OBSESSIVE-COMPULSIVE DISORDER: DEFINING THE ROLE OF GENE-BASED VARIANTS AND IMMUNOLOGICAL FACTORS

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Thesis presented for approval for the Master's degree of Science in Medical Science at the Faculty of Health Sciences, University of Stellenbosch

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March 2001

DECLARATION

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

Signature. . Date.

SUMMARY

Obsessive-compulsive disorder (OCD) is a common psychiatric condition characterised by recurrent and disabling obsessions and compulsions. Although the pathogenesis of the disease is unclear, studies have consistently provided evidence for the involvement of genetic mechanisms. One strategy used to identify genetic susceptibility factors has been case-control association studies between polymorphisms in candidate genes and OCD.

Two promising OCD candidate genes are those encoding catechol-O-methyl transferase (COMT) and the serotonin transporter (the *COMT* and *SLC6A4* genes, respectively). Association studies have implicated functional polymorphisms in *COMT* and *SLC6A4* (*5-HTTLPR*) in the development of OCD in various populations. Futhermore, studies have shown that COMT activity, which is regulated by oestrogen, is under the control of oestrogen response elements (*ERE*s). The *ERE6* in the *COMT* promoter has been shown to play an important role in hormone-dependent regulation of *COMT* expression.

Immune-based theories of OCD pathogenesis have also been proposed. These stem from the observed similarities between OCD and the immunological disorder Sydenham's chorea (SC). Furthermore, patients with SC or childhood onset OCD have increased expression of the B-lymphocyte marker, D8/17. However, despite their possible phenomenological and psychobiological overlap, there have been no extensive studies to investigate D8/17 expression levels in other anxiety disorders, such as panic disorder (PD) or social phobia (SP) or putative OCD spectrum disorders, such as trichotillomania (TTM).

Aim:

The aim of the present study was to use case-control association studies in the genetically homogeneous Afrikaner sub-population to 1) reassess the roles in OCD pathogenesis of the previously described functional polymorphisms in *COMT* and *SLC6A4*; 2) determine if *ERE6* is polymorphic, and, if so, to extend the association

analysis using this variant and 3) assess the role of D8/17 in development of OCD, PD, SP and TTM.

Methods:

All case and control subjects were of Afrikaner descent and underwent a structured clinical interview, following their informed consent to take part in the study. The polymorphisms in *COMT* and *SLC6A4* were genotyped following published methods based on polymerase chain reaction (PCR) amplification of specific exons and restriction enzyme (*COMT*) or direct fragment size analysis (*SLC6A4*) of the products. Oligonucleotide primers were designed to amplify a 150 base pair fragment of the *COMT* promoter region containing *ERE6*, which was screened for sequence variation using PCR- single strand conformational polymorphism (SSCP) analysis. Fragments showing mobility shifts were sequenced using an automated sequencer (University of Stellenbosch). Genotype data was analysed by Chi-square analysis using the SPSS software package.

Analysis of D8/17 expression was by indirect immunofluorescence assay. B cell were labelled with D8/17 antibody and fluoresced with antimouse IgM and DR HLA class staining. The cells were then analysed by flow cytometry. D8/17 data was analysed using analysis of variance (ANOVA) and the post-hoc Tukey procedure was used to correct for multiple testing.

Results:

A statistically significant association was shown between *COMT* and OCD, but not between *SLC6A4* (*5-HTTLPR*) and the disease. A meta-analysis, pooling the results of *5-HTTLPR* genotyping in the present study and that of a study conducted in a North American population, also generated no significant result. A single nucleotide polymorphism (SNP) was detected 8 bases 5-prime to *ERE6* in *COMT*, but did not show association with OCD development.

Expression levels of the B lymphocyte marker D8/17 revealed no significant association with OCD or TTM subjects, when compared to controls. Nor were differences observed when the OCD group was stratified according to age of onset, or history of various comorbid disorders. However, the PD/SP group had significantly lower levels of D8/17 expression than the other groups.

Conclusion:

Case:control association studies frequently produce discrepant results. The data generated in the present study supports a role for *COMT* in the development of OCD, but provides evidence that *SLC6A4* may not play a significant role. Furthermore, the previously reported association of OCD with higher levels of D8/17 expression may not be general and may be related to the age of onset of the disease. The lower levels reported in the present study in the PD/SP group warrants further investigation.

Identifying gene variants and other factors that play a significant role in the development of OCD is important for the design of further studies to understand the molecular pathophysiology of the disease. In turn, it is envisaged that this will lead to better patient management and treatment strategies.

OPSOMMING

Agtergrond:

Obsessiewe kompulsiewe steuring(OKS) is 'n algemene psigiatriese kondisie / toestand wat gekarakteriseer word deur herhaaldelike obsessies en konpulsies. Alhoewel die oorsaak van die toestand nie bekend is nie, het studies herhaaldelik bewys dat genetiese faktore 'n rol speel. Hierdie genetiese faktore word onder andere geidentifiseer deur assosiasie studies (gevalle vs kontroles) tussen polimorfismes in kandidaat gene en OKS.

Die gene wat onderskeidelik kodeer vir katesjol-O-metieltransferase (COMT) en die serotonien draer (die *COMT* en *SLC6A4* gene onderskeidelik), is geï dentifeseer as twee belowende kandidaat gene. Assosiase studies het geïmpliseer dat funksionele polimorfismes in *COMT* and *SLC6A4* (*5-HTTLPR*) 'n rol gespeel het in die onstaan van OKS in verskeie populasies. Studies het ook aangetoon dat COMT aktiwiteit, wat gereguleer word deur estrogeen, onder beheer is van estrogeen respons elemente (*ERE*s). Die *ERE6* in die *COMT* promotor speel 'n belangrike rol in hormoon-afhanklike regulasie van COMT uitdrukking/ekspressie.

Daar is ook voorstelle van immuun gebasseerde teorïee van OKS patogenese. Dit spruit uit die ooreenkoms tussen OKS en die immunologiese afwyking Sydenham-chorea (SC). Daar is ook waargeneem dat pasïente met SC of OKS, met aanvang in kinderjare, 'n verhoogde uitdrukking het van die B-limfosiet merker D8/17. Ten spyte van 'n moontlike fenotipiese en psigobiologiese ooreenkoms, is daar nog geen deeglike studies gedoen om D8/17 uitdrukkingsvlakke in ander angsafwykings, naamlik paniek afwyking (PA) of sosiale fobia (SF), of moontlike OKS spektrum afwykings soos trigotillomanie (TTM), aan te dui nie.

Doelwit:

Die doel van hierdie studie was om geval-kontrole assosiasie studies aan te wend in die geneties homogene Afrikaner sub-populasie om 1) die rolle van die voorheen

beskryfde funksionele polimofismes in COMT en SLC6A4 te ondersoek; 2) om te bepaal of ERE6 polimorfies is, en indien wel, om die assosiasie analises uit te brei deur gebruik te maak van die polimorfisme en 3) om die rol van D8/17 te bepaal in die ontwikkeling van OKS, PA, SF en TTM.

Metodes:

Alle gevalle en kontrole pasïente was van Afrikaner afkoms en het, na ingeligte toestemming om deel te neem aan die studie, 'n gestruktureerde kliniese onderhoud ondergaan. Die polimorfismes in *COMT* en *SLC6A4* is gegenotipeer deur gebruik te maak van gepubliseerde medtodes. Hierdie metodes was gebaseer op die polimerase ketting reaksie (PKR) amplifikasie van spesifieke eksons gevolg deur restriksie ensiem verterings (*COMT*) of direkte fragment grootte analises (*SLC6A4*) van die PKR produkte. 'n EEN honderd en vyftig basis paar fragment van die *COMT* promotor area bevattende *ERE6* is geamplifiseer deur gebruik te maak van spesifieke oligonukleotied inleiers. Hierde fragment is geanaliseer vir nukleotiedvolgorde variasie deur gebruik te maak van PKR enkelstring konformasionele polimorfismes (SSCP's). Fragmente wat 'n verandering in grootte getoon het se nukleotiedvolgorde is bepaal deur gebruik te maak van 'n outomatiese nukleotiedvolgorde bepaler (Universiteit van Stellenbosch). Genotipiese inligting is geanaliseer deur Chi-kwadraat analises deur gebruik te maak van die SPSS sagteware pakket.

D8/17 uitdrukking is geanaliseer deur direkte immunofluoressensie toetse. B- selle is gemerk met D8/17 antiliggame en gefluoriseer met anti-muis IgM en DR HLA klas kleuring. Die gemerkte selle is geanaliseer deur gebruik te maak van vloeisitometrie. Die D8/17 data is geanaliseer deur gebruik van ANOVA, en die post-hoc Tukey prosedure is gebruik om veelvoudige toetse te korrigeer.

Resultate:

'n Statisties beduidende assossiasie is aangetoon tussen *COMT* en OKS, maar geen assossiase is aangetoon vir *SLC6A4* (*5-HTTLPR*) en OKS nie. Meta-analises, 'n kombinasie van die resultate van *5-HTTLPR* genotipering in hierdie studie en van 'n studie in 'n Noord-Amerikaanse populasie, het geen betekenisvolle resultate

opgelewer nie. 'n Enkel- nukleotied polimorfisme (ENP) is waargeneem 8 basisse van die 5' van *ERE6* in *COMT*, maar het geen assosiasie getoon in die ontwikkeling van OKS nie.

Geen beduidende assosiasie is aangetoon in die uitdrukkingsvlakke van die B-limfosiet merker D8/17 tussen OKS en TTM pasïente en kontroles nie. Verkleining van die OKS groep, deur gebruik te maak van aanvangs ouderdom of geskiedenis van verskeie ander afwykings het ook geen verskille opgelewer nie. Die PA/SF groep het egter beduidende laer vlakke van D8/17 uitdrukking as die ander twee groepe.

Gevolgtrekking:

Gevalle:kontrole assosiasie studies lewer in baie gevalle teenstrydige resultate. Die inligting wat gegenereer is in hierdie studie dui op 'n rol vir COMT in die ontwikkeling van OKS, maar dien ook bewyse dat SLC6A4 nie 'n beduidende rol speel nie. Die voorheen beskryfde assosiasie tussen OKS en hoër uitdrukkingsvlakke van D8/17 is moontlik nie 'n algemene verskynsel nie, en mag verband hou met die aanvangsouderdom van die afwyking/toestand. Die laer uitdrukkingsvlakke van D8/17 in hierdie studie vir die PA/SF groep noodsaak verdere navorsing.

Identifisering van geen polimorfismes and ander faktore wat 'n rol speel in die ontwikkeling van OKS is belangrik vir verdere navorsing om die molekulêre patofisiologie van die afwyking te verstaan. Die resultate sal ook aanleiding gee tot beter hantering van pasïente en nuwe behandeling strategïee.

ACKNOWLEDGEMENTS

To my supervisor, Dr Valerie Corfield, thank you for all the scientific and linguistic contributions you made in the compiling of this thesis. I appreciate all your support and for sacrificing so much of your time. I also thank you for all your career advice and helping me with important decisions concerning my future. But most of all, I thank you for believing in me and for your continued encouragement.

I would also like to thank the following people and institutions for their contributions.

- To Dr Dana Niehaus, for introducing me to the field of psychiatric genetics, for all the clinical support and scientific input into the project.
- To Dr Hanlie Moolman-Smook, for all your technical support and scientific input and for always putting me at ease during the oral presentations of this project.
- To Prof Paul Brink, for all the statistical input and intellectual contributions you made to this project.
- To the people in lab 445 and offices 407/408, for providing me with an enjoyable working environment and for all your the support.
- To my family, for taking an active interest in my studies, for supporting me (financial and moral), for believing in my abilities and for your continued encouragement.
- To the US/MRC Centre for Molecular and cellular biology, where all the genetic work was done, the MRC research Unit on Anxiety and Stress Disorders, for providing me with the clinical data and the Department of Medica| Microbiology, where the immunological work was done.
- To the National Research Foundation and the Obsessive-compulsive Disorder Foundation of South Africa, for their financial support.
- To my loving wife, Hildegard, for putting up with me these last few months, for always cheering me up when I felt overwhelmed by this thesis and for all your support and understanding.

Finally, I would like to thank God for bringing me this far.

LIST OF ABBREVIATIONS

5-HIAA : 5 hydroxyindoleacetic acid
5-HT : Serotonin
5-HT1a : Serotonin receptor 1a
5-HT2 : Serotonin receptor 2
5-HT2c : Serotonin receptor 2c
5HTR2C : Serotonin receptor 2c gene
5-HTT : Serotonin transporter protein
5HTTLPR : Serotonin transporter promoter-liked polymorphism
A : Adenosine
AgNo3 : Silver nitrate
ANOVA : Analysis of Variance
ASP : Affected sib pair
APM : Affected pedigree method
ASREA : Allele specific restriction enzyme analysis
BG : Basal ganglia
bp : Base pair
C : Degree Celsius
C : Cytosine
cM : Centimorgan
COMT : Catechol-O-methyltransferase
dATP : Deoxy-adenosine triphosphate
dCTP : Deoxy-cytosine triphosphate
DDC : Dopa decarboxylase
dGTP : Deoxy-guanosine triphosphate
DMSO : Dimethyl sulphoxide
DNA : Deoxyribonucleic acid
DOPEG : Dihydroxyphenylglycol
DSM-IV : Diagnostic and Statistical Manual of Mental Disorders
dTTP : Deoxy-thymidine triphosphate
EDTA : Ethylene-diamine-tetra-acetic acid

EEG : Electroencephalogram
ERE : Oestrogen response elements
FACS : Fluorescence activated cell sorting
Fig : Figure
FL : Fluorescence
FSC : Forward scatter
GABHS : Group A B-haemolytic streptococcus
HRR : Haplotype relative risk
kb : Kilobase
LOD : Logarithm of odds
M : Molar
MAO : Monoamine oxidase
MAO-A : Monoamine oxidase A
MAO-B : Monoamine oxidase B
MB-COMT : Membrane-bound COMT
ml : Millilitre
mM : Millimolar
MOPEG : 3-methoxy-4-hydroxyphenylethyleneglycol
NAT : Negative automatic thought
OCD : Obsessive-compulsive disorder
OC : Obsessive-compulsive
PANDAS : Paediatric autoimmune neuropsychiatric disorder associated with streptococcal infection
PCR : Polymerase chain reaction
PCR-SSCP : Polymerase chain reaction single strand conformational polymorphism
PITANDS : Paediatric infection-triggered autoimmune neuropsychiatric disorder
RET : Rational emotive therapy
RF : Rheumatic fever
SC : Sydenham's chorea
SCID-I : Structured clinical interview for diagnosis
S-COMT : Soluble COMT
sec : Seconds
SIT : Self-instructional training

SLC6A4 : Solute carrier protein family 6 member 4

SSC : Side scatter

SSRI : Selective serotonin reuptake inhibitors

T : Thiamine

TA : Annealing temperature

TBE : Tris, boric acid and EDTA buffer

TD : Denaturing temperature

TDT : Transmission disequilibrium test

TE : Extension temperature

TH : Tyrosine hydroxylase

TPH : Tryptophan hydroxylase

TS : Tourette's syndrome

TTM : Trichotillomania

VMA : Vanillylamandelic acid

W : Watts

www : World wide web

Y-BOCS : Yale-Brown obsessive-compulsive scale

YGTSS : Yale global tic severity scale

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CHAPTER 1

INTRODUCTION

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CHAPTER 1 : INTRODUCTION

1.1. Genetics and psychiatry

Many diseases run in families, but that, in itself, is not enough evidence to invoke the involvement of genes, since infection and environmental factors may also be familial. However, the use of studies of adopted siblings and of twin pairs provides evidence for genetic involvement in familial diseases. Such adoptive studies have shown that the risk of an adopted child developing a psychiatric disorder is significantly more dependant on the mental health of the biological parents than that of the adoptive parents (Kendler and Diehl, 1993).

In twin studies, the investigative strategy is to compare the concordance rate, that is the chance of both twins being afflicted with the same disorder, among monozygotic and dizygotic twin pairs. If the concordance rate for a particular disorder is similarly high for both mono- and di-zygotic twin pairs, a shared environment is likely to be the major factor inferring disease susceptibility, while a similarly low concordance rate would suggest similar, non-shared environmental conditions to be the major factor. A genetic component for disease is implicated when the concordance rate for monozygotic twins is significantly higher than for dizygotic twins. If only one gene contributes to the disorder in a family, the concordance rate for monozygotic twins would be two-fold higher than in dizygotic twins.

Twin study data have implicated genetic factors in a variety of psychiatric disorders (Plomin, 1994). The concordance rates generated indicate that some psychiatric disorders may have a stronger genetic component than many other complex diseases, although generally the concordance rates between monozygotic and dizygotic twins are less than two-fold, suggesting that both genetic and shared environmental factors contribute to the disorder (Plomin, 1994).

1.1.1. Many psychiatric diseases are multifactorial disorders.

Multifactorial disorders are disorders which are caused by the interaction of a variety of susceptibility genes and environmental factors. Diabetes mellitus, cancer and

schizophrenia are examples of multifactorial disorders which run in families, but have a rather complex pattern of inheritance (Carter, 1972).

With the availability of detailed genetic maps in humans, and many other organisms (Hudson, 1998), various strategies have been developed to unravel the complex genetic basis of multifactorial diseases. Many of these methods have been used in identifying possible genetic loci for the development of psychiatric disorders such as schizophrenia (Bailer *et al.*, 2000; Brzustowicz *et al.*, 2000), Tourette's syndrome (TS) (Barr *et al.*, 1999) and bipolar affective disorder (Detera-Wadleigh *et al.*, 1999; Friddle *et al.*, 2000). These molecular genetic strategies and their application to psychiatric disorders will be discussed briefly in the following section.

1.1.2. Genetic search strategies.

1.1.2.1.Parametric linkage analysis

Linkage analysis is the first step in positional cloning, which is a two-step genetic search strategy to find disease-causing genes. This method is used to identify alleleic variations of genes or genetic markers of known location that are co-inherited with the disease-causing gene and thus are located on the same chromosomal segment (reviewed by Keating, 1992). When performing linkage analysis, it is important, firstly, to identify a suitable family, in which the disease is segregating with a known mode of inheritance. Secondly, the phenotype of the disease must be carefully classified in the family members. This is crucial in linkage analysis, since this technique deals with the comparison of genotype and phenotype and an incorrect diagnosis could lead to incorrect interpretation of linkage data. (reviewed by Keating, 1992). Careful genotyping of each family member using deoxyribonucleic acid (DNA) markers of known position is then performed. Currently, the most commonly used DNA markers are short tandem repeats (STR). These STRs are simple sequences that are repeated a variable number of times, resulting in length differences that are polymorphic and stably inherited. The most commonly used STRs are the dinucleotide CA repeats, which are spaced on average every 50000 base pairs (bp) throughout the genome (Weissenbach *et al.*, 1998).

The logic behind linkage analysis is as follows: if a disease-causing mutation is in a gene on, for example, chromosome 6, then all affected members of the family used in the study should carry an identical region of chromosome 6 around that mutant allele (identity by descent) (fig 1). The chromosomal locus of a disease-causing gene mutation is mapped by determining genetic linkage, or co-inheritance, between the inheritance of disease within a family and DNA markers of known chromosomal location. If a DNA marker is closely linked to the disease-causing gene, affected individuals in the family would be expected to inherit the same allele of the DNA marker from an affected parent (fig 1 allele b). If, however, the marker is on the same chromosome, but distant from the disease-causing gene, the alleles at the marker locus will sometimes be separated from the disease-causing gene during meiotic recombination (fig 1. II-3 allele A). The further away the marker from the disease-causing mutation, the more often recombination events occur. Therefore, the rate of recombination is a measure of distance on a chromosome between the marker and the disease causing mutation (reviewed Burmeister, 1999). If two loci are less than 1 centimorgan (cM) (1cM=approximately 1 million base pairs) apart, their alleles are co-inherited 99% of the time. However, if two loci are 50 cM or more apart, their alleles are co-inherited in only 50% of individuals with the disease, which is equal to free recombination.

Computational analysis is used in linkage studies to identify co-segregation of the alleles of a marker of known chromosomal location and a locus that causes disease within a family. These linkage programmes analyse the genotype:phenotype data of the family members and calculate the odds that a DNA marker and disease gene are linked, ie., they are co-inherited more often than would be expected by chance (reviewed by Burmeister, 1999). These odds are represented in a logarithmic form known as the LOD (logarithm of odds) score. A lod score of +3, which represents odds of 1000:1, is considered as good evidence for linkage, whereas a LOD score of –2, which represents odds of 1:100, means that a locus can effectively be excluded. (reviewed by Burmeister, 1999).

Locus heterogeneity, which is when the same clinical disease in different families is caused by mutations in different genes, is a major complication in linkage mapping. A prime example of this is inherited deafness, for which over 30 different loci have been

identified (Petit, 1996). Linkage analysis for genetically heterogeneous, multilocus disorders such as deafness is usually established in single large families studied one family at a time. Alternatively, families living in remote areas, or in cultural isolation are studied, where only one deafness gene may be present and the population represents a large extended pedigree. This approach was effectively used in the field of psychiatric genetics in the study of bipolar affective disorder in Costa Rican and Amish populations (Freimer *et al.*, 1996; Ginns *et al.*, 1996).

Once linkage analysis has localised a gene to a specific chromosomal region, it may be isolated by cloning the region (positional cloning) (Botstein et al., 1980), although, more recently, sequence and related information generated by the Human Genome Project often obviate this step (Cheung *et al.*, 2001). Subsequently, the gene, its mutations and the protein product it encodes can be identified (reviewed by Marian and Roberts, 1995).

1.1.2.2. Non-parametric linkage analysis

The success of linkage analysis is largely due to the fact that in monogenic disorders the mode of inheritance is known. To avoid some of the problems of selecting a specific mode of inheritance for complex disorders, while still employing some of the power of linkage analysis, non-parametric linkage analyses, which are mode-of-inheritance-independent, methods were developed. One such method is affected sib pair (ASP) or, more generally, affected pedigree member (APM), analysis. In this method, only sibpairs or other pairs of affected relatives are studied. This, however, means that the power of observing alleles segregating in large families is lost. The basis behind this strategy is that, independently of whether a disease is dominant, recessive or complex, if there is a disease-causing mutation in a specific chromosomal region, two affected members of a family can be expected to share an allele of a marker in a gene involved in disease development, or adjacent to it, more often than can be expected by chance. With this method, one is able to design studies that include very specific diagnosis, eg., only bipolar I disorder with exclusion of bipolar II within a family, thus avoiding unclear phenotypes. Also, no genetic model of the disease needs to be specified and linkage can be detected in the presence of genetic heterogeneity (reviewed by Burmeister 1999).

