The development and application of a polymerase chain reaction (PCR) based assay to determine the impact of genetic variation in South African patients diagnosed with depression

BY

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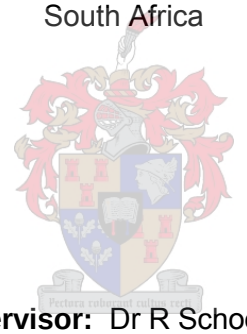

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DECLARATION

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Summary

Major Depressive Disorder (MDD) is a severe debilitating medical condition that may lead to suicide. Due to a poor understanding of the biological mechanisms underlying the disease process therapeutic decisions are usually taken using a 'trial and error' approach. This is not ideal since many treatments do not work as expected for all individuals. Studies have shown that only half of MDD patients receive the appropriate treatment, whereas many patients have adverse response to anti-depressants. These may include weight gain and raised homocysteine levels that may further compromise the health status of MDD patients and may partly explain the link with cardiovascular disease.

The objective of the study was to identify genetic risk factors interacting with environmental factors implicated in MDD that may be of relevance to the South African population. Polymorphisms in the MTHFR (677 C>T, rs1801133 and 1298 A>C, rs1801131), COMT (472G>A, rs4680), CYP2D6 (6937G>A, rs3892097), ASMT (24436 G>A, rs4446909) and SLC6A4 (43 bp ins/del, rs4795541) genes were genotyped in 86 MDD patients and 97 population-matched controls. The specific aims were 1) to analytically validate high throughput real-time polymerase chain reaction (RT-PCR) genotyping assays for the selected SNPs against direct sequencing as the gold standard for 2) possible integration into a pathology-supported genetic testing strategy aimed at improved clinical management of MDD.

A total of 183 unrelated Caucasians participated in the study, including 69 females and 17 males with MDD and 57 female and 40 male controls without a personal and family medical history of overlapping stress/anxiety and depressive disorders. All study participants were genotyped for the six selected SNPs considered clinically useful based on international data. The allelic distribution of the SNPs, single or combined into a genotype risk score after counting their minor alleles, did not differ between MDD patients and controls. Homocysteine levels were determined and correlated with body mass index (BMI) and other variables known to influence these phenotypes. The folate score assessed with use of the study questionnaire was significantly lower in the patient group compared with controls ($p=0.003$) and correlated significantly with BMI, particularly in females ($p=0.009$). BMI was on average 8% higher in the MDD patients compared with controls ($p=0.015$) after adjustment for age and sex. The MTHFR rs1801133 677 T-allele was associated with a 14% increase in BMI in MDD patients but not controls ($p=0.032$), which in turn was associated with significantly increased homocysteine levels ($p<0.05$).

The aims of the study were successfully achieved. Identification of the MTHFR rs1801133 677 T-allele reinforces the importance of adequate folate intake in the diet due to increased risk of obesity and depression found to be associated with low dietary intake. Evidence of shared genetic vulnerability for many chronic diseases and drug response mediated by the MTHFR 677 T-allele support the clinical relevance of this low-penetrance mutation.

Opsomming

Major depressie (MD) is ‘n aftakelende siektetoestand wat tot selfdood kan lei. Onkunde oor die siekte se onderliggende biologiese meganismes lei dikwels tot ‘n lukrake terapeutiese benadering. Dit is ‘n onbevredigende situasie aangesien indiwidue verskillend reageer op die middels wat voorgeskryf word. Navorsing toon dat slegs ongeveer die helfte van MD pasiënte toepaslike behandeling kry, terwyl anti-depressante ‘n nadelige uitwerking het op baie pasiënte. Dit sluit massatoename en verhoogde homosisteïenvlakke in wat MD pasiënte se gesondheid bykomend nadelig kan beïnvloed en die verband met kardiovaskulêre siekte gedeeltelik kan verklaar.

Hierdie studie poog om MD verwante genetiese risikofaktore en omgewingsfaktore wat mekaar beïnvloed en moontlik op die Suid Afrikaanse bevolking betrekking het, te identifiseer. Polimorfismes in die MTHFR (677 C>T, rs1801133 en 1298 A>C, rs1801131), COMT (472G>A, rs4680), CYP2D6 (6937G>A, rs3892097), ASMT (24436 G>A, rs4446909) en SLC6A4 (43 bp ins/del, rs4795541) gene is geanaliseer in 86 MD pasiënte en 97 kontroles geselekteer van dieselfde populasie. Die spesifieke doelwitte was om 1) hoë deurset direkte polimerase kettingreaksie (RT-PCR) genotiperingstoetse vir die 6 gekose polimorfismes met direkte volgordebepaling as maatstaf analities te valideer vir 2) moontlike insluiting in ‘n patologie-ondersteunde genetiese toetsstrategie met die oog op beter kliniese hantering van MD.

Altesaam 183 Kaukasiërs het aan die studie deelgeneem. Die MD pasiënte het uit 69 vroue en 17 mans bestaan. Die kontroles (57 vroue en 40 mans) het geen mediese geskiedenis (persoonlik of familie) van oorvleuelende stress/angstigheid of depressie gehad nie. Gebaseer op internasionale data, is al die deelnemers vir die 6 gekose, potensieel klinies-bruikbare polimorfismes getoets. Die alleliese verspreiding van die polimorfismes enkel of gekombineer (uitgedruk as ‘n genotipe-risiko-syfer nadat minor allele getel is), was dieselfde in MD-pasiënte en kontroles. Homosisteïenvlakke is bepaal en gekorreleer met die liggaamsmassa-indeks (BMI) en ander veranderlikes wat bekend is vir hulle invloed op hierdie fenotipes. In teenstelling met die kontroles, was die folaat telling, soos bepaal met die studievraelys, betekenisvol laer in die pasiënte ($p=0.003$). Die korrelasie met die liggaamsmassa-indeks, spesifiek by vroue, was ook betekenisvol ($p=0.009$). Na aanpassings vir ouderdom en geslag, is gevind dat die liggaamsmassa-indeks gemiddeld 8% hoër was in die die MD pasiënte teenoor die kontroles. By MD-pasiënte, maar nie by die kontroles nie, is die MTHFR rs1801133 677 T-alleel geassosieer met ‘n 14% toename in liggaamsmassa-indeks ($p=0.032$), wat ook geassosieer was met betekenisvolle verhoogde homosisteïenvlakke ($p<0.05$).

Die doelwitte van die studie is bereik. Identifisering van die MTHFR rs1801133 677 T-alleel beklemtoon hoe belangrik dit is om voldoende folaat in te neem, veral omdat ‘n verhoogde risiko vir vetsug en depressie met ‘n lae folaatinname in die diet geassosieer word. Die kliniese belang van die MTHFR 677 T-alleel word beklemtoon deur toenemende bewyse wat daarop dui dat gedeelde

genetiese vatbaarheid vir ‘n verskeidenheid van kroniese siektes asook middelrespons aan bemiddeling deur hierdie lae penetrasie mutasie toegeskryf kan word.

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List of Abbreviations and Symbols

5’	5-prime
3’	3-prime
α	Alpha
β	Beta
©	Copyright sign
°C	Degrees Celsius
>	Greater than
<	Less than
µg/L	Microgram per litre
µl	Micro litre
%	Percentage
®	Registered trademark
=	Equal to
kg/m2	Kilogram per square meter
mg	Microgram
µg/L	Microgram per litre
µL	Micro litre
µmol/L	Micromole per litre
-	Minus
%	Percentage
+	Plus
±	Plus-minus
5-MTHF	N-5-methyltetrahydrofolate
5, 10-MTHF	N-5, 10-methylenetetrahydrofolate
ATP	Adenosine 5’-triphosphate
ADHA	Attention Deficit Hyperactivity Disorder
ADR’s	adverse drug reactions
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
CI	Confidence Interval
CNS	Central Nervous System

CVD	Cardiovascular disease
COMT	Catechol-O-Methyl Transferase
CRF	Corticotropin-releasing factor
dATP	2'dioxy-adenosine-5'triphosphate
DA	Dopaminergic pathways
dCTP	2'dioxy-cytosine-5'triphosphate
ddH_2O	Doubled distilled water
dGTP	2'dioxy-gaunosine-5'triphosphate
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DM	Diabetes Mellitus
DNA	Dioxyribonucleic acid
DNMTs	DNA methyltransferases
dNTPs	Dioxyribonucleotide triphosphates
dsDNA	Double stranded DNA
dTTP	2'dioxy-thymidine-5'triphosphate
DLPFC	Dorsolateral prefrontal cortex
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
F Primer	Forward primer
Fmri	Functional magnetic resonance imaging
g	Gram
GC	Guanine-Cytosine
H_2O	Water
H_3BO_3	Boric acid
HWE	Hardy Weinberg equilibrium
HPA	Hypothalamic-pituitary-adrenal axis
k	Kilo
kb	Kilobases
M	Molar

M (Met)	Methionine
MB-COMT	Membrane Bound Catechol-O-Methyl Transferase
MDD	Major Depressive Disorder
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
mM	Milli-molar
mmol/L	millimol per litre
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MTHFR	Methylenetetrahydrofolate reductase
MAOIs	Monoamine Oxidase Inhibitors
MetS	Metabolic syndrome
NCBI	National Centre for Biotechnology Information
ng	Nanogram
ng/µl	Nanogram per micro litre
NF H_2O	Nuclease free water
NTC	Non-template control
NE	Noradrenergic pathway
nAChRs	Nicotinic acetylcholine receptors
OCD	Obsessive Compulsive Disorder
PAR	Pseudoautosomal region
pmol	Picomole
PCR	Polymerase Chain Reaction
PET	Position emission tomography
PM	Poor metabolizer
RefSeq	Reference Sequence
rpm	Revolutions per minute
R primer	Reverse primer
RNA	Ribonucleic Acid
RT-PCR	Real-Time Polymerase Chain Reaction

S-COMT	Soluble Catechol-O-Methyl Transferase
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SNP(s)	Single nucleotide polymorphism(s)
ssDNA	Single stranded DNA
SST	Social Skills Training
SNRIs	Selective Noradrenalin Reuptake Inhibitors
SSRIs	Selective Serotonin Reuptake Inhibitors
SGA	Second generation antipsychotic
T_A	Annealing temperature
TAE	Tris-acetate-EDTA buffer
Taq	*Thermus aquatics*polymerase enzyme
TBE	Tris-Borate-EDTA buffer
THF	Tetrahydrofolate
T_M	Melting temperature
TM	Trademark
tRNA	Transfer ribonucleic acid
TCAs	Tricyclic Antidepressants
TSH	Thyroid stimulating hormone
U	Units
UV	Ultraviolet
µl	Micro litre
V	Volts
V (Val)	Valine
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization ()
x	Times
Y (Tyr)	Tyrosine

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INTRODUCTION

The research question leading to this study was whether genotyping of functional polymorphisms previously implicated in the development and/or response to treatment of major depressive disorder (MDD) would be clinically useful in the South African context. The background to this translational research project is summarized as follow:

- MDD is a chronic, recurrent disease that is severely disabling
- MDD is important due to reduced quality of life, productivity and high risk of suicide
- MDD is caused by a complex interaction between genetic and environmental risk factors
- Treatment of MDD is hampered by paucity of reliable diagnostic and predictive biomarkers

The ultimate aim of this study is to reduce cumulative risk leading to the development of depression and adverse response to treatment, treatment failure or medication side effects. A translational research study was therefore undertaken which involved a multi-step process whereby candidate genetic markers were selected for study in the context of environmental triggers, following a review of the literature (chapter 1). Standard operating procedures (SOPs) were developed and documented as used in the study population (chapter 2). Analytical validation of the assay procedures verified the accuracy of the genotypes generated for comparison of allelic distribution between patients and controls (chapter 3). Finally, genotype-phenotype association studies were performed in South African patients with MDD in an attempt to replicate the effect of genetic and environmental factors on homocysteine levels as an important previously-identified intermediate phenotype between gene and disease (chapter 4).

Assessment of genetic risk factors separate from contributing environmental factors and the influence of co-morbidities such as obesity hinders our understanding of complex multifactorial disorders and the effect of genetic variation on treatment response. Therefore, environmental factors known to influence homocysteine levels - such as body mass index (BMI), folate intake, smoking, alcohol intake, and physical activity - were considered in this study not only as potential confounders that need to be adjusted for during statistical analysis to determine a gene effect, but also as modifiable contributors to the disease phenotype that may be useful to mitigate a gene effect in MDD patients. In conclusion (chapter 5), a pathology-supported genetic testing approach is therefore proposed to overcome the limitations of genetics alone to account for the phenotypic expression of low-penetrance mutations and functional single nucleotide polymorphisms (SNPs) implicated in disease susceptibility, treatment response and development of treatment side effects.

Chapter 1

Literature Review

1.1 Epidemiology of Major Depressive Disorder

Major depressive disorder (MDD) is a severe and disabling disorder, with a significant economic burden (DeRubeis et al. 2008, Stoudemire et al. 1998, Kiyohara et al. 2009). The lifetime prevalence of MDD is 7-16% (DeRubeis et al, 2008, Liu et al. 2013), of which one out of three patients do not recover (Schosser et al. 2012). Depression is characterized by a depressed mood, loss of interest, pessimism, reduced self-esteem and motivation, sleep disturbances and suicidal thoughts. Unipolar depression consists of depressive episodes which affect roughly 20% of females and 10% of males, with an estimated heritability of approximately 25% in less severe cases and 50% in severe recurrent cases (DeRubeis et al. 2008).

MDD has major global public health implications and the World Health Organization (WHO) estimates that 450 million individuals experience a mental or behavioural disorder (Kiyohara et al. 2009); however 60-70% of cases are under diagnosed (Nuyen et al. 2005). The WHO stated that depression is ranked the 3rd most disabling disorder world-wide (www.who.int). A study by Tomlinson et al. (2009) illustrated that in the South African population the lifetime prevalence for MDD is 9.8%, with the average age of onset for both males and females found to be 25.6 and 26 years respectively (Tomlinson et al. 2009). It has been estimated that in the first year of recovery 12% of patients relapse and 33% within 4 years (Weisze et al. 2006). Depression in children and adolescents are significant, continual, frequent and a major public health issue (Weisze et al. 2006). It is estimated that the prevalence for MDD in children is 2% and 5% in adolescents (Stoudemire et al. 1998) and by the age of eighteen 20% are diagnosed with depression (Weisze et al. 2006). MDD may cluster in families and an individual who has a parent or sibling with MDD has a 20-25% chance of sharing the condition (Klein et al. 1993). An earlier age of onset and chronic depression has been associated with a family history of MDD (Kornstein et al. 2001).

MDD is often undiagnosed and untreated regardless of the increasing prevalence of the disorder (Asch et al. 2003). Due to a lack of access to healthcare services the prevalence of MDD could be underestimated (Kiyohara et al. 2009, Cutrona et al. 2006). Another cause for not diagnosing MDD is due to the overlap between the somatic symptoms of depression and symptoms of general medical conditions (Barraclough 1997, Asch et al. 2003). It has been estimated that in primary care between one half and two thirds of patients are undiagnosed with MDD. This could contribute to adverse reactions, poor adherence and suicide (Asch et al. 2003).

In most cases MDD has an excellent recovery rate and may be settled without the administration of antidepressants (DeRubeis et al. 2008). However, at least three-quarters of depressed patients will experience recurrent episodes, which occur more frequently if the patient had an earlier onset or has a family history of the disorder (Liu et al. 2013). It is estimated that 85% of MDD patients experience recurrent episodes which increases by 16% after each consecutive episode, therefore prevention of recurrent episodes is essential (Huijbers et al. 2012, Mann et al 2005). Relapse

occurs during the remission stage (before recovery) and when the patient fulfils all the criteria for a new episode, this is known as recurrence. Recovery is defined as being in remission for 6 months (Huijbers et al. 2012). Numerous factors are associated with relapse including gender, previous episodes, marital status, increased number of depressive episodes, and an extended episode (Mueller et al. 1999). Chronic depression is characterised by repetitive or extended episodes, associated with a decrease in functionality and cognition over an extended period. The pathogenesis of these subtypes (acute vs. chronic) differ (Bellmarker et al. 2008). Individuals are also likely to experience anxiety disorders, substance abuse, personality disorders and an elevated risk for suicide attempts (Kornstein et al. 2001).

Despite numerous research studies done on MDD little is known about the etiological risks and mechanisms behind the disorder (Liu et al. 2013, Mill et al. 2007). MDD is a complex systematic disorder which involves the interaction of genes, environmental factors and disease phenotypes, all of which contribute to the pathogenesis of the disorder (Manji et al. 2001). Numerous risk factors have been linked to MDD which include age, gender, psychosocial stressors and comorbid medical illnesses, genetic risk factors, smoking, obesity and substance abuse. MDD is known to influence various pathways including the central nervous system (CNS), immune, endocrine and cardiovascular systems (Kiecolt-Glaser et al. 2002).

The peak age of onset of MDD is generally late 20's, however it is not uncommon for older or younger generations to be affected (Kiyohara et al. 2009). Recent studies have shown that MDD is increasing in patients with an earlier age of onset (10-21 years) and these individuals are predisposed to additional episodes that are more severe and recurrent (Zisook et al. 2007, Kaplan et al. 1998). Pre-adult onset of MDD is more commonly associated with females who experience more severe episodes and symptoms in relation to the adult onset of MDD, although not all studies have replicated this finding (Zisook et al. 2007). Pre-adult onset has been linked with family history, substance abuse, as well as medical and psychiatric co-morbidity (Zisook et al. 2007).

MDD appears to be more prevalent in women than men (Piccinelli et al. 2000) and therefore gender may be a risk factor for MDD. It has been estimated that 20-25% of females will suffer at least one depressive episode in their lifetime compared to the 12% in males (Kiyohara et al. 2009). This is speculated to occur due to the unique anatomy of the female body, hormonal changes, ovarian steroid modifications, oral contraceptive usage, luteal phase of menstruation cycle, pregnancy, postpartum depression and menopause (Parry et al. 2001). Females are also more likely to seek treatment compared to males (Kiyohara et al. 2009). Gender variations were noted post puberty when neuroendocrine reproductive modifications occur (Parry et al. 2001). Ovarian hormones could influence mood by effecting neurotransmitters, neuroendocrine systems or circadian rhythmicity (Parry et al. 2001). Females are more likely to suffer from pre-existing anxiety

disorders, whereas males are more prone to substance abuse or antisocial tendencies (Piccinelli et al. 2000).

1.2 Diagnosis of Major Depressive Disorder

The Diagnostic and Statistical Manual of mental disorders (DSM) classifies MDD as major depressive episodes without showing signs of manic, hypomanic or mixed episodes and is characterized by one or more major depressive episodes occurring consistently for more than 2 weeks (DSM-IV-TR 2000). A major depressive episode is characterised is defined as a depressed mood and /or loss of interest in almost all activities together with five additional symptoms which as described in table 1.1 (DSM-IV-TR, 2000).

Table 1.1. Criteria for a Major Depressive Episode

(A) According to the DSM-IV five or more of the symptoms listed below have to be present in an individual consistently for 2 weeks. One of these symptoms has to be either (1) a depressed mood or (2) a loss of interest or pleasure in almost all activities. Symptoms due to medical conditions, delusions or hallucinations must be excluded.

- Depressed mood - an individual will feel depressed daily for a large portion of the day. This can either be noted as a personal report (e.g. feelings of unhappiness or meaninglessness) or witnessed by others (e.g. tearful). In children and adolescents moods such as irritability should be taken into account.
- Loss of interest or pleasure - an individual will feel a loss of interest and activities daily for a large portion of the day.
- Weight loss/gain - A major weight loss will be noted in an individual (not dieting) or gain (e.g. body weight changes by > 5% in a month). Reduction or increase of appetite daily. Children may fail to gain the expected weight.
- Insomnia or hypersomnia daily.
- Psychomotor agitation or retardation daily
- Fatigue or loss of energy daily
- Feelings of worthlessness or guilt daily
- A reduced ability to think/reason or concentrate daily
- Consistent thoughts of death and suicide, attempted suicide or specifically plans to commit suicide.

(B) The symptoms are not those that are seen for mixed episode.

(C) The symptoms listed above results in distress and diminish an individual's social, career or other important areas of life.
(D) The symptoms due to physiological effects such as substance abuse (e.g. medication) or medical conditions (e.g. hypothyroidism) should be excluded

MDD is a complex multifactorial disorder instigated by numerous factors (Kiyohara et al. 2009). This heterogeneous disorder exhibits a broad range of psychopathological indicators with diverse clinical symptoms, which range in severity (Mill et al. 2007, Schroeder et al. 2010). Due to this variation, symptoms and experiences are unique to each individual with this disorder (Kiyohara et al. 2009) and as the degree of depression is amplified, the more severe the depressive symptoms are (Piccinelli et al. 2000).

Patients with depression have a reduced ability to experience satisfaction, happiness or pleasure (Kaplan et al. 1998) and clinical symptoms include cognitive, psychomotor and emotional distress (Sun et al. 2013). It is estimated that 90% of patients with MDD suffer from anxiety (Kaplan et al. 1998). Indicators for depression in children include a phobia for school, excessive clinginess, irritability, complains about headaches and stomach aches and children may fail to gain weight (Kaplan et al. 1998, Stoudemire et al. 1998, Moore et al. 1996). In adolescents, symptoms such as a reduction in academic performance, an increase in social isolation, truancy, loss of interest in hobbies and sports, development of physical complaints (no medical reason), moodiness, low self-esteem, misconduct, rebelliousness, irritability behaviour or running away may be warning signs of MDD (Kaplan et al. 1998, Moore et al. 1996, Weisze et al. 2006). Younger patients are also predisposed to substance abuse and tend to attempt or complete suicide, which is the 3rd most common cause of death (Weisze et al. 2006).

1.3 Aetiology of Major Depressive Disorder

MDD is a complex multifactorial disease (Kiyohara et al. 2009). Predisposing factors include genetics (e.g. family history), while precipitating factors often include psychosocial stressors.

1.3.1 Biological

1.3.1.1 Brain Pathology and Biochemical Contributions

Postmortem and neuroimaging studies reported alterations in cognition, emotional and reward systems in MDD patients (Sun et al. 2013). Changes in grey matter volume of the brain (striatal-limbic circulating cortex and hippocampus), morphology of the spine and neurons, neurochemistry,

intracellular signalling, regulation of gene expression, neuroplasticity, cellular resilience (Tsankova et al. 2007, Sun et al. 2013, Manji et al. 2001) and hypermetabolism in amygdala and frontal cortical regions (Sun et al. 2013) were noted. Cell damage may also occur in the subgenul prefrontal cortex, atrophy dorsolateral prefrontal cortex and orbital frontal cortex, with elevated cells in the hypothalamus and dorsal raphe nuclease. These symptoms are similar to individuals with Cushing's disease (Bellmaker et al. 2008).

Today the hypothesis suggests that MDD could be associated with a shortage of signal transduction occurring between a neurotransmitter and its postsynaptic neuron, with normal amounts of neurotransmitters and receptors (Kiyohara et al. 2009). However, studies have yet to find definitive support for the deficiency claim (Bellmark et al. 2008). Another essential neurotransmitter in the brain is dopamine which regulates feelings, incentive, and reinforcement behaviour through the mesocorticolimbic pathway. Depressive symptoms are a result of the hypofunction of the dopaminergic system in some MDD patients (Kiyohara et al. 2009).

The amygdale is situated in the limbic region of the human brain, which is responsible for processing and producing emotions. Therefore, abnormalities in the amygdala region could affect both the neural and cognitive processes in the brain (DeRubeis et al. 2008). Functional magnetic resonance imaging (fMRI) and position emission tomography (PET) illustrated elevated activity in the amygdala was associated with pessimistic emotions (Krishnan et al. 2008).

Findings in PET imaging and fMRI studies support the theory that reduced prefrontal activity occurs in MDD. One of the functions of the prefrontal cortex is to inhibit the effects of the amygdala activity (DeRubeis et al. 2008). The dorsolateral prefrontal cortex (DLPFC) is responsible cognitive tasks such as control and working memory. In depressed individuals the DLPFC activity is reduced, which supports the theory that elevated limbic activity interferes with the prefrontal control (DeRubeis et al. 2008).

Glucocorticoid regulates both physical and psychological stress and has been associated with MDD (Krishnan et al. 2008, Manji et al. 2001). Therefore, acute or chronic stress will elevate the glucocorticoid levels leading to atrophic changes in the hippocampus region, resulting in neurogenesis in MDD patients (Manji et al. 2001). Reduced hippocampus regions could result in a loss of neurons (Mann et al 2005). Elevated glucocorticoids could explain metabolic abnormalities in MDD such as diabetes or metabolic syndrome (Krishnan et al. 2008). It has also been shown that patients with severe MDD episodes may have elevated cortisol levels (Mann et al 2005).

Over the past four decades neuroscience research has aimed to identify the causative factors for deficiency of the neurotransmitters serotonin (from dorsal raphe), noradrenalin (from locus coeruleus) and dopamine (from ventral tegmental area). However, little is still known about their role in the pathogenesis of MDD (Manji et al. 2001, Krishnan et al. 2008). The monoaminergic

neurotransmitter systems have been associated with MDD for two reasons. Firstly the monoamine system is distributed throughout the limbic, striatal and prefrontal cortical regions and secondly due to the effective regulation of serotonin and noradrenalin by antidepressants (Manji et al. 2001).The optimal functioning of neurotransmitters and circadian rhythms are influenced by the gonadal hormones, which are required for stress management (Piccinelli et al. 2000). Monoamine oxidase catabolizers serotonin and noradrenalin inhibit the neurotransmitters and elevate the accessibility (Bellmark et al. 2008). The serotoninergic and noradrenergic pathway expands the majority of the human brain and monitors and controls the areas of emotion, thought and behaviour (Bellmark et al. 2008). Rat models have shown that serotonin and noradrenalin antidepressant treatments elevate dopamine levels in the hippocampus and frontal cortex, respectively (Benedetti et al. 2009).

These findings led to the monoamine hypothesis which originally stated that depression occurs due to a deficiency of neurotransmitters serotonin and noradrenalin (Kiyohara et al. 2009). This deduction was made due to antidepressant treatments stimulating the depleted concentrations of these neurotransmitters in patients with depression (Krishnan et al. 2008). The serotoninergic system plays a pivotal role in mood disturbances, appetite and stress, all of which could lead to a functional deficiency of serotonin levels. During stressful periods the serotonin levels are degraded in the body, and could explain the association between a deficiency of this neurotransmitter and MDD (Kiyohara et al. 2009, Piccinelli et al. 2000).

Recent neuroscience studies focuses on intracellular signalling pathways involved in neuroplastic events, which controls the processing of neurons and could modify signals produced by neurotransmitters (Manji et al. 2001). Stress modifies the mechanisms of neuroplasticity in the hippocampus and prefrontal cortex, which are functionally abnormal in MDD patients (Pittenger et al. 2008). Stress results in atrophy and deterioration of apical dendrites and pyramidal cells in the hippocampus resulting in impaired neuroplasticity, which could lead to recurrent depressive episodes and disease progression (Manji et al. 2001, Pittenger et al. 2008). However, the exact mechanism underlying the pathogenesis of MDD and abnormal neuroplasticity is unknown and has mostly been conducted in animal studies (Pittenger et al. 2008). Neuroplasticity regulates appetite, sleep disturbances and psychosocial and cognitive processes (Manji et al. 2001).

MDD could disrupt the immune system leading to immune and endocrine changes (Kiecolt-Glaser et al. 2002). The hypothalamic-pituitary-adrenal (HPA) axis is constantly activated which compromises the immune system of individuals, elevates DNA damage and inhibits apoptosis. This HPA axis disruption may result in a worse prognosis of additional comorbid diseases (Degi et al. 2010). Corticotropin-releasing factor (CRF) regulates the secretion of corticotropin, which elevates cortisol levels in (stressed) patients suffering with depression (Kay et al. 2000; Murray et al. 2008). This hypercolesterolaemia could lead to the development of insulin resistance (type 2

diabetes), which has been linked previously to depression (Hotopf et al. 2008). Hypercortisolemia dysfunction of the hypothalamic-pituitary-thyroid axis results in an insufficient release of the thyroid stimulating hormone (TSH). It is for this reason that thyroid hormone could be used as augmentation strategy to patients with partial response to antidepressants (Kay et al. 2000; Kaplan et al. 1998). The neurotoxicity hypothesis suggests that elevated glucocorticoids levels (over an extended period) could lead to neuronal damage (Sheline et al. 2011) or reduced hippocampal volumes in patients with recurrent MDD and hypothalamic-pituitary-adrenal axis abnormalities (Sheline et al. 2011). Since cholesterol is a key component in the development of cell membranes and lipoproteins, both elevated and reduced cholesterol concentrations have been linked to depression (Ledochowski et al. 2003).

In recent years, circadian rhythm disturbances became an area of focus for the treatment of MDD. The majority of MDD patients have psychomotor dysfunction, altered circadian rhythms and sleep disturbances (Kasper et al. 2010), which include modifications in REM sleep (reduced REM latency, elevated interval of first REM cycle and broken delta sleep), cortisol secretion and body temperature (Kay et al. 2000, Kaplan et al. 1998). The biological clock is situated in the suprachiastmatic nuclei of the anterior hypothalamus which is essential for the regulation of the circadian rhythm (Quera Salva et al. 2011). Abnormalities in sleeping patterns such as insomnia (Waldinger et al. 1997) are one of the major symptoms in MDD and 90% of patients report that these disturbances affect daytime functioning (Kasper et al. 2010).

MDD patients can experience either initial insomnia (difficulty falling asleep), middle insomnia (wake up at night and then struggle to fall sleep again) or terminal insomnia (early morning waking and cannot fall asleep again) (Waldinger et al. 1997, Andreasen et al. 2006). Sleep abnormalities result in lingering symptoms which elevate disease progression and increase the risk for recurrent episodes (Kasper et al. 2010). Most antidepressants improve sleep disturbances. Unfortunately, SSRIs and SNRIs may change sleep patterns or disrupt sleep initially, therefore up to 35% of patients initially receive a hypnotic drug to assist in sleeping while circadian rhythms normalise (Kasper et al. 2010, Waldinger et al. 1997).

1.3.1.2 Genetic contributions

A current hypothesis states that the behavioural symptoms associated with MDD could be explained by predisposed genes and that environmental variables hijack the epigenetic profile in the human brain (Sun et al. 2013). Therefore, it has become increasingly apparent that analyzing genetic factors separately from contributing environmental factors hinders our understanding of complex multifactorial disorders. Therefore, the interplay of these factors in addition to identifying

the epigenetic mechanisms is crucial, to ascertain the aetiology, mechanism and susceptibility of MDD (Mill et al. 2007, Champagne et al. 2009).

Epigenetics has been associated with neurogenesis, neuronal plasticity, learning and memory dysfunction, MDD, addiction, schizophrenia and cognitive dysfunction (Tsankova et al. 2007). The mechanism of epigenetics involves altering the gene expression (DNA methylation, histones and protein interactions) without changing the genetic code itself (Schroeder et al. 2010, Tsankova et al. 2007, Mill et al. 2007). The process is produced during mitosis and due to tissue specificity and environmental variables it is unique to every individual (Mill et al. 2007), therefore epigenetics is both heritable and acquired (Schroeder et al. 2010, Tsankova et al. 2007). It regulates genomic functions interceded by modifications in DNA methylation and chromatin configuration. The function of chromatin (histone and non-histone proteins) is to compress and compact double stranded DNA in order to generate a barrier for repair, transcription, replication, and recombination (Sun et al. 2013). Chromatin is generally in the inactive form (heterochromatin, deacetylated) and once activated (euchromatin, acetylated) transcription of genes are possible (Mill et al. 2007). The nucleosome is responsible for folding and packaging DNA into the nucleus of cells, which guarantees contact of DNA for transcription (Tsankova et al. 2007).

