

**Development of a high-throughput genotyping assay
for detection of functional polymorphisms involved in
homocysteine metabolism and the methylation
process implicated in multiple sclerosis**

BY

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Summary

The aetiology of multiple sclerosis (MS) remains largely unknown due to the multifactorial nature of disease susceptibility determined by both environmental and genetic factors. Progress has been made in identifying the genetic component of MS, as well as the possible interactions with the environment. In this study single nucleotide polymorphisms (SNPs) in the *FTO* (rs9939609, Intron 1 T>A), *MTR* (rs1805087, 2756 A>G), *MTRR* (rs1801394, 66 A>G), *MTHFR* (rs1801133, 677 C>T and rs1801131, 1298 A>C) and *COMT* (rs4680, 472 G>A) genes involved in the methylation metabolic pathway were studied in the context of MS.

The overall objective of this study was to elucidate the mechanism underlying raised homocysteine levels in MS patients. The specific aims were 1) to analytically validate high throughput real-time polymerase chain reaction (RT-PCR) genotyping assays for the 6 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 MS. The study population included a total of 114 unrelated Caucasian MS patients (98 females and 16 males) and 195 unrelated Caucasian control individuals without a diagnosis of neurological disease (128 females and 67 males).

A novel finding of this study was that the risk-associated *FTO* rs9939609 A-allele was associated with raised homocysteine levels ($p=0.003$) in patients diagnosed with MS, but not in controls. Furthermore, homocysteine levels correlated significantly with body mass index (BMI) ($p=0.046$) and total cholesterol levels ($p=0.048$). Both homocysteine ($p=0.011$) and BMI ($p=0.017$) were significantly reduced with increasing intake of folate in the diet, while high saturated/trans fat intake correlated significantly with increased BMI ($p<0.001$). High physical activity correlated with reduced BMI ($p<0.006$) in the study population, adjusted for age, gender and disease status. Daily intake of at least five fruit and vegetable portions and the *COMT* rs4680 (472 G>A) AA genotype had a favourable lowering effect on MS disability as assessed by the expanded disability status scale (EDSS) ($p=0.035$), while smoking increased MS disability significantly ($p<0.001$). All SNPs studied were found to be in Hardy-Weinberg equilibrium (HWE), with no significant differences detected between patients and control individuals in genotype distribution or allele frequencies.

This study has shown for the first time that the underlying disease process of MS moderates the effect of the *FTO* rs9939609 polymorphism on homocysteine levels, which is consistent with the role of *FTO* in demethylation and epigenetic changes. Identification of *FTO* rs9939609 reinforces the importance of adequate folate intake in the diet that can be assessed accurately with use of the Medical History and Lifestyle Questionnaire applied in this study.

Finally, the finding that raised homocysteine levels and BMI are significantly influenced by lifestyle factors such as diet and physical activity in our study cohort, offers a solution to counteract the detrimental effects of genetic risk factors contributing to the development of these established vascular risk factors for MS. Combining this information with *FTO* rs9939609 and *COMT* rs4680 genotyping may in future translate into a comprehensive pathology supported genetic testing strategy aimed at improved risk management and quality of life in MS patients.

Opsomming

Die etiologie van meervoudige sklerose (MS) is grootliks onbekend as gevolg van die multifaktoriale aard van siekte vatbaarheid wat bepaal word deur beide genetiese en omgewingsfaktore. Vordering is reeds gemaak in die identifisering van die genetiese component van MS, asook moontlike interaksie met die omgewing. In hierdie studie is enkel nukleotied polimorfismes (SNPs) in die *FTO* (rs9939609, Intron 1 T > A), *MTR* (rs1805087, 2756 A > G), *MTRR* (rs1801394, 66 A > G), *MTHFR* (rs1801133, 677 C > T en rs1801131, 1298 A > C) en *COMT* (rs4680, 472 G > A) gene, wat betrokke is in die metilering metaboliese padweg, in die konteks van MS bestudeer.

Die oorhoofse doel van hierdie studie was om die onderliggende meganisme betrokke by verhoogde homosisteïen vlakke in MS pasiënte uit te lig. Die spesifieke doelwitte was 1) om die analitiese geldigheid van die hoë deurvoer riëltyd polymerase kettingreaksie (RT-PCR) genotiperings metode soos toegepas vir die 6 geselekteerde SNPs te bevestig teen direkte DNA volgorde bepaling as die goue standaard, vir 2) moontlike integrasie in 'n patologie-gesteunde genetiese toetsing (PSGT) strategie wat gemik is op verbeterde kliniese hantering van MS. Die studiepopulasie bestaan uit 'n totaal van 114 nie-verwante Kaukasiese MS pasiënte (98 vroue en 16 mans) en 195 nie-verwante Kaukasiese kontroles sonder 'n diagnose van neurologiese siektes (128 vroue en 67 mans).

'n Nuwe bevinding van hierdie studie was dat die risiko-verwante *FTO* rs9939609 A-alleel geassosieer was met verhoogde homosisteïen vlakke ($p = 0,003$) in pasiënte gediagnoseer met MS, maar nie in kontroles nie. Homosisteïen vlakke was verder beduidend geassosieer met liggaamsmassa-indeks (BMI) ($p=0,046$) en totale cholesterol vlakke ($p=0,048$). Beide homosisteïen ($p=0,011$) en BMI ($p=0,017$) het aansienlik verminder met 'n hoër inname van folaat in die dieet, terwyl 'n hoë versadigde/trans vet en koolhidrate inname beduidend gekorreleer het met 'n verhoogde BMI ($p < 0,001$). Hoë fisiese aktiwiteit was gekorreleer met 'n verminderde BMI ($p < 0,006$) in die gekombineerde groep, aangepas vir die ouderdom, geslag en MS diagnose. Daaglikse inname van ten minste vyf vrugte en groente porsies en die *COMT* rs4680 (472 G>A) AA genotipe het 'n gunstige uitwerking op vermindering van gestremdheid gehad, soos bepaal deur die uitgebreide gestremdheid status skaal (EDSS) ($p=0,035$), terwyl rook MS gestremdheid beduidend verhoog het ($p < 0,001$). Alle SNPs bestudeer was in Hardy-Weinberg ewewig (HWE), met geen

beduidende verskille waargeneem in genotipe verspreiding of alleelfrekwensies tussen pasiënte en kontroles nie.

Hierdie studie het vir die eeste keer aangetoon dat 'n diagnose van MS die effek van die *FTO* rs9939609 polimorfisme op homosisteïen vlakke modereer, wat ooreenstem met die rol van *FTO* in demetilering en epigenetiese veranderinge. Identifikasie van *FTO* rs9939609 versterk die belangrikheid van genoegsame folaat inname in die dieet wat akkuraat gemeet kon word deur gebruik te maak van die Mediese Geskiedenis en Leefstyl Vraelys soos toegepas in hierdie studie.

Ten slotte, die bevinding dat verhoogde homosisteïen vlakke en BMI statisties betekenisvol beïnvloed word deur leefstylfaktore soos dieet en fisiese aktiwiteit in ons studie populasie, verskaf 'n oplossing om die genetiese bydrae tot hierdie gevestigde vaskulêre risikofaktore vir MS teen te werk. Kombinasie van hierdie inligting met *FTO* rs9939609 en *COMT* rs4680 genotipering kan moontlik in die toekoms benut word as deel van 'n omvattende patologiese-gesteunende genetiese toetsing strategie wat daarop gemik is om die risikobestuur en kwaliteit van lewe te verbeter in MS pasiënte.

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List of Abbreviations and Symbols

3'	3-prime
5'	5-prime
25(OH)D	25-hydroxyvitamin D
5MTHF	N-5-methyltetrahydrofolate
5,10-MTHF	N-5,10-methylenetetrahydrofolate
°C	degrees Celsius
=	equal to
kg/m ²	kilogram per square meter
>	larger than
µg	microgram
µg/L	microgram per litre
µL	micro litre
µmol/L	micromole per litre
-	minus
%	percentage
+	plus
±	plus-minus
®	registered trademark
<	smaller than
A	adenine
A (Ala)	alanine
APC	antigen presenting cells
ATP	adenosine 5'-triphosphate
BBB	blood brain barrier
bp	base pair

BHMT	betaine-homocysteine methyltransferase
BLAST	basic local alignment search tool
BMI	body mass index
C	cytosine
CI	confidence interval
CIS	clinically isolated syndrome
CNS	central nervous system
COMT	catechol-o-methyl transferase
CRP	c-reactive protein
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
D (Asp)	aspartic acid
dATP	2'deoxy-adenosine-5'triphosphate
dCTP	2'deoxy-cytosine-5'triphosphate
ddH ₂ O	double distilled water
dGTP	2'-deoxy-guanosine-5'-triphosphate
dH ₂ O	distilled water
DMA	disease-modifying agents
DNA	deoxyribonucleic acid
DNMTs	DNA methyltransferases
dsDNA	double stranded DNA
dTMP	2'deoxy-thymidine-5'monophosphate
dTTP	2'-deoxy-thymidine-5'-triphosphate
dUMP	2'deoxy-uridine-5'monophosphate
EAE	experimental allergic encephalomyelitis
EBV	Epstein-Barr virus
EDSS	expanded disability status scale
EDTA	ethylenediaminetetraacetic acid
EFAs	essential fatty acids

EtBr	ethidium bromide
FAD	flavin adenine dinucleotide
FFS	functional system score
FTO	fat mass and obesity associated
g	gram
G (Gly)	glycine
G	guanine
H ₂ O	water
H ₃ BO ₃	boric acid
HERV	human endogenous retrovirus
HERV-W	human endogenous retrovirus type W
HHV-6	human herpes virus type 6
HIOMT	hydroxyindole-O-methyltransferase
HLA	human leukocyte antigen
HSV-1	herpes simplex virus type 1
HWE	Hardy Weinberg equilibrium
ICAM-1	Intercellular Adhesion Molecule 1
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IM	infectious mononucleosis
M (Met)	methionine
M	molar
MAT	methionine adenosyltransferase
MB-COMT	membrane bound catechol-o-methyl transferase
mg	milligram
MGB	minor groove binder

MgCl ₂	magnesium chloride
mg/L	milligram per litre
ml	millilitre
mM	millimolar
mmol/L	millimol per litre
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	Multiple Sclerosis
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
N (Asn)	asparagines
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Innovation
ng	nanogram
ng/μL	nanogram per micro litre
NHANES	National Health and Nutrition Examination Study
NTC	non-template control
OPC	oligodendrocyte precursor cells
OR	odds ratio
PAR	pseudoautosomal region
PCR	polymerase chain reaction
PLP	peridoxal-5'-phosphate
pmol	picomole
PNMT	phenylethanolamine n-methyltransferase
PP-MS	primary-progressive multiple sclerosis
PR-MS	progressive-relapsing multiple sclerosis

q	long arm of chromosome
RDA	recommended daily allowance
RNA	ribonucleic acid
rpm	revolutions per minute
RR-MS	relapse-remitting multiple sclerosis
RT-PCR	real-time polymerase chain reaction
S (Ser)	serine
S-COMT	soluble catechol-o-methyl transferase
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SH2D2A	SH2 domain-containing protein 2A
SNP(s)	single nucleotide polymorphism(s)
SP-MS	secondary-progressive multiple sclerosis
T	thymine
T _A	annealing temperature
<i>Taq</i>	<i>Thermus aquaticus</i> polymerase enzyme
TBE	Tris-Borate-EDTA buffer
TE	Tris-EDTA buffer
THF	tetrahydrofolate
TLR	Toll-like receptor
T _M	melting temperature
TM	trademark
TNF-α	tumor necrosis factor-alpha
U	units
UV	ultraviolet

V (Val)	valine
V	volts
VLA-4	very late antigen 4
v/v	volume per volume
VZV	varicella zoster virus
w/v	weight per volume
x	times
x g	times gravity
Y (Tyr)	tyrosine

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Chapter 1

Literature Review

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is primarily a chronic inflammatory disease and the most common disabling neurological disease affecting middle-aged and young adults (Feinstein 1999). It is generally believed that MS is an immune-mediated disorder that occurs in genetically susceptible individuals, in which the fatty myelin sheaths around the axons of the brain and spinal cord are damaged, leading to demyelination and scarring as well as a broad spectrum of signs and symptoms. MS favours women over men by a ratio of almost 2:1, and it most often strikes between the ages of 20 and 40 with Caucasians being especially vulnerable (Loren and Rolak, 2002).

1.2 Pathological Hallmarks of Multiple Sclerosis

The brain consists of white and grey matter and contains various highly specialised cell types including neurons and glial cells. Neurons are crucial and responsible for brain function. The glial cells include astrocytes and oligodendrocytes. In MS a principal target of immune attack is the oligodendrocytes that synthesise and maintain the myelin sheaths of up to 40 neighbouring nerve axons (Compston and Coles 2002). Myelin is a dielectric (electric insulating) material synthesised by mature oligodendrocytes and forms the myelin sheath around the axon of a neuron. This myelin sheath is essential for the proper functioning of neurons, and thus the nervous system, by allowing impulses to propagate through the neurons at high speed (Compston and Coles 2002).

The pathological trademark of chronic MS is the formation of a demyelinated sclerotic plaque, which represents the end stage of a process involving inflammation, demyelination, remyelination, astrocytosis, and axonal degeneration. The order in which these processes take place however, is still unknown (Noseworthy *et al.* 2000). Lesions have a tendency to occur in the periventricular white matter, cerebellum, optic nerves, spinal cord, brain stem, and often surround one or several medium sized vessels (Noseworthy *et al.* 2000).