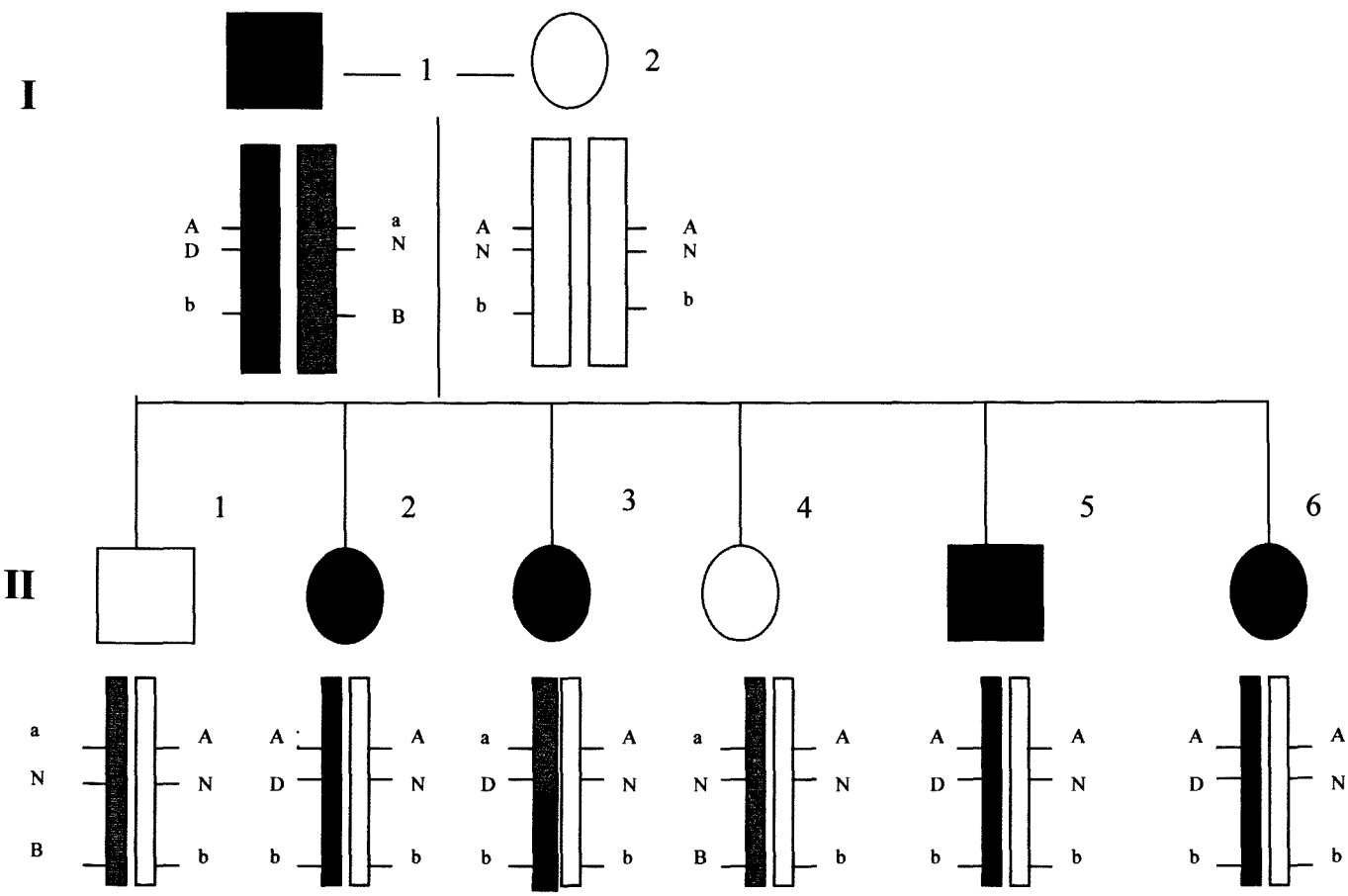


Fig 1: Linkage analysis and recombination. Individuals indicated by a solid symbol are affected with dominant disease due to the presence of disease allele D. N allele is the normal allele at this locus. Most affected offspring inherit allele A and b at the flanking marker from their affected father because these alleles are linked to disease-causing allele D. Individual II-3 shows a recombination event, in which allele A was separated from the disease causing allele. The more tightly linked alleles A and b are to the disease-causing gene, the less likely the chances of recombination. Thus, recombination frequency is the measure of the distance markers and the disease-causing gene. Squares and circles represent male and female, respectively. (Adapted from Burmeister, 1999).

1.1.2.3. Case-Control Association studies

Both parametric and non-parametric linkage analyses are based on following marker alleles that are close to a disease-causing mutation on a chromosome though a family. They are therefore family-based and rely on a number of affected individuals within a two or multi-generation family. Linkage analysis can be conducted with no knowledge of pathophysiology of the disorder and is therefore not hypothesis driven. When a chromosomal region is shown to be linked to a phenotype, it may be quite extensive and may contain hundreds of genes. The ultimate identification of the variants that are contributing to the disease phenotype may be laborious and resource intensive (reviewed by Malhotra and Goldman, 1999).

The disadvantage of using parametric linkage analysis in identifying genes involved in complex disorders includes the necessity to specify a genetic model, the difficulties involved to identify large families with multiple affected individuals and the low power to detect genes of small effect.

In contrast to linkage analysis, association studies are methods that are able to identify susceptibility genes in a more direct manner, using knowledge of the disease pathogenesis. These studies rely on the hypotheses of the molecular causes of the disorder being investigated and, unlike linkage analysis, they are not necessarily family-based. In association studies, an individual is the basic unit of analysis and no knowledge of the genetic mode of inheritance is needed. Therefore, these studies are well-suited to investigate genetic influences in complex disorders.

Genetic case-control association studies compare the frequency of alternative polymorphic forms (alleles) of a specific candidate gene within a patient group with that of a control group, with statistical support provided. The generation of a statistically significant difference between allele frequencies and genotype distribution in patient and control groups suggests either a causative role for a functional polymorphism tested or the presence of another functional variant in the same gene or in a linked one. Since psychiatric disorders are common and it is assumed that multiple genes play a role in the development of these disorders, the

predisposing alleles are expected to be fairly common polymorphisms rather than rare mutations (Burmeister et al., 1999).

When designing genetic case-control association studies, many factors need to be taken into account. As the present study utilizes this method, the following sections will deal with some of these factors.

Selection of subjects

One major pitfall of case-control association studies is the potential for ethnic stratification between subjects. If a particular allele occurs at a different frequency across ethnic groups and cases and controls are not precisely ethnically matched, one might find differing allele frequencies between the two groups, thus observing an association with the phenotype of interest that, in fact, does not exist (reviewed by Malhotra and Goldman, 1999).

One method to control for genetic complexity among population groups is to conduct association studies in ethnically homogeneous population groups. Populations that are either physically or culturally isolated are frequently also genetically isolated (Sheffield *et al.*, 1998). The present study makes use of the Afrikaner sub-population of South Africa.

The Afrikaner sub-population was formed by a group of European immigrants, primarily of Dutch, German and French descent, who began settling in Cape Town in 1652 (Torrington *et al.*, 1984; Jenkins, 1990). At the turn of the 18th century, there were 1265 Europeans in Cape Town. In the 18th century, European immigration to South Africa was small and by the first half of the 19th century, the Afrikaners began the "Great Trek", which were a series of migrations of Europeans away from the coastal regions to establish farming communities in the interior of the country (Simonic *et al.*, 1998). The lack of large waves of immigration from Europe to South Africa, coupled with the "Great Trek" and the small number of original founders, has lead to the relative homogeneity of the Afrikaner sub-population. Furthermore, the early geographical isolation of the Afrikaner settlements and the cultural isolation of the sub-population, mainly due to the Afrikaans language and the religious beliefs (Dutch Reformed), further contributed to their homogeneity.

As discussed above, conducting association studies in homogeneous population groups is a way to overcome the problem of genetic complexities between different populations. Another way to overcome this population stratification problem in case-control studies is by using family-based approaches known as haplotype relative risk (HRR) and transmission disequilibrium test (TDT) (Fig2 b-d).

In HRR, the alleles that are not transmitted to the affected child by the parents are used as control samples (Falk and Rubinstein, 1987; Terwilliger and Ott, 1992) (fig2c). A 2x2 contingency table is made of allele frequencies in the proband and controls and, by using chi-square (χ^2) analysis, the presence or absence of a significant difference between the two groups is determined. The disadvantage of this method is that no distinction is made between probands homozygous or heterozygous for a particular marker in affected and unaffected individuals.

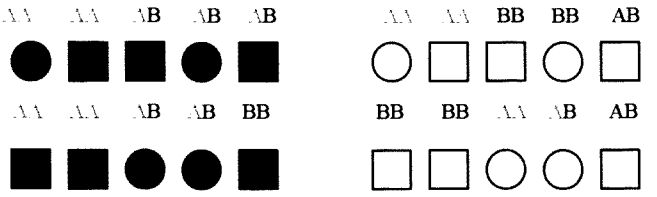
The basis for TDT is the use of a family with one or more affected offspring where at least one of the parents is heterozygous for the marker suspected of being involved the disease. It is expected that one of the marker alleles will be transmitted to the affected child and one not. The TDT compares the frequency of transmitted versus non-transmitted parental alleles by using χ^2 analysis (Spielman and Ewens, 1996) using the non-transmitted allele from the parents of an affected child as an internal control (fig 2b).

Selection of candidate genes and polymorphisms

Candidate genes are selected for their possible involvement in the disease pathology. Firstly, a candidate gene can be identified by its position in a chromosomal area that has been implicated in prior linkage studies. Alternatively, a candidate gene can be selected, irrespective of position, based upon evidence that the gene plays a role in the pathophysiology of the disease. In the study of neuropsychiatric disorders, many candidate genes are chosen based on research implicating specific systems, of which their gene products form part, in a specific condition (reviewed by Malhotra and Goldman, 1999).

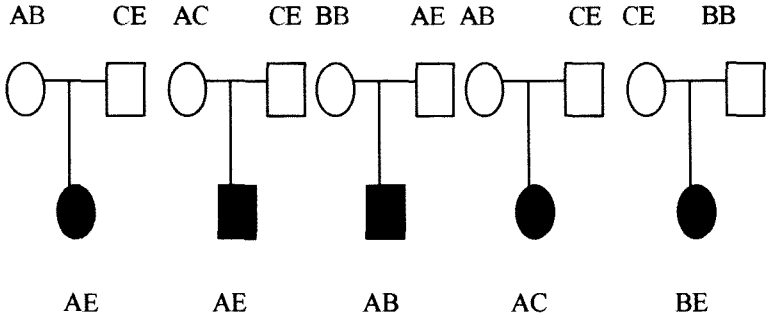
Population based case-control association studies

A



Family-based association studies

B



Haplotype Relative Risk (HRR)

C

Alleles in affected	Alleles in unaffected
AE, AE, AB, AC, BE	BC, CC, BE, BE, BC

Transmission disequilibrium test (TDT)

D

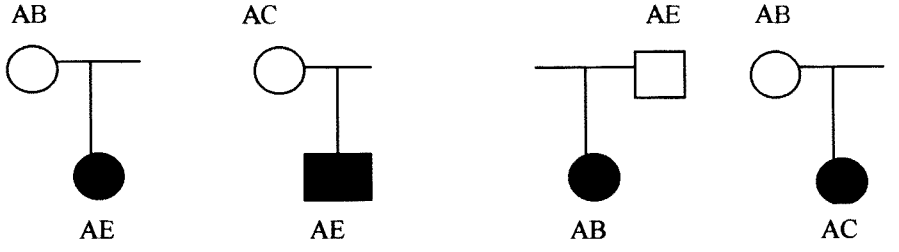


Fig 2 Categories of association studeis. (**A**) Case-control association compares genotype distribution and allele frequencies of patient and appropriately matched control group. The figure clearly shows that allele A occurs more frequently in the patient group than in the control group. (**B**) Family based association studies use non-transmitted alleles (shown in red) as control samples. (**C**) HRR uses non-transmitted alleles (shown in red in 2B) from parents as controls. (**D**) TDT compares the frequency of transmitted and non-transmitted parental alleles from heterozygous parents (Adapted from Burmeister 1999).

After selecting an appropriate candidate gene, the next step is to select a suitable variation (or polymorphism) within the gene to test the association. Most genes contain a number of sequence variations. The selection of an appropriate sequence variant is crucial in the design of case-control association studies and several factors need to be taken into consideration.

Firstly, it is important to determine the frequency of the allele (reviewed by Malhotra and Goldman, 1996). Studies have shown that the polymorphic variants with low frequencies of one allele may provide little statistical power to detect significant associations unless the study group's size is large enough, or the variant has a strong influence on phenotype.

Secondly, the number of alleles at a specific locus also needs to be taken into account. Variants with several relatively frequent alleles provide a number of potential genotypes, thereby increasing the informativeness of the locus, but complicating the statistical analysis (Chang *et al.*, 1996).

Thirdly, the functionality of the selected polymorphism must also be considered. Generally intronic sequence variants do not affect gene splicing or gene expression. Many naturally occurring sequence variants have been identified within the coding region of genes, but again this does not necessary alter gene expression or amino acid sequence (reviewed by Malhotra and Goldman, 1996) Association studies employing non-functional polymorphisms have a significantly lower prior probability of detecting valid associations, than studies using functional variants (reviewed by Malhotra and Goldman, 1996). Although they may indicate the presence of a closely linked functional polymorphism, positive findings with non-functional variants may be as a result of inadvertent ethnic stratification of cases and controls. Thus, the most critical criterion in selecting a candidate polymorphism within a candidate gene is the careful examination of the functional effects of each variant.

Sample size and statistical power

In order to successfully analyse association studies, the sample size and statistical power of the study need to be carefully evaluated. In designing these studies, the sample size needs to be sufficiently large to create enough statistical power to reduce the probability of generating false-positive associations (Type I errors) (Berry *et al.*, 1998). In cases where association studies may lack sufficient power to prove or disprove an association, a meta-analysis can be performed. A meta-analysis is a statistical procedure that integrates the results of several independent studies of a particular disease provided that the investigators 1) have followed the same diagnostic criteria, 2) followed the same methodology and 3) have conducted the study in sufficiently similar population groups.

The ability to conduct a successful meta-analysis relies on the availability of all the relevant data. The inclusion of only a subset of all the available data in a meta-analysis may introduce biases and threaten its validity. This is a major disadvantage of meta-analyses since many studies, especially negative association studies, remain unpublished. Other studies may have limited distribution, or may be published in journals not currently on the bibliographical databases, making their detection and inclusion into a meta-analysis rather difficult (McAuley *et al.*, 2000).

In summary, there are many factors to take into account when designing case-control association studies. It is firstly very important to choose a suitable patient group and a ethnically well-matched control group. Failure to do this could lead to spurious results because of genetic stratification among population group. The effect of population stratification can be minimized by the used of genetically homogeneous populations or employing family-based association studies. Secondly, care should be taken when selecting a specific candidate gene, as well as candidate polymorphism within the selected candidate gene. Candidate genes should be chosen on the basis of knowledge of disease pathology and candidate polymorphisms should preferably have a functional effect on the gene product. Finally, the sample size and statistical power of the study should be sufficiently large in order to reduce the risk of false associations.

Investigations of the molecular causes of psychiatric disorders has often produced controversial or non-reproducible results (Burmeister, 1999). As the present study will

involve association studies to delineate the role of certain candidate genes in the pathogenesis of obsessive-compulsive disorder (OCD), the following section of this review will discuss OCD in some detail.

1.2. OBSESSIVE-COMPULSIVE DISORDER

Obsessive-compulsive disorder has been described as a prevalent, disabling psychiatric disorder (Murray and Lopez., 1996). It is characterised by recurrent obsessions or compulsions that are time consuming, ie., take more than one hour per day, or are severe enough to cause marked distress or significant social and work impairment.

Obsessions are persistent ideas, thoughts, impulses or images that are experienced as intrusive and inappropriate and that cause marked anxiety and distress. The most common obsessions are: i) repeated thoughts about contamination, eg., being contaminated by shaking hands with another person, ii) repeated doubts, eg., wondering whether one has performed some act, such as having hurt someone in a traffic accident or not having turned off the stove at home, iii) a need to have things arranged in a particular order, eg., intense distress when objects are not ordered or symmetrical, iv) aggressive impulses, eg., recurrent thoughts of hurting one's own child and sexual imagery, eg., a recurrent pornographic thought.

Compulsions are repetitive behaviours or mental acts, which a sufferer is driven to perform to reduce or prevent anxiety and distress often caused by obsessions. Adults suffering from OCD, at some point during their illness, recognise that their obsessions or compulsions are excessive or unreasonable. This, however, does not apply to children, since they lack significant cognitive awareness to make this judgement.

A number of clinical syndromes and OCD overlap in terms of phenomenological factors, clinical course and treatment. These disorders form a distinct category of inter-related disorders referred to as obsessive-compulsive (OC) spectrum disorders and may share a common pathophysiologic and genetic basis with OCD (Hollander, 1993; Rasmussen, 1994; McElroy *et al.*, 1994).

Obsessive-compulsive (OC) spectrum disorders

Obsessive-compulsive spectrum disorders include impulse control disorders (eg., trichotillomania [TTM], pathological gambling and compulsive buying), somatoform disorders (eg., body dimorphic disorder), eating disorders (eg., anorexia and binge eating), compulsive sexual disorders, Tourette's syndrome (TS) and other movement disorders (reviewed by Ravindran, 1999).

The phenomenological similarities between OCD and OC spectrum disorders have been well documented (McElroy *et al.*, 1994; Hollander and Benzaquen, 1997). These similarities include the nature and content of thoughts, the associated distress caused by these thoughts, the frequent self-evaluation and the demand for reassurance (reviewed by Rivindran, 1999).

Obsessive-compulsive spectrum disorders, like OCD, often have onset in childhood or early adult life. Comorbidity data is perhaps the strongest evidence for a link between OCD and OC spectrum disorders (Barsky *et al.*, 1986; Hollander, 1993; Rubenstein *et al.*, 1993) and there have been reports of high rates of OC spectrum disorders in patients with OCD (Rasmussen and Tsuag, 1987; Tamburrino *et al.*, 1994). Further support for this comes from the reports on high rates of OCD in family members of probands with OC spectrum disorders (Pauls *et al.*, 1995).

The pathophysiology of OCD spectrum disorders still remains unclear, and it has been proposed that they form an overlapping, but heterogeneous group, of disorders with multifactorial aetiology (Rasmussen, 1999).

1.2.1. THE DIAGNOSTIC AND STATISTICAL MANUAL OF MENTAL DISORDERS (4TH ED) DIAGNOSTIC CRITERIA FOR OCD

The Diagnostic and Statistical Manual of Mental Disorders (4th Ed) (DSM-IV) (American Psychiatric Association, 1994) is the diagnostic tool most frequently used in the clinical diagnosis of mental disorders, including OCD. The criteria for OCD, as laid out in the DSM-IV, define OCD in terms of its characteristic symptoms and not,

as in the case with depression, in terms of a syndrome. The diagnostic criteria for OCD defined by the DSM-IV are as follows:

The obsessions must be recurrent or persistent or the images, which are experienced, must be intrusive and inappropriate. They should also cause marked distress and anxiety. The intrusiveness should be greater than that experienced by normal worry. The obsessions and compulsions must be a product of the patient's own mind and not due to an outside stimulus (this excludes thought insertion or schizophrenia). The patient must attempt to suppress or ignore these obsessions or attempt to neutralize them with some other thought or action.

The DSM-IV defines compulsions as repetitive mental acts or behaviour which a patient is driven to perform in response to an obsession or according to rules that must be very strictly applied. The repetitive behaviours or mental acts must be performed in order to reduce or prevent anxiety or prevent some feared event or situation; they should, however, be clearly excessive or have no real connection to the anxiety that they are intended to neutralise.

At some point during the illness, the patient should recognise that the obsessions and compulsions are excessive or unreasonable. These obsessions and compulsions should also be time-consuming, i.e., more than 1 hour a day or significantly interfere with the patient's normal functioning. The DSM-IV also excludes a diagnosis of OCD in the presence of another Axis I disorder. So, if a patient with affective disorder experiences an obsessive component that is clearly depressive and completely constrained within the duration of affective disorder, this would not be regarded as OCD. Moreover, there are cases in depression where a patient experiences intrusive images that are obsessional in nature, but that are clearly related to the depression.

1.2.2. EPIDEMIOLOGY OF OCD

A number of studies have attempted to determine the prevalence of OCD. These studies can be divided into the earlier studies in which diagnosis of OCD was solely based on clinical judgement and the modern studies, in which structured or semi-

structured instruments were used in the diagnosis. Roth and Luton carried out the first of the earlier studies in Tennessee in 1942 (Roth and Luton, 1942). In their study, it was found that 0.3% of 1700 people in the sample had a condition that would be diagnosed as OCD. Brunetii in France carried out a second study in 1977. In this study, again, only clinical judgement was used and it was revealed that approximately 1% of the population had OCD (Brunetti, 1977).

More recent surveys have identified OCD patients using standardised diagnostic tools. These studies can be divided into three groups:

1. The 14 worldwide studies based on the Present State Examination (PSE) (reviewed by Wing *et al.*, 1974).
2. Studies based on the Diagnostic Interview Schedule (DIS), of which the most significant are the Epidemiological Catchments Area (ECA) surveys carried out in the United States in the 1980's (Robbins and Reiger., 1991).
3. The British National Survey of Psychiatric Morbidity (Jenkins *et al.*, 1997, Meltzer *et al.*, 1995).

Overall results from these epidemiological surveys suggest that OCD is prevalent in approximately 2-3% of the population, which makes it more common than schizophrenia (1%) and less prevalent than depressive disorders (Karno *et al.*, 1988).

1.2.3. TREATMENT OF OCD

Patient response to a variety of pharmacological treatments for OCD has given investigators clues concerning metabolic pathways, which may be involved in OCD pathogenesis. The drugs that are used in OCD treatment target a variety of proteins and enzymes involved in neurotransmission. The targets of these medications also serve as ideal candidate genes for OCD susceptibility. Furthermore, the pathways they act upon may also harbour many strong candidate genes. For these reasons, the following sections will deal with the pharmacological treatment of OCD.

1.2.3.1. Pharmacotherapy

Manipulations of serotonin (5-HT) are pivotal in pharmacological treatment of OCD. Drugs used in the treatment of OCD typically act by increasing 5-HT levels within the nerve-synapse in one of four ways: (i) by increasing 5-HT synthesis, (ii) inhibiting the reuptake of 5-HT from the presynaptic neuron, (iii) increasing the release of 5-HT from the presynaptic neuron and (iv) inhibiting the metabolism of 5-HT in the presynaptic neuron.

Selective serotonin reuptake inhibitors

Several placebo-controlled studies have demonstrated the efficacy of selective serotonin reuptake inhibitors (SSRIs) (Chouinard *et al.*, 1992; Goodman *et al.*, 1989) in OCD and other anxiety disorders such as panic disorder (PD) and social phobia (SP) (Davidson, 1998; Katzelnick *et al.*, 1995; Taylor, 1998). It is believed that the therapeutic effects of SSRIs are to block the serotonin transporter (5-HTT) protein, responsible for the reuptake of 5-HT into the presynaptic neuron, leading to an increase in synaptic levels of 5-HT. This, in turn, leads to an increase in the stimulation of 5-HT receptors (5-HT_2 and 5-HT_{1a}) on the post-synaptic neurons on a variety of brain regions. It is this cascade of events that is believed to lead to the improvement of symptoms.

Potent SSRIs such as fluoxitine, paroxetine and fluvoamine have been well established as the monotherapy of choice for OCD. These drugs are well tolerated by patients, even in large doses. Fortunately, when one SSRI fails to improve symptoms, several others are available and may be beneficial.