Epigenetics is necessary for optimal cellular development, differentiation and gene function regulation (Mill et al. 2007), which is determined by how accessible a DNA sequence is to the transcription factors (Champagne et al. 2009). DNA sequences determine the structure of proteins, whereas epigenetics manages superiority, locality and the phase of gene expression (Mill et al. 2007).

Two major mechanisms are involved in the epigenetic process namely:

- DNA methylation that affects a gene over an extended period and occurs when a methyl group is added to the promoter region of the gene sequence. This reduces the accessibility of DNA and ‘silence’ gene expression, while instigating transcription of the adjoining gene (Champagne et al. 2009, Schroeder et al. 2010).
- Cytosine and Guanine islands (CpG) are responsible for the methylation of the CpG promoter region by methylation of the CpG binding proteins in order to attach and suppress gene expression (Schroeder et al. 2010).

Depression is commonly associated with learning and memory abnormalities, which results in a variation of DNA methylation and histone modifications. This hinders the methylation process in the hippocampus and deteriorates memory (Tsankova et al. 2007, Champagne et al. 2009). Epigenetic profiles are influenced by environmental toxins, which modify DNA methylation and histones. Dietary intake such as folate status influences DNA methylation and methamphetamines drugs. An

interplay between genetic polymorphisms and environmental factors may lead to an increased risk of developing MDD and may explain why certain individuals are susceptible to adverse life events and others not (Mill et al. 2007), Liu et al. 2013).

It is estimated that approximately 10 million polymorphisms exist in the human body, which is divided into two subclasses, namely tandem repeats and single nucleotide polymorphisms (SNPs) (Kiyohara et al. 2009). A SNP is defined as a variation in a base pair between two or more nucleotides which alters the function of a gene (Kiyohara et al. 2009). SNPs occur in more than 1% of the general population and may affect the expression of the protein, which may in turn affect disease development or progression (Kiyohara et al. 2009). Numerous studies have reported that genetic factors could contribute to the disease burden of MDD (Piccinelli et al. 2000). The heritability of MDD is 40% (Tamatam et al. 2012), which was corroborated by findings of Sun et al. (2013) indicating that the heritability is approximately 31-42%. This may predisposes an individual to experience an earlier onset of disease, recurrence and more severe symptoms (Mill et al. 2007).

Genetic twin and adoption studies have reported an association between MDD and genetic etiology (Kaplan et al. 1998). Twin studies have shown that in monozygotic twins, when one has MDD the other has a 50% chance of also developing the condition. Whereas with dizygotic twins, when one has MDD the other has a 10-25% chance of developing the condition (Kaplan et al. 1998). The 50% discordance rate is less than the desired 100% heritability; however it indicates a genetic component (Mill et al. 2007, Sun et al. 2013). The 50% discordance rate could be as a result of non-shared environmental or epigenetic factors. These findings support the interaction between genetic polymorphisms and environmental variables (stressful life events), both of which contribute to the increased risk of MDD (Sun et al. 2013). Only a small number of adoption studies have been performed to confirm a potential link between MDD and genetic aetiology (Mill et al. 2007). These studies showed that biological children of depressed parents have an increased risk for depression even if they are raised by non-affected adoptive families (Kaplan et al. 1998). However, discrepancies have been noted and could be as a result of the methodologies used in different studies (Mill et al. 2007).

Genetic counselling is important in patients with MDD since the disorder may be inherited (Smoller et al. 2008). Children were shown to be at an increased risk for MDD when a parent suffers from the disorder, with an estimated 3-fold increased risk (Smoller et al. 2008). First degree relatives of patients with MDD have a 2-3-fold increased risk for developing the disorder compared to normal control relatives (Moore et al. 1996). The population frequency for MDD is estimated to be 15% for first-degree relatives.

Despite numerous genetic studies performed world-wide, no success has been made in associating a single major gene with MDD and the outcome of antidepressant treatment cannot

always be replicated across the various samples (Klengel et al. 2012). This may be due to the fact that MDD is multifactorial and genetically complex disorder, with numerous genes involved in several different modes of inheritance (Klengel et al. 2012, Tamatam et al. 2012). Interaction between genes and modification of gene expression due to environmental influences obscure efforts to understand the disorder (Lesch 2004) and most likely explain the 'missing heritability' (Klengel et al. 2012).

The human genome consists of 3 billion base pairs which are packaged in various genes. A permanent change in structure or genetic information may result in altered or abnormal protein production, this is known as a mutation (genetic variation) (Richard et al. 2005). Numerous genes and/or genetic variations have been linked to MDD, however the mechanisms, aetiology, clinical relevance and risk factors of each gene have yet to be determined. Understanding the mechanism and functionality of genetic variations could lead to improved treatment programs (Richard et al. 2005) and for this reason functional polymorphisms in the MTHFR (rs1801133 and rs1801131), COMT (rs4680), ASMT (rs4446909), CYP2D6 (rs3892097) and SLC6A4 (rs4795541) genes were evaluated further for potential clinical application.

5, 10-methylenetetrahydrofolatereductase (MTHFR) gene

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme required for the one-carbon metabolism pathway. The 5, 10-methylenetetrahydrofolatereductase (MTHFR) gene is crucial for optimal functioning of the folate-mediated methylation pathway, DNA methylation and the formation of neurotransmitters (Peerbooms et al. 2011). The function of MTHFR is to convert folate to 5-methyltetrahydrofolate (MTHF), for the remethylation of homocysteine to methionine (Kungi et al. 1998, Devlin et al. 2012). 5-MTHF is the primary circulating form of folate and it transfers a methyl group to homocysteine forming S-adenosylmethionine (SAM) (Arinami et al. 1997, Gilbody et al. 2007), a precursor molecule required for DNA methylation (Toffoli et al. 2003).

The most commonly studied SNP in psychiatric disorders is the MTHFR rs1801133 positioned in exon 4, which results in an amino acid change from alanine to valine. This change inhibits the re-methylation of homocysteine to methionine (Toffoli et al. 2003). This may lead to hyperhomocysteinaemia and reduced DNA methylation, especially with inadequate folate intake. A second SNP, the MTHFR rs1801131 variant positioned in exon 7, results in a change from glutamate to alanine (Toffoli et al. 2003). Each copy of the 677T allele in a homozygous genotype leads to a decline in enzymatic activity by 35% compared to the heterozygous genotype, which displays ~65% of normal enzymatic activity (Lea et al. 2004). Both MTHFR rs1801133 and homocysteine play a vital role in the methylation process (Bjelland et al. 2003). Reduction of enzymatic activity is found with every copy of the 1298C allele, but to a lesser extent (Peerbooms

et al. 2011). Approximately 10% of Caucasian individuals are homozygous for the MTHFR rs1801133 677 T-risk associated allele.

Studies by Arinami et al. (1997) showed that individuals homozygous for the MTHFR T allele have an increased risk for depression as evidenced by an increased frequency of the T allele seen in depressed patients. Gilbody et al. (2007) corroborated this finding showing an association between the MTHFR C667T polymorphism and MDD. The study also showed an association with the MTHFR A1298C polymorphism however, this variant still needs further studies in the context of MDD (Gilbody et al. 2007). The MTHFR 677 T-risk associated allele is known to influence total plasma homocysteine levels (increase) and DNA methylation (dysfunction) and may affect anti-psychotic treatment response in children (Devlin et al. 2012).

Over recent years there has been increasing evidence that a disruption of the one-carbon metabolic pathway results in elevated homocysteine levels, which may contribute to the development of MDD and treatment response (Coppen and Bolander-Gouaille 2005). However, it is still unclear whether homocysteine is a causal factor or merely a biomarker for deficiencies in folate and other B vitamins (Bottiglieri 2005). Numerous studies have tested for association between the MTHFR rs1801133 polymorphism and MDD (Devlin et al. 2012, Peerbooms et al. 2011). Despite inconsistent results, many studies have found an association between MTHFR rs1801133 and psychiatric disorders (Arinami et al. 1997; Bjelland et al. 2003; Gilbody et al. 2007). The MTHFR 677T risk allele may be associated with cardiovascular disease, hypertension, congenital abnormalities, spontaneous abortions (Toffoli et al. 2003, Devlin et al. 2012) and neural tube defects (Mayor-Olea et al. 2008). It could also increase the risk for acute leukaemia, esophageal squamous cell and gastric carcinoma, as well as other types of cancers when folate status is low (Toffoli et al. 2003). Other clinical associations include mental retardation, motor dysfunction, seizures and thrombosis (Arinami et al. 1997). This dysfunction could affect the epigenetic process disrupting the gene expression and ultimately could contribute to the development of MDD; however the mechanism and its components remain unclear.

Homocysteine is a sulphurated amino acid produced from methionine and is generated from the ingestion of foods such as cheese, eggs, fish, meat and poultry. Increased plasma homocysteine levels are toxic to both neurons and the blood vessels in the human body and may result in the degradation of DNA, oxidative stress and apoptosis (Folstein et al. 2007). In addition to MDD raised homocysteine levels have been associated with numerous other diseases and neurological disorders including seizures, Alzheimer's disease, schizophrenia and Parkinson's disease (Folstein et al. 2007, Gu et al. 2012).

MDD patients with reduced folate levels experience prolonged and severe depressive episodes and responds poorly to treatment compared to those with normal folate levels. Therefore, folate

supplementation may improve treatment response (Bjelland et al. 2003). Increased total plasma homocysteine can be used as a biological marker for deficiencies in both folate and vitamin B12 (Ebesunun et al. 2012). These micronutrients play an essential role in the production of monamine neurotransmitters (Tiermeier et al. 2002). Deficiencies in folate and vitamin B12 underlying high homocysteine levels have been convincingly been linked to MDD (Tiermeier et al. 2002, Dimopoulos et al. 2007). Therefore, it may be possible to extrapolate that cerebral vascular disease and /or a deficiency in neurotransmitters may lead to depression. A study by Ebesunun et al. (2012) showed raised homocysteine levels in conjunction with reduced vitamin B12 levels in Nigerian depression patients.

In addition to variation in methylation pathway genes such as MTHFR, environmental factors such as smoking and excessive alcohol consumption may also increase homocysteine levels. Modifications in homocysteine levels are also influenced by ageing, deteriorating physiological changes, drug-drug interactions and medical conditions, including hypothyroidism, rheumatoid arthritis, systemic lupus erythematosis and diabetes (Coppen and Bolander-Gouaille 2005; Gu et al. 2012).

Catechol-O-methyltransferase (COMT) gene

Catechol-O-methyltransferase (COMT) plays a role in the catabolic pathways responsible for neurotransmission (Schosser et al. 20120). The enzyme is required for the break-down of catecholamine neurotransmitters by methylating dopamine, epinephrine and norepinephrine (Massat et al. 2005, Kiyohara et al. 2009, Bendetti et al. 2009). The enzymatic activity varies between ethnic groups at least in part due to differences in allele frequencies that may vary amongst population groups (Palmatier 1999).

COMT affects the prefrontal cortex, dopamine regulation and may modify cognition, emotions and behaviour (Lohoff et al. 2008). The COMT protein has two variants namely soluble and membrane COMT (S-COMT and MB-COMT respectively) encoded by a single gene on chromosome 22 (Palmatier. 1999). The two variants are identical with the exception of 50 amino acids on the N-terminal region of the MB-COMT (Palmatier. 1999). In tissue these two variants are expressed from two mRNA transcripts namely a long and short mRNA. The long m-RNA produces both S-COMT and MB-COMT, whereas the short m-RNA only produces the S-COMT. The short m-RNA is present in all tissue except the brain, which is the most abundant (Palmatier. 1999).

The most extensively studied SNP in the COMT gene is the rs4680 (Val158Met) polymorphism (Volavka et al. 2004). This variant is characterized by an amino acid change from Val (472G) to Met (472A) at codon 158. COMT Met/Met causes reduced enzymatic activity 3-4 times lower, whereas COMT Met/Val has intermediary activity and COMT Val/Val increased activity (Lohoff et al. 2008, Kiyohara et al. 2009, Ill et al. 2010, Bendetti et al. 2009). COMT is a candidate gene for

neurological disorders involved in noradrenergic and dopaminergic systems (Palmatier. 1999, Schosser et al. 2012), and has been linked to MDD (Kiyohara et al. 2009) and SSRIs treatment response (Ill et al. 2010). Homozygosity for COMT Val/Val is associated with decreased availability of dopamine and nor epinephrine and impaired pharmacological efficacy of serotonergic and noradrenergic antidepressants during the first 6 weeks of treatment (Baune et al. 2008). It therefore seems likely that antidepressive add-on therapy with substances increasing dopamine availability may be beneficial in MDD patients homozygous for the high-activity COMT allele.

Ohara et al. (1998) reported a significant association between MDD and COMT Met/Met, however not all studies replicated this finding (Kungai et al. 1997, Frisch et al. 1999, Serretti et al. 2006). In addition to MDD, variation in the COMT gene has also been associated with Parkinson's disease, obsessive compulsive disorder (OCD), schizophrenia, bipolar disorder, attention deficit hyperactivity disorder (ADHA), substance abuse, violence, phobic anxiety and panic disorder (Palmatier 1999, Hoth et al. 2006, Lohoff et al. 2008). It has also been associated with suicide as indicated in a meta- analysis by Kia-Keating et al. (2007) with apparent gender differences. However it could also be indirectly involved in suicide due to an effect on various personality traits (Schosser et al. 2012). Peerbooms et al. (2011) showed that patients with a T allele for MTHFR C677T and COMT Met/Met displayed an increase in psychotic symptoms when experiencing stress. This interaction was not observed in healthy control individuals.

Acetylserotoninmethyltransferase (ASMT) gene

The function of the acetylserotoninmethyltransferase (ASMT) gene is to convert N-acetlyserotonin to melatonin and increase serotonin levels. Pacchierotti et al. (2001) argues the reason melatonin can be used as a marker for depression is due to the modified circadian rhythms and the reduced secretion of melatonin (Pacchierotti et al. 2001). Reduced melatonin levels could increase the risk for recurrent depression, however this has not been proved or disproved (Kripe et al. 2011, Galecki et al. 2010).

Melatonin is a pineal hormone coordinated by the light/dark cycle (Pacchierotti et al. 2001) and plays an essential role in the regulation of sleep and circadian rhythms (Srinivasan et al. 2006). The circadian clock controls both the synthesis and secretion of melatonin cAMP signal transduction cascade (Eser et al. 2009). Daylight inhibits the synthesis of melatonin that occurs after sunset, climaxes at 2 am and regresses by morning (Pacchierotti et al. 2001). Patients suffering from MDD and bipolar disorder have altered melatonin secretion (Srinivasan et al. 2006), which could explain the sleep disturbances and insomnia. Certain authors believe that pineal dysfunction occurs due to a deficiency in the neurotransmitters serotonin and norepinephrine levels in the brain (Pacchierotti et al. 2001). Melatonin may be synthesised in the retina, intestines, bone marrow and lymphocytes. The ASMT enzyme is located in the pseudoautosomal region (PAR) 1 of

the X and Y sex chromosomes (Galecki et al. 2010, www.ncbi.nlm.gov). The recently identified ASMT rs4446909 SNP occurs in the promoter region of the gene and affects the expression of ASMT enzymatic activity. The ASMT rs4446909 A-allele may reduce the risk for recurrent depression, therefore it is regarded as protective (Galecki et al. 2010), whereas the G allele is associated with an increased risk of MDD (Kripe et al. 2011).

Altered melatonin levels may occur due to genetic regulation, age, diet and seasonal change (Pacchierotti et al. 2001), whereas secretion may be diminished due to neoplasia, neurological disorders, migraines, dizziness, epilepsy and Alzheimer's disease. Other studies showed that melatonin may correlate with ideas of suicide (Srinivasan et al. 2006).

Cytochrome P450 family 2 subfamily D polypeptide (CYP2D6) gene

Cytochrome P450 family 2 subfamily D polypeptide is a highly polymorphic metabolic enzyme which was first discovered in the 1970's. The function of this enzyme is to oxidise the metabolism of drugs and catalyzes about 90% of all drugs. A defect of this enzyme may reduce an individual's ability to metabolize certain drugs (Zanger et al. 2008). This trait is inherited autosomal recessively and may lead to over-reaction, toxicity or a lack of response to certain drugs (Zanger et al. 2008). CYP2D6 plays a role in the oxidative metabolism of various drugs such as neuroleptics, antidepressants (TCA's and SSRI's) (Steijns et al. 1998), adrenergic-blocking drugs (metoprolol), anti-arrhythmic drugs (sparteine and propafenone) and opiods (codeine) (Zanger et al. 2008).

The CYP2D6 gene is situated in close proximity to two cytochrome pseudogenes namely the CYP2D7 and CYP2D8P on chromosome 22q13.1. More than 60 different alleles have been discovered for this enzyme (Zanger et al. 2008). The CYP2D6 allele 4 (rs3892097) is the most common variant occurring in Caucasians with a heterozygote frequency of 20-25%. In this population group the null allele for CYP2D6 allele 4 occurs due to a premature stop codon. A change occurs in the slice site acceptor site of intron 3 (slicing defect), which results in a base change from a G (wild type) to A at nucleotide position 1846 in intron 3. Between 5-10% of the Caucasian population have the null-allele compared to 1-3% in other ethnic groups (Zanger et al. 2008). Individuals who inherit two copies of CYP2D6 allele 4 have the poor metabolizer (PM) phenotype. Carriers are more susceptible to severe adverse drug reactions (ADR's) such as hyponatraemia and reduced serum sodium concentrations (Kwadijk-de Gijsel et al. 2009). PMs metabolize drugs slower whereas the ultrarapid metabolizers (UM) with high enzymatic activity metabolize drugs at a rapid rate (Steijns et al. 1998).

Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 gene (SLC6A4)

Serotonergic (5-HTT, SLC6A4) neurotransmission plays an essential role in various physiological functions such as appetite, sleep and anxiety (Wilhelm et al. 2006). More than 40 years ago Alec

Coppen (1967) proposed the 5-HTT transporter hypothesis. The hypothesis stated that reduced activity of the 5-HTT pathway could lead to MDD (Cowen 2008). This evidence was based on TCAs inhibiting the reuptake of 5-HTT, thus increasing the 5-HTT activity in depressed individuals. The use of SSRI's was further proof that enhancing the 5-HTT function may improve symptoms of depression (Cowen 2008). Due to the effectiveness of SSRI treatment in certain patients numerous studies have explored the potential association between MDD and 5-HTT, however the mechanism remains unclear with mixed results reported (Kiyohara et al. 2009). The SLC6A4 gene is located on chromosome 17 and plays a key role in the regulation of serotonergic neurotransmitters in brain and peripheral systems (Bondy et al. 2006, Wilhelm et al. 2006). The function of 5-HTT is to reuptake serotonin in pre-synapses in order to terminate and regulate serotonergic neurotransmission (Nakamura et al. 2000).

The most common genetic alteration in the system is the 5-HTTLPR rs4795541 polymorphism in the promoter region of the 5-HTT gene, which reduces the ability of the brain to re-uptake serotonin efficiently (Kiyohara et al. 2009, Nakamura et al. 2000, Wilhelm et al. 2006). 5-HTTLPR rs4795541 is a functional polymorphism in the 5'-regulatory region, which modifies gene transcription due to a 44bp deletion. Two alleles of 14 repeats (short allele) and 16 repeats (long allele) were identified (Kiyohara et al. 2009, Bondy et al. 2006, Heuzo-Diaz et al. 2009). The short allele (S allele) is associated with reduced gene expression and transcriptional efficacy of serotonin (Kiyohara et al. 2009, Bondy et al. 2006). The short allele appears to increase an individual's susceptibility to develop MDD with adverse life events; this is known as the gene x environment interaction hypothesis (Caspi et al. 2003, Wilhelm et al. 2006), meaning that individuals with the two copies of the short allele are more susceptible to experience MDD due to adverse life event than individuals with one or no copy of the short allele. This was confirmed in a meta-analysis performed by Lotrich at al. (2004) which indicated that individuals with the SS genotype are more susceptible to developing MDD compared to those with the LL genotype. Adverse life events including childhood trauma and/or maltreatment may contribute to the gene-environmental interaction and MDD onset (Wilhelm et al. 2006).

The SLC6A4 gene has been associated with several other psychiatric disorders in addition to depression, including anxiety, obsessive-compulsive disorder, substance abuse disorders and suicide (Nakamura et al. 2000, Wilhelm et al. 2006, Bondy et al. 2006, Smoller et al. 2008). It may also play an important role in eating disorders, attention deficit hyperactivity disorder (ADHD), autism, schizophrenia, Alzheimer's and Parkinson's disease (Serretti et al. 2006).

1.3.2 Psychosocial

Personal and Environmental Contributions

MDD is influenced by numerous psychosocial stressors which triggers the disorder and contributes to its severity (Mill et al. 2007). MDD is associated with environmental triggers and adverse life events, which reduces an individual's ability to function optimally in their careers, relationships (separation or divorce etc.) or in social activities (Klein et al. 1993, Kiyohara et al. 2009). Environmental triggers may increase the risk of psychiatric disorders and need to be taken into account to provide a more accurate estimate of risk (Peerbooms et al. 2011).

Epidemiological studies have confirmed that adverse life events such as social isolation and difficulties, death of a spouse or loved one, sexual assault, violent crimes, prolonged medical conditions, injuries and disabilities, absence of social support, grief, natural disasters, war, physical abuse, low self-esteem and/or marital discord are all associated with the onset of MDD (Mill et al. 2007, Kiyohara et al. 2009, Piccinelli et al. 2000, Liu et al. 2013). Studies have also indicated that a low socioeconomic status has been associated with MDD, due to psychosocial stressors such as unemployment, low education status, poverty, housing and financial difficulties (Stromberg et al. 2011). However, not all individuals are sensitive to adverse life events. Therefore, understanding the genetic contribution and environmental triggers will help comprehend the pathophysiology of MDD (Klengel et al. 2012).

Various studies during the past 15 years have reviewed the association between stress and depression (Kiyohara et al. 2009). Stressful life events such as bereavement or loss of a career may cause a depressive episode or vice versa (Moore et al. 1996). However, it is unclear whether stressful life events play a minor or major role in MDD (Kaplan et al. 1998). Stress is activated and triggered by a stress response that stimulates the physiological systems in the body to release neurotransmitters and hormones, due to stressor stimulation in the brain (Kiyohara et al. 2009). This stimulus is received by the cortex region of the brain and transmitted to the hypothalamus, where cortico-tropin-releasing hormone (CRH) is secreted and transported to the pituitary receptors (Belmaker et al. 2008).

In most individuals stress is harmless and can be seen as a stimulant (positive stress or acute stress), however certain individuals experience negative stress or chronic stress. Chronic stress is consistent and occurs due to the dysfunction of the cortisol rhythm in the body (Kiyohara et al. 2009). In animal studies acute stress was demonstrated when rodents were forced to swim and the coping response was assessed. With chronic stress rodents were exposed to physical pain or social isolation for an extended period to produce anhedonia symptoms and reduce the reward system (Sun et al. 2013). One needs to consider the possibility that antidepressants may not heighten an individual's mood but rather reduces secondary stress, which could explain the clinical

relevance and usefulness of antidepressant treatments (Belmaker et al. 2008). Due to the variation in stress types each response to stress may differ between individuals, therefore stress could be a contributing factor to a depressed mood or a consequence of a depressive mood (Belmaker et al. 2008).

1.4 Medical comorbidity

Numerous medical conditions contribute to the risk of MDD, with co-morbid diseases such as cardiovascular disease (CVD), cancer, diabetes mellitus and the metabolic syndrome found to be frequently associated with depression (Kiyohara et al. 2009; DeRubeis et al, 2008). Studies have estimated that 78% of MDD patients have chronic medical illnesses (Dwight-Jonson et al. 2000). It is for this reason that long term medical conditions need to be taken into account when diagnosing and treating MDD. Table 1.2 indicates other neurological and/or general medical disorders and certain drugs or medications respectively, which contribute to development of MDD.

Table 1.2. Neurological and/or general medical conditions which may lead to MDD-like symptoms

Neurological Disorders	Infectious Disorders	Cardiovascular Disease	Endocrine Disorders	Vitamin Deficiencies
Parkinson's disease	Syphilis	Cardiomyopathy	Hypoglycaemia	Folate
Huntington's disease	Hepatitis	Cerebral ischemia	Postpartum	Vitamin B12
Alzheimer's disease	AIDS	Congestive heart failure	Cushing's or Addison's disease	Niacin
Dementia	Tuberculosis	Myocardial infarction	Hyperaldosteronism	Vitamin C
Multiple sclerosis	Influenza		Hypothyroidism	Thaimine
Wilson's disease			Hyperthyroidism	Iron
Brain tumors				Riboflavin
Narcolepsy				Pyridoxine

An increased risk of depression has been noted in individuals with cardiovascular disease (CVD) since the late 1960's (Rudisch et al. 2003). This has been corroborated in epidemiological studies which have reported a significant association between MDD and CVD, in which the disorder elevates the morbidity and mortality of CVD (Bellmaker et al. 2008). In the majority of patients CVD precedes MDD; however the reverse is also a possibility (Bellmaker et al. 2008, Assies et al. 2004, Musselman et al. 1998). Patients who experienced an acute myocardial infarction (MI) are approximately three times more likely to develop depressive symptoms, with 15-20% of patients who experienced an MI meeting the DSM-IV criteria for depression (Lichtman et al. 2008). It has been shown that individuals with either moderate or severe depression have a 69% increased risk for cardiac death and 78% for all caused deaths. Therefore, it is essential to accurately diagnose and treat these patients at an early stage of the disorder in order to reduce health care costs, improve treatment response and quality of life (Lichtman et al. 2008).

MDD has also been associated with cancer with an estimated 60% of cancer patients reporting depressive symptoms (Degi et al. 2010). Cancer patients with MDD are more likely to experience elevated suicidal thoughts and attempts and scepticism about treatment efficacy and recovery, which contribute to disease progression, treatment response and reduced quality of life (Degi et al. 2010). Depression reduces natural killer cells, which could elevate the development of cancer (Degi et al. 2010). The relationship between depression and cancer is influenced by numerous risk factors such as smoking, gender and age, adverse life events, low education status, social isolation, alcohol consumption and body mass (Degi et al. 2010).

An association was reported between depression and diabetes (Gavard et al. 1993) as evidenced by a meta-analysis performed by Anderson et al. (2001). This study concluded that patients with diabetes are twice as likely to develop MDD, with the odds being more prevalent in females with diabetes compared to males. This association increases the risk of hyperglycaemia, complications, functional impairment and mortality in patients (Gonzalez et al. 2008). Both direct and indirect effects could explain the poor glycaemic status noted (Kornstein et al. 2001). This relationship between diabetes and MDD may be due to reduced self-care and treatment adherence (Gonzalez et al. 2008).

Patients with MDD and CVD frequently show signs of the metabolic syndrome (MetS). MetS is a grouping of cardiovascular risk factors including insulin resistance (most common feature), central obesity, hypertension, glucose intolerance, diabetes mellitus, dyslipidaemia, elevated triglycerides and reduced HDL cholesterol, all of which elevates an individual's chance for the development of CVD ((Assies et al. 2004, Loprinzi et al . 2012). Skilton et al. (2007) showed a significant association between depression and MetS in both men (22.5% with MetS and depressed and 15.5% controls) and women (38.6% with MetS and depressed and 23.3% for controls). MetS occurs in nearly 40% of the adult population in the US (Loprinzi et al. 2012). Certain studies have

indicated that side effects of second generation antipsychotic (SGA) treatment could lead to MetS, whereas 37.3% of adults report MetS. This association was also confirmed in studies of children linked to genetic variation in the MTHFR gene (Devlin et al. 2012).

Depression can increase mortality rate both by the interaction with medical conditions as noted above, but also by suicide. Suicide is a deliberate and premeditated self-inflicted act as a result of the accumulation of consistent thoughts about attempting completed suicide, which is mostly triggered by psychological distress (Bondy et al. 2006, Kaplan et al. 1998). Patients perceive this as the key solution for a desperate need to escape unbearable pain (Kaplan et al. 1998). According to the WHO 2% of deaths worldwide are suicide related, accounting for approximately 1 million deaths per year worldwide and predicted to increase to 1.5 million by 2020 (Kapur et al. 2008). MDD increases the risk of suicidal tendencies with a mortality rate of 242 per 100 000, with the prevalence of death by suicide found to be 15% (Kiyohara et al. 2009). About 2/3 of patients with depression have suicidal ideation, 10% attempt suicide, and 1% successfully commit suicide (www.who.int, Bondy et al. 2006).

Variations such as climate, age, gender, ethnicity, occupation, marital status, physical and mental health, methods, substance abuse, religion, social, political or genetic risk could be important contributing factors to suicide (Bondy et al. 2006, Kaplan et al. 1998). It is more prevalent in males compared to females, with the exception of China were more females are prone to suicide (Bondy et al. 2006, Kapur et al. 2008). Suicide is most commonly noted in Caucasian males at an average age of 45 years due to marital conflicts (Andreasen et al. 2006). The frequency of suicidal thoughts are doubled in females compared to males, however violent suicidal methods are more frequently noted in males (Bondy et al. 2006, Waldinger et al. 1997). Suicide attempts are more prevalent in females; however males are more successful at completing the attempt (Kiyohara et al. 2009, Waldinger et al. 1997). Males are most likely to commit suicide using violent methods such as suicide by firearms, hanging or jumping, whereas females are more likely to overdose or consume toxic substances (Kaplan et al. 1998, Andreasen et al. 2006). Alcoholics are at an increased risk of committing suicide (15%) of which 80% are males (Kaplan et al. 1998) and 2-4% of chronic alcoholics will commit suicide (Andreasen et al. 2006). It has been estimated that 90% of suicide victims have psychiatric conditions; therefore suicide needs to be supervised in hospital where the patient are consistently monitored with limited access to sharp objects, belts or any other potentially lethal objects (Andreasen et al. 2006).

1.5 Lifestyle Factors

Psychoactive substances have been associated with MDD, which changes and modifies an individual's state of mind (Andreasen et al. 2006). Substance abuse is defined as excessive

consumption of potentially toxic substances such as alcohol, nicotine, prescription medication and illegal substances (www.medicine.net.com). It could result in the destruction of an individual's profession related to reduced work performance, physical harm such as driving while intoxicated, legal implications or social problems within the family (Andreasen et al. 2006). While many MDD patients use alcohol to self-medicate, alcohol addiction could also be the cause of MDD (Waldinger et al. 1997). Excessive alcohol intake could lead to physical, psychiatric and social morbidity (Puri et al. 2002). Approximately 76% of males and 65% of females suffer from both alcohol addiction and a psychiatric disorder, with the peak comorbid rates influenced by the most potent substance i.e. opioids, cocaine and alcohol (Kaplan et al. 1998). An increase of substance abuse over the years may be due to wider availability of these substances. It is most commonly associated with adolescents and young adults, however other groups can be affected as well (Andreasen et al. 2006). The lifetime prevalence for alcohol addiction is 14%, whereas the lifetime prevalence for drug addiction is 14.6% in males and 9.5% in females. It is recommended that patients diagnosed with depression should refrain from psychoactive substances, which are likely to elevate MDD symptoms, increase the risk of suicide and potentially could cause adverse reactions with antidepressant drugs (Kay et al. 2000). Table 1.3 provides a summary of various drugs associated with MDD.

Table 1.3. Psychoactive and other drugs associated with depressive symptoms

Narcotics	Antihypertensive drugs	Gastrointestinal drugs	Other drugs
Marijuana	Reserpine	Cimetidine	Cytotoxic agents
Amphetamines	Propranolol		Corticosteroids
Cocaine	Methyldopa		Oral contraceptives
Opiates	Clonidine		
Alcohol	Guanethidine		

Globally smoking is a major health concern and tobacco is one of the most misused substances worldwide. According to the WHO roughly one billion males currently smoke and 250 million females smoke (Bruijnzeel 2012). Each year 5.4 million people die from tobacco usage and 600 000 from secondary exposure (Bruijnzeel 2012). In the USA the prevalence of smoking is 20-25%,

however in patients with disabling mood disorders the frequency is 35-65% (Mineur et al. 2009). Nicotine is considered to be the addictive substance in smoking (Mineur et al. 2009). During smoking nicotine binds to both the central and autonomic nervous system, which triggers and desensitizes nicotinic acetylcholine receptors (nAChRs). These receptors are stimulated by the acetylcholine neurotransmitter or nicotine with results in neuronal firing (Mineur et al. 2009).