1.3 Signs and Clinical Symptoms of Multiple Sclerosis

The onset of MS may differ in severity from person to person, being insidious in some patients to alternately abrupt in others with the initial symptoms varying from trivial to severe (Kremenutzky *et al.* 2006). MS is characterised by CNS dysfunction with persistent remissions and exacerbations and individual patients may present with a broad spectrum of neurological impairments at different times. The

most common of these symptoms are: weakness or clumsiness of an extremity; paresthesia in one or more extremity, trunk or face; and/or visual disturbances that include partial blindness and pain in one eye (due to retrobulbar optic neuritis), dimness of vision, scotomas, or diplopia (Compston *et al.* 2006; Noseworthy *et al.* 2000).

Other important signs include: transient weakness of an extremity, gait disturbances, fleeting ocular palsy, difficulties with bladder control, stiffness or fatigability of a limb, and vertigo. These wide varieties of signs are evidence of widespread CNS involvement. A phenomenon known as Uthoff's syndrome may accentuate the signs and symptoms when exposed to heat (Compston *et al.* 2006; Noseworthy *et al.* 2000). Debilitating fatigue is also often reported by MS patients and this can be attributed to sympathetic vasomotor involvement (Flachenecker *et al.* 2003), or biochemical deficiencies (Van Rensburg *et al.* 2012). Both sensory and motor involvement are significant in the signs and symptoms of MS. Inattention, lack of judgement and apathy may occur with emotional lability being relatively common and patients reporting reactive depression and/or euphoria.

The Kurtzke Expanded Disability Status Scale (EDSS) published in 1983 is a method of quantifying disability in MS. The EDSS measures disability severity in eight functional systems namely cerebellar, pyramidal, sensory, brainstem, bowel and bladder, sensory, cerebral, visual and other, allowing neurologists to assign a functional system score (FSS) in each category. People who are fully ambulatory fall in the EDSS range of 1.0 to 4.5. Impairment to ambulation is defined in EDSS steps 5.0 to 9.5 with 10 being death due to MS (Kurtzke 1983).

1.4 Disease Classification

MS was first described as a distinct disease in 1868 by the French neurologist Jean-Martin Charcot (1825-1893) calling it "sclerose en plaques" (Compston 1988; Roncaroli 2005).

In 1996 the United States National MS Society standardized four clinically distinct subtypes of MS. They are: relapsing-remitting MS (RR-MS), primary progressive MS (PP-MS), secondary progressive MS (SP-MS) and progressive relapsing MS (PR-MS) (Lublin and Reingold, 1996). In addition, some people diagnosed with MS experience no disability progression and are regarded as having "benign MS".

1.4.1 Relapsing Remitting Multiple Sclerosis

Relapsing-remitting MS is defined as “clearly defined disease relapses with full recovery or with sequelae and residual deficit upon recovery; periods between disease relapses characterized by a lack of disease progression” (Lublin and Reingold, 1996). This describes the initial disease course of 80% of patients diagnosed with MS. Relapsing-remitting MS is characterised by unpredictable episodes of severe worsening of neurologic functions followed by periods of months to years of remission with no new signs of disease activity (Compston and Coles 2008; Lublin and Reingold, 1996).

1.4.2 Primary Progressive Multiple Sclerosis

Primary progressive MS is defined as “disease progression from onset with occasional plateaus and temporary minor improvements allowed”. This kind of MS is characterised by a gradual, almost continuously worsening baseline accompanied by small fluctuations but no distinct relapses (Lublin and Reingold, 1996). Approximately 15-20% of patients develop the primary progressive form of MS (Disanto *et al.* 2011; Noseworthy *et al.* 2000).

1.4.3 Secondary Progressive Multiple Sclerosis

Secondary progressive MS is defined as an “initial relapsing-remitting disease course followed by progression with or without occasional relapses, minor remissions, and plateaus”. SP-MS describes around 70-80% of initial RR-MS and may also be seen as a long term outcome of the latter. This is because most SP-MS patients originally begin with a RR-MS diagnosis, but as the baseline between relapses begins to progressively worsen the patient is switched to a SP-MS diagnosis (Compston and Coles 2008; Lublin and Reingold, 1996; Noseworthy *et al.* 2000).

1.4.4 Progressive Relapsing Multiple Sclerosis

Progressive relapsing MS is described as a “progressive disease from onset, with clear acute relapses, with or without full recovery; periods between relapses characterised by continuing progression” (Lublin and Reingold, 1996). It is a rare form of MS, affecting fewer than 5% of patients (Hauser and Goodwin, 2008).

1.4.5 Benign Multiple Sclerosis

Benign MS was described as a “disease in which the patient remains fully functional in all neurological systems 15 years after disease onset” (Lublin and Reingold, 1996). Pittock *et al.* found in 2004 that patients with benign MS with an EDSS score of ≤ 2 for 10 years or longer had more than 90% chance of remaining stable (Pittock *et al.* 2004). Consensus on the definition of benign MS has varied with most considering it to be an EDSS score of ≤ 3 10 years after disease onset while others suggest an EDSS ≤ 2 after 10 years (Pittock *et al.* 2004; Thompson 1999).

1.4.6 Malignant Multiple Sclerosis

Lublin *et al.* (1996) defined malignant MS as a “disease with a rapid progressive course, leading to significant disability in multiple neurologic systems or death in a relatively short time after disease onset” (Lublin and Reingold, 1996).

1.5 Diagnosis

There are no clinical findings that are unique to MS, but some are highly characteristic. The differential diagnosis of MS is not straightforward and several conditions such as autoimmune diseases, cerebrovascular diseases, vitamin B12 deficiency and infections can mimic the white matter changes and clinical features of MS (Alexander *et al.* 1986; Böttcher *et al.* 2013; Brinar and Habek, 2010; Calabresi, 2004; Reynolds, 2006).

The McDonald criteria were first developed in 2001 and revised in 2005 and again in 2010. These diagnostic criteria for MS include clinical and paraclinical laboratory assessments, with the core requirement of diagnosis being the objective demonstration of dissemination of the central nervous system (CNS) lesions in both time and space. This can be done based on either a combination of clinical and MRI findings or clinical findings alone (Polman *et al.* 2011).

The patients in the present study were diagnosed using the 2005 revised McDonald criteria. The panel stated that “the 2010 revisions to the McDonald criteria will in some instances allow a more rapid diagnosis of MS, with equivalent or improved specificity and/or sensitivity compared with past criteria and will in many instances clarify and simplify the diagnostic process with fewer required MRI [magnetic resonance imaging] examinations”.

One of the most significant changes made to the new criteria will allow some patients to be diagnosed when they present with symptoms for the first time, unlike previously when patients had to wait for a new disease to develop. Table 1 summarises the McDonald criteria revised in 2010 for the diagnosis of MS.

Table 1.1: The 2010 McDonald Criteria for Diagnosis of MS	
Clinical Presentation	Additional Data Needed for MS Diagnosis
≥2 attacks; objective clinical evidence of ≥2 lesions of objective clinical evidence of 1 lesion with reasonable historical evidence of prior attack	None
≥2 attacks; objective clinical evidence of 1 lesion	Dissemination in space (DIS), demonstrated by: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, of spinal cord); or Await a further clinical attack implicating a different CNS site
1 attack; objective clinical evidence of 1 lesion	Dissemination in time (DIT), demonstrated by: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack
1 attack; objective clinical evidence of 1 lesion (clinically isolated syndrome)	Dissemination in space and time, demonstrated by: For DIS: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, of spinal cord); or Await a further clinical attack implicating a different CNS site; and For DIT: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack
Insidious neurological progression suggestive of MS (PPMS)	1 year of disease progression (retrospectively or prospectively determined) plus 2 or 3 of the following criteria: 1. Evidence for DIS in the brain based on ≥1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions 2. Evidence of DIS in the spinal cord based on ≥2 T2 lesions in the cord 3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)

(Polman *et al.* 2011)

1.6 Possible Causes of Multiple Sclerosis

MS is thought to be an immune-mediated disorder that occurs in genetically predisposed people, however the sequence of events that initiate the disease remains largely unknown (Noseworthy *et al.* 2000). Studies performed in the South African population highlighted the possible significance of folate and iron deficiency in a subgroup of patients with MS (Van Rensburg *et al.* 2006; Van Rensburg *et al.* 2012).

1.6.1 MS and Autoimmunity

For many years it has been believed that MS is an autoimmune disease, although this continues to be a subject of debate in the scientific community. Autoimmunity means that the immune system is reacting against normally-occurring antigens in the body, as if these antigens were foreign. Antigens are generally proteins that stimulate an immune response and the exact cause of the immune response in MS is currently still unknown (Diaz-Villoslada *et al.* 1999; Sprent and Kishimoto, 2001).

The most popular autoimmune theory is that abnormal T-helper cells are formed that are auto reactive against and target a component of myelin in the central nervous system. These T-cells that became sensitized to myelin cross the blood-brain barrier (BBB) with the help of adhesion molecules such as very late antigen 4 (VLA-4). B-Cell activation and pro-inflammatory cytokine production take place once the CNS is stimulated by antigens that mimic myelin proteins presented by local antigen presenting cells (APCs) (Hogquish *et al.* 2005). The pro-inflammatory cytokines tumor necrosis factor-alpha (TNF- α) and interferon-gamma (IFN- γ) are thought to be key contributors to neuroinflammation. This process, along with activation of macrophages, cytotoxic T-cells and B-cells, is hypothesised to result in inflammation and damage to oligodendrocytes, myelin and the axons causing demyelination (Zamvil and Steinman, 2003).

Currently the autoimmune model is considered controversial (Rodriguez 2009; Wootla *et al.* 2012). This model was derived from an animal model, experimental allergic encephalomyelitis (EAE) presenting brain inflammation, which according to Sriram and Steiner (2005) is not an ideal model for human MS in all respects.

1.6.2 Infections and Viral Factors Concerning MS

Evidence exists to suggest a temporal relationship between environmental infectious triggers and the onset of neurological symptoms. Some of these proposed infectious agents in humans implicated in MS include Epstein-Barr virus (EBV), Herpes Simplex Virus type 1 (HSV-1), varicella zoster virus (VZV), human herpes virus 6 (HHV-6), Chlamydia pneumonia and endogenous retroviruses such as the human endogenous retrovirus (HERV). These viruses can make intrusions through the blood-brain barrier but perivascular brain macrophages normally intercept them, in which they cannot replicate and generate abortive infection. (Christensen 2007; Perron and Lang, 2010; Sotelo *et al.* 2008; Wootla *et al.* 2012).

Peron and Lang (2009) described a previously unknown member of the HERV group, endogenous retrovirus type W (HERV-W), shown to trigger potent activation of innate immunity that leads to the release of pro-inflammatory cytokines, through Toll-like receptor (TLR-4) initial antagonistic effect. This reaction appears to be the most upstream effect on the immune system activation (Peron and Lang, 2009).

1.6.3 Oligodendrocyte Apoptosis

Another hypothesis for the aetiology of MS is the apoptotic (controlled) cell death of oligodendrocytes, the myelin producing cells, leading to extensive demyelination (Barnet and Prineas 2004). In early lesions demyelination is initiated by microglia, the resident immune cells in the brain, and not by peripheral immune cells. During apoptosis, phosphatidylserine is externalised on cell membranes of oligodendrocytes and acts as an “eat me” signal to phagocytes such as microglia (Barnett *et al.* 2006). The demised oligodendrocytes and the resulting dysfunctional myelin from the axons are stripped away by the activated microglia. Subsequently, peripheral immune cells such as macrophages penetrate the BBB as scavengers, amplifying the inflammatory response in the brain (Barnet and Prineas 2004; Barnett *et al.* 2006). According to this hypothesis, the immune system is thus not the primary cause of the disease but acts to remove dead or damaged tissue.

Adult stem cells, resident in the brain and acting as oligodendrocyte precursor cells (OPCs), were observed in the immediate vicinity of the apoptotic process and will under the right circumstances mature into new oligodendrocytes and remyelinate the axons (Barnet and Prineas 2004). This remyelination of axons by OPCs would induce restoration of function and subsequently remission, whilst lack of survival of

these OPCs would lead to continued myelin loss leading to neural injury and clinical disability (Van Rensburg *et al.* 2012).

Oligodendrocyte apoptosis may be caused by several factors including infective agents, inflammatory mediators such as cytokines (Cammer 2002), deficiencies of essential nutrients (Van Rensburg *et al.* 2006), poisons or toxins e.g. components of cigarette smoke (Healy *et al.* 2009), mitochondrial failure (Ly *et al.* 2003), oxygen radicals (Kim and Kim 1991), oxidative stress and depletion of antioxidants leading to the release of ceramide (Jana and Pahan 2007) and iron depletion or overload (Fassl *et al.* 2003).

1.7 Therapy and Management

Although there is no known cure for MS, several therapies may improve the quality of life. The use of disease-modifying agents (DMAs) has drastically increased throughout the world while controversy still exists regarding how early in the disease DMAs should be used and whether all patients should be treated.

As of April 2013, eight DMAs have been approved by regulatory agencies of different countries. 1) Interferon beta-1a (Avonex, Rebif, CinnoVex, ReciGen) injected once or three times per week; 2) Interferon beta-1b (Betaseron) injected every second day; 3) Glatiramer acetate (Copaxone) injected daily; 4) Mitoxantrone (Novantrone) intravenous infusion every three months; 5) Natalizumab (Tysabri) intravenous infusion at monthly intervals; 6) Fingolimod (Gilenya) daily single oral dose; 7) Teriflunomide (Aubagio) daily single oral dose; and 8) Dimethyl fumarate (BG12, Tecfidera) twice daily oral dose. Methylprednisolone is used for management of acute attacks (Fox *et al.* 2012; Gold *et al.* 2012).