Tricyclic antidepressants

Tricyclic antidepressants (TCA) have been extensively studied in a wide variety of anxiety disorders (Casacalenda and Boulenger, 1998; Lydiard *et al.*, 1996; Taylor, 1998). Clomipramine, which is a potent 5-HT selective TCA, has been found to be effective in the treatment of OCD (The Clomipramine Collaborative Study Group, 1991; De Veaugh-geiss, 1993). Studies comparing the efficacy of clomipramine and

SSRIs have shown that clomipramine may be slightly more effective than SSRIs in the treatment of OCD, but this benefit is offset by an associated increase in the incidence of adverse side-effects (Carpenter *et al.*, 1996).

Administration of medication

The first step in treating OCD patients is to make a positive diagnosis according to DSM-VI criteria (Appendix IV). Current evidence suggests that OCD is commonly misdiagnosed and therefore often left untreated (Stein, 2000). Also, various disorders with intrusive symptoms, such as post traumatic stress disorder or generalised anxiety disorder, are often misdiagnosed as OCD (Stein, 1996).

Once a DSM-IV diagnosis of OCD is made, certain complications need to be considered as they may have an effect on the pharmacotherapy of OCD. The severity of symptoms needs to be assessed as patients with severe symptoms may require hospitalisation in order to help contain symptoms.

Comorbid medical disorders and medication is another complication that needs to be considered when treating OCD patients. Clinicians need to be aware of the multiple interactions between medications that are used in the treatment of OCD and other medical disorders. Fortunately, SSRIs have relatively few interactions with other medications (Leonard, 1997). Many patients with TS, a disorder characterised by vocal and motor tic, frequently suffer from comorbid OCD. Although the comorbid OCD may respond to conventional OCD treatment, additional medication that targets the tics is necessary to relieve many symptoms of TS (Swedo and Leonard, 1994). During pregnancy, lactation and menopause, pharmacotherapy should ideally be avoided. However, where clinical considerations outweigh the risk of medication, such intervention is considered only after consultation with a specialist. Studies have shown that fluoxitine is relatively safe during pregnancy (Nulman and Koren, 1997).

For reasons, such as those outlined above, the first line of medication in OCD treatment is SSRIs. The safety and efficacy of SSRIs has been well established with studies showing that up to 50% of patients respond to these agents (Hirschfeld, 1999). When SSRI treatment is successful in a particular patient, treatment is maintained for

at least one year. The maintenance dose of SSRI required may be slightly lower than is required for acute treatment. When it is decided to discontinue treatment, medication is slowly tapered off. Patients who are intolerant to one SSRI are switched to another SSRI or chlomipramine. Duration and dosage of medication are optimised in patients who partially respond to treatment.

Response to SSRIs in OCD may take longer than in many other disorders (up to twelve weeks), thus, it is important that each patient be given a trial of medication of adequate duration. Patients should be reassessed at the end of each trial. Cognitive behavioural therapy is advisable prior to and during withdrawal of medication in order to maintain gain.

Augmenting medication is administered to patients who respond poorly after optimum trial of medication and to patients with comorbid tics. Studies demonstrate that patients with comorbid tics show improvement of symptoms after augmentation of SSRI with dopamine blockers (McDougle *et al.*, 1994). Another option, which is becoming more common, is to supplement SSRI treatment with a low dose of chlomipramine (Hollander *et al.*, 1991).

When OCD does not respond to a clinical trial of optimal dose and duration, a number of factors are taken into consideration. These include patient compliance, comorbid substance use, comorbid personality disorders and underlying medical conditions. These factors are assessed in unresponsive patients as they may impact on the choice of subsequent intervention. A different agent (other than SSRIs and chlomipramine) is used in these unresponsive patients where assessment of these factors sheds no light on their condition.

For patients who have failed multiple medication and behavioural treatments, and in whom the disorder is quite severe, neurosurgery is considered. Several studies have suggested that specific lesions to the cortico-striatal pathways may lead to significant reduction in OCD symptoms in treatment refractory patients (Jenike *et al.*, 1998).

1.2.3.2. Cognitive behavioural therapy of anxiety disorders

The core-element of anxiety disorders such as OCD is a cognitive process in the form of automatic thoughts or images which suddenly manifest themselves in response to a primary stimulus, followed by intense fearfulness (Beck *et al.*, 1994). According to the cognitive model of anxiety disorders, it is not certain events that contribute to the development of anxiety, but rather how these events are interpreted.

Within the cognitive model of anxiety disorders, two levels of dysfunctional thoughts have been identified, namely negative automatic thoughts (NATs) and dysfunctional assumptions and beliefs (Moller, 1990). Negative automatic thoughts refer to those thoughts that are triggered spontaneously in response to a specific situation. The contents of these thoughts involve the threat of possible physical or psychological trauma. In most cases., these thoughts are so rapid that the patient is only aware of the possible consequences and subsequent feelings of distress, but not of the thought itself. This makes it rather difficult for a patient to objectively evaluate the validity of the NATs.

Dysfunctional assumptions and beliefs refer to general assumptions and beliefs which patients have about themselves and the world around them. These beliefs make the patient vulnerable to interpret specific situations in an unusual, dysfunctional manner (Beck *et al.*, 1994). These assumptions and rules form the basic structural components of a patient's cognition and are as a result of early life events (Hawton *et al.*, 1996). They are the foundation that determines the patient's behaviour.

Behavioural therapy of OCD is a method whereby patients learn to perform adaptive behaviours, instead of pathological ones in response to an intrusive thoughts or images which comprise the core symptoms of OCD. In order to successfully complete therapy, patients must tolerate the uncomfortable feelings as a result of OCD psychopathology. The term 'exposure response management' was coined in order to describe how patients could learn cognitive strategies to cope with the intense feeling of discomfort that arises during exposure and response prevention treatment.

Cognitive therapy techniques

The literature describes three basic techniques of cognitive therapy for OCD: challenging the obsessional thought, thought-stopping and challenging the negative automatic thought.

Many techniques have been developed to challenge the obsessional thought. These techniques typically involve either rational emotive therapy (RET) (Ellis and drysden, 1987) or self-instructional training (SIT) (Meichenbaum, 1974). Rational emotive therapy is focused on challenging the belief in the obsessional thought through rational disputations. Self-instructional training teaches patients to determine the level of their anxiety, to observe and record their obsessional thoughts and replace them with productive statements.

Thought-stopping is a process that involves interrupting the intrusive thought process by using a cue word. Patients are instructed to use a cue word whenever an intrusive thought presents itself. Also, patients are instructed to imagine a pleasant scene after saying the cue word. Challenging the negative automatic thoughts is a technique that differs from the two previously mentioned techniques in that it does not focus on the intrusive thoughts.

1.2.4. PATHOGENESIS

Although the pathogenesis of OCD remains largely unknown, several models have been put forward. These models include the 1) involvement of certain brain regions, 2) genetic factors and 3) immunological factors. The following section describes these three models.

1.2.4.1. NEUROBIOLOGICAL AETIOLOGY OF OCD

Brain regions of interest

Currently, there are several brain regions that are prime candidates as possible sites of dysfunction in OCD.

Basil Ganglia

The basal ganglia (BG) are subcortical nuclei that comprise the extrapyramidal system and are closely associated with the cortical and limbic structures. They consist of the caudate nucleus, putamen and the globus pallidus (together, these three make up the corpus striatum) and the amygdaloid complex. Historically, the BG have been attributed a role in the modulation of corticospinal outflow or movement.

There is some evidence linking BG dysfunction with TS (Devinsky, 1983) as well as Sydenham's chorea (SC) (Swedo *et al.*, 1989), a disease which is closely related to OCD. Tourette's Syndrome is a disorder that typically develops in childhood and is characterized by tics manifested as involuntary movements or vocalizations. Just like a compulsion, these tics tend to be exacerbated in times of stress and can be suppressed for a short while, during which time a patient's anxiety escalates. Many patients with TS experience concurrent symptoms of OCD. Also, there is a disproportionately higher incidence of OCD in the family members of TS sufferers (Lenane *et al.*, 1990). Similarly, OCD patients are more likely to suffer from tics or have family members who have tics than control individuals (Pittman, 1989).

Patients with SC, which is a movement disorder that afflicts up to 20% of patients suffering from rheumatic fever (RF), have a higher prevalence of OCD symptoms than individuals with RF without SC (Swedo *et al*, 1989).

The BG are implicated in OCD beyond the above mentioned mutual relationship between movement disorders. The BG nuclei participate in cognitive aspects of behaviour, whereas the motor functions of the BG are carried out predominantly by the putamen, the cognitive functions are thought to be carried out by the caudate

nucleus (Cote *et al*, 1995) (fig 3a). Thus, the BG are involved in thought, and abnormalities in these structures could result in obsessions and repetitive patterns of behaviour.

Computerized tomography has suggested structural differences in the brains of OCD patients and control individuals. Ventricular/brain ratios have been shown to be enlarged in OCD patients (Behar, *et al*, 1984) and caudate volumes were found to be reduced in OCD patients as compared to normal control individuals (Luxenberg, *et al*, 1988).

Positron emission tomography (PET) studies have revealed increased metabolic activity of the caudate bilaterally in the brains of OCD patients compared to control individuals (Baxter *et al*, 1987) and in two separate case reports, OCD patients were found to have local lesions involving the caudate and putamen as determined by MRI (Weilburg *et al*, 1989; Williams *et al*, 1988).

Frontal Cortex

The frontal lobes are associated with neuropsychological functions of programming, regulating, controlling and verifying behaviour. Therefore, frontal lobe pathology correlates with inflexibility, decreased response inhibition, preservation and stereotypy, characteristics which are reminiscent of OCD symptoms (Otto, 1990).

Frontal lobe dysfunction has been implicated in some neuropsychological studies of OCD (Flor-Henry *et al*, 1985; Behar *et al.*, 1984; Malloy *et al.*, 1989). For example, PET studies have consistently shown increased frontal cortical metabolism in OCD patients compared to control individuals (fig 3b). Positron emission tomography analysis of ten OCD subjects and ten controls demonstrated increased metabolism in the orbital gyrus and orbital gyrus/ipsilateral hemispheric ratio bilaterally in the patient group (Baxter, 1992). These findings have been replicated (Nordahl *et al.*, 1989). Similarly, PET investigation of 18 adult childhood-onset OCD subjects, versus control individuals, revealed increased metabolism of the bilateral prefrontal areas and an increase in left orbitofrontal cortex (Swedo *et al.*, 1989).

Limbic Structures

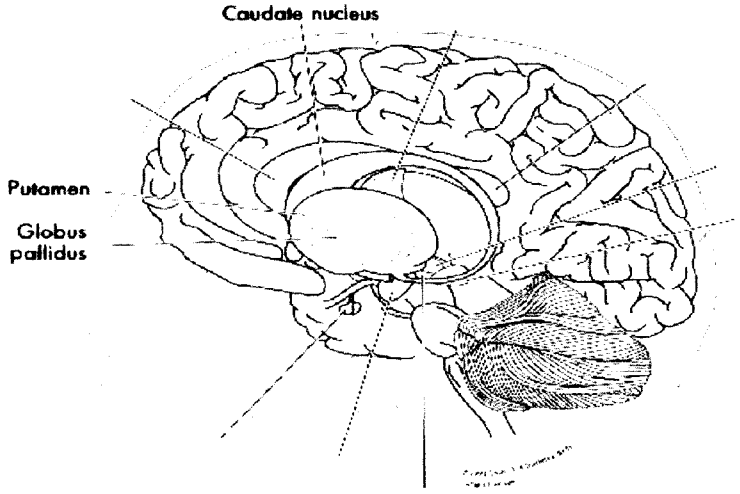
The limbic system consists of several interconnected structures that are believed to serve as the neural substrate for drive-related and emotional behaviour. Limbic structures include the septal area, hippocampus, cingulum, amygdala and certain portions of the temporal lobe (particularly the parahippocampus gyrus).

Animal models of OCD include bilateral hippocampectomised rats that display repetitive behaviours, invariability, enhanced avoidance and excessive behaviour (Pitman, 1989). One model provides data suggesting that the septohippocampus interacts with cortical outputs to compare sensory information with anticipated conditions. It then modulates behaviour accordingly. This 'comparative' pathway responds to novel or threatening stimuli with behavioural inhibition, enhanced attention and arousal. So the repetitive behaviour seen in OCD may be due to a malfunction in the septohippocampus.

The cingulum is closely related to the septohippocampal system and the thalamocortical tracts. Positron emission tomography analysis of OCD subjects has shown increased metabolic activity bilaterally in anterior cingulated cortex (Swedo *et al.*, 1989). Also, it has been shown that cingulated lesions ameliorate OCD symptoms in some patients (Martuza *et al.*, 1990; Jenike *et al.*, 1991; Kelly *et al.*, 1973) and may act by disrupting the thalamocortical tract, as seen in leucotomy (Rees, 1973). It has, therefore, been proposed that cingulum hyperactivity may play a role in compulsive behaviour.

The temporal lobes were initially thought to be involved in OCD because of the clinical similarities between OCD and temporal lobe epilepsy. Complex partial seizures are characterised behavioural manifestations which include "forced thinking" that is practically indistinguishable from obsessions experienced in OCD (Bear, 1985). In addition to this, several reports have demonstrated that temporal lobe electroencephalogram (EEG) abnormalities coincide with OCD (Jenike, 1984). There are, however, studies that found no significant EEG abnormalities in OCD patients compared to control individuals.

A



B

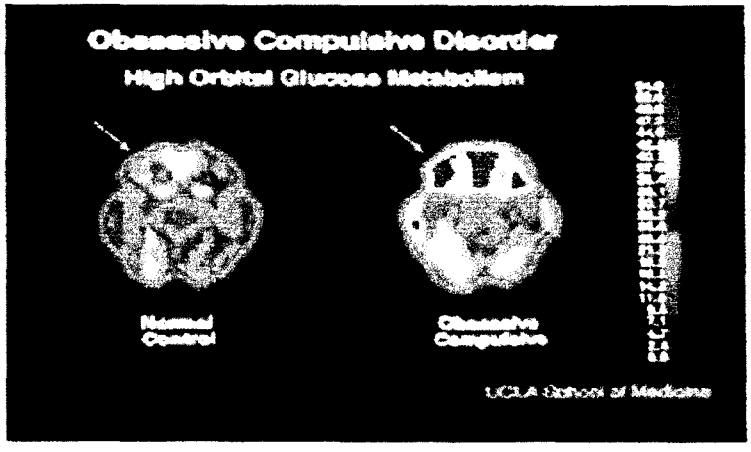


Fig 3: Brain regions involved in OCD pathogenesis. 3A: The structure of the basal ganglion showing the caudate nucleus, which is responsible for cognitive functions, the globus pallidus and the putamen, which is responsible for motor function (taken from www.ncbi.nih.gov/publicat/ocdbrain.htm). **3B:** PET scan of a control individual's brain (left) and an OCD patient's brain (right). These scans show an increased frontal cortical metabolism in OCD patient compared to the control individual (taken from www.ncbi.nih.gov/publicat/ocdbrain.htm).

In summary, by using a variety of brain scanning techniques, researchers have shown anatomical and metabolic variations in various brain regions (BG; frontal cortex and limbic structures) of OCD patients compared to control individuals, thereby implicating these regions in OCD pathogenesis.

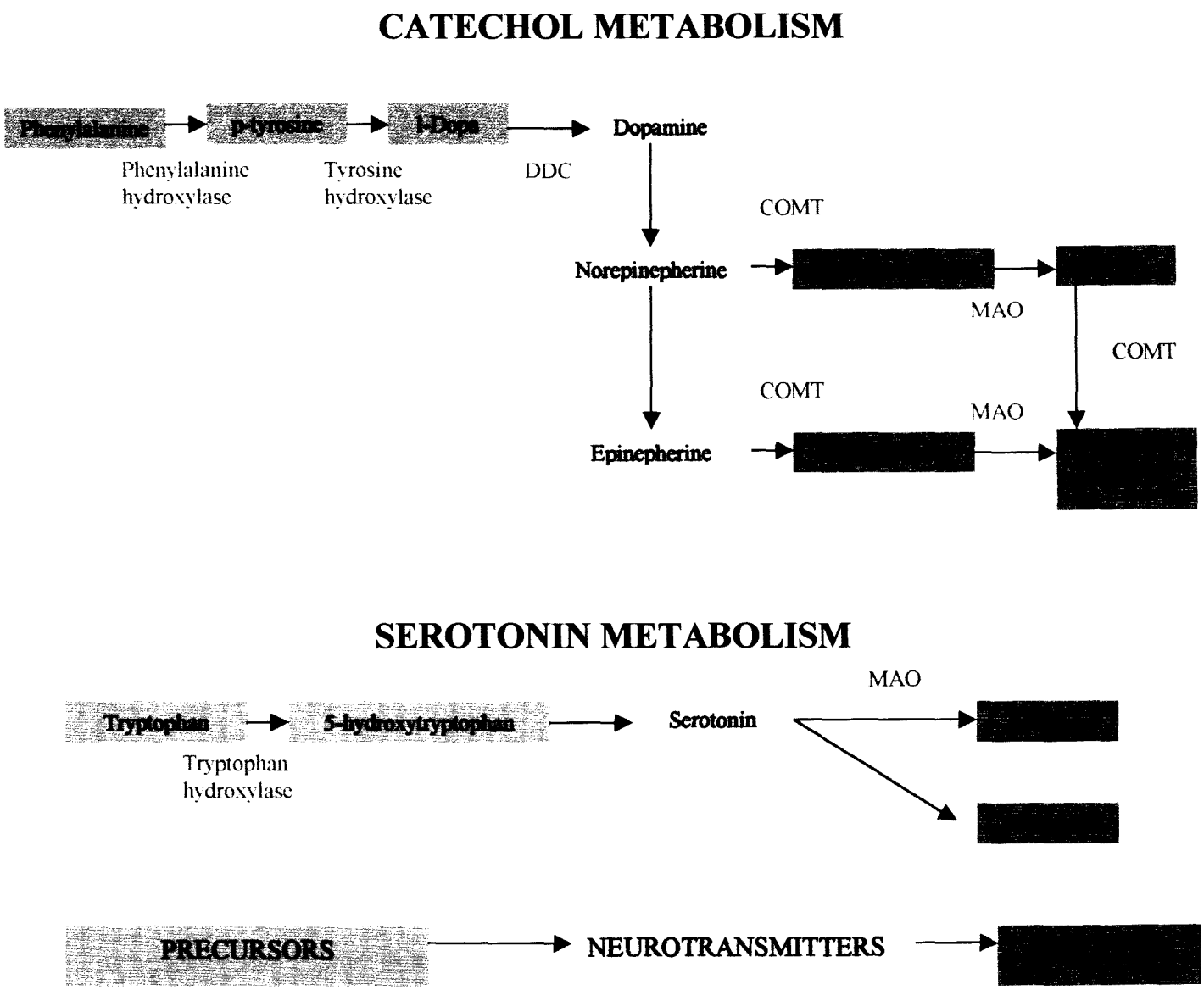
1.2.4.2. GENETIC AETIOLOGY OF OCD

The genetic model of OCD is largely based on the pathways of neurotransmission and neurotransmitter metabolism (fig 4). These pathways are regulated by a host of enzymes and receptors, functional variants of which many may be considered good candidates for increased susceptibility to OCD. The following section will deal with the components of the neurotransmitter metabolic pathways and, specifically, the enzymes involved in these pathways, as well as the 5-HTT, which facilitate neurotransmission. For the purpose of this thesis, the catechol-O-methyltransferase (*COMT*) and the serotonin transporter (*SLC6A4*) genes will be discussed in detail, while other possible candidates genes whose products are involved in these pathways will be mentioned briefly. Firstly, however, family studies of OCD will be discussed, since they provide some evidence for the genetic aetiology of OCD.

1.2.4.2.1. Family studies of OCD

Although the aetiology of OCD remains largely unknown, several investigations in the past have indicated that OCD is familial. Several twin studies have found that concordance for OCD is substantially greater in monozygotic twins (53%-87%) than dizygotic twins (22%-47%) (Rasmussen and Tsuang, 1986). These findings have provided limited evidence for the importance of genetic factors, and, although a number of family studies on OCD have been completed, the subject remains controversial.

Some studies found rates of OCD as high as 35% (Lenane *et al.*, 1990) among first-degree relatives of OCD probands, whereas other studies found no increase in rates (Rosenberg 1967; Insel *et al.*, 1983). There are many reasons for these discordant results, such as differences in diagnostic criteria and methods of assessment. Most of the earlier studies did not directly interview relatives of OCD sufferers, while others



COMT:	Catecol-O-methyltransferase
DDC:	Dopadecarboxylase
DOPEG:	Dihyroxyphenylglycol
5-HIAA:	5-hydroxyindoleacetic acid
MAO:	Monoamine oxidase
MOPEG:	3-methoxy-4-hydroxyphenylethyleneglycol
VMA:	Vanillylamandelic acid

Fig 4: Schematic representation of the synthesis and degradation pathways of neurotransmitters. Enzymes shown in red are those enzymes used in the present association study, while those shown in purple are the subject of a concurrent study.

failed to include control groups. During the early 1990's, five studies addressed the shortcomings of the earlier studies (Lenane *et al.*, 1990; Riddle *et al.*, 1990, Bellodi *et al.*, 1992; Black *et al.*, 1992; Leonard *et al.*, 1992). Findings from these studies provided further support for the hypothesis that there is a familial component in some forms of OCD. More recently, two further family studies of OCD have emerged (Pauls *et al.*, 1995; Nestadt *et al.*, 2000), both of which provide further evidence for the familial nature of OCD and which have lead the authors to speculate on the presence of a gene of major effect which contributes to the pathogenesis of OCD.

While these findings are consistent with a genetic aetiology of OCD, familial studies by themselves are not able to determine whether genetic factors play a major role in the manifestation of OCD. Data generated from such family studies are used to examine specific genetic hypotheses using segregation analysis. If segregation analysis reveals that patterns within families are consistent with a fairly simple mode of inheritance, the results can then be taken as further evidence for the importance of genetic factors in OCD pathogenesis The mode of inheritance of OCD has been investigated by means of segregation analysis in four independent studies (Nicolini *et al.*, 1990, 1993; Cavallini *et al.*, 2000; Nestadt *et al.*, 2000). Two of these studies (Nicolini *et al.*, 1991; Cavallini *et al.*, 2000) suggested a gene of major effect, but failed to show any distinct mode of inheritance. The study by Nestadt *et al.*, (2000), investigating 80 case families and 73 control families, showed strong evidence supporting a mendelian dominant or codominant mode of inheritance.

Additional support for the involvement of specific genes in OCD comes from twin and family studies of TS, which have shown a high rate of OCD among TS families (Pauls *et al.*, 1986). As the latter familial condition appears to have a substantial genetic basis (Pauls, 1992, Simonic *et al.*, 1998), the higher rate of OCD among the relatives of TS probands suggests that certain forms of OCD might be genetically related to TS.

In summary, in the studies discussed above, the genetic basis of OCD remains a contentious issue and, although family and twin studies provided some evidence for genetic factors involved in OCD, conclusive evidence is still lacking.

1.2.4.2.2 The Serotonin Hypothesis

The 5-HT system is consistently implicated in the development of OCD and the most consistent pathophysiological finding in OCD points toward abnormalities in 5-HT neurotransmission. Neurotransmission mediated by 5-HT contributes to many physiological functions such as motor activity, food intake, sleep and reproductive activity. It also contributes to cognition and emotional states including mood and anxiety (Ramboz *et al.*, 1998).