In chronic smokers nicotine affects numerous brain circuits causing neuroadaptions, which could explain why after smoking depressed individuals experience elevated moods compared to normal non-smoking individuals (Mineur et al. 2009). This positive reinforcement may explain why numerous depressed individuals self-medicate with nicotine (Mineur et al. 2009). Positive reinforcements include mild exhilaration, leisure and enhanced concentration, thought and functioning memory (Bruijnzeel 2012). Therefore, MDD smokers are unlikely to refrain from smoking and more often than not experience withdrawal side effects (Mineur et al. 2009) such as depressed mood, elevated anxiety and impaired thought and functioning memory.

Despite the positive reinforcements of smoking, chronic smoking elevates a depressed mood and ultimately death (Mineur et al. 2009). This finding was supported by Luk et al. (2010), which reported that current and passive smoking are linked to raised MDD symptoms more so in adolescents and the elderly. Although smoking has frequently been linked to various psychiatric disorders including MDD, the mechanisms underlying this relationship remain unclear (Bruijnzeel 2012, Pasco et al. 2008, Luk et al. 2010). Twin studies have implicated predisposed genes for both depression and smoking (Mineur et al. 2009).

The underlying mechanism that links MDD and obesity has yet to be identified. Obesity is associated with a continuous state of low–grade inflammation associated with abnormalities in central nervous system (CNS) (Hryhorczuk et al. 2013). Over the past 20 years obesity has become increasingly frequent and a problematic health concern, with a higher prevalence in females (24.9%) than males (19.9%) (Dong et al. 2004, Carpenter et al. 2000). Although various studies have linked obesity with hypertension, coronary heart disease, diabetes mellitus, chronic back pain, degenerative joint disease and other chronic medical conditions, little is known about its role in MDD (Onyike et al. 2003, Dong et al. 2004).

Obesity could increase an individual's risk for psychological distress which increases the chances of developing MDD, therefore numerous studies have postulated an association between the two disorders (Carpenter et al. 2000, Lett et al. 2004). However, not all studies have consistently found a direct link between MDD and obesity (Dong et al. 2004). These discrepancies could be due to variation in psychiatric diagnostic criteria, the definition of obesity, the time frame, obesity levels, gender, and not taking covariates (race, socioeconomic status, BMI level) and genetic variation into account as potential confounders or contributing factors (Dong et al. 2004, Onyike et al. 2003).

A meta-analysis by Luppino et al. (2010) confirmed an association between MDD and obesity. It was predicted that 55% of obese individuals may develop MDD and conversely, 58% of MDD patients may become obese. Not all concur that the association between MDD and obesity is casual (Roberts et al. 2003). Biological mechanisms, psychological distress due to low self-esteem and/or unhealthy lifestyle could be the reason obesity leads to MDD. Increased weight gain is believed to be a late consequence of MDD (Roberts et al. 2003). Testing the relevance between obesity and MDD could be clinically relevant to implement weight reduction programs. A link between obesity and a family history of MDD has been noted but data is limited (Dong et al. 2004, Onyike et al. 2003). Failure to identify a positive link between MDD and obesity in some studies may be related to the definition of obesity level as the association may be limited to extreme obesity. Onyike et al. (2003) showed an association between past month MDD in both males and females who had a BMI of 40 kg/m2 or higher.

The consumption of foods high in fat and salt content has become more prevalent and could explain the increase of obesity rates (Bodnar et al. 2005). Such poor dietary choices over time may modify mood and behaviour via an influence on the CNS, energy metabolism, endocrine function or immunity (Hryhorczuk et al. 2013). Nutrition, dietary intake, obesity and low physical activity have also been associated with MDD (Bodnar et al. 2005, Hryhorczuk et al. 2013). Therefore, improving diet quality could improve nutrintional deficiencies (modifications in absorption, transportation or storage), the nutritional milieu of the human brain, as well as treatment response (Bodnar et al. 2005). Folate is crucial for optimal CNS functioning and is required for the production of neurotransmitters and catecholamines (Bodnar et al. 2005). Folate deficiency may occur due to a reduced appetite leading to weight loss frequently noted in MDD patients (Young et al. 2007). Reduced folate levels and elevated homocysteine levels could play a role in the pathogenesis of MDD by increasing disease severity and prolonging MDD episodes (Bodnar et al. 2005). Fava et al. (1997) reported that MDD patients with a folate deficiency had poor treatment response compared to MDD patients with sufficient folate levels (35% vs. 20%). This finding was corroborated by Papkostas et al. (2004) who reported that only 7% of MDD patients with a folate deficiency had adequate treatment response when compared to those patients with sufficient folate levels (45%). Augmentation of antidepressants with folic acid could improve treatment response and prevent recurrence (Young et al. 2007, Nelson et al. 2012). Raised homocysteine levels and reduced folate levels have been associated with elevated BMI (Mojtabai 2004; Narin et al 2005; Lawrence et al. 2006). This was corroborated in a local study by Davis et al. (2013), who found an inverse correlation between folate status and BMI.

Physical activity is believed to improve mood status by reducing pessimism, helping patients develop a new skill, and improve social interaction (Byrne et al. 1993, Cooney et al. 2013). On a biochemical level physical activity could modify endorphin, monoamine and cortisol levels to

improve mood (Cooney et al. 2013). A study by De Moor et al. (2006) reported that physical activity reduced anxiety and depression (effect sizes from -0.18 to -0.29 SD) when compared to non-exercisers. Loss of interest characteristic of depression leads to inactivity levels and modifies dietary intake that may result in inflammation and obesity, all which have been associated with MDD. Therefore, obese patients could benefit from physical activity included in the treatment management of MDD (Shelton et al. 2011, Cooney et al. 2013

1.6 Management of Major Depressive Disorder

Once a diagnosis of MDD is made, appropriate treatment is be selected. The goal of treatment is firstly acute stabilization. Once a patient is stabilised, the focus of treatment is to attain remission and maintenance thereof, and thereafter relapse prevention. Treatment should be holistic and include medication, psychotherapy, addressing the social stressors, and lifestyle changes (avoiding substances, exercise, and a healthy diet). Once treatment is prescribed the treating clinician will monitor and measure the effectiveness of treatment, including drug response. If initial poor response to treatment is evident, the treatment plan needs to be re-evaluated and adjusted. Medication adjustments can be implemented which could include dose adjustment, switching, augmentation, or combination treatment.

1.6.1 Antidepressant pharmacotherapy

A primary mechanism whereby antidepressants target the underlying pathogenesis in MDD and serve to limit or suppress symptoms is by correcting the dysfunctional regulation of monoamine neurotransmitter metabolism in the central nervous system (CNS). This process involves modification of both the degradation and reuptake of neurotransmitters such as noradrenaline, serotonin and dopamine in the limbic system (DeRubeis et al. 2008). Antidepressants elevate synaptic levels of these molecules, increase postsynaptic neuron stimulation, improve hippocampal synaptic plasticity and regulate cell survival and apoptosis (Manji et al. 2001; Bellmark et al. 2008). Genetic variations affecting these processes could potentially affect the therapeutic response to antidepressant therapy (Benedetti et al. 2009). It should be emphasized that treatment is also only effective with continued usage and does not diminish the risk of episode recurrence (DeRubeis et al. 2008). Numerous antidepressants are currently available on the market, with the most commonly prescribed ones and the individual classes to which they belong summarized in Table 1.4.

Table 1.4. Classes of antidepressant medications

Class	Compound Name
Monoamine Oxidase Inhibitor (MAOI)	Tranylcypromide, moclobemide
Tricyclic antidepressants (TCA)	Imipramine, amitriptyline, clomipramine
Tetracyclic antidepressant	Maprotiline, mianserine
Selective Serotonin Reuptake Inhibitor (SSRI)	Fluoxetine, citalopram, paroxetine, sertraline, fluvoxamine, escitalopram
Noradrenergic and Specific Serotonin Antagonists (NASSA)	Mirtazapine
Serotonin and Noradrenaline Reuptake Inhibitor (SNRI)	Venlafaxine, duloxetine
Selective Noradrenaline Reuptake Inhibitor (NARI)	Reboxetine
Noradrenaline and Dopamine Reuptake Inhibitor (NDRI)	Bupropion
Melatonergic antidepressant	Agomelatine

Although SSRIs and SNRIs are often considered first-line treatment options in MDD, which class of antidepressant should preferentially be prescribed remains a topic of debate (Kennedy et al. 2009). While these drugs show an improved therapeutic tolerance when compared to older/classical antidepressants such TCAs and MAOIs, their use as well as discontinuation may still be associated with a variable host of usually benign but often troublesome side-effects, including gastrointestinal distress, weight gain, daytime sleepiness and sexual dysfunction (Kennedy et al. 2006). In a recent meta-analysis, Cipriani et al. (2009) aimed to identify which antidepressants currently available are the most efficacious and tolerable. The authors illustrated that mirtazapine (24.4%), escitalopram (23.7%), venlafaxine (22.3%) and sertraline (20.3%) were the most effective drugs when compared to citalopram (3.4%), milnacipran (2.7%), bupropion (2%), duloxetine (0.9%), fluvoxamine (0.7%), paroxetine (0.1%), fluoxetine (0%) and reboxetine (0%). The antidepressant drugs associated with the highest tolerance rate were escitalopram (27.6%), sertraline (21.3%), bupropion (19.3%) and citalopram (3.4%), whilst those showing the least tolerability were milnacipran (7.1%), mirtazapine (4.4%), fluoxetine (3.4%), venlafaxine (0.9%), duloxetine (0.7%), fluvoxamine (0.4%), paroxetine (0.2%) and roboxetine (0.1%). This study

therefore seemingly indicates that escitalopram and sertraline are the best first-line treatment options in MDD, whereas venlafaxine, duloxetine, fluvoxamine, paroxetine and reboxetine should be avoided where possible (Cipriani et al. 2009).

The STAR*D study conducted by Rush et al. (2009), aimed to identify effective and tolerable antidepressants used for the treatment of MDD in adult outpatients (n=4041) using various treatment steps. In the first step, 50% of patients remitted with citalopram treatment. Low socioeconomic burden, reduced quality of life and nervous, despondent and unhappy features were present in those with poorer outcomes. In the second step, no variation in remission was observed when treatments was switched (sertraline 27%, bupropion sustained release 26% and venlafaxine extended release 25%) or citalopram therapy augmented with other medications (bupropion sustained release 39% and buspirone 33%). However, augmentation was observed to be a better option than switching (Rush et al. 2009). In the third step, no change in remission was observed when treatments were switched (mirtazapine 8% or notriptyline 12%) or augmented with lithium, which was discontinued due to (a high prevalence of) adverse side effects. In the fourth step, no variation in remission was again observed when treatments were switched (tranylcypromine 14% or venlafaxine extended release with mirtazapine 16%). The study concluded that 67% of patients who participated until the fourth step reached remission (Rush et al. 2009).

More recently, research has focused on novel drugs rectifying circadian rhythm disturbances in depression, e.g. agomelatine, (a novel antidepressant with melatonergic MT1 and MT2 receptor antagonists in addition to serotonin receptor antagonistic properties in the CNS) (Eser et al. 2009). Animal, preclinical and clinical studies have illustrated reduced somatic complaints and sleep disturbances with use of these drugs and therefore may be a novel method for remedying MDD episodes (Eser et al. 2009, Kennedy et al. 2006). Kennedy et al. (2006) reported that a raised response rate was noted in patients on agomelatine (49.1%) compared to those on placebo treatment (34.4%).

Despite evidence showing that antidepressant pharmacotherapy is highly efficacious (75-90% success rate) in MDD, application is hampered by a delayed onset of clinically appreciable improvement (up to 3 months) as well as a series of unfavourable and debilitating potentially lethal side-effects, including gastrointestinal discomfort, insomnia, anxiety, sexual dysfunction and suicide ideation or attempt (Moore et al. 1996; Illman et al. 2004). Furthermore, many MDD patients will also show a suboptimal response to antidepressant therapy, and may experience either treatment intolerance or therapeutic failure (Coppen et al. 2005, Kennedy et al. 2006). Of particular concern is the risk of remission or the development of treatment-resistant depression (TRD), which is defined as the inability to respond to antidepressants despite numerous attempts

at treatment (Schosser et al. 2012). In addition to these shortcomings, MDD patients show marked heterogeneity in their response to antidepressant treatment.

In light of these observations, there is a pressing need to develop novel management strategies that are safe, clinically effective, tolerable, flexible and cost-effective, which could translate into a diminished disease burden as well as improved quality of life for both MDD patients and family members. Considering the numerous variables which contribute to the aforementioned inter-individual variance in treatment response observed in patients with MDD, including personal (age, treatment preference and compliance), clinical (depression sub-type and episode severity, general medical conditions), environmental (lifestyle and dietary habits), genetic and therapeutic (drug availability, safety, cost, other medications) factors, could be of benefit in this process (Danileviciute et al. 2002; Kennedy et al. 2006; Eichelbaum et al. 2006). It is therefore crucial that treatment be individualised by matching the patient profile, diseases profile, and medication profile.

The current process applicable for medication selection (Moore et al. 1996; Kornstein et al. 2001; Puri et al. 2002; Mann et al 2005; Andreasen et al. 2006) should take note of the following, but is not limited to these considerations:

- Patient Profile: The Relevant socio-demographic information of the patient should be recorded with reference to age, ethnicity, gender and history of substance abuse (Kornstein et al. 2001, Puri et al. 2002, Andreasen et al. 2006). All personal and relevant family medical and psychiatric conditions should be documented. Assessment of first-degree relatives is important as psychiatric disorders may be inherited (Puri et al. 2002, Andreasen et al. 2006). A complete general physical examination of all bodily systems as well as a standard neurological examination is generally performed (Andreasen et al. 2006) and the type of psychiatric disorder identified since this will influence treatment choice (Puri et al. 2002, Andreasen et al. 2006). The patient's current complaints and core symptoms leading to current referral, such as lack of interest, pessimism, reduced motivation, lack of appetite, sleep disturbances and suicidal thoughts should also be documented (Puri et al. 2002, Andreasen et al. 2006, DeRubeis et al. 2008, Kaplan et al. 1998).

- Disease Profile: After conducting a neuropsychiatric assessment, the chronological history of the patient's prior episodes should be identified and recorded. These include chronicity, nature, severity and response to previous antidepressant therapy (including tolerance), with the aim of reducing the risk of side-effects or hypersensitivity reactions (Kornstein et al. 2001, Puri et al. 2002; Andreasen et al. 2006). The clinician will attempt to identify possible precipitating factors for the current symptoms, such as adverse psychosocial stressors, pre-existing medical conditions or substance abuse (Kornstein et a. 2001; Puri et al. 2002; Andreasen et al. 2006).

- Treatment Profile: A detailed history of all previous treatment modalities considered should be recorded, including a description of the quantity, interval, efficacy, tolerance, side-effect profiles and hypersensitivity reactions associated with antidepressant medications previously prescribed. In addition to documentation of current or prior use of other non-psychiatric medications, the risk of intolerance and possible drug-drug reactions arising from the new treatment regimen can be considered (Kornstein et al. 2001; Andreasen et al. 2006).

1.6.2 Psychotherapy

Pharmacotherapy is an essential component of any management plan devised for MDD. However, the clinical utility of psychotherapy as either first-line treatment or adjunct, particularly in those presenting with mild depression or known with a history of severe psychosocial stressors or interpersonal difficulties, has repeatedly been demonstrated, and should thus always be considered in these individuals (Stoudemire et al. 1996; Mann et al. 2005). In fact, many patients prefer psychotherapy to pharmacotherapy, while combination treatment could potentially most effectively reduce depressive episodes, anxiety, improve coping with illness and improve the quality of life (Mann et al. 2005; Huijbers et al. 2012).

While individual therapy is considered most effective, group therapy may also be considered (Robinson et al. 1990). The effectiveness of psychotherapy has been thoroughly examined over the past 30 years, with mixed results. In a recent meta-analysis, Weisze et al. (2006) reported that Cohen's effect sizes ranged from small to medium for different psychotherapeutic strategies. Cognitive Behavioural Therapy (CBT) is generally considered the psychotherapeutic intervention treatment of choice for MDD with efficacy demonstrated in various clinical trials (Andreasen et al. 2006) Haby et al. 2005, Butler et al. 2006). CBT is a brief and structured method (Andreasen et al. 2006) and the treating clinician will aim to reduce and alter pessimistic thoughts ultimately to restructure future thoughts (Cuijpers et al 2008). A meta-analysis by (Haby et al. 2005) found a medium to large effect size of 0.68 (95% CI=0.51-0.84) in MDD patients treated with CBT. The variation in the efficacy of CBT could be explained by treatment, duration of psychotherapy, severity of MDD patients included, country and control group included (Haby et al. 2005).

1.6.3 Personalized medicine

A number of factors could influence and change the expression of genes namely mRNA (changes in impact factor of gene), innumerable factors (one gene increases or decreases the expression of

others), epigenetics and/or environmental factors (sleep, emotions, exercise, drugs or diet) (Kalow et al. 2006). Normal healthcare practices involve identifying single drug treatments specific for the general population. A clinician will diagnose patients based on their symptoms and if the patient does not respond or experiences adverse side effects, the clinician may then modify the dose or switch to a different drug treatment. This process will be repeated until a significant treatment choice is discovered (Aspinall and Hamermesh. 2007). Individuals who respond poorly to initial treatment could respond to secondary treatment prescribed at a follow up visit. Regrettably only 50% of the patients return thus reducing the benefit of second-line treatment (Simon et al. 2010). Therefore, it is becoming apparent that the one size fits all approach may not always be feasible as clinicians are becoming more aware of how complex the human body is and how much individuals differ genetically (Gordon et al. 2007, Gurwitz et al. 2004). In reality a variety of major drug treatments are specific and effective for only 25-60% of individuals (Wilkinson 2005), whereas the rest of the population will experience no benefit or toxicity response to treatment (Hulot 2010), which can be fatal in certain patients (Kalow 2005). Variation in treatment response can be caused by age, weight, gender, ethnicity, certain medical conditions, environmental factors and genetic background (Wilkinson 2005).

It is estimated that adverse drug reactions account for 2.5-12% of hospital admissions and 0.4-0.5% of deaths (Hulot 2010). Meta-analyses have shown that the incidence of hospital admissions is between 2-6% due to adverse drug reactions (Pouyanne et al. 2000). Treatments are usually prescribed based on the trial and error method, which results in increased patient morbidity, over-servicing and increase of healthcare costs (Gordon et al. 2007).

To overcome the adverse affects occurring in some patients the concept of personalised medicine is accepted as a solution for the future (Wilkinson 2005; Hulot 2010). The definition of personalized medicine is the use of genomic and molecular analysis aimed at improved healthcare by uncovering and clinically testing new treatments based on an individual's predisposition to a medical condition (Cascorbi et al. 2010). The benefits of personalized medicine involving the identification of various genes associated with MDD, include increased quality of life, reduced hospitalization, reduced adverse drug effects and reducing treatment costs (Gurwitz et al. 2004, Hulot 2010). Although application of pharmacogenetics can improve both drug safety and efficacy it is not 100% accurate due to variation in gene expression over time (Kalow 2005, Aspinall and Hamermesh. 2007). The FDA has recently modified their treatment labels to recommend or encourage genetic testing before treatment of certain drugs (Hulot et al. 2010).

Despite the extra time it may take in terms of patient diagnosis and treatment, personalized medicine could be seen as an incentive to refine diagnostic methods and improve treatment efficacy using pharmacogenomics techniques (Gurwitz et al. 2004, Aspinall and Hamermesh. 2007).

There is a lack of reliable predictive biomarkers for psychiatric disorders due to our poor understanding of the biology of these disorders. The development for personalized medicine has been up-and-down over the past few years, due to the complexity of genetic disorders (Gurwitz et al. 2004). Over the years researchers involved in personalized medicine translation have made great strides at identifying biomarkers and developing screening and diagnostic tests. So far these diagnostic tests have been used mostly in cancer and HIV patients, but the field is now moving beyond these diseases (Allison 2008). Gordon et al. 2007 believes we have surpassed the first stage (proof of concept) and are currently in the second stage, which involves distinguishing the biological markers with the best cost benefit scale. The success of this phase will require the selection of cost effective markers and availability of relevant tests that can be implemented effectively. Genomic markers used include assessment of genes, drug metabolizing enzymes, drug membrane transporters and drug receptors (Gordon et al. 2007). However, failure to translate research into clinically useful information makes the personalized medicine approach difficult to implement into a clinical setting (Cascorbi et al. 2010). Several hurdles exist such as important ethical issues, the incorporation of pharmacogenomics into both medical studies and practice, the lack of interest by medical insurers, lack of effective business models, poor quality and validation of tests that may require large scale investments (Gurwitz et al. 2004, Allison 2008). Therefore understanding the genetic, environmental and epigenetic processes involved in depression could advance personalized medicine as an effective treatment option aimed at improved quality of life.

1.7 Aims and Objectives

The long-term objective of the research program is to improve the clinical management of patients living with depression, based on a scientific understanding of the genetic contribution to drug response and variability in dopamine, melatonin, serotonin, and homocysteine levels implicated in the disease process. This study focused specifically on the development of a pathology-supported genetic testing (PSGT) strategy (Kotze et al. 2013) that can be applied in routine clinical practice to guide intervention and to monitor response to treatment in a subgroup of patients with altered nutritional requirements due to a genetically induced disturbance in the methylation pathway.

The specific aims were as follows:

- Analytical validation of high-throughput real-time polymerase chain reaction (RT-PCR) assays for functional polymorphisms in the MTHFR (rs1801133, rs1801131), COMT (rs4680), ASMT (rs4446909), CYP2D6 (rs3892097) and SLC6A4 (rs4795541) genes using bidirectional DNA sequencing as the gold standard.

- Comparison of the allele frequencies and genotype distribution between patients diagnosed with depression and unaffected controls.
- Determination of the effects of clinical characteristics, relevant environmental factors and genotype on homocysteine levels and BMI in patients and controls.

This study involves the translation of research for clinical application using a two-step process that involves 1) the identification of clinically useful genetic biomarkers and 2) development of laboratory SOPs evaluated as part of this study prior to the implementation phase. This approach is important as failure to translate research into clinical practice will prevent implementation of personalised medicine into daily medicine (Cascorbi et al. 2010). Preliminary studies have shown that patients with MDD may benefit from the implementation of pathology supported genetic testing (PSGT), which combines relevant clinical and laboratory information for clinical decision-making (Kotze et al. 2013) to improve diagnosis and treatment response.

Chapter 2

Materials and Methods

2.1 Ethical Approval

The Ethics Review Committee of the University of Stellenbosch granted ethical clearance for this study protocol under project number N09/08/224. Written informed consent was obtained from each of the study participants.

2.2 Study Population

The study population consisted of 183 unrelated Caucasians, including 86 patients (69 females and 17 males) clinically diagnosed with MDD and 97 controls (57 females and 40 males). Patients were selected for inclusion in the study based on an objective clinical diagnosis of MDD according to the DSM-IV-TR criteria. In the patient study group 72 were currently on treatment, with 32 receiving single anti-depressant treatment and 40 using a combination of antidepressants. The control group was selected from the Gknowmix database (http://www.gknowmix.org) based on the absence of any self-reported personal or family history of anxiety and/or depression.

2.3 Biochemical and Questionnaire-Based Assessments

Plasma homocysteine levels were measured in 183 Caucasian participants using blood drawn after an overnight fasting period. DNA extraction was done on saliva swabs and/or whole blood. Each of the 86 MDD patients and 97 control individuals completed the Gknowmix™ Medical History and Lifestyle Questionnaire available at https://www.gknowmix.com. The demographic status and clinical characteristics of each of the study participants were obtained, which included:

- Age and gender
- The medication use/side effects
- Family history of anxiety/depressive disorders
- Lifestyle factors, such as alcohol consumption, physical activity, smoking status and body mass index (BMI) was included

A calculated folate score was determined for each participant based on the amount of days per week eating certain foods rich in folate over the previous three months. A calculated physical activity score was obtained using two assessments namely day-time activity and weekly structured exercises (occasionally/none, 2-3 times or 4 or more times). The clinical characteristics of the study population is summarised in table 2.1

Table 2.1. Clinical characteristics of the study population

Characteristics	Controls (n=97)		MDD patients (n=86)	
	Females	**Males**	**Females**	**Males**
Number	57	40	69	17
Age, years	46 (13)	48 (14)	44 (10)	39 (11)
Body Mass Index (kg/m^2):	25 (23-28)	27 (24-30)	27 (23-33)	27 (24-32)
Normal weight (%)	30 (53%)	12 (30%)	27 (39%)	6 (35%)
Overweight (%)	16 (28%)	18 (45%)	16 (23 %)	4 (24%)
Obese (%)	11 (19%)	10 (25%)	26 (38%)	7 (41%)
Homocysteine (µmol/L)	9 (7-11)	10 (9-13)	9 (7-11)	13 (11-14)
Folate SCORE	7 (4-10)	9 (6-11)	5 (4-7)	6 (3-7)
Low (%)	47 (82%)	26 (65%)	65 (94%)	15 (88%)
Moderate (%)	4 (7%)	11 (28%)	2 (3%)	1 (6%)
High (%)	6 (11%)	3 (8%)	2 (3%)	1 (6%)
Current Smoking	4 (7%)	2 (5%)	10 (15%)	5 (29%)
Alcohol intake:				
Abstain (%)	16 (28%)	7 (18%)	24 (35%)	6 (35%)
Occasionally (%)	17 (30%)	8 (20%)	28 (41%)	2 (12%)
1-13 units per week (%)	23 (40%)	22 (55%)	15 (22%)	7 (41%)
14-21 units per week (%)	1 (2%)	3 (8%)	2 (3%)	0 (0%)

≥ 22 units per week (%)	0 (0%)	0 (0%)	0 (0%)	2 (12%)
Lifestyle - Physical Activity				
Low (%)	23 (40%)	15 (38%)	36 (52%)	8 (47%)
Moderate (%)	23 (40%)	11 (28%)	26 (38%)	4 (24%)
High (%)	11 (19%)	14 (35%)	7 (10%)	5 (29%)
Family History of MDD	Excluded		30 (43%)	7 (41%)

Table 2.2 Antidepressants used by the 71 MDD patients grouped according to class of therapy

Class	**Active substance**
SSRI	Paroxetine
	Citalopram
	Escitalopram
	Fluoxetine
	Sertraline
SNRI	Duloxetine
	Venlafaxine
NASSA	Mirtazapine
SARI	Trazodone
NDRI	Bupropion

2.4 DNA Extraction

2.4.1 DNA Extraction from Whole Blood using the QIAGEN QIAamp® DNA Blood Midi Kit

The purpose of this method was to extract purified genomic DNA from2 ml whole blood. To initiate the DNA extraction process 200 µl of QIAGEN Protease stock solution was added to separate 1.5 ml microcentrifuge tubes for each sample analyzed. Protease enzyme caused cell lysis releasing cellular DNA into solution. Using a filter tip 2 ml of whole blood was added into each of the microcentrifuge tubes and mixed briefly via pulse vortex for a few seconds. This vigorous mixing caused dispersal of the protease enzyme throughout the solution and increased the efficiency of the lysing effect. Buffer AL (2.4 ml) was then added to each tube and was inverted 15 times and mixed via pulse vortex for one minute to produce a homogeneous solution. This guaranteed sufficient lysing of all samples. The samples were then incubated on a dry heating block at 70°C for 10 minutes. After the incubation step to all samples were removed from the dry block and briefly centrifuged to remove any sample residue left on the sides. After centrifugation, 2 ml of ethanol (100%) was added to each of the tubes and inverted 10 times, before mixing the samples via pulse vortex for 15 seconds. The vigorous mixing of the homogenized samples allowed for the lysate binding to the membrane in the QIAamp® Mini column.

One half of each of the samples was carefully transferred into a QIAamp® Midi column inside a 15 ml microcentrifuge tube. The samples was centrifuged for 3 minute at 3 000 revolutions per minute (rpm). The QIAamp® Midi column was removed from the centrifuge and transferred into a new 15 ml microcentrifuge tube, with the surplus discarded as bio-hazardous waste. The remaining half of the solution was added into the individual columns and centrifuged for 3 minute at 3 000 rpm. The filtrate was discarded as mentioned above. The removal of the surplus is an essential step to prevent an obstruction within the column, which could diminish the washing process thereby reducing the purity of the extracted DNA.

This step was followed by the addition of 2 ml of Wash buffer 1 (AW1) to the QIAamp® Midi columns and centrifuged for 1 min at 5 000 rpm. The column was transferred to a new microcentrifuge tube and the surplus discarded. 2 ml of Wash buffer 2 (AW2) was added and centrifuged for 15 min at 5 000 rpm to remove any traces of the buffer. After centrifugation the QIAamp® Mini column was transferred into a clean 15 ml microcentrifuge tube, while the surplus was once again discarded. Next, 200 µl nuclease free water at room temperature (15°C -25°C) was added to each of the QIAamp® Mini columns and incubated for 5 min at room temperature, followed by centrifugation for 2 min at 5 000 rpm.

To obtain maximum DNA yield the eluted samples (200 µl) was reloaded into the QIAamp® Mini columns and incubated for 5 min at room temperature (15°C -25°C) and centrifuged once again for 2 min at 5 000 rpm. To ensure maximum DNA yield the newly eluted DNA samples were placed on

a shaker overnight at room temperature. For frequent use the DNA samples were stored at 4°C whereas, the DNA samples were stored in small aliquots at -20°C for long term.

2.4.2 DNA Extraction from Saliva using the Oragene-DNA / Saliva Kit

The purpose of this method was to extract purified genomic DNA from a saliva swab samples. Saliva samples collected in the Oragene®-DNA vials were inverted for several seconds to allow for thorough mixing. The samples were incubated in a water bath set to 50°C for 2 hours. This step allowed for permanent inactivation of nucleases. 500 µl of the Oragene®-DNA/saliva sample was transferred into a 1.5 ml microcentrifuge tube. 20 µl of Oragene-DNA purifier (OG-L2P) was added to the 1.5 ml microcentrifuge tube and mixed via vortexing for a few seconds. Each sample was incubated for 10 min on ice and centrifuged for 10 min at 13 000 rpm at room temperature. This step was essential for the impurities to be separated from the DNA (supernatant- DNA and pellet-impurities). The supernatant was transferred into a fresh 1.5 ml microcentrifuge tube and the pellet discarded. 600 µl of ethanol (100%) at room temperature was added to 500 µl of clear supernatant for each of the samples and mixed by inversion 10 times.

The samples were left to incubate for 10 min at room temperature, allowing the DNA to fully precipitate. The tubes were centrifuged for 2 min at 13 000 rpm at room temperature and were loaded in such a fashion to know the exact location of the DNA pellet. By taking care not to disturb the pellet, the supernatant was removed and discarded. 250 µl of 70% ethanol was added to each of the tubes containing the pellets and was left to stand on the bench for 1 min at room temperature (start of wash step). The ethanol was removed carefully for each of the samples as to not disturb the pellet. To complete the wash step 100 µl of nuclease free water was added to each of the tubes and vortexed for 5 seconds, allowing for the DNA pellet to dissolve. The samples were then incubated for 1 hour in a water bath set to 50°C and vortexed in between occasionally, allowing for the DNA to completely hydrate. The samples were finally incubated overnight at room temperature on a shaker allowing for homogenization. On the completion of this step the samples was stored at 4°C.

