992) compared to western populations (25.0%) (Rittmannsberger and Schöny, 1986). The prevalence of this disorder in individuals of African descent has been found to be higher compared to other populations (van Harten *et al*., 1996). However, results are not consistent and the prevalence of TD for example in the Xhosa population was shown to be 28.4% (Patterson *et al*., 2005), which falls within the range (20-30%) of average TD prevalence (APA, 1994). The elderly also seem to be more susceptible to developing TD and show a prevalence of up to 50% (APA, 1994). The incidence of TD in the elderly lies between 25-30% after an average of 1 years cumulative exposure to neuroleptic medication (APA, 1994; Jeste *et al*., 1995), while younger individuals show an incidence of 3-5% per year (APA, 1994). Several cases of spontaneous tardive dyskinesia development have been reported (McCreadie *et al*., 1996; Sachdev, 2000; Kane, 2004). The calculation of the incidence and prevalence of drug-induced TD may therefore be confounded

by the occurrence of these spontaneous dyskinesias, but also by the use of different antipsychotics and other drugs, diagnostic criteria, symptom fluctuations and duration of antipsychotic treatment (Sachdev, 2000; Kane, 2004).

### 1.3.5 Different hypotheses

Drug induced tardive dyskinesia is potentially irreversible (Basile *et al*., 2002) and several changes (neuroplasticity, neurotoxicity, apoptosis) that may occur in the brain due to antipsychotic treatment have been investigated (reviewed in Dean, 2005). Still, the pathophysiology of TD is not well understood, however several theories have been proposed to explain its possible mechanism (Basile *et al*., 2002; Casey, 2004).

#### 1.3.5.1 Dopamine supersensitivity hypothesis

The most popular theory for TD development so far has been that of dopamine receptor hypersensitivity/supersensitivity (Klawans and Rubovits, 1972; Casey, 2004). It speculates that due to the chronic antipsychotic treatment (mainly dopamine antagonists), dopamine receptors will become supersensitive towards dopamine, which in the end results in TD development (Klawans and Rubovits, 1972; Sachdev, 2000; Casey, 2004).

The dopamine supersensitivity hypothesis is supported by research on rodents, where D2 receptor occupancy levels induced by haloperidol treatment had to be high and sustained before vacuous chewing movements were observed (Turrone *et al*., 2003). In addition, an increased number of dopamine D2 receptors have been correlated with behavioural changes in animal studies (Casey, 2004). However, data on humans does not seem to support this (Sachdev, 2000). For example, Adler *et al*. (2002) did not find striatal dopamine binding differences between patients with either schizophrenia, schizoaffective disorder, major depression or bipolar affective disorder, and TD or AIMS scores. Furthermore, this hypothesis does not explain age as a risk factor for TD development, as well as the formation of spontaneous dyskinesias (Sachdev, 2000).

Since different frequencies of TD are observed in typical and atypical antipsychotic treated patients, other hypotheses have been proposed based on the various receptors the two types of drugs bind to (Casey, 2004). Atypical antipsychotics block serotonin 2A receptors (HTR2A) in addition to D2 receptors, compared to typical antipsychotics, which mainly block D2 receptors (Meltzer, 1999; Casey, 2004). Serotonin may influence the dopaminergic pathway, by inhibiting dopamine release

from dopaminergic axon terminals. HTR2A blockade by the atypical antipsychotics may result in less dopamine receptor binding and hence reduce chances of TD development (Casey, 2004).

A further hypothesis focuses on different receptor-binding behaviour by the two types of antipsychotics. It is thought that atypical antipsychotics may not block D2 receptors long enough for EPS and other adverse affects to develop, but long enough for antipsychotic action to take place. The theory is difficult to prove or disprove, however it was found that rapid dissociation from the D2 receptor is correlated with lower EPS development (Casey, 2004).

#### 1.3.5.2 Neurodegeneration hypothesis

Neurodegeneration has been hypothesised to result in TD development (Andreassen and Jørgensen, 2000; Sachdev, 2000). This is also supported by risk factors for TD such as age and brain damage, as well as the disorder's persistent nature (Andreassen and Jørgensen, 2000). In addition atypical and typical antipsychotics were shown to result in cerebral cell death (Bonelli *et al*., 2005). Several hypotheses have been put forward that imply a neurodegenerative mechanism for TD development (Andreassen and Jørgensen, 2000).

##### 1.3.5.2.1 *Excitotoxicity*

TD development may also be explained by the excitotoxicity hypothesis (Andreassen and Jørgensen, 2000). It proposes that long-term neuroleptic treatment may increase the release of glutamate from the cortico-striatal terminals or result in decreased neuronal uptake of glutamate, which then results in excitotoxicity (Andreassen and Jørgensen, 2000, Burger *et al*., 2005a). Excitotoxic lesions have been implicated in the pathogenesis of TD (Gunne and Andren, 1993) and an association has been found between reduced glutamate transport and TD development in rats (Burger *et al*., 2005a), and this may be taken as evidence for an excitotoxic mechanism for TD development. The exact mechanism of the excitotoxicity is unknown (Andreassen and Jørgensen, 2000). However, an impaired cellular energy metabolism through long-term exposure to typical neuroleptics, also known as "indirect excitotoxicity" (Andreassen and Jørgensen, 2000), as well as reduced glutamate transporter activity resulting in excitotoxicity (Burger *et al*., 2005a), has been proposed as a possible mechanism.

1.3.5.2.2 *Free radical damage*

Another mechanism, which could result in neurodegeneration and possibly TD development, is free radical damage (Cadet and Kahler, 1994; Andreassen and Jørgensen, 2000; Sachdev, 2000). The sources of free radicals as well as the specific vulnerability of neurons to free radical damage were discussed earlier. It is thought that an increase in dopamine turnover due to long-term neuroleptic treatment (blocks dopamine receptors) may result in the higher free radical production, which in turn may lead to the development of TD (See, 1991; Andreassen and Jørgensen, 2000; Hori *et al*., 2000). Mainly typical antipsychotics seem to have an oxidative effect (Cadet and Perumal, 1990; Lezoualc'h *et al*., 1996; Maurer and Möller, 1997; Parikh *et al*., 2003; Polydoro *et al*., 2004). The free radicals produced may then result in oxidative stress and possibly neurodegeneration and TD (Andreassen and Jørgensen, 2000). Findings that support this hypothesis are higher levels of free radicals such as TBARS and conjugated dienes in the brain of rats (Naidu *et al*., 2002; Burger *et al*., 2005b) after neuroleptic treatment and also in the blood plasma and cerebrospinal fluid of TD patients (Lohr *et al*., 1990; Brown *et al*., 1998). Furthermore, abnormal antioxidant enzyme levels have been found in haloperidol-treated rat cell lines (Sagara, 1998), the brain of the TD rat model (Cadet and Perumal, 1990; Naidu *et al*., 2002), as well as the plasma of schizophrenic patients with TD (Zhang *et al*., 2003a). In addition, vitamin E (free radical scavenger) has been reported to reduce TD (Lohr *et al*., 1996; Andreassen and Jørgensen, 2000; Lohr *et al*., 2000). Some studies, however, could not confirm vitamin E's protective role in TD development (Andreassen and Jørgensen, 2000). The finding that cerebrospinal fluid of patients showed signs of lipid peroxidation (hence oxidative stress) after treatment with phenothiazines, provides further evidence for the free radical hypothesis (Pall *et al*., 1987). Also the fact that some antipsychotics result in free radical production supports the notion of a free radical mechanism in TD development (Cadet and Perumal, 1990; Lezoualc'h *et al*., 1996; Maurer and Möller, 1997; Parikh *et al*., 2003; Polydoro *et al*., 2004).

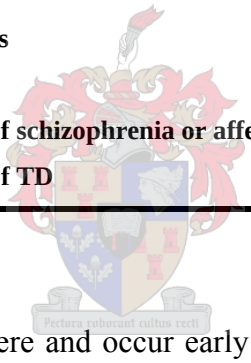
Movement disorders are thought to be heterogeneous and several mechanisms may result in TD development (Klein, 2005). The combination of a few hypotheses may be able to explain TD pathophysiology (Andreassen and Jørgensen, 2000; Sachdev, 2000; Margolese *et al*., 2005).

## 1.3.6 Risk factors

Several risk factors have been associated with TD development (Table 3). Older age has consistently been found to be a risk factor for TD development (Kane and Smith, 1982; Lieberman *et al*., 1984; Jeste, 2004; Kasper *et al*., 2006). TD in the elderly is more likely to be in the orofacial

**Table 3:** Some possible risk factors for TD. Reprinted with permission from Macmillan Publishers Ltd: The Pharmacogenomics Journal (Müller DJ, Shinkai T, De Luca V, Kennedy JL Clinical implications of pharmacogenomics for tardive dyskinesia. 4:77-87), copyright (2004).

| |
|---|
| **Advanced age** |
| **Female (or male) gender** |
| **Ethnicity (higher in African-Americans)** |
| **Presence of (early) extrapyramidal symptoms** |
| **Dose and duration of exposure to classical antipsychotics** |
| **Anticholinergic drugs, lithium** |
| **Organic brain dysfunctions and neurological deficits** |
| **Affective symptoms/disorders** |
| **Negative symptoms** |
| **Cognitive symptoms** |
| **Alcohol/drug misuse** |
| **Smoking** |
| **Diabetes mellitus** |
| **Menopause** |
| **Family history of schizophrenia or affective disorder** |
| **Family history of TD** |



area, to be irreversible, to be more severe and occur early during neuroleptic treatment (Sachdev, 2000). Changes in the central nervous system as well as to other biological processes could lead to older medicated patients being more prone to develop TD (Goldberg, 2003). Segman and Lerer (2002) put forward the hypothesis that certain genes that predispose to TD may in some way interact with age, resulting in a higher TD incidence in older patients. They did find an interaction between a *HTR2A* polymorphism and age. In addition, evidence suggests reduced serotonin receptor density, as well as serotonin-receptor-mediated prolactin response with age (Segman and Lerer, 2002). They propose that genetic polymorphisms associated with TD development may become more relevant with age-related changes in for example receptor density.

Another known risk factor is ethnicity, with African Americans having a higher and Asians having a lower risk of developing TD (Morgenstern and Glazer, 1993; Glazer *et al*., 1994). Furthermore, African Americans showed a poorer course of TD than did European Americans (Wonodi *et al*., 2004). European Americans also tended to show more dyskinetic movements in the upper and lower extremities, compared to African Americans, who showed more dyskinetic movements in the

trunkal area. Genetic factors have been postulated to explain these ethnic differences (Wonodi *et al*., 2004).

Duration of treatment with antipsychotics and its dose are also considered risk factors for TD development (APA, 1994; Jeste *et al*., 1995; Kapitany *et al*., 1998). However, this is also dependent on the type of antipsychotic prescribed (typical or atypical), since atypical antipsychotics seem to have a better outcome regarding TD development (Kawanishi *et al*., 2000; Abidi and Bhaskara, 2003; Casey, 2004; Tenback *et al*., 2005) and typical antipsychotics have generally been found to be a risk factor for the development of TD (Kawanishi *et al*., 2000; Abidi and Bhaskara, 2003; Gharabawi *et al*., 2005; Kasper *et al.*, 2006). In the Xhosa population duration of treatment and cumulative antipsychotic dose were also found to be risk factors for TD development (Patterson *et al*., 2005).

Gender also has been associated with TD development. While TD severity and spontaneous dyskinesia development were associated with female gender (Yassa and Jeste, 1992), Kasper *et al.* (2006) actually found that males are more at risk of developing TD. It seems, though, that in younger individuals gender is not a risk factor, while elderly females are more prone to develop TD (Yassa and Jeste, 1992; APA, 1994; Kasper *et al*., 2006).

Other risk factors that have been associated with TD development include duration of schizophrenia diagnosis, organic brain abnormalities, diagnosis with diabetes mellitus, mood disorders, drug or alcohol abuse and previous or early development of TD (also see Table 3) (Kane *et al*., 1988; APA, 1994; Sachdev, 2000; Basile *et al*., 2002; Jeste, 2004; Kasper *et al.*, 2006). In addition, anticholinergic drugs have been suggested to worsen, but not cause TD (Sachdev, 2000).

### 1.3.7 Treatment

So far no effective treatment for TD exists. There have been several studies on the possible treatment of TD with vitamin E. Results are, however, inconclusive, with some reporting reduced TD symptoms with vitamin E treatment (Elkashef *et al*., 1990; Lohr *et al*., 1996), some only if treatment occurs early during TD development (Boomershine *et al*., 1999; Pham and Plakogiannis, 2005), and no effect at all (Adler *et al*., 1999). Essential fatty acids have also been suggested to reduce symptoms of TD (Mellor et al., 1995, 1996), but results have varied (Emsley *et al*., 2006). In addition, long-term atypical antipsychotic treatment may also possibly reduce TD severity (Louzã and Bassitt, 2005). Other substances that have been mentioned as potential treatment, but need

further investigation, are: the antioxidant N-acetyl cysteine amide (AD 4) (Sadan *et al*., 2005), diphenyl diselenide (Burger *et al*., 2004), sarizotan (Rosengarten *et al*., 2006) and benzodiazepines (Bhoopathi and Soares-Weiser, 2006). In general a preventative strategy is adopted, and if TD develops, efforts are made to minimise the symptoms. This is done by prescribing the smallest antipsychotic dose, without risking relapse of the patient. Constant assessment of the patient for signs of TD during the treatment period should also be done to ensure that symptoms do not worsen, by for example discontinuing treatment with the specific antipsychotic or reducing the dose (Sachdev, 2000). In addition, anticholinergic drug treatment should be discontinued and dental status evaluated and corrected if need be (since badly fitted dentals may increase discomfort and involuntary movements (Blanchet *et al*., 2005)) (Sachdev, 2000). If possible, atypical antipsychotics should be used, since they seldom seem to result in TD or EPS development (Kawanishi *et al*., 2000; Abidi and Bhaskara, 2003; Casey, 2004; Tenback *et al*., 2005). The problem with this strategy is that atypical antipsychotics are generally more expensive than typical antipsychotics and therefore not a viable option in third world countries such as South Africa (Müller *et al*., 2004).

### 1.3.8 Genetics and TD

TD seems to have a genetic basis (Weinhold *et al*., 1981; Waddington and Youssef, 1988). Possible candidate genes for pharmacogenetic studies on TD have been chosen based on the pharmacokinetic characteristics of the antipsychotic (e.g. which enzyme metabolises the drug), or on the theory used to explain TD pathophysiology.

#### 1.3.8.1 Dopamine theory

Based on this theory (Discussed in section 1.3.5.1), genes coding for proteins in the dopamine pathway have been focused on. Genetic variants of the dopamine genes may affect dopamine receptor density (e.g. affects mRNA expression) (Arinami *et al*., 1997) or dopamine binding (Lundstrom and Turpin, 1996). The dopamine D2 receptor (DRD2) has been studied extensively in connection with TD, since it is the main site of action for typical antipsychotics. Yet in general no associations have been found between this gene and vulnerability to TD (Table 4) (Segman *et al*., 2003). Only the dopamine D3 receptor (DRD3) gene Ser9Gly polymorphism has been repeatedly associated with susceptibility to TD (Table 4).

DRD3 (OMIM number: *126451), a G-protein coupled receptor, belongs to the D2-like subfamily. It serves as an autoreceptor as well as a postsynaptic receptor (Schwartz *et al*., 1993) and is

**Table 4:** Association studies between tardive dyskinesia and several genetic variants.

| Gene | **Polymorphism** | **Author** | **Number of subjects (ethnic origin)** | **Results** |
|---|---|---|---|---|
| *MnSOD* | Ala-9Val | Akyol *et al.* (2005) | 153 (Turkish) | No association |
| *MnSOD* | Ala-9Val | Zhang *et al.* (2002a) | 151 (Chinese) | No association |
| *MnSOD* | Ala-9Val | Hori *et al.* (2000) | 333 (Japanese) | Possible association between -9Ala allele & protection against TD |
| *MnSOD*<br><br>*DRD3* | Ala-9Val<br><br>Ser9Gly | Zhang *et al.* (2003b) | 101 (Chinese) | Significant association of both variants combined with TD |
| *DRD3* | Ser9Gly | Woo *et al.* (2002) | 113 (Korean) | Significant association between Gly/Gly genotype & TD, but not with Ser9Gly alleles or AIMS score |
| *DRD3* | Ser9Gly | Garcia-Barcelo *et al.* (2001) | 131 (Chinese) | No association |
| *DRD3* | Ser9Gly | Segman *et al.* (1999) | 233 (Ashkenazi Jews, non-Ashkenazi Jews) | Significant association between Gly allele & TD |
| *DRD3* | Ser9Gly | Liao *et al.* (2001) | 115 (Chinese) | Significant association between heterozygotes & TD |
| *DRD3* | Ser9Gly | Rietschel *et al.* (2000) | 157 (German, Caucasian) | No association |
| *DRD3* | Ser9Gly | Steen *et al.* (1997) | 100 (Scottish, Caucasian) | Significant association between Gly homozygotes & TD |
| *DRD3* | Ser9Gly | Basile *et al.* (1999) | 112 (Caucasian, African American, Asian) | Significant association between: Gly allele & TD, & also between Gly homozygotes & AIMS scores |
| *DRD3*<br><br>*DRD2* | Ser9Gly<br><br>Ser311Cys | Chong *et al.* (2003b) | 317 (Chinese) | No association, except between *DRD3* Ser/Ser genotype & TD |
| *DRD3*<br><br>*DRD2*<br><br>*DAT* | Ser9Gly<br><br>His313His<br><br>40 bp tandem repeat | Inada *et al.* (1997) | 105 (Japanese) | No association |
| *DRD1*<br>*DRD2*<br>*DRD3*<br>*DRD4*<br>*DAT*<br>*COMT* | 24 polymorphisms | Srivastava *et al.* (2005) | 335 (North Indian) | Association between genetic variants in *DRD4* & *COMT* & TD. No association for other variants |
| *DRD3*<br><br>*DRD2*<br><br>*GSTM1*<br><br>*GSTT1*<br><br>*MDR1*<br><br>*CYP3A5* [1]<br><br>*CYP2D6* [1] | Ser9Gly<br><br>Ser311Cys<br>-141C Ins/del<br><br>Deletions<br><br>Deletions<br><br>C3435T<br><br>*3 *6<br><br>*2 *3 *4 *5 *6 *7 *8 *9 *10 *11 *14 *15 *17 *18 *19 *20 *25 *26 *29 *30 *31 *35 *36 *37 *40 *41 *43 *45 | De Leon *et al.* (2005) | 516 (American) | Significant association between severe TD & *DRD3*'s Ser9Gly;<br>Significant association between TD & *GSTM1* deletion specifically in white women;<br>Nonsignificant trend between TD & *CYP2D6* & *CYP3A5* absence, particularly in white men;<br>No significant association for *GSTT1*, *MDR1*, *DRD2* & TD |
| *DRD3 (meta-analysis)* | Ser9Gly | Bakker *et al.* (2006) | 1610 (various) | Significant association between *DRD3* Gly allele & TD; however possible publication bias |
| *DRD3 (meta-analysis)* | Ser9Gly | Lerer *et al.* (2002) | 780 (Caucasian, African American, German, Israeli Ashkenazi & non-Ashkenazi Jews, Austrian) | Significant association between *DRD3* Gly allele carrier & TD, & also between *DRD3* genotype & TD; significant association between Gly homozygotes & AIMS scores |

[1] The specific genetic variants which make up each individual allele are presented on the home page of the human cytochrome P450 (CYP) allele nomenclature committee: http://www.cypalleles.ki.se