The efficacy of SSRIs, and the absence of improvement of OCD symptoms after treatment with norepinepherine reuptake inhibitors and dopamine antagonists, presents strong evidence for the involvement of 5-HT in the pathophysiology of OCD (Barr *et al.*, 1992; Goodman *et al.*, 1990; Murphy *et al.*, 1989).

Taking into account that 5-HT neurotransmission could play a major role in the pathogenesis of OCD, genes in the serotoneric pathway, such as the 5-HT transporter (*SLC6A4*) and serotonin receptors (*5-HTR*), are good candidate genes for conferring susceptibility to OCD.

1.2.4.2.3. Catecholamine metabolism

Several studies have implicated catecholamine metabolism (fig 4) in psychiatric disorders and, more specifically, anxiety disorders (Mallet *et al.*, 1994). This metabolic pathway harbours several key enzymes and many genes encoding these enzymes, eg., *COMT* and monoamine oxidase A (*MAO-A*), are considered plausible candidate genes for increased susceptibility to OCD.

1.2.4.2.4. Candidate Genes

Catechol-O-methyltransferase (COMT)

Catechol-O-methyltansferase is a physiologically important enzyme that catalyses the transfer of the methyl group from S-adenosyl-L-methionine to a phenolic hydroxyl group of catechol neurotransmitters, catechol steroids and catechol drugs (fig 5) (Axelrod, 1966; Campbell *et al.*, 1984). The gene encoding COMT has been localised

to chromosome 22q11.1-q11.2 by Grossman *et al.*, (1985). In the brain, COMT degrades catechol amines such as norephinepherine, epinephrine and dopamine (Fig 5) into O-methyl esters (Guldberd and Marsden, 1975).

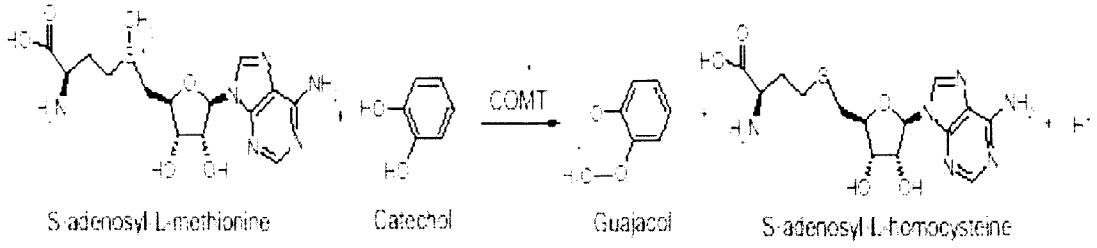


Fig 5. Methyl transfer from S-adenosyl-L-methionine to catechol in a *COMT* catalysed reaction

Cell fractionation and immunological studies have shown that the COMT enzyme occurs as two distinct forms in mammals: in the cytoplasm as a soluble form (S-COMT) and associated with membranes as a membrane-bound form (MB-COMT) (Assicot and Bohuon, 1971). S-COMT activity is the more prevalent form in all tissues, while MB-COMT generally represents less than 5% of the total COMT activity (Guldberg and Marsden, 1975; Jeffery and Roth, 1984; Grossman *et al.*, 1985). However, in the brain, MB-COMT activity has been reported to be higher than in other tissues (Rivett *et al.*, 1982).

Rat and human *S-COMT* and *MB-COMT* cDNAs have been cloned and characterised (Bertocci *et al.*, 1991; Lundström *et al.*, 1992; Tilgmann *et al.*, 1992) (fig 6). Both S-COMT and MB-COMT are encoded by one gene, but are transcribed using two distinct separate promoters (Tenhunen *et al.*, 1994). The *S-COMT* promoter contains a 633 base pair (bp) open reading frame (ORF) encoding the S-COMT protein (Lundström *et al.*, 1992; Salminen *et al.*, 1990). The translation initiation codon for the *MB-COMT* form in humans is located 150 bp upstream from the *S-COMT* form in the same ORF. According to these results, the S-COMT (25 kDa) and the MB-COMT (30kDa) polypeptides are partly identical, but with MB-COMT containing an amino (N)-terminal extension that carries a stretch of hydrophobic amino acids which is not present in the S-COMT sequence. This hydrophobic N-terminal end of MB-COMT is responsible for cotranslationally directing the MB-COMT protein to the membrane by acting as a signal-anchor domain (Ulman and Lundström, 1991).

The *COMT* gene is of interest in the pathogenesis of OCD for three reasons:

Firstly, the argumentation of SSRI treatment with dopamine agonists appears to be useful in a subset of OCD patients (McDougle *et al.*, 1994) implicating the involvement of dopaminergic pathways in OCD pathogenesis.

Secondly, it has been previously reported that patients with 22q11 microdeletions manifest a number of psychiatric phenotypes (Bassett et al., 1999; Karayiorgou *et al.*, 1995; Pulver *et al.*, 1994). Deletions in this region are responsible for chromosome 22 deletion syndrome (22qDS), the second most common genetic syndrome that affects approximately one in 4000 individuals world-wide (du Montcel *et al.*, 1996). The syndrome encompasses velocardiofacial syndrome (VFCS), DiGeorge syndrome and conotruncal anomaly face syndrome (CTAFS), all due to chromosome 22q11.2 microdeletions (Demczak and Aurius *et al.*, 1995), which generally occur *de novo* (Deczak and Aurius *et al*, 1995; Leana-Cox *et al.*, 1994). Learning disabilities, palatal anomalies, cardiac defects and atypical facial features are common, although the presentation is highly variable (Yamagisgi *et al.*, 1998). It is inferred that the symptoms associated with the deletions, which vary in extent in different patients, are associated with the loss of particular genes harboured in the missing region. Most interestingly, a review of 22qDS found that psychiatric disorders, of which schizophrenia was the most frequent, were reported in 53% of cases of 22qDS that were not ascertained from psychiatric sources (Papalos *et al.*, 1996; Pulver *et al.*, 1994) and that another group of patients showed obsessive-compulsive symptoms (Karayiorgou *et al.*, 1997) A follow-up study (Papolos *et al.*, 1996) in patients with 22q11 deletions reported OC symptoms in a majority of these patients. These findings provide strong evidence that genes involved in increased OCD susceptibility may be harboured at the 22q11 region. The gene encoding COMT maps to this region and is frequently deleted in patients with 22qDS and has also been linked to schizophrenia susceptibility in various studies (Liou *et al.*, 2001; de Chadlee *et al.*, 1999; Lachman *et al.*, 1998).

Thirdly, a functional polymorphism in *COMT*, in which the high (*H*) and low (*L*) activity alleles encode a valine or methionine amino acid residue, respectively, at

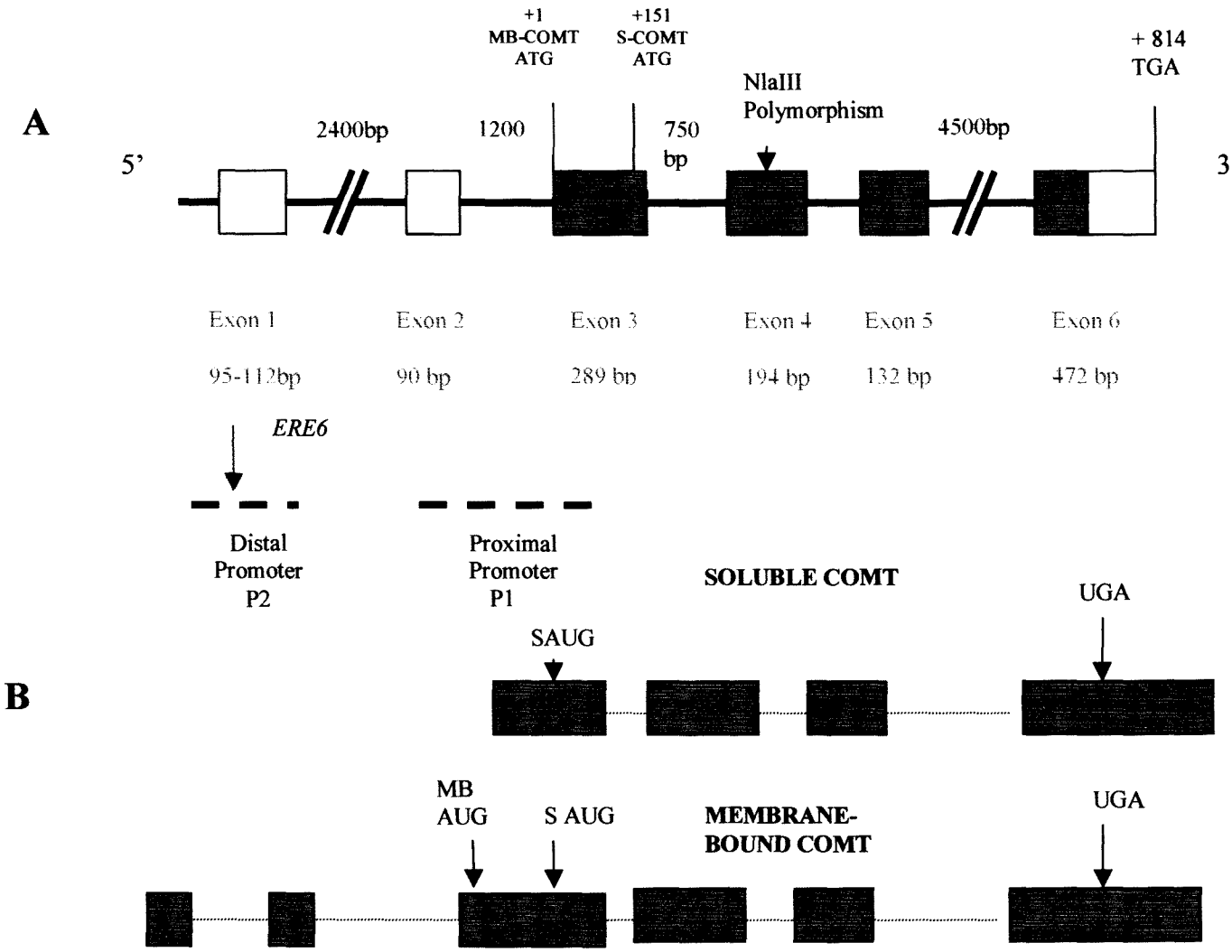


Fig 6. A: Exon-intron organization of the human *COMT* gene. The *COMT* gene consists of 6 exons indicated by open and blue rectangles. Solid blue rectangles represent transcribed regions, whereas open rectangles represent untranslated regions. Intron sizes are indicated in red font, while exon sizes are indicated in blue font. The gene codes for two distinct forms of the enzyme (MB-COMT and S-COMT) and each form is transcribed from a distinct promoter indicated by dashed lines. The red arrow shows the relative position of *ERE6*. The *MB-COMT* start codon is located at position +1 while the *S-COMT* start codon is located at position +151. The stop codon for both forms is located at position +814. The *Nla*III polymorphism is located in exon 4 as shown by the black arrow (adapted from Lotta *et al.*, 1995)

B: mRNA species for both S-COMT and MB-COMT. (Adapted from Lotta et al., 1995). S UAG represents the start codon for the soluble form of COMT, while MB AUG represents the membrane-bound form start codon.

position 158 of the protein, has been described. There is a three- to four-fold reduction in enzyme activity between the variants encoded by the *H/H* and *L/L* genotypes, with heterozygotes (*H/L*) showing intermediate enzyme activity. This polymorphism is represented by a G to A transition resulting in the creation of an *Nla*III restriction enzyme site (Lachman *et al.*, 1996) and was used in two North American studies to determine the role of *COMT* in OCD. Both of these studies showed an association between OCD and the *L* allele of *COMT* (Karayiorgou *et al.*, 1997; Karayiorgou *et al.*, 1999).

Furthermore, there is evidence that oestrogen downregulates *COMT* transcription. Therefore, high levels of oestrogen may directly lower its expression (Xie *et al.*, 999). Several half-palindromic oestrogen response elements (*ERE*s) have been identified within the promoter regions of *COMT* (Xie *et al.*, 1999), of which *ERE6* was found to have a major influence on the oestrogen-dependant regulation of *COMT*.

Many genes containing *ERE*s have been identified, with many of them containing one or more polymorphism within a 13 nucleotide consensus sequence (Driscoll *et al.*, 1998). Although there is no published data which suggests that *ERE6* is polymorphic, it is possible that a polymorphism in this element could have an effect on *COMT* transcription and expression levels and thus be implicated in OCD pathogenesis.

Serotonin Transporter (5-HTT)

Serotonergic neurotransmission is terminated by the active transport of 5-HT back into the presynaptic neuron by the serotonin transporter protein (5-HTT). The latter regulates the magnitude and duration of serotonergic responses by mediating the sodium-dependant re-uptake of 5-HT into presynaptic nerves, and, in so doing, it is central to the fine-tuning of brain serotonergic neurotransmission and peripheral actions of 5-HT (Lesch *et al.*, 1996). The serotonin transporter protein expression in the brain is notably abundant in the cortical and limbic areas involved in the emotional aspects of behaviour. It is also expressed in blood and expression levels are similar to that found in the brain (Greenberg *et al.*, 1999).

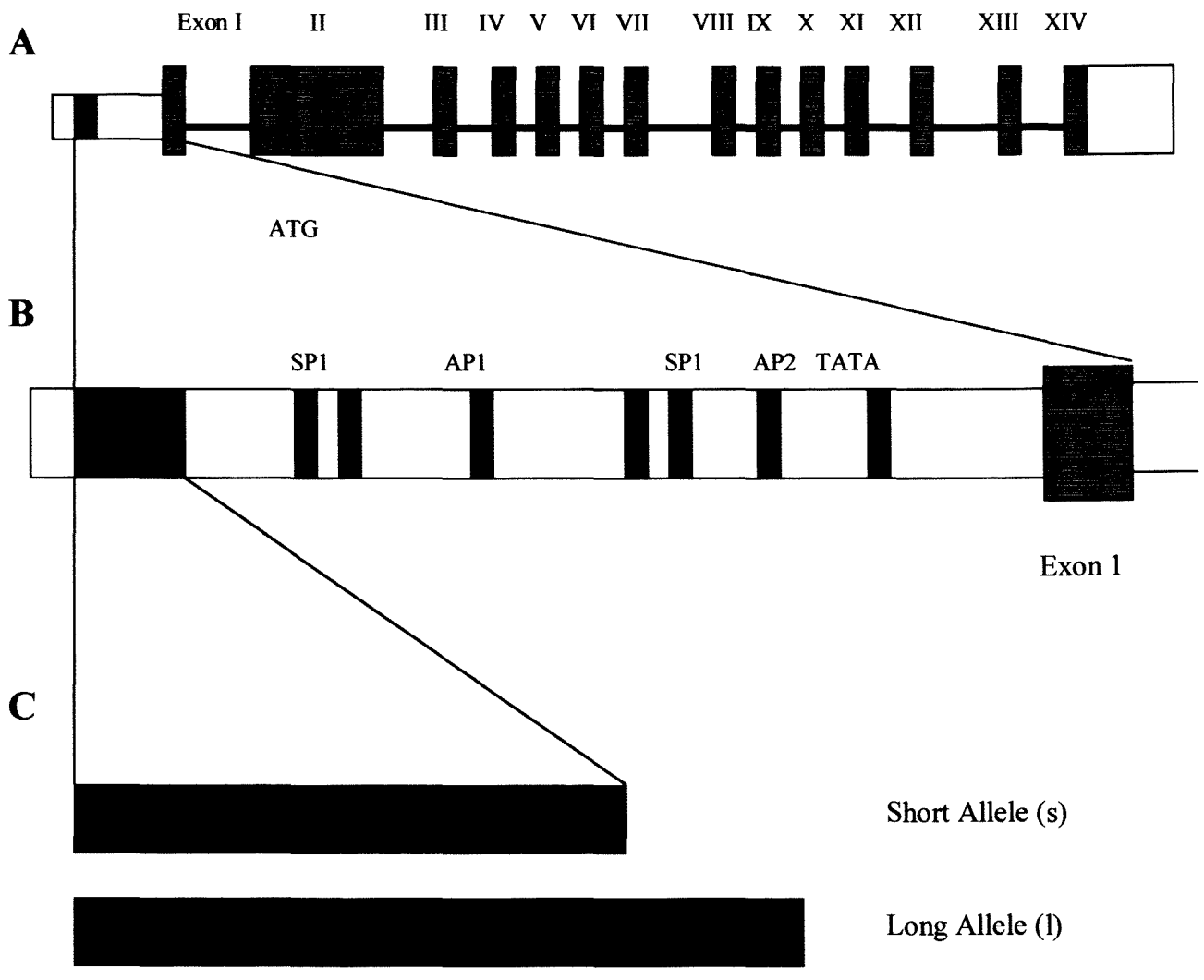


Fig 8. The serotonin transporter gene (*SLC6A4*). A: Organization of the human 5-HTT gene and its flanking regulatory region on chromosome 17q11.2. Solid rectangles/ open rectangles represent coding regions. The bold horizontal line represent intronic sequence. **B:** The 5-HTT 5' flanking region. The 5-HTT promoter is defined by a TATA-like motif, and several potential binding sites for transcription factors, including AP1, AP2 and SP1, are present in the 5' flanking region. The position of the *5-HTTLPR* is indicated by the purple rectangle. **C:** The *5-HTTLPR* showing the presence of the 44bp deletion (short allele) and the presence of the 44bp insertion (long allele). Adapted from Heils *et al.*, 1996.

The 5-HTT protein is one of several structurally similar sodium-dependant transporter proteins that contain 12 putative membrane-spanning regions (Amara and Kahar, 1993) (fig 7). The human 5-HTT is encoded by a single gene on chromosome 17q12, which is organised into 14 exons spanning approximately 35 kilobases (kb). (Ramamoorthy *et al.*, 1993) and has the official gene symbol *SCL6A4* (solute carrier family 6 [neurotransmitter serotonin], member 4). A unique GC-rich repetitive sequence is located in the proximal 5' regulatory region of *SCL6A4*. Heils *et al.*, (1996) revealed a common polymorphism in this region (*5-HTTLPR*) generated by a 44bp insertion/ deletion sequence (*l/s*) (Fig 8).

SCL6A4 is of interest for its involvement in OCD for a number of reasons. Firstly, there is a substantial body of work that implicates the 5-HT system in the development of OCD, including the finding that OCD responds selectively to 5-HT reuptake inhibitors (Murray *et al.*, 1996). Secondly, the *5-HTTLP* (*l/s*) polymorphism in *SCL6A4* has been shown to have a significant effect on blood 5-HT content (Hanna *et al.*, 1998 Lesch *et al.*, 1996).

In an association study between *SCL6A4* and OCD, McDougle *et al.*, (1998) employed a TDT design (section 1.1.2.3.) in 34 European-American triads and found that the *l* allele of *5-HTTLPR* was significantly more commonly transmitted by heterozygous parents to their OCD affected offspring. In a population-based association study, Bengel *et al.*, (1999) demonstrated that OCD patients were more likely to be homozygous for the *5-HTTLPR l* allele than control individuals. Another study, however, found that the *5-HTTLPR* polymorphism did not appear to influence response to pharmacotherapy in OCD, thus raising some doubt about the functional importance of the above-mentioned findings (Billet *et al.*, 1997). Similarly, early reports of an association between *SCL6A4* alleles and anxious and depressive traits (Lesch *et al.*, 1996) have not been replicated (Gelernter *et al.*, 1998).

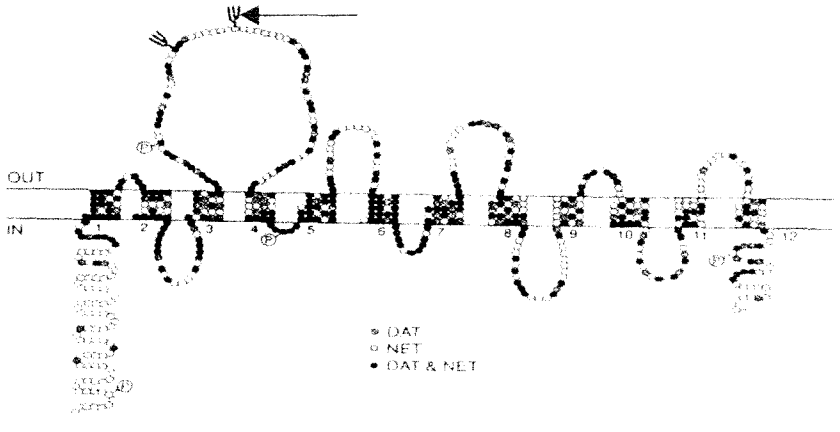


Fig 7. The structure of rat 5-HTT. The rat 5HTT has been studied extensively. This schematic representation of rat 5HTT shows homologous amino acids with the rat dopamine transporter (DAT) and human norepinepherine transporter (NET). P indicates possible phosphorylation sites while the blue arrow shows possible glycosylation sites (Adapted from Frazer *et al.*, 1999).

Monoamine Oxidase A (MAO-A)

Monoamine oxidase (MAO) is a flavin containing enzyme that degrades a variety of biogenic amines, including the neurotransmitters norepinepherine, dopamine and 5-HT (Weyler *et al.*, 1994) (fig 4). Two forms of the enzyme, MAO-A and MAO-B, have been identified on the basis of the difference in their molecular weight, substrate specificity, substrate affinities, inhibitor sensitivities and immunological properties (Garrick and Murphy, 1982). In the human brain, MAO-B is the prominent form (Garrick and Murphy, 1982) expressed at the highest levels in the astrocytes and serotonergic neurons. In contrast, MAO-A is expressed at highest levels in the catecholaminergic neurons (Fowler *et al.*, 1987; Thorpe *et al.*, 1987).

Variations in MAO activity appear to affect neurophysiological and behavioural traits in humans and in animals. In *MAOA* knock-out mice, 5-HT levels in the brain have been shown to be increased up to nine-fold, with the mice exhibiting distinct behavioural changes (Cases *et al.*, 1998).

In humans, moderate inhibition of MAOA and MAOB activity can lead to mood elevation, loss of 'rapid eye movement' (REM) sleep, motoric hyperactivity,

orthosomatic hypertension and hyperflexia without spasticity (Kupfer and Bowers 1972; Murphy *et al.*, 1983; Squires *et al.*, 1978).

The two protein-encoding genes, *MAO-A* and *MAO-B*, have been mapped to the p11.23-11.4 region of the X chromosome (Ozelius *et al.*, 1988; Levy *et al.*, 1989). They each comprise 15 exons with identical intron-exon organisation but encode proteins with different polypeptide compositions. Brunner *et al.*, (1993) studied a Dutch family, with five male cases of X-linked borderline mental retardation and behavioural disturbances, screening for *MAO* gene mutations and assaying MAO-A activity levels. A point mutation was detected in exon 8 of *MAOA*, resulting in a truncated and non-functional MAOA protein. All of the affected males exhibited abnormal and often overly aggressive behaviour including arson, rape of a female relation, exhibitionism, voyeurism and one, after being criticised, attempted to murder his supervisor. This study offers a correlation between inappropriate emotional responses and the elimination of MAOA activity via a genetic mutation.

1.4.2.4.5 Other candidate OCD susceptibility genes

As the candidate genes discussed below do not form part of the present study, but rather of a concurrent study, they will be discussed briefly.