DMAs have shown to have modest benefits in terms of short-term disability, however, the long term benefits remain unproven and no benefit has been found in primary progressive MS (Pittock and Rodriguez 2008). The positive outcome of DMAs includes a reduced number of relapses, reduced number of brain lesions and reduced disability progression. The treatments are very expensive, have numerous adverse effects and do not attenuate or reverse disability progression (Noseworthy *et al.* 2005). According to Noseworthy and co-workers (2005) the long term benefits of DMAs remain unclear, with no benefit found in treating primary progressive MS and the benefit of treating secondary progressive MS remains uncertain.

A major limitation with IFN β is that 30 to 50% of MS patients do not respond to IFN β therapy (Axtell *et al.* 2010). It is not clear why these facts differ from clinical data gathered from clinical trials. Filippini *et al.* (2003) found that most trials had major weaknesses with the most common flaw being high dropout rates after randomisation, combined with failure to do an intention-to-treat analysis (Filippini *et al.* 2003).

1.8 Epigenetic mechanisms in Multiple Sclerosis

MS is a multi-factorial disease with environmental, genetic and lifestyle components playing important roles. Susceptibility to MS is thought to be determined by both environmental and genetic factors, and progress has been made in identifying some of these genetic associations, as well as their possible interactions with the environment.

An explanation why some genetically susceptible people stay healthy whereas others develop MS might be found in epigenetics (Poser, 2004). Epigenetics refer to mechanisms underlying changes in gene expression of cellular phenotype due to environmental influences, gene dosing control, parent of origin effects, X-chromosome inactivating and imprinting. On a molecular level epigenetics involves post-translational modification of histones, DNA methylation, modifications of DNA base pairs, and the effects of non-coding RNAs. The differentiation of progenitors into myelin-forming cells is an example of epigenetic regulation of gene expression. Changes in transcription that lead to myelination are characterized by activating epigenetic marks being present at myelin genes (Liu *et al.* 2010), repression marks being present at the transcriptional inhibitors of myelin genes (He *et al.* 2007; Shen *et al.* 2008) and fine tuning of transcription by specific microRNAs (Douas *et al.* 2010; Lau *et al.* 2008; Zhao *et al.* 2010).

DNA methylation is one of the best characterized examples of epigenetic modification and refers to the process of adding methyl groups to cytosines by DNA methyltransferases (DNMTs) (Bestor, 2000). These methylated cytosines occurring near the transcriptional start site interfere with transcription factors recognizing the start sequence leading to stable transcriptional repression (Takizawa *et al.* 2001; Watt and Molloy, 1988). It has been shown through genome-wide sequencing that approximately 70% of annotated gene promoters are associated with CpG islands, making these areas the most common promotor type in the vertebrate genome (Saxonov *et al.* 2006).

1.8.1 Genetic Factors and MS

The potential role of genetics in the etiology of MS has been the focus of numerous studies. Attempts to distinguish the exact genes involved in MS susceptibility have not been successful. Genetic susceptibility is shown through studies indicating that 20% of people with MS have a familial history of the disease. It was also found that the risk of inheriting the disease in closely related relatives, which includes first, second and third degree relatives, is about 5% (Comston and Coles, 2005). Through twin studies genetic susceptibility is also indicated with a concordance rate of about 30% and an index of heritability of 0.25-0.76 in monozygotic (identical) twins. The genetic effect is proven to be higher in monozygotic twins than dizygotic (fraternal) twins (Hawkes and Macgregor, 2009).

Most of the studies done regarding inheritance point to genetic susceptibility rather than Mendelian inheritance, but the specific genes involved remain elusive. There are many genes that seem to be associated with MS. These include, but are not limited to, HLA classes I and II, T-cell receptor β , *CTLA4*, *ICAM1*, and *SH2D2A*. HLA allele DRB1*1501 was found to contribute the highest genetic risk of MS development in patients of Northern European ancestry (Ramagopalan *et al.* 2009). However, genetics alone does not explain the complex disease susceptibility of this heterogeneous disease.

The observation that some races such as Caucasians from Scotland and Scandinavia are more susceptible to the disease also supports a genetic component in the etiology of MS. MS was found to be rare in Chinese, Japanese, American Indians, Mongolians (Rosati 2001) and Eskimos (Chan 1977). It is also found less frequently in Aboriginal (Miller *et al.* 1990), African blacks (Morario and Linden 1980), Gypsies (Rosati 2001) and Norwegian Lapps (Gronlie *et al.* 2000).

1.8.2 Environmental Factors and MS

Similar to other multi-factorial diseases, there is increasing evidence to support the notion that different combinations of lifestyle and environmental risk factors could cause MS to develop in genetically susceptible individuals. Any environmental factor is likely to be ubiquitous and act on a population-basis rather than within the family microenvironment.

Incidence of MS was shown to be related to geographical latitude and generally increases with the distance from the equator, suggesting that regions further away

from the equator (both south and north), were at higher risk to develop MS. North America, Australia and Northern Europe have a similar prevalence in the general population of about >100-200 cases per 100,000 people while incidence is much lower in the South Americas, Asia, and Arabian Peninsula (<5 cases per 100,000 people). (Comston *et al.* 2006; Herna *et al.* 1999; Kurtzke 2005; Rosati 2001). Figure 1 shows the geography of MS and prevalence per 100,000 population. In South Africa the risk is 5 - 30 per 100,000 (Modi *et al.* 2008).

Interestingly, the risk of developing MS after immigrating to a lower risk area remains unchanged if the person is older than 15 years of age. Immigrating before puberty will result in the individual adopting the same risk of developing the disease of the population of the country of destination (Alter *et al.* 1971; Gale and Martin, 1995; Hammond *et al.* 2000; Kurtzke, 1985).

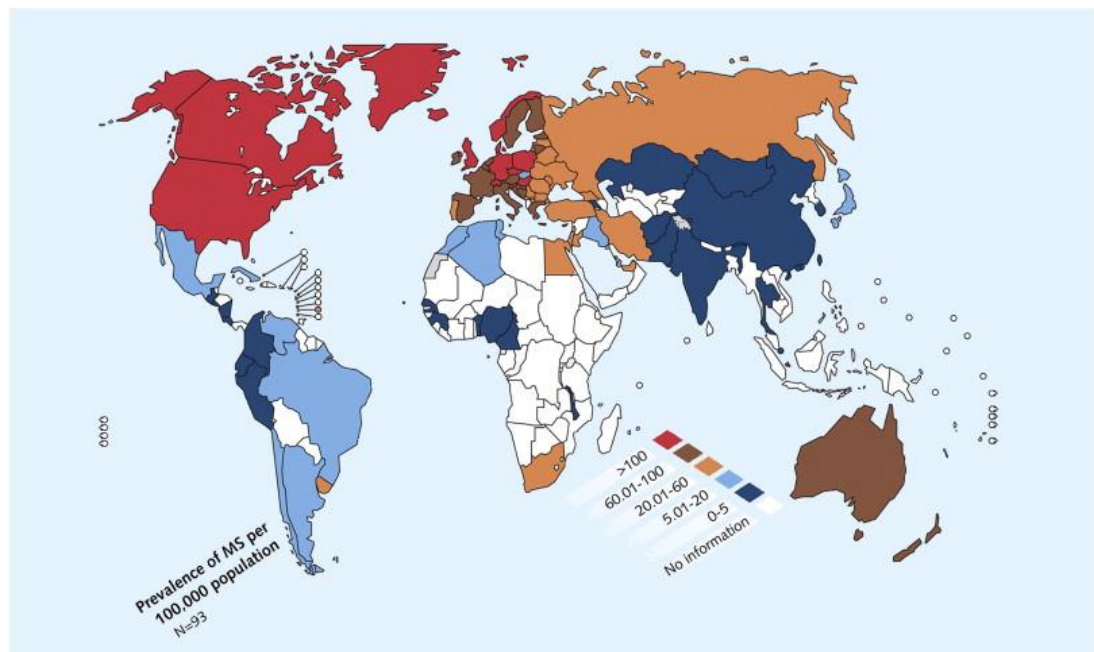


Figure 1.1: The geography of multiple sclerosis: prevalence per 100,000 population. (From Atlas multiple sclerosis resources in the world 2008, p15)

1.8.3 Infectious agents

Amongst the long list of proposed pathogens, including various herpes viruses, human endogenous retrovirus, Chlamydia, varicella zoster virus, Torque teno virus and other bacterial agents, the Epstein-Barr virus (EBV) has gained the most notable interest amongst scientists. The agent is widely spread and in the industrialised world, about half of the population acquires EBV before 5 years of age. Another large percentage of the population contracts the virus later in adolescence and for about

three to five out of ten the virus would lead to symptomatic primary infection known as infectious mononucleosis (IM) (Luzuriaga and Sullivan, 2010). The risk for MS is associated with IM, which is characterised by glandular fever and considerable expansion of virus-specific T lymphocytes that decrease with the resolving of the infection (Luzuriaga and Sullivan, 2010). A recent meta-analysis showed that the combined relative risk for developing MS after IM was estimated at 2.17 (95% CI 1.97-2.39; $P < 10^{-54}$) (Handel *et al.* 2010). HLA-DRB1*15 carriers with a history of IM showed a sevenfold increase in combined relative risk of developing MS than normal individuals (Nielsen *et al.* 2009). This epidemiological evidence linking MS development with symptomatic EBV infection in genetically predisposed individuals makes the virus a major candidate for MS initiation.

1.8.4 Lifestyle Factors and MS

Vitamin D

Several biochemical and nutritional deficiencies have been linked to the development and progression of MS. Among the non-infectious environmental factors, vitamin D levels stand out in research studies as a possible factor contributing to MS pathogenesis. As a potent immunomodulator, vitamin D affects the number and activity of regulatory T cells (Pierrot-Deseiligny and Souberielle, 2010) as well as proinflammatory pathways (May *et al.* 2004; Lemire 1995). Epidemiological studies have shown that an increasing distance from the equator, inversely correlated with intensity and duration of sunlight, correlated with an increase in MS frequency (Acheson *et al.* 1960; Kurtzke *et al.* 1979; Miller *et al.* 1990; Simon *et al.* 2010; Vukusic *et al.* 2007). It was also noted that populations situated at high latitudes but having a vitamin D rich diet, showed a reduced MS prevalence against expectations (Goldberg 1974; Swank 1952; Westlund 1970). A longitudinal prospective, nested case-control study, including more than 7 million US military personnel, investigated serum samples and found that high serum levels of 25-hydroxyvitamin D [25(OH)D] were associated with a reduced incidence of MS (Munger *et al.* 2006). 25(OH)D₃ is the metabolite routinely used to evaluate the nutritional vitamin D status of an individual. A study by Smolders *et al.* (2008) found association of low serum vitamin D levels with both the degree of disability as measured by the EDSS and the relapse rate. The exact molecular mechanisms underlying the effect of vitamin D in MS is still elusive, but many studies showing correlation between low vitamin D and MS

advocate the beneficial role of supplement action as treatment and/or prophylactic agent.

Smoking

Cigarette smoking has been almost unanimously associated with increased susceptibility to MS in various studies as emphasized by a recent meta-analysis including more than 3000 cases and 450,000 controls (Handel *et al.* 2011). A Swedish study found a significant increased risk to develop MS among those who had, at some point in their life, smoked compared to individuals who had never smoked (OR 1.5 [95% CI 1.3-1.8]) (Henderström *et al.* 2009). Similar results were found in populations from Norway (OR 1.81 [95% CI 1.1-2.9]) (Riise *et al.* 2003) and the UK (OR 1.3 [95% CI 1.0-1.9]) (Hernan *et al.* 2005). Hedstrom *et al.* (2011) showed a significant interaction between smoking and two genetic risk factors for MS: the presence of HLA-DRB1*15 and the absence of HLA-A*02. This interaction was observed for non-smokers. Smoking individuals with both genetic risk factors had a 2.8-fold increased MS risk, and for those without the susceptibility alleles a 1.4-fold risk was observed. In a 2009 study Healy *et al.* (2009) found a positive association between smoking and EDSS scores in MS patients. Those who smoked had higher EDSS scores, an indication of disease severity.

A case-control study in Belgrade, consisting of 210 cases with clinically proven and/or laboratory-confirmed MS with age and sex matched controls, assessed the risk of developing MS associated with certain lifestyle factors. These risk factors included cigarette smoking and alcohol and coffee consumption. They found that smoking was significantly more frequent in the MS patients than controls (OR 1.6, $p=0.021$). A dose-response relationship between both the number of cigarettes smoked daily ($p=0.021$) and duration (years) of smoking ($p=0.027$) and the risk of MS was observed. Coffee consumption was found to be significantly more frequent in the MS group (OR 1.7, $p=0.047$), and a dose-relationship was also observed. The daily consumption of hard liquor was shown to be significantly associated with risk of MS (OR 6.7, $p=0.024$) (Pekmezovic *et al.* 2006). However, some studies from the Netherlands found no influence of cigarette smoking on MS (Koch *et al.* 2007; Jafari *et al.* 2009).