**Table 4:** continued.

| Gene | Polymorphism | Author | Number of subjects (ethnic origin) | Results |
|---|---|---|---|---|
| *DRD3*<br>*HTR2C* | Ser9Gly<br>Cys68Ser | Segman *et al. (*2000) | 212 (Ashkenazi Jews, non-Ashkenazi Jews) | Significant association between *HTR2C* together with *DRD3* & TD |
| *DRD3*<br>*CYP-17* | Ser9Gly<br>Promoter T→C polymorphism | Segman *et al. (*2002a) | 113 (Ashkenazi Jews, non-Ashkenazi Jews) | Significant association between A2-A2 genotype together with *DRD3* Gly allele & susceptibility to abnormal orofacial distal involuntary movements & be incapacitated by these movements |
| *CYP2D6* [1] | *2 | Ohmori *et al. (*1999) | 99 (Japanese) | No association |
| *CYP2D6* [1] | A, B<br>(*3 *4) | Arthur *et al. (*1995) | 16 (Swedish, Caucasian) | No association between genotype & TD; but possible association between PM & AIMS score |
| *CYP2D6* [1] | *3 *4 | Jaanson *et al. (*2002) | 52 (Estonian, Russian) | Significant association between at least one PM allele & TD |
| *CYP2D6* [1] | *3 *4 *5 | Kapitany *et al. (*1998) | 45 (Austrian, Caucasian) | Significant association between heterozygotes & TD |
| *CYP2D6* [1] | A, B, D<br>(*3 *4 *5) | Armstrong *et al. (*1997) | 76 (Caucasian, 1 Asian) | Non-significant trend between PM & TD |
| *CYP2D6* [1] | *3 *4 *10 | Ohmori *et al. (*1998) | 100 (Japanese) | Significant association between *10 & AIMS; modest association between *10 & TD |
| *CYP2D6* [1] | *10 (C188T) | Liou *et al. (*2004) | 216 (Chinese) | Modest association between C100T & TD (especially in males); also between AIMS scores & C100T |
| *CYP2D6* [1] | *1*3 *4 *5 *6 *7 | Andreassen *et al. (*1997) | 100 (Scottish, Caucasian) | Non-significant tendency between PM & TD |
| *CYP2D6* [1] | *2 *3 *4 *10 *12 | Inada *et al. (*2003) | 516 (Japanese) | No association |
| *CYP2D6* [1] | *1 *2 *3 *4 *5 *6 *7 *8 *9 *10 *11 *14 *18 *19 *25 *26 *31 *36 *41 | Nikoloff *et al.* (2002) | 202 (Korean) | Significant association between males with at least one decreased or loss of function allele & TD |
| *CYP2D6* [1]<br>*CYP3A4* [1] | *4<br>*1B | Tiwari *et al. (*2005a) | 335 (North Indian) | No significant association with TD, but trend between *CYP2D6*4* & severity of TD |
| *CYP1A2* [1] | C→A in intron 1 | Chong *et al. (*2003a) | 291 (Chinese) | No association; but association between smokers & TD |
| *CYP1A2* [1] | C→A in intron 1 | Basile *et al. (*2000) | 85 (Caucasian, African American) | Significant association between polymorphism & TD |
| *CYP1A2* [1] | C734A<br>G-2964A | Matsumoto *et al. (*2004a) | 199 (Japanese) | No association |
| *CYP1A2* [1] | *1C<br>*1F | Tiwari *et al. (*2005b) | 335 (Indian) | Significant association between *1C (smokers) & TD severity |
| *DRD2*<br>*DAT*<br>*DRD4*<br>*HTR6*<br>*5-HTT*<br>*TPH* | *Taq*I A<br>-141C Ins/Del<br>Ser311Cys<br>3' VNTR<br>G2319A<br>Exon 3 VNTR<br>Promoter 120 bp repeat<br>C267T<br>5-HTTLPR (promoter)<br>Intron 7 A218C | Segman *et al. (*2003) | 122 (Ashkenazi Jews, non-Ashkenazi Jews) | No association |
| *DRD2* | Ser311Cys<br>-141C Ins/Del<br>*Taq*I A | Hori *et al. (*2001) | 200 (Japanese) | No association |
| *DRD2* | Ser311Cys<br>-141C Ins/Del<br>*Taq*I A<br>*TaqI* B<br>*TaqI* D | Liou *et al. (*2006) | 253 (unknown) | Significant association of *TaqI A* & *TaqI B* with TD development |

[1] The specific genetic variants which make up each individual allele are presented on the home page of the human cytochrome P450 (CYP) allele nomenclature committee: http://www.cypalleles.ki.se

**Table 4:** continued.

| Gene | Polymorphism | Author | Number of subjects (ethnic origin) | Results |
|---|---|---|---|---|
| *DRD2* | A-241G<br>-141C Ins/Del<br>*Taq*I A<br>*Taq*I B<br>*Taq*I D<br>Pro310Ser<br>Ser311Cys<br>Val96Ala<br>Leu141Leu | Kaiser *et al.* (2002) | 665 (European, Caucasian) | No association |
| *5-HTT* | 5-HTTLPR (promoter) | Chong *et al.* (2000) | 188 (Chinese) | No association |
| *HTR2A*<br><br>*5-HTT*<br><br>*COMT* | A-1438G<br>T102C<br><br>VNTR<br>5-HTTLPR (promoter)<br><br>Val158Met | Herken *et al.* (2003) | 222 (Turkey) | No association |
| *HTR2A* | T102C,<br>His452Tyr,<br>A-1438G | Segman *et al.* (2001) | 217 (Ashkenazi Jews, non-Ashkenazi Jews) | No association for His452Tyr; Significant association of T102C & A-1438G with TD |
| *HTR2A* | T102C | Tan *et al.* (2001) | 318 (Chinese) | Significant association between variant & TD |
| *HTR2A* | T102C,<br>His452Tyr,<br>A-1438G | Basile *et al.* (2001b) | 136 (Caucasian, African American) | No association |
| *HTR2A*<br><br>*HTR2A*<br><br>*HTR2C* | A-1438G<br><br>T102C<br><br>C68G (Cys23Ser) | Deshpande *et al.* (2005) | 335 (unknown) | No association |
| *HTR2C* | C-759T<br>G-697C | Zhang *et al.* (2002b) | 92 (Chinese) | Significant association between G-697C in males +TD |
| *ACE* | 287 bp Ins/Del in intron 16 | Segman *et al.* (2002b) | 200 (Ashkenazi Jews, non-Ashkenazi Jews) | No association |
| *COMT* | Val158Met | Lai *et al.* (2004) | 299 (Chinese) | No association |
| *COMT*<br><br>*MAOA*<br><br>*MAOB* | Val158Met<br><br>30 bp repeat (promoter)<br><br>A113546G (A/G in intron 13) | Matsumoto *et al.* (2004b) | 206 (Japanese) | No association |
| *ESR1* | *Pvu*II polymorphism (~400 bp upstream of exon 2)<br><br>*Xba*I polymorphism (~350 bp upstream of exon 2 | Lai *et al.* (2002) | 246 (Chinese) | Marginal association between *Pvu*II & TD, but no association for *Xba*I |
| *GSTP1* | Ile105Val | Shinkai *et al.* (2005) | 225 (Caucasian, African American) | No association |
| *GSHPx* | Pro197Leu | Shinkai et al. (2006) | 68 (Caucasian, African American) | No association |
| *NOS1* | C267T | Wang *et al.* (2004) | 251 (Chinese) | No association |
| *PAH* | 12 variants | Richardson *et al.* (2006) | 123 (unknown) | No association |
| *OPRM* | A118G | Tan *et al.* (2003) | 303 (Chinese) | No association, but trend of protective effect by 118G allele |
| (whole genome) | CAG repeat expansions | Lowrimore *et al.* (2004) | 19 (African American, Hispanic, Caucasian) | No association |

Pectora roborant cultus recti

expressed scarcely in the brain and selectively expressed in the nucleus accumbens and olfactory tubercle (Steen *et al*, 1997). Suzuki *et al*. (1998), however, found the D3 mRNA more widely spread throughout the human brain, with intense D3 mRNA expression in the islands of Calleja, the ventral striatum, and the dentate gyrus. DRD3 mRNA has also been localised through autographic studies to the ventral side of the globus pallidus, which is a brain region involved in motor control (Liao *et al*., 2001). Generally the D3 receptor is associated with the limbic and basal ganglia regions

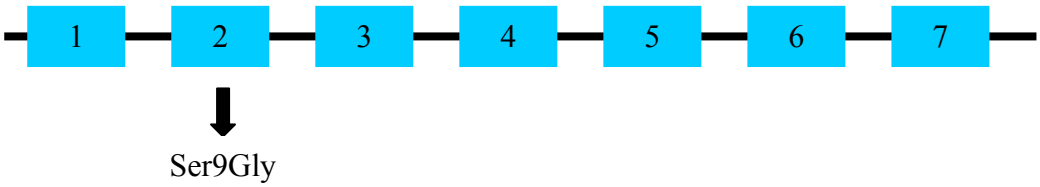
(Sokoloff *et al*., 1990; Suzuki *et al*., 1998). These areas are involved in cognitive, emotional, endocrine and motor functions (Suzuki *et al*., 1998). DRD3 seems to play a role in motor control, since for example DRD3 antagonists increase locomotor activity in rats (Kling-Petersen *et al*., 1995) and *DRD3* knockout mice show hyperactivity (Accili *et al*., 1996). The D3 receptors also have a high affinity for antipsychotics and a higher dopamine affinity than the D1 and D2 receptors (Sokoloff *et al*., 1990; Suzuki *et al*., 1998). The *DRD3* gene (see Figure 2) is located on chromosome 3q13.3 (Le Coniat *et al*., 1991) and consists of 7 exons (Genatlas, Université René Descartes, Paris, http://www.genatlas.org). A genetic variant located in exon 2 in the *DRD3* gene results in an amino acid change from serine to glycine in the N-terminal extracellular domain of the D3 receptor (Lannfelt *et al*., 1992). The Ser9Gly polymorphism was found to affect dopamine binding affinity, with the Gly homozygote having a higher binding affinity than the heterozygote or the Ser homozygote (Lundstrom and Turpin, 1996).

Several studies have performed association studies between the Ser9Gly polymorphism (Table 4) and susceptibility to TD (Segman *et al*., 1999; Woo *et al*., 2002). Most studies did confirm an association between the Ser9Gly polymorphism and vulnerability to TD, however, Inada *et al*. (1997), Rietschel *et al*. (2000) and Garcia-Barceló *et al*. (2001) did not. Eichhammer *et al*. (2000) also found a link between Ser9Gly homozygosity and the development of acute akathisia, which can be regarded as a forerunner of TD, in 150 Caucasian schizophrenics. In general, Gly homozygosity was found to be associated with either risk of TD or severity of AIMS scores. However, Chong *et al*. (2003b) reported an association between Ser homozygosity and risk of TD. In a meta-analysis of 780 patients from six research centres, Lerer *et al*. (2002) confirmed an association between DRD3 Gly allele carrier status as well as genotype and TD, with Gly homozygotes having higher AIMS scores than heterozygotes and Ser homozygotes. They found that the polymorphism's effect is small but significant, and that other genetic variants as well as environmental factors may also contribute to the development of TD. Ethnic differences in the genetic variant frequency as well as vulnerability to TD were also observed in this study. Segman *et al*. (2002a) also reported an interactive effect of the Gly allele and homozygosity for the mutant form of a CYP17α-hydroxylase polymorphism in the development of abnormal orofacial and distal involuntary movements as well as incapacitation by these movements, when chronically exposed to typical antipsychotics. In addition, the same group investigated a 5-HT2C receptor gene (*HTR2C*) polymorphism and the possibility of an interactive effect with *DRD3* Ser9Gly in the susceptibility to TD (Segman *et al*., 2000). They found that these two polymorphisms contribute in an additive but only small way to the susceptibility to TD. An interactive effect between *DRD3* Ser9Gly and *MnSOD* Ala-9Val polymorphism was also observed in a study by Zhang *et al*. (2003b), where the

***DRD3***

Length: 50.1 kb

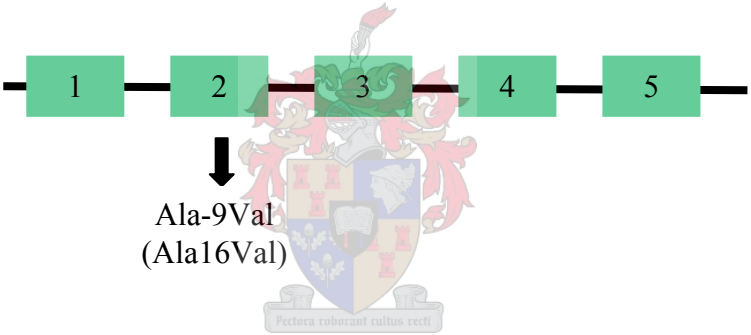
Chromosome location: 3q13.3




***MnSOD***

Length: 11.03 kb

Chromosome location: 6q25.2




***CYP2D6***

Length: 5 kb

Chromosome location: 22q13.1

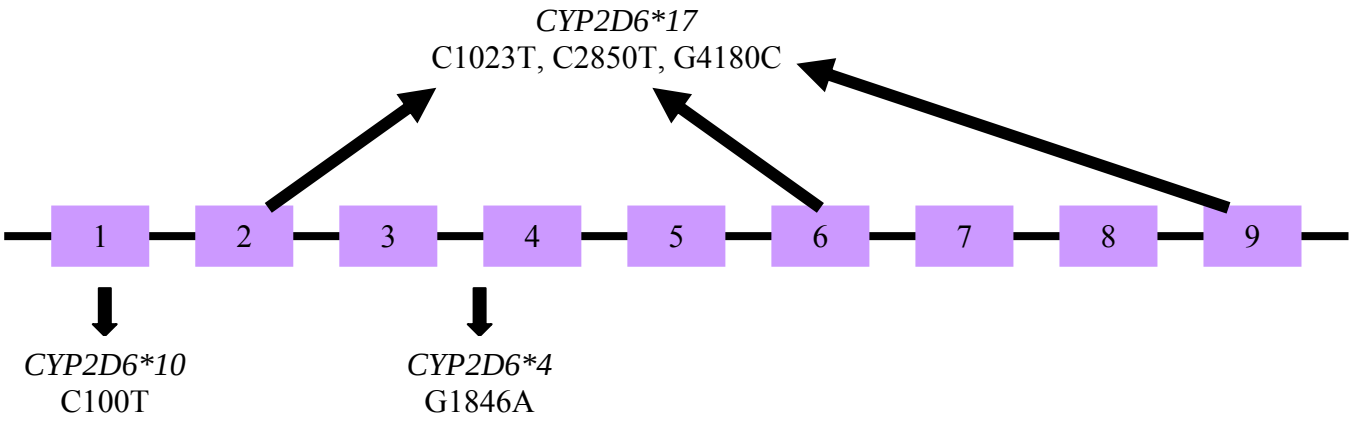



**Figure 2:** Size, location and structure of *DRD3*, *MnSOD* and *CYP2D6* (not drawn to scale).

Ser and the Val alleles respectively were associated with the development of TD. Another study looked at the *DRD3* Ser9Gly polymorphism in conjunction with the occurrence of spontaneous TD development in drug naive schizophrenics, but did not find any association (Løvlie *et al.*, 2001). *DRD3's* Ser9Gly polymorphism has also been studied in conjunction with schizophrenia development. However, results have been contradictory (Dubertret *et al*., 1998; Jönsson *et al*., 2003; Ambrósio *et al*., 2004; Staddon *et al*., 2005).

Genes in the serotonin pathway have been investigated for their role in TD development as well. Serotonin receptor genetic variant association studies have shown little results, mainly finding no association with TD development (Table 4). In addition, genetic variants involved in serotonin and dopamine synthesis, degradation, re-uptake and transport have been studied, however no association between these variants and TD development could be confirmed (Table 4) (Segman *et al*., 2003; Herken *et al*., 2003). Mainly studies have focused only on single or small numbers of SNPs and their association with TD. This in association with population heterogeneity has posed a problem in the search for genetic variants giving rise to TD susceptibility (Malhotra *et al*., 2004).

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1.3.8.2 Free radical theory

Based on the free radical theory genes involved in the antioxidant pathway have been examined, such as *GSHPx*, *CAT* and *MnSOD.* One study investigated the role of the Pro197Leu genetic variant in the glutathione peroxidase gene, which codes for an enzyme involved in the antioxidant pathway (Shinkai *et al*., 2006). Yet, no association was found. For the main part studies have focused on *MnSOD* (see Figure 2) where a functional polymorphism was discovered (Ala-9Val). MnSOD was discussed earlier in the context of schizophrenia development (See section 1.2.7.1), and, as mentioned, is involved in superoxide radical detoxification (Fridovich, 1974; Chance *et al*., 1979). A role of this enzyme in the pathophysiology of tardive dyskinesia has been suggested (Hori *et al*., 2000; Zhang *et al*., 2003b). It was shown that the Val allele resulted in less efficient transport compared to the Ala allele in rat liver cells and HuH7 human hepatoma cells (Sutton *et al*., 2003, 2005). Hori *et al*. (2000) found a significant association between Ala-9Val and TD in a Japanese cohort of 233. The mutant –9Ala allele was found less in TD patients compared to healthy controls, indicating that this allele may have a protective effect in the development of TD. Zhang *et al*. (2002a) confirmed an association between MnSOD activity and AIMS scores, however no association was found between the Ala-9Val polymorphism and TD in a cohort of 115 Chinese subjects consisting of schizophrenic patients with and without TD and healthy control subjects. Akyol *et al*. (2005) could also not confirm an association between the polymorphism and TD in a

Turkish population. Zhang *et al*. (2003b), however, found an interactive effect between *DRD3* Ser9Gly and *MnSOD* Ala-9Val polymorphism, where the Ser and the Val allele was associated with the development of TD. While MnSOD scavenges free radicals, it also produces $H_2O_2$, which, if it is not eliminated, can interact with transition metals to form free radicals and hence result in cell damage (Chance *et al.*, 1979). This highlights the relevance of other antioxidant enzymes, such as GSHPx and CAT in removing reactive oxygen species.

#### 1.3.8.3 Drug metabolising enzymes

Drugs are metabolised by several mechanisms: by phase I (oxidative) or phase II (conjugative) reactions (Linder *et al*., 1997; Wolf and Smith, 1999). Antipsychotics are metabolised by members of the cytochrome P450 (CYP) family *via* oxidation (Wilkinson, 2005). The CYP family is responsible for metabolising about 50% of drugs (Bertz and Granneman, 1997). Several genetic variants that result in altered activity of the enzyme have been observed in these genes (Homepage of the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee, http://www.cypalleles.ki.se/cyp2d6.htm). Since antipsychotic dosage and duration of treatment has been shown to be a risk factor for TD (Kapitany *et al*., 1998), any factor that increases the plasma level of antipsychotic medication may contribute to the risk of developing TD (Liou *et al*., 2004). Hence these enzymes have been studied in the context of TD development. CYP enzymes that have been investigated include CYP1A2, CYP2D6 and CYP17, with varying results (Table 4). However, the focus in regard to TD development has mainly been on CYP2D6 (Table 4).

CYP2D6 (OMIM number: +124030) metabolises several drugs (about 25% of all CYP-metabolised drugs), including neuroleptics and antidepressants, as well as other foreign chemicals (e.g. environmental pollutants), arachidonic acid and eicosanoids by oxidative metabolism (Arthur *et al*., 1995; Nebert and Russell, 2002; Ingelman-Sundberg, 2005b), and is found in the liver, the gastrointestinal tract, the brain, and possibly the lung (Zanger *et al*., 2004). It is considered a high-affinity, low capacity metabolic enzyme, since it only constitutes about 2% (Shimada *et al*., 1994) of the CYP enzymes found in the liver (Malhotra *et al*., 2004). The *CYP2D6* gene (see Figure 2), consisting of 9 exons, lies on chromosome 22 together with its pseudogenes *CYP2D7P* and *CYP2D8P* (97 % and 92 % exonic nucleotide similarity to *CYP2D6* respectively). *CYP2D7P* is similar to *CYP2D6*, but has a reading frame-disrupting insertion within exon one. *CYP2D8P* on the other hand contains several insertions, deletions and terminations within its exons (Kimura *et al*., 1989). CYP2D6 activity can be determined in two ways, namely *via* phenotyping or genotyping.

Phenotyping involves the intake of a probe drug (only metabolised by CYP2D6), such as debrisoquine, and later the calculation of the metabolic ratio (ratio of parent compound to metabolite in urine or serum) (MR) (Linder *et al*., 1997; McKinnon and Evans, 2000; Abraham and Adithan, 2001).