Tryptophan hydroxylase

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the synthesis of 5-HT (fig 4). Abnormalities in 5-hydroxyindoleacetic acid (5-HIAA) concentrations, the major metabolite of serotonin in the brain and cerebrospinal fluid, have been described in depression, schizophrenia and impulsive aggression (Tuckwell and Kozoil, 1996). Tryptophan hydroxylase may be an important contributor to treatment response, given the fact that SSRI's depend on the availability of serotonin to exert their effect via transport inhibition. Therefore, this enzyme is an ideal candidate for OCD susceptibility.

Tyrosine hydroxylase

Tyrosine hydroxylase (TH) is a rate-limiting enzyme in the synthesis of catecholamines (fig 4). A deficiency of TH leads to decreased cerebrospinal fluid levels (CSF) of homovanillic acid and 3-methoxy-4-hydroxyphenylethyleneglycol, together with normal CSF tyrosine and 5-hydroxy-indole-acetic acid concentrations (Wevers *et al* 1999). Functional polymorphisms of the *TH* gene may therefore be involved in the pathogenesis of both manic depressive episodes, panic disorder and OCD.

Serotonin Receptors

Since many studies indicate that abnormalities in the serotonergic system play a major role in the development of OCD, the 5-HT receptor genes are very attractive candidate genes. The 5-HT receptor family is divided into seven main branches that each contain 1- 4 individual receptor genes (Peroutka, 1994). All 5-HT receptors are coupled to G-proteins linked to an adenyl cyclase or to the phospho-inositol system, except 5-HT receptor 3 (5-HT_3) which is an ion channel. Many 5-HT receptor genes have been implicated in a variety of psychiatric disorders and personality traits. The 5-HT receptor 2C (5-HT2_c) gene (*5-HTR2C*) has been associated with the personality trait of reward dependence (Ebstein *et al.*, 1997), while 5-HT2_a receptor densities have been shown to be decreased in patients with neuroleptic-naïve schizophrenia (Ngan *et al.*, 2000). Also, a 5-HT2_a receptor polymorphism has been implicated in the development of bipolar affective disorder (Massat *et al.*, 2000).

1.2.4.3 IMMUNOLOGICAL AETIOLOGY OF OCD

Immune-based theories of OCD pathogenesis stem from the observed similarities between its symptoms and those of SC. Sydenham's Chorea is a disorder developed in a subset of patients with RF, and is thought to be an immunological illness in which infection with group A β-haemolytic streptococci (GABHS) induces the production of antibodies that cross-react with neuronal proteins (Taranta *et al.*, 1956).

Swedo *et al.*, (1989) showed that patients with SC might show OC symptoms in up to 75% and OCD in 33% of cases. Furthermore, a symptom pattern of sudden dramatic

onset and slow waxing and waning over a period can be found in SC and a subgroup of OCD (Leonard, 1993; Swedo *et al.*, 1989; Swedo *et al.*, 1994). This symptom pattern was described in four paediatric patients by Allen *et al.*, 1995. These observations provided a diagnostic criterion for a subgroup of patients called paediatric, infection-triggered, autoimmune neuropsychiatric disorders (PITANDs) (Allen *et al.*, 1995). Subsequently, many cases of paediatric autoimmune neuropsychiatric disorders associated with streptococcal infection have been described (PANDAS) (Swedo *et al.*, 1997) The diagnostic criteria of PANDAS include evidence of adventitious movements (eg., mild chorea of motor hyperactivity) (Swedo *et al.*, 1998). The presence of abnormal movements (eg., choreiform) has been reported in OCD patients (Hollander *et al.*, 1989), while tic-like movements have been reported in streptococcal infection and SC (Kiessling *et al.*, 1993).

The involvement of the BG in both OCD and SC has been well documented (Berthier *et al.*, 1996, Giedd *et al.*, 1995; Hebebrand *et al.*, 1993; Heye *et al.*, 1993). In a recent study, BG enlargements were found among patients with PANDAS (Giedd *et al.*, 2000). This result was found to be similar to a report of BG enlargements in patients with SC (Giedd *et al.*, 1995) Taken together, these studies are consistent with the hypothesis of cross-reactive antibody-mediated inflammation of post-streptococcal OCD or tics in some patients. These studies, however, did not demonstrate a relationship between BG size and symptom severity, which may indicate that BG size and pathophysiology are not directly related.

D8/17

D8/17 is a monoclonal IgM antibody that was isolated by Khanna *et al.*, (1989). This 84 kDa antibody is B-cell specific and has not been detected on T-cells or macrophages. Kemeny *et al.*, (1994) showed that D8/17 recognises a helical-coiled-coil structure on the surface of B-cells that is similar to molecules such as myosin, tropomyosin and the M6 protein of group A streptococci. It cross-reacts with triophosphate isomerase and binding of D8/17 to frozen heart sections can be absorbed by antigens isolated from some strains of group A streptococci, but not by others.

The non-HLA B cell surface antigen, recognised by D8/17, was found to be expressed at higher levels in patients with RF compared to controls (Khanna *et al.*, 1989). Since this marker was not found at elevated levels in post-streptococcal glomerulonephritis or other autoimmune disorders, it may indicate that D8/17 might be an indicator of vulnerability to specific complications of RF, like SC (Gibofsky *et al.*, 1991). Follow-up studies, using a rapid testing procedure, found that patients with a history of RF showed elevated D8/17 levels although they had no active RF at the time of testing (Herdy *et al.*, 1992).

Among psychiatric disorders, childhood-onset OCD and TS were associated with higher expression of D8/17 than control subjects (Chapman *et al.*, 1998; Murphy *et al.*, 1997; Swedo *et al.*, 1997). Niehaus *et al.*, (1999) also reported a significant group difference in the mean expression of D8/17 in a small sample of OCD patients, trichotillomania (TTM) patients and controls (Niehaus *et al.*, 1999). This study showed that South African patients with OCD had significantly higher average percentage of B-cells expressing D8/17; however, because of the relatively small sample size, and high mean percentage of B cells expressing D8/17 in the TTM group and control individuals, a follow up study was needed. The follow-up study forms part of this thesis.

1.2.5. THE PRESENT STUDY

The pathogenesis of OCD still remains largely unknown, with many theories and models being proposed. The present study investigated two proposed mechanisms of OCD pathogenesis, broadly classified as the genetic and the immunological aetiologies of the disease.

The strategy used in the genetic approach, which investigated the role of two candidate genes, namely *COMT* and *SLC6A4* in the pathogenesis of OCD, was a population-based case-control association study. Previously described polymorphisms implicated in the development of OCD, ie., the *COMT/Nla*III and the *SLC6A4/5-HTTLPR* polymorphisms, were used in a comparison of the genotype distribution and allele frequencies between a group of unrelated OCD affected individuals and a group of unrelated control individuals and thus assess the role of these genes in OCD

pathogenesis. In order to reduce genetic complexity, this study was conducted in the homogeneous Afrikaner sub-population of South Africa.

The *COMT/Nla*III polymorphism was genotyped using allele specific restriction enzyme analysis, (ASREA), while the *SLC6A4/5-HTTLPR* polymorphism was genotyped using polymerase chain reaction (PCR) and direct assessment of the size of the amplified products.

A recent report showing that the promoter regions of *COMT* contain several *ERE*s prompted a search for possible polymorphisms within *ERE6*, which has been shown to be important in oestrogen-dependant regulation of *COMT*, in the present study. PCR-single stranded conformational polymorphism (PCR-SSCP) was the method of choice used to determine whether *ERE6* was polymorphic. Upon the detection of a mobility shift within the amplified PCR fragments containing *ERE6*, the fragments were sequenced to identify the variation. The sequence variation thus identified was then genotyped in the study population to determine whether it was associated with OCD.

Furthermore, the role of an immunological marker, D8/17, in OCD was investigated, as previous studies have shown that D8/17 may be a useful trait marker for early onset OCD (Swedo *et al.*, 1997; Murphy *et al.*, 1997) Thus this study aimed to determine whether this marker could possibly be used as a trait marker for late-onset OCD and to evaluate its specificity within the spectrum of anxiety disorders.

CHAPTER 2

MATERIALS AND METHODS

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CHAPTER 2 MATERIALS AND METHODS

PART I: GENETIC STUDY

2.1 STUDY SUBJECTS

All patients and control subjects participating in this study were of Afrikaner descent based on Afrikaans being their home language and at least three of their four grandparents being of Afrikaner descent.

Unrelated OCD patients and controls were recruited through the Medical Research Council (MRC) Unit on Anxiety and Stress Disorders from throughout South Africa by trained clinical psychologists and via media advertisements. These controls were matched to the case subjects according to ethnicity, gender and age. The University of Stellenbosch Ethics Committee approved the protocol and all subjects provided written informed consent.

All patients underwent a structured interview, which included the SCID-I, the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) and the Yale Global Tic Severity Scale (YGTSS) (First *et al.*, 1994; Goodman *et al.*, 1989; Leckman *et al.*, 1989), and which was conducted by a trained research clinician.

All patients met DSM-IV criteria for OCD and had no history of psychotic disorder. Controls underwent a semi-structured interview (SCID-I screening). Control individuals included in the genetic study had no reported history of an anxiety, mood or psychotic disorder. The control group used in the immunological study was divided into three groups namely, an anxiety disorder group, in which subjects met DSM-IV criteria for either SP or PD, a OC spectrum disorder group, in which subjects met DSM-IV criteria for TTM, and a healthy control group, in which subjects had no reported history of any anxiety or mood disorder. All subjects included in the immunological study also had no history of RF or SC.

2.2 BLOOD COLLECTION

Blood samples were drawn from both patients and control individuals by means of venous puncture and collected into 5 ml etheylene-diamine-tetra-acetic acid (EDTA) tubes. The EDTA tubes were collected immediately if blood was drawn at the MRC Unit on Anxiety and Stress Disorders, while blood from patients from around South Africa was couriered to the research laboratory within 24 hours of sampling.

2.3 DNA EXTRACTION

2.3.1. Extracting nuclei from whole blood

Blood from three 5ml EDTA tubes per patient was transferred into a 50ml Falcon tube. The tube was then filled to 20 ml with ice-cold lysis buffer (appendix I). After gently inverting the tubes a few times, the sample was incubated on ice for 5-10 minutes. The sample was then centrifuged at 2500-3000 rpm at room temperature in a Beckman model TJ-6 centrifuge (Scotland, UK). The supernatant was discarded and the pellet was resuspended in 20ml, ice-cold lysis buffer, followed by another round of incubation and centrifugation. The supernatant was then discarded and the pellet resuspended in 900µl sodium-EDTA (appendix I) and 100µl 10% sodium dodecyl sulphate (SDS) (appendix I). The nuclei were then either immediately used for DNA extraction or stored at -70°C until DNA was required.

2.3.2. Extracting DNA from nuclei.

To the freshly prepared or thawed nuclei, 100µl of proteinase K (10µg/ml) was added and the mixture was incubated overnight at 37°C. After this step, 2ml distilled water, 500µl 3M sodium-acetate (appendix I) and 25µl phenol/chloroform (appendix I) were added to the sample. The tubes were subsequently inverted and mixed gently for 10 minutes on a Voss rotator (Voss of Maldon, England) at 4°C. The mixture was then transferred to a glass Corex tube so that the aqueous phase could be clearly distinguished from the organic phase, followed by centrifugation in a Sorvall RC-5B

refrigerated super-speed centrifuge (rotor SS 34, Dupont Instruments) at 8000 rpm for 10 minutes at 4°C.

The upper aqueous phase containing the DNA, was transferred to a clean Corex tube using a sterile plastic pasteur pipette, while taking care not to disturb the interface or the organic phase. Approximately 25ml chloroform/octanol (appendix I) was added to the aqueous phase after which the tube was closed with a polypropylene stopper and gently inverted for 10 minutes. This mixture was then centrifuged at 4°C, followed by the removal of the upper aqueous phase as described earlier. The DNA was then ethanol precipitated by adding two volumes of ice-cold 96% ethanol and inverting gently until DNA strands appeared as a white precipitate.

The DNA strands were removed using a yellow tipped Gilson pipette and placed in a clean, 1.5ml Eppendorf microfuge tube. One millilitre 70% ethanol was then added to the DNA and the mixture centrifuged in a Beckman microfuge for 3 minutes at 13000 rpm. The ethanol was carefully decanted and the 70% ethanol wash repeated one more time in order to remove any excess salts. After careful removal of most of the ethanol, the DNA pellet was air-dried for 30-60 minutes at room temperature by inverting the Eppendorf microfuge tube on Carlton paper. Two hundred microlitres Tris-EDTA (appendix I) buffer was added and the DNA was resuspended initially by stationary incubation at 37°C overnight and subsequently by gentle mixing in a Voss rotator (Voss of Maldon, England) at 4°C for a further 3 days. This was followed by stationary incubation at 4°C until the DNA has been resuspended.

After 1-3 weeks, when the DNA had completely resuspended in the buffer, the optical density (OD) of the DNA was determined in a Milton Roy series 120i spectrophotometer (USA) at 260nm (OD_{260}). The DNA concentration was determined by diluting 10µl of DNA in 500µl of TE and multiplying the measured OD_{260} by a factor of 2.5. This gave the DNA concentration in µg/ µl. The purity of the DNA was determined by calculating the ratio of the OD_{260} and the OD_{280}, which should be approximately 1.8 for pure DNA.

2.4. POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) is a technique which provides a rapid means of exponentially amplifying a specific target region of DNA flanked by unique regions to which oligonucleotide primers are designed (Saiki *et al.*, 1985) (fig 9). The PCR technique was used to amplify the *Nla*III polymorphic (Karayiorgou *et al.*,1997) region and the region containing *ERE6* of *COMT* (Xie et al., 1999), as well as *5-HTTLPR* of *SCL6A4* (Gelernter *et al.*, 1997).

2.4.1. Oligonucleotide primers

Oligonucleotide primers are important in the PCR reaction as they are homologous to the unique sequence flanking the area being amplified, which makes PCR very specific. The DNA polymerase finds where these primers bind to the single stranded DNA before forming a new double strand in the 5'-3' direction during amplification of the area of interest (fig 9). Oligonucleotide primers were synthesised according to standard phosphoramidite methodology at the Department of Biochemistry, University of Cape Town (UCT), Cape Town, South Africa. Primers for the *COMT/Nla*III (Karayiorgou *et* al., 1997) polymorphism and *5-HTTLPR* (Gelernter *et al.*, 1997) were synthesised according to published sequence, while oligonucleotide primers for *COMT/ERE6* were designed from published *COMT* promoter sequence using the DNAMAN (Lynnon Biosoft Copyright©) computer programme. The primer sequences for each polymorphic site genotyped are shown in table 1.

2.4.2. PCR conditions

DNA amplification was performed in a 50µl reaction containing 0.1µg genomic DNA as template, 75µM of each of dATP, dCTP, dGTP and dTTP (Promega Corp., Madison Wisconsin USA), 5µl of a 10x Taq DNA polymerase buffer (Bioline UK Ltd, London, UK), 150ng of each oligonucleotide primer (Table 1), 0.5U Taq DNA polymerase (Bioline UK Ltd, London, UK), 5% glycerol (Sigma chemical company, St Louis, Missouri, USA) and water to a final volume of 50µl. Mineral oil was added

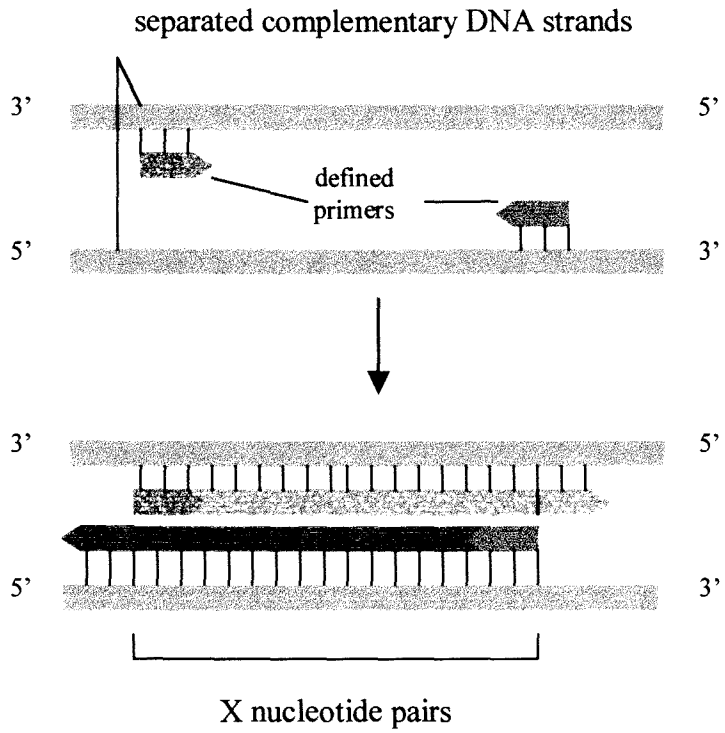

Fig 9a. Amplification of specific nucleotide sequences *in vitro* using PCR. DNA isolated from cells is heated to 94°C in order to separate its complementary strands. Oligonucleotide primers (one complementary to each strand) that have been chemically synthesized according to known sequence flanking the region to be amplified, are then annealed to the single stranded DNA by lowering the temperature from 94°C to a previously determined annealing temperature. The two oligonucleotide primers designed are specific to the target region to be amplified. A DNA polymerase finds the binding site of the primers to the DNA strand, and creates a new double strand using nucleotide bases in the PCR reaction mixture. The newly synthesized strands are complementary to the original separated DNA strands, thus creating two new double-stranded DNA molecules. This cycle is repeated 30 times and the amount of DNA produced by this reaction doubles in each cycle. Adapted from Molecular Biology of the Cell, 3rd edition.

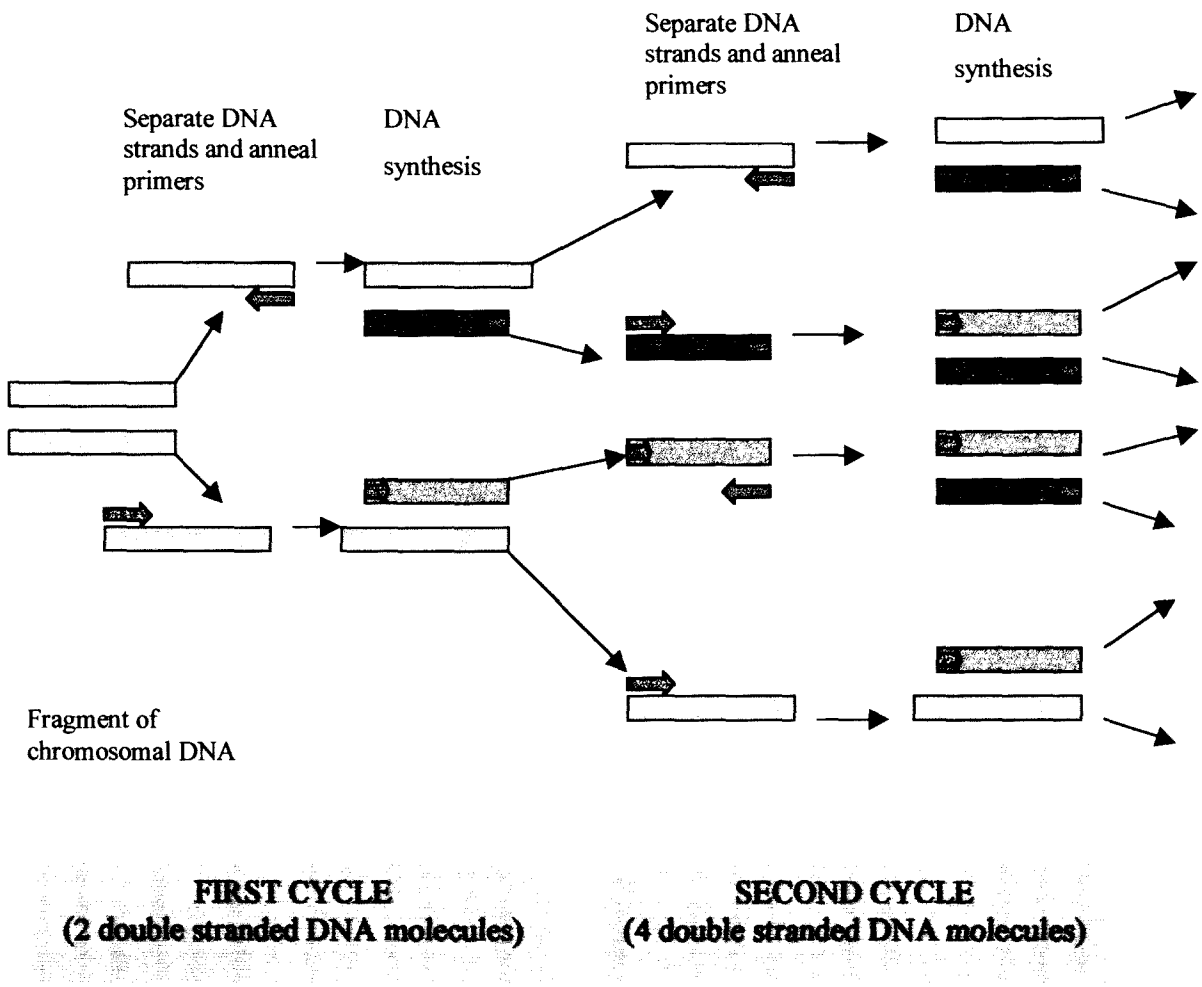

Fig 9b. PCR amplification. PCR produces an amount of DNA that doubles in each cycle of DNA synthesis and includes a uniquely sized DNA species. After numerous cycles of reaction, the population of DNA in the reaction mixture becomes dominated by a single DNA fragment of X nucleotides long (depending on the position of the primer sequences). Adapted from Molecular Biology of the Cell 3rd edition.

to each tube to avoid evaporation during thermal cycling. Amplification was performed in a thermal cycler (Gene E, Techne Ltd., Cambridge, England). The cycling parameters and the PCR conditions for each set of oligonucleotide primers are shown in table 2.

Table 1: Primer sequences used in the amplification of the polymorphic sites in *COMT* and *SCL6A4*.

Gene/ Polymorphism	Primer name	Length bp	Sequence	Reference
COMT/NlaIII	CFP	20	5'-tcaccatcgagatcaacccc-3'	Karayiourgou et al., 1997
	CRP	19	5'-acaacgggtcaggcatgca-3'	
COMT/ERE6	ERE6-F	20	5'-agagtctaggggtctgggga-3'	Present Study
	ERE6-R	20	5'-aaggtcctgctgtgctgact-3'	
SLC6A4/5-HTTLPR	JP	24	5'-atgccagcacctaacccctaatgt-3'	Gelernter et al., 1997
	GR	20	5'-cgaccgcaaggtgggcggga-3'	

Abbreviation

COMT: catechol-O-methyltransferase

ERE6: oestrogen response element 6

5-HTTLPR: serotonin transporter-linked polymorphic region.

bp: base pair

Table2: PCR conditions used in amplification of polymorphic sites in *COMT* and *SCL6A4.*

Primer set	Polymorphism	[$MgCl_2$] mM	TD °C	Time sec	TA °C	Time sec	TE °C	Time sec
CFP/CRP	*COMT/NlaIII*	1.5	94	30	60	30	72	30
ERE6 F/R	*COMT/ERE6*	1.5	94	30	62	30	72	30
JP/GR	*5-HTTLPR*	1.5	94	30	66	30	72	30

The thermocycler was set to perform 30 amplification cycles for all polymorphisms genotyped in the present study.

Abbreviation

COMT: catechol-O-methyltransferase

ERE6: oestrogen response element 6

5-HTTLPR: serotonin transporter linked polymorphic region.