Vascular Comorbidities

Vascular risk factors and obesity have been implicated in disease development and progression in MS. Marrie *et al.* (2010) highlighted the connection between vascular risk factors and MS. The study addressed five vascular risk factor categories that included hypertension, hypercholesterolemia, diabetes, heart disease and peripheral vascular disease. They concluded that “vascular comorbidity was associated with greater disability progression in MS, whether present at diagnosis or later in the disease course”. Participants in the study who had reported one or more vascular comorbidities at the time of diagnosis had a more than 1.5-fold increase in risk of ambulatory disability. This translated into a substantial difference of up to 6 years in the time from diagnosis to needing a walking stick for ambulation. Vascular comorbidity occurring during the disease course was also found to be associated with an increase in the risk of disability progression, which increased by more than 200% in individuals with 2 comorbidities. Trials investigating the effects of disease modifying therapies in MS showed none to 42% reduction in probability of disability progression (The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group; Polman *et al.* 2006). The average delay of disease modifying drugs in disability progression translated into less than 12 months over a 2- to 3-year study, with less pronounced benefits on disability progression later in the disease (European Study Group on interferon beta-1b in secondary progressive MS; PRISMS study group). According to Marrie *et al.* (2010) “this suggests that comorbidity has a substantial, important effect on outcome, possibly greater than the effect (in the opposite direction) of disease modifying drugs used for MS”. As some vascular conditions can change through treatment, more aggressive treatment of these comorbidities could improve disability progression in MS.

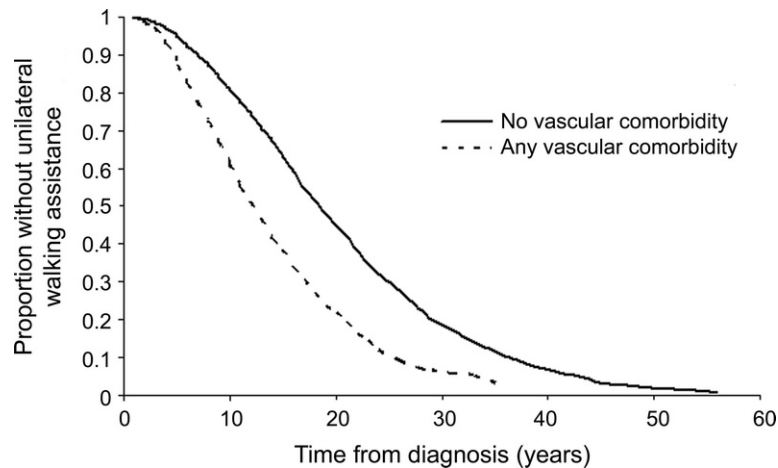


Figure 1.2: Time from diagnosis of MS to needed unilateral assistance to walk in North American Research Committee on Multiple Sclerosis participants with (n=572) and without (n=2,286) vascular comorbidity at diagnosis. Any vascular comorbidity = any of diabetes, hypertension, heart disease, hypercholesterolemia, and peripheral vascular disease (Used with permission Marrie *et al.* 2010).

Diet

Although no specific benefit from a particular diet has been proven, various clinical, epidemiological and experimental studies propose that nutritional factors may influence the course as well as the incidence of MS.

Through epidemiological studies it was shown that the risk of MS is low in countries with a high intake of polyunsaturated fat and high in countries with an increased intake of saturated fat (Lauer 1997; Swank and Dugan, 1990). Swank (1950) suggested a direct relationship of consumption of saturated fatty acid of animal origin to the frequency of MS and was supported by a study of the nutrition and incidence of MS patients in Norway (Swank *et al.* 1952).

Both omega-3 (n-3) and omega 6 (n-6) essential fatty acids (EFAs) are of structural importance to the CNS as components of the myelin sheath and brain tissue. Lipids make up 70% of the myelin sheath and a third of that is polyunsaturated with equal n-3 and n-6 (Crawford *et al.* 1979; Hunter and Laing 1996). Swank and Dugan (1990) reported less deterioration and a lower death rate, over a period of 34 years, in a group of MS patients taking a very low-fat diet of less than 20g per day, compared to MS patients with a higher fat intake of more than 20g per day. A meta-analysis of three controlled trials showed a beneficial effect of 17-23g of linoleic acid per day on the severity of relapses in patients with mild RRMS, but little effect in SPMS was found (Dworkin *et al.* 1984)

Vitamin B₁₂ and MS

Several studies have reported that people with MS present with a significantly higher rate of vitamin B₁₂ deficiency than in people without MS. A link between MS and vitamin B₁₂ deficiency has been suspected due to the fact that the illness is often accompanied by macrocytosis (Miller *et al.* 2005). Vitamin B₁₂ plays a fundamental role in the metabolism of fatty acids vital for maintaining myelin and thus helps maintain the myelin sheath. A person with vitamin B₁₂ deficiency shows damage to both the underlying axon and the myelin covering it. This may present as MS in severe cases of vitamin B₁₂ deficiency and even a slight deficiency may exhibit symptoms like depression and fatigue (Kocher *et al.* 2009; Zhu *et al.* 2011).

Defects in vitamin B₁₂ metabolism (whether due to low B₁₂ levels, oxidation of the cobalt atom or due to genetic variations) contribute to defective myelination as a result of inappropriate fatty acid synthesis, resulting in the incorporation of odd-chain and methyl branched fatty acids into myelin (Kishimoto *et al.* 1973; Ramsay *et al.* 1977).

Homocysteine Metabolism

Homocysteine is a non-protein-forming sulphur amino acid. Its metabolism is at the intersection of two metabolic pathways, namely remethylation and transsulfuration. In remethylation, homocysteine acquires a methyl group from N-5-methyltetrahydrofolate (5MTHF) or from betaine to form methionine. The reaction with 5MTHF occurs in all tissues and is vitamin B₁₂ dependent, while the reaction with betaine is confined mainly to the liver and is vitamin B₁₂ independent. A considerable proportion of methionine is activated by ATP to form S-adenosylmethionine (SAM), which serves as a universal methyl donor to a variety of acceptors. S-adenosylhomocysteine (SAH), the by-product of these methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine, which then becomes available to start a new cycle of methyl-group transfer. It is important to note that this hydrolysis is a reversible reaction that favours the synthesis of SAH, and that elevated cellular concentrations of this metabolite are likely to precede and accompany all forms of hyperhomocysteinemia (Selhub 1999).

In the transsulfuration pathway, homocysteine combines with serine to form cystathionine in an irreversible reaction catalyzed by the pyridoxal-5'-phosphate (PLP)-containing enzyme, cystathionine β -synthase. Cystathionine is hydrolyzed by a second PLP-containing enzyme, γ -cystathionine, to form cysteine and α -

ketobutarate. Excess cysteine is used for the synthesis of glutathione, an important antioxidant, or oxidized to taurine or inorganic sulphates or excreted in the urine. Thus, in addition to synthesis of cysteine, this transsulfuration pathway effectively catabolises excess homocysteine, which is not required for methyl transfer. The two pathways are coordinated by SAM, which acts as an allosteric inhibitor of the methylenetetrahydrofolate reductase (MTHFR) reaction and as an activator of cystathionine β -synthase (Selhub 1999).

Hyperhomocysteinemia is a medical condition characterized by an abnormally high level of homocysteine in the blood and usually arises from disrupted homocysteine metabolism. Plasma homocysteine levels increase with age (Triantafyllou *et al.* 2008) and are higher in men than in women (Zoccolela *et al.* 2011). High total homocysteine levels are associated with high plasma creatinine, impaired renal function, coffee consumption, alcoholism, smoking, and certain drugs, including folate antagonists, nitrous oxide, and L-DOPA (Matthews 2001; Selhub 1999). As a consequence of the biochemical reactions in which homocysteine is involved, deficiencies of the vitamins folic acid (B₉), pyridoxine (B₆), or cobalamin (B₁₂) can also lead to high homocysteine levels (Selhub 1999; Miller 1994). A positive correlation between increased levels of cholesterol and high plasma levels of homocysteine has been observed in patients with hyperhomocysteinemia (Olszowski *et al.* 1989; O *et al.* 1998). Conflicting results between BMI and homocysteine levels have been reported (Hultdin *et al.* 2005; Nakazato *et al.* 2011; Osganian *et al.* 1999).

Hyperhomocysteinemia is frequently reported due to genetic variation in genes which affect the breakdown of homocysteine. This may be due to reduced activity of one or more of the enzymes involved in folate metabolism, including the extensively studied methylenetetrahydrofolate reductase (*MTHFR*) as well as methionine synthase (*MTR*) and methionine synthase reductase (*MTRR*).

Elevated homocysteine concentrations have been found in cerebrospinal fluid and plasma of MS patients (Ramsaransing *et al.* 2006; Vrethem *et al.* 2003). Genetic variations in genes found in the single carbon transfer pathway have been reported to result in elevated intracellular plasma homocysteine leading to cerebrovascular and neurodegenerative diseases as well as CNS dysfunction (Ramsaransing *et al.* 2006).

1.9 Genetic Factors in the Methylation Metabolic Pathway and Associated Polymorphisms

A requirement for myelin synthesis and maintenance is the optimal functioning of the folate-vitamin B12-methyl transfer cycle constantly providing activated methyl groups. Long-term deficiencies of this pathway, such as low folate and cobalamin levels, cause demyelinating diseases of the spinal cord and brain (Lever *et al.* 1986; Selzer *et al.* 2003; Surtees 1998).

Single-carbon groups enter this pathway from the cytoplasmic pool and are bound by tetrahydrofolate (THF) to form N-5,10-methylenetetrahydrofolate (5,10-MTHF). MTHFR is a multi-functional enzyme encoded by the *MTHFR* gene and catalyzes the conversion of 5,10-MTHF, the major intracellular form of folate, to N-5-methyltetrahydrofolate (5-MTHF), the major circulatory form of folate and co-substrate for homocysteine remethylation to methionine. The enzyme contains noncovalently bound flavin adenine dinucleotide (FAD) as a cofactor. The flavin accepts reducing equivalents from NAD(P)H and transfers them to methylenetetrahydrofolate (Matthews 2001; Surtees 1998; Zhou *et al.* 2012).

Methionine synthase (MTR), also known as 5-methyltetrahydrofolate-homocysteine methyltransferase, is an enzyme encoded by the *MTR* gene and catalyses the final step in methionine biosynthesis. This methylcobalamin-dependent enzyme catalyses the remethylation of 5-MTHF and homocysteine to THF and methionine. Methionine is an essential amino acid required for protein synthesis and one-carbon metabolism. Due to the oxidation of its cob(I)alamin cofactor, methionine synthase eventually becomes inactive. The protein encoded by the *MTRR* gene, methionine synthase reductase or 5-methyltetrahydro-folate-homocysteine methyltransferase reductase, regenerates a functional methionine synthase via reductive methylation using SAM as methyl donor, thus maintaining methionine synthase in its active state. Methionine can also be synthesised in the liver via an alternate pathway, when betaine is hydrolysed to dimethylglycine by the enzyme betaine-homocysteine methyl transferase (BHMT) (ncbi.nlm.nih.gov; uniport.org; Leclerc *et al.* 1996; Leclerc *et al.* 1998; Van Der Veyver 2002; Wilson *et al.* 1999).

Methionine is further activated to SAM, which has an anti-inflammatory action, by methionine adenosyltransferase (MAT). SAM also plays a very important role in the synthesis of phospholipids and CNS myelination. Under normal conditions, most of the SAM generated per day is used in transmethylation reactions in which methyl

groups are added to compounds and SAM is converted to S-adenosylhomocysteine (SAH), a potent competitive inhibitor of transmethylation reactions. SAH is recycled to homocysteine by the enzyme s-adenosylhomocysteine hydrolase (SAHH). The SAM:SAH ratio (methylation ratio) is a key determinant of the rate of transmethylation, as it represents the balance between the methyl donor for methyltransferases and their key inhibitor (Lu 2000; Rosenblatt 1995; Surtees *et al.* 1991).

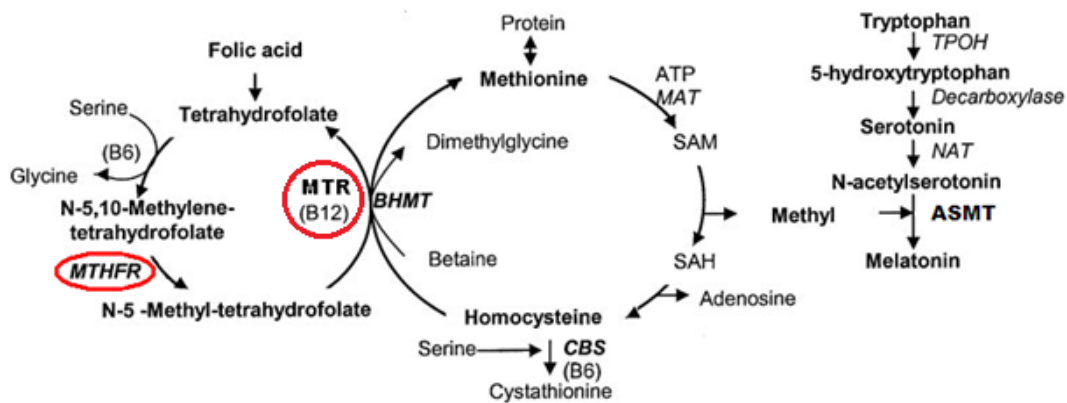


Figure 1.3: Methylation pathway. Homocysteine (below middle) is converted into methionine by two pathways and converted to SAM to act as universal methyl donor. MTHFR and MTR are highlighted in the Folic Acid pathway. (Modified with permission from Fournier *et al.* 2002)

The Methyl Folate Trap

The levels of SAM and SAH regulate THF formation, so that when SAH is high or SAM is low, MTHFR is activated and folate is directed from DNA synthesis to the remethylation pathway. Insufficient methionine synthase, or low vitamin B₁₂, limits the methionine supply needed to generate the methylation donor SAM. This will stimulate MTHFR to generate more 5MTHF in an attempt to recycle folate towards methionine synthesis. However with inadequate methylcobalamin (vitamin B₁₂) function, the 5MTHF accumulates while the intracellular folate pool available for methylation reactions decreases. The effect of vitamin B₁₂ deficiency on this pathway is thus a functionally deficient folate state with hyperhomocysteinemia with a low methionine level (Van der Veyver 2002).