Genotyping involves the identification of known mutations which affect enzyme activity (McKinnon and Evans, 2000; Abraham and Adithan, 2001), as described below. Several genetic variants have been described for the *CYP2D6* gene, some of which result in lower CYP2D6 levels, or a total lack thereof (Homepage of the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee, http://www.cypalleles.ki.se/cyp2d6.htm). Individuals with a MR≥20 (phenotype) who are also homozygous for the alleles that result in a lack of enzyme activity (null alleles) (genotype), and hence have elevated drug plasma levels, are referred to as poor metabolisers (PMs) (Kirchheiner *et al*. 2004, Zanger *et al*., 2004). These individuals may be at a higher risk of adverse side effects, such as tardive dyskinesia (Kapitany *et al*., 1998). About 5-10% of Caucasians (Alván *et al*., 1990; Andreassen *et al*., 1997; Sachse *et al*., 1997) and 0-1% of Asians (Sohn *et al*., 1991) are poor metabolisers. The PM frequency of Africans varies between populations (0-19%) (Evans *et al*., 1993; Masimirembwa *et al*., 1996a; Panserat *et al*., 1999; Xie *et al*., 2001). For example, the San Bushmen have a higher PM frequency (19%) (Sommers *et al*., 1988) compared to population groups from Ghana (3%) (Griese *et al*., 1999) and Zimbabwe (2%) (Masimirembwa *et al*., 1996a). In general, though, it seems that African populations have a somewhat lower PM frequency (1-9%) compared to Caucasians (Bradford *et al*., 1998). Hence the prevalence of PMs between different populations may vary, with Caucasians showing the highest PM frequency. Individuals with 1.2<MR<20 (phenotype) who are also homozygotes for a deficient enzyme activity allele or a carrier of a null allele (genotype), have intermediate drug plasma levels (Panserat *et al*., 1999; Zanger *et al*., 2004; Kirchheiner *et al*. 2004). These are the so-called intermediate metabolisers (IM). IMs are quite frequent in the Asian (38.1-70.0% of *CYP2D6*10* allele) (Xie *et al*., 2001) and African populations (9.0-34.0% of *CYP2D6*17* allele) (Panserat *et al*., 1999; Xie *et al*., 2001) compared to Caucasians (Sachse *et al*., 1997; Xie *et al*., 2001). Individuals with 0.15≤MR≤1.2, or who are wildtype homozygotes or heterozygotes for a deficient function allele, are referred to as extensive metabolisers (EMs) and are considered to have "normal" enzyme activity (Kirchheiner *et al*., 2004; Zanger *et al*., 2004). Ultrarapid metabolisers (UMs) are individuals that show a MR<0.15 (phenotypically) and gene duplications (genotypically) and hence show a lower plasma drug level (Bertilsson *et al*., 1993; Zanger *et al*., 2004). This phenomenon has mainly been observed in Caucasian (Masimirembwa and Hasler, 1997) and North African populations (Aklillu *et al*., 1996). It has been proposed that the higher frequency of *CYP2D6* duplications, specifically in the

Ethiopian population, may have resulted as a consequence of selection pressure during starvation, allowing more efficient detoxification of plant toxins and hence wider selection of food (Ingelman-Sundberg, 2005a). It should be noted that other *CYP2D6* classification systems are being developed, due to the discrepancies regarding the definition specifically of IM status (Steimer *et al.*, 2004). While genetics clearly influences CYP2D6 activity, other factors may also influence CYP2D6 metabolism such as environmental factors, drug interactions and enzyme inhibition (Caraco, 2004; Wilkinson, 2005). Through the latter two mechanisms an individual may be converted from an EM into a PM, independent of genotype, this is termed phenocopying (Kroemer and Eichelbaum, 1995; Wilkinson, 2005). Although EM women may have a slightly higher *CYP2D6* activity compared to men, *CYP2D6* cannot be induced by hormones nor regulated by any other known environmental substance (Meibohm *et al*., 2002; Ingelman-Sundberg, 2005a).

Several association studies have been done on *CYP2D6* genetic variants or PM status and susceptibility to TD (Table 4). In this context, *CYP2D6*3* and *CYP2D6*4* have been studied most often. While several studies report a significant association or non-significant trend between PM status and TD, few could confirm a link between one specific variant and susceptibility to TD. Ohmori *et al*. (1998) did find *CYP2D6*10* (mainly present in Asians) to be significantly associated with risk of TD in the Japanese population. Liou *et al*. (2004) could only confirm this association in males, and also found a significant association between AIMS score and *CYP2D6*10* in a Chinese population. Association studies between *CYP2D6* and TD were performed on different populations, and since it is clear that some *CYP2D6* genetic variants are more frequent in certain populations, it is important to study the effects of the population relevant variants in the appropriate population. Based on the allele frequencies of *CYP2D6* variants in African populations (Xie *et al*., 2001), some of these alleles will be discussed in more detail below.

#### 1.3.8.3.1 *CYP2D6*4*

The variant characterising this allele is G1846A (end of intron 3; see Figure 2), which results in a splicing defect, and in homozygous individuals in the absence of CYP2D6 (PM status) (Kagimoto *et al*., 1990). This variant is found less in the African (0.9-9.3%) and almost never in the Asian population (0.0-0.8%) (Evans *et al*., 1993; Masimirembwa and Hasler, 1997; Bathum *et al*., 1999; Xie *et al*., 2001), and occurs more frequently in Caucasians (11.3-28.6%) (Xie *et al*., 2001).

1.3.8.3.2 *CYP2D6*10*

This allele is made up primarily by the C100T mutation (exon 1; see Figure 2). It results in an amino acid change from proline to serine. Functional studies showed that C100T results in the expression of a more unstable gene product (hence less CYP2D6 enzyme) (Johansson *et al*., 1994). Homozygosity for this variant results in the IM state. *CYP2D6*10* is mainly found in the Asian population (38.1-70.0%), whereas it is found less in the African (1.0-8.6%) and Caucasian (1.4-6.1%) populations (Xie *et al*., 2001).

1.3.8.3.3 *CYP2D6*17*

Three variants make up the *CYP2D6*17* allele (see Figure 2), C1023T (exon 2), C2850T (exon 6) and G4180C (exon 9). These variants result in the amino acid changes Thr107Ile, Arg296Cys and Ser486Thr respectively, of which the last two variants are also present in *CYP2D6*2* (Masimirembwa *et al.*, 1996b). The presence of C1023T and C2850T specifically results in the IM phenotype in homozygous individuals by lowering the metaboliser rate (Oscarson *et al.*, 1997). *CYP2D6*17* is mainly found in African populations (9.0-34.0%) (Masimirembwa *et al.*, 1996b; Panserat *et al.*, 1999; Xie *et al*., 2001), and its presence explains the high amount of intermediate metabolisers in this population (Bathum *et al*., 1999; Panserat *et al.*, 1999; Dandara *et al.*, 2001; Xie *et al*., 2001). This allele is very seldom found in Caucasians (0.0-1.1%) as well as Asian populations (0.0%) (Xie *et al*., 2001).

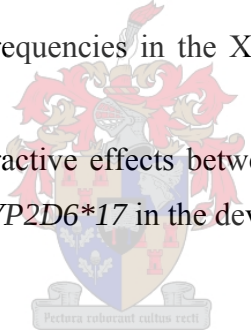
## 1.4 Aim of study

The mechanism of AIM/TD is not well understood and possibly heterogeneous of nature. Furthermore it may also differ between populations. Several association studies have implicated specific variants in the development of this disorder, however, mainly in Caucasian or Asian populations. Very few studies have focused on African populations and then mainly on African Americans in combination with Caucasians (Table 4). The Xhosa population is unique to South Africa and is one of the largest population groups here. As mentioned earlier, a study on a Xhosa schizophrenia population found a prevalence of 28.4% for TD (Patterson *et al*., 2005). Our study aimed at determining the prevalence of specific genetic variants in this population and attempted to replicate associations found between certain genetic variants and TD in the unique African Xhosa population. Since the diagnosis of TD requires very stringent criteria, patients were at first only diagnosed as having abnormal involuntary movements from the clinical data available. Therefore, this study focused on the link between abnormal involuntary movements (which includes TD) and certain genetic variants. However, a *post hoc* TD classification was later performed on patients with

abnormal involuntary movements and hence associations between certain genetic variants and TD were still investigated. This study is unique in that it focuses on AIM as well as TD development and severity. In the future genetic testing to determine the optimal treatment for a patient may become a reality, but is dependent on various factors, including the determination of the genetic components that result in AIM/TD in the various populations (since different genetic components may contribute to the development of the disorder in different populations). This study attempted to find these possible genetic components in the Xhosa schizophrenia population.

The specific objectives of this study were:

- Investigate the role of the following genetic variants in the development and severity of AIM and TD:
    - *MnSOD* Ala-9Val ;
    - *DRD3* Ser9Gly;
    - *CYP2D6*4*, *CYP2D6*10* and *CYP2D6*17;*
- Investigate the role of *MnSOD* Ala-9Val in the development and severity of schizophrenia;
- Compare allele and genotype frequencies in the Xhosa schizophrenia population to those reported in the literature;
- Determine if there are any interactive effects between *MnSOD* Ala-9Val, *DRD3* Ser9Gly, *CYP2D6*4*, *CYP2D6*10* and *CYP2D6*17* in the development of AIM/TD.



# Chapter Two

# Association between the *MnSOD* Ala-9Val polymorphism and development of schizophrenia and abnormal involuntary movements in the Xhosa population

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## 2.1 Abstract

Reactive oxygen species (ROS)-mediated damage has been hypothesized to play a role in the development and poor outcome of schizophrenia, as well as the development of neuroleptic-induced abnormal involuntary movements. Recently, the functional polymorphism (Ala-9Val) in the manganese superoxide dismutase (*MnSOD*) gene (part of the antioxidant defence mechanism) was found to be associated with schizophrenia in a Turkish population. This study was aimed at replicating this finding in a Xhosa population. In addition, the role of Ala-9Val in abnormal involuntary movement and tardive dyskinesia development in the Xhosa population was also investigated. The schizophrenic patient group (n=286) and a healthy control group (n=243) were genotyped for the Ala-9Val polymorphism using heteroduplex-single stranded conformational polymorphism (HEX-SSCP) analysis. No significant difference in genotype or allele frequency could be observed between the schizophrenia and control group ($P$=0.294 and $P$=0.528 respectively). In addition no association could be found between the polymorphism and symptom severity (SANS and SAPS). The Xhosa schizophrenia patient group with abnormal involuntary movements (n=54) and a subgroup with tardive dyskinesia (n=30) was found to significantly differ in Ala-9Val genotype frequency ($P$=0.008 and $P$=0.011 respectively) compared to the Xhosa schizophrenia patient group without abnormal involuntary movements (n=204). However, no significant difference was found for the allele frequencies ($P$=0.955 and $P$=0.161). Further, using ANCOVA no association was found between AIMS score and genotype in the group with abnormal involuntary movements ($P$=0.1234). However, in the patient group with tardive dyskinesia an association was observed between genotype and AIMS score ($P$=0.0365). These results do not support a major role of the *MnSOD* Ala-9Val polymorphism in the development of schizophrenia or symptom severity in the Xhosa population. Yet it seems to be involved in the development of

abnormal involuntary movements and tardive dyskinesia and may even modulate the severity of tardive dyskinesia.

## 2.2 Introduction

Reactive oxygen species (ROS) are generated *in vivo* by several mechanisms including auto-oxidation and electron leakage in the respiratory chain in the mitochondria (Halliwell, 1997; Fridovich, 1983). The antioxidant defence mechanism of the body consists of (a) enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT), and (b) antioxidant compounds such as α-tocopherol (vitamin E) and ascorbic acid (vitamin C) (Mahadik and Mukherjee, 1996; Rao and Balachandran, 2002). Excess ROS that are not scavenged by the antioxidant defence mechanism can interact with lipids, proteins and nucleic acids, resulting in cellular dysfunction or cell death (Halliwell, 1991; Mahadik and Mukherjee, 1996). Aberrations, whether linked to diet or genetic alterations in the antioxidant defence mechanism, may therefore result in an excess of ROS and hence damage to the cells.

Neural cells seem to be highly susceptible to oxidative damage (Mahadik *et al.*, 2001; Rao and Balachandran, 2002), and there is increasing evidence that ROS-mediated damage in the central nervous system (CNS), through an inefficient antioxidant defence mechanism and/or increase in ROS production, may play a role in the development of schizophrenia (reviewed in Yao *et al.*, 2001).

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Individuals with schizophrenia seem to have higher superoxide anion levels (Melamed *et al.*, 1998; Sirota *et al.*, 2003) as well as increased levels of lipid peroxidation products, which are a measure of oxyradical-mediated damage (Mahadik *et al.*, 1998; Dakhale *et al.*, 2004), compared to healthy controls. Furthermore, low levels of membrane phospholipid PUFAs have consistently been observed in drug-naïve and medicated schizophrenic patients (Khan *et al.*, 2002; Arvindakshan *et al.*, 2003a). Evidence, albeit ambiguous, also exists for altered levels (Ranjekar *et al.*, 2003; Michel *et al.*, 2004) and activity (Ravikumar *et al.*, 2000; Kuloglu *et al.*, 2002) of the antioxidant enzymes SOD, CAT and GSHPx in the blood or CNS of drug-naïve or antipsychotic-treated patients. Other antioxidant compounds such as albumin, bilirubin, uric acid, and ascorbic acid have also been shown to be lower in drug-naïve schizophrenic patients compared to healthy controls (Reddy *et al.*, 2003; Dakhale *et al.*, 2004). While some antipsychotics may have pro-oxidant qualities (Parikh *et al.*, 2003; Polydoro *et al.*, 2004) which could confound findings, drug-naïve patients have also been shown to have aberrant levels of antioxidant enzymes (Mukherjee *et al.*, 1996), lipid peroxidation products (Mahadik *et al.*, 1998) as well as antioxidant compounds (Reddy *et al.*, 2003). This has

lead to the hypothesis that ROS-mediated damage may be involved in the pathophysiology of schizophrenia (Mahadik and Mukherjee, 1996). Nevertheless, it is thought that oxidative damage may not be the primary event resulting in schizophrenia, but may affect deterioration and poor outcome of the disorder (Mahadik *et al.*, 2001; Arvindakshan *et al.*, 2003a). For example, an excess of ROS or low total antioxidant status during a three month drug-free (not drug-naïve) period have been positively correlated with negative symptoms (PANSS, BHPR, BPRS, SANS) in patients with schizophrenia (Yao *et al.*, 1998a; Sirota *et al.*, 2003). In addition some evidence exists that worsening negative symptoms in psychotic patients is linked to the development of tardive dyskinesia (TD) (Van Os *et al.*, 2000), a neuroleptic-induced involuntary movement disorder (Sachdev, 2000). Other studies, however, did not confirm this (Chiu *et al.*, 1993).

It has been postulated that oxidative damage, due to an increased dopamine turnover, may also play a role in the development of TD (See, 1991; Cadet and Kahler, 1994; Andreassen and Jørgensen, 2000). Evidence that supports this hypothesis comes from higher levels of ROS indices (such as TBARS and conjugated dienes) in the brain of rats after neuroleptic treatment (Naidu *et al.*, 2002; Burger *et al.*, 2005b) and in the plasma and cerebrospinal fluid of TD patients (Lohr *et al.*, 1990; Brown *et al.*, 1998). Neuroleptics used in the treatment of schizophrenia, specifically typical antipsychotics, have been shown to induce oxidative stress (Cadet and Perumal, 1990; Maurer and Möller, 1997). Abnormal antioxidant enzyme levels have been found in haloperidol-treated (typical antipsychotic) rat cell lines (Sagara, 1998), the brain of the TD rat model (Cadet and Perumal, 1990; Naidu *et al.*, 2002), as well as the plasma of schizophrenic patients with TD (Zhang *et al.*, 2003a). In line with this hypothesis a diet rich in antioxidants is suggested to be protective against the development of TD (Lohr *et al.*, 2000).

One of the enzymes which is part of the antioxidant defence system is SOD. It plays a role in neurodevelopment, specifically growth termination and differentiation initiation (Mahadik and Mukherjee, 1996). Three types of SOD enzymes can be distinguished, namely copper-zinc SOD in the cytosol (McCord and Fridovich, 1969), extracellular SOD (Marklund, 1982), and manganese SOD (MnSOD) that is localized in the mitochondria (Weisiger and Fridovich, 1973). MnSOD (OMIM number: *147460) converts $O_2^-$ to $H_2O_2$ (Fridovich, 1974). A functional polymorphism in the *MnSOD* gene, due to a change of one nucleotide in the mitochondrial targeting sequence (MTS), results in an amino acid change from alanine (G<u>C</u>T) to valine (G<u>T</u>T) at residue 16 of the protein, and position –9 in the signal peptide (hence Ala-9Val) (Rosenblum *et al.*, 1996; Shimoda-Matsubayashi *et al.*, 1996). Computer models predict the formation of a partial α-helix structure by the alanine allele and a β-sheet structure by the valine allele (Shimoda-Matsubayashi *et al.*, 1996).

The Ala-9Val polymorphism may therefore influence mitochondrial transport. Indeed, the Val allele resulted in less efficient transport of MnSOD into the mitochondrion compared to the Ala allele in rat liver cells and HuH7 human hepatoma cells (Sutton *et al.*, 2003, 2005). Since MnSOD plays a vital role in the antioxidant defence system as well as neurodevelopment, a change in enzyme concentration through the action of Ala-9Val, may result in ROS accumulation and hence cell injury.

Two previous studies did not find an association between Ala-9Val and schizophrenia in Chinese and Japanese populations (Hori *et al.*, 2000; Zhang *et al.*, 2002a). Recently though, Akyol *et al.* (2005) observed a significant difference between the genotypes of Turkish schizophrenia patients and healthy controls, with the Ala/Ala and Val/Val genotype frequency lower, and the Ala/Val genotype frequency higher in the schizophrenic group. Association studies on TD and Ala-9Val also show contradictory results, with Hori *et al.* (2000) confirming an association in a Japanese population, while Zhang *et al.* (2002a) and Akyol *et al.* (2005) did not find such an association in a Chinese and Turkish population respectively.

Ethnicity has been suggested to be a risk factor for the development or the course of TD, with for example African Americans having a higher risk compared to Caucasians (Morgenstern and Glazer, 1993; Wonodi *et al.*, 2004). TD prevalence may therefore vary between populations, (Chiu *et al.*, 1992), and hence it is important to investigate the genetic basis of TD in different populations. Patterson *et al.* (2005) found the prevalence of TD in the Xhosa population to be 28.4%, which is in the range of other reports (~20-30%) (Kane and Smith, 1982; Holden, 1987; Kane *et al.*, 1988). The Xhosa population is the Southern-most group of the Nguni-speaking Africans and one of the largest population groups in South Africa. TD development in this unique population was associated with the total numbers of treatment years and cumulative antipsychotic dose. The study also suggested a protective role of an antioxidant rich diet against TD development. Unfortunately the Patterson study did not examine the role of negative symptoms in the development or degree of TD.

The *MnSOD* Ala-9Val polymorphism has not been studied in association with TD development in the Xhosa population. Since the risk of TD development varies depending on ethnicity, we wanted to investigate the effect of Ala-9Val polymorphism on abnormal involuntary movement (AIM) (which encompasses TD) development specifically in a South African Xhosa population. In addition, the role of this polymorphism in schizophrenia development, as well as symptom severity was examined in this population.

## 2.3 Materials and Methods

### 2.3.1 Clinical

#### 2.3.1.1 Patient population

Patients were recruited from in- and outpatient hospital services and community clinics throughout the Western Cape Province of South Africa as part of a large multi-site genetic study. All possible participants were screened for suitability and diagnosed according to the diagnostic and statistical manual of mental disorders, fourth edition (DSM-IV) criteria (APA, 1994). Unrelated controls (matched for ethnicity, gender and age within 5 years) were recruited from local communities by word of mouth. The study was approved by the ethics committee of Stellenbosch University and informed, written consent was obtained from participants and where applicable from their caregiver. Inclusion criteria for patients comprised a diagnosis of schizophrenia (DSM-IV Criteria) and for patients and controls Xhosa ethnic origin (4/4 grandparents reported as of Xhosa origin).

#### 2.3.1.2 Assessment

Each patient was interviewed utilizing the standardized Diagnostic Interview for Genetic Studies version 2.0 [DIGS] (Nurnberger *et al.*, 1994), including the Schedule for the Assessment of Negative Symptoms (SANS) and the Schedule for the Assessment of Positive Symptoms (SAPS) (Andreasen, 1989; Andreasen, 1995). For the investigation of involuntary movements, the Abnormal Involuntary Movement Scale (AIMS) was used. This scale measures seven domains of involuntary movements on a severity scale of 0 to 4 where 0 = absent and 2-4 = presence of involuntary movement (Guy, 1976). Interviews were conducted in Xhosa, the native language of all participants. All participants reported on for this article were recruited at a single study site, using the same interviewers who held regular calibration meetings. Supplemental information was obtained from Hospital chart records and family members where available.

### 2.3.2 Laboratory

#### 2.3.2.1 Genotyping

Primers encompassing the Ala-9Val polymorphism were designed for PCR amplification of exon 2 (Forward primer: 5'-GCTTTCTCGTCTTCAGC-3', Reverse primer: 5'-CTCCT CGGTGACGTTC-3'). Amplification was performed in a 25 µl reaction containing 25 ng genomic DNA, 0.5 units of Biotaq$^{TM}$ DNA polymerase (Bioline, Canton, MA, USA), 3 mM $MgCl_2$ (Bioline,

Canton, MA, USA), 1X $NH_4$ buffer (Bioline, Canton, MA, USA), 0.4 µM of each primer, 0.1 mM of each dNTP (Fermentas, Ontario, Canada), with a GeneAmp PCR System 2700 (Perkin Elmer, Applied Biosystems, Warrington WA, Great Britain). The PCR program used included an initial denaturation step of 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 20 seconds and elongation at 72°C for 30 seconds, with a final elongation step at 72°C for 7 minutes. The 194 bp fragment was visualized under UV light on a 1.5% (w/v) agarose gel stained with ethidium bromide (0.01%; v/v). The samples were screened for the Ala-9Val polymorphism using heteroduplex-single strand conformational polymorphism (HEX-SSCP) analysis. Electrophoresis was performed in 12% (w/v) polyacrylamide gels, containing 7.5% (w/v) urea at 250V for 17 hrs at 4°C. The gels were silver stained to resolve the genotype of each sample. Samples of which the DNA sequence had been determined, served as controls to enable accurate scoring of the genotypes from the polyacrylamide gels. Please also see Appendix A and B for more information regarding DNA extraction and genotyping.