2.5 DNA Quantification

The Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA) with the v3.5.2 software package was used to measure the concentration and purity of all the extracted DNA samples used in this study. The process to determine the concentration and purity was performed after initially cleaning the pedestal of the instrument with 70% ethanol and then distilled water. To initialize the instrument, 2µl Nuclease Free Water was loaded onto the pedestal. To blank the

instrument 2µl Nuclease Free Water was loaded onto the pedestal. Once the blanking process was completed each of the study samples were measured and stored. Upon completion of the experiment the cleaning process was performed once again using 70% ethanol and distilled water. To determine to purity of the DNA samples the absorbance ration was done at a reading of 230 nm, 260 nm and 280 nm. All the purity values were within the acceptable range (260/280 absorption ratio: 1.6-1.9; 260/230 absorbance ratio: >1.9) indicating that no contaminants such as proteins, salts or phenols were present in the samples. All samples except for those required for genotyping of the SLC6A4 43 bp ins/del (rs4795541) were diluted with nuclease free water to obtain a 10 ng/µl final concentration. This was required to complete Real-Time Polymerase Chain Reaction amplification.

2.6 Polymerase Chain Reaction (PCR) amplification

2.6.1 Oligonucleotide Primers

Forward and reverse oligonucleotide primers were designed to amplify the following single nucleotide polymorphisms (SNP's): MTHFR 677C>T rs1801133, MTHFR 1298A>C rs1801131, COMT 472G>A rs4680, CYP2D6 6937G>A rs3892097 allele 4, SLC6A4 3595_3596 rs4795541 and ASMT 24436G>A rs4446909. Amplification was performed using the LightCycler® Probe Design 2.0, version 1.0.R.36 software package (F. Hoffmann-La Roche Ltd, Switzerland), whereas genotyping the SLC6A4 gene was performed using conventional PCR. The reference sequences required for the each of the genes were obtained from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.giv). Information on the primers are summarised in table 2.3.

Table 2.3. Oligonucleotides primers used for conventional PCR amplification

Gene	SNP	Primer	Nucleotide sequence (5'-3')	GC Content (%)	T_M (°C)	T_A (°C)	Amplicon Size (bp)
MTHFR 677 C>T	rs1801133	F	ATCCCTCGCCTTGAACA	52.9	53.60	56	256
		R	TCACCTGGATGGGAAAGAT	47.3	53.10		
MTHFR 1298 A>C	rs1801131	F	CTCTGTCAGGAGTGTGC	58.8	52.40	61	383
		R	GGTGGAGGTCTCCCAACTTA	55.0	56.10		
COMT 472 G>A	rs4680	F	GGACCAGCGTGAGCATA	58.8	54.6	56	647
		R	GCTGTGAGACCCTCACT	58.8	53.9		
CYP2D6 6937 G>A, allele 4	rs3892097	F	AGGGAGCAAGGTGGATGCAC	60.0	60.1	61	515
		R	TAGGTCCAGCAGCCTGAG	61.1	56.4		
SLC6A4 3595_3596	rs4795541	F	ATGCCAGCACCTAACCCCTAATGT	50	60.5	65	375
		R	GGACCGCAAGGTGGGCGGGA	75	67.7		
ASMT	rs4446909	F	CATGCCTGTCTTCCCAGCAC	60.0	58.7		

24436 G>A		R	TGAGGATCCCATCTTGTCTC	50.0	53.5	60	780

Abbreviations: TM and GC content was made available by Whithead Scientific (Pty) Ltd; °C- degrees Celsius; bp- base pairs; GC- Guanine-Cytosine; TA- annealing temperature; TM- melting temperature; F- forward; R- reverse.

Table 2.4. Reagent volumes used for PCR amplification of selected SNPs

Names of PCR Reagents	Volume of reagents (µl) (x1) MTHFR 677 C>T	Volume of reagents (µl) (x1) MTHFR 1298 A>C	Volume of reagents (µl) (x1) COMT G>A	Volume of reagents (µl) (x1) CYP2D6*4 G>A	Volume of reagents (µl) (x1) SLC6A4 43 bp ins/del	Volume of reagents (µl) (x1) ASMT G>A
NF H_2O	14.675	13.275	14.675	14.675	13.175	15.425
5x Buffer	5	5	5	5	5	5
$MgCl_2$(25mM)	1.5 (1.5mM)	1.5 (1.5mM)	1.5 (1.5mM)	1.5	0.75	1.5
dNTPs (10mM)	0.5 (0.2mM)	0.5 (0.2mM)	0.5 (0.2mM)	0.5	0.5	0.5
F primer (10µM)	0.6 (0.24µM)	1.3 (0.24µM)	0.6 (0.24µM)	0.6	0.6	0.6
R primer (10µM)	0.6 (0.24µM)	1.3 (0.24µM)	0.6 (0.24µM)	0.6	0.6	0.6
***GoTaq® DNA Polymerase* (Promega) (50ng/µl)**	0.125	0.125	0.125	0.125	0.125	0.125
DNA	2	2	2	2	2	2
Total	25	25	25	25	23+1.25 DMSO	25

Abbreviations: PCR- polymerase chain reaction; NF H_2O-nuclease free water; dNTPs-deoxyribonucleotide triphosphates; DNA-deoxyribonucleic acid; µl- micro litre.

2.6.2 PCR Reaction mixture and Thermal Cycling conditions

All SNPs were amplified using the PromegaGoTaq® Flexi DNA Polymerase PCR Kit. The Applied Biosystems Gene Amp® PCR System 2700, 9700 and 2720 thermal cyclers were used for amplification of the target sequences. All reaction mixtures were a total of 25 µl and the components of these mixtures are summarised in table 2.4.

The amplification run used the following thermal cycling conditions: 30 cycle repeat of the denaturation step at 95°C for 30 seconds, an annealing step for 30 seconds, an extension step at 72˚C for 30 seconds, a final extension at 72 °C for 5 minutes and a cooling step at 22˚C for 1 minute. The ASMT (rs4446909) and SLC6A4 (rs4795541) primers were optimized at 35 cycles and the annealing temperatures are summarized in table 2.3.

2.7 Gel Electrophoresis

In order to determine successful amplification the PCR products were loaded onto a 2% (w/v) agarose gel. The gel was prepared using 2 g of agarose in 100 ml of 1x TBE buffer solution (90 mMTris-HCl, 90 mMBoric acid and 2.2 Mm Ethylenediaminetetraacetic Acid [EDTA]). This consisted of 700 µl of 1xTBE and 70 µl of ethidium bromide (visualise the PCR products). A total sample volume of 10 µl of the PCR product was loaded into each well. One lane was reserved for the molecular size marker (SimplyLoad® 100bp DNA ladder), which underwent electrophoresis along with the PCR products. This was done to verify that the correct PCR product was amplified based on fragment size. The PCR products underwent electrophoresis for ±1 hour at 100 V in the electrophoretic buffer and were visualised using a trans-illuminator radiating ultraviolet light at wavelength of 100 (Life Technologies TFX-35M trans-illuminator).

2.8 DNA Sequencing

Successfully amplified PCR products were submitted for post PCR clean up and bi-directional sequencing at the Central DNA Sequencing Facility of Stellenbosch University. Analysis of sequencing electropherograms was done using FinchTV version 1.4.0 (developed by the Geospiza Research Team).

2.9 Real-Time Polymerase Chain Reaction (RT-PCR) Amplification

2.9.1. Applied Biosystems® *Taq*Man® SNP Genotyping Assays

All RT-PCR runs were done using Applied Biosystems® *Taq*Man® SNP Genotyping Assays. Pre-designed assays namely MTHFR rs1801133, MTHFR rs1801131, COMT rs4680, CYP2D6 rs3892097 allele 4 and ASMT rs4446909 were used for end-point genotyping by allelic discrimination analysis. The assays consisted of unlabelled and labelled PCR primers TaqMan® Minor Groove Binder (MGB) probes (FAM™ and VIC® dye-labelled), which was diluted (20x) in sterile SABAX double distilled water. For this study the Corbett Rotor-Gene™ 6000 real-time PCR instrument was used.

2.9.2. Corbett Rotor-Gene™ 6000/ QIAGEN Rotor-Gene Q

The Rotor-Gene ™ 6000 series Multiplexing System, 5-Plex HRM model (Corbett Research, Australia) was used in conjunction with the ABI™ *Taq*Man® SNP Genotyping Assays. Genetic variations were detected using both the allelic discrimination and scatter plot analysis, which was generated during the RT-PCR experiment using fluorescent technology. The total reaction volume of 10 µl was used for each sample, which consisted of 5µl *Taq*Man® universal genotyping PCR master mix (P/N 4371355), 10ng/µl template specific DNA (2.0 µl), 20x *Taq*Man® SNP genotyping assay (0.5 µl) and 2.5 µl SABAX (nuclease free).

The amplification run used the following thermal cycling conditions: 45 cycle repeat of the initial hold step at 95°C for 10 minutes, denaturation at 92°C for 15 seconds, an annealing and final extension at 60 °C for 1 minute.

2.10 Statistical Analysis

Using allele counts, the population frequencies and genotype distribution of the selected SNPs namely MTHFR rs1801133 and rs1801131, COMT rs4680, CYP2D6 rs3892097 allele, SLC6A4 rs4795541 and ASMT rs4446909 were estimated and the Hardy-Weinberg equilibrium was assessed using the exact test. To analyze the single and combined allelic effects, the potential confounding factors were identified and adjusted for. The qualitative characteristics (i.e. gender) were described using cross tabulation and frequency tables whereas the median and interquartile range was used for the quantitative phenotypes (i.e. homocysteine). Logistic regression models were done to compare pairs of groups, such as patients to controls, whereas linear regression models were used to compare the quantitative characteristics between the genotypes. Quantitative outcomes with non-symmetric distributions were log-transformations for analyses. The R software

and R package genetics, freely available from http://www.r-project.org, were used for analyses. Results corresponding to p-values below 0.05 are described as significant.

Chapter 3

Detailed Laboratory Results and Discussion

3.1.1 Genetic studies

Functional polymorphisms in the MTHFR (rs1801133 and rs1801131), COMT (rs4680), ASMT (rs4446909), CYP2D6 (rs3892097) and SLC6A4 (rs4795541) genes were evaluated in 86 clinically diagnosed MDD patients and 97 control individuals. Based on the literature review, the polymorphisms shown in table 3.1 were considered appropriate for inclusion in this study due to their potential role in MDD.

Table 3.1. Summarizes the single nucleotide polymorphisms analysed in this study.

Biological pathway	Gene	Genetic Variation
Homocysteine	MTHFR	677 C>T , rs1801133 1298 A>C , rs1801131
Dopamine	COMT	472G>A , rs4680
Melatonin	ASMT	24436 G>A , rs4446909
Drug resistance	CYP2D6	6937 G>A , rs3892097 (allele 4)
Serotonin	SLC6A4	3595_3596, rs4795541 43bp ins/del

3.1.2 Conventional PCR and Bidirectional Sequencing

PCR was performed using pre-designed primers (table 2.2) for the MTHFR (rs1801133 and rs1801131), COMT (rs4680), CYP2D6 6937 (rs3892097, allele 4), ASMT (rs4446909) and SLC6A4 (rs4795541) genes. Agarose gel electrophoresis was conducted to ascertain successful amplification of the PCR products. The PCR products were visualized on a 2% agarose gel with Ethidium Bromide, which are presented as figures 3.1, 3.5, 3.9, 3.13 and 3.19 respectively. A molecular size marker (Promega 100 bp DNA ladder) was used to confirm that the correct fragment of interest was amplified during PCR. To exclude to possibility of contamination a non-template control (NTC) was included in all genotyping runs.

The electropherograms represents the sequenced genotype results for the control samples. Figures 3.2, 3.3, 3.4, 3.6, 3.7, 3.8, 3.10, 3.11, 3.12, 3.14, 3.15, 3.16, 3.17, 3.18, 3.20, 3.21 and 3.22 represent each of the three genotype sequencing results for the controls for each of the genetic polymorphisms analysed in this study. Bidirectional sequencing was performed (forward and reverse) to analytically validate each genotype. The reverse sequence is not shown here that confirmed the forward result.

Figure 3.1 shows the successful amplification of the MTHFR 677 C>T primer set. Lanes 2-4 represent the amplification of the MTHFR 677 C>T primer set; each produced single bands with an amplification size of 256bp that is supported by the 100bp DNA molecular ladder represented in lane 1. Lane 5 represents the negative control and showed that no contamination occurred during the PCR amplification.

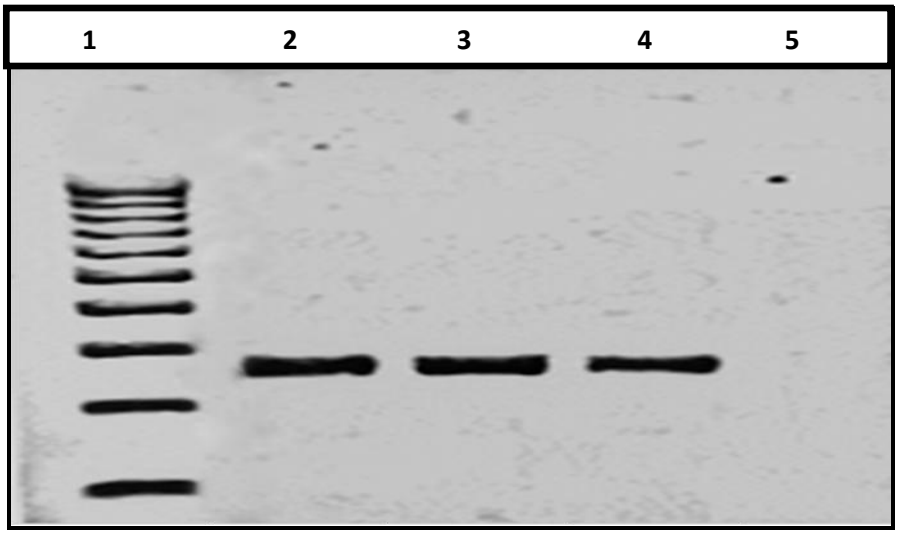

Figure 3.1. A 2% (w/v) agarose gel representing the PCR amplicons generated with the MTHFR 677 C>T primer sets.

Figure 3.2 illustrates the electropherogram generated for the MTHFR 677 C>T primer set. The highlighted region (blue) indicates the position of the SNP. The C indicated corresponds to the CC genotype, which depicts the wild type genotype.

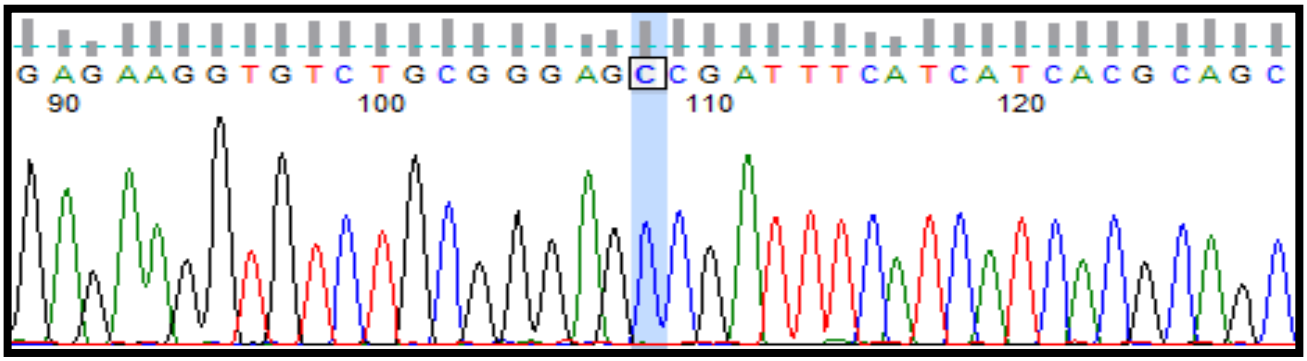

Figure 3.2. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the MTHFR 677 C>T primer set for the wild type genotype.

Figure 3.3 illustrates the electropherogram generated for the MTHFR 677 C>T primer set. The highlighted region (blue) indicates the position of the SNP. The S indicated corresponds to the CT genotype, which depicts the heterozygous genotype.

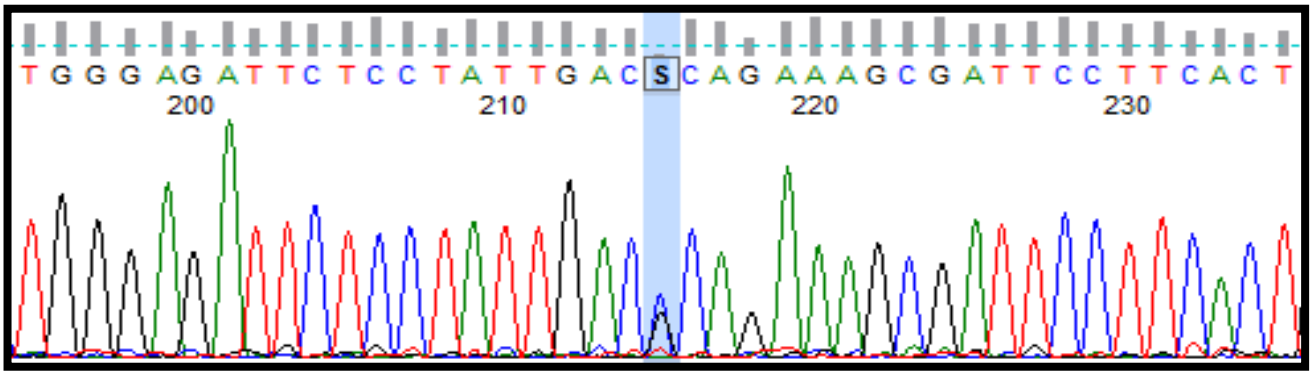

Figure 3.3. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the MTHFR 677 C>T primer set for the heterozygous genotype.

Figure 3.4 illustrates the electropherogram generated for the MTHFR 677 C>T primer set. The highlighted region (blue) indicates the position of the SNP. The T indicated corresponds to the TT genotype, which depicts the homozygous genotype.

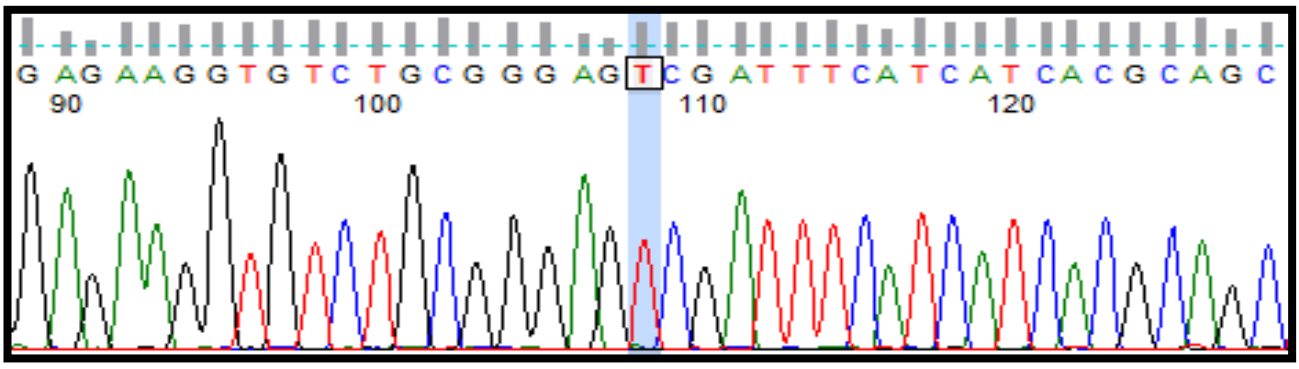

Figure 3.4. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the MTHFR 677 C>T primer set for the homozygous genotype.

Figure 3.5 shows the successful amplification of the MTHFR 1298 A>C primer sets. Lanes 2-4 represent the amplification of the MTHFR 1298 A>C primer sets each produced single bands with an amplification size of 383bp, which is supported by the 100bp DNA molecular ladder represented by lane 1. Lane 5 represents the negative control and showed that no contamination occurred during the PCR amplification.

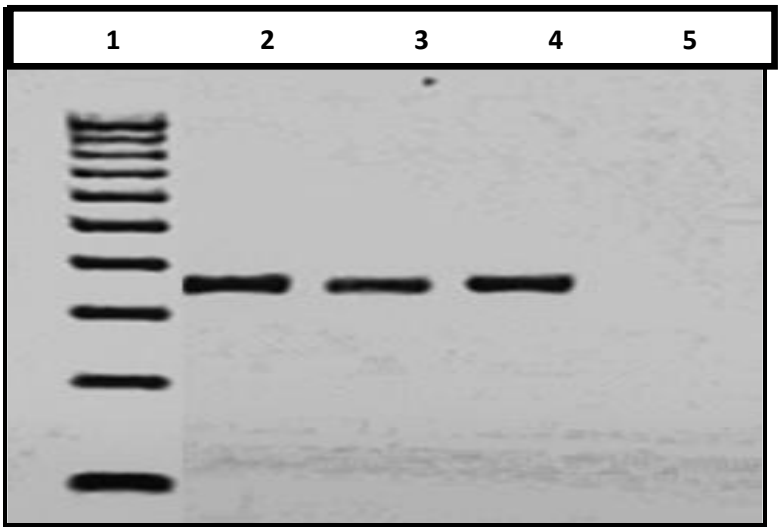

Figure 3.5. A 2% (w/v) agarose gel representing the PCR amplicons generated with the MTHFR 1298 A>C primer sets.

Figure 3.6 illustrates the electropherogram generated for the MTHFR 1298 A>C primer sets. The highlighted region (blue) indicates the position of the SNP. The A indicated corresponds to the AA genotype, which depicts the wild type genotype.

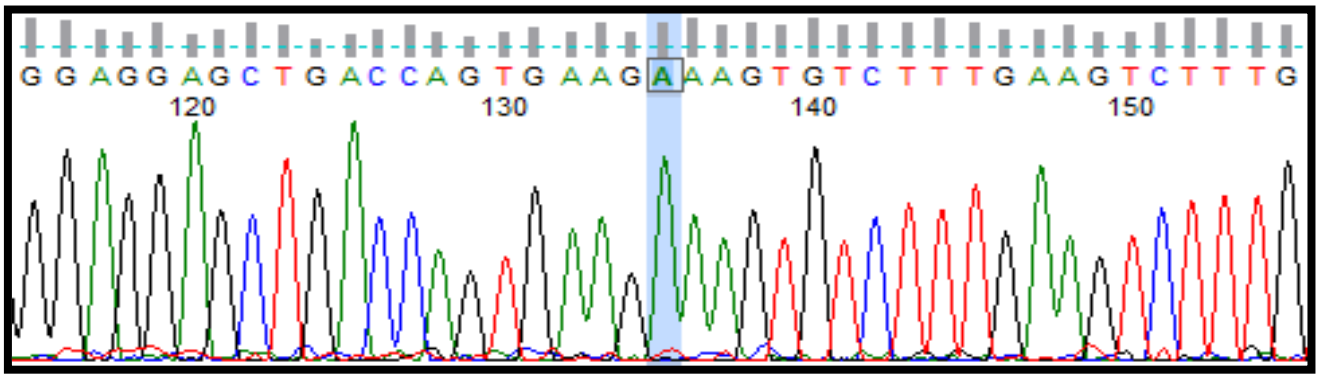

Figure 3.6. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the MTHFR 1298 A>C primer set for the wild type genotype.

Figure 3.7 illustrates the electropherogram generated for the MTHFR 1298 A>C primer sets. The highlighted region (blue) indicates the position of the SNP. The M indicated corresponds to the AC genotype, which depicts the heterozygous genotype.

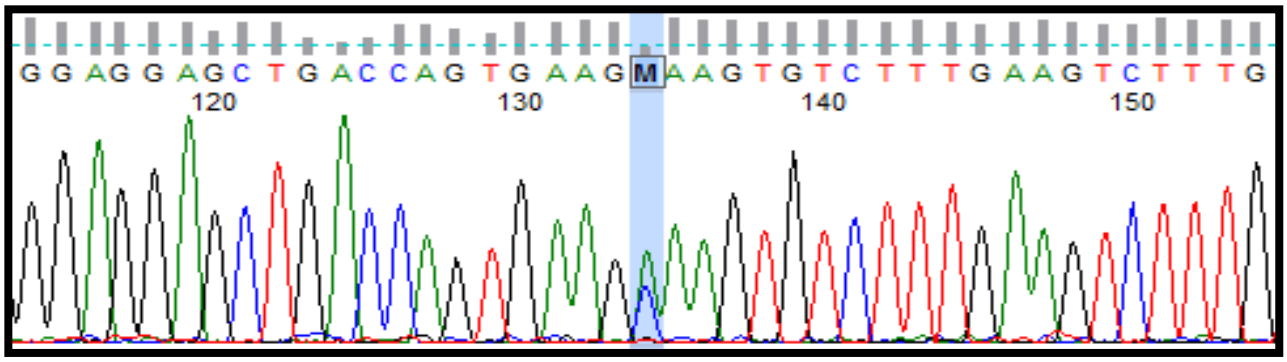

Figure 3.7. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the MTHFR 1298 A>C primer set for the heterozygous genotype.

Figure 3.8 illustrates the electropherogram generated for the MTHFR 1298 A>C primer sets. The highlighted region (blue) indicates the position of the SNP. The C indicated corresponds to the CC genotype, which depicts the homozygous genotype.

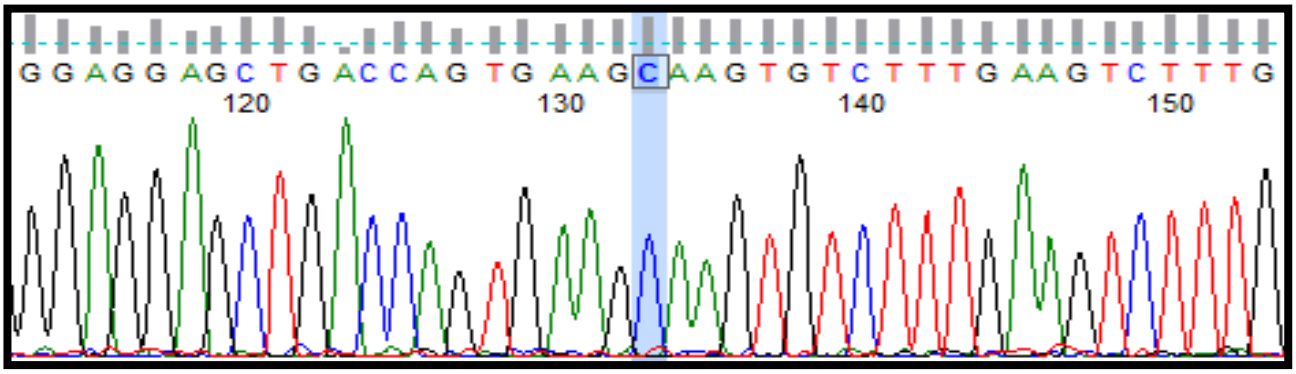

Figure 3.8. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the MTHFR 1298 A>C primer set for the homozygous genotype.

Figure 3.9 shows the successful amplification of the COMT 472 G>A primer sets. Lanes 2-4 represent the amplification of the COMT 472 G>A primer sets each produced single bands with an amplification size of 647bp, which is supported by the 100bp DNA molecular ladder represented by lane 1. Lane 5 represents the negative control and showed that no contamination occurred during the PCR amplification.

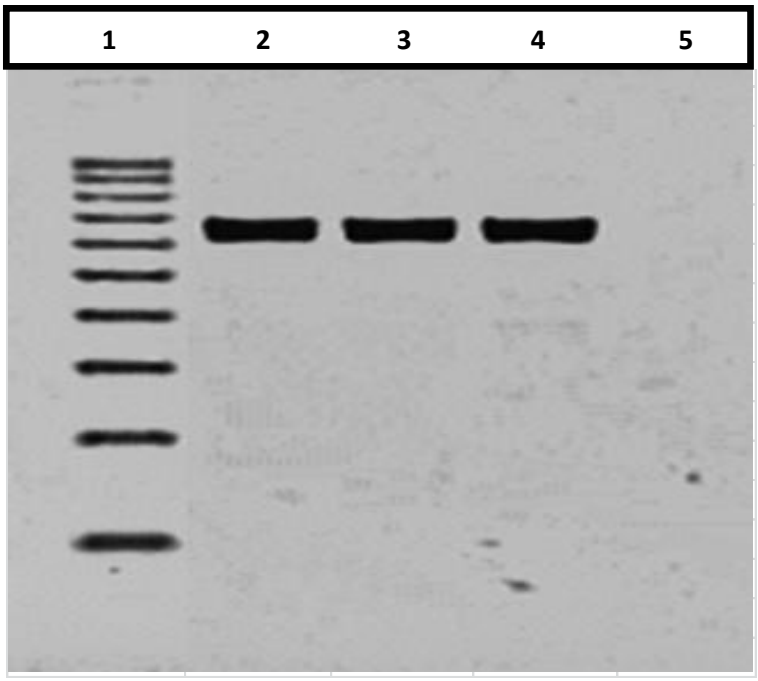

Figure 3.9. A 2% (w/v) agarose gel representing the PCR amplicons generated with the COMT 472 G>A primer sets.

Figure 3.10 illustrates the electropherogram generated for the COMT 472G>A primer sets. The highlighted region (blue) indicates the position of the SNP. The G indicated corresponds to the GG genotype, which depicts the wild type genotype.

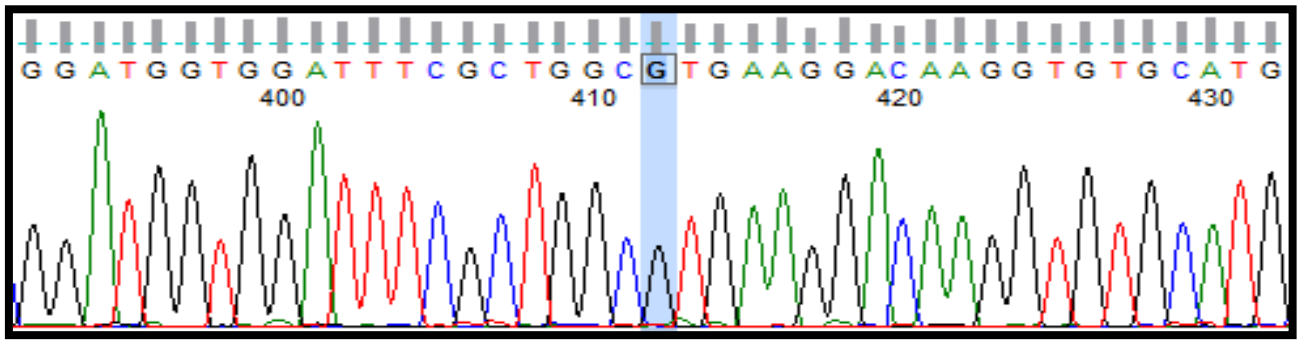

Figure 3.10. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the COMT 472G>A primer set for the wild type genotype.

Figure 3.11 illustrates the electropherogram generated for the COMT 472G>A primer sets. The highlighted region (blue) indicates the position of the SNP. The R indicated corresponds to the GA genotype, which depicts the heterozygous genotype.

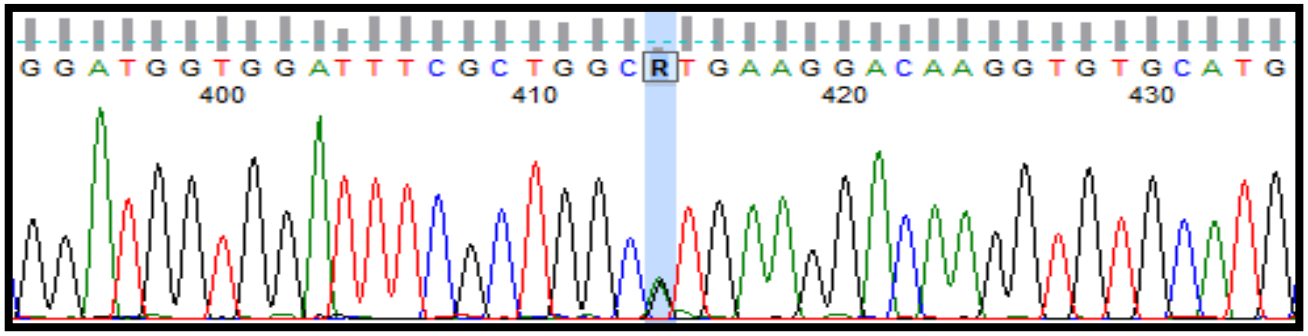

Figure 3.11. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the COMT 472G>A primer set for the heterozygous genotype.