1.9.1 Methylene tetrahydrofolate Reductase (MTHFR)

As MTHFR function is important in these pathways it makes the gene a good candidate for analysis of functional genetic variants. *MTHFR*, the gene that codes for the MTHFR enzyme, is located at position p36.3 on chromosome one (Goyette *et al.* 1994) and contains 11 exons (Frosst *et al.* 1995). There are many DNA sequence variants (genetic polymorphisms) associated with altered expression of MTHFR. Two of the most investigated single nucleotide polymorphisms (SNPs) are rs1801133 (also known as 677C>T or A222V) and rs1801131 (1298A>C or E429A).

The *MTHFR* C>T polymorphism in exon 4 of the *MTHFR* gene results in a nucleotide change at position 677 from cytosine to a thymine resulting in a thermolabile enzyme with reduced activity. This *MTHFR* C>T change has the effect of an amino acid substitution of alanine by valine at codon 222. Compared to the wild type (CC), homozygous C>T (TT) individuals only have ≈30% of the expected MTHFR enzyme activity and heterozygous C>T (CT) individuals ≈65% of normal enzyme activity (www.snpedia.com; Zhou *et al.* 2012).

The T-allele frequency of the C>T polymorphism is approximately 30% in Caucasians, while 10% of this population is homozygous for the TT genotype. The rare allele frequency of the C>T polymorphism in sub-Saharan Africans is 6% while the frequency in East Asians is very similar to that in European whites (Matthews 2001). In the South African population a significant difference was observed between different population groups, with the lowest allele frequency detected in the Black population (4%), intermediate in the Coloured population (18%) and highest in the Caucasian population (36%) (Scholtz *et al.* 2002).

MTHFR 677C>T was found to be associated with hyperhomocysteinemia. Plasma total homocysteine levels in heterozygous (CT) and homozygous (TT) knockout mice were found to be 1.6- and 10-fold higher respectively than those in wild type (CC) littermates. Both heterozygotes and homozygotes had decreased levels of SAM and elevated levels of SAH, and their DNA was hypomethylated (Miller *et al.* 1994).

Another *MTHFR* polymorphism in exon 7 of the *MTHFR* gene, 1298A>C (rs1801131), results in a substitution of glutamate by alanine at codon 429 and can lead to a decrease of 40% in enzyme activity. The result is an increase in 5,10-MTHF concentration during nucleic acid synthesis and cell proliferation in the course of inflammation (Linnebank *et al.* 2004). This causes the levels of SAM to decrease,

diminishing the regeneration of phospholipids and myelination, a pathophysiological effect implicated in demyelination (Surtees *et al.* 1991).

In a Swedish study the minor C-allele frequency was found to be 33% with 12% of the Caucasian control population being homozygous (CC) for the *MTHFR* 1298A<C (rs1801131) polymorphism and 46% wild type (AA) (Guelpen *et al.* 2006). Black or African American populations showed a lower minor allele frequency of about 14% with only 2% homozygous for the SNP (Guéant-Rodriguez *et al.* 2006; Hughes *et al.* 2006).

MTHFR 1298A>C (rs1801131) was first identified by Van der Put *et al.* (1998) in patients with neural tube defects with elevated homocysteine that could not be explained by the presence of the *MTHFR* 677C>T polymorphism. A German cross-sectional study of 138 patients with clinically defined MS found the *MTHFR* 1298A>C (rs1801131) polymorphism to influence the incidence rate of MS and neurodegeneration (Klotz *et al.* 2010).

MTHFR and Disease Association

Homocysteine and the vitamins involved in 1-carbon metabolism such as folate and vitamin B₁₂ have been associated with a variety of diseases including MS. Low-penetrance mutations in *MTHFR* (rs1801133 and rs1801131) that lead to reduced enzyme function are associated with elevated levels of homocysteine in human plasma (hyperhomocysteinemia). People with hyperhomocysteinemia are more likely to have strokes, Alzheimer's disease, dementia, kidney disease, diseases of the eye, erectile dysfunction and cardiovascular disease (De Bree *et al.* 2002; Matthews 2001). A study furthermore found that women with the minor *MTHFR* 677T allele have an increased risk of invasive breast cancer (Ericson *et al.* 2009). This allele also increases the risk of breast cancer in BRCA1 mutation carriers, and possibly also in patients with a genetic predisposition for ovarian cancer (Jakubowska *et al.* 2007; Pepe *et al.* 2007).

Folate metabolism appears to be associated with cancer risk due to its important role in DNA methylation. Notably, impaired function of various risk alleles of the vitamin-dependent *MTHFR* enzyme can be restored to normal functionality by elevating intracellular folate levels (Marini *et al.* 2008). Documentation of disease status, however, is important as folic acid, the synthetic form of folate, may enhance carcinogenesis. While supplementation was shown to be an effective chemopreventive agent in animal models if given prior to the establishment of early

lesions, tumour growth may be enhanced by folate once a preneoplastic lesion is present. The role of folate in tumour growth is exemplified by the use of folate antagonists (e.g. methotrexate, 5-fluorouracil) in cancer treatment, which target folate metabolism. Functional polymorphisms in genes encoding key enzymes involved in folate metabolism can predict treatment response to these chemotherapeutics. Severe toxicity may be experienced with a single dose of methotrexate (12.5 mg) in the presence of two copies of SNP 677C>T in the *MTHFR* gene (Przekop *et al.* 2007). It was noted that routine pre-treatment *MTHFR* gene testing could have prevented the adverse drug response in the patient, as either a reduced dose or alternative agent would have been indicated.

Polymorphisms in the *MTHFR* gene could also be one of the factors leading to increased risk of developing schizophrenia. Schizophrenic patients with the *MTHFR* 677 TT genotype show more deficiencies in executive function tasks (Roffman *et al.* 2007). This SNP has also been linked to autism, depression, neural tube defects, cleft lip and cleft palate in the developing foetus and migraine (Matthews 2001). It has become common practice to prescribe folate supplementation as part of a nutritional support program for pregnant women to prevent birth defects and to reduce the risk of thrombophilia and miscarriage. In a similar way, individuals with a lifelong increased requirement for folate and other B-vitamins above the RDA due to their genetic background may need to be encouraged to ensure adequate intake of these nutrients on a daily basis to reduce the disease burden and improve health and well-being at the population level.

1.9.2 Methionine Synthase (MTR)

Methionine synthase (MTR), which requires vitamin B₁₂ as a cofactor for the transfer of a methyl group from 5-methylenetetrahydrofolate (5MTHF) to homocysteine by the reduction of 5MTHF to tetrahydrofolate (THF), is the most important of the three enzymes known to be necessary for synthesis of methionine (Van Der Veyver 2002).

A common *MTR* variant, 2756A>G (rs1805087), contains a change from adenine to guanine at nucleotide position 2756 and converts into an aspartate to glycine at codon 919. The A-allele (wild type) frequency of the 2756A>G polymorphism was found to be 84% in a European population with the GG homozygous genotype making up 2 % of the population (ncbi.nlm.nih.gov; opensnp.org). Another study investigating *MTR* 2756A>G (rs180508) and head and neck cancer risk in 805

individuals found the genotype distribution to be 65% AA, 30.6% AG and 4.4% GG with the minor G allele frequency 23.4% (Galbiatti *et al.* 2010).

Some functional studies have indicated that the variant G allele is associated with higher folate concentrations (Chen *et al.* 2001), lower homocysteine levels (Chen *et al.* 2001; Dekou *et al.* 2001; Harmon *et al.* 1999; Tsai *et al.* 2000), decreased colorectal cancer risk for men (Ma *et al.* 1999) and decreased adenoma risk for women (Chen *et al.* 1998) as risk implications are dependant on environmental exposure (e.g. vitamin B₁₂ status). Other case control studies of the *MTR* 2756A>G (rs180508) polymorphism have suggested increased risk for colorectal (Le Marchad *et al.* 2002) and adenoma cancer (Pufulete *et al.* 2003), for which hyperhomocystenemia is a risk factor, with no reported sex differences.

The lack of data regarding the functional impact of *MTR* 2756A>G (rs180508) on protein function contributes to the difficulty in interpreting the observed associations. Some studies of homocysteine levels suggesting that this might be an activating polymorphism consisted of more than 75% men (Tsai *et al.* 2000) or only male participants (Chen *et al.* 2001; Dekou *et al.* 2001; Harmon *et al.* 1999). Studies including both male and female participants did not observe this association (D'Angelo *et al.* 2000; Jacques *et al.* 2003; Morita *et al.* 1999; van der Put *et al.* 1997). One study even reported an increase in homocysteine levels only among the male individuals with the G allele and no difference among the females (Wang *et al.* 1999).

The known influence of *MTR* 2756A>G (rs180508) on folate and homocysteine metabolism raises the possibility that it may have a protective effect against neurodegenerative, oncological and vascular diseases. Linnebank *et al.* (2005) investigated whether this polymorphism might have an epidemiological impact on disease-free longevity. They analysed 329 healthy individuals and found the prevalence of the mutant G-allele to be significantly higher in the older than the younger individuals ($p=0.005$). This finding appears to support the hypothesis that *MTR* rs1805087 is beneficial to disease free longevity. When male and female subjects were analysed separately, it was shown that the *MTR* genotype influence became relevant at a younger age in male subjects as opposed to their female counterparts, suggesting a gender dependent effect.

1.9.3 Methionine Synthase Reductase (MTRR)

Methionine synthase reductase (MTRR) maintains adequate levels of activated cobalamin, the enzyme bound cofactor for MTR, and consequently may be an important determinant of total homocysteine concentrations.

The most common SNP found in the *MTRR* gene is 66A>G (rs1801394) resulting in an adenine to guanine nucleotide change at position 66. This results in an isoleucine-to-methionine change at codon 22 and has an inversely associated relationship with plasma homocysteine. It was also found that the protein had a lower affinity for MTR resulting in a decrease in its function to maintain methionine synthase in its active state (Gaughan *et al.* 2001; Wilson *et al.* 1999). In a European population the A-allele frequency was found to be 47% with the AA genotype (wild type) present in only 23% of the population (ncbi.nlm.nih.gov; opensnp.org).

Gaughan *et al.* (2006) found that the *MTRR* rs1801394 polymorphism is a risk factor for hyperhomocysteinemia by having a modest but significant effect on total homocysteine concentration in males aged 30-46. The homozygous GG genotype was found to be a determinant of elevated total homocysteine, with a relative risk of 1.59 (95% CI 1.10-2.25) of being in the top half of total homocysteine distribution ($p=0.03$). The homozygous genotype was associated with a 0.7 μ mol/L higher total homocysteine concentration than compared with the wild type genotype.

1.9.4 Fat Mass and Obesity Associated Polymorphisms

The fat mass and obesity associated protein (FTO), first discovered by Peters *et al.* (1999) is an enzyme that affects development of human obesity and energy homeostasis. Also known as alpha-ketoglutarate-dependant dioxygenase, the protein is encoded by the *FTO* gene located on chromosome 16q12.2. It is widely expressed but especially in the hypothalamus, the most critical component to appetite control in the brain.

The T to A base change (rs9939609) in the first intron of the *FTO* gene causes an increase in gene transcription (Frayling *et al.* 2007). The frequency of the *FTO* rs9939609 risk-associated minor A-allele in East-Asians were shown to be between 12-20% and in South-Asians 30-33% (Li *et al.* 2012). In Western and Central Europeans (46%) and West Africans (51%) this allele occurred at a higher frequency in comparison with Chinese individuals (16%) (Ho *et al.* 2010). In white Europeans approximately 18% carry both risk alleles with 66% carrying at least one A-allele.

Despite the genetic background being different, the effect of FTO on obesity related traits in Caucasian Europeans were similar to South- and East Asians.

The biochemical function of FTO has not been fully elucidated, forming part of a complex process involving methylation and demethylation of nucleic acids. The site of action includes regions of the brain stem and hypothalamus related to feeding behaviour and satiety (Fredriksson *et al.* 2008). FTO forms part of the AlkB family of enzymes that depend on alpha-ketoglutarate, oxygen and divalent iron (Fe²⁺) as cofactors (Niu *et al.* 2013). FTO was the first RNA demethylase discovered, but more of these factors have been found since (Sibbritt *et al.* 2013, Zheng *et al.* 2013).

On a behaviour level, a recent study showed that subjects homozygous for the FTO AA risk allele had dysregulated circulating levels of acyl-ghrelin, which led to attenuated postprandial appetite reduction. Peripheral blood cells from these subjects exhibited reduced ghrelin mRNA N6-methyladenosine methylation (Karra *et al.* 2013). Acyl-ghrelin impacts brain regions that regulate appetite, reward processing, and incentive motivation. Data from the second National Health and Nutrition Examination Study (NHANES II) showed that serum homocysteine concentration was positively related ($p=0.02$) to the intake of energy-dense foods such as visible fats, nutritive sweeteners and sweetened beverages, desserts and snacks (Kant 2000).