TD: denaturing temperature

TA: annealing temperature

TE: extension temperature

sec: seconds

mM millimolar

°C degrees Celcius

2.5. GEL ELECTROPHORESIS

2.5.1. 12% non-denaturing polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gels were used for size separation of PCR products (section 2.4) and restriction enzyme digests (section 2.6.1), using a 12% polyacrylamide solution (Appendix I).

2.5.1.1. Casting the gel

The gel (Appendix I) was cast between two 100mm x 80mm glass plates. The plates were washed with Cal-liquid hand soap (Cal-Chem, R.S.A.), rinsed with tap water and dried using paper towel. The surfaces of the two plates were then sprayed with 70% ethanol and dried again with paper towel. One millimetre spacers were placed on either side of the back plate and the front plate was then placed on top of the spacers. The plates were held in place using clamps, and the sides and bottom of the plates were then sealed using gel-sealing tape (SIGMA, Germany). The gel solution (appendix I) was then poured between the assembled plates and the well-forming comb was inserted. The gel was left to set for about 20 minutes before electrophoresis.

2.5.1.2. Electrophoresis

After the gel had set, the gel-sealing tape around the sides and bottom of the gel was removed. The gel was then clamped into the gel electrophoresis apparatus (Scigen verticle mini apparatus, Whitehead Scientific, R.S.A.) and the buffer chambers filled with 1 x TBE electrophoresis buffer (appendix I).

Bromophenol blue dye (2µl) was added to each of the samples to be electrophoresed. The samples were then electrophoresed for 1½ - 2 hours at 150 volts.

2.5.1.3. Silver staining

After completion of electrophoresis, the electrophoresis apparatus was dismantled and the gel removed. The gel was then placed in a container containing 50ml of solution B (0.1% $AgNO_3$) and gently shaken for 10 minutes. Solution B was carefully decanted

and the gel rinsed with water. Fifty millilitres of Solution C (appendix I) was then poured into the container, to cover the gel, which was gently agitated until stained bands could be seen. The gel was then viewed on a white light illuminator (Lauda thermostat, Germany) and photographed using a video printer (Sony, Japan).

2.5.2. Mutation analysis by PCR- single strand conformational polymorphism (PCR-SSCP) analysis.

Single-strand conformational polymorphism analysis is a rapid and sensitive method of detecting sequence variation (Orita *et al.*, 1989). Single stranded DNA has a folded conformation, stabilised by intrastrand interactions, which determines its mobility under PCR-SSCP conditions. Consequently, the conformation, and thus the mobility of the single stranded DNA under PCR-SSCP conditions, is dependant on the sequence (Orita *et al.*, 1989). A single base change is enough to change the folded conformation of the single stranded DNA and hence alter its mobility under SSCP conditions. Thus, PCR-SSCP involves the detection of electrophoretic shifts of single stranded DNA with sequence variation from the wild type sequence.

For each panel of samples analysed, a 10% mildly denaturing polyacrylamide gel with 5% glycerol (appendix I) was used for PCR-SSCP mutation screening.

2.5.2.1. Casting the gel

The gel (Appendix) was cast between 390mm x 290mm glass plates, which were first washed with Cal-liquid hand soap (Cal-Chem, R.S.A), rinsed in tap water and wiped dry with paper towel, after which the surfaces were sprayed with 70% ethanol and wiped clean as before. Gelbond™ PAG polyester film (FMC, Bioproducts, Rockland, Maine, USA) was attached to the back plate by its hydrophobic side, to facilitate this, 70% alcohol was lightly sprayed onto this plate. The hydrophilic side of the Gelbond™ was exposed so that, when the gel solution was poured, the two would bond covalently, thus creating a solid support for the gel for subsequent silver staining. The front plate was silinised with paper towel spotted with Wynn's C-THRU windshield rain dispersant (Wynn Oil S.A. Pty Ltd,SA).

One-millimetre spacers were placed on either side of the Gel bond-covered back plate and the front plate then placed on top with the C-THRU-coated side on the inside. The edges, on the sides and bottom of the plates, were sealed with gel-sealing tape (SIGMA, Germany), after which the casting rubber boot (S2 casting boot, Life™ Technologies, UK) was fitted for further sealing. The gel solution was poured with the glass plate assembly slanted at an angle. Immediately thereafter, the glass plates assembly was laid down horizontally, a square-tooth, well-forming comb fitted, the plates clamped and the gel allowed to set for ~1hr.

2.5.2.2. Electrophoresis

Thereafter, the casting boot, the sealing tape at the bottom of the plates and the comb were removed, in this order. The glass plate assembly was mounted vertically on the electrophoresis apparatus (Omeg Scientific, R.S.A), moved to the cold room (4°C) and 0.5xTBE (appendix I) buffer added to the reservoir tanks. The wells were washed with buffer to remove any residues, and the denatured PCR sample (denatured at 94°C for 2 minutes) immediately loaded. Electrophoresis was carried out in 0.5x TBE buffer, at a constant power of 50W overnight at 4°C.

2.5.2.3. Silver Staining

Following electrophoresis of samples on the gels, the glass assembly was dismantled and the gel still supported by the Gelbond™, was silver stained as discussed in section 2.5.1.3.

2.6. GENOTYPING

Polymorphisms can be detected in a variety of ways, depending on the type of polymorphism. In the present study, two known polymorphisms were investigated. Each of these polymorphisms required a different detection method.

The *COMT/Nla*III polymorphism is the result of a G to A transition and which creates a restriction site for the restriction endonuclease *Nla*III (Karayoirgou *et al.*, 1997). It is, therefore, possible to detect alleles of this polymorphism based on the presence or absence of the restriction enzyme site. This technique is referred to as Allele Specific Restriction Enzyme Analysis (ASREA).

The *5-HTTLPR* polymorphism, on the other hand, does not affect a restriction enzyme site. This polymorphism is represented by a 44 bp insertion or deletion. Detecting the different alleles in this type of polymorphism involves PCR amplification of the target region, followed by gel electrophoresis. The allele containing the 44bp insertion yields a larger PCR fragment than the allele without the insertion. Thus the detection of *5-HTTLPR* polymorphism is purely based on the differences in size between the generated PCR fragments representing each allele.

Another aim of this study was to determine whether *ERE6* in *COMT* is polymorphic. In order to detect an unknown polymorphism or mutation, PCR-SSCP can be used. This technique involves PCR amplification of the target region of interest followed by SSCP analysis.

2.6.1. ALLELE SPECIFIC RESTRICTION LENGTH POLYMORPHISM (ASREA) ANALYSIS OF *COMT/NlaIII* POLYMORPHISM

PCR-based allele specific restriction enzyme analysis was used to screen OCD patients and control individuals for the previously reported *Nla*III polymorphism in exon 4 of the *COMT* gene.

Five microlitres of the amplified PCR-product was aliquoted into a 500µl eppendorf microfuge tube. To this, 1µl of a 10x *Nla*III buffer (New England Bioloabs), 0.5U *NlaIII* restriction enzyme (New England Biolabs) and water to a final volume of 10µl were added. The mixture was incubated at 37°C for 3 hours to allow digestion to proceed. Fig 10 shows the expected sizes of the digested products.

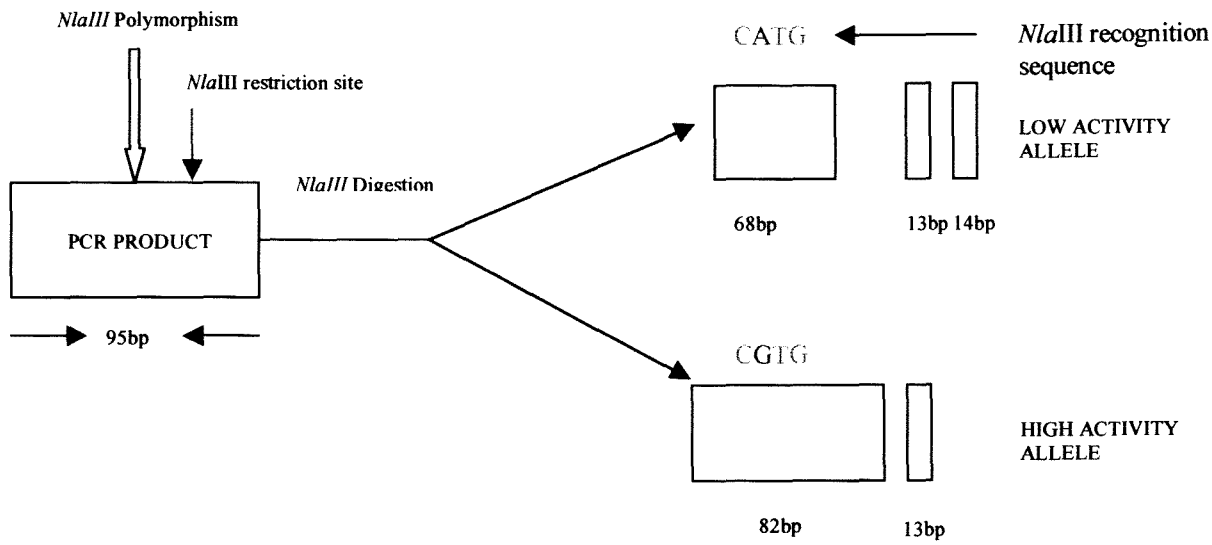

Fig 10: Expected size fragments generated by digestion of *COMT/NlaIII* amplified product with *NlaIII* restriction enzyme. The red arrow indicates the position of a non-polymorphic *Nla*III restriction site in the amplified region

2.6.2. Genotyping of *5HTTLPR* polymorphism

The region containing the 44bp (*l/s*) polymorphism was amplified from genomic DNA using the primer set *5-HTTLPR* (JP and GR) shown in table 1 under the PCR conditions shown in table 2. Alleles were then size-separated by electrophoresis in 12% polyacrylamide gels. Each sample was then genotyped according to the presence of an insertion or deletion.

Meta-analysis of *SLC6A4/5-HTTLPR* polymorphism.

The meta-analysis of the 5HTTLPR polymorphism was performed using a combination study of subjects shown in table 3 and subjects from a study by Bengel *et al* (1999), which consisted of 75 unrelated Caucasian OCD patients (39 male and 36 female), all of whom underwent the Structured Clinical Interview for DSM-III-R as well as the Yale-Brown Obsessive-compulsive scale (Y-BOCS), and 397 unrelated

healthy controls (Bengel *et al.*, 1999). The data from the present study and the above mention study was pooled and statistical analysis was performed.

2.6.3. PCR-SINGLE STRAND CONFORMATIONAL POLYMORPHISM (PCR-SSCP) ANALYSIS OF *COMT/ERE6*

The region of *COMT* containing *ERE6* was subjected to PCR-SSCP analysis to determine whether a polymorphic site within *ERE6* existed (section 2.5). Mobility shifts detected were then sequenced (discussed in the next section).

2.6.4. SEQUENCING

A mobility shift detected by PCR-SSCP analysis indicated the presence of sequence variation in the *COMT/ERE6* PCR-amplified product. In order to confirm the presence of a sequence variation and determine what the variation was, three samples with mobility shifts were sequenced.

2.6.4.1. Purification of PCR-fragment.

Before sequencing any PCR product, it is important to purify the product in order to avoid generation of incorrect sequence data.

The *COMT/ERE6* PCR products were purified using the QIA quick gel extraction kit (Qiagen, Germany), following manufacturer's instructions. The *COMT/ERE6* amplified products were loaded onto a 2% agarose gel, containing ethidium bromide, and electrophoresed for 1 hour in 1x TBE running buffer at room temperature. The DNA was visualised on a longwave transilluminator (3UV transilluminator, California USA) and the correctly sized fragment (as indicated by the λ*Pst*I molecular weight marker) excised from the gel using a sterile scalpel blade. The gel slice was placed into a 1.5ml Eppendorf microfuge tube and weighed. Three volumes of the QX1 buffer was added to 1 volume gel.

The gel slice in the QX1 buffer (Qiagen, Germany) mixture was incubated for 10 minutes at 50°C or until the gel has completely dissolved. One gel volume of

isopropanol was added to the sample and gently mixed (in order to increase the yield of small DNA fragments). A Qiaquick spin column was placed into a provided 2ml collection tube and the sample was applied to the column and centrifuged in a Beckman microfuge (Germany) at 13000rpm for one minute, in order for the DNA to bind the column. The flowthrough was discarded and the column was replaced in the same collection tube. Buffer QX1 (500µl) was then added to the column and centrifuged at 13000 rpm for one minute to remove all traces of agarose. Fifty microlitres distilled water was added to the column, in order to elute the DNA. The DNA was subsequently dried in a Speedivac (Savant, New York, USA) and was resuspended in 10µl of distilled water.

2.6.4.2. Automated sequencing

Both coding and non-coding strands of the PCR-fragment containing *ERE6* was directly sequenced using primers ERE6-F and ERE6-R (table 1) at the Department of Genetics at the University of Stellenbosch, Stellenbosch, South Africa, using an ABI Prism^{TM} 377 automated sequencer.

2.6.4.3.Genotyping

As the polymorphism detected in this region did not affect any restriction enzyme site, the SSCP banding pattern was used to separate the alleles. The SSCP banding pattern was compared to sequence data, to determine which banding pattern represented which allele.

2.7. STATISTICAL ANALYSIS

2.7.1. Calculating allele frequencies

The frequency of high (*H*) and low (*L*) alleles of the *COMT/NlaIII* polymorphism, long (*l*) and short (*s*) alleles of *5-HTTLPR* and *C* and *T* alleles of *COMT/ERE6* alleles were calculated by dividing the total number of a particular allele by the total number of chromosomes in the study group.

2.7.2. Calculating expected allele frequencies

In order to predict the expected distribution of alleles at each of the loci in the study, the Hardy-Weinberg formula was used. This formula predicts that $p^2+2pq+q^2=1$ (where p= frequency of allele A and q= frequency of allele B), if the genotypes in the population are in Hardy-Weinberg equilibrium. The observed allele frequencies at each locus were inserted into the Hardy-Weinberg equation, while the expected number of genotypes was calculated by multiplying the values of p^2 (AxA), 2pq [2(AxB)] and q^2 (BxB) by the total number of individuals genotyped in the control and OCD patient groups.

2.7.3. χ^2 analysis of the expected versus observed genotype frequencies

To determine whether the genotype frequencies were in Hardy-Weinberg equilibrium, the expected and observed genotype frequencies for OCD patients and control individuals were displayed in a 3X2 contingency table and χ^2 analysis performed using the Software Programme for Social Science (SPSS).

2.7.4. Determination of the association between OCD and the polymorphisms at the various loci

The frequency of genotypes observed at each locus for OCD patients and that of the control individuals were displayed in a 3x2 contingency table. Chi-square analysis was performed using the SPSS to determine the statistical significance differences between these values.

PART II: IMMUNOLOGICAL STUDY

2.8. STUDY SUBJECTS

Subjects recruited for this part of the study were between the ages of 9 and 65 years. Patients with OCD, TTM, PD and SP were recruited from the MRC unit on Anxiety

and Stress Disorders. Control individuals were recruited as described in section 2.1. Diagnostic criteria for each subjects is as described in section 2.1. Inclusion criteria for affected individuals were a predominant DSM-IV diagnosis of OCD, TTM PD or SP. The control group had no past or current psychiatric disorder, RF or SC. The psychiatric control were further stratified into two groups namely, the anxiety disorder group, in which patients had a predominant DSM-IV diagnosis of either SP or PD, and a OC spectrum disorder group, in which patients had a predominant diagnosis of TTM.

2.9. BLOOD COLLECTION

Blood was collected as described in section 2.2.

2.10. FLOURESCENCE ACTIVATED CELL SORTING (FACS)

Flow cytometry allows investigators to analyse cells at the single cell level by measuring specific characteristics of the cells. On each cell, several characteristics can be measured. These include the forward scatter (FSC), side scatter (SSC) and fluorescence (FL). Prior to analysis, the cells are stained with two antiboby-conjugated dyes, which have the same excitation range, but different emission maxima.

During analysis, cells are separately sucked though a thin capillary tube and shot with an argon laser beam with a wavelength of 488 nm. The antibody-conjugated dyes on stained cells become excited and emit a light signal. In a flow cytometer, these signals are proportional to the amount of light detected. The FSC and SSC detectors measure the scatter of the light signal. Forward scatter is a measure of cell size, while SSC is the measure of the granularity of the cell population. All these data are then represented on dot plots or histograms (fig 17) using Cell Quest software.

2.10.1. LABELLING OF CELLS

Labelling of cells always took place within 6 hours of blood being drawn. Fifty microlitres of whole blood was aliquoted into a 3ml plastic Falcon FACS tube. To the

blood, 20µl of phycoerythin-conjugated monoclonal HLA-DR (Anit-MHC Class II) was added to identify B cells. The mixture was incubated in the dark at room temperature for 20 minutes, after which 2ml ice cold phosphate buffered saline (PBS) was added. The sample was gently mixed by carefully inverting the tube. This was followed by centrifugation at 300rpm for five minutes. The clear supernatant was discarded and to the pellet, 20µl FITC-conjugated antimouse IgM antibody was added for detection. The tube was again incubated in the dark for 20 minutes at room temperature, followed by another PBS wash cycle (addition of PBS, centrifugation, discarding of supernatant). Fifty microlitres D8/17 antibody was aliquoted into the tube, which was again incubated in the dark for 20 minutes. This was followed by another PBS wash cycle. To the pellet, 2ml FACS lysing solution was added and the sample was mixed by vortexing (Vortex Genie2). The sample was incubated in the dark for 10 minutes at room temperature followed by centrifugation at 300rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 500µl FACS fixing solution. The sample was then either immediately used for flow cytometry or stored at 4 C until needed.

2.10.2. FLOW CYTOMETRY

The analysis of the preparation was based on the lymphocyte scatter gate (low side scatter and high forward scatter) and within this gate, positivity for MHC Class II was determined. This identified B-cells falling within the gate. Within this gate the expression of D8/17 positive B cells was determined. The percentage of B-cells expressing D8/17 was determined using a Facscalibre^{TM} flow cytometer (Beckton Dickenson) and analysed by the Cell Quest software program at the Department of Medical Microbiology, University of Stellenbosch.

2.10.3. STATISTICAL ANALYSIS

Dr. Carl Lombard at the Medical Research Council, South Africa, performed analysis of variance and post-hoc Tukey procedure for correction for multiple testing on the D8/17 data generated.

CHAPTER 3

RESULTS

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CHAPTER 3: RESULTS

3.1. Recruitment of subjects

The numbers of subjects analysed in each section of this study are shown in tables 3 and 4.

The *COMT* and the *SLC6A4* studies were run concurrently, therefore, each study group genotyped for *COMT/Nla*III was also genotyped for *5-HTTLPR*. The DNA samples used in these two investigations were also used by other researchers for different studies. These studies included association studies of other candidate genes by another investigator in our laboratory and, furthermore, 500µl of each sample was couriered to investigators in the United States for collaborative studies. As a result of this, 14 OCD patients' DNA samples were depleted by the time the *COMT/ERE6* study had started. This accounts for the unequal number of patients analysed in each study. Subsequent results (see section 3.2.1.) obviated the need to collect further control samples for the *COMT/Nla*III study, but required the recruitment of more control individuals for the *5-HTTPLR* study (section 3.3.). The same control samples used in the *COMT/Nla*III study were also used in the *COMT/ERE6* study, however, six of these control samples were also depleted because of their use in concurrent studies. For these reasons, there are discrepancies between the numbers of control individuals used in each study.

When the immunological study started, many patients had already been recruited for the genetic study and had donated blood. Subsequently, many of these patients were either unwilling or unavailable to donate more blood for the immunological study. This accounts for the discrepancy between the patient numbers in the genetic and immunological studies.

Table 3. Number of OCD patients and control individuals recruited for genetic studies

	Number of study subjects					
	OCD patients			Control individuals		
Polymorphism Studied	Total	Male	Female	Total	Male	Female
*COMT/Nla*III	54	26	28	54	26	28
COMT/ERE6	40	25	15	48	20	28
5-HTTLPR	54	26	28	82	40	42

OCD: Obsessive-compulsive disorder

Table 4. Number of OCD patients and control individuals assessed in immunological study

	Number of study subjects		
	Total	Male	Female
OCD	26	12	14
Healthy control	26	10	16
Psychiatric controls: *SP* and *PD*	16	10	6
TTM	22	3	19

OCD: Obsessive-compulsive disorder

SP: Social phobia

PD: Panic disorder

TTM: Trichotillomania

3.2. Genotyping of polymorphisms in *COMT*

3.2.1. ASREA of *COMT/Nla*III polymorphism

PCR amplification of the region flanking the *COMT/Nla*III polymorphic site in exon 4 of *COMT* yielded a 95 bp fragment (fig. 11). This fragment also contains a non-variant *Nla*III restriction enzyme site, which served as an internal control to monitor enzyme activity and as an indication of incomplete digestion (fig 10). Therefore, digestion of this product with *Nla*III restriction enzyme yielded three fragments of 68 bp, 14 bp and 13 bp in size for the *L* allele or two fragments of 82 bp and 13bp for the *H* allele. Consequently, in an *H/L* heterozygous individual, four fragments are expected. However, the 13 bp and the 14 bp fragments were not visualised since they were electrophoresed from the gel because of their small size (fig 12).

In the present association study, both patient and control groups were in Hardy-Weinberg equilibrium (table 5). A statistically significant difference was detected in genotype distribution of the *COMT/Nla*III polymorphism between the two groups, with a predominance of the *H/L* genotype in the OCD patient group (p=0.0017) (table 6). When the study sample was stratified according to gender, a statistically significant difference was generated between either male or female patients and controls (p=0.043 and 0.029, respectively) (table 6). There was, however, no statistical difference between the distribution of *H* and *L* alleles in OCD and control groups (0.44 *H*: 0.56 *L*, OCD; 0.46 *H*: 0.54 *L*, control) (table 7).

3.2.2. *COMT/ERE6* analysis

3.2.2.1. Detection of novel sequence variant

The amplified 150 bp PCR product containing *COMT/ERE6* is shown in figure 13. The PCR fragments were subjected to PCR-SSCP analysis to detect the presence of any sequence variation. A frequently occurring mobility shift was detected in both OCD and control groups (fig 14). Subsequent sequence analysis of three individuals

revealed a single nucleotide change 8 bp 5' to *ERE6* (fig 15), in which the variation is represented by a C-T transition. Single nucleotide polymorphism (SNP) database

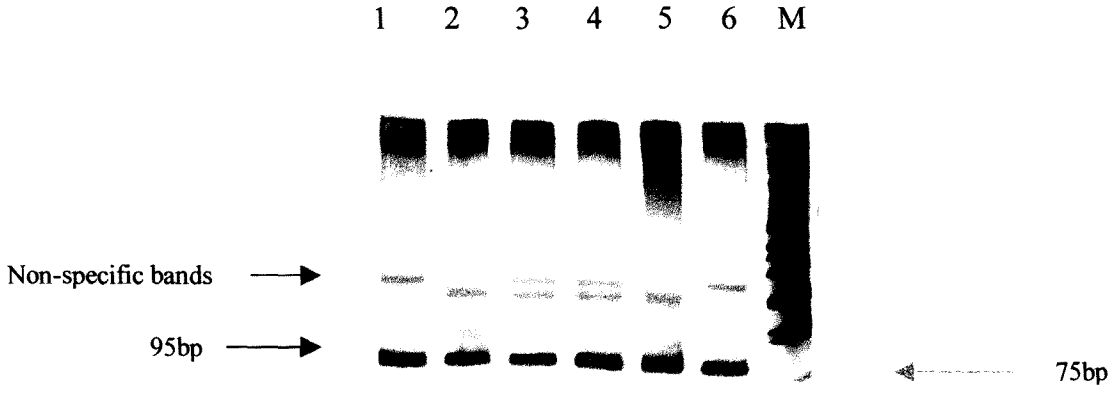


Fig 11: PCR amplification of exon 4 containing *COMT/Nla*III polymorphism.
12% polyacryalamide gel showing the fragment size generated by PCR using the primers 5'-tcaccatcgagatcaacccc-3' and 5'-acaacgggtcaggcatgca-3'. Lane M: Molecular weight marker (λDNA digested with *Pst*I restriction enzyme; the 75bp fragments indicated by blue arrow). Lanes 1-6: 95 bp PCR fragment (indicated by black arrow) containing the *Nla*III polymorphism. Because of the sensitivity of silver staining, many non-specific DNA bands (indicated by red arrow) are visualised, which would not normally be visible on ethidium bromide-stained gels.