Figure 3.12 illustrates the electropherogram generated using the COMT 472G>A primer sets. The highlighted region (blue) indicates the position of the SNP. The A indicated corresponds to the AA genotype, which depicts the homozygous genotype.

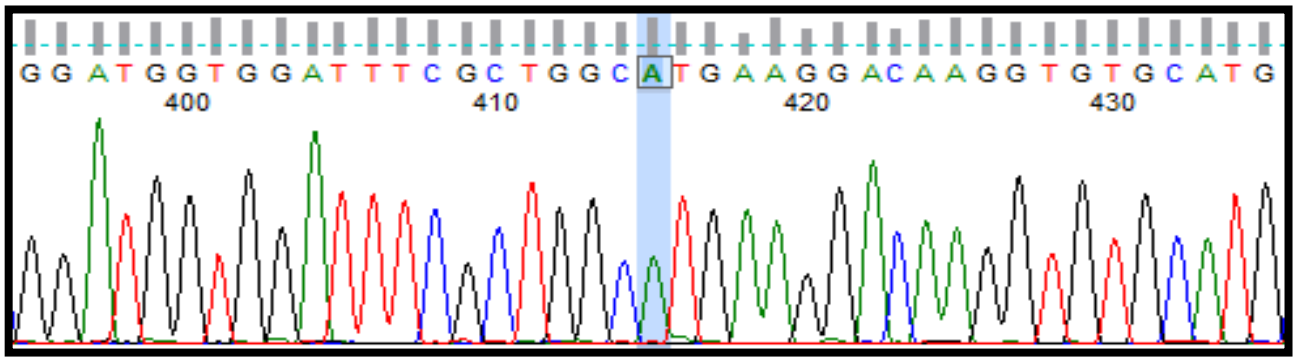

Figure 3.12. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the COMT 472G>A primer set for the homozygous genotype.

Figure 3.13 shows the successful amplification of the ASMT 24436 A>G the CYP2D6 6937 G>A primer sets. Lanes 2-4 represent the amplification of the ASMT 24436 A>G primer sets and lanes 7-9 represent the amplification of the CYP2D6 6937 G>A allele 4 primer sets each produced single bands with an amplification size of 780bp and 515bp respectively, which is supported by the 100bp DNA molecular ladder shown in lane 1. Lane 5 represents the negative control and showed that no contamination occurred during the PCR amplification.

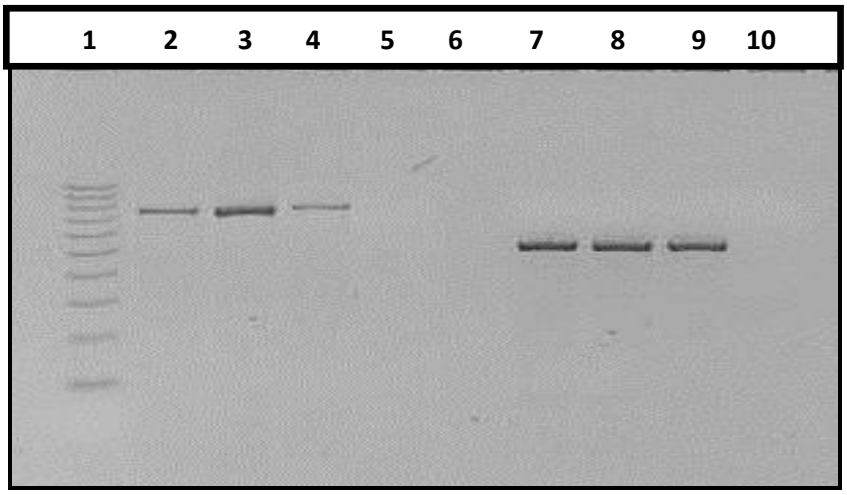

Figure 3.13. A 2% (w/v) agarose gel representing the PCR amplicons generated with the ASMT 24436 A>G the CYP2D6 6937 G>A allele 4 primer sets.

Figure 3.14 illustrates the electropherogram generated using the CYP2D6 6937 G>A primer sets. The highlighted region (blue) indicates the position of the SNP. The G indicated corresponds to the GG genotype, which depicts the wild type genotype.

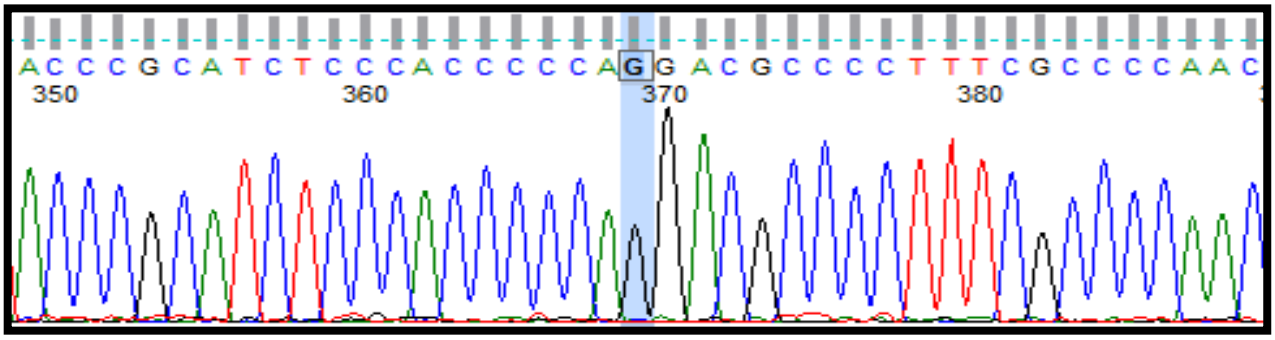

Figure 3.14. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the CYP2D6 6937 G>A primer set for the wild type genotype.

Figure 3.15 illustrates the electropherogram generated using the CYP2D6 6937 G>A primer sets. The highlighted region (blue) in figure 3.15 indicates the position of the SNP. The R indicated corresponds to the GA genotype, which depicts the heterozygous genotype.

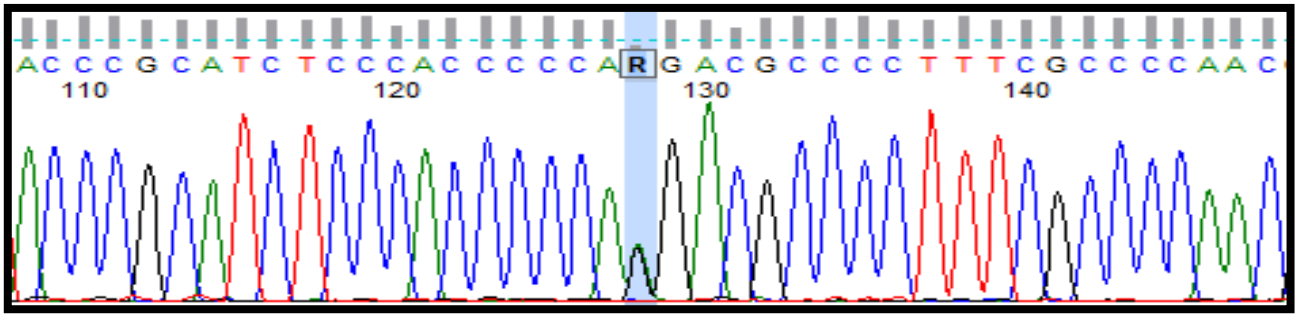

Figure 3.15. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the CYP2D6 6937 G>A primer set for the heterozygous genotype.

Figure 3.16 illustrates the electropherogram generated using the CYP2D6 6937 G>A primer sets. The highlighted region (blue) indicates the position of the SNP. The A indicated corresponds to the AA genotype, which depicts the homozygous genotype.

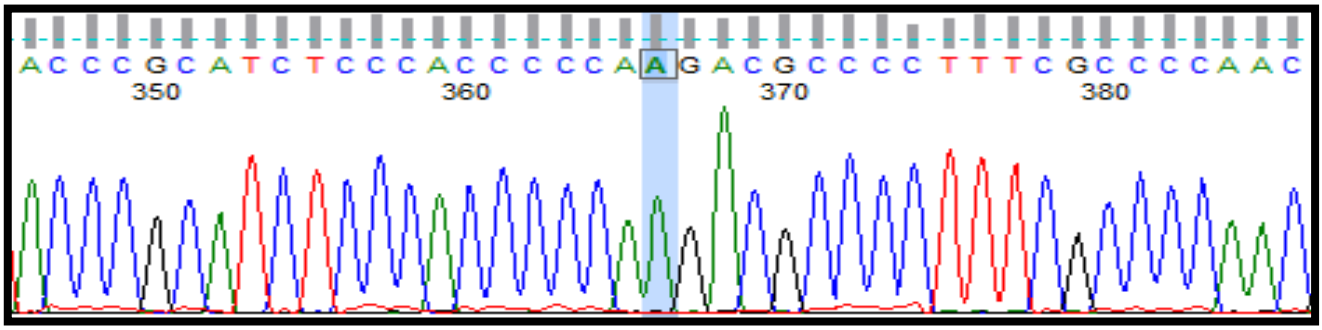

Figure 3.16. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the CYP2D6 6937 G>A primer set for the homozygous genotype.

Figure 3.17 illustrates the electropherogram generated for the ASMT 24436 G>A primer set. The highlighted region (blue) indicates the position of the SNP. The G indicated corresponds to the GG genotype, which depicts the wild type genotype.

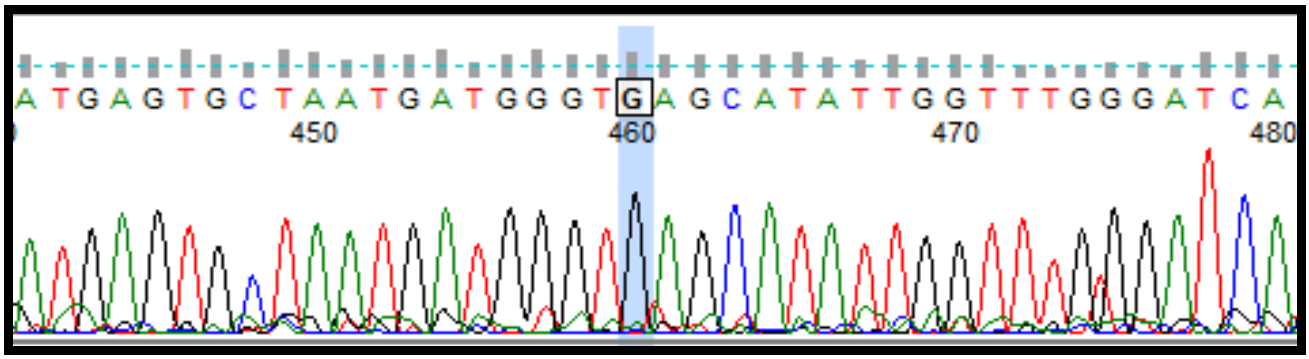

Figure 3.17. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the ASMT 24436 G>A primer set for the wild type genotype.

Figure 3.18 illustrates the electropherogram generated using the ASMT 24436 G>A primer set. The highlighted region (blue) indicates the position of the SNP. The R indicated corresponds to the GA genotype, which depicts the heterozygous genotype.

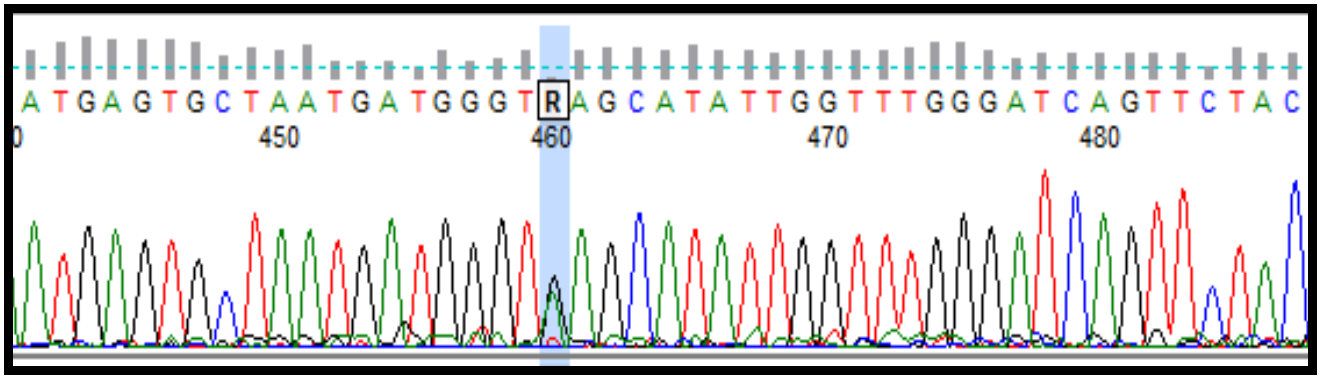

Figure 3.18. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the ASMT 24436 G>A primer set for the heterozygous genotype.

SLC6A4 gene

The SLC6A4 gene polymorphism as previously mentioned, results in the formation of a 43 bp deletion/insertion and a Taqman RT-PCR assay was available commercially for analysis of this structural gene alteration. Genotyping was therefore performed using conventional PCR including positive and negative controls for analytical validation. Each of the three genotypes observed on a 2% agarose gel was sequenced to obtain positive controls for use during extended genotyping in the study population. The figures below illustrate the electrophoresis and sequencing results obtained in this study from representative samples. All patients and controls samples were ran on a 2% agarose gel for approximately 50 min using the forward and reverse primers (primers) as described in table 2.3.

Figure 3.19 shows the successful amplification of the SLC6A4 L>S 43bp ins/del primer set. Lanes 2-19 represent the amplification of the SLC6A4 L>S rs4795541 primer set primer sets each produced single bands with an amplification size of 375bp, which is supported by the 100bp DNA molecular ladder represented by lane 1. Lane 5 represents the negative control and showed that no contamination occurred during the PCR amplification. Lanes 6-19 represent the results generated in the DNA samples of study participants with positive controls show in lanes 2-4. Lane 2 illustrates the wild type allele also known as the long (L/L) allele/genotype. Lanes 3 and 4 illustrate the heterozygous genotype (long (L)/short (s) allele). The homozygous (mutant) allele known as the short (S/S) allele/genotype is indicated in the patient sample in lane 7.

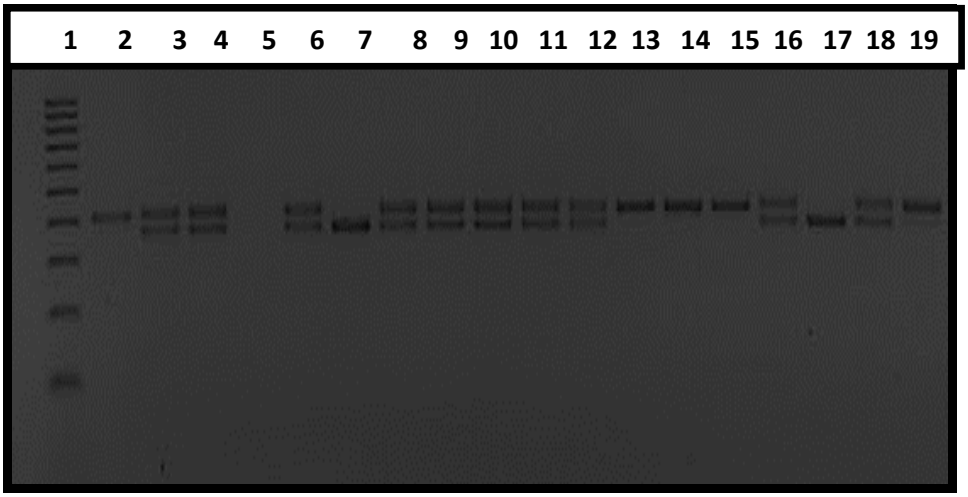

Figure 3.19. A 2% (w/v) agarose gel representing the PCR amplicons generated with the SLC6A4 L>S 43bp ins/del primer set.

Figure 3.20 illustrates the electropherogram generated using the SLC6A4 L>S 43bp ins/del primer set. The highlighted region (blue) indicates a position of the gene. The indicated region (146 bp to 186 bp) depicts a region of the wild type genotype.

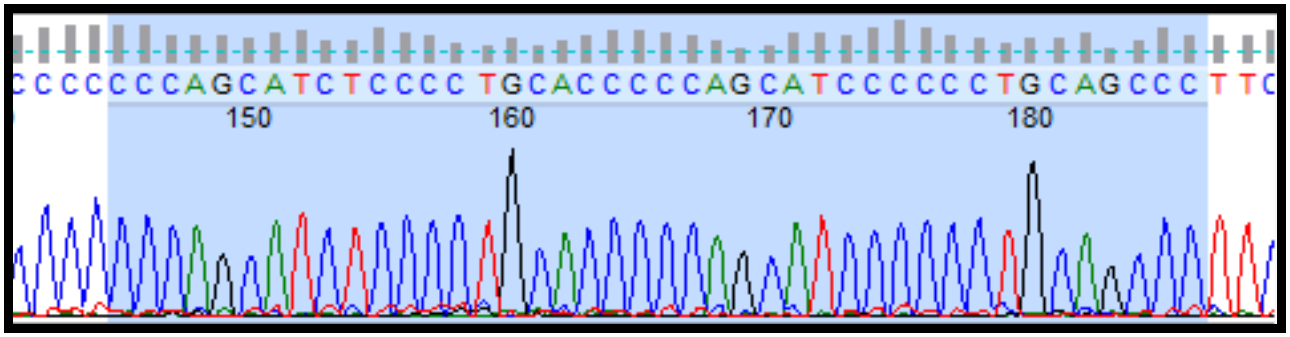

Figure 3.20. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the SLC6A4 L>S 43bp ins/del primer set for the wild type genotype.

Figure 3.21 illustrates the electropherogram generated for the SLC6A4 L>S 43bp ins/del primer set. The highlighted region (blue) indicates a position of the gene. The indicated region (146 bp to 186 bp) depicts the heterozygous genotype.

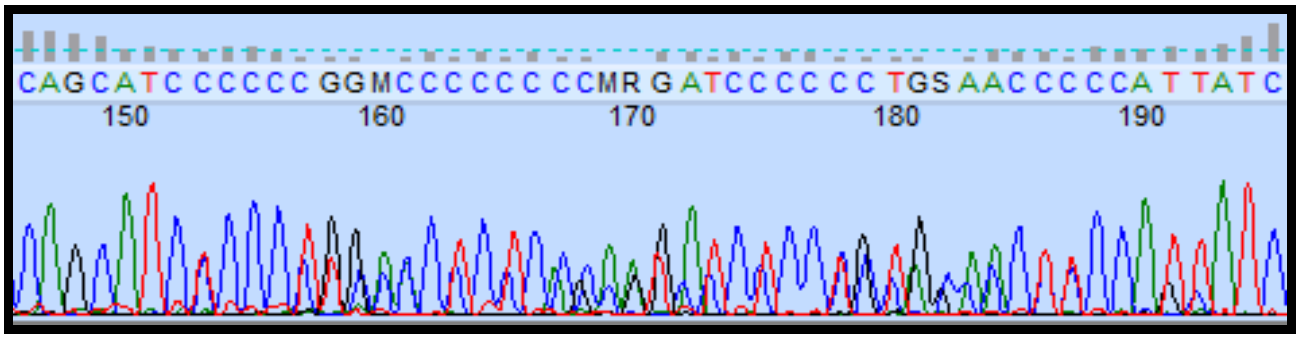

Figure 3.21. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the SLC6A4 L>S primer set for the heterozygous genotype.

Figure 3.22 illustrates the electropherogram generated using the SLC6A4 L>S 43bp ins/del primer set. The highlighted region (blue) indicates a position in the gene. The indicated region (146 bp to 186 bp) depicts the homozygous genotype. This is known due to the same position as indicated in the figure 3.22 as being different therefore indicating the 43 bp deletion.

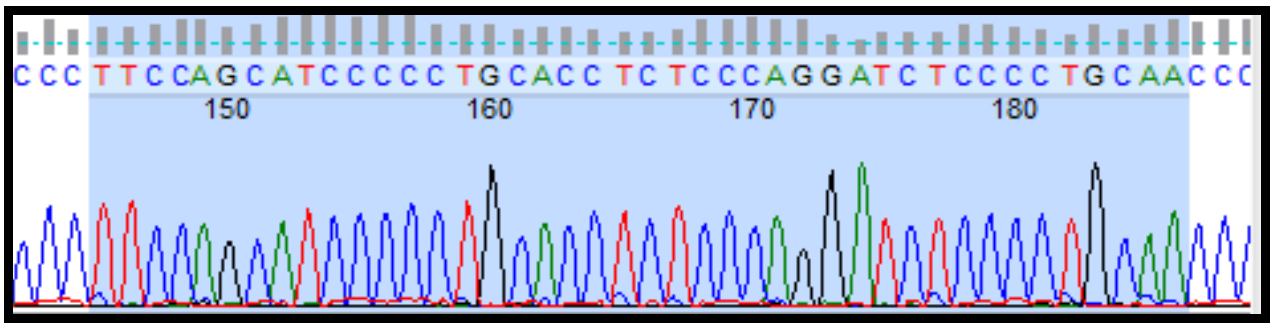

Figure 3.22. Electropherogram of the forward (sense) sequencing reaction for the amplified PCR product of the SLC6A4 L>S primer set for the homozygous genotype.

3.2 RT-PCR genotyping with the Corbett Rotor-Gene ™ 6000/QIAGEN Rotor-Gene Q

In this study the Corbett Rotor-Gene™ 6000 multiplexing system (5-Plex HRM model) was used in conjunction with the ABI™ *Taq*Man® SNP genotyping assay for high-throughput genotyping of the patients and controls, with the generation of allelic discrimination and scatterplot analysis. Allelic discrimination analysis was performed using real-time kinetic data from multiple channels. The targeted sequence was amplified using the ABI™ *Taq*Man® SNP genotyping assay, which consisted of unlabelled primers and the allelic discrimination was determined using the dual labelled *Taq*Man ® MGB probes (VIC® and FAM™).

These dual-labelled probes consisted of specific fluorophores (allele specific fluorescent dyes), which differentiates between the various alleles of the polymorphisms studied. The Rotor-Gene™ instrument has a fluorometer employed to detect the yellow and green channels. The yellow channel was operational for the detection of the VIC® fluorescent probe which is excited at a wavelength of 530 nm and emitted at 555 nm. The green channel was operational for the detection of the FAM™ fluorescent probe, which is excited at a wavelength of 470 nm and emitted at 510 nm.

A scatterplot analysis was generated for both patient and control samples using both yellow and green channels. The differences in genotype expression were determined, based on the regions defined in the scatterplots and the expression of both channels after normalization.

All five assays (MTHFR rs1801133 and rs1801131, COMT rs4680, ASMT rs4446909 and CYP2D6 rs3892097) performed on the Rotor-Gene™ were successful in the mutation determination process, indicating clear and precise amplification of the targeted polymorphic region for each individual sample. For each run internal controls of known genotype were used to verify the sequencing data generated. A threshold was generated to quantify the data obtained during each run and also to exclude any small changes in the fluorescence. Non-template controls were included in each run and no amplification suspicious of contamination was observed. This indicated that no cross contamination occurred during the procedure and that the results generated in this study are accurate and reliable.

Due to the massive amount of genotyping data generated in this study only one sample batch for each assay is shown, to illustrate the results obtained on the Rotor-Gene™.

The allelic discrimination analysis for the MTHFR rs1801133 assay is illustrated in figure 3.23 with the genotypes and legends illustrated in table 3.24. The FAM™ labelled probes detected the (C) allele indicated by the lines without intermitted circles. The VIC® labelled probes detected the homozygous mutant (T) allele indicated by the lines with intermitted circles. Both FAM™ and VIC® detected the heterozygous (C/T) genotype indicated by the alleles amplifying in both channels. A threshold level (>0.1) was set up to indicate in which channel each of the specific allele fluoresced.

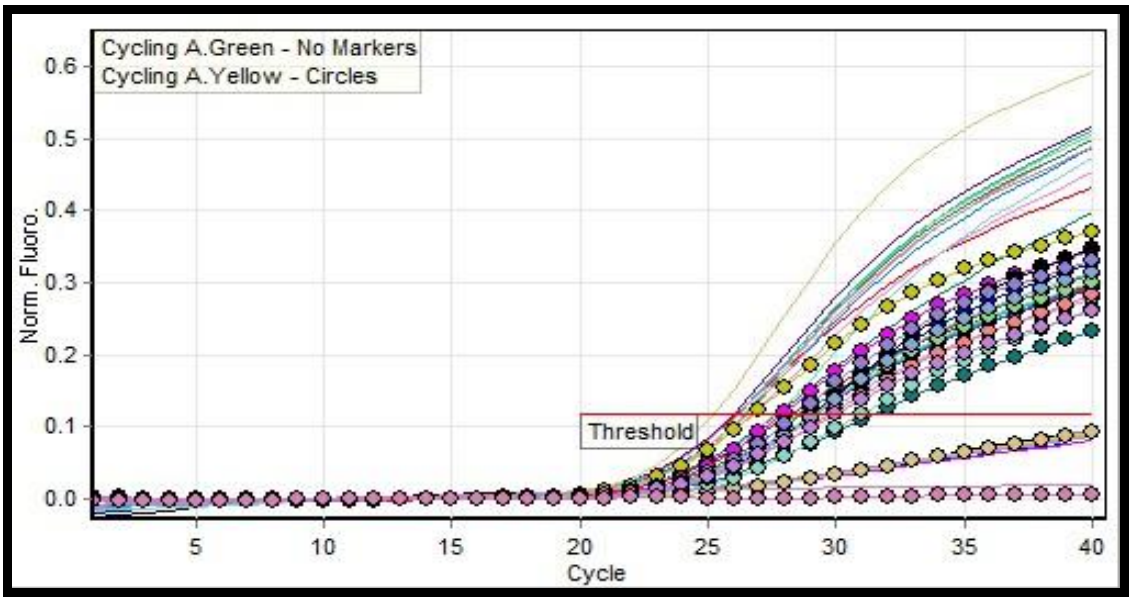

Figure 3.23. The allelic discrimination analysis of the MTHFR rs1801133 polymorphism.

To further validate the RT-PCR results a scatterplot analysis was done in addition to the allelic discrimination analysis. The scatterplot was generated using both the FAM™ and VIC® channel simultaneously, respectively. None indicates the blank used as the control. Y axis indicates the FAM™ channel and VIC® channel represents the x axis. The legend for the scatterplot is indicated in table 3.12

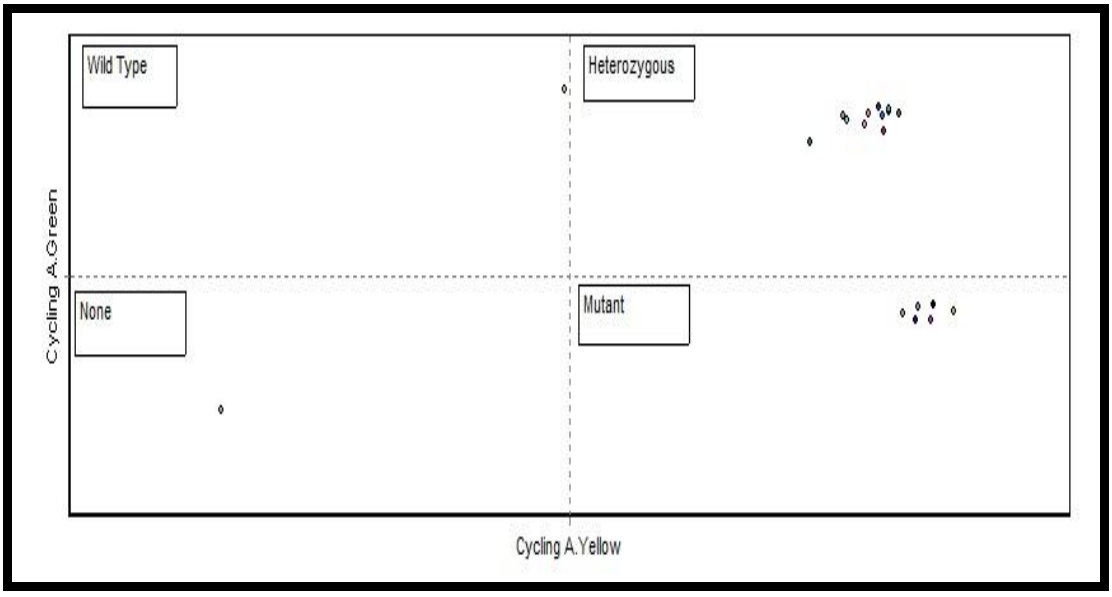

Figure 3.24. The scatterplot analysis generated for the MTHFR rs1801133 polymorphism.

Table 3.1.2. Legends for figures 3.23 and 3.24

No.	Colour	Name	Genotype	Cycling A.Green (FAM™)	Cycling A.Yellow (VIC®)
1		Sample	Heterozygous	Reaction	Reaction
2		Sample	Mutant	No Reaction	Reaction
3		Sample	Mutant	No Reaction	Reaction
4		Sample	Heterozygous	Reaction	Reaction
5		Sample	Heterozygous	Reaction	Reaction
6		Sample	Heterozygous	Reaction	Reaction
7		Sample	Heterozygous	Reaction	Reaction
8		Sample	Heterozygous	Reaction	Reaction
9		Sample	Heterozygous	Reaction	Reaction
10		Sample	Mutant	No Reaction	Reaction
11		Sample	Mutant	No Reaction	Reaction
12		Sample	Heterozygous	Reaction	Reaction
13		Sample	Wild Type	Reaction	No Reaction
14		Sample	Heterozygous	Reaction	Reaction
15		Sample	Heterozygous	Reaction	Reaction
16		Sample	Mutant	No Reaction	Reaction
17		Sample	Mutant	No Reaction	Reaction
18		Sample	Heterozygous	Reaction	Reaction
19		Blank	None	No Reaction	No Reaction

The allelic discrimination analysis for the MTHFR rs1801131 assay is illustrated in figure 3.25 with the genotypes and legends illustrated in table 3.26. The FAM™ labelled probes detected the (A) allele indicated by the lines without intermitted circles. The VIC® labelled probes detected the homozygous mutant (C) allele indicated by the lines with intermitted circles. Both FAM™ and VIC® detected the heterozygous (A/C) genotype indicated by the alleles amplifying in both channels. A threshold level (>0.1) was set up to indicate in which channel each of the specific allele fluoresced.