### 2.3.3 Statistical analysis

The genotype and allele frequencies for the Xhosa control and schizophrenia groups, and the subgroups within the schizophrenic group (with AIM [AIM+] and without AIM [AIM-]) were calculated, and tested for departure from Hardy-Weinberg equilibrium (HWE) by means of a chi-square-goodness-of-fit-test with Tools for Population Genetic Analysis (TFPGA) version 1.3 (Miller, 1997). The classifications towards AIM status of patients were done according to AIMS score counts, where patients with an AIMS of two and above were considered to have an AIM. The choice of a positive score on the AIMS is broader and more inclusive than the accepted TD criteria (for example Schooler-Kane). It is however much less stringent and could be over-inclusive of movements that would not necessarily lead to functional impairment. However, the potential outcome of this study had to consider the structure and availability of health resources within South Africa. As the majority of mental health treatment takes place in a community setting under the care of nursing personnel the use of our criteria (presence of any positive score on AIMS) will be within the abilities and scope of the available health resources. Although the primary aim of this study was to investigate abnormal involuntary movements in a broader context, a post-hoc classification was done according to the Schooler-Kane criteria (Schooler and Kane, 1982) to determine the presence of tardive dyskinesia. These criteria require a score of “mild” on at least two items or “moderate” on any one item in categories 1 to 7. Possible differences in genotype or allele frequencies between the control, patient group, and the subgroups were determined using an analog of a Fisher’s exact test on a contingency table (2X2 or 2X3 as appropriate) with Excel.

Clinical demographic data was only available for the schizophrenic group. The data was tested for normality by means of the Kolmogorov-Smirnov test and normality plots using Statistical Package for the Social Sciences (SPSS Inc., Chicago IL) version 14 for Windows. Testing for possible differences in gender, antipsychotic drug type and smoking status among the schizophrenic subgroups (AIM+ and AIM-) was accomplished by way of an analog to Fisher's exact test (on a contingency table) with Excel. All subsequent analyses were performed using Statistical Package for the Social Sciences (SPSS Inc., Chicago IL) version 14 for Windows. Possible differences in age, duration of illness, onset age, SAPS and SANS scores among the different groups were assessed by t-test. Correlation between SANS and SAPS score and the different genotypes (schizophrenic group) was examined by making use of the Spearman rank correlation coefficient (r), a non-parametric test, corrected for ties with a two-tailed significance ($\alpha = 0.05$). Analysis of covariance (ANCOVA) was used to assess the relationship between AIMS score and different genotypes of the AIM+ schizophrenic subgroup. Age was used as covariate. This analysis was performed using SAS software version 9.1. Copyright, SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA. The same statistical analysis was performed for the TD vs. the AIM- group. For the ANCOVA age and onset age were used as covariates.

## 2.4 Results

### 2.4.1 *MnSOD* Ala-9Val and schizophrenia

An association between the *MnSOD* Ala-9Val polymorphism with schizophrenia and AIM development was investigated, by genotyping Xhosa schizophrenia patients (with and without AIM) as well as healthy Xhosa controls for this variant using HEX-SSCP analysis (Figure 3). The genotype and allele frequencies for this polymorphism in a South African Xhosa schizophrenic (n=286) and a healthy control (n=243) group are given in Table 5. Also shown are the frequencies for the subgroups within the schizophrenia group, AIM+ and AIM-. Both the Xhosa control and schizophrenia group were found to be in HWE ($P$=0.7813 and $P$=0.0756). However, due to the low $P$ value for HWE in the schizophrenic population, a Type II error cannot be excluded**.** For the schizophrenic patients the Ala/Val genotype was numerically more frequent compared to the control group (Table 5). Slight differences in the allele frequencies, with the schizophrenia group having a higher Ala and a lower Val allele frequency compared to the controls were also observed (Table 5). However, the difference was not statistically significant for either the genotype

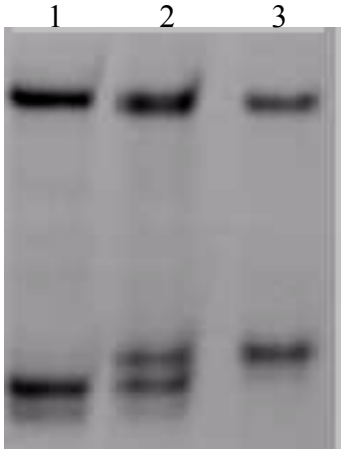


**Figure 3:** Silver-stained HEX-SSCP gel, showing the different genotypes for *MnSOD* Ala-9Val. The sample in Lane 2 is heterozygous for Ala-9Val; the samples in lane 1 and 3 are homozygous for the Val and the Ala alleles respectively.

($\chi^2$=2.445, d.f.=2, *P*=0.294), or the allele frequencies ($\chi^2$=0.398, d.f.=1, *P*=0.528) of the two groups. Furthermore, the Spearman rank correlation coefficient showed no significant correlation between the different genotypes and SANS score (r=-0.086, *P*=0.150) or SAPS score (r=0.021, *P*=0.737) in the schizophrenic patient group.

**Table 5:** *MnSOD* Ala-9Val genotype and allele frequencies for the control and schizophrenia group with subgroups for AIM+ and AIM-.

| | | Schizophrenic patients | | | Controls |
|---|---|---|---|---|---|
| | | AIM+ n (%) | AIM- n (%) | Total n (%) | n (%) |
| Genotypes | Ala/Ala | 12 (22) | 22 (11) | 41 (14) | 46 (19) |
| | Ala/Val | 20 (37) | 121 (59) | 153 (53) | 117 (48) |
| | Val/Val | 22 (41) | 61 (30) | 92 (32) | 80 (33) |
| | P-value | 0.008 | | 0.294 | |
| Allele frequencies | Ala | 0.41 | 0.40 | 0.41 | 0.43 |
| | Val | 0.59 | 0.60 | 0.59 | 0.57 |
| | P-value | 0.955 | | 0.528 | |

### 2.4.2 *MnSOD* Ala-9Val and AIM

Classification towards AIM status of patients was done according to AIMS score counts (patients with AIMS>1 were considered as having AIM). Missing data points on the AIMS excluded 28 patients from this sub-analysis. Where data points were missing for the individual categories (age, gender, onset age, antipsychotic type, smoking status, SANS and SAPS score and duration of illness), patients were also excluded for the calculation of the mean for each category. The AIM+ group was found to be in HWE ($P$=0.0869). The AIM- group, however, showed a significant departure from HWE (P = 0.0010). AIM+ patients exhibited a higher Ala/Ala and Val/Val genotype, but a less frequent Ala/Val genotype count compared to the AIM- group. The Chi-square test indicated that statistically significant differences exist between the genotypes ($\chi^2$=9.675, d.f.=2, $P$=0.008), but the allele frequencies ($\chi^2$=0.003, d.f.=1, $P$=0.955) of the two groups did not show similar results. Information on the clinical demographics of the subjects used is shown in Table 6.

**Table 6:** Demographic characteristics of the South African Xhosa schizophrenic patient group (patients without the relevant information are excluded).

| | AIM+ | AIM- |
|---|---|---|
| Number | 54 | 204 |
| Age (years) | 39.52 +/-11.36 | 32.72 +/-10.12 |
| Gender (male:female) | 42:12 | 164:40 |
| Antipsychotic type (typical:atypical:clozapine:unknown) | 43:0:6:5 | 164:2:18:20 |
| Smoking status (yes:no:unknown) | 34:19:1 | 148:50:6 |
| SAPS | 4.78 +/-4.56 | 5.17 +/-4.91 |
| SANS | 9.28 +/-4.90 | 9.57 +/-4.23 |
| Onset age (years) | 25.16 +/-9.08 | 23.04 +/-6.42 |
| Duration of illness (years) | 13.66 +/-9.05 | 9.50 +/-8.27 |

No significant differences were shown to exist between the AIM- and AIM+ groups as far as antipsychotic treatment ($\chi^2$=0.786, d.f.=3, $P$=0.853), smoking status ($\chi^2$=2.562, d.f.=2, $P$=0.278) and gender distribution ($\chi^2$=0.181, d.f.=1, $P$=0.670) were concerned. The t-tests performed for age ($P$=0.000) and duration of illness ($P$=0.002) showed statistically significant difference to exist between the AIM+ and AIM- groups. SANS scores ($P$=0.661), SAPS scores ($P$=0.616) and onset age ($P$=0.057), however, showed no statistically significant difference to exist between the two groups. The relationship between AIMS score and genotypes of the AIM+ schizophrenic subgroup

was used to perform an ANCOVA. Age was used as covariate (due to the statistically significant difference previously shown to exist). No significant differences were observed between the three genotypes (F=2.18, d.f.=2, *P*=0.1234), and no association between AIMS score and genotype could therefore be elucidated.

Within the AIM+ group, patients were classified *post hoc* for TD (n=30) according to Schooler-Kane criteria. This group was compared to the AIM- group. No significant difference regarding SANS and SAPS scores (*P*=0.367 and *P*=0.597 respectively), type of antipsychotics ($\chi^2$=2.096, d.f.=3, *P*=0.553), gender ($\chi^2$=0.146, d.f.=1, *P*=0.703) and smoking ($\chi^2$=3.831, d.f.=2, *P*=0.147) was observed. Yet age (*P*=0.000), onset age (*P*=0.009) and duration of illness (*P*=0.013) differed significantly between the two groups. With regard to genotype, a significant differences was observed ($\chi^2$=9.006, d.f.=2, *P*=0.011), though not for the allele frequency ($\chi^2$=1.966, d.f.=1, *P*=0.161). In addition, *MnSOD* genotype did seem to influence AIMS scores (F=3.86, d.f.=2, *P*=0.0365) using age and onset age as covariates. A t-test was performed comparing AIMS scores between the three genotype classes. The Ala/Ala genotype had significantly lower scores than heterozygotes (*P*=0.0155) or the Val/Val genotype group (*P*=0.0406).

## 2.5 Discussion

### 2.5.1 *MnSOD* and schizophrenia

Oxidative processes may contribute to the pathophysiology of schizophrenia (Mahadik and Mukherjee, 1996; Mahadik *et al.*, 2001). While MnSOD is an important enzyme involved in the antioxidant pathway, and the Ala-9Val polymorphism affects mitochondrial import efficiency of MnSOD (Sutton *et al.*, 2003, 2005), in this study no association could be illustrated between this polymorphism and susceptibility to schizophrenia in this Xhosa sample. Our findings are in contrast to those of Akyol *et al.* (2005) in a Turkish population, and in accordance with Zhang *et al.* (2002a) in a Chinese population and with Hori *et al.* (2000) in a Japanese population (Table 7). Akyol *et al.* (2005) observed a statistically significant difference for the genotype frequencies, with higher Ala/Val (69.3%) and lower Ala/Ala (9.2%) and Val/Val (21.6%) genotype frequencies in the schizophrenia group compared to the control group (Ala/Val: 42.3%; Ala/Ala: 23.5%; Val/Val: 34.2%). Other studies in the Chinese and Japanese populations showed somewhat different genotype frequencies (Table 7). In our cohort we observed a similar trend to Akyol *et al.* (2005) (Table 7), however, these differences were not statistically significant. The allele frequency in the Xhosa population was similar to that of Akyol *et al.* (2005) (Table 7) and we also did not find a significant difference between the allele frequencies of the Xhosa schizophrenia and the healthy

control group (Table 5). The Val allele was the more common allele, which is in accordance with above-mentioned studies. Heterogeneity, and interpopulation differences in the development of the disorder; as well as population stratification (although this is unlikely) could explain the contrasting results between Akyol *et al.* (2005) and our study. In addition, our study cohort may not have been large enough to be able to detect a small effect of the Ala-9Val polymorphism on schizophrenia development, though it was larger than several other studies shown in Table 7. Another explanation may be that Ala-9Val is in linkage disequilibrium with a variant that results in schizophrenia development in the Turkish population.

**Table 7:** Genotype and allele frequencies as well as cohort numbers for studies on *MnSOD* Ala-9Val and schizophrenia and AIM development.

| | **Hori *et al.* (2000)** | | **Zhang *et al.* (2002a)** | | **Akyol *et al.* (2005)** | | **Our findings** | |
|---|---|---|---|---|---|---|---|---|
| **Population** | Japanese | | Chinese | | Turkish | | Xhosa | |
| | Patients (n=192) | Controls (n=141) | Patients (n=101) | Controls (n=50) | Patients (n=153) | Controls (n=196) | Patients (n=286) | Controls (n=243) |
| **Ala/Ala (%)** | 1.6 | 1.4 | 0 | 0 | 9.2 | 23.5 | 14.3 | 18.9 |
| **Ala/Val (%)** | 20.3 | 19.2 | 33 | 34 | 69.3 | 42.3 | 53.5 | 48.2 |
| **Val/Val (%)** | 78.1 | 79.4 | 67 | 66 | 21.6 | 34.2 | 32.2 | 32.9 |
| **Ala** | 0.12 | 0.11 | 0.16 | 0.17 | 0.46 | 0.44 | 0.41 | 0.43 |
| **Val** | 0.88 | 0.89 | 0.84 | 0.83 | 0.54 | 0.56 | 0.59 | 0.57 |

## 2.5.2 SANS score and *MnSOD*

We did not find an association between SANS or SAPS scores and Ala-9Val genotype. Several factors may influence negative symptom severity. These include age, diet, smoking status, duration of untreated psychosis, type of medication treated with, and age of onset. Samples were not matched according to these factors and this could have influenced results. Smoking has been suggested to exacerbate oxidative damage (Morrow *et al.*, 1995). Yet, some studies did not find an association between smoking status and a decrease in antioxidant compounds (Yao *et al.*, 1998a, 1998b; Reddy *et al.*, 2003), or increase in lipid peroxidation product levels (Akyol *et al.*, 2002) in schizophrenic patients. Diet may also influence the antioxidant defence system. For example, supplementing schizophrenic patients with essential polyunsaturated fatty acids and/or antioxidant

compounds in their diet, impacted on outcome of schizophrenia in some studies (reviewed in Mahadik *et al.*, 2001; Arvindakshan *et al.*, 2003b). Hence any differences in diet between patients may have affected negative symptom severity. In this study, patients were mainly treated with typical antipsychotics (typical: 81%; atypical and clozapine: 10%; unknown or refusing medication: 9%). The frequent use of typical antipsychotics can be expected as access to newer generation antipsychotics were still very limited at the time of recruitment for this study. A very small percentage thus received newer generation atypical antipsychotics (0.7%). A relationship was also not found between SANS score and genotype when the group receiving only typical antipsychotics was analyzed (r=-0.094, *P*=0.155). In this study a correlation was observed between onset age and SANS score (r=-0.124, *P*=0.04), therefore potentially affecting our results. The duration of untreated psychosis was, however, not examined, and differences here may well have influenced results regarding negative symptoms and genotype. In addition to the factors mentioned that may have influenced the results of this study, population differences and a heterogeneous mechanism for negative symptoms and schizophrenia development may further explain the non-association of Ala-9Val and SANS scores. Furthermore, our study cohort may not have been large enough to be able to detect a small effect of Ala-9Val on negative symptom severity.

### 2.5.3 *MnSOD* and AIM

Apart from the role of oxidative processes in the development of schizophrenia, an oxidative role in TD development has also been suggested (Cadet and Kahler, 1994; Sachdev, 2000). We did find a significant difference for the genotype frequencies between the AIM+ and AIM- groups, yet no significant difference for the allele frequencies between the AIM+ and AIM- groups was found. The two groups did not differ significantly in terms of antipsychotic type, onset age, smoking status, SANS and SAPS score and gender distribution. A difference regarding age and duration of illness, with the AIM+ group being older and duration of illness being longer, was observed in this study. While drug dosage is also an important factor to look at in conjunction with AIM, equivalency calculations would likely be inaccurate as patients were receiving a range of typical depot injections, oral medication, or a combination of the two. Age was included as a covariate in the ANCOVA, when looking at genotype and AIMS score (duration of illness was not included since it directly correlates with age). However, no association was found. Hori *et al.* (2000) also observed a significant difference for the Ala-9Val genotype between Japanese schizophrenic patients with and without TD. In addition, the allele frequencies differed significantly between the two groups in that study, with the -9Val allele being more common in the TD schizophrenic group. There was also a non-significant trend for the Val/Val genotype group to have higher AIMS scores. In contrast,

Zhang *et al.* (2002a) did not find a significant difference for genotype or allele frequencies between a Chinese schizophrenic TD and nonTD group nor an association with AIMS score. However, they did observe a positive correlation between plasma MnSOD activity and AIMS score. Akyol *et al.* (2005) also did not confirm an association between *MnSOD* Ala-9Val and TD development. The advantage of our study was the use of structured assessment tools in a relatively homogenous (schizophrenia) clinical population. Nevertheless, our study criteria for involuntary movement are inclusive and clearly not limited to TD. This could account for some of the differences in findings. We therefore performed a *post hoc* analysis of patients in the AIM+ group who could be classified as having TD (TD+) according to the criteria of Schooler and Kane. Like the AIM+ group, no significant difference for gender, SANS scores, SAPS scores, smoking and antipsychotic type were observed between the TD+ and the AIM- group. Age as well as duration of illness did however differ between the two groups, as had also been noted when comparing AIM+ and AIM-. Where onset age had not shown a significant difference between the AIM+ and the AIM- group, it did differ when comparing TD+ and AIM-. It was therefore also included as a covariate for the ANCOVA together with age (once again duration of illness was not used as covariate due to its correlation with both age and onset age). In contrast to the AIM+ group, the TD+ group did show a relationship between genotype and AIMS score. The Ala/Ala genotype showed a protective effect in that patients had lower AIMS scores in this group compared to the other two genotype groups. This is in accordance with the nonsignificant trend between Val/Val genotype and higher AIMS scores noted by Hori *et al.* (2000). The protective effect of Ala/Ala on TD severity may be due to too little mitochondrial MnSOD content in patients with the Ala/Val or Val/Val genotype. Results for the genotype and allele frequencies, when comparing TD+ and AIM-, were similar to what we had observed earlier for the AIM+ and AIM- group, with a significant difference being observed for genotype only.

A significant difference between the genotypes of the AIM+ as well as TD+ and AIM- group (Table 5) was found in this study. From a subsequent analysis we could deduce that Xhosa schizophrenic patients who had at least one Val allele were less likely to have AIM ($P$=0.0155) or TD ($P$=0.0249). From our data, the presence of the –9Val allele could be suggested to protect against the development of AIM as well as TD. This would be in accordance with other studies that found the Ala allele to be associated with for example Parkinson's disease (Shimoda-Matsubayashi *et al.*, 1996), macular degeneration (Kimura *et al.*, 2000) and motor neuron disease (Van Landeghem *et al.*, 1999). They suggested that since the Ala allele results in more efficient transport of MnSOD into the mitochondria, this would result in more production of $H_2O_2$, which can result in ROS-induced cell damage. However this is in contrast to the theory proposed by Hori *et al.* (2000) on

Ala-9Val and TD development, where the Val allele is considered to result in less MnSOD content in the mitochondria, and hence inefficient protection against ROS. It is interesting to note that the Ala/Ala genotype seemed to decrease the severity of TD (in terms of total AIMS score) in our TD+ group, however, the presence of at least one Val allele showed a protective effect in the development of AIM or TD in the AIM+ and TD+ group. These seemingly contradictory results may be due to the complexity of the enzymatic antioxidant defence system. MnSOD may remove and at the same time produce ROS. Antioxidant enzymes downstream of MnSOD may therefore play a role in combination with MnSOD in determining TD development as well as severity.

### 2.5.4 Limitations and future perspectives

Importantly, schizophrenia is a complex disorder, and it is more likely that its pathophysiology involves more subtle effects and the interaction of several genetic variants in different genes as well as the environment and, possibly, even epigenetic factors, as has been suggested by Sullivan (2005) and Sharma (2005). The same approach should be applied in the genetic analysis of AIM/TD development, considering for example the finding of a small interactive effect of *DRD3* Ser9Gly and *MnSOD* Ala-9Val in the development of TD (Zhang *et al.*, 2003b). Hence, the sample number (schizophrenic group: n=286; control group: n=243; AIM+: n=54; AIM-: n=204) in this study may still have been insufficient (even though it is comparable to other studies shown in Table 7) to be able to observe the effects of only a single polymorphism, Ala-9Val, on AIM development and severity, schizophrenia development, as well as negative symptom severity (Sullivan, 2005). The fact that we did find a relationship between AIMS score and *MnSOD* genotype for the TD+ group, which had not been observed for the AIM+ group, emphasizes the importance of strict TD classification.

MnSOD works in combination with other enzymes, CAT and GSHPx, to protect against ROS. $H_2O_2$, which is produced by MnSOD, can form $^{\cdot}OH$ in the presence of transition metal ions. CAT and GSHPx are responsible for the removal of $H_2O_2$ to form $H_2O$ and therefore prevent $H_2O_2$ forming ROS (Chance *et al.*, 1979). For that reason it is vital that a balance exists between these enzymes, to ensure that intermediate products are not converted to ROS. The possibility exists that genetic variants located in *CAT* or *GSHPx* may individually, or in combination with *MnSOD`s* Ala-9Val polymorphism, influence schizophrenia or AIM/TD development and/or severity of symptoms. This study did not focus on *CAT* or *GSHPx* and further studies are warranted. Furthermore other genetic variants located in the coding or regulatory regions of *MnSOD* were not screened in this study. Changes in MnSOD expression may compromise the antioxidant defence

system. Hence, genetic variants located in the promoter and/or regulatory region, as well as epigenetic factors, may affect *MnSOD* expression, and will need to be investigated in the future for their possible function in schizophrenia or AIM/TD development and symptom severity. The fact that, in contrast to Akyol *et al.* (2005), we did not find an association between Ala-9Val and schizophrenia development, again demonstrates the importance of replicating studies in different populations.