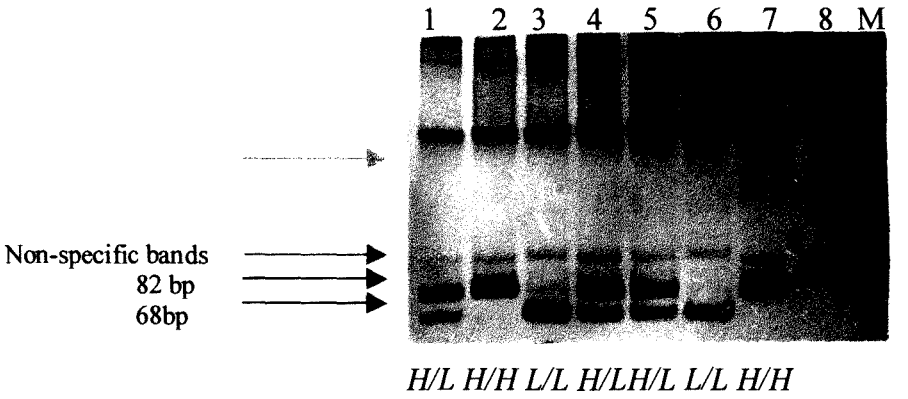


Fig 12: ASREA of reported *COMT/Nla*III polymorphism.

A representative 12% polyacrylamide gel of *Nla*III restriction enzyme digest of *COMT* exon 4 digested PCR amplified products. Lane M: Molecular weight marker (λDNA digested with *Pst*I); Lane1: undigested PCR product; Genotypes in Lanes 2 and 7: *H/H*; Lanes 1,4 and: *H/L*; Lanes 3 and 6: *L/L*. Higher molecular weight non-specific bands can also be seen (red arrow). The band indicated by the blue arrow is bovine serum albumin, which is present in the restriction enzyme reaction mix.

Table 5. Comparison of expected and observed distribution of *COMT/Nla*III genotypes in OCD patients and control individuals by χ^2 analysis.

Study group	Number	*H/H*	*H/L*	*L/L*	P-value
OCD	Expected	10	27	17	0.09
	Observed	5	38	11	
Control	Expected	11	27	16	0.38
	Observed	15	20	19	

Table 6: *COMT* genotype distribution in patients and controls

	OCD Patients (N=54)			Control Individuals (N=54)		
*COMTNla*III Genotype	Total	Male	Female	[1]Total	[2]Male	[3]Female
H/H	5 (9.3%)	4 (15.4%)	1 (3.6%)	15 (28%)	9 (34.6%)	6 (21.4%)
H/L	38 (70.4%)	18 (69.2%)	20 (71.4%)	20 (34.6)	9 (34.6%)	11 (39.3%)
L/L	11 (20.3%)	4 (15.4%)	7 (25%)	19 (35%)	8 (30.8%)	11 (39.3%)

[1] χ^2= 12.72; 2 df; **p=0.0017**

[2] Male OCD versus control **p=0.043**

Genotype percentages shown in brackets

[3] Female OCD versus control **p=0.029**

Table 7: *COMT/Nla*III allele frequencies in patient and control individuals

	H allele	*L* allele
OCD patients	0.44	0.56
Control individuals	0.46	0.54
	χ^2= 0.2; 2df; p=0.89	

searches at http://www.ncbi.nlm.nih.gov/SNP/get_html revealed that the detected sequence variation (hereafter refered to as *COMTP2-1040-C/T)* had not previously been reported.

3.2.2.2. Genotyping of novel sequence variant

Since the *COMTP2-1040-C/T* polymorphism did not affect any restriction enzyme site, patient and control samples were genotyped for the C/T change using PCR-SSCP banding patterns (fig 14). Both OCD and control groups were in Hardy-Weinberg equilibrium (table 8) as indicated by the p-values of 0.96 (OCD) and 0.41 (control), when comparing observed and expected genotype distribution.

Chi-square analysis, applied to the data to determine whether this variation was associated with OCD, revealed no significant difference in the genotype distribution (p=0.45) or allele frequency (p=0.26) between patient and control groups (table 9). Allele frequency data suggested that this polymorphism is commonly occurring within the study population (i.e., in more than 1% of the population) and therefore the *COMTP2-1040-C/T* polymorphism can be classified as an SNP. The *COMTP2-1040-C/T* polymorphism was submitted to the SNP database at http://www.ncbi.nlm.nih.gov/SNP/get_html (Accession number: AF001102).

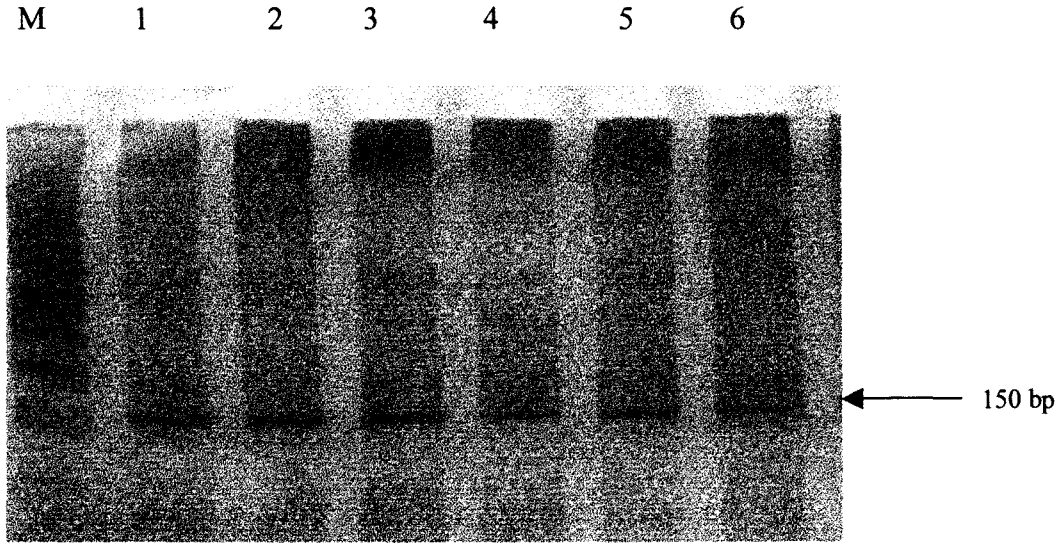


Fig 13: PCR amplification *COMT* promoter region 2 containing *ERE6.*

12% polyacrylamide gel showing fragment size generated by using primers 5'-agagtctaggggtctgggga-3' and 5'-aaggtcctgctgtgctgact-3' in PCR. Lane M: Molecular weight marker (λDNA digested with *Pst*I restriction enzyme); Lanes 1-6: 150 bp PCR fragment of *COMT* promoter region 2 containing *ERE6*

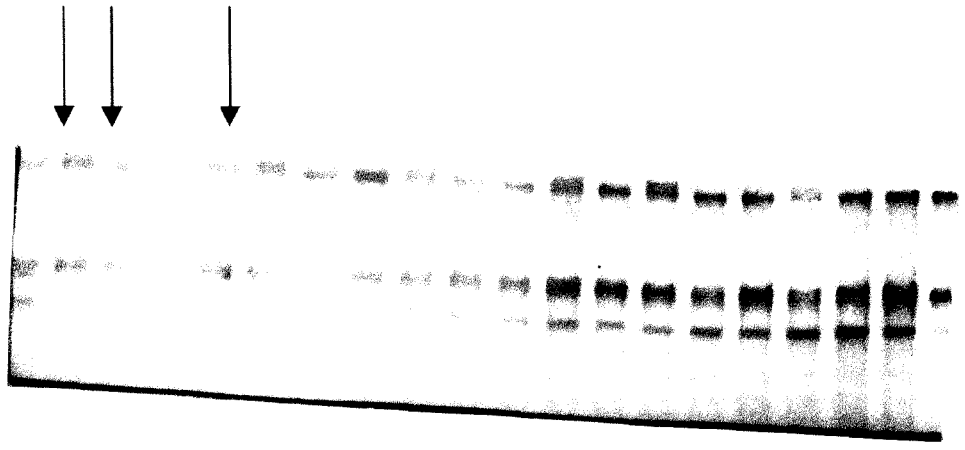


Fig 14: PCR-SSCP analysis of *ERE6*-containing region on a 10% mildly denaturing polyacrylamide gel with 5% glycerol. SSCP mobility shifts are indicated by arrows above the lanes. The arrows each indicate a different genotype of the detected sequence variation. The purple arrow shows the C/C genotype (C allele shown in fig 15 A), the red arrow shows the T/T genotype (T allele shown in fig 15 B) and the black arrow shows the C/T heterozygote genotype

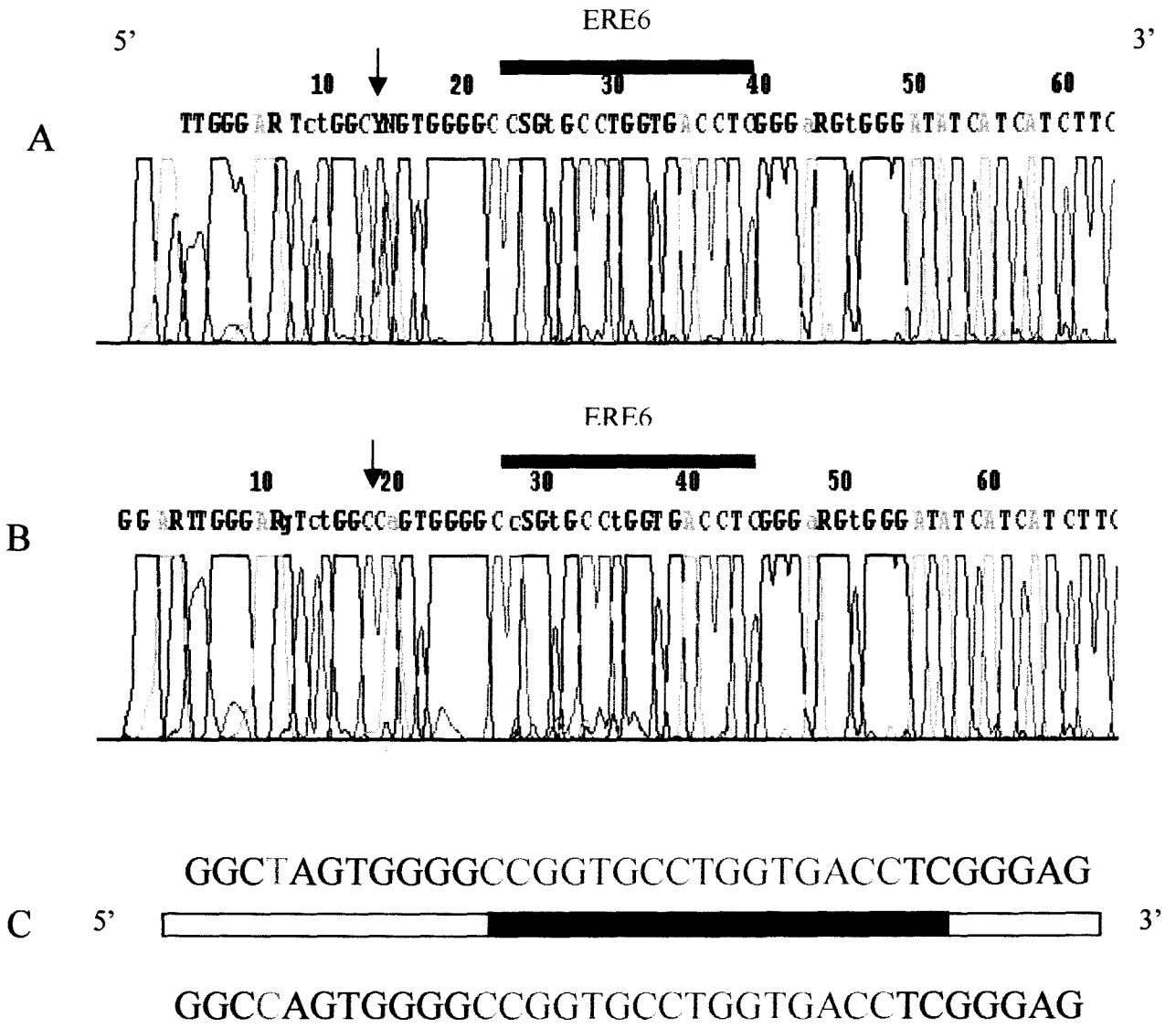


Fig 15: Partial sequence of promoter region of *COMT* containing *ERE6*. Vertical arrows show the position of the novel C-T transition. Magenta bars represent the position of the *ERE6*. **A** represents the *T* allele, while **B** represents the *C* allele on the coding strand. In A, the sequence text shows a 'Y' at the position of the polymorphism. Looking at the sequence trace, however the red peak below the 'Y' indicates the presence of a 'T' at that position. **C** shows a schematic representation of the sequence trace. The purple rectangle indicates the position of the half-palindromic *ERE6*. The magenta font indicates the half-palindrome *ERE* consensus sequence. The top sequence represents the T allele, while the bottom sequence represents the C allele (as indicated by the blue font).

Table 8. Comparison of expected and observed distribution of *COMTP2-1040 – C/T* genotypes in OCD patients and control individuals by χ^2 analysis

		Number of subjects			
Study group	Number	*C/C*	*C/T*	*T/T*	P-value
OCD	Expected	29	10	1	0.96
	Observed	30	9	1	
Control	Expected	24	20	4	0.41
	Observed	30	16	2	

OCD: Obsessive-compulsive disorder

Table 9: *COMTP2-1040-C/T* genotype distribution and allele frequencies in OCD patients and control individuals

	[1] Genotype distribution			[2] Allele frequency		
	C/C	*C/T*	*T/T*	Total	*C*	*T*
OCD	30 (75%)	9 (22.5%)	1 (2.5%)	40	0.86	0.14
Control	30 (62.5%)	16 (33.3%)	2 (4.2%)	48	0.79	0.21

[1] χ^2= 1.58; 2df; p=0.45 [2] χ^2= 1.25; 2df; p=0.26

Genotype percentage shown in brackets

OCD: Obsessive-compulsive disorder

3.3. Genotyping of *5-HTTLPR* polymorphism

3.3.1. Analysis of data generated in the present study

PCR amplification of the region of *SLC6A4* encompassing *5-HTTLPR* yielded a 419 bp fragment for the *l/l* genotype and a 375 bp fragments for the *s/s* genotype, with the *l/s* heterozygote yielding two fragments 419bp and a 375 bp in size (fig 16). Both experimental and control groups were in Hardy-Weinberg equilibrium (table 10).

Table 11 shows the distribution of genotypes and allele frequencies observed in the present study. No statistical differences between the observed genotype frequencies (p=0.19) or allele frequencies (p=0.067) were detected between the OCD and control groups.

3.3.2. Meta-analysis of *5-HTTLPR*

Because of the relatively small sample size analysed in the present study, a meta-analysis of *5-HTTLPR* was performed, in order to improve the power to detect a statistically significant difference between patients and controls. The meta-analysis consisted of the present study (table 11) and data generated by Bengel *et al.*, (1999) (table 12), thereby examining a total of 129 OCD patients and 479 control individuals (table 13).

The results of the meta-analysis showed that there was no significant difference in the distribution of *5-HTTLPR* genotypes (p=0.11) and allele frequencies (p=0.56) between patient and control groups.

Fig 16 PCR of *SLC6A4/5-HTTLPR* . 12% polyacrylamide gel showing fragments generated by PCR using primers 5'-agagtctaggggtctgggga-3'and 5'-aaggtcctgctgtgctgact-3'. Lane M: molecular weight marker (λDNA digested with PstI); Lanes 1,6,9 and 10: s/s; Lanes 2,3,5 and 7: *l/s; Lanes 4 and 8: l/l*

Table 10: Comparison of expected and observed distribution of *5-HTTLPR* genotypes in OCD patients and control individuals by χ^2 analysis

		Number of subjects			
Study group	Numbers	*l/l*	*l/s*	*s/s*	P-value
OCD	Expected	11	27	16	1.00
	Observed	11	27	16	
Control	Expected	27	40	15	0.98
	Observed	27	39	16	

Table 11: Distribution of *5-HTTLPR* genotypes and allele frequencies in OCD patients and control individuals of Afrikaner descent

	[1]Genotype distribution				[2]Allele frequency	
	l/l	*l/s*	*s/s*	Total	*l*	*s*
OCD	11 (20.4%)	27 (50%)	16 (29.6)	54	0.45	0.55
Control	27 (32.9%)	39 (47.6)	16 (19.5%)	82	0.57	0.43

[1] $\chi^2 = 3.29$; 2df; p=0.19 [2] $\chi^2 = 2.88$; 2df; p=0.067

Genotype percentage shown in brackets

Table 12. Distribution of *5-HTTLPR* genotypes in study by *Bengel et al* (1999)

	[1] Genotype distribution				[2] Allele frequency	
Study group	*l/l*	*l/s*	*s/s*	Total	*l*	*s*
OCD	35 (46%)	29 (38.7%)	11 (14.7%)	75	0.66	0.34
Control	98 (24.7%)	214 (53.9)	85 (21.4%)	397	0.51	0.49

[1] $\chi^2 = 5.19$; 2df; p=0.023 [2] $\chi^2 = 4.04$; 2df; **p=0.044**

Genotype percentage shown in brackets

Table 13: Distribution of *5-HTTLPR* genotypes and allele frequency in meta-analysis

	[1]Genotype distribution				[2]Allele frequency	
	l/l	*l/s*	*s/s*	Total	*l*	*s*
OCD	46 (35.7%)	56 (43.4%)	27 (20.9%)	129	0.57	0.43
Control	125(26.1%)	253(52.8%)	101(21.1%)	479	0.53	0.47

[1] $\chi^2 = 4.29$; 2df; p=0.11 [2] $\chi^2 = 0.32$; 2df; p=0.56

Genotype percentages shown in brackets

3.4. Analysis of D8/17 expression

Figure 17 shows a representative FACS analysis data set determining the percentage D8/17 expressing B-lymphocytes. Analysis of variance of mean D8/17 expression showed a significant difference between groups ($F=131.6$, $df=84$, $p=0.01$), with no age ($p=0.39$) or sex ($p=0.81$) effect. D8/17 levels were found to be significantly higher in the OCD patient group (22.95%) than in the group with anxiety disorders (9.97%)($p < 0.0001$), or the normal control group (16.09%) ($p = 0.041$), but not the TTM group (16.05%)($p = 0.097$)(Table 14).

Post-hoc Tukey procedure for correcting for multiple comparisons revealed only one significant difference; D8/17 expression was significantly higher in the OCD group than in the anxiety disorder group ($p=0.006$) (table 15).

The OCD study group was stratified according to age of onset of disease. Fifteen of the twenty-six OCD patients had an onset of symptoms after the age of 10 years (late onset). The expression levels of D8/17 showed no significant difference between early and late onset of disease (table 16). An association with past or present motor or vocal tics was also evaluated, therefore the OCD study group was restratified according to whether they had a history of comorbid tic disorders. The data shows that vocal or motor tics (3/26) in OCD patients were also not associated with higher D8/17 expression (table 16).

Table 14: Analysis of expression of D8/17 in OCD, psychiatric and non-psychiatric control groups by Analysis of Variance (ANOVA)

	OCD	Normal Controls	SAD/PD	TTM
% D8/17 expression	22.95 (±14.2)	16.09 (±8.5)	9.97 (±4.3)	16.05 (±13.9)
[1]p-values		**p = 0.041**	**p = 0.0001**	p = 0.097

[1] p-value of OCD versus other groups

Table 15: Expression of D8/17 in OCD, psychiatric and non-psychiatric control groups corrected for multiple testing by the post-hoc Tukey procedure

	Study groups			
	OCD	Normal Control	SP/PD Control	TTM Control
Number of subjects	26	26	16	22
% D8/17 expression	22.95 (±14.2)	16.09 (±8.5)	9.97 (±4.3)	16.05 (±14)
p- value [1]		0.20	**0.006**	0.26

[1] p-value of OCD versus other groups

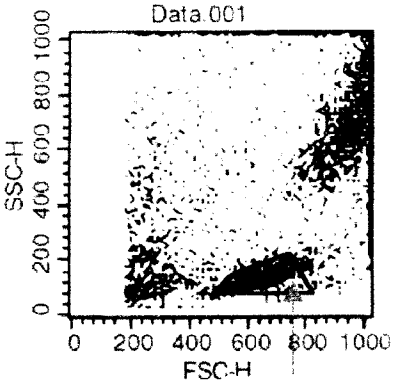
Table 16: D8/17 expression in the OCD group, stratified according to age of onset and lifetime history of tics

	Age of onset[1]		Tics[2]	
	Early	Late	Present or history	Absent
Number of subjects (26)	11	15	3	23
% D8/17 expression (mean)	21.85 (±15.5)	23.76 (±13.7)	12.93 (±12.6)	24.26 (±14.1)
p-value		0.74		0.20

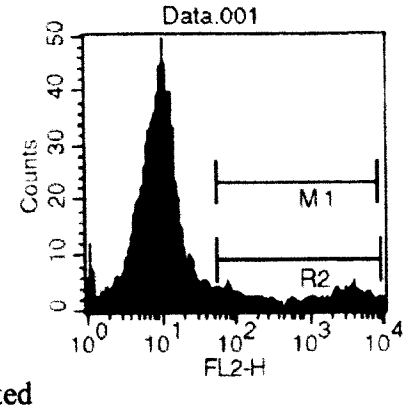
[1] Late onset defined as onset after the age of 10 years.

[2] Lifetime history of vocal or motor tics.

A

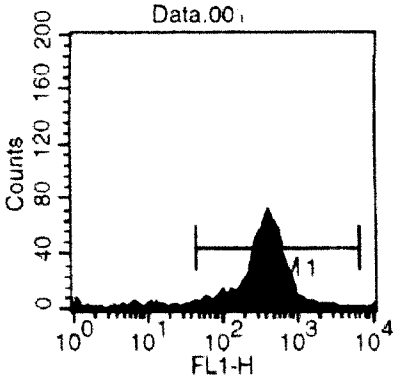


B



Lymphocytes gated

C



Acquisition Date: 7-Oct-98 Gate: G1
Gated Events: 4682 Total Events: 17640
X Parameter: FL2-H (Log)

D

Marker	Left, Right	Events	% Gated	% Total	Mean	Ge
All	1, 9910	4682	100.00	26.54	162.86	
M1	55, 8354	396	8.46	2.24	1575.30	

E

Acquisition Date: 7-Oct-98 Gate: G2
Gated Events: 7617 Total Events: 17640
X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Ge
All	1, 9910	7617	100.00	43.18	409.33	
M1	44, 6552	7320	96.10	41.50	408.46	

Fig 17. A typical flow cytometric measurement of the level of D8/17 expression.