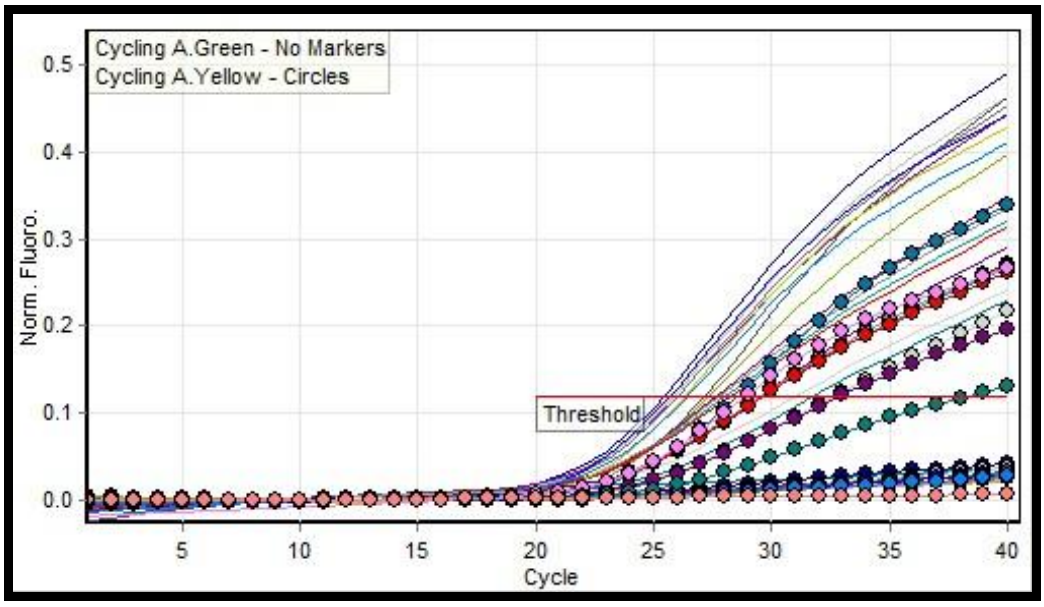

Figure 3.25. The allelic discrimination analysis of the MTHFR rs1801131 variant

To further validate the RT-PCR results a scatterplot analysis was done in addition to the allelic discrimination analysis. The scatterplot was generated using both the FAM™ and VIC® channel simultaneously, respectively. None indicates the blank used as the control. Y axis indicates the FAM™ channel and VIC® channel represents the x axis. The legend for the scatterplot is indicated in table 3.1.3.

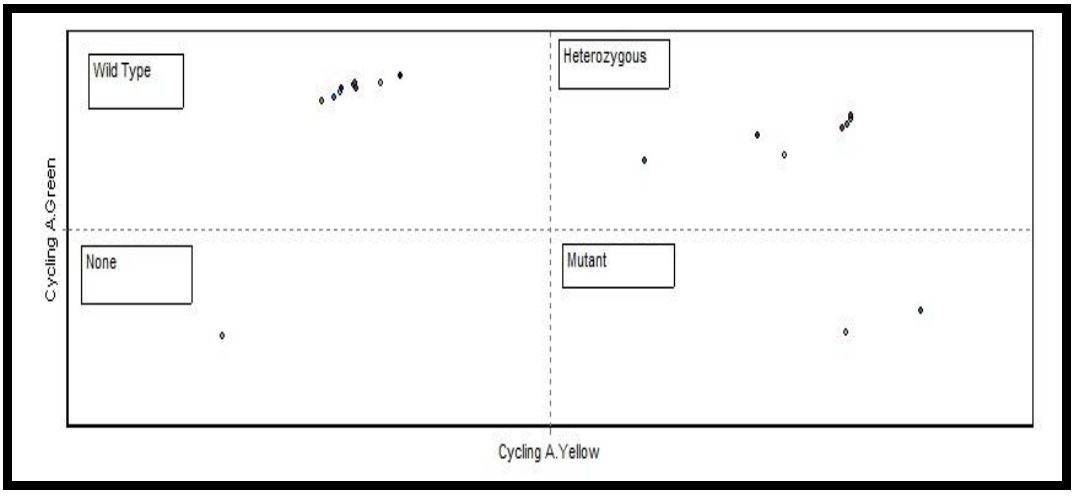

Figure 3.26. The scatterplot analysis generated for the MTHFR rs1801131 genotype.

Table 3.1.3. Legends for figures 3.25 and 3.26

No.	Colour	Name	Genotype	Cycling A.Green (FAM™)	Cycling A.Yellow (VIC®)
1		Sample	Wild Type	Reaction	No Reaction
2		Sample	Heterozygous	Reaction	Reaction
3		Sample	Mutant	No Reaction	Reaction
4		Sample	Wild Type	Reaction	No Reaction
5		Sample	Wild Type	Reaction	No Reaction
6		Sample	Heterozygous	Reaction	Reaction
7		Sample	Heterozygous	Reaction	Reaction
8		Sample	Wild Type	Reaction	No Reaction
9		Sample	Heterozygous	Reaction	Reaction
10		Sample	Wild Type	Reaction	No Reaction
11		Sample	Wild Type	Reaction	No Reaction
12		Sample	Heterozygous	Reaction	Reaction
13		Sample	Wild Type	Reaction	No Reaction
14		Sample	Wild Type	Reaction	No Reaction
15		Sample	Heterozygous	Reaction	Reaction
16		Sample	Mutant	No Reaction	Reaction
17		Sample	Wild Type	Reaction	No Reaction
18		Sample	Heterozygous	Reaction	Reaction
19		Blank	None	No Reaction	No Reaction

The allelic discrimination analysis for the COMT rs4680 assay is illustrated in figure 3.27 with the genotypes and legends illustrated in table 3.28. The VIC® (Cycling A Yellow) labelled probes detected the (G) allele indicated by the lines without intermitted circles. The FAM™ (Cycling A Green) labelled probes detected the homozygous mutant (A) allele indicated by the lines with intermitted circles. Both FAM™ and VIC® detected the heterozygous (G/A) genotype indicated by the alleles amplifying in both channels. A threshold level (0.1) was set up to indicate in which channel each of the specific allele fluoresced.

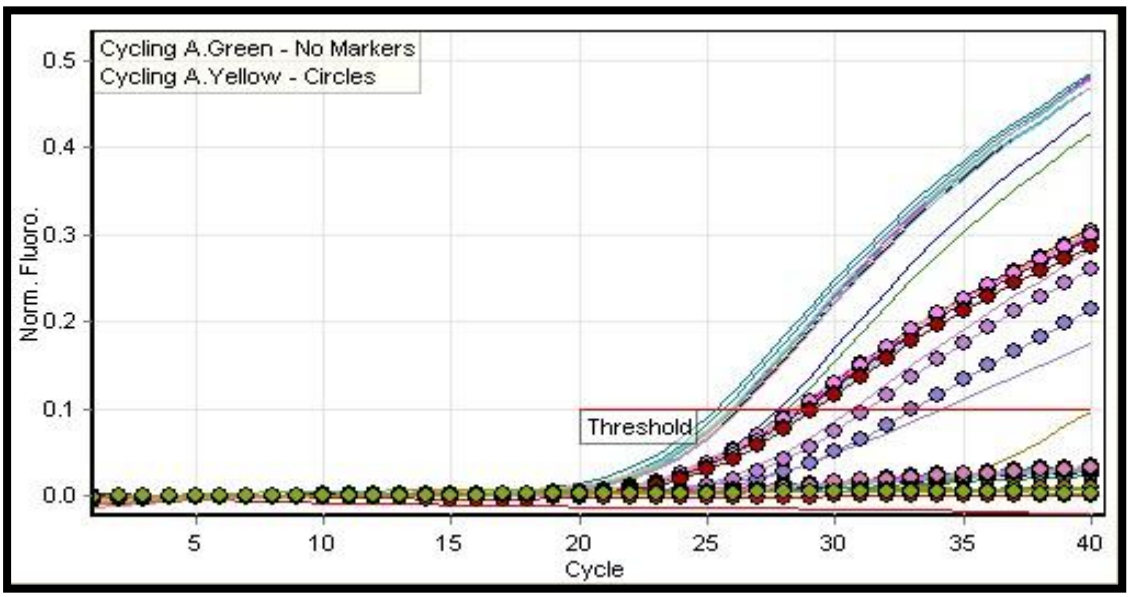

Figure 3.27. The allelic discrimination analysis of the COMT rs4680 polymorphism.

To further validate the RT-PCR results a scatterplot analysis was done in addition to the allelic discrimination analysis. The scatterplot was generated using both the FAM™ and VIC® channel simultaneously, respectively. None indicates the blank used as the control. Y axis indicates the FAM™ channel and VIC® channel represents the x axis. The legend for the scatterplot is indicated in table 3.1.4.

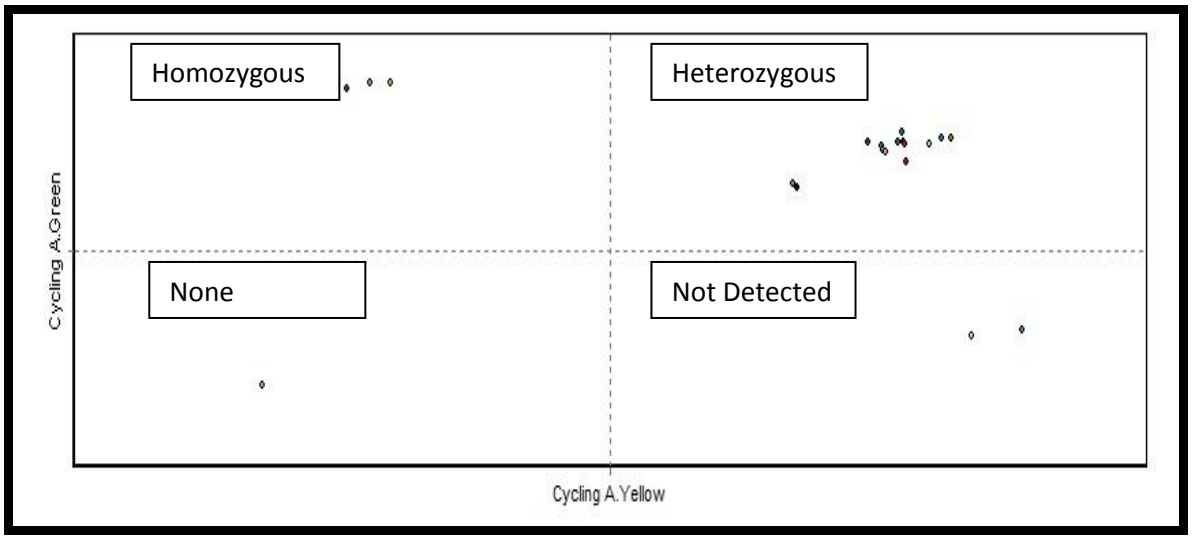

Figure 3.28. The scatterplot analysis generated for the COMT rs4680 genotype.

Table 3.1.4. Legends for figures 3.27 and 3.28

No.	Colour	Name	Genotype	Cycling A.Green (FAM™)	Cycling A.Yellow (VIC®)
1		Sample	Heterozygous	Reaction	Reaction
2		Sample	Heterozygous	Reaction	Reaction
3		Sample	Wild Type	No Reaction	Reaction
4		Sample	Heterozygous	Reaction	Reaction
5		Sample	Heterozygous	Reaction	Reaction
6		Sample	Mutant	Reaction	No Reaction
7		Sample	Heterozygous	Reaction	Reaction
8		Sample	Wild Type	No Reaction	Reaction
9		Sample	Heterozygous	Reaction	Reaction
10		Sample	Mutant	Reaction	No Reaction
11		Sample	Heterozygous	Reaction	Reaction

No.	Colour	Name	Genotype	Cycling A.Green (FAM™)	Cycling A.Yellow (VIC®)
12		Sample	Heterozygous	Reaction	Reaction
13		Sample	Heterozygous	Reaction	Reaction
14		Sample	Mutant	Reaction	No Reaction
15		Sample	Heterozygous	Reaction	Reaction
16		Sample	Heterozygous	Reaction	Reaction
17		Sample	Heterozygous	Reaction	Reaction
18		Sample	Heterozygous	Reaction	Reaction
19		Sample	Heterozygous	Reaction	Reaction

The allelic discrimination analysis for the CYP2D6 rs3892097 4 assay is illustrated in figure 3.29 with the genotypes and legends illustrated in table 3.30. The VIC® (Cycling A Yellow) labelled probes detected the (G) allele indicated by the lines without intermitted circles. The FAM™ (Cycling A Green) labelled probes detected the homozygous mutant (A) allele indicated by the lines with intermitted circles. Both FAM™ and VIC® detected the heterozygous (G/A) genotype indicated by the alleles amplifying in both channels. A threshold level (0.1) was set up to indicate in which channel each of the specific allele fluoresced.

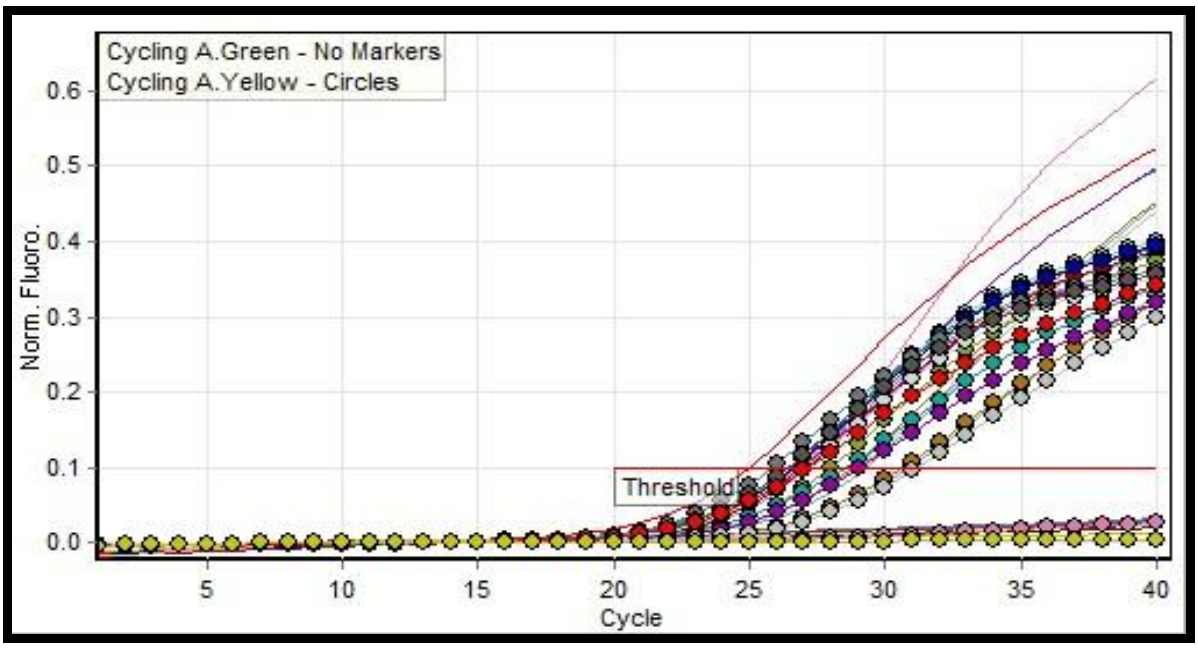

Figure 3.29. The allelic discrimination analysis of the CYP2D6 rs3892097 polymorphism.

To further validate the RT-PCR results a scatterplot analysis was done in addition to the allelic discrimination analysis. The scatterplot was generated using both the FAM™ and VIC® channel simultaneously, respectively. None indicates the blank used as the control. Y axis indicates the FAM™ channel and VIC® channel represents the axis. The legend for the scatterplot is indicated in table 3.1.5.

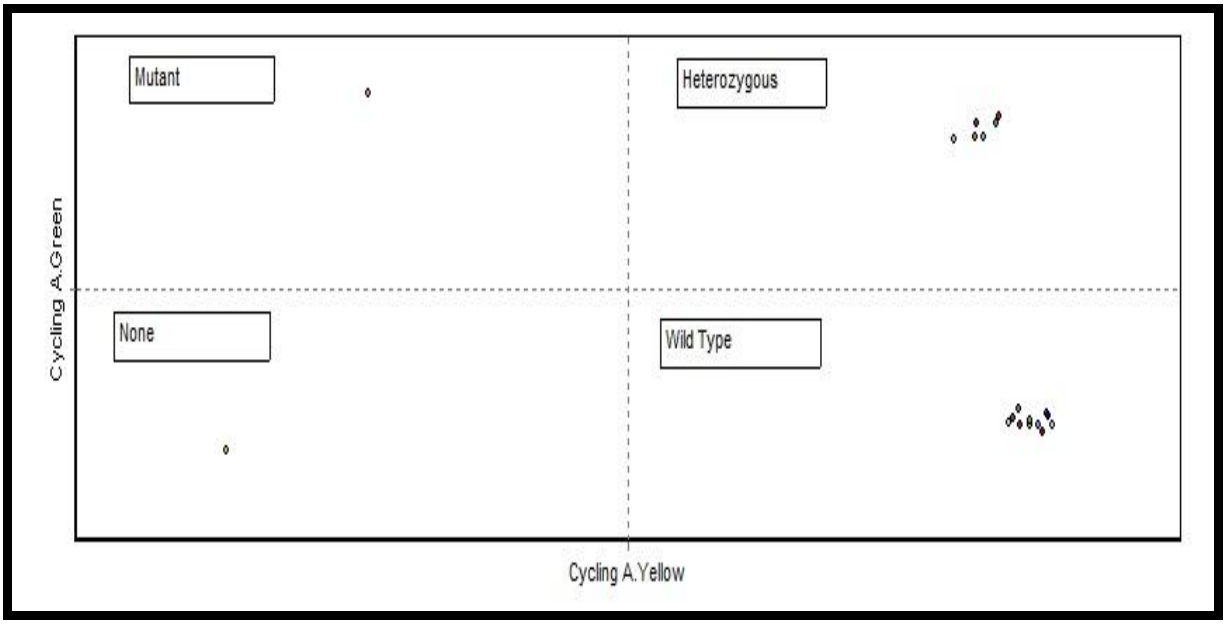

Figure 3.30. The scatterplot analysis generated for the CYP2D6 rs3892097 polymorphism.

Table 3.1.5. Legends for figures 3.29 and 3.30

No.	Colour	Name	Genotype	Cycling A.Green (FAM™)	Cycling A.Yellow (VIC®)
1		Sample	Wild Type	No Reaction	Reaction
2		Sample	Heterozygous	Reaction	Reaction
3		Sample	Wild Type	No Reaction	Reaction
4		Sample	Wild Type	No Reaction	Reaction
5		Sample	Mutant	Reaction	No Reaction
6		Sample	Wild Type	No Reaction	Reaction
7		Sample	Heterozygous	Reaction	Reaction
8		Sample	Wild Type	No Reaction	Reaction
9		Sample	Heterozygous	Reaction	Reaction
10		Sample	Wild Type	No Reaction	Reaction
11		Sample	Wild Type	No Reaction	Reaction
12		Sample	Heterozygous	Reaction	Reaction
13		Sample	Wild Type	No Reaction	Reaction
14		Sample	Wild Type	No Reaction	Reaction
15		Sample	Heterozygous	Reaction	Reaction
16		Sample	Wild Type	No Reaction	Reaction
17		Sample	Wild Type	No Reaction	Reaction
18		Sample	Heterozygous	Reaction	Reaction
19		Blank	None	No Reaction	No Reaction

The allelic discrimination analysis for the ASMT rs4446909 assay is illustrated in figure 3.31 with the genotypes and legends illustrated in table 3.32. The VIC® (Cycling A Yellow) labelled probes detected the (A) allele indicated by the lines without intermitted circles. The FAM™ (Cycling A Green) labelled probes detected the homozygous mutant (G) allele indicated by the lines with intermitted circles. Both FAM™ and VIC® detected the heterozygous (A/G) genotype indicated by the alleles amplifying in both channels. A threshold level (<0.1) was set up to indicate in which channel each of the specific allele fluoresced.

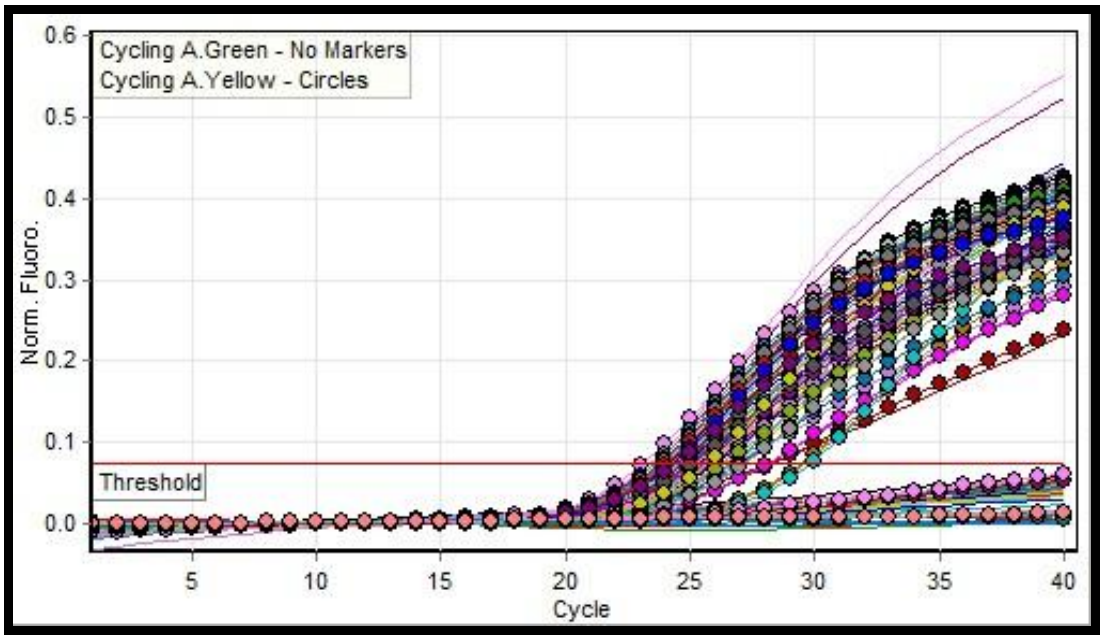

Figure 3.31 The allelic discrimination analysis of ASMT 24436G>A rs4446909 polymorphism.

To further validate the RT-PCR results a scatterplot analysis was done in addition to the allelic discrimination analysis. The scatterplot was generated using both the FAM™ and VIC® channel simultaneously, respectively. None indicates the blank used as the control. Y axis indicates the FAM™ channel and VIC® channel represents the x axis. The legend for the scatterplot is indicated in table 3.1.6.

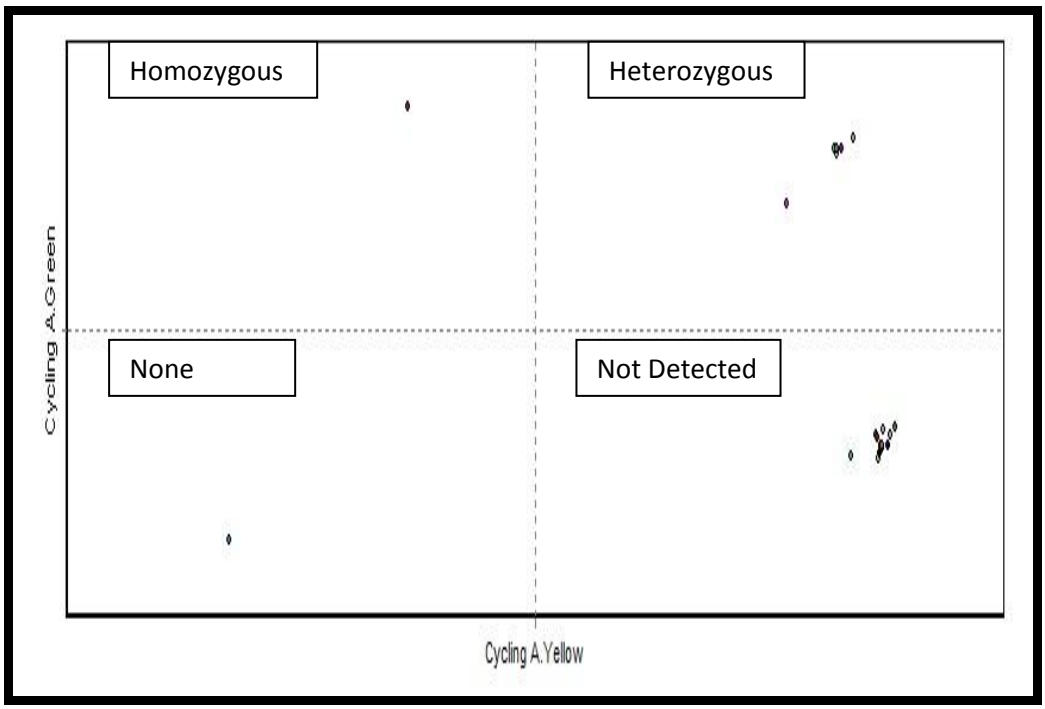

Figure 3.32. The scatterplot analysis generated for the ASMT rs4446909 polymorphism.

Table 3.1.6. Legends for figures 3.31 and 3.32

No.	Colour	Name	Genotype	Cycling (FAM™) A.Green	Cycling (VIC®) A.Yellow
1		sample	Wild Type	No Reaction	Reaction
2		sample	Heterozygous	Reaction	Reaction
3		sample	Wild Type	No Reaction	Reaction
4		sample	Wild Type	No Reaction	Reaction
5		sample	Heterozygous	Reaction	Reaction
6		sample	Wild Type	No Reaction	Reaction
7		sample	Wild Type	No Reaction	Reaction
8		sample	Wild Type	No Reaction	Reaction
9		sample	Wild Type	No Reaction	Reaction

No.	Colour	Name	Genotype	Cycling (FAM™) A.Green	Cycling (VIC®) A.Yellow
10		sample	Heterozygous	Reaction	Reaction
11		sample	Wild Type	No Reaction	Reaction
12		sample	Wild Type	No Reaction	Reaction
13		sample	Wild Type	No Reaction	Reaction
14		sample	Heterozygous	Reaction	Reaction
15		sample	Heterozygous	Reaction	Reaction
16		sample	Wild Type	No Reaction	Reaction
17		sample	Wild Type	No Reaction	Reaction
18		sample	Mutant	Reaction	No Reaction
19		Blank	None	No Reaction	No Reaction

3.3 Genotyping distribution According to the Allele Frequency of the Control and MDD populations

The genotype distribution for the MTHFR rs1801133 assay is summarised in figure 3.33. Of the 183 samples included (86 MDD patients and the 97 controls), 40 (47%) presented with the wild Type CC genotype, 37 (43%) with the heterozygous CT genotype, and 9 (10%) with the homozygous TT genotype. Amongst the 97 control samples 37 (38%) presented with the Wild Type CC genotype, 50 (52%) with the Heterozygous CT genotype and 10 (10%) with the Homozygous TT genotype.

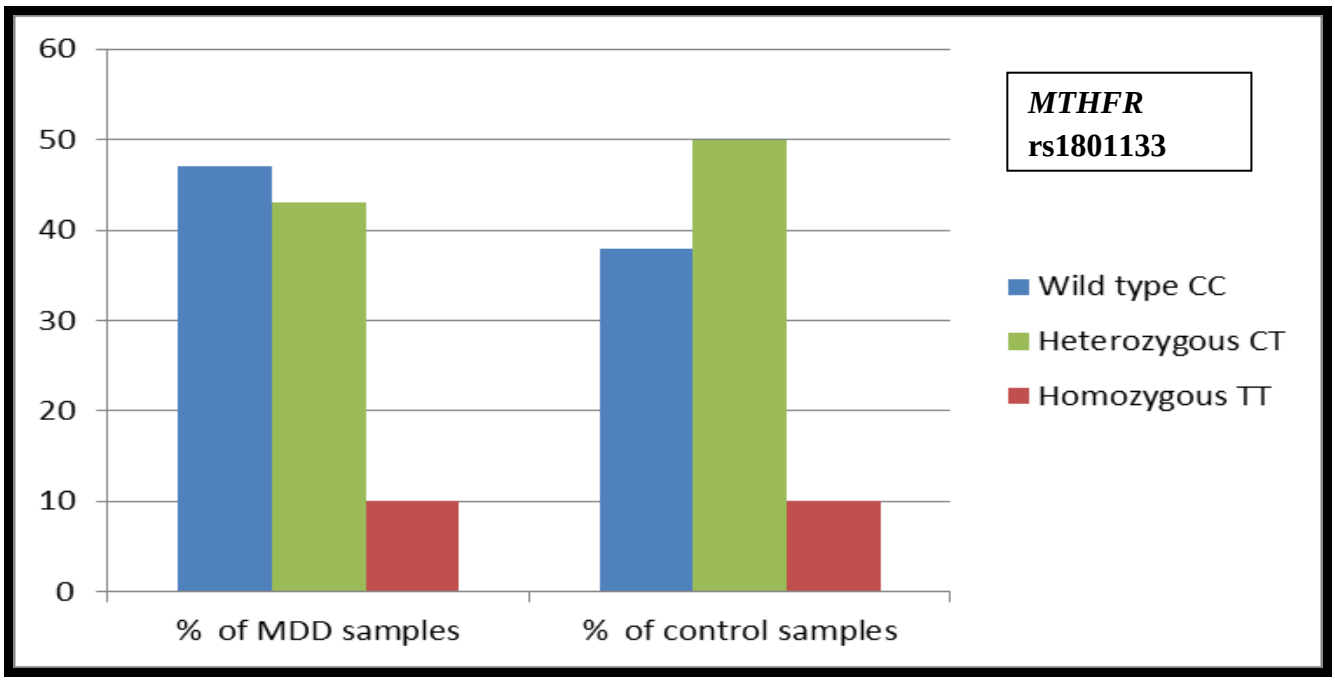

Figure 3.33. Genotype distribution obtained using the ABI™ *Taq*Man® *MTHFR* rs1801133 assay.

The genotype distribution for the MTHFR rs1801131 assay is summarised in figure 3.34. Of the 183 samples included (86 MDD patients and the 97 controls), 46 (54%) presented with the wild Type AA genotype, 37 (43%) with the heterozygous AC genotype, and 3 (3%) with the homozygous CC genotype. Amongst the 97 control samples 54 (56%) presented with the Wild Type AA genotype, 39 (40%) with the Heterozygous AC genotype and 4 (4%) with the Homozygous CC genotype.

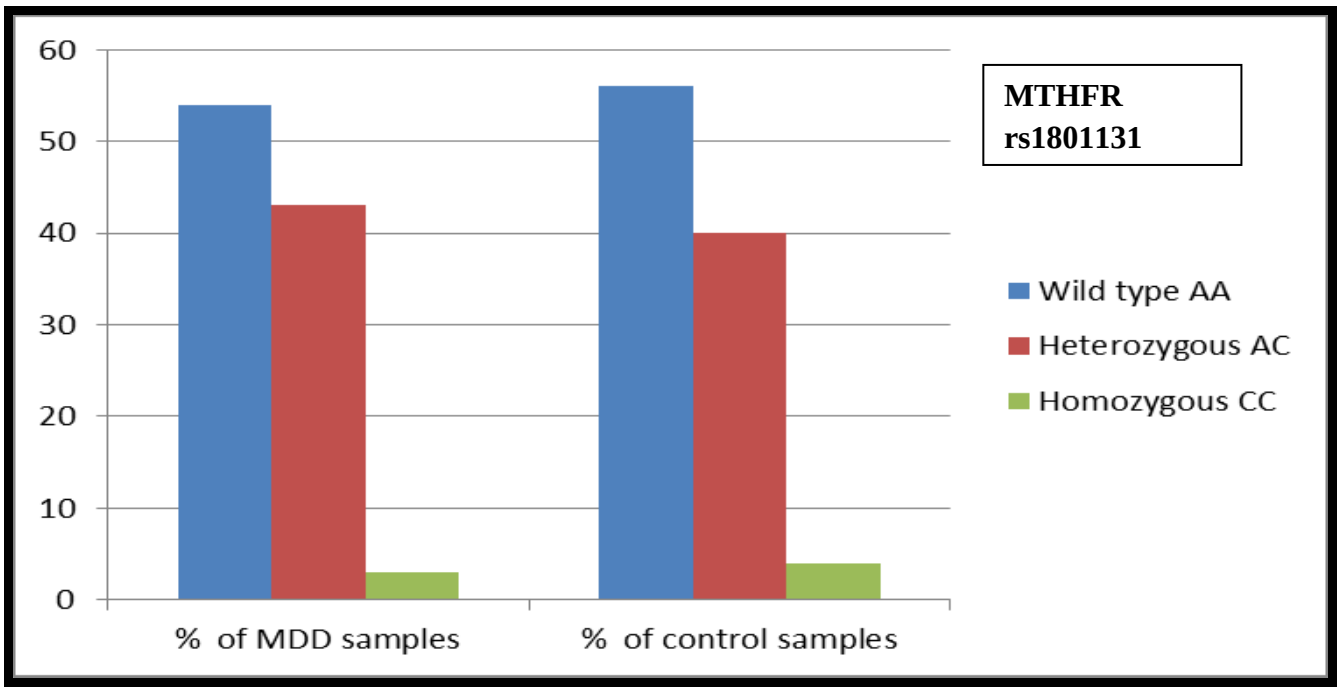

Figure 3.34. Genotype distribution obtained using the ABI™ *Taq*Man® MTHFR rs1801131 assay.