## 2.6 Conclusion

In conclusion, our study does not support a major role of *MnSOD* in schizophrenia or symptom severity, but suggests a possible role in AIM development as well as the development of TD, with the Ala/Val genotype and the presence of at least one Val allele seemingly having a protective role. This supports the theory that the presence of the Ala allele may result in excess $H_2O_2$ production and ultimately ROS damage to the cell. In contrast, the Ala/Ala genotype seems to result in less severe TD, supporting the notion that higher mitochondrial MnSOD content and hence ROS removal may lessen the severity of TD. Further association studies on other genetic variants downstream of the antioxidant pathway combined with MnSOD may give more insight on the mechanism of ROS damage and TD.

# Chapter Three

# Association between the *DRD3* Ser9Gly polymorphism and abnormal involuntary movement development in a Xhosa population

## 3.1 Abstract

No hypotheses exist that could explain the development of abnormal involuntary movements (AIM) in general. But for several disorders such as tardive dyskinesia (TD), which show AIM as a symptom, hypotheses do exist. Based on the dopamine supersensitivity hypothesis of TD, genetic variants in the dopamine receptors have been investigated in the development of this disorder. Previous studies have implicated the *DRD3* Ser9Gly polymorphism in the development of TD in various population groups. However, these studies have not extended to African populations. This study aimed at investigating the role of Ser9Gly in the development of AIM in a Xhosa schizophrenia population, and also aimed at comparing the allele and genotype frequencies observed in this population to those of other populations described in the literature. A possible interactive effect between *MnSOD* Ala-9Val and *DRD3* Ser9Gly in the development of AIM was also investigated. Schizophrenia patients with AIM (n=55) as well as without AIM (n=68) were genotyped for Ser9Gly using restriction enzyme analysis. Subsequently, genotype and allele frequencies were compared, as well as an association between AIMS score and genotype investigated. The interactive effect between Ala-9Val and Ser9Gly in the development of AIM was determined using chi square analysis and ANOVA. The genotype and allele frequencies compared to those of the African American population (higher Gly allele frequency), but differed to those of other populations (higher Ser allele frequency). No significant difference between genotype or allele frequency ($P$=0.252 and $P$=0.240 respectively) was observed for the two patient groups. In addition, no association between AIMS score and Ser9Gly genotype was found, nor could an interactive effect between Ala-9Val and Ser9Gly in the development of AIM be determined. A subgroup of the group with AIM with TD (n=27) was also analysed and compared with the group without AIM and also analysed for possible interactive effects by Ser9Gly and Ala-9Val. The results were similar to the group with AIM. This study does not support a role of the Ser9Gly polymorphism in the development or severity of AIM or TD in a Xhosa schizophrenia population, nor does it support a relationship between *MnSOD* Ala-9Val and *DRD3* Ser9Gly in the

development of either disorder. Possible interactions of this polymorphism with other genetic variants in the development of AIM/TD, however, cannot be excluded.

## 3.2 Introduction

Little is known about the exact pathophysiology of abnormal involuntary movements (AIM) and no hypotheses exist. Tardive dyskinesia (TD), which shows AIM as a symptom, has been studied more extensively and several hypotheses exist for this disorder (Basile *et al.*, 2002; Casey, 2004). The reader is referred to section 1.3 for more information regarding the diagnosis, symptoms, prevalence, risk factors and genetic basis of TD. One of the hypotheses that may explain TD development is that of dopamine supersensitivity, and suggests that due to chronic antipsychotic treatment (mainly dopamine antagonists), dopamine receptors will become supersensitive towards dopamine and result in TD (Klawans and Rubovits, 1972). Hence, the dopamine receptors have long been investigated for their possible role in TD development, yet, results have varied (Basile *et al.*, 1999; Segman *et al.*, 1999, 2003; Srivastava *et al.*, 2005). Of all the dopamine receptors investigated, the dopamine D3 receptor (DRD3) Ser9Gly variant has most consistently been associated with the development of TD, as well as with increased abnormal involuntary movements scale (AIMS) scores (summarized in Table 8).

For more information regarding DRD3 (OMIM number: *126451) and its gene please see section 1.3.8.1. As mentioned in that section *DRD3* Ser9Gly results in a functional change in that Gly homozygotes have a higher dopamine binding affinity compared to Ser homozygotes and heterozygotes (Lundstrom and Turpin, 1996). Most studies, including meta-analyses, on TD development and severity and Ser9Gly do show an association, generally with the Gly allele (See Table 8). The effect of this gene on TD development seems to be small, with other variants and environmental factors probably also playing a role (Lerer *et al.*, 2002). The pathophysiology of TD or AIM is complex and most likely involves several genetic variants in different genes. Some studies have focused on possible interactions between certain genetic variants and the development of TD (See Table 8). Importantly, Zhang *et al.* (2003b) observed an interactive effect of *MnSOD* Ala-9Val polymorphism and *DRD3* Ser9Gly.

A difference in Ser9Gly frequency specifically between African Americans and other ethnicities, as well as ethnic differences in vulnerability to TD was previously noted by Lerer *et al*. (2002). Hence it is important to replicate studies in different populations. So far the Ser9Gly variant and its role in TD development has only been investigated in Caucasian, Chinese, Jewish, African American and

**Table 8:** Association studies of TD and *DRD3* Ser9Gly.

| Gene | Polymorphism | Author | Number of subjects (ethnic origin) | Results |
|---|---|---|---|---|
| *DRD3* | Ser9Gly | Woo *et al.* (2002) | 113 (Korean) | Significant association between Gly/Gly genotype & TD, but not with Ser9Gly alleles or AIMS score |
| *DRD3* | Ser9Gly | Garcia-Barcelo *et al.* (2001) | 131 (Chinese) | No association |
| *DRD3* | Ser9Gly | Segman *et al.* (1999) | 233 (Ashkenazi Jews, non-Ashkenazi Jews) | Significant association between Gly allele & TD |
| *DRD3* | Ser9Gly | Liao *et al.* (2001) | 115 (Chinese) | Significant association between heterozygotes & TD |
| *DRD3* | Ser9Gly | Rietschel *et al.* (2000) | 157 (German, Caucasian) | No association |
| *DRD3* | Ser9Gly | Steen *et al.* (1997) | 100 (Scottish, Caucasian) | Significant association between Gly homozygotes & TD |
| *DRD3* | Ser9Gly | Basile *et al.* (1999) | 112 (Caucasian, African American, Asian) | Significant association between: Gly allele & TD, & also between Gly homozygotes & AIMS scores |
| *DRD3* | Ser9Gly | Chong *et al.* (2003b) | 317 (Chinese) | Significant association between *DRD3* Ser/Ser genotype & TD |
| *DRD3* | Ser9Gly | Inada *et al.* (1997) | 105 (Japanese) | No association |
| *DRD3* | Ser9Gly | De Leon *et al.* (2005) | 516 (American) | Significant association between severe TD & *DRD3* Ser9Gly |
| *MnSOD*<br>*DRD3* | Ala-9Val<br>Ser9Gly | Zhang *et al.* (2003b) | 101 (Chinese) | Significant association of both variants combined with TD |
| *DRD3*<br>*HTR2C* | Ser9Gly<br>Cys68Ser | Segman *et al.* (2000) | 212 (Ashkenazi Jews, non-Ashkenazi Jews) | Significant association between *HTR2C* together with *DRD3* & TD |
| *DRD3*<br>*CYP-17* | Ser9Gly<br>Promoter T→C polymorphism | Segman *et al.* (2002a) | 113 (Ashkenazi Jews, non-Ashkenazi Jews) | Significant association between A2-A2 genotype together with *DRD3* Gly allele & susceptibility to abnormal orofacial distal involuntary movements & be incapacitated by these movements |
| *DRD3 (meta-analysis)* | Ser9Gly | Bakker *et al.* (2006) | 1610 (various) | Significant association between *DRD3* Gly allele & TD; however possible publication bias |
| *DRD3 (meta-analysis)* | Ser9Gly | Lerer *et al.* (2002) | 780 (Caucasian, African American, German, Israeli Ashkenazi & non-Ashkenazi Jews, Austrian) | Significant association between *DRD3* Gly allele carrier & TD, & also between *DRD3* genotype & TD; significant association between Gly homozygotes & AIMS scores |

Indian populations, but not yet in African populations (Table 8). For this reason we wanted to investigate the role that the *DRD3* Ser9Gly polymorphism plays in the development and severity of abnormal involuntary movements (AIM) and TD in the unique South African Xhosa population and compare the allele and genotype frequencies observed to those reported in the literature. Since Zhang *et al.* (2003b) had found an interactive effect between *MnSOD* Ala-9Val and *DRD3* Ser9Gly, we further wanted to investigate this potential relationship in the development of AIM and TD in the Xhosa schizophrenia population.

## 3.3 Materials and Methods

### 3.3.1 Clinical

#### 3.3.1.1 Patient population

The patient population was the same as described in Chapter Two. Briefly, patients were recruited from the Western Cape Province of South Africa, were diagnosed according to the DSM-IV criteria (APA, 1994) and were of Xhosa origin. Informed, written consent was obtained from the patient or when appropriate their caregivers, and the study was approved by the ethics committee of Stellenbosch University.

#### 3.3.1.2 Assessment

Please see Chapter Two for a more detailed description of the assessment tools used. Patients were interviewed according to the standardized Diagnostic Interview for Genetic Studies version 2.0 [DIGS] (Nurnberger *et al.*, 1994) and AIM determined using AIMS (Guy, 1976).

### 3.3.2 Laboratory

#### 3.3.2.1 Genotyping

The genotyping protocol was adapted from Segman *et al*. (1999). Primers specifically amplified *DRD3*'s exon 2 and hence the Ser9Gly variant (Forward primer: 5'-GCTCTATCTCCAACTCTCACA-3'; Reverse primer: 5'-AAGTCTACTCACCTCCAGGTA-3'). Amplification occurred in a total volume of 25 µl, containing 25 ng genomic DNA, 0.5 U Biotaq$^{TM}$ DNA polymerase (Bioline, Canton, MA, USA), 3 mM $MgCl_2$ (Bioline, Canton, MA, USA), 1X $NH_4$ buffer (Bioline, Canton, MA, USA), 0.3 µM of each primer, 0.1 mM of each dNTP (Fermentas, Ontario, Canada), with a GeneAmp PCR System 2700 (Perkin Elmer, Applied Biosystems, Warrington WA, Great Britain). PCR conditions were as follows: 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and elongation at 72°C for 45 seconds, with a final elongation step at 72°C for 7 minutes. Successful amplification was monitored by electrophoresis of the PCR product on a 1.5% (w/v) agarose gel, stained with ethidium bromide (0.01%; v/v), followed by UV light visualisation of the 463 bp long amplicon. Samples were tested for the Ser9Gly variant using restriction endonuclease analysis. PCR product was cut with *MscI* at 37°C overnight whereafter the enzyme was deactivated at 65°C for 30 seconds. Genotypes were then visualised on a 2% (w/v) agarose gel stained with

ethidium bromide (0.01%; v/v). Gly homozygotes resulted in the presence of 207 bp, 111 bp, 98 bp, and 47 bp bands, while Ser homozygotes showed 305 bp, 111 bp and 47 bp bands. The 111 bp and 98 bp band migrated as one on a 2% (w/v) agarose gel. Samples of which the DNA sequence had been determined, served as controls to enable accurate scoring of the genotypes from the agarose gels. Addendum A and B give a detailed description of DNA extraction and the genotyping method used.

### 3.3.3 Statistical analysis

The genotype and allele frequencies of the AIM+ and AIM- group were calculated and then tested for departure from Hardy-Weinberg equilibrium (HWE) using a chi-square-goodness-of-fit-test with Tools for Population Genetic Analysis (TFPGA) version 1.3 (Miller, 1997). Patients were considered to have AIM if their AIMS score was above one. Classification according to AIMS score may be broader and more inclusive than accepted TD criteria such as those by Schooler and Kane (1982). This, on the other hand, means that the criteria used in this study are less stringent and may include movements that would not necessarily lead to functional impairment. This study had to consider the structure and availability of resources within South Africa. Since the majority of mental health treatment is conducted under the care of nursing personnel within a community setting, the criteria used are within the abilities and scope of the available health resources. Nonetheless, patients within the AIM+ group were classified *post hoc* for TD according to the criteria of Schooler and Kane (1982) and analysed.

Allele and genotype frequencies between the AIM+ and AIM- group were compared using an analog of a Fisher's exact test on a contingency table (2X2 or 2X3 as appropriate) with Excel. The data was tested for normality using the Kolmogorov-Smirnov test and normality plots. The two groups were then compared in regard to gender, antipsychotic drug type and smoking status by making use of an analog to Fisher's exact test (on a contingency table) with Excel. Subsequent statistical analyses were executed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago IL) version 14 for Windows. Possible differences in age, duration of illness, onset age, SAPS and SANS score between the two groups were determined *via* t-test. To evaluate the relationship between AIMS score and genotype in the AIM+ group analysis of covariance (ANCOVA) was performed, with age and onset age as covariates. This analysis was performed using SAS software version 9.1. Copyright, SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA. The same analyses as for the AIM+ group were conducted for the TD+ group (n=27).

A possible interactive effect between *MnSOD* Ala-9Val (data was obtained from previous study, see Chapter Two) and *DRD3* Ser9Gly was determined using a $\chi^2$ test as described by Zhang *et al.* (2003b) using either the Val allele or the Ala allele as "high risk" allele combined with the Gly/Gly "high risk" genotype (Analysis shown in Addendum C). This interaction was further investigated using ANOVA, by testing the combined effect of the two variants on AIMS score. Once again this analysis was performed using SAS software version 9.1. Copyright, SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

## 3.4 Results

Samples were genotyped for the *DRD3* Ser9Gly polymorphism as can be seen in Figure 4. In this study we compared the Ser9Gly genotype and allele frequency between Xhosa schizophrenia patients that were subdivided into the AIM+ (n=55) and AIM- (n=68) groups (Table 9). Both the AIM+ and AIM- group were found to be in HWE (*P*=0.175 and *P*=0.947). The demographics of either group are shown in Table 10. The groups did not differ significantly in terms of antipsychotic type ($\chi^2$=0.436, d.f.=3, *P*=0.933), smoking ($\chi^2$=4.576, d.f.=2, *P*=0.101), gender ($\chi^2$=1.047, d.f.=1, *P*=0.306) and SAPS (*P*=0.245) and SANS score (*P*=0.887). Age (*P*=0.000), onset age (*P*=0.027) and duration of illness (*P*=0.000), however, differed significantly between the two groups. Genotype and allele frequency comparisons between AIM+ and AIM- did not show any significant difference ($\chi^2$=2.757, d.f.=2, *P*=0.252; and $\chi^2$=1.378, d.f.=1, *P*=0.240). In addition, no association

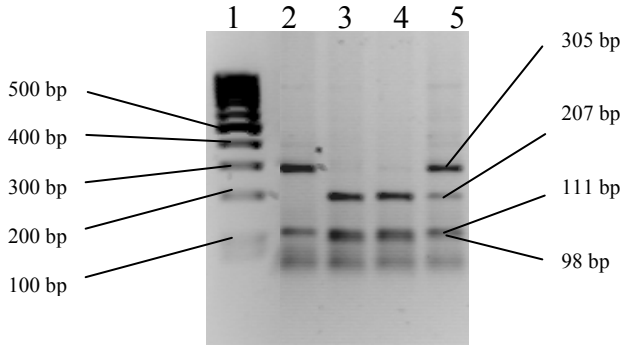


**Figure 4:** A 2% (w/v) agarose gel stained with ethidium bromide (0.01%; v/v), showing the different genotypes for *DRD3* Ser9Gly. The Ladder shown in lane 1 is O'Generuler 100 bp DNA ladder (Fermentas, Ontario, Canada**).** The sample in Lane 2 is homozygous for the Ser allele, samples in lane 3 and 4 are homozygous for the Gly allele, and the sample in lane 5 is heterozygous for Ser9Gly.

**Table 9:** *DRD3* Ser9Gly genotype and allele frequencies of AIM+ and AIM-.

| | Ser/Ser | Ser/Gly | Gly/Gly | Ser | Gly |
|---|---|---|---|---|---|
| AIM+ (n=55) | 0 | 17 | 38 | 0.155 | 0.845 |
| AIM- (n=68) | 3 | 23 | 42 | 0.213 | 0.787 |
| | | *P* =0.252 | | *P* =0.240 | |

between AIMS score and genotype was found (F=0.27, d.f.=1, *P*=0.6074), using age and onset age as covariates (duration of illness was not included since it directly correlates with age and onset age). When comparing the TD+ (n=27) with the AIM- group similar results were observed. Once again groups only differed in terms of duration of illness (*P*=0.004), onset age (*P*=0.001) and age (*P*=0.000). Genotype ($\chi^2$=1.469, d.f.=2, *P*=0.480) and allele frequencies ($\chi^2$=0.021, d.f.=1, *P*=0.884) were not significantly different between the TD+ and AIM- group. Furthermore no association was found between the AIMS score and genotype (F=0.45, d.f.=1, *P*=0.5118) within the TD+ group.

**Table 10:** Demographics of AIM+ and AIM-.

| | AIM+ | AIM- |
|---|---|---|
| Number | 55 | 68 |
| Age (years) | 41.91±11.560 | 30.97±9.146 |
| Gender (male:female) | 43:12 | 58:10 |
| Antipsychotic type (typical:atypical:clozapine:unknown) | 44:0:4:7 | 51:0:6:11 |
| Smoking (yes:no:unknown) | 40:15:0 | 52:12:4 |
| SANS | 7.93±4.674 | 8.05±4.474 |
| SAPS | 6.44±5.747 | 7.59±4.793 |
| Duration of illness (years) | 16.02±10.803 | 8.48±8.099 |

When the relationship between *MnSOD* Ala-9Val and *DRD3* Ser9Gly and the development of AIM was investigated, no significant interaction was observed when the Gly/Gly "high risk" genotype was combined with either the Ala allele (*P*=0.458 and *P*=0.603) or the Val allele (*P*=0.067 and *P*=0.589) (See Addendum C), depending on what is considered to be the "high risk" allele in the *MnSOD* gene (See section 2.5.3). The same applied for the combined effect of the variants on TD development ("high risk" Ala allele: *P*=0.351 and *P*=1.000; "high risk" Val allele: *P*=0.062 and

*P*=0.336) (See Addendum C). When an ANOVA was performed to investigate the interactive effect of the two variants on AIMS score, once again, no significant relationship was observed in the AIM+ (*P*=0.9819) nor in the TD+ group (*P*=0.5865).

## 3.5 Discussion

This study investigated the role of the *DRD3* Ser9Gly variant and its role in the development of AIM in a Xhosa schizophrenia population. The two groups, AIM+ and AIM-, did not differ significantly for gender, antipsychotic type, smoking status, SAPS and SANS score, but differed in terms of age, onset age and duration of illness (age and onset age included as covariates in ANCOVA). The AIM+ group was generally older, had a later onset age and a longer duration of illness than the AIM- group. This is in accordance with the finding that age and indirectly duration of illness (schizophrenia has a certain onset age and therefore high age means long duration of illness) are risk factors for TD (Kane and Smith, 1982; Sachdev, 2000). Antipsychotic dosage is an important factor to consider in association studies on TD, since it is a known risk factor for TD (Jeste *et al*., 1995; Kapitany *et al*., 1998). However, patients in this cohort received a variety of typical depot injections, oral medication, or a combination of the two and thus equivalency calculations would have been inaccurate.

Similar results were obtained for the TD+ group compared to the AIM+ group. The TD+ group also was significantly older, had a later onset age and longer duration of illness than the AIM- group. Furthermore, genotype and allele frequencies were not significantly different between the TD+ and AIM- group, and no association was observed between AIMS scores and genotype.

Previous studies mainly found an association with the Gly allele or Gly homozygotes and TD development and/or TD severity (Table 8) in various populations including African Americans (Basile *et al.*, 1999). However, Inada *et al.* (1997), Rietschel *et al.* (2000), and Garcia-Barcelo *et al.* (2001) did not confirm this association in a Japanese, German and Chinese population respectively. Our study also could not confirm an association between Ser9Gly (genotype or allele) and AIM or TD development nor its severity in a Xhosa population.

While our study cohort was relatively homogenous regarding ethnicity, our study criteria for involuntary movement not only includes patients who have TD but also those which may not develop TD though having AIM. This therefore could explain the differences in findings. Yet, we did perform a *post hoc* TD classification according to Schooler Kane criteria (Schooler and Kane, 1982) and when this subgroup was analysed, results did not change.