A: Dot plot showing the forward scatter (FSC) (x-axis) and the side scatter (SSC) (y-axis) of each cell analysed in this representational FACS analysis. Lymphocytes are characterised by low SSC. The lymphocytes are gated for analysis (blue arrow).
B: Histogram showing the gated lymphocytes in **A**. The number of cells (x100) is shown on the Y-axis, while the HLA-DR fluorescence (FL2-H) is shown on X-axis. HLA-DR is B-cell specific and thus all B-cells are stained with this dye. The region of the histogram marked M1 and R1 shows the amount of B-lymphocytes among the lymphocyte population gated in **A**, while the region to the left of M1 and R1 indicate T-lymphocytes. **C:** Histogram showing the number of D8/17 expressing B-lymphocytes (M1) within the population of B-lymphocytes under regions M1 and R1 in **B**. **D:** A tabular representation of the histogram in **B**. **E:** A tabular representation of the histogram shown in **C**. The blue box indicates the percentage D8/17 expressing B-lymphocytes.

CHAPTER 4

DISCUSSION

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CHAPTER 4: DISCUSSION

PART I: GENETIC STUDY

In the present study, the possible roles of two candidate genes in OCD pathogenesis were investigated using a case-control association study design. The first candidate gene screened was *COMT*, which encodes the MB-COMT, and S-COMT, that is responsible for the degradation of a variety of catechol neurotransmitters (section 1.2.4.2.4). The second gene investigated was *SLC6A4* that encodes the 5HTT protein responsible for the reuptake of 5HT during neurotransmission (section 1.2.4.2.4). The following section discusses the results of the present association study between these two genes and OCD in the Afrikaner population.

4.1. *COMT/Nla*III

The results generated by the present association study showed an association between the *H/L* genotype and OCD in the Afrikaner population and this association was found to be gender independent (table 6). Interestingly, these results differ from those of a previously reported association study, where homozygosity for the *L* allele was associated with OCD in males (Karayiourgou *et al.*, 1997). The biological basis for the observed sex-selective association reported by Karayiorgou and colleagues is still unclear. The authors speculated that since studies have shown that oestrogens can reduce COMT activity epigenetically, females may have evolved mechanisms compensating for their lower COMT activity levels. Therefore, they are relatively less vulnerable to developing OCD in association with lower enzyme activity.

Explanations for the predominance of the heterozygous genotype in the present study are also speculative. One possible explanation is that genetic or environmental interacting modulators influence this association. There is an increasing body of evidence that suggests that specific environmental determinants are important mediators of OCD (Swedo *et al.*, 1998, Niehaus *et al.*, 1999). Another possibility, which could account for the association seen in the present study, is that there may be

another genetic susceptibility factor present in this homogeneous population that predisposes individuals with intermediate COMT enzyme activity levels to develop OCD.

However, although the data generated in the present study and the work of Karayiorgou *et al.*, (1997) differ in respect to OCD susceptible gender and genotype, they both implicate the *COMT* gene in the pathogenesis of the disorder.

It is important to re-evaluate the findings of the present association study in a follow-up study employing a second group of OCD patients and control individuals of Afrikaner descent. Furthermore, the relatively small sample size may have lead to a TypeI error in the present study, thus re-evaluation studies will need to recruit more samples in order to overcome this problem. Replication of the association generated in the present study would confirm the involvement of COMT in OCD pathogenesis in the Afrikaner population.

4.2. *COMT/ERE6*

Gender differences in clinical manifestations have been described for several psychiatric disorders, including OCD (Castle *et al.*, 1995; Lensi *et al.*, 1996). These differences have been attributed to the epigenetic hormonal influences that affect disease processes.

Studies have shown that COMT activity is regulated by oestrogens via *EREs* within the *COMT* gene promoter region (Xie *et al.*, 1999). In the present study, it was hypothesised that if a variation occurs within *ERE6*, an *ERE* which is crucial in *COMT* regulation, COMT activity may be affected thereby increasing the risk for OCD. The results of the present study identified a novel sequence variation 8 bp 5' to *ERE6* (fig 15), designated *COMTP2-1040-C/T*, which, however, did not reveal an association with OCD pathogenesis in the Afrikaner population (table 8).

Since an association was observed between OCD and the *COMT/Nla*III functional polymorphism, one would have expected to observe a similar association with *COMTP2-1040-C/T*. As these two polymorphisms occur within the same gene, they

would be expected to be in linkage disequilibrium (LD) in the genetically homogeneous population studied. In the present study, this was, however, not observed.

The lack of information content of *COMTP2-1040-C/T* may explain why this polymorphism and *COMT/Nla*III do not appear to be in LD. In a biallelic system, such as the one investigated, SNP markers are generally informative enough to use in analysis if the rarer allele has a frequency of at least 0.2 (Read and Strachan, 1996). Upon genotyping 48 OCD patients and 40 control individuals for *COMTP2-1040-C/T*, it was found that the C:*T* allele distribution was 0.82: 0.18 (table 9). Therefore, this polymorphism may lack the necessary information content to detect any significant association in the present study. Therefore it could be speculated that *COMTP2-1040-C/T*, may be in LD with *COMT/Nla*III, but did not generate the statistical power to prove or disprove this hypothesis in the present study.

4.3. *SLC6A4*

The data generated in the present case-control study of OCD in the Afrikaner population did not support the hypothesis of an association between genotype distribution of the functional *5-HTTLPR* in *SLC6A4* and OCD (table 11).

As the present study may have lacked sufficient power to detect minor effects of *SLC6A4* on OCD pathogenesis, a meta-analysis of *5-HTTLPR* genotype distribution was performed that included data from the present study, as well as the study of Bengel *et al.*, 1999). Data generated in an association study by McDougle *et al.*, (1998) was excluded from the meta-analysis as it employed a TDT design. The studies by Ohara *et al.*, (1998) and Nicollini *et al.*, (1999) were also excluded from the meta-analysis because they made use of a Japanese and a Mexican population, respectively. The use of the latter two studies would confound the meta-analysis as the frequency of *l* and *s* alleles in the Japanese and Mexican populations differ significantly from that of the Afrikaner and North American Caucasian populations (Ohara *et al.*, 1998. Nicollini *et al.*, 1999).

If, as suggested by the data generated by Bengel *et al.*, (1999), *5-HTTLPR* is involved in OCD pathogenesis, the meta-analysis would have been expected to strengthen this previous association. The fact that the combination of data yielded a non-significant result could be attributed to one of a number factors. Firstly, it may be ascribed to the underlying genetic differences between the two populations studied. Even though both populations studied were Caucasian, the Afrikaner population of South Africa originated from a group of primarily Dutch, German and French descent (section 1.1.2.3), whereas the North American Caucasian population is made up largely of Irish, Italian and eastern European settlers (http://www.oneworld.org/)

Alternatively, it is possible that the role played by *SLC6A4* in the pathogenesis of OCD may be negligible, or even non-existent. At a low level of gene involvement, it is possible that factors, which cannot be controlled in the experimental design, may generate discrepant results in consecutive studies.

4.4. ASSOCIATION STUDIES IN PSYCHIATRY

As indicated in the previous section, population-based association studies often generate conflicting results (Ohara *et al.*, 1998; Bengel *et al.*, 1999), which authors generally attribute to a variety of confounding factors although they frequently omit to include study design or chance as explanations.

4.4.1. Underlying genetic differences

Difficulties in confirming positive or negative associations detected between a specific candidate gene and a disease, in different studies, are often ascribed to underlying genetic differences between populations. Indeed this is one explanation offered in the present study (section 4.3). Our current understanding of complex disorders, such as OCD, is that several mechanisms (genetic and environmental) may play a role in the presentation of disease phenotype in different individuals (Zohar et al., 1987; Stein 2000). Taking into account the genetic and environmental differences among population groups, it is possible that different combinations of environmental and genetic mechanisms could contribute to disease pathogenesis in each population.

The present study is being run concurrently with studies at the Genealogical Institute of South Africa (GISA), who are tracing many of the Afrikaner cases and control families, used in this study, back to their original founders. The study subjects recruited in this study were classified as being Afrikaner if at least three of their grandparents were of Afrikaner descent; which may have lead to genetic admixture because of the ethnicity of the fourth grandparent. This raises the question of whether the study population was sufficiently genetically homogeneous and this question is currently being addressed by the GISA investigation.

Furthermore, it has been proposed (Stoltenberg and Burmeister, 2000) that the interaction of a variety of genetic loci (some of major effect and others of minor effect) contributes to disease pathogenesis in a variety of psychiatric disorders, including OCD. It is therefore likely that different combinations of allelic variation in different genes, interacting with one another and the environment, may lead to the disorder in different population groups. This could account for the inconsistent data generated in studies assessing the role of *SLC6A4* and *COMT* in various association studies using different population groups.

4.4.2. Statistical power

Statistical power and sample size are other confounding factors that could account for the inconsistencies between association studies of specific candidate genes and complex diseases.

The sample size required to reach statistical significance in genetic case-control association studies is dependent on a number of interacting factors. These include i) the power of the study (ie., the expression of the ability of the study to detect a true effect if one exists and the ability to differentiate between a significant negative association and an inconclusive result) and ii) the specific effect size (ie. the contribution to disease pathogenesis made by the specific allelic variation under investigation) (Berry *et al.*, 1998).

It has been proposed as a convention that, when an investigator has no other basis for choosing the desired power of the study, a value of 80% be used, that is the ability to

detect a 20% difference between two values. (Berry *et al.*, 1998). It is also common practice that the level of significance (ie., the p-value) be set at 0.05, that is with a 95% confidence level.

Therefore, in studying diseases of complex aetiology, it follows that the sample size required to detect an association between disease and genes of major effect would be considerably less than the sample size required to detect an association with genes of minor effect. This explains why the present study was able to detect an association between *COMT/Nla*III and OCD using a relatively small sample. The association study of *COMT/Nla*III and OCD by Karayiorgou *et al*, (1997) used a sample of 78 OCD patients and 148 control individuals to show an association between *COMT* and OCD. The results of the two above mentioned case-control association studies together with the association found between *COMT* and OCD using family-based methods (Karyiorgou, *et al.*, 1998), using modest sample sizes, provides much evidence for *COMT* being a gene of major effect in OCD pathogenesis.

On the other hand, the non-significant finding generated in the present study of OCD and *5-HTTLPR* in *SLC6A4* may have been the result of analysing insufficient samples which did not allow the detection of a significant association (Type II error). As previously mentioned, the contribution made by this gene to OCD pathogenesis may be minor or non-existent. Under these circumstances, if one proposes that *SLC6A4* does play a minor role in OCD pathogenesis, the sample size required to detect the contribution made by this gene would be greater than the sample size used in the *COMT/Nla*III studies. In fact it can be calculated, the sample size required to detect a 20% difference the 5% significance level for the pooled 129 OCD samples used in the meta-analysis was 1444 control individuals. Thus, as only 479 controls were genotyped, it can be reasoned that even the two studies used in the meta-analysis were conducted using insufficient numbers to detect an association between OCD and *SLC6A4*, if in fact it does exist. Furthermore, the positive association reported by Bengel *et al*, (1999) may have been a spurious result, which may be attributed to a Type I error.

However, it is important to realise that although increasing the sample size of the study cohorts may generate statistically significant data, this may not always equate to clinical relevance (Berry *et al.*, 1998).

The Afrikaner population and future association studies

Association studies to test the candidature of *COMT* and *SLC6A4* as susceptibility factors in OCD development in the Afrikaner population have generated divergent findings in comparison with other study groups. This may be attributed to the inherent genetic differences among population groups used in various association studies. It is for this reason association studies between attractive OCD candidate genes and clinically well characterised and matched subjects from a genetically homogeneous population may help to further delineate the specific mechanism(s) that underpin this complex disorder. To this end, the genetically homogeneous Afrikaner sub-population of South Africa provides researchers with an invaluable investigative tool for further studies (Niehaus *et al.*, in press; Kinnear *et al.*, 2000).

PART II: IMMUNOLOGICAL STUDY

4.5. D8/17

Our finding that B-lymphocyte D8/17 expression levels are not significantly higher in OCD than in normal controls differs from previously reported data by Swedo *et al.*, (1997) and Murphy *et al.*, (1997). The latter two studies showed mean D8/17 expression to be significantly higher in the patient groups (mean, 22% and 29%, respectively) versus the healthy control groups (mean, 9% and 8%, respectively) (Swedo *et al.*, 1997; Murphy *et al.*, 1997). A preliminary study by our group has also showed an association between increase D8/17 expression levels and OCD in the Afrikaner population (Niehaus *et al.*, 1999).

It is possible that the divergent results generated in the present study may have resulted from important differences between the samples used in the two above mentioned studies and the present study. The studies of Swedo *et al.*, (1997) and Murphy *et al.*, (1997) focused on early onset OCD (11: childhood onset; 15: adult

onset), and both included a high prevalence of patients with concurrent tics (24/27 and 21/30). Our OCD sample did not focus on childhood onset OCD and consisted of only 3/26 patients with a history of tics (table 16). It might be hypothesized that OCD in patients with early onset and/or tics is particularly likely to be associated with auto-antibodies that cross-react with neuronal proteins on the basal ganglia. This could consequently result in striatal damage (Swedo *et al.*, 1994).

Although we were unable to find differences in mean levels of D8/17 expression when the OCD group was stratified into early versus late onset, those with or without a history of tics, and those with or without a history of different comorbid psychiatric disorders, the relatively small sample size may well have resulted in type II errors for these sub-analyses. As more OCD patients are recruited, the problem of insufficient sample size will be rectified.

Recent work by Peterson and colleagues (2000) has suggested that anti-Streptococcal antibodies may be associated with attention deficit hyperactivity disorder (ADHD), rather than with OCD or TS. Again, we were unable to adequately address this question with our sample, in which only 3 subjects (all in the PD/SP group) also had a history of ADHD.

Nevertheless, our study did find a significant different in D8/17 expression across the different groups. D8/17 was numerically, if not statistically, higher in OCD than in other groups, and there was a significant difference between OCD and other anxiety disorders (PD/SP group). No clear reason for the relatively decreased expression of D8/17 in this last group is apparent. It would seem that additional work on this marker in a range of psychiatric disorders remains necessary.

4.6. SUMMARY OF RESULTS OF THE PRESENT STUDY

1. The study showed a positive association between the previously described *COMT/Nla*III polymorphism and OCD in the Afrikaner population.

2. A novel SNP (*COMTP2-1040-C/T*) in the *COMT* gene was identified and deposited into an SNP database.
3. *COMTP2-1040-C/T* was shown not to be associated with OCD in the Afrikaner population. However, this non-significant finding may be the result of the low information content of this polymorphism.
4. *SLC6A4*, the gene encoding 5HTT, did not reveal any significant association with OCD in the Afrikaner population.
5. *SLC6A4* also showed no association with OCD when the results of the present study was pooled with the data generated in an association study by Bengel *et al.*, 1999.
6. The immunological study showed that the B-lymphocyte surface antigen marker D8/17 is not significantly associated with OCD in our sample population. We do, however, speculate that higher D8/17 levels may be associated with childhood onset OCD or history of tic disorders.

4.7. THE FUTURE OF THIS RESEARCH

The immediate objective for this project is to continue to recruit more patients of Afrikaner descent to improve the statistical power of these case-control association studies, thereby improving the ability to detect functional polymorphisms that have a major to moderate effect on OCD pathogenesis. This is already underway and many more patients have already been recruited through the MRC Unit on Anxiety and Stress disorders. By recruiting more patients and control individuals, it would also be possible to re-evaluate these candidate genes using a new sample of Afrikaner descent in an effort to replicate the findings of the present study. A larger study sample will also make a genome-wide scan for OCD susceptibility genes possible. We aim, in collaboration with the New York State Psychiatric Institute, to do a genome scan in the future. Furthermore, this project has recruited enough parents of OCD probands, which makes TDT analysis (section 1.1.2.2) possible and this is a priority in continuing studies.

In the past year, another researcher in our group has already extended this study to screen other candidate genes, namely, those encoding the dopamine transporter (*SLC6A3*), tyrosine hydroxylase (*TH*), the serotonin receptor 2A (*5HT2A*),

monoamine oxidase A (*MAOA*) and dopa-decarboxylase (*DDC*). Although, many candidate genes have been screened in this study and some significant findings have been made (S. Hemmings, personal communication) many other candidate genes remain to be screened.

It is important that researchers reach a consensus about particular genes and their allelic variants that are involved in OCD pathogenesis, so that future functional studies of the proteins they encode may be valid. Reaching such a consensus would involve many more candidate gene association studies that replicate previously found association between OCD and a particular gene, as well as new ones.

One of the primary goals in psychiatric genetics is to further the development of psychopharmacological genetics. Psychopharmacogenetics may improve patient care by helping the clinician to individualise the pharmacotherapy of each patient based on their genotype at specific genetic markers (Stoltenberg and Burmeister, 2000). The present study has gathered a huge amount of genotypic data on this carefully phenotyped patient sample; therefore this resource could be potentially used to correlate the generated genotypic data with specific response to pharmacological treatment. In the future, it is foreseeable that polymorphic markers in candidate genes screened in this study and studies around the world, as well as in genes still to be assessed, could help improve diagnoses of OCD, even subclinical OCD. Also, it may assist in providing patients with more specific, individualised treatment for OCD based on the genotype at susceptibility loci (Stoltenberg and Burmeister, 2000).

The role of D8/17 as a possible marker for susceptibility to OCD also warrants further investigation. As the sample population used in the present study was significantly different from the sample population used in previous studies, it is important to reassess the role of D8/17 in the Afrikaner population using a study population consisting of patients with childhood-onset OCD and a history of comorbid tic disorders. Replication of the association found in studies of Swedo *et al*, (1997) and Murphy *et al*, (1997) using such a sample population would strengthen the present study hypothesis that D8/17 only serves as a trait marker in patients with childhood-onset OCD or patients with a history of comorbid tic disorders.

This study formed part of an integrated approach to identify OCD genetic susceptibility factors in the Afrikaner population. The data generated in each different facet of this integrated study may help identify presymptomatic individuals and facilitate their management. Furthermore, the results may contribute to an understanding of the molecular pathophysiology of this disabling condition.

APPENDIX I

BUFFERS, MARKERS AND SOLUTIONS

1 BUFFERS

1.1. CELL LYSIS BUFFER

Sucrose	0.32M
Triton-X-100	1%
$MgCl_2$	5mM
Tris-HCl	10mM
H_2O	1l

1.2. DNA EXTRACTION BUFFER

NaCl	0.1M
Tris-HCl	0.01M
EDTA (pH8)	0.025M
SDS	0.5%
Proteinase K	0.1mg/ml

1.3. TBE-BUFFER (10x stock)

Tris-HCl	0.89M
Boric Acid	0.89M
Na_2EDTA (pH8)	20mM

1.4. TE-BUFFER (10x stock)

TrisOH	0.1M
EDTA(pH8)	0.01M
H_2O	150ml

1.5. *Taq* POLYMERASE BUFFER (Bioline)

Ammonium sulphate	160mM

Tris-HCl (pH 8.8)	670mM
Tween-20	0.1%

1.6. QX1 BUFFER (Qiagen)

Buffer QX1	500ml

2. SOLUTIONS

2.1. SOLUTIONS FOR POLYACRYLAMIDE GELS

2.1.1. 30% ACRYLAMIDE/0.8% BIS STOCK SOLUTION- FOR SSCP AND OTHER POLYACRYLAMIDE GELS

Bis-acrylamide	1.56g
Acrylamide	58.5g

Make up solution to 195 ml with H_2O after the acrylamide has gone into solution and store at 4°C.

2.1.2. 10% AMMONIUMPERSULPHATE (APS)

APS	2g
H_2O	20ml

2.2. SOLUTIONS FOR SILVER STAINING

2.2.1. 0.1% $AgNO_3$ (Solution B)

$AgNO_3$	1g
H_2O	1L

2.2.2. DEVELOPING SOLUTION (Solution C)

NaOH	15g
$NaBH_4$	0.1g
Formaldehyde	4ml
H_2O	1L

3. GELS

3.1. 12% POLYACRYLAMIDE GEL

30 acrylamide/0.8% bis-acrylamide stock	4ml
10xTBE	1ml
Distilled H_2O	5ml
APS	80µl
TEMED	30µl

3.2. 10% MILDLY DENATURING POLYACRYLAMIDE GEL WITH 5% GLYCEROL

30 acrylamide/0.8% bis-acrylamide stock	40.5ml
10xTBE	8ml
Urea	24g
Distilled H_2O	84ml
APS	1000µl
TEMED	160µl

4. LOADING DYES

4.1. BROMOPHENOL BLUE

Bromophenol blue	0.2%(w/v)
Glycerol	50%
Tris (pH8)	10mM

4.2. SSCP LOADING DYE

Formamide	95%
EDTA	20mM
Bromophenol blue	0.01%
Xylene cyanol	0.05g

5. MOLECULAR WEIGHT MARKER (LAMBDA *Pst*I)

Bacteriophage Lambda DNA (250µg)	100µl
Buffer M (Boerhinger Mannhein)	15µl
*Pst*I (Boerhinger Mannheim)	11µl
H_2O	32µl

Incubate at 37°C for 2 hours followed by heat inactivation at 65°C for 5 minutes. Load 2µl onto polyacrylamide gels.

6. SOLUTIONS FOR FACS ANALYSIS

6.1. PHOSPHATE BUFFERED SALINE

PBS	10%

6.2. FACS LYSING SOLUTION

Diethylene glycol	50%
Formadldehyde	15%

6.3 FACS FIXING SOLUTION

Formaldehyde	1%

APPENDIX II

LIST OF SUPPLIERS

Acrylamide	Merck
$AgNO_3$	Merck
Ammonium persulphate	Merck
Bis-acrylamide	Merck
Boric acid	Merck
Bromophenol blue	Merck
dGTP	Boerhinger Mannheim
dCTP	Boerhinger Mannheim
dTTP	Boerhinger Mannheim
dATP	Boerhinger Mannheim
*EcoR*V	Promega
EDTA	Boerhinger Mannheim
Ethanol	Boerhinger Mannheim
FACS Lysing Solution	Beckton Dickenson
Formamide	Merck
Formaldeyde	Merck
Gelbond	Merck
Glycerol	FMC
K-acetate	Sigma
KCl	Merck
Lambda DNA	Promega
Mineral oil	BDH Chemicals
NaAc	Merck
NaCl	BDH Chemicals
NaOH	Sigma
*Nla*III	New England Biolabs
Oligonucleotide primers	Department of Biochemistry, University of Cape Town School

Phenol	Merck
PBS	Sigma
Proteinase K	Sigma
Qiagen Kit	Stratagene
SDS	Sigma
Taq polymerase	Bioline
TEMED	Sigma
Tris	Merck
Tris-OH	Merck
Tris-HCl	Merck
Urea	BDH Chemicals

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