Frayling *et al.* (2007) established the effect of FTO on BMI through genome-wide association studies. It was found that the *FTO* gene was associated with normal variation in BMI, and was the first strong identification of a common polymorphism with BMI and obesity. The effect of FTO on BMI was found not to be present at birth, fully present by age 7, and was thought to be stable throughout adulthood. The effect on BMI was also found to be driven through adiposity and not skeletal or lean tissue mass. Elks *et al.* (2010) showed that the minor 'fat' A-allele was associated with an earlier sexual maturation in girls, an average of 0.5 months earlier at menarche. This can be explained through the well-known correlation between earlier age at puberty and increased adiposity.

The rs9939609 variant was shown to be associated with an intake of dietary macronutrients in Aboriginal and Europeans and in a multi-ethnic cohort there were positive associations between the risk A-allele and greater fat mass ($0.94\pm 0.56\text{kg}$, $p=0.045$), relative greater subcutaneous abdominal adipose tissue ($4.9\pm 2.8\%$, $p=0.039$), per cent body fat ($0.7\pm 0.4\%$, $p=0.031$) and per cent daily calories from fat ($0.4\pm 0.2\%$, $p=0.064$). The authors suggested that dietary intake in adults may be

associated with the A-allele of the *FTO* rs9939609 variant (Lear *et al.* 2013). A Brazilian study found that the effect of the *FTO* polymorphism on glucose metabolism and inflammatory status could be counteracted with a lifestyle intervention of lower energy intake and higher physical activity compared to controls (Curti *et al.* 2012).

The association between variation in the *FTO* gene and risk of obesity, shown to increase approximately 1.3 fold with each additional risk allele, has been confirmed across ethnic groups (Frayling *et al.* 2007; Adeyemo *et al.* 2010; Liu *et al.* 2010). A recent study by Almén *et al.* (2012) aimed at elucidation of the mechanism behind this association, showed a significant differential methylation level in carriers of the *FTO* rs9939609 risk-associated A-allele for at least six genes. In addition, 20 differentially methylated sites associated with obesity were identified, which suggests that the effect of the *FTO* rs9939609 polymorphism is mediated through epigenetic changes. In addition, *FTO* is the first RNA demethylase identified (Jia *et al.* 2011), specifically demethylating the nucleoside N6-methyladenosine in RNA. Of special interest is a recent study investigating the effect the *FTO* rs9939609 A-allele on weight gain and type 2 diabetes, showing that the adverse effects of *FTO* may be modulated by adherence to the Mediterranean diet, while increased folate intake had significant interaction effects on fasting plasma glucose levels in control subjects (Ortega-Azorín *et al.* (2012).

A recent study linked childhood obesity and risk of paediatric MS and clinically isolated syndrome (CIS) (Langer-Gould *et al.* 2013). Obesity was significantly associated with an increased risk of CIS/MS in girls ($p=0.005$) but not in boys ($p=0.93$). Moderate and extremely obese cases were also more likely to present with transverse myelitis compared to overweight or normal children ($p=0.003$). Another study in a Swedish population found that subjects (both men and woman) with a BMI exceeding 27 kg/m^2 at age 20 had a two-fold increased risk for developing MS compared to normal weight individuals (BMI between 18.8 and 21 kg/m^2) (Hedström *et al.* 2012). Conflicting results have been reported in relation to the link between BMI and homocysteine levels, not previously studied in MS patients in relation to genetic risk factors.

Methylation and demethylation reactions are ubiquitous in the body, involving DNA, RNA, proteins and lipids, and are of great significance for MS. The *FTO* gene is of particular interest due to its biological function involving DNA repair, posttranslational modifications, fatty acid metabolism and nucleic acid demethylation (Gerken *et al.*

2007). Apart from the role of methylation in the synthesis of nucleic acids, methylation is indispensable for myelin synthesis and maintenance, since myelin basic protein needs to be methylated (Kim *et al.* 1997), and phosphatidylcholine, a constituent of myelin, is synthesised from phosphatidylethanolamine via 3 methylation reactions (Voet and Voet 2004). In addition, the synthesis of sphingomyelin, an important myelin lipid, involves the transfer of phosphocholine to ceramide (Voet and Voet 2004).

1.9.5 Catechol-O-Methyl Transferase (COMT)

Catechol-O-methyl transferase (COMT) is one of several catabolic enzymes involved in the degradation of numerous bioactive molecules and is a key postsynaptic neuronal enzyme in the catabolic pathway of noradrenaline, a ubiquitous neurotransmitter of the CNS. Total homocysteine levels might be affected by COMT because during the metabolism of dopamine and noradrenaline, it converts SAM to SAH, which is reversibly converted to homocysteine.

The gene encoding COMT is genetically localized to chromosome 22q11.2 and transcribes two distinct isoforms of COMT, a membrane bound (MB-) and soluble (S-) form. MB-COMT predominates in the brain whilst S-COMT predominates in the periphery (Mannisto and Kaakkalo 1999; Tenhunen *et al.* 1994; Turnbridge *et al.* 2007). Exon 4 of the *COMT* gene contains a relatively common G to A polymorphism (rs4680, G472A, Val158Met). This base pair substitution results in a valine to methionine (Val/Met) amino acid change at codon 158 of MB-COMT that affects its thermolability, altering enzyme activity and protein abundance. The effect in enzyme activity is a three- to four-fold decrease of the MB-COMT enzyme activity leading to a reduction of noradrenaline and dopamine catabolism within synapses (Chen *et al.* 2004; Lachman *et al.* 1996; Lotta *et al.* 1995; Shield *et al.* 2004). The G- and A-alleles thus produce high and low activity COMT enzyme forms respectively. The G-allele frequency is approximately 49% in Caucasians, while 24% of this population is homozygous for the GG (high activity) genotype with 27% homozygous for the AA (low activity) genotype (Martinez *et al.* 2009).

Turnbridge *et al.* (2008) showed that the common Val158Met polymorphism influences total homocysteine levels. The high activity enzyme (GG genotype) carriers had approximately 1 $\mu\text{M/L}$ higher total homocysteine levels than the slow enzyme (AA genotype) individuals. They further showed an interaction with the *MTHFR* C677T SNP in that TT homozygous mutant *MTHFR* and COMT high activity

enzyme individuals had the highest total homocysteine levels, approximately 3 $\mu\text{M/L}$ greater than the other groups (Tunbridge *et al.* 2008).

The lowering of enzyme activity and the resulting reduction of noradrenaline catabolism within synapses is postulated to decrease inflammation occurring in neuroinflammatory diseases. Studies done on rodent and mouse astrocytes found that noradrenaline induced a decreased expression of MHC class II molecules (Frohman *et al.* 1988; Frohman *et al.* 1998). Noradrenaline may confer a natural immunosuppressive protection within the normal healthy brain environment by reducing the role of antigen-presenting cells (APCs), as brains from healthy individuals demonstrate a lower threshold of inflammation compared to other organs (Lassmann *et al.* 1991). The high activity COMT enzyme could result in a decreased amount of noradrenaline and thus lead to CNS inflammation in MS patients. Supporting this possibility a recent case-control study involving 108 Caucasian MS patients found that two bi-allelic polymorphisms within the promoter of phenylethanolamine N-methyltransferase (PNMT), a final enzyme involved in the biosynthesis of noradrenaline, were associated with MS (Mann *et al.* 2002).

1.10 Aim of the Study

The long-term objective of the MS research program is to improve the clinical management of MS patients based on a scientific understanding of the role of inflammation, oxidative stress, iron dysregulation and the methylation metabolic pathway in the disease process. This study focused on the development of a laboratory testing strategy 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 overall objective was to elucidate the mechanism underlying raised homocysteine levels in MS patients.

The specific aims of the study were as follows:

- Analytical validation of high-throughput real-time polymerase chain reaction (RT-PCR) assays for six selected SNPs in the *FTO* (rs9939609, Intron 1 T>A), *MTR* (rs1805087, 2756 A>G), *MTRR* (rs1801394, 66 A>G), *MTHFR* (rs1801133, 677 C>T and rs1801131, 1298 A>C) and *COMT* (rs4680, 472 G>A) genes, against direct sequencing as the gold standard.
- Comparison of genotype distribution and allele frequencies between MS patients and controls.
- Determination of the effects of clinical characteristics and genetic risk factors on homocysteine levels in MS patients and controls.
- Determination of the potential impact of genetic and environmental risk factors on disability assessed by EDSS in MS patients.

Chapter 2

Subjects and Methods

Ethical approval for this study was obtained from the Ethics Review Committee of Stellenbosch University under project number N07/09/203 (Appendix A). Written informed consent for participation in the study was obtained from all study participants.

2.1 Study Population

The study population consisted of 114 unrelated Caucasian MS patients: 98 females and 16 males. DNA samples of 48 patients were available from a previous study performed by Kotze *et al.* (2001), for whom age of onset of MS symptoms/diagnosis was documented at the time but none of the other (biochemical and environmental) variables were assessed. A total of 195 unrelated Caucasian control individuals without neurological disease (128 females and 67 males) were selected for this study from a secure online genetic database (<https://www.gknowmix.org>). The clinical characteristics of the study population are summarised in table 2.1.

2.2 Study questionnaire

A subset of 43 MS patients and 186 control individuals completed the Gknowmix™ medical history and lifestyle questionnaire available at <https://www.gknowmix.com>. Information on family history, own medical history, clinical symptoms (including age of onset/diagnosis of MS), medication use and lifestyle risk factors were recorded. The latter included smoking status (available for 56 patients), alcohol consumption and body mass index (BMI), representing well-established environmental risk factors for MS. The number of days per week of eating certain foods over the previous three months were recorded and scores were calculated for (i) saturated/trans fat, (ii) fruit, vegetables and fibre, as well as (iii) folate intake. Physical activity was documented for both day-time / occupational activity and participation in recreational sport (occasionally or at least 1 time per week), or structured exercise on a weekly basis (none, 2-3 times or 4 or more times) with each activity lasting more than 30 minutes. A physical activity score was calculated from these two assessments.

EDSS scoring was performed by a neurologist or a trained medical doctor in 51 patients to assess disability and quality of life at entry into the research program as well as during follow-up visits as previously described (van Rensburg *et al.* 2006). For the purpose of this study, only baseline EDSS values were evaluated in relation to diet and lifestyle factors, biochemical parameters and genotype in MS patients. The EDSS ranges from 0 to 10 and the individual scores are interpreted as summarised in table 2.2 (Kurtzke, 1983).

Table 2.1 Description of clinical characteristics in the Caucasian control and patient groups stratified by disease and gender according to available data sets.

	Controls (n=195)		MS Patients (n=114)	
	Female	Male	Female	Male
	128	67	98	16
Median (IQR)				
Age, years	51 (39-57)	49 (38-56)	45 (37-53)	42 (40-48)
Age of Symptoms, years			32 (24-40)	36 (30-44)
Age of Diagnosis, years			37 (27-45)	38 (33-44)
Body Mass Index (kg/m ²)	26 (23-31)	27 (24-30)	25 (22-28)	24 (22-25)
Cholesterol, total (mmol/L)	5.4 (4.8-6.6)	5.3 (4.3-5.9)	5.4 (5.0-6.0)	5.1 (4.7-6.5)
C-reactive Protein (mg/L)	4.0 (4.0-4.0)	4.0 (4.0-4.0)	4.0 (4.0-5.4)	4.0 (4.0-4.0)
Homocysteine (µmol/L)	7.9 (6.7-10.8)	9.9 (8.5-11.4)	8.6 (7.1-10.0)	11.4 (11.3-14.8)
Serum Folate (µg/L)	16 (13-18)	14 (7-22)	19 (14-24)	15 (9-17)
Vitamin B12 (ng/L)	360 (315-439)	494 (439-510)	620 (446-850)	489 (417-755)
Fat Score, saturated	14.0 (9.0-20.0)	16.0 (10.5-21.0)	15.0 (10.0-19.8)	11.0 (9.0-13.0)
Folate Score	8.0 (5.0-10.5)	8.0 (4.0-11.0)	6.0 (4.0-9.0)	6.0 (2.0-6.0)
Fruit/veg/fibre Score	14.0 (10.0-16.0)	12.0 (9.0-15.0)	13.0 (9.0-16.0)	12.0 (11.0-13.0)
≥ 5 fruits/vegetables daily	6.0 (4.0-7.0)	4.0 (3.0-6.0)	5.0 (3.0-7.0)	7.0 (4.0-7.0)
EDSS			3.5 (2.0-6.0)	4.8 (3.5-6.0)
Count (%)				
Family History of MS	1 (1)	1 (2)	3 (8)	0 (0)
Current Smoking	8 (6)	4 (6)	10 (18)	4 (57)
Current Alcohol intake				
Abstain	22 (9)	9 (4)	22 (9)	3 (1)
Occasionally	54 (23)	18 (8)	14 (6)	0 (0)
1-13 units per week	38 (16)	29 (12)	11 (5)	2 (1)
14-21 units per week	8 (3)	4 (2)	0 (0)	1 (0)
≥ 22 units per week	1 (0)	1 (0)	0 (0)	0 (0)
Physical activity Score				
High	35 (28)	21 (33)	5 (10)	0 (0)
Moderate	50 (40)	21 (33)	15 (29)	4 (57)
Low	40 (32)	21 (33)	31 (61)	3 (43)

For the 48 patients from a previous study (Kotze *et al.* 2001) the biochemical parameters and lifestyle factors were not documented at the time of sample collection.