Ethnic differences have been noted in the frequency of Ser9Gly, with the Gly allele the more common allele in African Americans compared to the Caucasian population (Basile *et al.,* 1999). This was also the case in this study in the Xhosa population with the Gly allele frequency significantly greater compared to other populations (See Table 11). Lerer *et al*. (2002) also noted ethnic differences in vulnerability to TD, and hence our finding that Ser9Gly does not play a role in AIM or TD, may well not apply to other populations. It must also be noted that our population size was probably not adequate in detecting small effects of the variant, however, it compares to several other previous association studies done on *DRD3* Ser9Gly (Table 8 and Table 11).

The interactive effect of *MnSOD* Ala-9Val and *DRD3* Ser9Gly on TD or AIM development in a Xhosa schizophrenia population was also investigated in this study. However, no significant relationship was observed. This is in contrast to Zhang *et al.* (2003b) who did find a relationship between the Gly allele together with the Val/Val genotype in a Chinese population. Once again different ethnic groups may vary in regard to genetic risk factors in the development of TD or AIM and this could possibly explain the difference in findings, while a small study cohort may mean that small effects of the genetic variant(s) may not be detectable. In addition, a range of other genetic variants may contribute to the development of TD or AIM. Other variants that have been investigated in combination with *DRD3* Ser9Gly and were found to have an interactive effect on TD development are cytochrome P450 17α-hydroxylase (*CYP-17*) (may indirectly affect dopamine levels in the brain) polymorphism (Segman *et al*., 2002a), as well as *HTR2C* Cys68Ser which may affect the serotonin receptor's binding affinity (Segman *et al*., 2000).

## 3.6 Conclusion

In conclusion, the Xhosa population clearly had a much greater Gly allele frequency than other populations (not of African descent). No significant association was observed between *DRD3* Ser9Gly variant and the development or severity of AIM or TD in a schizophrenic Xhosa population. Furthermore, no interactive effect between this variant and *MnSOD* Ala-9Val in the development of TD or AIM was observed. It remains to be investigated whether this genetic variant in combination with other variants may contribute to TD development and/or severity in this unique Xhosa population.

**Table 11:** *DRD3* Ser9Gly allele and genotype frequencies of various populations (TD association studies).

| | **This study** | **Basile *et al.* (1999)** | | **Steen *et al.* (1997)** | **Chong *et al.* (2003)** | **Garcia-Barceló *et al.* (2001)** | **Liao *et al.* (2001)** | **Inada *et al.* (1997)** | **Woo *et al.* (2002)** | **Segman *et al.* (1999)** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Population** | Xhosa | African American | Caucasian | Caucasian | Chinese | Chinese | Chinese | Japanese | Korean | Askenazi and non-Ashkenazi Jews |
| **AIM-/TD-** | **n=68** | | | **n=49** | **n=200** | **n=66** | **n=94** | **n=56** | **n=54** | **n=63** |
| Ser/Ser (%) | 4.4 | | | 57.1 | 44.5 | 63.6 | 58.5 | 58.9 | 38.9 | 46.0 |
| Ser/Gly (%) | 33.8 | | | 38.8 | 44.0 | 27.3 | 30.9 | 33.9 | 61.1 | 46.0 |
| Gly/Gly (%) | 61.8 | | | 4.1 | 11.5 | 9.1 | 10.6 | 7.1 | 0.0 | 8.0 |
| Ser | 0.21 | | | 0.77 | 0.66 | 0.77 | 0.74 | 0.76 | 0.69 | 0.69 |
| Gly | 0.79 | | | 0.23 | 0.34 | 0.23 | 0.26 | 0.24 | 0.31 | 0.31 |
| **TD+** | **n=27** | | | **n=51** | **n=117** | **n=65** | **n=21** | **n=49** | **n=59** | **n=53** |
| Ser/Ser (%) | 0.0 | | | 45.1 | 51.3 | 55.4 | 28.6 | 51.0 | 42.4 | 24.5 |
| Ser/Gly (%) | 40.7 | | | 33.3 | 39.3 | 35.4 | 66.7 | 34.7 | 47.5 | 69.8 |
| Gly/Gly (%) | 59.3 | | | 21.6 | 9.4 | 9.2 | 4.8 | 14.3 | 10.0 | 5.7 |
| Ser | 0.20 | | | 0.62 | 0.71 | 0.73 | 0.62 | 0.68 | 0.66 | 0.59 |
| Gly | 0.80 | | | 0.38 | 0.29 | 0.27 | 0.38 | 0.32 | 0.34 | 0.41 |
| **Total group[2]** | **n=123[1]** | **n=25** | **n=85** | **n=100** | **n=317** | **n=131** | **n=115** | **n=105** | **n=113** | **n=116** |
| Ser/Ser (%) | 2.4 | 0.0 | 38.8 | 51.0 | 47.0 | 59.5 | 53.0 | 55.2 | 40.7 | 36.2 |
| Ser/Gly (%) | 32.5 | 36.0 | 50.6 | 36.0 | 42.3 | 31.3 | 37.4 | 34.3 | 54.0 | 56.9 |
| Gly/Gly (%) | 65.0 | 64.0 | 10.6 | 13.0 | 10.7 | 9.2 | 9.6 | 10.5 | 5.3 | 6.9 |
| Ser | 0.19 | 0.18 | 0.64 | 0.69 | 0.68 | 0.75 | 0.72 | 0.72 | 0.68 | 0.65 |
| Gly | 0.81 | 0.82 | 0.36 | 0.31 | 0.32 | 0.25 | 0.28 | 0.28 | 0.32 | 0.35 |

[1] Total of AIM+ and AIM- group, AIM+ is not shown in this table. [2]Total of TD+ and TD- group.

# Chapter Four

# Abnormal involuntary movement development and the role of *CYP2D6*4*, *CYP2D6*10* and *CYP2D6*17* in a Xhosa schizophrenia population

## 4.1 Abstract

Drug metabolising enzymes have been implicated in the development of tardive dyskinesia. Specifically *CYP2D6* genetic variants have been investigated in this regard in various population groups. This study aimed at determining the association between *CYP2D6*4*, *CYP2D6*10* and *CYP2D6*17* and abnormal involuntary movements (AIM) or tardive dyskinesia (TD) development in a Xhosa schizophrenia population. Different methodological approaches were attempted in this study with only partial success, which meant the relationship between the *CYP2D6* variants and AIM or TD could not be investigated. Possible reasons for the lack of results obtained and future recommendations for the genotyping of *CYP2D6* are discussed.

## 4.2 Introduction

Once again the reader is referred to section 1.3 for information regarding the diagnosis, symptoms, prevalence, risk factors, genetics, and hypotheses of tardive dyskinesia (TD). A higher antipsychotic dosage may result in TD development (see section 1.3.8.3), and therefore genetic variants in drug metabolising enzymes have been investigated for their potential role in the development and severity of this disorder (Table 12). CYP2D6 (OMIM number: +124030) is one of the major drug metabolising enzymes that have been investigated in this regard. More information regarding the function, location, and gene of this enzyme, as well as its involvement in TD is given in section 1.3.8.3. Briefly, genetic variants in *CYP2D6* may result in either of four phenotypes: extensive metaboliser (EM), intermediate metaboliser (IM), poor metaboliser (PM) and ultrarapid metaboliser (UM). Duplications of CYP2D6 result in the UM phenotype and low plasma drug levels (Bertilsson *et al*., 1993; Zanger *et al*., 2004). Homozygotes for a null allele (genetic variant(s) resulting in inactive gene product) develop the PM phenotype. Individuals heterozygous for a null allele or homozygous for a reduced function allele generally show the IM phenotype (Kirchheiner *et al*. 2004, Zanger *et al*., 2004). EMs are considered to have "normal" CYP2D6 activity (Kirchheiner *et al*., 2004).

In general, no single polymorphism has been associated with TD development (Table 12), though, the PM phenotype or the presence of at least one null allele was associated with the development of the disorder. However, results have been inconclusive.

The role of *CYP2D6* alleles in the development of TD has been investigated in various populations, but very few studies have focused on African populations. Yet it is known that the frequencies of the *CYP2D6* alleles differ between populations. For example, *CYP2D6*17* is mainly found in African populations, while *CYP2D6*10* is predominantly observed in the Chinese population (Xie *et al*., 2001). Hence it is important to investigate the effects of the population specific genetic variants in the appropriate population. This study, therefore, investigated the role of *CYP2D6*4*

**Table 12:** Association studies between *CYP2D6* genetic variants and TD development.

| Gene | Polymorphism | Author | Number of subjects (ethnic origin) | Results |
|---|---|---|---|---|
| *CYP2D6*[1] | *2 | Ohmori *et al.* (1999) | 99 (Japanese) | No association |
| *CYP2D6*[1] | A, B (*3 *4) | Arthur *et al.* (1995) | 16 (Swedish, Caucasian) | No association between genotype & TD; but possible association between PM & AIMS score |
| *CYP2D6*[1] | *3 *4 | Jaanson *et al.* (2002) | 52 (Estonian, Russian) | Significant association between at least one PM allele & TD |
| *CYP2D6*[1] | *3 *4 *5 | Kapitany *et al.* (1998) | 45 (Austrian, Caucasian) | Significant association between heterozygotes & TD |
| *CYP2D6*[1] | A, B, D (*3 *4 *5) | Armstrong *et al.* (1997) | 76 (Caucasian, Asian) | Non-significant trend between PM & TD |
| *CYP2D6*[1] | *3 *4 *10 | Ohmori *et al.* (1998) | 100 (Japanese) | Significant association between *10 & AIMS; modest association between *10 & TD |
| *CYP2D6*[1] | *10 (C188T) | Liou *et al.* (2004) | 216 (Chinese) | Modest association between C100T & TD (especially in males); also between AIMS scores & C100T |
| *CYP2D6*[1] | *1*3 *4 *5 *6 *7 | Andreassen *et al.* (1997) | 100 (Scottish, Caucasian) | Non-significant tendency between PM & TD |
| *CYP2D6*[1] | *2 *3 *4 *10 *12 | Inada *et al.* (2003) | 516 (Japanese) | No association |
| *CYP2D6*[1] | *1 *2 *3 *4 *5 *6 *7 *8 *9 *10 *11 *14 *18 *19 *25 *26 *31 *36 *41 | Nikoloff *et al.* (2002) | 202 (Korean) | Significant association between males with at least one decreased or loss of function allele & TD |
| *CYP2D6*[1] | *4 | Tiwari *et al.* (2005a) | 335 (North Indian) | No significant association with TD, but trend between *CYP2D6*4* & severity of TD |
| *CYP2D6*[1] | *2 *3 *4 *5 *6 *7 *8 *9 *10 *11 *14 *15 *17 *18 *19 *20 *25 *26 *29 *30 *31 *35 *36 *37 *40 *41 *43 *45 | De Leon *et al.* (2005) | 516 (American) | Nonsignificant trend between TD & *CYP2D6* absence, particularly in white men |

[1]The specific genetic variants which make up each individual allele are presented on the home page of the human cytochrome P450 (CYP) allele nomenclature committee: http://www.cypalleles.ki.se

(G1846A, results in null allele), *CYP2D6*10* (C100T, results in reduced function allele) and *CYP2D6*17* (C1023T and C2850T, results in reduced function allele) in the development of abnormal involuntary movements (includes TD) and its severity in a Xhosa schizophrenia population. The selection of these alleles was based on their relevant frequencies in African populations. Information regarding each allele (exact mutation, effect on CYP2D6, and population frequencies) is given in section 1.3.8.3.1, 1.3.8.3.2, and 1.3.8.3.3.

## 4.3 Materials and Methods

### 4.3.1 Clinical

#### 4.3.1.1 Patient population

The patient population studied was the same described in section 2.3.1.1. Briefly, patients were recruited within the Western Cape Province of South Africa, diagnosed according to the DSM-IV and were of Xhosa origin. Patients or their caregiver (where appropriate) gave informed, written consent and the study was approved by the ethics committee of Stellenbosch University.

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#### 4.3.1.2 Assessment

The same assessments performed in section 2.3.1.2 were performed in this study, with interviews conducted according to DIGS (Nurnberger *et al*., 1994) and the presence of AIM determined using the AIMS (Guy, 1976). For more information please see section 2.3.1.2. Please also see the statistical analysis section in Chapter Two (section 2.3.3) for the division into AIM+ or TD+ and AIM-.

### 4.3.2 Laboratory

This study tried two different approaches in an attempt to amplify the specific regions of interest. The first approach considered the amplification of either one exon at a time (exon 2 and exon 5&6), or the amplification of several exons (exon 1-2 and exon 3-6). Nested PCR would then be performed on the multi-exonic fragments to obtain smaller fragments suited for restriction enzyme digestion. The second approach used long range PCR technology to amplify the whole *CYP2D6* gene and a nested PCR to amplify specific *CYP2D6* exons of interest, which once again are suitable for restriction enzyme digestion. It should be noted that for all the nested PCRs the template was diluted 1 in 500 or 1 in 200 depending on band intensity and that all amplification reactions were performed using a GeneAmp PCR System 2700 (Perkin Elmer, Applied Biosystems, Warrington

WA, Great Britain). Please also refer to addendum A and B regarding more information about the DNA extraction protocol and genotyping methods. Primers were designed using Primer3 (Rozen and Skaletsky, 2000), while BLAST (Altschul *et al.,* 1990) was used to determine whether primers bind to pseudogenes or any other genes. Please see Figure 5 and Table 13 for the position and sequence of the individual primers.

#### 4.3.2.1 First approach

Primer sequences and PCR conditions for the amplification of exon 1, exon 2, exon 1-2, exon 4 fragment, exon 5-6 and exon 3-6 are shown in Table 13 and Table 14 respectively. The primers for exon 1 and the exon 4 fragment bind to the pseudogenes, therefore only exon 2 and exon 5-6 were amplified at first using primers that did not bind to the pseudogenes.

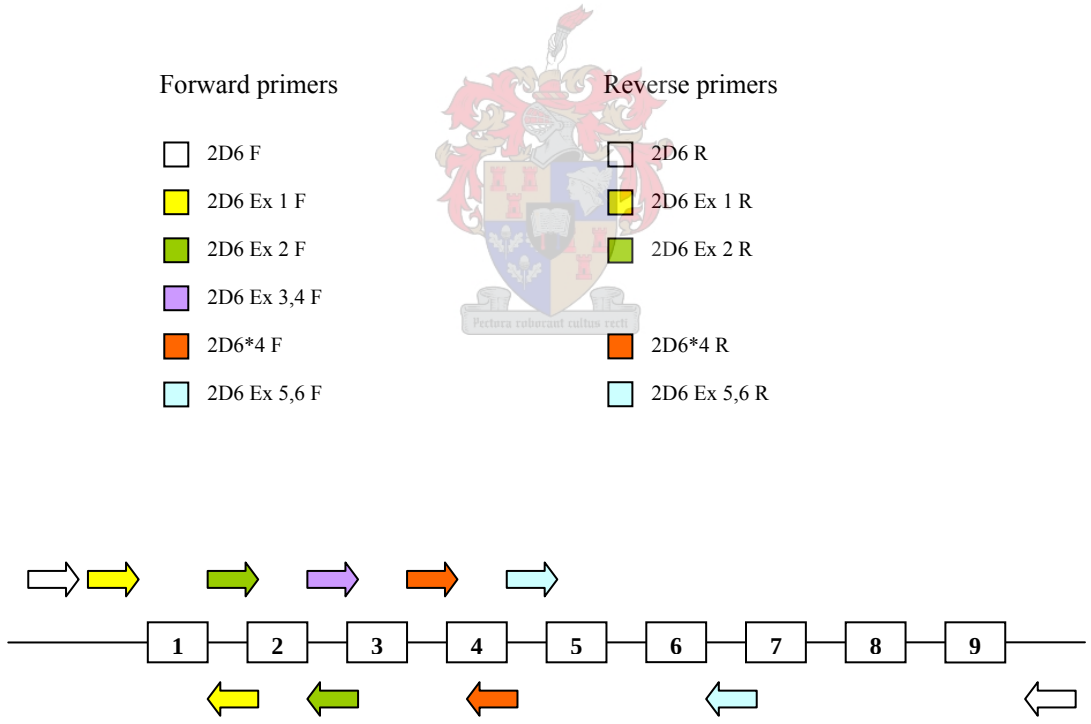


**Figure 5:** Diagram of the various primer positions in the *CYP2D6* gene (Not drawn to scale).

**Table 13:** *CYP2D6* primer information.

| Primer name | Primer orientation | Primer sequence | Amplification of |
|---|---|---|---|
| 2D6 F[a] | Forward | 5'-CCAGAAGGCTTTGCAGGCTTCA-3' | *CYP2D6* and Exon 1-2 |
| 2D6 R[a] | Reverse | 5'-ACTGAGCCCTGGGAGGTAGGTA-3' | *CYP2D6* |
| 2D6 Ex 1 F | Forward | 5'-TCTGGAGCAGCCCATACCCG-3' | Exon 1 and Exon 1-2 |
| 2D6 Ex 1R | Reverse | 5'-TAAATGCCCTTCTCCAGGAC-3' | Exon 1 |
| 2D6 Ex 2 F | Forward | 5'-TCCTCCTTCCACCTGCTCAC-3' | Exon 2 |
| 2D6 Ex 2 R | Reverse | 5'-CTTCGACACCGGATTCCAGC-3' | Exon 2 and Exon 1-2 |
| 2D6*4 F | Forward | 5'-GTGGGTGATGGGCAGAAG-3' | Exon 4 fragment |
| 2D6*4 R | Reverse | 5'-GAGGGTCGTCGTACTCGAA-3' | Exon 4 fragment |
| 2D6 Ex 5,6 F | Forward | 5'-ACAGGCAGGCCCTGGGTCTA-3' | Exon 5-6 |
| 2D6 Ex 5,6 R | Reverse | 5'-GGCCCTGACACTCCTTCTTG-3' | Exon 5-6 and Exon 3-6 |
| 2D6 Ex 3,4 F | Forward | 5'-AGCTGGAATCCGGTGTCGAA-3' | Exon 3-6 |

[a]Lundqvist *et al.* (1999)

#### 4.3.2.1.1 *Amplification of exon 2*

For the detection of *CYP2D6*17* C1023T a 561 bp fragment was amplified using primers 2D6 Ex 2 F and 2D6 Ex 2 R. This fragment would then be subjected to restriction enzyme digestion using *FokI* (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions, resulting in a 561 bp band in wildtype homozygotes and a 313 bp and 248 bp band in mutant homozygotes.

#### 4.3.2.1.2 *Amplification of exon 5 to exon 6*

The amplification of exon 5 and 6 (772 bp; primers: 2D6 Ex 5,6 F and 2D6 Ex 5,6 R) was performed for the detection of *CYP2D6*17* C2850T. The amplicon was then digested according to manufacturer's instructions using *HhaI* (New England Biolabs, Ipswich, MA, USA). Wildtype homozygotes showed a 372 bp, 261 bp and a 139 bp band, and mutant homozygotes only a 633 bp and 139 bp band (See Figure 6).

#### 4.3.2.1.3 *Amplification of exon 1 to exon 2*

Due to the high homology between *CYP2D6* and its pseudogenes, specifically in the exon 1 region, and the problems encountered amplifying exon 2 on its own (discussed in results section), exon 1 and exon 2 were amplified together (reverse primer does not bind to pseudogenes) either using 2D6 F or 2D6 Ex 1 F as forward primer (optimization attempts were always tried for both primer options). The 1664 bp (2D6 F – 2D6 Ex 2 R) or 1544 bp (2D6 Ex 1 F – 2D6 Ex 2 R) fragment would therefore be used as template for a nested PCR of exon 1 (551 bp; primers: 2d6 Ex 1 F and 2D6 Ex 1 R) and exon 2 (561 bp). Using *HphI* (New England Biolabs, Ipswich, MA, USA) to cut the exon 1 PCR product, the presence of C100T could be detected, since wildtype homozygotes

presented with a 413 bp, 71 bp and 67 bp band, and mutant homozygotes with a 313 bp, 100 bp, 71 bp and 67 bp band. *CYP2D6*17* C1023T was detected as described previously (See amplification of exon 2).

Table 14: ***CYP2D6* PCR conditions and optimization attempts.**Error! Not a valid link.