The genotype distribution for the COMT rs4680 assay is summarised in figure 3.35. Of the 183 samples included (86 MDD patients and the 97 controls), 24 (28%) presented with the wild Type GG genotype, 46 (53%) with the heterozygous GA genotype, and 16 (19%) with the homozygous AA genotype. Amongst the 97 control samples 26 (56%) presented with the Wild Type GG genotype, 48 (49%) with the Heterozygous GA genotype and 23 (24%) with the Homozygous AA genotype.

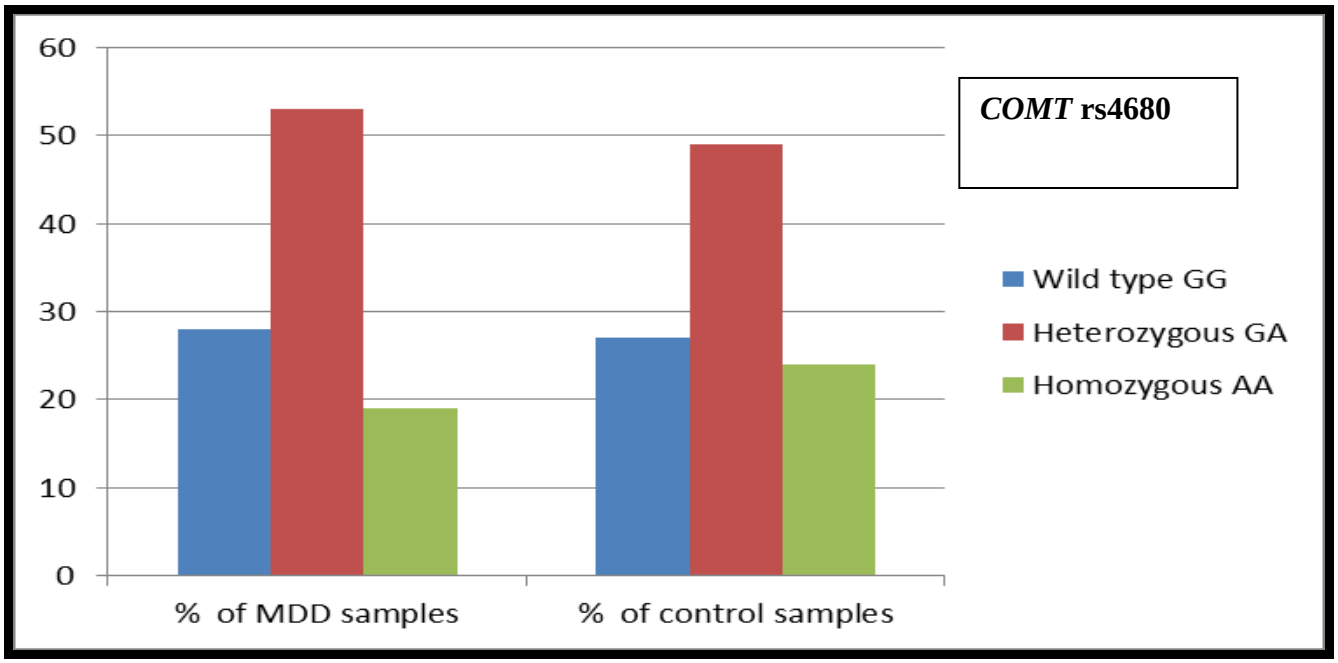

Figure 3.35. Genotype distribution obtained using the ABI™ *Taq*Man® COMT rs4680 assay.

The genotype distribution for the CYP2D6 rs3892097, allele 4 assay is summarised in figure 3.36. Of the 183 samples included (86 MDD patients and the 97 controls), 60 (70%) presented with the wild Type GG genotype, 23 (27%) with the heterozygous GA genotype, and 3 (3%) with the homozygous AA genotype. Amongst the 97 control samples 67 (69%) presented with the Wild Type GG genotype, 25 (26%) with the Heterozygous GA genotype and 5 (5%) with the Homozygous AA genotype.

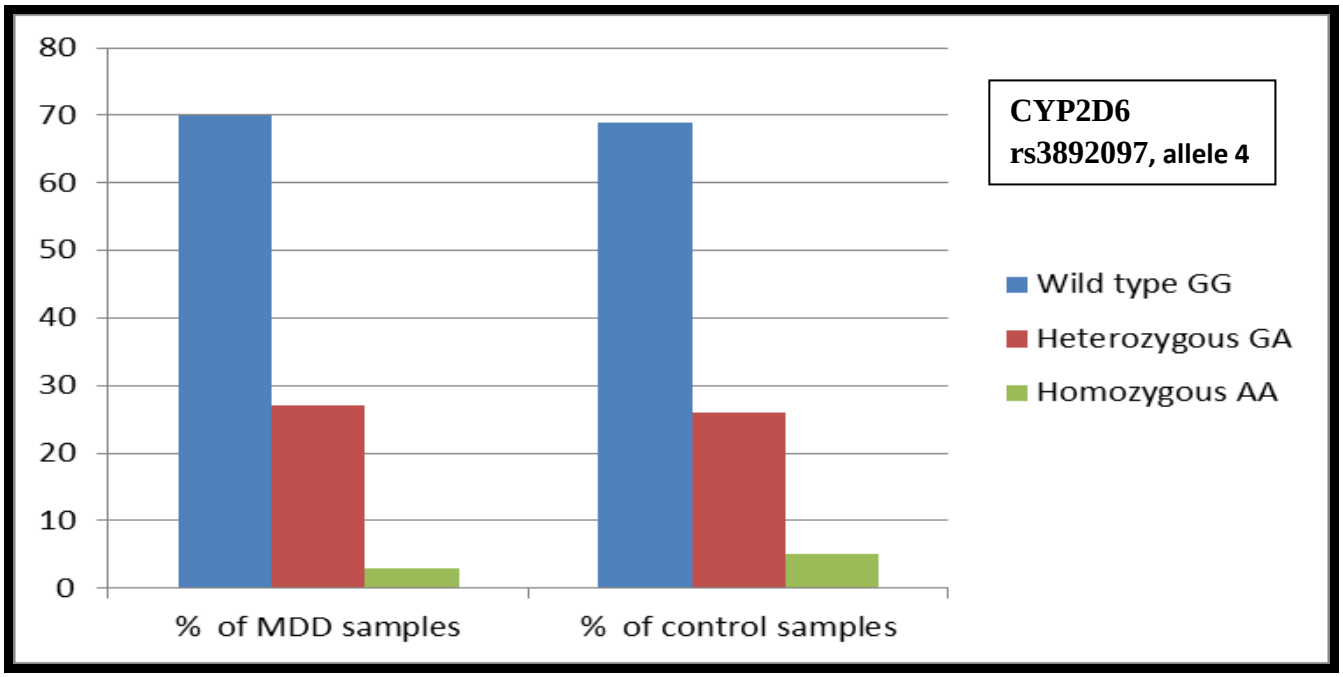

Figure 3.36. Genotype distribution obtained using the ABI™ *Taq*Man® CYP2D6 rs3892097 assay.

The genotype distribution for the ASMT rs4446909 assay is summarised in figure 3.37. Of the 183 samples included (86 MDD patients and the 97 controls), 43 (50%) presented with the wild Type GG genotype, 40 (47%) with the heterozygous GA genotype, and 3 (3%) with the homozygous AA genotype. Amongst the 97 control samples 50 (52%) presented with the Wild Type GG genotype, 45 (46%) with the Heterozygous GA genotype and 2 (2%) with the Homozygous AA genotype.

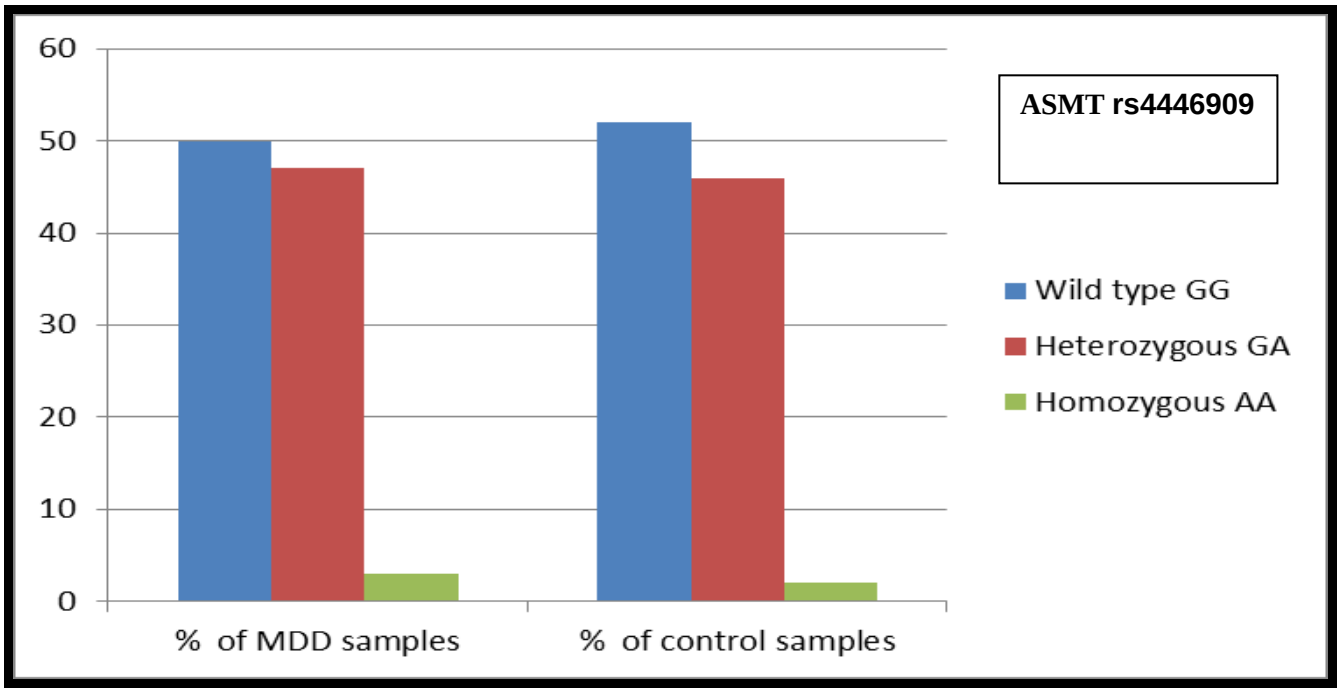

Figure 3.37. Genotype distribution obtained using the ABI™ *Taq*Man® ASMT rs4446909 assay.

The genotype distribution for the SLC6A4 rs4795541 assay is summarised in figure 3.38. Of the 106 samples included (48 MDD patients and the 58 controls), 15 (31%) presented with the wild Type GG genotype, 27 (56%) with the heterozygous GA genotype, and 6 (13%) with the homozygous AA genotype. Amongst the 97 control samples 20 (34%) presented with the Wild Type GG genotype, 29 (50%) with the Heterozygous GA genotype and 9 (16%) with the Homozygous AA genotype.

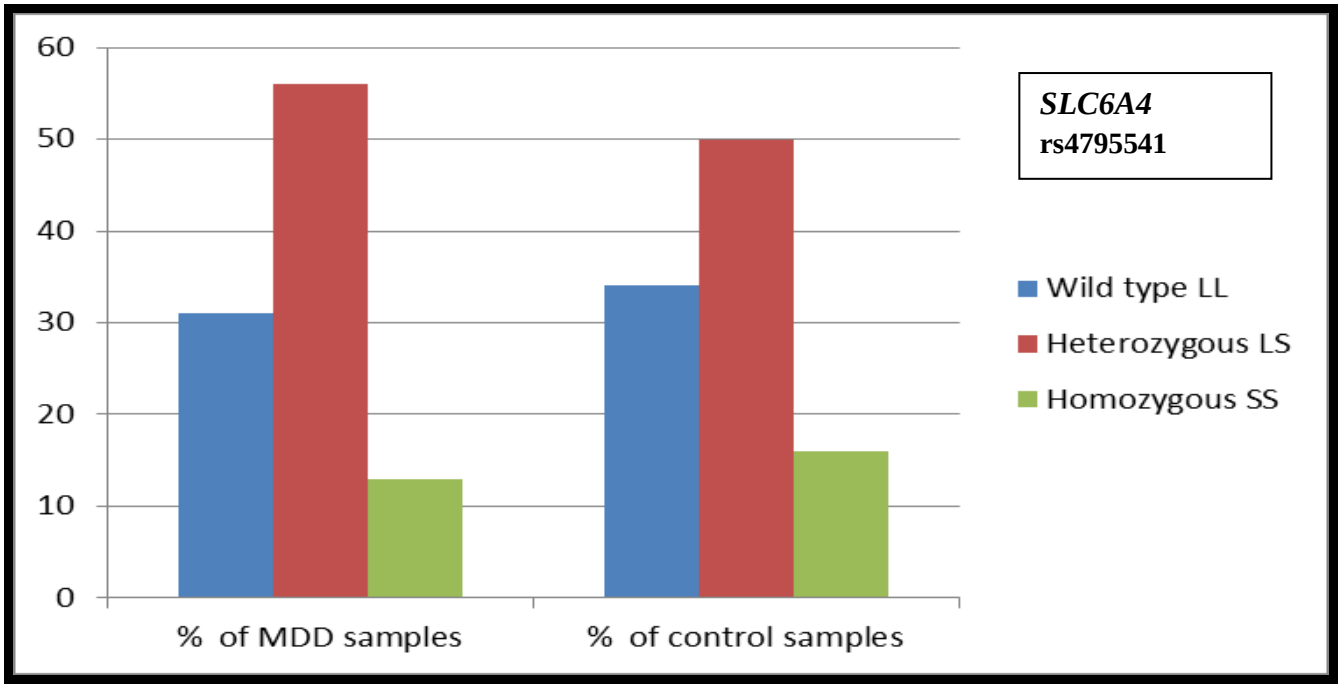

Figure 3.38. Genotype distributions obtained using the SLC6A4 rs4795541 conventional primers.

The genotype distribution and minor allele frequencies obtained from RT-PCR and conventional PCR are summarised in Table 3.3.1. Except for ASMT rs4446909 in the control group, all other polymorphisms were in Hardy-Weinberg equilibrium (HWE) in both MDD patients (86) and control (97) individuals. Although difficult to explain, this deviation may be related to the fact that the common ASMT rs4446909 G-allele is considered the risk allele and not the minor A-allele. The genotype distribution and allele frequencies were not significantly different between study groups.

Table 3.2.1 Comparison of genotype distribution and minor allele frequencies between patients and controls

Genotype	Controls	Patients	Patients vs. Controls P-values
n= 183	**MTHFR rs1801133**		
C/C	37 (38%)	40 (47%)	0.4620
C/T	50 (52%)	37 (43%)	
T/T	10 (10%)	9 (10%)	
T	0.36	0.32	0.3477
HWE	0.3771	1.0000	
n= 183	**MTHFR rs1801131**		
A/A	54 (56%)	46(53%)	0.8805
A/C	39 (40%)	37 (43%)	
C/C	4 (4%)	3 (4%)	
C	0.24	0.25	0.7081
HWE	0.5781	0.2522	
n= 183	**COMT rs4680**		
G/G	26 (27%)	24 (28%)	0.3135
G/A	48 (49%)	46 (53%)	
A/A	23 (24%)	16 (19%)	
A	0.48	0.45	0.3396
HWE	1.0000	0.5207	
n= 183	**CYP2D6 rs3892097**		
G/G	67 (69%)	60 (70%)	0.9237
G/A	25 (26%)	23 (27%)	
A/A	5 (5%)	3 (3%)	
A	0.18	0.17	
HWE	0.1850	0.6983	0.8249
n= 183	**ASMT rs4446909**		
G/G	50 (52%)	43 (50%)	0.7200
G/A	45 (46%)	40 (47%)	
A/A	2 (2%)	3 (3%)	
A	0.25	0.27	0.6929
HWE	0.0308	0.1053	
n= 106	**SLC6A4 rs4795541**		
L/L	20 (34%)	15 (31%)	0.5988

L/S	29 (50%)	27 (56%)	
S/S	9 (16%)	6 (13%)	
S	0.41	0.41	0.7197
HWE	1.0000	0.3707	

For each study participant a calculated genotype risk score (1-10) was calculated by counting the minor alleles for *MTHFR* rs1801133 and rs1801131, *COMT* rs4680, *ASMT* rs4446909 and *CYP2D6* rs3892097 as illustrated in figure 3.39. In both the control and MDD patient groups a minimum risk score of 0 and maximum score of 7 was obtained. Neither of the two groups had a maximum risk score of 10 for any of the 5 selected SNPs. No significant difference was noted between patients with depression and controls for each of the individuals SNPs or combined genotype risk score. The *SLC6A4* gene was excluded from the risk score analysis due to insufficient samples which reduced the statistical power. Although the ASMT A-allele is considered protective according to the literature, it was included as part of the risk score as the frequency was not different in patients and controls.

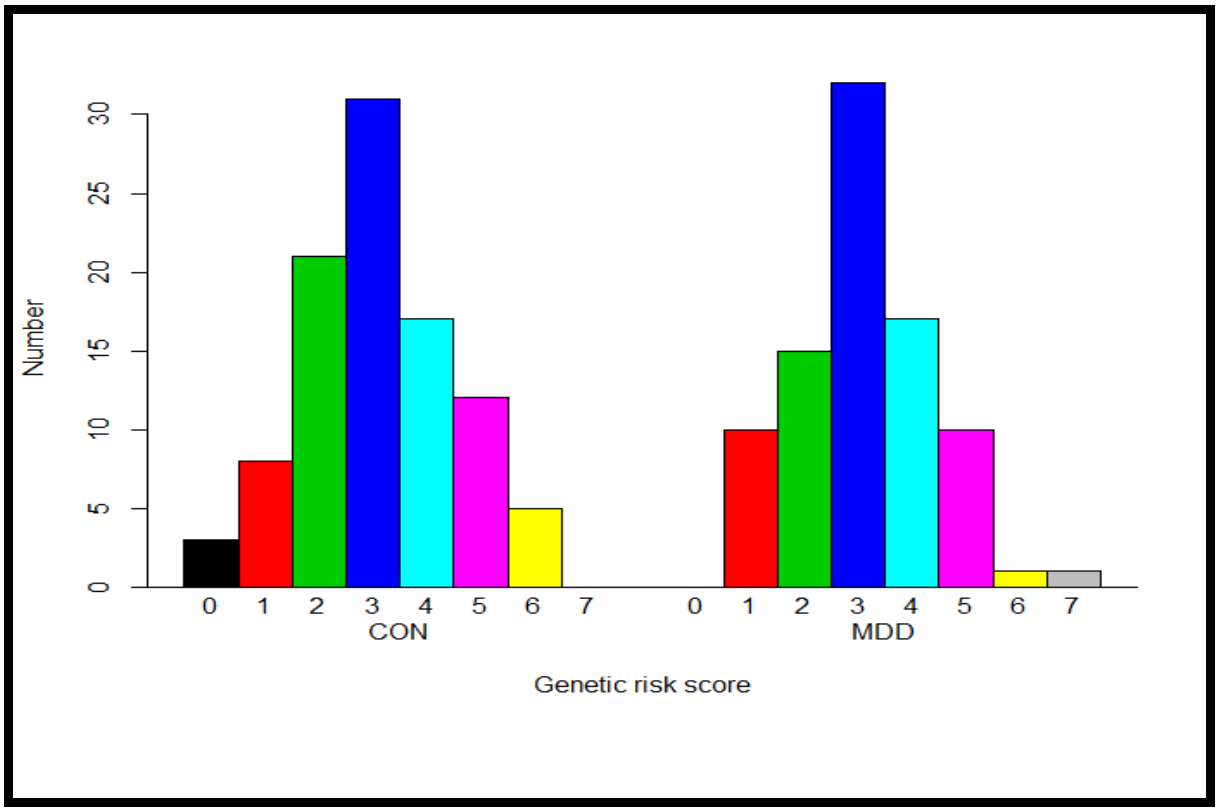

Figure 3.39. Comparison of the genotype risk score between patients (MDD) and controls (CON)

3.4 Discussion

Analytical validation

The DNA samples extracted from 97 unrelated Caucasian control individuals were used to conduct the initial analytical validation process of the methods used in this study and to generate genotype results for comparative studies in patients diagnosed with MDD. The SNPs analysed using polymerase chain reaction (PCR)-based methods was first verified in 5 internal control samples (K1-5). For each SNP three genotypes were identified, namely the wild type (SNP not detected), heterozygous and homozygous genotypes.

DNA samples of known genotype were used for analytical validation of high-throughput genotyping and were included as internal controls during each real-time PCR (RT-PCR) amplification process. The internal control samples for the MTHFR rs1801133 and rs1801131, COMT rs4680, ASMT rs4446909 and CYP2D6 rs3892097 polymorphisms were successfully genotyped using fluorescence resonance energy transfer probes. The generated RT-PCR results were compared against direct sequencing as the gold standard. This corroborated our findings and confirmed that each RT-PCR assay has been analytically validated. The 5-HTT polymorphism rs4795541 was genotyped using PCR and agarose gel electrophoresis. Due to the relatively large deletion of 43 bp, genotyping with high throughput RT-PCR was not possible since no *Taqman* assay was commercially available for this SNP. This method was the most time consuming and required extensive man power, which could be problematic since rapid turn-around time is important for routine diagnostic testing.

Conventional PCR is very similar to RT-PCR with the exception of the reagents used, reaction mixtures and detection technology. RT-PCR uses fluorescence resonance energy transfer probes, with oligonucleotide primers labelled with florescent reporters. Detection and amplification occurs once the probe hybridizes to a complementary DNA site in a head to tail formation

The PCR technology applied in this study is a method whereby a specific DNA region is amplified using specific pre-designed oligonucleotide primers. Once the reaction had been completed, successful amplification was detected using gel electrophoresis. UV technology was used to ensure that the fragment size corresponded with the fragment size of the reference sequence.

After each amplification cycle the fluorescent data was collected. This allowed for the sample detection to be both specific and sensitive for the assays when compared to the controls. RT-PCR was done using the ABI™ *Taq*Man® pre-designed SNP Genotyping Assays, which consisted of two primers to amplify the targeted polymorphic sequence and 2 *Taq*Man® MGB allele probes for differentiation. Both probes contained a reporter dye at the 5'of the allele probe 1, VIC® dye and the FAM™ dye is linked to the 5' end of the allele probe 2. All samples tested were found to be specific and sensitive for the assays employed.

Direct DNA sequencing is considered the gold standard and is the preferred method used routinely in research and diagnostic laboratories to compare and corroborate high throughput assays such as RT-PCR. Our results confirmed that the combination of conventional PCR and direct sequencing is reliable and analytically sound to validate high throughput genotyping assays. RT-PCR was found to be rapid and cost effective for genotyping large sample numbers and therefore was an appropriate method to use in this study.

Genotype distribution and allele frequencies in patients and controls

Except for ASMT rs4446909, all other SNPs included in this study were in Hardy-Weinberg equilibrium (HWE). The reason for this deviation of ASMT rs4446909 from HWE in the controls is unclear as the genotype distribution seems similar in patients (GG 50%, GA 47%, AA 3% with a minor allele frequency of 27%) and controls (GG 52%, GA 46%, AA 2% with a minor allele frequency of 25%). The possibility of a genotyping error is excluded by the findings of Kripe et al. (2011), who reported a minor allele frequency of 24%.

No statistically significant difference was noted between the MTHFR rs1801133 genotype distribution in MDD patients (CC 47%, CT 43%, TT 10%) and controls (CC 38%, CT 52%, TT 10%). A previous study in the South African population (Scholtz et al. 2002) demonstrated that the minor allele frequency of the MTHFR rs1801133 polymorphism differed significantly between different ethnic groups. The authors found MTHFR 677 T-allele frequency of 4% in the South African Black population, 18% in the Coloured population and 36% in the Caucasian population. A similar finding was noted in our study, where the minor allele frequency was 32% in the Caucasian MDD patients and 36% in the controls. This is in agreement with a mutant MTHFR 677 TT genotype frequency of approximately 10% in the general population (Mathews 2001, Gueplen et al. 2006).

The genotype distribution for the COMT rs4680 polymorphism in our sample of patients with MDD (GG 28%, GA 53%, AA 19%) and controls (GG 27%, GA 49%, AA 24%), which was similar to the distribution described by Matinez et al. (2009) (GG 28%, GA 53%, AA 19%). The minor allele frequency in our patients and controls were 53% and 48% respectively, which was similar to the 51% reported by Matinez et al. (2009).

The genotype distribution of the CYP2D6 rs3892097 polymorphism in our MDD patients (GG 70%, GA 27%, AA 3%) and controls (GG 69%, GA 26%, AA 5%) were not significantly different. The minor allele frequency in our patients (17%) and controls (18%) was similar to the 16% reported by Steimer et al. (2004).

No significant differences were furthermore noted in the genotype distribution of the SLC6A4 rs4795541 polymorphism between patients and controls. The minor allele frequency was 41% in

both MDD patients and controls. A similar noted was reported by Hahn et al. (2008) who reported a minor allele frequency of 42%.

Finally, a genotype risk score was calculated for all study participants by counting the minor alleles of MTHFR rs1801133 and rs1801131, COMT rs4680, ASMT rs4446909 and CYP2D6 rs3892097. Due to problems experienced with the method used for detection of this polymorphic deletion variant (which resulted in failed genotyping in a large number of study participants), the SLC6A4 rs4795541 polymorphism was not included in this analysis. . Minimum and maximum risk scores (0 and 7 respectively) were established for both patient and control participants. None of the study participants were homozygous for all the SNPs tested, therefore a maximum risk score of 10 was not observed. No difference was noted in the risk score between patients and controls as a possible indication of susceptibility to depression.

Strengths and Limitations

To our knowledge this was the first study assessing a genetic link between MDD, homocysteine levels and BMI for the panel of SNPs analysed in the South African population. Analytical validation of high throughput genotyping provided a framework for development of standard operating procedures (SOPs) that can in future be applied in extended comparative studies towards development of a routine pathology-supported genetic testing service for patients with depression. Limitations of this study include a relatively small sample size that may have limited the statistical power to detect well-established genotype-phenotype associations. Psychiatric patients are less likely to participate in studies, which could explain the small sample size. Few male participants were included in this study, which could be due to fewer males reporting depression and/or being willing to participate in research studies. Not all susceptible genes, environmental factors or biochemical parameters implicated in previous MDD studies were analysed in this study. Some of the SNPs were previously implicated in disease susceptibility, some in drug response and some in both, which may explain why the genotype risk score was uninformative.

Chapter 4

Genotype-phenotype Association Study

TITLE: Significance of dietary folate intake, homocysteine levels and MTHFR 677 C>T genotyping in South African patients diagnosed with depression: Test development for clinical application

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Abstract

Low folate intake in the presence of the functional MTHFR 677 C>T (rs1801133) polymorphism is an important cause of elevated homocysteine levels previously implicated in major depressive disorder (MDD) and many other chronic diseases. In this study the clinical relevance and inter-relationship of these aspects were evaluated in 86 South African patients diagnosed with MDD and 97 population-matched controls participating in a chronic diseases screening program. A questionnaire-based clinical and nutrition assessment was performed, homocysteine levels determined, and all study participants genotyped for MTHFR 677 C>T (rs1801133) using allele-specific TaqMan technology. The folate score was found to be significantly lower in the patient group compared to controls (p=0.003) and correlated with increased body mass index (BMI), particularly in females with MDD (p=0.009). BMI was significantly higher in the MDD patients compared with controls after adjustment for age and sex (p=0.015), but this association was no longer significant after further adjustment for the level of folate intake in the diet. In MDD patients but not controls, the minor T-allele of MTHFR 677 C>T was associated with increased BMI (p=0.032), which in turn correlated significantly with increased homocysteine levels. The significant association between BMI and homocysteine levels was observed in both the MDD patient (p=0.049) and control (p=0.018) study groups. The significantly higher homocysteine levels observed in MDD patients compared to controls after adjustment for age and sex (p=0.030), therefore appears to be mediated by the effects of MTHFR 677 C>T and low folate intake on BMI. Detection of the low-penetrance MTHFR 677 C>T mutation reinforces the importance of folate intake above the recommended daily dose to prevent or restore dysfunction of the methylation pathway.

Introduction

Major depressive disorder (MDD) is a severe psychiatric illness ranked the 3rd most disabling disorder world-wide (World Health Organisation, http://www.who.int). In South Africa the lifetime prevalence of MDD is 9.8% across different population groups (Tomlinson et al. 2009). Efforts to understand MDD is complicated by the genetically complex, multi-factorial aetiology of this condition (Lesch 2004). Elucidation of the genetic component is important as first-degree relatives of patients diagnosed with depression have a nearly 3-fold increased risk for developing MDD compared to the general population (Li et al. 2008).

Since diagnostic methods used in patients with MDD frequently fail to determine cause or origin, genomic discoveries have raised expectations that current “trial and error” therapeutic decisions will in future be replaced by truly personalised medicine (Gurwitz and Weizman 2004; Hulot 2010). However, genetic biomarkers for psychiatric disorders found to be indicative of risk for disease development or medication side effects, have yet to reach full predictive value. Due to epigenetic

involvement in the pathophysiology of psychiatric and many other chronic diseases, reaching this goal may require an integrative, pathway-based approach applicable across diagnostic boundaries (Peerbooms et al. 2011; Kotze et al. 2013). Extensive research in the genetically distinct populations of South Africa provided a model to facilitate the integration of personalized genomics into the scope of current clinical practice, with a strong focus on shared disease pathways relevant to both current and future health status (Kotze and van Rensburg 2012). This pathology-supported genetic testing (PSGT) service linked to the development of a growing database for translational research (Kotze et al. 2013) was developed over more than a decade, after careful consideration of potential ethical implications (Kotze et al 2004).

Environmental and genetic risk factors may interact to cause disruption of the one-carbon metabolic pathway, resulting in elevated homocysteine levels implicated in many chronic diseases including MDD (Coppen and Bolander-Gouaille 2005; Sugden 2006; Gu et al. 2012). Although it remains unclear whether raised homocysteine is a causal factor or merely a biomarker for deficiencies in folate and other B vitamins, inadequate intake of these nutrients has convincingly been linked to MDD in some patients (Tiemeier et al. 2002; Bottiglieri 2005). Tiemeier et al. (2002) provided evidence that vitamin B12 may be causally related to depression, while the effect of folate deficiency may be mediated via cardiovascular risk and associated co-morbidities. This finding may be particularly relevant in the local population (Kotze and van Rensburg 2012), with advanced aging known to be associated with alterations in plasma levels of these nutrients and homocysteine levels (Dimopoulos et al. 2007).

In addition to age, gender and various modifiable environmental factors such as diet, smoking, alcohol consumption, physical activity and anti-folate drugs, homocysteine concentration is also influenced by genetic variation. The extensively studied cytosine to thymidine (C>T) base change at nucleotide position 677 of the methylenetetrahydrofolate reductase (MTHFR) gene was found to be associated with a range of psychiatric disorders (Peerbooms et al. 2011). Homozygotes with the MTFR 677 TT genotype display approximately 30% of normal enzyme activity, while heterozygotes with the MTHFR 677 CT genotype have approximately 60% of the normal enzyme activity (Bailey and Gregory 1999). Importantly, enzyme function can be restored to normal with adequate availability of vitamin co-factors such as folate (Marini et al. 2008).