EDSS=Expanded Disability Scale Score.

Table 2.2 Expanded Disability Scale Score (EDSS) used to assess disease progression in MS patients

Score	Level of Disability in Multiple Sclerosis
0.0	Normal neurological exam
1.0	No disability, single sign present
1.5	No disability, minimal signs present
2.0	Minimal disability
2.5	Mild disability
3.0	Moderate disability
3.5	Fully ambulatory despite some disabilities
4.0	Fully ambulatory without aid despite relatively severe disability
4.5	Fully ambulatory with relatively severe disability
5.0	Disability impairs full daily activities
5.5	Disability precludes full daily activities
6.0	Requires intermittent or unilateral constant assistance to walk
6.5	Requires constant bilateral support to walk
7.0	Unable to walk and essentially restricted to a wheelchair
7.5	Restricted to wheelchair and unable to take more than a few steps
8.0	Essentially restricted to a wheelchair with generally effective use of arms
8.5	Essentially restricted to bed much of day with some effective use of arms
9.0	Confined to bed, still able to communicate and eat
9.5	Bedridden and unable to communicate effectively or eat and swallow
10.0	Death due to MS

(Kurtzke, 1983)

2.3 Biochemical Analysis

Blood was drawn for biochemistry testing in the morning between 9h00 and 10h30 to standardise for diurnal variation. Plasma homocysteine levels were measured using a Siemens Centaur XP auto-analyser in 60 patients and 87 controls and correlated with selected vascular risk factors. Serum total cholesterol, folate, vitamin B12 and C-reactive protein levels were determined using a Siemens Advia 1800 auto-analyser. Several studies have shown that overnight fasting prior to measurement of lipid (Sidhu and Naugler, 2012) and homocysteine (Fokkema *et al.* 2003) levels are unnecessary; therefore patients were not required to fast overnight before blood was collected for biochemical determinations. There was also an ethical incentive to

expidite phlebotomy procedures, since some of the patients presented with fragile veins, possibly due to vascular risk factors and/or immunosuppressant therapy.

2.4 Genetic Analysis

In addition to the biochemical measurements of total cholesterol, C-reactive protein, homocysteine, folate and vitamin B₁₂ levels, 6 SNPs known to influence these blood levels were genotyped using DNA samples extracted from whole blood or saliva.

2.4.1 DNA extraction from Whole Blood using the QIAGEN QIAamp[®] DNA Blood Midi Kit (Spin Protocol)

This protocol is for purification of genomic DNA from up to 2 ml of whole blood. Blood samples were equilibrated to room temperature (15-25°C) before starting the DNA extraction process with the addition of 200 µl of QIAGEN Protease into the bottom of a 15 ml centrifuge tube. QIAGEN Protease (or protease K) is an enzyme responsible for lysing the cells in the sample to release the DNA into the solution. The blood sample of 2 ml was then added and mixed briefly through vigorous shaking (i.e. vortexing). Buffer AL (2.4 ml) was added to tube and mixed thoroughly by inverting the tube 15 times, followed by additional vortexing for at least 1 minute. To ensure adequate lysis, the sample must be mixed thoroughly with Buffer AL to yield a homogenous solution. The homogenous sample was then incubated at 70°C for 10 minutes. After incubation 2 ml of ethanol (96-100%) was added to the sample, mixed by inverting the tube 10 times, followed by additional vortexing.

Half of the homogenous solution was carefully transferred onto the QIAamp[®] Midi column placed in a 15 ml centrifuge tube, taking care not to moisten the rim, and centrifuged for 3 minutes at 3000 rpm (1850 x g). The QIAamp[®] Midi column was removed, the filtrate discarded, and the column was placed back into the 15 ml centrifuge tube. The remaining half of the homogenous solution was loaded onto the QIAamp[®] Midi column and centrifuged for 3 minutes at 3000 rpm (1850 x g). The QIAamp[®] Midi column was removed, the filtrate discarded, and the column was placed back into the 15 ml centrifuge tube. Removal of the filtrate is essential to prevent the nozzle of the QIAamp[®] Midi column from being submerged in the filtrate, which would reduce the washing efficacy.

Wash buffer AW1 was carefully added to the column (2 ml) without wetting the rim and centrifuged at 5000 rpm (4500 x g) for 1 minute. Wash buffer AW2 was added to the column (2 ml) and centrifuged at 5000 rpm (4500 x g) for 15 minutes to remove

all traces of buffer AW2 from the QIAamp[®] Midi column before elution. After centrifugation the QIAamp[®] Midi column was removed and put into a clean 15 ml centrifuge tube and the filtrate was discarded. Distilled water (200 µl), equilibrated to room temperature (15-25°C), was pipetted directly onto the membrane of the QIAamp[®] Midi column and incubated at room temperature for 5 minutes before being centrifuged at 5000 rpm (4500 x g) for 2 minutes. The final solution, containing purified genomic DNA, was incubated on a shaker at room temperature (15-25°C) overnight to ensure homogenization of the DNA in the buffer.

2.4.2 DNA extraction from saliva using the Oragene-DNA / Saliva Kit

The Oragene[®]-DNA/saliva kit was used to isolate the relatively large amount of DNA present in saliva. When saliva is mixed with the Oragene[®]-DNA buffer during the collection process, the DNA is immediately stabilized and remains stable at room temperature for at least 5 years without processing.

Samples collected in the Oragene[®]-DNA vial were mixed by inversion and gentle shaking for a few seconds. This was done to ensure that the viscous saliva is properly mixed with the Oragene[®]-DNA solution. The sample was then incubated in a waterbath at 50°C for 2 hours. This heat-treatment step was to ensure permanent inactivation of nucleases. The Oragene[®]-DNA/saliva sample (500 µl) was transferred to a (1.5 ml) microcentrifuge tube. For 500 ml of Oragene[®]-DNA/saliva, 20 µl (1/25th volume) of Oragene[®]-DNA Purifier (OG-L2P), was added to the microcentrifuge tube and mixed by vortexing for a few seconds. The sample was incubated on ice for 10 minutes and centrifuged at room temperature at 13,000 rpm (15,000 x g) for 5 minutes. The clear supernatant was then transferred into a fresh microcentrifuge tube, without disturbing the pellet containing impurities. The tube containing the pellet was discarded. To 500 µl of Oragene[®]-DNA/saliva, 500 µl 95-100% ethanol was added and mixed gently by inversion. The sample was incubated at room temperature for 10 minutes to allow precipitation. The tube was then placed in a centrifuge in a known orientation and centrifugation was carried out at room temperature for 2 minutes at 13,000 rpm (15,000 x g). The supernatant was carefully removed without disturbing the DNA pellet and discarded. Thereafter 250 µl of 70% ethanol was carefully added to the tube and incubated at room temperature for 1 minute before completely removing the ethanol without disturbing the pellet. This was done to remove residual inhibitors. Nuclease-free water (100 µl) was finally added to dissolve the pellet through vortexing for 5 seconds. To ensure complete hydration of the DNA the samples were incubated overnight on a shaker.

2.4.3 DNA Quantification

The NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, USA) and its v3.5.2 software package was used to measure the DNA quality and quantity. All genomic DNA samples were diluted to a final concentration of 10ng/µl using nuclease-free water. The ratio absorbance reading at 260/280 for all the samples ranged from 1.6 to 1.9. Any values within this range indicate absence of contaminants such as salts or phenols in a sample. (www.nanodrop.com/techsupport/nd-1000-users-manual.pdf).

2.4.4 Polymerase Chain Reaction Amplification

Oligonucleotide Primers

Oligonucleotide primers were designed to detect 6 SNPs in the *FTO* (rs9939609; intron 1 T>A), *MTR* (rs1805087; 2756 A>G), *MTRR* (rs1801394; 66 A>G), *MTHFR* (rs1801133; 677 C>T and rs1801131; 1298 A>C) and *COMT* (rs4680; 472 G>A) genes using the LightCycler® Probe Design 2.0, version 1.0.R.36 software package (F. Hoffmann-La Roche Ltd, Switzerland). The reference sequences for the *FTO* (NG_012969.1), *MTR* (NG_008959.1), *MTRR* (NG_008856.1), *MTHFR* (NG_013351.1) and *COMT* (NG_011526.1) genes were obtained from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). Primers used in conventional PCR experiments and direct DNA sequencing reactions are summarised in table 2.3.

Table 2.3: Oligonucleotide primers used for conventional PCR amplification

Gene	dbSNP ID#	Primer	Nucleotide Sequence (5' to 3')	GC Content (%)	T _M (°C)	T _A (°C)	SNP Position(s)	Amplicon Size (bp)
<i>FTO</i>	rs9939609	Forward	CTGGCTCTTGAATGAAATAGGA	40.9	52.4	54	Intron 1	275
		Reverse	CTTAAAGTTAATGGCTTCAGGG	40.9	51.5			
<i>MTR</i>	rs1805087	Forward	GAACATCCCAAGCCCAC	58.8	53.5	48.5	2756	595
		Reverse	CACCTGTTTCCCTGCTG	58.8	53.4			
<i>MTRR</i>	rs1801394	Forward	GTTTCATTCGTACACTCTCC	45.0	50.6	45.6	66	616
		Reverse	CAGCATATGCTACTTCTGTC	45.0	50.5			
<i>MTHFR</i>	rs1801133	Forward	ATCCCTCGCCTTGAACA	52.9	53.6	56.0	677	256
		Reverse	TCACCTGGATGGGAAAGAT	47.3	53.1			
	rs1801131	Forward	CTCTGTCAGGAGTGTGC	58.8	52.4	62	1298	383
		Reverse	GGTGGAGGTCTCCCAACTTA	55.0	56.1			
<i>COMT</i>	rs4680	Forward	GGACCAGCGTGAGCATA	58.8	54.6	56	472	647
		Reverse	GCTGTGAGACCCTCACT	58.8	53.9			

bp = base pair, dbSNP = Database of Single Nucleotide Polymorphisms, SNP = Single Nucleotide Polymorphism, T_M = melting temperature, T_A = annealing temperature

PCR Reaction Mixture and Thermal Cycle Program

A Promega GoTaq[®] Flexi DNA Polymerase PCR kit and the Applied Biosystems GeneAmp[®] PCR System 2700 thermal cycler were used for amplification of the target sequence in 25 µl reactions. The reaction mixture consisted of 100 ng of template DNA, 1x Colourless GoTaq[®] Flexi buffer (Promega), 0.2 mM of each dNTP (dATP, dTTP, dGTP, dCTP) (Applied Biosystems), 1.5 mM MgCl₂ (Promega), 60 pmol of each primer and 1.25 U GoTaq[®] DNA Polymerase (Promega).

The PCR thermal cycling conditions used were: an initial denaturation step at 94°C for 5 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at T_A°C for 30 seconds and extension at 72°C for 2 minutes; followed by a final extension at 72°C for 10 minutes and a soak or cooling step at 22°C for 1 minute.

2.4.5 Gel Electrophoresis

Gel electrophoresis using a 2% (w/v) agarose gel was used, consisting of 2 g agarose dissolved in 100 ml 1xTBE [90 mM Tris-HCl, 90 mM boric acid (H₃BO₃) and 2.2 mM EDTA (pH 8.0)]. Thereafter 70 µL 0.0001% (v/v) Ethidium bromide (EtBr) was added to 700 ml 1xTBE electrophoretic buffer, to enable visualization of the PCR products. A total volume of 15 µl consisting of 5 µL PCR product and 10 µl Ficoll Orange G loading buffer [0.1% (w/v) Orange G, 20% (w/v) Ficoll, 10 mM EDTA at pH 7.0], was loaded into each well of the gel. To determine size of the amplified DNA fragment, a molecular size marker (Promega 100 bp DNA ladder) was also loaded onto the gel. Electrophoresis was performed for 1 hour at 100 V in the 1xTBE electrophoretic buffer. Visualization was achieved by ultraviolet (UV) light transillumination using a TFX-35M transilluminator (GibcoBRL Life Technologies, USA).

2.4.6 DNA Sequencing and Analysis

Sequencing was performed at the Central Analytical Facility of Stellenbosch University and the electropherograms were analysed using FinchTV v1.4.0 (Geospiza Research Group).

2.4.7 Real Time Polymerase Chain Reaction Amplification

2.4.7.1 Applied Biosystems® TaqMan® SNP Genotyping Assays

Applied Biosystems® (ABI™) TaqMan® SNP Genotyping Assays were used for the real time PCR (RT-PCR) runs. The pre-designed 40X assay mix, consisting of unlabelled primers and TaqMan® Minor Groove Binder (MGB) probes (VIC® and FAM™ dye-labelled), was diluted to 20x using sterile SABAX water (double distilled water). These assays were used for end-point genotyping by allelic discrimination analysis for SNPs on the Corbett Rotor-Gene™ 6000 / QIAGEN® Rotor-Gene™ Q. The assays employed in this study are presented in table 2.4.

Table 2.4: Genetic variants investigated in this study with their respective ABI™ TaqMan® SNP Genotyping Assay ID numbers.