**Table 14:** continued.

| **Amplification of** | **Exon 1-2** | **Exon 3-6** | ***CYP2D6*** |
|---|---|---|---|
| Polymerase | 0.5 U GoTaq® Flexi[a] | 0.5 U GoTaq® Flexi[a] | 1.25 U GeneAmp® High Fidelity PCR system[d] |
| Buffer | 1X GoTaq® Flexi Buffer | 1X GoTaq® Flexi Buffer | 1X GeneAmp® High Fidelity PCR buffer |
| Forward primer | 0.8 µM 2D6 Ex 1 F or 2D6 F | 0.4 µM 2D6 Ex 3-4 F | 0.3 µM 2D6 F |
| Reverse primer | 0.8 µM 2D6 Ex 1 R | 0.4 µM 2D6 Ex 5-6 R | 0.3 µM 2D6 R |
| [$MgCl_2$] | 3 mM | 1.5 mM | 1.5 mM |
| [dNTP][ab] | 0.1 mM | 0.1 mM | 0.2 mM |
| DNA | ~50 ng | ~50 ng | ~30 ng |
| Reaction volume | 25 µl | 25 µl | 25 µl |
| PCR program | 94°C 3 minutes<br>94°C 20 seconds<br>62°C 20 seconds } X15<br>72°C 2 minutes<br>94°C 20 seconds<br>59°C 20 seconds } X20<br>72°C 2.5 minutes<br>72°C 4 minutes | 94°C 3 minutes<br>94°C 20 seconds<br>55°C 20 seconds } X15<br>72°C 2 minutes<br>94°C 20 seconds<br>52°C 20 seconds } X20<br>72°C 2.5 minutes<br>72°C 4 minutes | 94°C 2 minutes<br>94°C 15 seconds<br>64°C 30 seconds } X9<br>68°C 4 minutes<br>94°C 15 seconds<br>64°C 30 seconds } X21<br>68°C 4 minutes +5 seconds/cycle<br>72°C 7 minutes |
| **Optimisation attempts** | | | |
| Increase [$MgCl_2$] | | | **A)** 2 mM; **B)** 2.5 mM; **E)** 1-4 mM ($MgCl_2$ range); **F)** 1.35 mM |
| Decrease [$MgCl_2$] | **A & C)** 2.5 mM | | |
| Increase DNA | | **A)** >80 ng | **B), D), E) & F)** ~60 ng; **C)** 90 ng |
| Increase [dNTP][ab] | | | **C)** 0.26 mM; **E) & F)**: 0.28 mM |
| Increase [primer] | **E)** 1.98 µM | | **C)** 0.6 µM; **E)** 0.4 µM |
| Different polymerase | | | **D)** TaKaRa Ex Taq™ [e] |
| Increase [polymerase] | | | **F)** 2.5 U |
| **Change PCR program** | | | |
| Cycle number | | | **C) & F)**[f] 35 cycles |
| Annealing temperature | **A & B)** 63°C and 60°C<br>**D)** 55°C and 52°C<br>**E)** 57°C and 54°C | | **A) & B)** 60°C<br>**E)** 54°C-64°C (temperature range)<br>**F)** 68°C[f] |
| Denaturation time | | | **B)** 20 seconds; **E)** 30 seconds; **F)** 12 seconds[f] |
| Annealing time | | | **F)** 7 minutes (annealing and elongation time and no increase in time/cycle)[f] |
| Elongation time | | | **C)** 5 minutes (no increase in time/cycle) |

[a]Product of Fermentas, Ontario, Canada; [b]Concentration given for each dNTP; [c]Product of Bioline, Canton, MA, USA; [d]Product of Applied Biosystems, Foster City, CA, USA; [e]Product of Takara Bio inc., Otsu, Japan; [f]According to Lundqvist *et al.* (1999)

4.3.2.1.4 *Amplification of exon 3 to exon 6*

For the detection of *CYP2D6*4* G1846A and as an alternative strategy for the amplification of *CYP2D6*17* C2850T (only gave partial results in previous approach; see section 4.4.2) exon 3 to 6 was amplified as one fragment (1809 bp; primers: 2D6 Ex 3,4 F and 2D6 Ex 5,6 R). PCR product obtained was then diluted and used as template for a nested PCR of an exon 4 fragment (primers: 2D6*4 F and 2D6*4 R) and exon 5 to 6. For the exon 4 fragment (184 bp) a restriction enzyme digest using *MvaI* (Fermentas, Ontario, Canada) (according to manufacturer's instructions) detected the presence of wildtype homozygotes (98 bp and 86 bp band), mutant homozygotes (184 bp band) and heterozygotes (184 bp, 98 bp and 86 bp band) for G1846A (See Figure 7). *CYP2D6*17* C2850T was detected as described under amplification of exon 5 to exon 6 (section 4.3.2.1.2).

4.3.2.2 Second approach

4.3.2.2.1 *Amplification of entire CYP2D6 gene*

Here it was attempted to amplify the entire *CYP2D6* gene (~5kb). Primers used by Lundqvist *et al.* (1999) were used in this study. PCR product obtained would once again be diluted and used as template for the amplification of exon 1, exon 2, exon 4 fragment and exon 5 to 6 and genotypes determined using restriction enzyme digests as described previously.

4.3.2.3 Visualisation

PCR product was visualised after 40 minutes on a 1.5% (w/v) agarose gel stained with ethidium bromide (0.01%; v/v), subjected to electrophoresis at 100V, while restriction enzyme digest product was visualised on a 1.5% (w/v) or 2% (w/v) agarose gel, stained with ethidium bromide (0.01%; v/v), after an hour of electrophoresis at 80V. Detection of the relevant product was performed under UV light.

## 4.4 Results

### 4.4.1 Amplification of exon 2

Amplification of control samples (unrelated anonymous control DNA samples) worked well, yet when performed with the Xhosa schizophrenia DNA samples either extremely light bands with non-specific product or no amplification at all were observed. Hence, several optimization attempts were made as shown in Table 14. This included the reordering of the primers (not shown in Table 14). However, no results were obtained.

### 4.4.2 Amplification of exon 5 to exon 6

Of the 123 Xhosa schizophrenia samples to be genotyped, amplification was only possible for about half of these samples. Even after several optimization attempts shown in Table 14, no progress was made. See amplification of exon 3 to exon 6 for information regarding the genotyped samples.

### 4.4.3 Amplification of exon 1 to exon 2

After several attempts to optimize this reaction using BioTaq™ (Bioline, Canton, MA, USA) at first (not shown in Table 14), amplification was finally possible using GoTaq® Flexi (Fermentas, Ontario, Canada) and control DNA. Non-specific bands were also visible, though they almost disappeared when using the PCR conditions shown in Table 14. However, once this reaction was performed on Xhosa patient samples, only non-specific bands were observed. Steps taken to optimize the reaction for the Xhosa patient DNA samples are shown in Table 14, and once again new primers were ordered, to make sure the primers had not degenerated. However, this also proved unsuccessful.

### 4.4.4 Amplification of exon 3 to exon 6

Amplification of exon 3 to exon 6 only resulted in partial success and using more DNA (shown in Table 14) did not make a difference. For the nested PCR of exon 5 to 6 also only about half of the samples could be amplified. Together with the previous attempt to amplify exon 5 to 6 (see amplification of exon 5 to exon 6 section), in total only 63 out of possible 123 Xhosa patient samples were genotyped (Figure 6). The nested PCR of the exon 4 fragment was also only partly successful, and only 44 Xhosa schizophrenia patients could be genotyped (Figure 7), even though amplification of control samples had worked well.

### 4.4.5 Amplification of entire *CYP2D6* gene

Numerous attempts were made to optimise this reaction (see Table 14), however, the correct fragment could not be amplified, with either no product or only non-specific bands being observed.

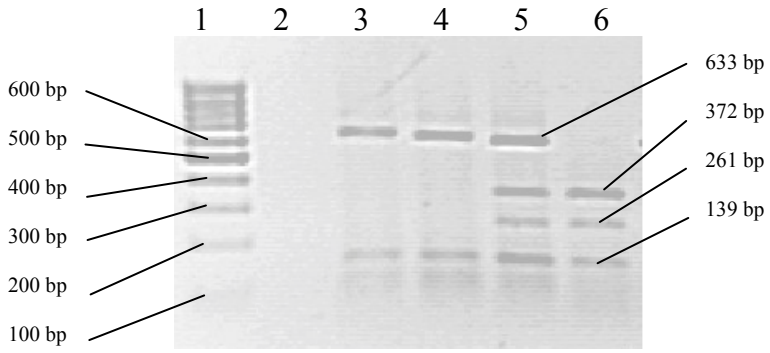


**Figure 6:** *CYP2D6* C2850T genotype determination by *HhaI* digestion of exon 5 to 6, visualised on a 1.5% (w/v) agarose gel. Lane 1: Ladder: O'Generuler 100 bp DNA ladder (Fermentas, Ontario, Canada); lane 3 and 4: mutant allele homozygotes; Lane 5: Heterozygote; Lane 6: Wildtype allele homozygote.

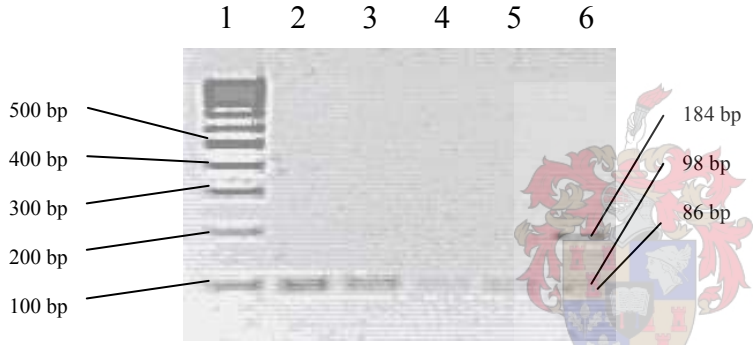


**Figure 7:** *CYP2D6* G1846A genotype determination by digestion of the exon 4 fragment using *MvaI*, visualised on a 2% (w/v) agarose gel. Lane 1: Ladder: Hyperladder IV (Bioline, Canton, MA, USA); Lane 2-5: Wildtype allele homozygotes; Lane 6: Heterozygote.

## 4.5 Discussion

This study focused on the association between several *CYP2D6* genetic variants, namely *CYP2D6*4* (G1846A), *CYP2D6*10* (C100T) and *CYP2D6*17* (C1023T and C2850T), and the development and severity of AIM or TD. However, only 63 Xhosa schizophrenia patients could be genotyped for C2850T and 44 for G1846A. For the latter variant only two heterozygotes were observed (both in the AIM- group), while the rest were all homozygous for the wildtype allele (Table 15). Caucasians show a higher *CYP2D6*4* allele frequency (11.3-28.6%) compared to African populations (0.9-9.3%) and Asian populations (0.0-0.8%) (Evans *et al*., 1993; Masimirembwa and Hasler, 1997; Bathum *et al*., 1999; Xie *et al*., 2001). The allele frequency of 2.3% in our genotyped sample group (Table 15) (4.2% in the AIM- group), therefore, corresponds

with this finding. Caucasians had higher allele frequencies and Chinese showed even lower allele frequencies than the Xhosa population (Table 15). Crawley (2006) found the *CYP2D6*4* allele frequency to be 6.3% in a healthy Xhosa population. This figure is higher than found in this study, but falls within the allele frequency range described for African populations (0.9-9.3%).

In general, the trend has been that at least one loss of function (PM) allele is associated with TD development. We only found two heterozygotes, and these were in the AIM- group, theoretically not indicating an association between this variant and AIM development. However, the number of samples genotyped was of course too small to be able to perform association analyses and results obtained only give an idea of the effect of *CYP2D6*4* on AIM development in the Xhosa schizophrenia population.

**Table 15:** *CYP2D6*4* (G1846A) allele and genotype frequencies in various populations (TD association studies).

| | **This study** | **Kapitany *et al.* (1998)** | **Andreassen *et al.* (1997)** | **Jaanson *et al.* (2002)** |
|---|---|---|---|---|
| **Population**[1] | Xhosa | Caucasian | Caucasian | Russian |
| **Total**[2] | **n=44**[3] | **n=45** | **n=100** | **n=52** |
| GG (%) | 95.5 | 66.7 | 91.0 | 67.3 |
| GA (%) | 4.5 | 31.1 | 4.0 | 32.7 |
| AA (%) | 0.0 | 2.2 | 5.0 | 0.0 |
| G | 0.98 | 0.82 | 0.93 | 0.84 |
| A | 0.02 | 0.18 | 0.07 | 0.16 |

[1]Studies on Asian populations either did not find the A allele or only found one heterozygote (Nikoloff et al., 2002; Inada *et al.*,2003; Ohmori *et al.*,1998) and therefore were not included in this table; [2]Total of TD+ and TD- group; [3]Total of AIM+ and AIM- group.

No statistical analysis was performed for the 63 samples genotyped for C2850T, even though the sample number was reasonable. This is due to the fact that this variant is not only present in *CYP2D6*17* but also in various other alleles such as *CYP2D6*2*, *CYP2D6*8* and *CYP2D6*11* (Homepage of the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee, http://www.cypalleles.ki.se/cyp2d6.htm). Only in combination with C1023T does it result in a functional effect, and classification as *CYP2D6*17* is only possible if both of these variants are present in the same individual (even then one needs to check for variants unique to *CYP2D6*40* and *CYP2D6*58* such as 1863_1864insTTTCGCCCC, since both of these also contain C1023T and C2850T). Hence, it is not surprising that the allele frequency of C2850T was 51.6% in our cohort, 45.8% in the AIM+ and 59.6% in the AIM- group. Since C1023T could not be genotyped, no interpretation regarding *CYP2D6*17* and its association with AIM or TD can be made in this study.

Various attempts were made to amplify exon 1, exon 2, an exon 4 fragment and exon 5 to 6. Partial results were only achieved for the exon 4 fragment and exon 5 to 6. The possible reasons for the non- or partial amplification are numerous.

Several optimisation steps were taken to successfully optimise the various exons and fragments (See Table 14 for all optimisation attempts). These included increasing or decreasing the $MgCl_2$ concentration, for optimal amplification. The DNA concentration in a PCR was also changed. The logic behind increasing the concentration was of course to increase the amount of possible template the primers could bind to and hence better amplification, while a decrease in DNA concentration might reduce possible PCR inhibitory substances present in the patient DNA samples and therefore, once again, result in better amplification.

In addition, annealing temperature(s) for various PCRs were changed. Initial annealing temperatures had been set at about 5°C below the melting temperature, as is generally recommended. Annealing temperatures were adjusted from there for optimal amplification. In some cases the annealing temperature was lowered somewhat in the hope of obtaining the right fragment, even in the presence of non-specific bands. The theory behind this was that once the right fragment was observed, small optimisation steps could be taken to eliminate non-specific amplification.

Furthermore, the primer concentration was increased for some PCRs. Due to the high homology between *CYP2D6* and its pseudogenes, and since one of the primers (of course never both) generally also bound to the pseudogenes, it was thought that possibly too few primers (either forward or reverse) were available for amplification and the primer concentration therefore increased.

One other option contemplated was the use of a different DNA polymerase (see Table 14), though again no success was attained.

Most optimisation attempts did not yield results. Strangely, for control samples used for the initial optimisation of the reaction correct amplification did occur for some reactions (exon 2 and exon 1-2), yet, when the same reaction was tried on several different patient samples no results were obtained. The argument that non-optimal cycling conditions may be the cause of the nonamplification hence does not apply. Of course it could mean that possibly the patient DNA samples were either of low quality and/or had low DNA concentrations. To address the latter, all

sample concentrations had been measured (done by GeneCare Molecular Genetics (Cape Town, South Africa) who had been contracted to extract DNA) and confirmed for the majority of samples in our laboratory. Generally about 50 ng of DNA was used in a 25 µl PCR reaction. In addition, as mentioned before, the DNA concentration was increased for some PCRs (also see Table 14). This study only had limited amounts of DNA available, and therefore no further measurements regarding purity were performed. This limitation also did not allow for any purification attempts to be made. When the same patient samples which did not amplify for any of the *CYP2D6* PCRs were subjected to a PCR of another gene fragment (*DRD3* as described in Chapter Three) amplification worked well (data not shown). Furthermore, the same patient sample had successfully been used for the amplification of various other genes such as *MnSOD* (see Chapter Two), *CYP-17*, *CYP1A2*, *CYP3A4*, *CYP3A5* and *MDR1* in our laboratory.

Due to the high homology of the pseudogenes and the numerous genetic variants of this gene, primers designed were not optimal regarding GC content, annealing temperature or self-complimentarity. This may well have resulted in poor or no amplification. Yet, this does not explain the amplification in control samples.

Another theory explaining nonamplification was that primers had degenerated over time. For this reason primers were reordered, though this also did not prove successful.

There was a general trend for PCR reactions to decrease in effectivity over time, which could be related to primer degeneration (see above) or to possible PCR inhibitory substances in the pipette. These substances may end up in the PCR reactions resulting in poor amplification or none at all. Pipettes were cleaned at several stages (rinsed with autoclaved distilled water after cleaning) and put under UV light which should have eliminated any inhibitory substances.

The amplification of the entire *CYP2D6* gene followed by any of several different genotyping procedures is the approach taken by most studies focusing on this gene (Hersberger *et al.*, 2000; Müller *et al.*, 2003; Sistonen *et al.,* 2005). This study also attempted this strategy, without success even after numerous optimisation steps (see Table 14). An honours project performed in our laboratory focused on the detection of the *CYP2D6* deletion (Ricketts, 2006). It incorporated the amplification of the entire *CYP2D6* gene, used as internal control for reaction failure and the detection of heterozygotes. While in that study the correct 5 kb fragment was observed using Fermentas *Taq* DNA polymerase (Fermentas, Ontario, Canada), replication proved problematic.

Due to time limitations, no further optimisation attempts based on the findings of the honours project could be made in this study.

Another factor which may explain the partial amplification or nonamplification of *CYP2D6* in this study is the possibility of the presence of genetic variants specific to the Xhosa population, which have not been documented yet. These may hamper primer binding if they lie within the primer binding site. Partial deletions of the *CYP2D6* gene may also exist, which could hinder amplification or result in the misinterpretation of results (smaller fragment seen as non-specific amplification). As mentioned, *CYP2D6* genetic variant frequencies do differ between populations. To our knowledge no studies have focused on *CYP2D6* variants in the Xhosa population and further investigations are therefore warranted. However, it is unlikely that all patients in this study had a variant or deletion that would result in nonamplification. For that reason the possibility of as yet unknown genetic variants alone cannot explain nonamplification.

In addition, there exists the possibility that due to factors such as coiled secondary structures, certain areas of the genome amplify better than others. Possibly the *CYP2D6* region is one of those areas that is more tightly coiled in the Xhosa population, hence less accessible to DNA polymerases and therefore amplifies with difficulty.

In our laboratory an honours study focused on the frequency determination of *CYP2D6*4* and *CYP2D6*17* (C2850T) in a healthy Xhosa population (Crawley, 2006). Out of 70 samples, 63 could be genotyped for *CYP2D6*4*, and only 33 for *CYP2D6*17* (C2850T) using the method described in section 4.3.2.1.4. This study was clearly more successful in genotyping *CYP2D6*4*, but faired the same in regard to *CYP2D6*17* (C2850T). Differences may be due to different DNA samples used (healthy Xhosa versus schizophrenia Xhosa population), minor differences in the method used, or possibly the presence of novel amplification-affecting genetic variants in either of the populations.

There are probably still numerous optimisation steps that could have been performed for each individual PCR, however, time and DNA limitations did not allow for further optimisation attempts. Various methods exist for the genotyping of *CYP2D6*. Some studies use *CYP2D6* specific ARMS reactions (Heim and Meyer, 1990), while other methods are based on the amplification of the entire *CYP2D6* gene, which is then followed by either ARMS (Hersberger *et al.*, 2000), detection through fluorescent technology (Sistonen *et al.*, 2005), or real-time PCR (Müller *et al.*, 2003). A relatively new and easy method makes use of microarray technology to genotype individuals (Nikoloff *et al.*, 2002; Heller *et al.*, 2006). This method does not limit genotyping to a few specific genetic variants

and further allows genetic screening of various populations, independent of ethnic genetic differences (Heller *et al.*, 2006). Roche's AmpliChip® CYP450 test, for example, is commercially available and tests for numerous *CYP2D6* and *CYP2C19* genetic variants (the first to be cleared by the FDA) (AmpliChip® CYP450 test, http://www.amplichip.us). In the future, opting for a probably more expensive but easier, faster and effective method may be a better choice for genotyping individuals for *CYP2D6*.

Pharmacogenetic tests for personalized therapy may become a reality in the future. *CYP2D6* is an important drug metabolizing enzyme and therefore is a likely candidate to be screened during pharmacogenetic tests. However, ethnic genetic differences should be investigated thoroughly, before implementation of *CYP2D6* genotype screening, since some variants may be unique to specific populations (e.g. *CYP2D6*17)*. The importance of this gene in TD development in the Xhosa population remains to be investigated, and further studies should focus on determining the association between known genetic variants and TD, as well as screening for novel genetic variants in this unique African Xhosa population.

# Chapter Five

## Conclusion

In general, genetic association studies on TD show contradictory findings. This can be explained by several factors and the complexity of the disorder. For example, the strictness regarding diagnosis of TD is a factor that may influence results. In addition, we found that some published *CYP2D6* studies used primers that did bind to pseudogenes (not used in this study) or only differed by one base pair, located in the centre of the primer. The findings of these studies may therefore be spurious. It may also be that the pathophysiology of TD differs between populations, and hence different genetic variants could lead to the development of the disorder in the one population but not in others.