Several meta-analyses have convincingly proven the association between the MTHFR 677 C>T mutation and depression (Gilbody et al. 2007; Peerbooms et al. 2011; Wu et al. 2013). Almeida et al. (2008) demonstrated that the odds ratio of prevalent depression increases 4% with every unit increase in homocysteine levels and MTHFR 677 TT homozygotes are 22% more likely than individuals without the risk-associated allele to have current depression or a history of depression. A triangular association was observed between the MTHFR genotype, homocysteine and depression, implying that raised levels of homocysteine increase the risk of depression while lowering of these levels by approximately 0.2 mg/L could reduce the odds of depression by up to

20%. These findings support the recommendation that patients with depression should be given 2 mg of supplemental folate during the acute, continuation, and maintenance treatment phases to enhance the therapeutic effect of anti-depressants (Abou-Saleh and Coppen 2006). The importance of normal folate status in the prevention and treatment of a spectrum of psychiatric conditions (Gilbody et al. 2007; Peerbooms et al. 2011; Stahl 2010) may also apply to patients who suffer from migraine, which often coexists with depression across population groups (Samaan et al. 2011).

In light of the association between folate deficiency and both the development of depression and reduced response to antidepressants (Alpert and Fava 1997; Fava and Mischoulon 2009), we aimed to confirm the deleterious effect of low dietary folate intake in South African patients diagnosed with MDD. A pathology-supported genetic testing approach (Kotze et al. 2013) was used to identify a subgroup of MDD patients with increased requirements for folate due to variation in the MTHFR gene (677 C>T, rs1801133) that may interact with lifestyle risk factors to increase homocysteine levels, considered an important intermediate phenotype between gene and disease.

Subjects and Methods

Ethical approval for the study protocol was obtained from the Ethics Review Committee of the University of Stellenbosch under project number N09/08/224. Written informed consent for participation in this translational research study was obtained from all study participants. A combined service and research approach was applied to allow integration of new genetic knowledge into clinical care based on the need to fill remaining information gaps through ongoing data collection and health outcome studies (Khoury et al. 2010; Kotze et al. 2013).

Study Population

The study population included 183 Caucasian individuals selected from the Gknowmix database (http://www.gknowmix.org) according to the presence (86) or absence (97) of depression. No specific exclusion criteria were applied during patient selection that was based only on a clinical diagnosis of MDD according to the DSM-IV-TR criteria. Of the 72 MDD patients (17 males, 69 females) using anti-depressants (84%) at the time of blood sampling, 32 received mono-treatment and 40 used a combination of two or more antidepressants. The control group (40 males, 57 females) was randomly selected from individuals participating in a chronic diseases screening program with a similar data set available as recorded in the patients, after exclusion of individuals with a personal or family history of overlapping stress/anxiety and depressive disorders.

Biochemical and questionnaire-based assessments

Blood was drawn after an overnight fasting period for determination of plasma homocysteine levels and DNA extraction, using standard techniques. The Gknowmix™ Medical History and Lifestyle Questionnaire was used to document the demographic and clinical characteristics of study participants, as well as medication use/side effects and lifestyle factors such as alcohol consumption, physical activity, smoking status and body mass index (BMI). The physical activity score was determined by using two assessments, namely day-time activity (sedentary, moderate or intensive) and weekly structured exercise (occasionally/none, 2-3 times or 4 or more times a week). The questionnaire also recorded the number of days per week of eating certain foods rich in folate over the previous three months. This information was used to calculate a folate score, which was considered to be low when the score is less than 11, moderate between 11 to 13, and high above 13.

MTHFR genotyping

DNA was extracted from whole blood using the QIAamp® DNA Midi-kit or from saliva using the Oragene® reagents. Conventional polymerase chain reaction (PCR) followed by direct DNA sequencing was performed for detection of the wild type, heterozygous and homozygous genotypes of the functional single nucleotide polymorphism (SNP) MTHFR 677 C>T (rs1801133) also referred to as a low-penetrance mutation. These internal control samples were subsequently used for analytical validation of high throughput genotyping performed on DNA samples extracted from the study participants, using the Applied Biosystems™ (ABI) *Taq*Man® SNP Genotyping Assays on the Corbett Rotor-Gene™ 6000 series Multiplexing System.

Statistical analysis

Genotype distribution and allele frequencies of MTHFR 677 C>T were estimated from allele counts and Hardy-Weinberg equilibrium assessed. Prior to inclusion of the genotyping results in the statistical model used for testing association, age and sex were identified as confounders and adjusted for in all analyses. Other variables including the folate score, smoking, alcohol consumption, physical activity, disease status (of medical conditions frequently associated with depression including breast cancer, multiple sclerosis and diabetes) and drug side effects reported with use of antidepressants were evaluated as potential confounders and adjusted for as appropriate. The qualitative characteristics were described as counts and frequencies whereas the median and interquartile ranges were used for the quantitative characteristics. Logistic regression models were applied to compare pairs of groups, such as patients to controls, whereas linear regression models were used to compare the quantitative characteristics of the genotypes. Quantitative characteristics with non-symmetric distributions were log-transformed for analyses. The R software and R package genetics, freely available from http://www.r-project.org, were used for analyses. Results corresponding to p-values below 0.05 are described as significant.

Results

Comparison of patients and controls

Table 1 summarises the clinical and biochemical characteristics of the study participants. The data is presented separately in males and females based on the knowledge that MDD is more prevalent in females compared to males, also apparent in this study as the number of females compared to males was significantly lower (p=0.002) in controls (57/97, 59%) than in the MDD patients (69/86, 80%). The controls were on average 3.5 years older than the MDD patients (p=0.046), whereas no age difference was observed between males and females among patients or controls.

A family history of MDD was reported by 43% (37/86) of patients, while those with a family history of anxiety and depression were excluded from the control group. The folate score assessed with the study questionnaire was significantly lower (1.8 units on average) in the patient group compared to controls (p=0.003). Males diagnosed with MDD consumed alcohol reportedly once or more a week more often than female patients (p=0.027). A significantly higher proportion of patients diagnosed with depression smoked compared with controls (p=0.019), while physical activity levels were found to be similar between patients and controls.

BMI was on average 6% higher in the MDD patients compared with controls (p=0.015) after adjustment for age and sex. When adjusted also for folate intake in the diet known to affect body weight the difference in BMI between patients and controls was no longer significant (p=0.064). After adjusting for age and sex, homocysteine levels were found to be significantly higher (9% on average) in MDD patients compared to the controls (p=0.030), but was not significant after further adjustment for alcohol intake, smoking, physical activity and the folate score (p=0.108) known to influence homocysteine levels.

Table 1. Clinical characteristics of the Caucasian control and MDD patient study groups stratified by sex. Values are median (IQR) unless otherwise indicated. P-values are for comparing characteristics of genders inside groups and also between groups after adjusting for age and gender where appropriate.

Characteristics	**Controls (n=97)**			**MDD Patients (n=86)**			**Patients vs. controls**
	Female	**Male**	**P-value**	**Female**	**Male**	**P-value**	**P-value**
Number	57	40		69	17		0.0023
Age, years	46 (13)	48 (14)	0.4149	44 (10)	39 (11)	0.0726	0.0459
Homocysteine (μmol/L)	9 (7-11)	10 (9-13)	0.0033	9 (7-11)	13 (11-14)	0.0002	0.0302
Body Mass Index (kg/m^2):	25 (23-28)	27 (24-30)	0.1974	27 (23-33)	27 (24-32)	0.572	0.0154
Normal weight (%)	30 (53%)	12 (30%)		27 (39%)	6 (35%)		
Overweight (%)	16 (28%)	18 (45%)		16 (23 %)	4 (24%)		
Obese (%)	11 (19%)	10 (25%)		26 (38%)	7 (41%)		
Folate Score (units)	7 (4-10)	9 (6-11)	0.2501	5 (4-7)	6 (3-7)	0.8388	0.0034
Very low (%)	19 (33%)	9 (23%)		33 (48%)	8 (47%)		
Low (%)	28 (49%)	17 (43%)		32 (46%)	7 (41%)		
Moderate (%)	4 (7%)	11 (28%)		2 (3%)	1 (6%)		
High (%)	6 (11%)	3 (8%)		2 (3%)	1 (6%)		
Alcohol intake			0.1383			0.0266	0.1103
Abstain (%)	16 (28%)	7 (18%)		24 (35%)	6 (35%)		
Occasionally (%)	17 (30%)	8 (20%)		28 (41%)	2 (12%)		
1-13 units per week (%)	23 (40%)	22 (55%)		15 (22%)	7 (41%)		
14-21 units per week (%)	1 (2%)	3 (8%)		2 (3%)	0 (0%)		
≥ 22 units per week (%)	0 (0%)	0 (0%)		0 (0%)	2 (12%)		
Current Smoking	4 (7%)	2 (5%)	0.6813	10 (15%)	5 (29%)	0.1677	0.0197
Physical Activity			0.1835			0.1404	0.1384
Low (%)	23 (40%)	15 (38%)		36 (52%)	8 (47%)		
Moderate (%)	23 (40%)	11 (28%)		26 (38%)	4 (24%)		
High (%)	11 (19%)	14 (35%)		7 (10%)	5 (29%)		
Family History of MDD (%)	Excluded			30 (43%)	7 (41%)	0.8635	

Homocysteine and body mass index (BMI)

A positive correlation was observed between homocysteine levels and BMI in both the control and patient study groups after adjustment for age and sex ($p<0.05$). No significant associations were observed between either of these characteristics and alcohol intake, smoking and the folate score within the patient and control groups, while increased physical activity was associated with significantly reduced BMI in patients with MDD after adjustment for age and sex ($p=0.013$) and in females only ($p=0.040$).

When further tests of association was performed in the female population only, a significant association was also found with smoking in the MDD patients ($p=0.016$), with smokers showing a 22% higher BMI compared to non-smokers. In females with MDD a low folate score correlated significantly with increased BMI ($p=0.009$), with a similar trend noted in the combined male and female control group ($p=0.078$).

Genotyping results

The genotype distribution and allele frequencies of MTHFR 677 C>T were similar in 86 MDD patients and 97 controls (Table 2). This functional polymorphism was in Hardy-Weinberg equilibrium (HWE) in both MDD patients and the controls. Genotype distribution was similar among the 12 MDD patients not using anti-depressants at entry into the study compared to those on treatment (data not shown).

Table 2. Genotype distribution and minor allele frequencies of MTHFR 677 C>T (rs1801133) shown to be in Hardy-Weinberg equilibrium (HWE) in both patients and controls.

Genotype	Controls	Patients	Patients vs. Controls P-values
n= 183	**n=97**	**n=97**	
C/C	37 (38%)	40 (47%)	0.4620
C/T	50 (52%)	37 (43%)	
T/T	10 (10%)	9 (10%)	
T	0.36	0.32	0.3477
HWE	0.3771	1.0000	

Genotype association

BMI was significantly higher in the presence of each minor T-allele of MTHFR 677 C>T in MDD patients (p=0.032), after adjusting for age and sex. After adjusting also for the significant effect of physical activity on BMI in patients with MDD (see above), to investigate whether differences in physical activity might affect this association, the additive allelic effect of MTHFR 677 C>T became more significant (p=0.020) (Figure 1). Each minor MTHFR 677 T allele increases BMI by 9.2%. This means that BMI is 9.2% higher in CT heterozygotes than CC homozygotes; and another 9.2% higher in TT homozygotes than CT heterozygotes. Since this association was not observed in controls the possibility that medication side effects may be confounding the relationship between MTHFR and BMI due to gene-drug interaction was also considered. Only three of the nine patients found to be homozygous for the MTHFR 677 C>T mutation (with BMI's of 20.9 kg/m2, 25.7 kg/m2 and 34.1 kg/m2) reported side effects with use of antidepressants, while a low folate score (<11) was found in all of these patients. Eight of the nine MTHFR 677 C>T homozygotes used antidepressants at entry into the study.

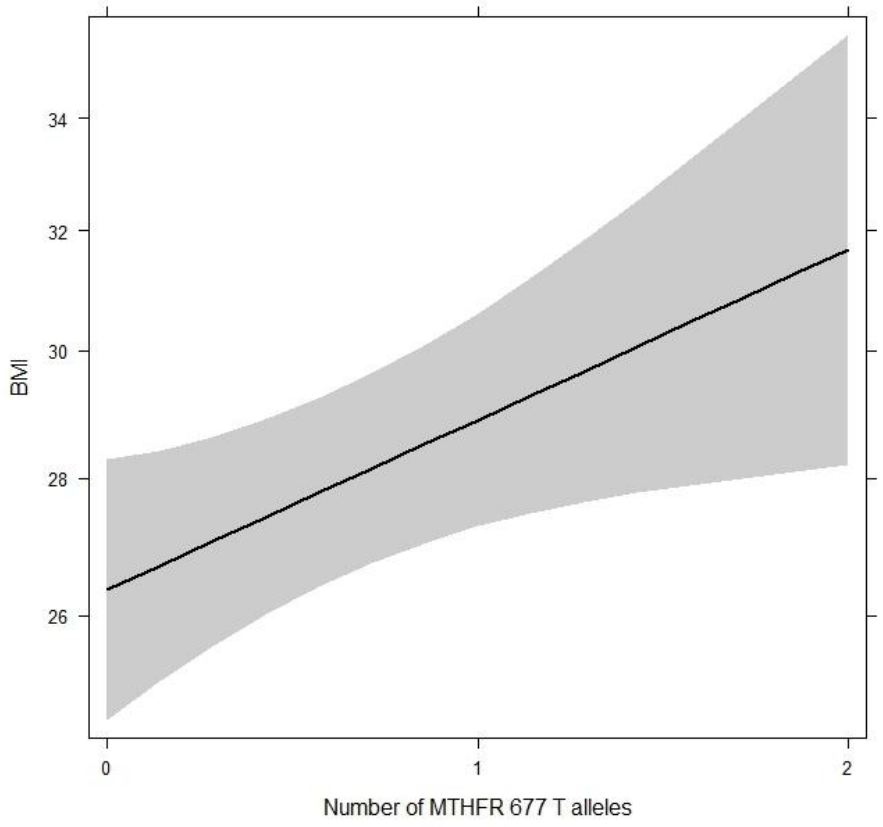

Figure 1. Estimated effect (line) with 95% confidence intervals (shaded area) of the minor T-allele of MTHFR 677 C>T on body mass index (BMI) in patients with depression, after adjusting for age, gender and physical activity (p=0.020).

Discussion

Treatment of MDD is hampered by paucity of reliable diagnostic and predictive biomarkers. The clinical relevance of MTHFR 677 C>T genotyping was confirmed in our study population consisting of 86 Caucasian patients diagnosed with MDD and 97 controls without a personal and family medical history of overlapping stress/anxiety and depressive disorders. The statistically significant effect of MTHFR 677 C>T on increased BMI was confined to patients with MDD. This finding may be related to the availability of folate required as a co-factor for MTHFR enzyme function and synthesis of serotonin, norepinephrine and dopamine in the brain, as the dietary folate score was significantly lower in the MDD patients studied compared to controls (p=0.003).

Replication of the association between BMI and MTHFR 677 C>T (Di Renzo et al. 2013) supports mutation detection as part of our integrative PSGT service that could be used as a clinical and genetic screen (Kotze et al. 2013) prior to recommendation of a suitable diet and/or folate supplementation shown to augment therapy and prevent recurrence in MDD, as well as improve BMI status (Young et al. 2007; Nelson et al. 2012; Di Renzo et al. 2013). Folate augmentation may enhance efficacy of antidepressants in non-responders, lead to remission in patients who partially respond to antidepressant monotherapy and alleviate residual symptoms during the treatment phase. In patients with the MTHFR 677 C>T mutation supplementation with L-methylfolate (5-MTHF) is considered the treatment of choice due to its greater bioavailability in these genetically predisposed individuals and reduced risk of specific side effects associated with folic acid, the synthetic form of folate (Papakostas et al. 2012).

The relatively high proportion (43%) of South African patients with a family history of MDD participating in this study supports the strong genetic component of this disorder, found to have a heritability of nearly 40% (Sullivan et al. 2000). No significant difference in age was observed between males and females within the South African patient and control groups, while individuals included in our control group were on average 3.5 years older than the MDD patients. Similar to previous findings (Tomlinson et al. 2009; Luk and Tosh 2010), MDD was more prevalent in females (69/86, 80%) than males (57/97, 59%). Based on these findings, all association studies were performed after adjustment for age and sex, or in females only.

The inverse correlation between the folate score and BMI observed in our female MDD patient cohort (p=0.009) is supported by similar findings reported recently by Davis et al. (2013), who used the same diet questionnaire as employed in the current study. These authors reported a significantly reduced occurrence of being overweight/obese in the presence of a high folate score (≥ 14) in females, with no difference between the cases (patients diagnosed with multiple sclerosis) and controls studied. In addition to the well-established link between low folate status and raised homocysteine levels, folate deficiency may also result in deleterious effects independent of the concentration of homocysteine (Kolb and Petrie 2013). This may be related to chronic inflammation

characteristic of obesity/high BMI found to be significantly associated with the MTHFR 677 T-allele in South African MDD patients (p=0.020), independent of physical activity adjusted for due to the significant inverse correlation noted between BMI and physical activity for males and females combined after adjustment for age and sex (p=0.013) and in females only (p=0.040).

Since no difference in allelic distribution of the MTHFR 677 C>T mutation was observed in our study between MDD patients and controls it is clear that this functional polymorphism in itself is not sufficient to cause the disorder, although it may elicit an unfavourable effect on BMI and homocysteine levels when folate status is low. This finding may be ascribed to the selection criteria used in this study and a relatively small sample size that may be inadequate to detect genetic effects of polymorphic variants that only become clinically relevant in a high-risk environment due to gene-environment and/or gene-drug interaction. Previous studies have consistently associated MDD with elevated homocysteine levels (Tiemeier et al. 2002; Bjelland at al. 2003), which is in accordance with our results showing on average 9% lower levels in controls when compared with MDD patients. After adjustment for alcohol intake, smoking, physical activity and the folate score this association disappeared, which confirms that these environmental factors individually or collectively have a significant effect on plasma homocysteine levels in the South African population. It is therefore important to identify and modify these environmental risk factors in MDD patients, particularly those who test positive for the minor T-allele of the low-penetrance mutation MTHFR 677 C>T due to hyper-responsiveness to environmental influences over the life span.

Variation in individual response to drug treatment (Pouyanne et al. 2000; Wilkinson 2005) underscores the importance of identifying subgroups of the population with genetically increased requirements for folate and other enzyme co-factors (Moriyama et al. 2002; Herrmann et al. 2003). MDD patients with low folate status may experience reduced response to antidepressants and relapse of depressive episodes, compared to those with normal folate status (Coppen and Bailey 2000; Stahl 2010). At least three randomized controlled trials demonstrated superior response with antidepressant-folate/methylfolate combinations from treatment initiation compared to the use of antidepressants alone. Notably, a wide variety of antipsychotics may cause side effects such as weight gain and development of the metabolic syndrome (Devlin et al. (2012; Kao and Müller 2013). Our observation that the MTHFR 677 C>T is associated with increased BMI in MDD patients but not controls, confirms the importance of the gene effect as a contributing factor to cumulative risk that may be exacerbated by the underlying disease process. In accordance with the findings of Tiemeier et al. (2002), the effect of folate deficiency appears to be mediated by obesity identified as a major co-morbidity in the MDD patients studied. MDD patients are also at increased cardiovascular risk due to the high proportion of smokers compared to controls (p=0.019), particularly in female smokers shown to have significantly higher BMI compared to non-smokers in this study (p=0.016). These findings are in accordance with evidence from both cross-sectional and longitudinal data suggesting that smoking increases the risk of MDD in women.

Pasco et al. 2008 demonstrated that the risk for development of depression is increased by 93% in smokers, independent of physical activity and alcohol consumption. High alcohol intake seems to be more of a problem in males than females with depression, as evidenced by alcohol use once or more a week much more often by male (41%) compared to female (22%) patients (p=0.027).

Despite the limitations imposed by the relatively small sample size of this study our findings are in agreement with previous data and confirm the importance of adequate dietary folate intake and MTHFR 677 C>T genotyping in Caucasian MDD patients in relation to increased BMI, correlating positively with homocysteine linked to depression. An important strength of this study is that the effect of BMI and various other environmental factors known to influence homocysteine was evaluated not only as potential confounders that need to be adjusted for during statistical analysis, but also as potential modifiable contributors to the disease process that could also be useful to mitigate the MTHFR gene effect. After adjustment for the folate score the statistically significant difference in BMI between patients and controls (p=0.015) disappeared, thereby confirming the significant effect of this nutrient on risk of obesity irrespective of the use of certain medications that may be associated with weight gain and/or elevation of homocysteine levels. In support of our findings several lines of evidence have shown that low folate status is associated with a high BMI (Mojtabai 2004; Luppino et al. 2010).

Coppen and Bolander-Gouaille (2005) recommended the use of folate in the treatment and prevention of depression as a population-level strategy. The nutrition questionnaire applied in this study may provide a valuable clinical tool for identification of individuals with low dietary folate intake who may benefit from an integrative PSGT service. This approach focused on both current and future risk (Kotze et al. 2013) may be particularly beneficial to guide treatment in MDD patients who (1) possess the MTHFR 677 C>T mutation, (2) are overweight/obese, smoke, consume excessive alcohol, (3) have high homocysteine levels, and/or (4) have low plasma or red blood cell folate levels. Evidence of shared genetic vulnerability for many chronic diseases and drug response affected by MTHFR 677 C>T support the clinical validation and utility of this low-penetrance mutation in patients with MDD and associated co-morbidities.

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Disclosure

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CHAPTER 5

CONCLUSIONS

Clinically useful biomarkers that could assist in the early diagnosis of MDD, associated risk factors and improved treatment response have the potential to reduce morbidity and mortality risks associated with MDD. We performed genotyping in 86 unrelated clinically diagnosed Caucasian MDD patients and 97 control Caucasian participants without a personal or family history of stress/anxiety or depression, using the Applied Biosystems™ (ABI) *Taq*Man® SNP genotyping assays on the Corbett Rotor-Gene™ 6000 multiplexing system. The test development process applied in this translational research study included essential components such as assessment of analytical validation, clinical validation and clinical utility (Grosse and Khoury 2006). The genotyping assays used to detect functional polymorphisms in the MTHFR (rs1801133, rs1801131), COMT (rs4680), ASMT (rs4446909), CYP2D6 (rs3892097) and SLC6A4 (rs4795541) genes were successfully optimised.

All six polymorphisms included in this study are low-penetrance mutations that are insufficient to cause depression in the absence of relevant environmental triggers. Development of depression associated with these biomarkers result from a complex interaction between genetic and environmental risk factors. By understanding which genetic and environmental factors contribute to the disease mechanism, it is possible to not only reduce predisposing factors but to potentially prevent disease and/or adverse drug effects as well. The results presented in this study support the notion that implementation of a healthy diet with adequate intake of folate and other essential nutrients required for optimal functioning of enzymes such as MTHFR could reduce the risk associated with genetic variation associated with reduced enzyme activity. However, the severity of the disease and environmental triggers needs to be taken into account, since more severe depressive symptoms and environmental factors (excessive alcohol consumption, heavy smoking and extreme stressful conditions) could require more intensive intervention.

No significant difference was noted in genotype distribution and allele frequencies for any of the polymorphisms studied in MDD patients or controls, which confirms that the polymorphisms itself is not disease causing in the South African patients. Studies have reported that MDD patients are at an increase risk of developing a folate deficiency (Tiemeier et al. 2002, Kelly et al. 2004, Almeida et al. 2004), which was supported by the results obtained in this study. Of the six polymorphisms analysed only MTHFR rs1801133 was associated with increased BMI, which in turn correlated with increased homocysteine levels. Raised homocysteine levels have previously been associated with MDD (Tiemeier et al. 2002, Almeida et al. 2004, Bjelland at al.2003) and in this study homocysteine levels were on average 9% lower in controls when compared with MDD patients. The folate score was on average 1.8 units lower in depressed patients. Identifying individual's with the MTHFR rs1801133 677 T-allele to ensure increased intake of folate above the recommended daily dose therefore has the potential to reduce recurrence of depression. Reduced folate levels correlated with increased BMI, which was 14% higher in our MDD patients. This finding was only seen in patients with the minor MTHFR rs1801133 T-allele with BMI was on average 8% higher in

the South African Caucasian MDD patients compared to control individuals, after adjusting for age and gender. Smoking has been associated with MDD and this was corroborated in our study, where the odds of smoking found to be at least 3 times higher in MDD patients than controls.

A growing body of evidence suggests that the key to disease prevention and improved clinical management of patients with MDD lies in a better understanding of gene-environment interactions underlying the disorder and effective intervention based on this knowledge (Kotze et al. 2013). In this South African Caucasian MDD cohort studied the folate score and BMI confirmed the association between the MTHFR mutation and diet intake in relation to the multifactorial nature of MDD. Therefore, combining MTHFR genotyping, dietary assessment and determination of serum homocysteine levels could aid in identifying depressed individuals with a folate deficiency or tendency for this nutrient deficiency that can be corrected or prevented. Papakostas et al. 2012 recommended that supplementing with L-methylfolate (or 5-MTHF) could benefit depressed individuals with the MTHFR 677 risk associated T-allele. This may be most applicable to patients with raised homocysteine levels (that may be indicative of nutrient deficiencies or drug side effects) or who are overweight/obese, smoke or consume alcohol. Evidence of shared genetic vulnerability for many chronic diseases and drug response mediated by the MTHFR 677 T-allele support the clinical relevance of this low-penetrance mutation. We conclude that the clinical relevance of MTHFR genotyping was confirmed in South African patients with MDD in this translational or replication study.

Despite study limitations our results are in agreement with previous data and highlighted the importance of taking folate status, smoking, alcohol intake and obesity into account when MTHFR genotyping is performed. This should be considered in relation to homocysteine levels as a reflection of a functional methylation pathway. Analysis of the potential interaction between MTHFR and any of the classes or individual antidepressants used by study participants was outside the scope of this study.

Ethical considerations

An exponential increase in the number of different genetic tests currently available in the global context has led to direct-to-consumer (DTC) services that raised controversy with regards to the ethical implications of SNP genotyping for multi-factorial conditions such as MDD and other single gene tests already performed routinely in clinical practice. A pressing need exists to define exactly to what extent personalised genomics could add value to current clinical practice and how it may be integrated into conventional risk management of chronic multi-factorial psychiatric diseases. To bridge this gap, a novel approach to personalized genomics termed Pathology-Supported Genetic

Testing (PSGT), has been developed over the past 10 years following careful consideration of ethical issues (Kotze et al. 2013).

When performing genetic testing, it is important to adhere to strict ethical standards as reviewed by Kotze et al. (2004) in the context of potential genetic discrimination related to health insurance in the South African context. Identification of genetic alterations in individuals with a family history or clinical features of the associated disease will not impact further on insurance, while exclusion of a genetic defect in a family member could be beneficial for insurance purposes in some instances. While early detection or pre-clinical diagnosis of treatable or preventable genetic diseases may be beneficial, it may also lead to anxiety when genetic risk factors are identified in an individual without clinical symptoms of a disease. Handling of genetic material after the genetic test has been performed is an important issue and a choice should be given for destroying the sample or long-term storage and inclusion in a genetic database for future research. Consideration should also be given to testing of the same specimens at different laboratories or using different mutation detection methods as part of a quality control process. In the event that genetic testing is performed in families, non-paternity may be revealed and it is therefore important that adoption be reported at the time that specimens are obtained for genetic testing. A genetic test generally screen for specific genetic alterations expected to provide useful information in relation to treatment/diet intervention and a positive result would imply that other family members may also have the genetic change(s). A positive genetic test does not mean that the person has a genetic disease or will develop the condition, but it can increase the risk of disease in the absence of appropriate risk reduction intervention. Failure to detect a specific genetic alteration (negative test result) on the other hand does not exclude undefined gene mutations or other risk factors not tested for. Genetic counselling should therefore always be offered to explain to patients the potential benefits and limitations of the test requested, what a positive or negative result will mean and the implications it may have for treatment options.

Pathology-supported genetic testing

It is recognized that personalized genomics may allow for the early detection and evaluation of genetically susceptible individuals at cumulative risk of disease development or treatment-related side-effects. However, testing directed at consumers without the involvement of a clinician is associated with profound limitations. The integration of knowledge garnered from clinical and biochemical assessment in relation to lifestyle together with genetic results into our PSGT platform aims to overcome these limitations. PSGT is considered suitable to facilitate the integration of personalized genomics into the scope of current clinical practice with a strong focus on shared disease pathways relevant to both current and future health status (Kotze and van Rensburg

2012). By identifying health-promoting lifestyle habits that might mitigate the adverse effects of risk-associated low-penetrance mutations and functional SNPs, PSGT may provide a more comprehensive platform by which to formulate personalized harm reduction strategies aimed at lowering cumulative disease risk and drug failure in genetically susceptible individuals. This notion is in line with the growing appreciation of the benefits derived from a multi-disciplinary therapeutic approach to disease, as well as the value of practice that is personalized, preventative, pro-active and participatory, so-called P4 medicine (Hood and Flores 2012).

P4 medicine is applicable to the findings of this study noting that folate deficiency associated with hyperhomocysteinemia are involved in the pathogenesis of MDD as well as obesity, as well as a predisposition towards greater symptom severity, disease progression and chronicity in the former (Mojtabai 2004; Narin et al 2005; Bodnar et al. 2005; Lawrence et al. 2006). It is therefore encouraging that dietary or pharmaceutical folate supplementation may augment therapy and prevent recurrence in MDD as well as improve BMI status in obesity (Young et al. 2007, Nelson et al. 2012, Papakostas et al. 2012). It remains controversial whether all patients with these conditions require supplementation, particularly in view of its low cost and relative lack of associated side-effects, or whether treatment should be individualized. In this context, a PSGT platform may also benefit the development of a step-wise multi-disciplinary algorithm aimed at identifying which MDD patients are set to derive the greatest benefit from folate supplementation.

This should commence with the detailed and accurate clinical diagnosis of affective disorders based on DSM criteria with emphasis on whether symptoms (viewed in relation to functional impairment) befit a formal diagnosis of MDD, as well as if a specific sub-type of disease may be identified. This may be relevant due to a varied association with symptoms of underlying hypothalamic shift – specifically, alterations in appetite and eating habits could influence dietary intake of folate and reduced bioavailability. Assessment of nutritional status should then be performed, possibly as was done with use of a dietary questionnaire and expression of folate intake as a score for research use and further validation. When deficiency is indicated (whether or not confirmed biochemically), the question arises whether genotyping for the MTHFR 667C>T SNP as investigated in this study could be used or developed as a biomarker to stratify MDD patients based on folate requirements and treatment response. While results from this study indicate that T-allele carriers may be those in need of supplementation, further studies are needed to confirm this finding in a larger population group with additional consideration of other confounders which were seen as falling outside the scope of the current study. It seems obvious that availability of clinically useful genetic biomarkers should be translated into a cheap and rapid test to be used as an effective screening tool, in line with future development of bedside and point-of-care testing methods. Furthermore, future prospective studies following initiation of supplementation may provide further support as to the clinical efficacy of folate supplementation and utility of personalized genomics to benefit a screening and stratification method for MDD patients.

The prevalence of MDD is increasing in developing nations with this condition considered a major cause of morbidity and mortality. Additional evidence provided in this study on the role of folate in the disease process has broadened our understanding of this condition. Although many factors remain to be elucidated, the results from this study provided further evidence in support of the clinical utility of MTHFR genotyping using the PSGT approach in patients with MDD in the local population.

Chapter 6

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