This thesis focused on the association between *MnSOD* Ala-9Val, *DRD3* Ser9Gly, *CYP2D6*4* (G1846A), *CYP2D6*10* (C100T) and *CYP2D6*17* (C1023T and C2850T) and the development and severity of AIM as well as TD in a Xhosa schizophrenia population. Only partial results could be obtained for *CYP2D6* and no association analysis was performed. In regard to *DRD3* Ser9Gly, no association between this variant and TD or AIM development as well as severity was observed. We did, however, find an association between *MnSOD* Ala-9Val and TD or AIM development and TD severity in this Xhosa schizophrenia population. This variant on its own probably has a small effect on AIM/TD and only together with several other variants in different genes will result in AIM/TD development. We did test for a possible interactive effect between the *DRD3* Ser9Gly and *MnSOD* Ala-9Val in the development of AIM or TD, but no significant effect was observed. Future studies should therefore focus on possible interactive effects between numerous other variants, as well as the variants investigated in this study. In this Xhosa schizophrenia population, the genotype and allele frequencies of *MnSOD* Ala-9Val were found to be similar to those of the Turkish population and different to those of the Asian populations. *DRD3* Ser9Gly genotype and allele frequencies differed from those of Caucasian and Asian populations, but were similar to those of the African American population. From the samples that could be genotyped for *CYP2D6*4* (G1846A) the genotype and allele frequencies were determined, and fell into the range described for African populations. They did differ from those allele and genotype frequencies described for Caucasian populations, as has been reported for African populations in the literature.

This study fulfilled its aims in that it successfully determined the role of *MnSOD* Ala-9Val and *DRD3* Ser9Gly in the development of AIM and TD, as well as the role of *MnSOD* Ala-9Val in the

development and severity of schizophrenia in the unique Xhosa schizophrenia population. In addition, the *MnSOD* Ala-9Val, *DRD3* Ser9Gly and *CYP2D6*4* genotype and allele frequencies of the Xhosa schizophrenia population (AIM+ and AIM-) was compared to those described in the literature for other populations (TD+ and TD-). Lastly, this study was able to investigate the possibility of an interactive effect between *MnSOD* Ala-9Val and *DRD3* Ser9Gly in the development of AIM and TD. However, this study could not fulfil all of its aims in that it was not able to investigate the role of *CYP2D6*4*, *CYP2D6*10* and *CYP2D6*17* in the development of AIM and TD, nor an interactive effect of any of these variants and *MnSOD* Ala-9Val or *DRD3* Ser9Gly in the development of the mentioned disorders. The genotype and allele frequencies of *CYP2D6*10* and *CYP2D6*17* could also not be determined.

A novel approach was attempted in this study, in that it investigated the role of the mentioned genetic variants in the development of AIM. Previous studies have focused solely on TD. It is interesting to note, that while results generally were similar between the TD+ and AIM+ group, differences were observed for the analysis of *MnSOD* Ala-9Val, where an effect of the variant on AIMS score, therefore severity, was only observed in the TD+ group.

Only certain genetic variants, based on previous research on TD development in different populations, were investigated in this study. Yet, several other genetic variants may play a role in TD development, and investigations of these have been based on the specific hypothesis used to explain TD development. TD is bound to be a complex disorder and a multi-variant approach should be taken for the better understanding of the exact mechanism of TD. This was partly attempted in this study, by trying to establish any interactive effects between genetic variants. However, this should be extended to a much greater number of genetic variants, and eventually to a multi-genic, multi-variant investigation of TD development.

In the future, knowledge regarding the frequencies of specific variants as well as their involvement in drug-induced side effects in different populations will be important, for the development of accurate methods in the prediction of an individual's drug response. Prediction of antipsychotic side effects, such as TD, is still a long way off, and is complicated by the apparent differences between ethnic groups. Yet, it is hoped in the future that through the use of high throughput methods, such as the AmpliChip® CYP450 test (AmpliChip® CYP450 test, http://www.amplichip.us), screening for numerous variants in several different genes simultaneously will eventually lead to the better understanding of the pathophysiology of TD, and hence the development of preventative and/or curative strategies.

# Chapter Six

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# Appendix A

## Total genomic DNA extraction from whole blood

Prior to the commencement of this study, GeneCare Molecular Genetics (Cape Town, South Africa) was contracted to extract DNA for Xhosa schizophrenia patients and healthy Xhosa controls, using the method described by Miller *et al.* (1988). For those samples with low DNA concentrations our lab once again performed DNA extractions on whole blood (was only available for some samples) with a modified protocol of Miller *et al.* (1988). Blood was collected in BD Vacutainer™ tubes (BD Vacutainer™ Systems, Plymouth, UK) containing ethylenediamine tetra-acetic acid (EDTA, $C_{10}H_{16}N_2O_8$). Blood was stored at 4°C for extraction on the same day or at -20°C if the extraction was performed at a later stage.

Before placing about 10-20 ml of blood in a 50 ml falcon tube (Eurotubo, Deltalab, Barcelona, Spain), the vacutainer tube was shaken. Thirty ml of cold lysis buffer (0.155 M ammonium chloride ($NH_4Cl$), 0.01 M potassium hydrogen carbonate ($KHCO_3$), 0.0001 M EDTA) was added to the blood and mixed by inversion. The mixture was then put on ice for 20 minutes, shaking the sample every 5 minutes. Thereafter centrifugation occurred for 10 minutes at 365xg (UNICEN 20, Orto-Alresa, Madrid, Spain). The supernatant was then thrown away and 10 ml phosphate buffered saline (PBS) (0.027 M potassium chloride (KCl), 0.137 M sodium chloride (NaCl), 0.008 M di-sodium hydrogen orthophosphate anhydrous ($Na_2HPO_4$), 0.0015 M potassium dihydrogen orthophosphate ($KH_2PO_4$)) was added to the pellet. Another centrifugation step at 365xg for 10 minutes followed. The supernatant was again discarded and the pellet dissolved in 3 ml nuclear lysis buffer (0.01M Tris-Cl, 0.4M NaCl, 0.002M EDTA), 50 µl proteinase K (10 mg/ml) (Finnzymes, Espoo, Finland) and 300 µl 10% (w/v) sodium dodecyl sulphate (SDS, ($CH_3(CH_2)_{11}OSO_3Na$)). The tube containing the mixture was well shaken and then incubated overnight at 55°C in a waterbath.

After this period 2 ml 6 M NaCl was added and the mixture shaken vigorously for 1 minute. A centrifugation step at 1986xg for 30 minutes followed. Thereafter, the supernatant was transferred (without transferring foam or pellet) to a new tube (Eurotubo, Deltalab, Barcelona, Spain) and shaken for 15 seconds. The mixture was then centrifuged for 15 minutes at 1013xg, after which the supernatant was once again transferred to a new tube (Eurotubo, Deltalab, Barcelona, Spain) (without transferring any residual foam). Three volumes of ice-cold ±99% (v/v) ethanol (EtOH) were then added and the solution left to stand. After a few minutes the formed DNA spool was placed into a 1.5ml microcentrifuge tube (Brand GMBH + Co KG, Wertheim, Germany) and 500 µl

70% (v/v) ethanol were added. This was followed by centrifugation of the mixture for 10 minutes at 31774xg at 4°C. The ethanol was then drained off and the pellet allowed to air dry. Once dry, the pellet was dissolved, depending on the size of the pellet, in 200-1000 µl sterile SABAX water (Adcock Ingram, Johannesburg, RSA).

# Appendix B

# Genotyping

## ***MnSOD***

### Heteroduplex single stranded conformational polymorphism analysis (HEX-SSCP)

Exon two was amplified from the *MnSOD* gene using primers, PCR reagents and amplification conditions as specified in Chapter Two, section 2.3.2 (also see Table 16). Five µl of SSCP loading buffer (91% (v/v) formamide ($H_2NCHO$), 18 mM EDTA ($C_{10}H_{16}N_2O_8$), 0.05% (w/v) xylene cyanol ($C_{31}H_{28}N_2Na_4O_{13}S$), 0.05% (w/v) bromophenol blue ($C_{19}H_{10}Br_4O_5S$)) was then added to the amplified product. Thereafter the mixture was heated at 98°C for 5 minutes, after which it was immediately put on ice. Fifteen µl were loaded onto a 12% (w/v) polyacrylamide (PAA), 7.5% (w/v) urea ($(NH_2)_2CO$), 1% cross-linking heteroduplex SSCP gel (370mm (height) X 165mm (width) X 0.75mm (depth)). 4.5 g urea (7.5%; w/v), 18 ml of 40% (w/v) PAA stock, 18 ml 5X TBE, 24 ml $dH_2O$, 800 µl of 10% (w/v) ammonium persulphate (APS, $(NH_4)_2S_2O_8$), and 80 µl TEMED (N', N', N', N', -tetramethylethylenediamine) were mixed to make a 60 ml gel. 1.5X TBE was used as buffer. The gels were then run for 17 hours at 4°C at 250 V and thereafter removed from the electrophoresis apparatus for staining.

### Staining

The polyacrylamide gel was stained, depending on the intensity of the amplified bands, with either of two methods. The gels were placed in a solution consisting of 1.5X TBE and 0.01% (v/v) ethidium bromide (EtBr) and allowed to stain for a few minutes, whereafter the bands were visualised under UV light in the Syngene Vacutec Multigenius Bioimaging® using GeneSnap™ (Synoptics ltd. version 6.04.00). If the bands were not clearly visible with ethidium bromide staining, silver staining was performed. For this, the gel was firstly placed in a plastic container and covered with fixing solution (10% (v/v) EtOH, 0.5% (v/v) acetic acid ($CH_3COOH$)) and shaken on a rotating platform (Belly dancer, Stovall Life Science, Inc) for 10 minutes, after which the solution was drained. The gel was then rinsed in water for one minute. After discarding the water, the gel was covered in freshly made staining solution (0.1% (w/v) silver nitrate ($AgNO_3$)) and shaken on

the rotating platform for 10 minutes. The staining solution was then drained and the gel rinsed for 5 seconds with water. The water was discarded and developing solution (1.5% (w/v) sodium hydroxide (NaOH), 0.155% (v/v) formaldehyde (HCHO) added just before use) was added so it covers the gel and shaken on the rotating platform for 10 minutes (or until red brown DNA bands became visible). Again the developing solution was drained and the gel rinsed with water. Gels were stored in a plastic covering in the dark. Solutions were discarded into marked bottles for biohazard waste disposal.

## ***DRD3***

Exon one of the *DRD3* gene was amplified using primers, PCR reagents and amplification conditions as specified in Chapter Three, section 3.3.2 (also see Table 16). The amplicon was then subjected to restriction enzyme digestion by *MscI* (New England Biolabs, Ipswich, MA, USA). About 5 µl (depending on band intensity) of the amplified product was used in a 10 µl reaction containing 0.3 U *MscI* and 1X buffer 4 (New England Biolabs, Ipswich, MA, USA). The samples were then incubated overnight at 37°C and deactivated by incubation at 65°C for 30 seconds. DNA fragments were visualised on an ethidium bromide (0.01%; v/v) stained 2% (w/v) 1X TBE agarose gel (Bio Basic inc., Ontario, Canada) in the Syngene Vacutec Multigenius Bioimaging® using GeneSnap™ (Synoptics ltd. version 6.04.00). Gly homozygotes show bands of sizes 47 bp, 98 bp, 111 bp and 207 bp. The Ser homozygotes, however, only show bands of sizes 47 bp, 111 bp and 305 bp. The 47 bp fragment could not be clearly visualised on a 2% (w/v) 1X TBE agarose gel. In addition, the 111 and 98 bp fragments migrated as one band on the agarose gel.

**Table 16:** Primers, annealing temperatures and $MgCl_2$ concentration for exons amplified in the *MnSOD*, *DRD3* and *CYP2D6* genes.

| Gene and exon | Forward primer 5'-3' | Reverse primer 5'-3' | Annealing temperature (°C) | [$MgCl_2$] (mM) |
|---|---|---|---|---|
| *MnSOD* Exon 2 | GCTTTCTCGTCTTCAGC | CTCCTCGGTGACGTTC | 58 | 3 |
| *DRD3* Exon 2 | GCTCTATCTCCAACTCTCACA[1] | AAGTCTACTCACCTCCAGGTA[1] | 57 | 3 |
| *CYP2D6* Exon 4 fragment | GTGGGTGATGGGCAGAAG | GAGGGTCGTCGTACTCGAA | 62 | 0.5 |
| *CYP2D6* Exon 5-6 | ACAGGCAGGCCCTGGGTCTA | GGCCCTGACACTCCTTCTTG | 65, 62, 60 | 1 |

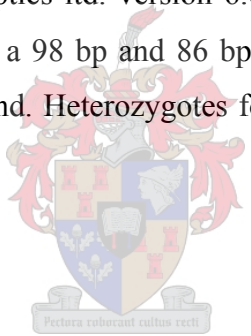
[1]Segman *et al.* (1999)

## ***CYP2D6***

Since exon 5 to 6 and the exon 4 fragment were the only exons that could at least partially be amplified (See Chapter Four, section 4.4), only genotyping method information for the variants G1846A and C2850T are given.

### *CYP2D6*4* (G1846A)

Amplification of the *CYP2D6* exon 4 fragment was performed as described in Chapter Four, section 4.3.2.1.4 (also see Table 16). This was followed by *MvaI* (Fermentas, Ontario, Canada) digestion to determine the G1846A genotype. About 5 µl (depending on band intensity) of PCR product was added to make a 10 µl reaction mix containing 2 U enzyme, and 1X buffer R (Fermentas, Ontario, Canada). Samples were then incubated overnight at 37°C followed by an inactivation step for 20 minutes at 80°C. DNA samples were visualised on an ethidium bromide (0.01%; v/v) stained 2% (w/v) 1X TBE agarose (Bio Basic inc., Ontario, Canada) gel in the Syngene Vacutec Multigenius Bioimaging® using GeneSnap™ (Synoptics ltd. version 6.04.00). Those samples homozygous for the wildtype allele of G1846A showed a 98 bp and 86 bp band, while those homozygous for the mutant allele only showed a 184 bp band. Heterozygotes for G1846A of course showed a 184 bp, 98 bp and 86 bp band.




### *CYP2D6*17* (C2850T)

Exon 5 to exon 6 was amplified as described in Chapter Four, section 4.3.2.1.2 (also see Table 16). The amplicon was then subjected to restriction enzyme digestion using *HhaI* (New England Biolabs, Ipswich, MA, USA) for the genotype determination of C2850T. In a 10 µl reaction, 5 µl of PCR product was added to 2 U enzyme, 1X buffer 4 and 100 µg/ml BSA (New England Biolabs, Ipswich, MA, USA). Digestion occurred overnight at 37°C. DNA fragments were visualised on a 1.5% (w/v) 1X TBE agarose (Bio Basic inc., Ontario, Canada) gel in the Syngene Vacutec Multigenius Bioimaging® using GeneSnap™ (Synoptics ltd. version 6.04.00). Wildtype homozygotes for C2850T showed a 372 bp, 261 bp and a 139 bp band, while mutant homozygotes only showed a 633 bp and 139 bp band. Heterozygotes showed a 633 bp, 372 bp, 261 bp and 139 bp band.

# Appendix C

**Table 17:** Interaction analysis according to Zhang *et al.* (2003b), when the *MnSOD* Val allele is considered to be the "high risk" allele and the *DRD3* Gly/Gly genotype as "high risk" genotype in the development of AIM.

| | ***MnSOD*Ala/Ala** | | ***MnSOD* Ala/Val Val/Val** | |
|---|---|---|---|---|
| | *DRD3* Ser/Gly n (%) | *DRD3* Gly/Gly n (%) | *DRD3* Ser/Gly n (%) | *DRD3* Gly/Gly n (%) |
| **AIM+ (n=38)** | 3 (7.9) | 5 (13.2) | 12 (31.6) | 18 (47.4) |
| **AIM- (n=55)** | 3 (5.5) | 2 (3.6) | 17 (30.9) | 33 (60.0) |
| **Total (n=93)** | 6 (6.5) | 7 (7.5) | 29 (31.2) | 51 (54.8) |
| Ala/Ala Gly/Gly versus Ala/Val Val/Val Gly Gly: $\chi^2$=3.358, d.f.=1, *P*=0.067 | | | | |
| Ala/Val Val/Val Ser/Gly versus Ala/Val Val/Val Gly/Gly: $\chi^2$=0.292, d.f.=1, *P*=0.589 | | | | |

**Table 18:** Interaction analysis according to Zhang *et al.* (2003b), when the *MnSOD* Ala allele is considered to be the "high risk" allele and the *DRD3* Gly/Gly genotype as "high risk" genotype in the development of AIM.

| | ***MnSOD* Val/Val** | | ***MnSOD* Ala/Val Ala/Ala** | |
|---|---|---|---|---|
| | *DRD3* Ser/Gly n (%) | *DRD3* Gly/Gly n (%) | *DRD3* Ser/Gly n (%) | *DRD3* Gly/Gly n (%) |
| **AIM+ (n=38)** | 7 (18.4) | 7 (18.4) | 8 (21.1) | 16 (42.1) |
| **AIM- (n=55)** | 6 (10.9) | 14 (25.5) | 14 (25.5) | 21 (38.2) |
| **Total (n=93)** | 13 (14.0) | 21 (22.6) | 22 (23.7) | 37 (39.8) |
| Val/Val Gly/Gly versus Ala/Val Ala/Ala Gly/Gly: $\chi^2$=0.550, d.f.=1, *P*=0.458 | | | | |
| Ala/Val Ala/Ala Ser/Gly versus Ala/Val Ala/Ala Gly/Gly: $\chi^2$=0.271, d.f.=1, *P*=0.603 | | | | |

**Table 19:** Interaction analysis according to Zhang *et al.* (2003b), when the *MnSOD* Val allele is considered to be the "high risk" allele and the *DRD3* Gly/Gly genotype as "high risk" genotype in the development of TD.

| | ***MnSOD*Ala/Ala** | | ***MnSOD* Ala/Val Val/Val** | |
|---|---|---|---|---|
| | *DRD3* Ser/Gly n (%) | *DRD3* Gly/Gly n (%) | *DRD3* Ser/Gly n (%) | *DRD3* Gly/Gly n (%) |
| **TD+ (n=22)** | 2 (9.1) | 3 (13.6)) | 8 (36.4) | 9 (40.9) |
| **AIM- (n=55)** | 3 (5.5) | 2 (3.6) | 17 (30.9) | 33 (60.0) |
| **Total (n=77)** | 5 (6.5) | 5 (6.5) | 25 (32.5) | 42 (54.5) |
| Ala/Ala Gly/Gly versus Ala/Val Val/Val Gly Gly: $\chi^2$=3.496, d.f.=1, *P*=0.062 | | | | |
| Ala/Val Val/Val Ser/Gly versus Ala/Val Val/Val Gly/Gly: $\chi^2$=0.925 d.f.=1, *P*=0.336 | | | | |

**Table 20:** Interaction analysis according to Zhang *et al.* (2003b), when the *MnSOD* Ala allele is considered to be the "high risk" allele and the *DRD3* Gly/Gly genotype as "high risk" genotype in the development of TD.

| | ***MnSOD* Val/Val** | | ***MnSOD* Ala/Val Ala/Ala** | |
|---|---|---|---|---|
| | *DRD3* Ser/Gly n (%) | *DRD3* Gly/Gly n (%) | *DRD3* Ser/Gly n (%) | *DRD3* Gly/Gly n (%) |
| **TD+ (n=22)** | 4 (18.2) | 3 (13.6) | 6 (27.3) | 9 (40.9) |
| **AIM- (n=55)** | 6 (10.9) | 14 (25.5) | 14 (25.5) | 21 (38.2) |
| **Total (n=77)** | 10 (13.0) | 17 (22.1) | 20 (26.0) | 30 (39.0) |

Val/Val Gly/Gly versus Ala/Val Ala/Ala Gly/Gly: $\chi^2$=0.871, d.f.=1, *P*=0.351

Ala/Val Ala/Ala Ser/Gly versus Ala/Val Ala/Ala Gly/Gly: $\chi^2$=0.000, d.f.=1, *P*=1.000

# Appendix D

## Oral presentation of work

South African Genetics Society Congress, April 2006:

Angelika Hitzeroth, Dana J.H. Niehaus, Liezl Koen, Willem C. Botes, Louise Warnich. *MnSOD* Ala-9Val polymorphism and the development of schizophrenia and tardive dyskinesia.

## Poster presentation of work

Faculty of Health Science, Academic Year Day 2005:

A Hitzeroth, D Niehaus, L Koen, L Warnich. No association between the *MnSOD* Ala-9Val polymorphism and development of schizophrenia in the Xhosa population.

## Articles submitted for publication

Angelika Hitzeroth, Dana J. H. Niehaus, Liezl Koen, Willem C. Botes, JF Deleuze, Louise Warnich. Association between the *MnSOD* Ala-9Val polymorphism and development of schizophrenia and abnormal involuntary movements in the Xhosa population. Submitted to *Progress in Neuro-Psychopharmacology & Biological Psychiatry* and accepted after minor revision.