Gene	Genetic Variant	dnSNP ID#	Assay ID number
<i>FTO</i>	intron 1 T>A	rs9939609	C_30090620_10
<i>MTR</i>	2756 A>G	rs1805087	C_12005959_10
<i>MTRR</i>	66 A>G	rs1801394	C_3068176_10
<i>MTHFR</i>	677 C>T	rs1801133	C_1202889_20
<i>MTHFR</i>	1298 A>C	rs1801131	C_850486_20
<i>COMT</i>	472 G>A	rs4680	C_25746809_50

2.4.7.2 Corbett Rotor-Gene™ 6000 / QIAGEN® Rotor-Gene™ Q

The Rotor-Gene™ 6000 series Multiplexing System, 5-Plex HRM model (Corbett Research, Australia) was used with ABI™ TaqMan® SNP Genotyping Assays. This system uses allelic discrimination and scatterplot analysis of the fluorescence data obtained from the RT-PCR run for SNP detection. A total reaction volume of 10 µl was used for each sample containing 10 ng/µl template DNA (2 µl), TaqMan® Genotyping Master Mix (P/N 4371355) (5 µl), 20x TaqMan® SNP Genotyping Assay (0.5 µl) and SABAX water (2.5 µl).

The thermal cycling conditions used were as follows: an initial hold step at 95°C for 10 minutes, followed by 40 cycles of annealing/extension at 92°C for 15 second and 60°C for 1 minute.

2.5 Statistical Analysis

Population frequencies of the selected SNPs in the *FTO* (rs9939609; intron 1 T>A), *MTR* (rs1805087; 2756 A>G), *MTRR* (rs1801394; 66 A>G), *MTHFR* (rs1801133; 677

C>T and rs1801131; 1298 A>C) and *COMT* (rs4680; 472 G>A) genes studied were estimated from allele counts. Hardy-Weinberg equilibrium was assessed using the exact test. Prior to inclusion of the genotyping results in the statistical model used for genotype-phenotype association studies, potential confounders were identified in the data set and adjusted for. For descriptive purposes, cross tabulation and frequency tables were used to denote occurrences of various qualitative attributes (such as gender, groups, etc.) whereas the median and interquartile range were used to describe quantitative phenotypes (Homocysteine, Serum Folate, Cholesterol, etc.). Log regression models were used to compare pairs of groups, while linear regression models were used to compare quantitative characteristics between diagnostic and genotypic groups and scores. These models were adjusted for possible confounders by including the confounders as factors. Log transformations were used when the distributions of quantitative traits were not symmetric. All p-values and effect sizes were derived from these models. All statistical analyses were done by a qualified biostatistician using functions from R software and R packages genetics and haplo stats, freely available from <http://www.r-project.org>.

Chapter 3

Results

Functional SNPs in the *FTO* (rs9939609, intron 1 T>A), *MTR* (rs1805087, 2756 A>G), *MTRR* (rs1801394, 66 A>G), *MTHFR* (rs1801133, 677 C>T and rs1801131, 1298 A>C) and *COMT* (rs4680, 472G>A) genes were evaluated in 114 MS patients and 195 control individuals. Non-modifiable variables such as age and gender known to affect homocysteine, cholesterol and possibly CRP levels, were correlated with the observed genotypes which in themselves may differ significantly between ethnic groups. A summary of the biochemical parameters and modifiable lifestyle risk factors evaluated in relation to the SNPs analysed is provided in table 3.1.

Table 3.1: Description of six SNPs studied in relation to known phenotypic expression and relevant clinical indicators also influenced by environmental factors.

Gene	Genetic Variation	dbSNP ID#	Amino Acid Change	Clinical Indicators of gene expression	Environmentally influenced
<i>FTO</i>	Intron 1 T>A	rs9939609	-	Cholesterol, BMI, CRP	Diet, physical activity
<i>MTR</i>	2756 A>G	rs1805087	Asp919Gly	Homocysteine	Smoking status Diet/B-vitamins Alcohol intake
<i>MTRR</i>	66 A>G	rs1801394	Ile22Met	Homocysteine	
<i>MTHFR</i>	677 C>T	rs1801133	Ala222Val	Homocysteine	
	1298 A>C	rs1801131	Glu429Ala		
<i>COMT</i>	472 G>A	rs4680	Val158Met	Homocysteine	Diet/B-vitamins

dbSNP = Database of Single Nucleotide Polymorphisms

DNA samples of the 195 Caucasian control individuals were initially used to perform analytical validation of the high-throughput RT-PCR genotyping method, against direct sequencing as the gold standard. Genotyping results of MS patients using the standardized RT-PCR assays for the six selected SNPs were subsequently compared with controls for all MS patients (n=114).

3.1 Conventional Sequencing – Agarose Gels and Electropherograms

The amplicons obtained after conventional PCR amplification of the *FTO* (rs9939609), *MTR* (rs1805087), *MTRR* (rs1801394), *MTHFR* (rs1801133 and rs1801131) and *COMT* (rs4680) gene regions are presented in figures 3.1, 3.3, 3.5, 3.7, 3.9 and 3.11 respectively. The PCR fragments were visualised with Ethidium Bromide after electrophoresis in a 2.5% agarose gel. To determine whether the DNA fragment of interest was amplified during PCR, a molecular size marker (Promega

100 bp DNA ladder) was used. A non-template control (NTC) was also included to rule out any contamination.

An example of the sequencing results is shown as an electropherogram for one of the control samples in figures 3.2 (*FTO* rs9939609), 3.4 (*MTR* rs1805087), 3.6 (*MTRR* rs1801394), 3.8 (*MTHFR* rs1801133), 3.10 (*MTHFR* rs1801131) and 3.12 (*COMT* rs4680). Forward (sense) and reverse (anti-sense) sequencing reactions were carried out after PCR clean-up, with only the forward shown as the reverse always corroborated the result obtained.

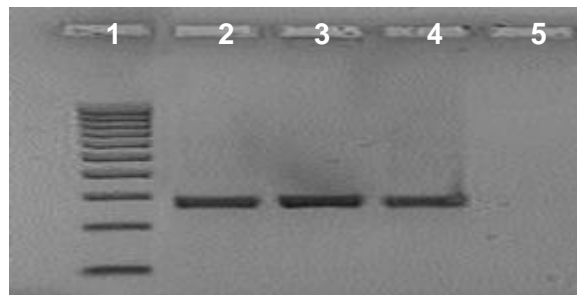


Figure 3.1: A 2.5% (w/v) agarose gel depicting the PCR amplicons synthesised with the *FTO* rs9939609 (Intron 1 T>A) primer set visualised with 0.0001% (v/v) Ethidium Bromide. Lane 1 contains a 100-bp DNA ladder. Lanes 2 to 4 contain amplicons of 275bp and lane 5 contains the NTC.

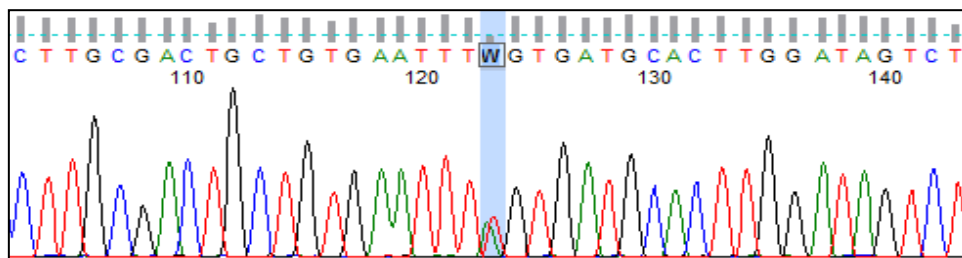


Figure 3.2: Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *FTO* rs9939609 (Intron 1 T>A) primer set. The highlighted area indicates the *FTO* (intron 1 T>A) nucleotide polymorphism position. The 'W' represents the TA Heterozygous genotype.

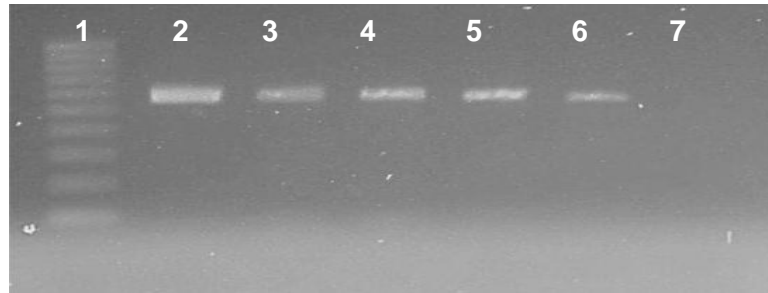


Figure 3.3: A 2.5% (w/v) agarose gel depicting the PCR amplicons synthesised with the *MTR* rs1805087 (2756 A>G) primer set visualised with 0.0001% (v/v) Ethidium Bromide. Lane 1 contains a 100-bp DNA ladder. Lanes 2 to 6 contains amplicons of 595bp and lane 7 contains the NTC. (bp=base pairs)

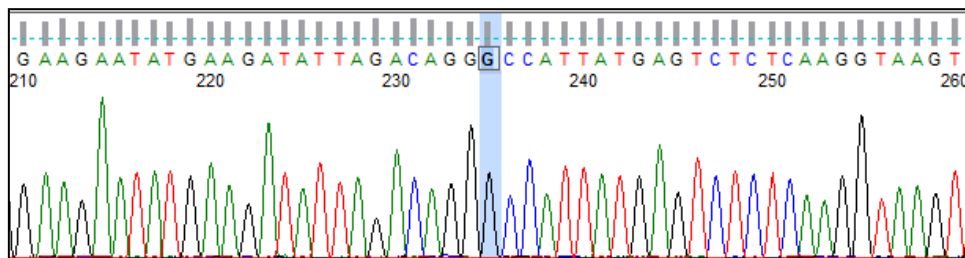


Figure 3.4: Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *MTR* rs1805087 (2756 A>G) primer set. The highlighted area indicates the *MTR* polymorphism position (nucleotide 2756). The 'G' represents the GG Homozygous genotype.

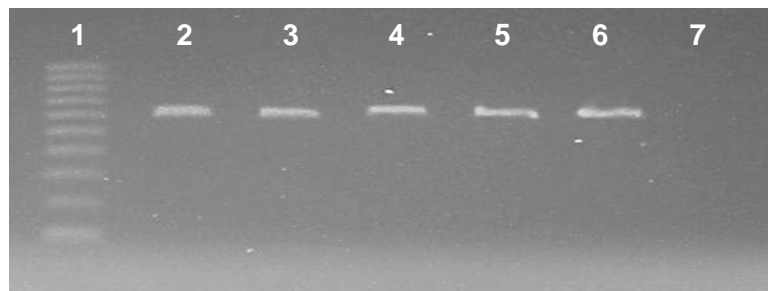


Figure 3.5: A 2.5% (w/v) agarose gel depicting the PCR amplicons synthesised with the *MTRR* rs1801394 (66 A>G) primer set visualised with 0.0001% (v/v) Ethidium Bromide. Lane 1 contains a 100-bp DNA ladder. Lanes 2 to 6 contains amplicons of 616bp and lane 7 contains the NTC. (bp = base pairs)

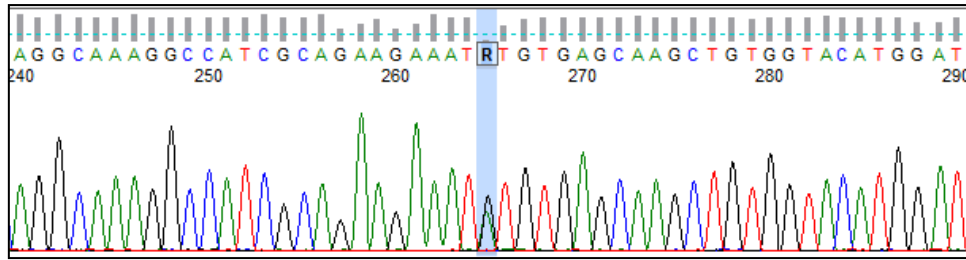


Figure 3.6: Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *MTRR* rs1801394 (66 A>G) primer set. The highlighted area indicates the *MTRR* polymorphism position (nucleotide 66). The 'R' represents the AG Heterozygous genotype.

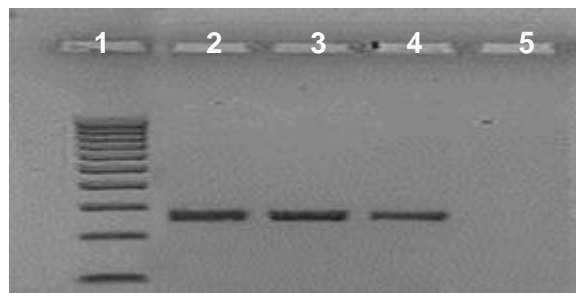


Figure 3.7: A 2.5% (w/v) agarose gel depicting the PCR amplicons synthesised with the *MTHFR* rs1801133 (677 C>T) primer set visualised with 0.0001% (v/v) Ethidium Bromide. Lane 1 contains a 100-bp DNA ladder. Lanes 2 to 4 contains amplicons of 256bp and lane 5 contains the NTC. (bp = base pairs)

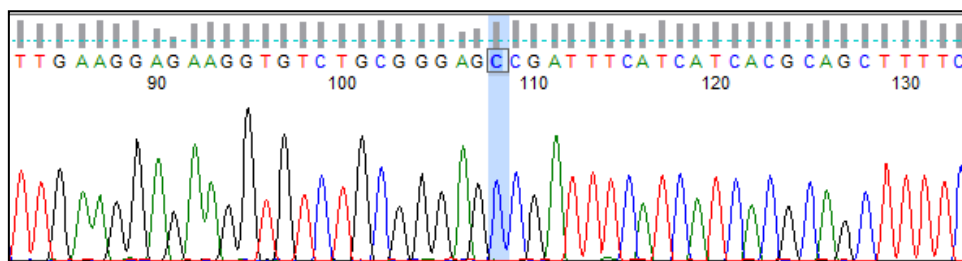


Figure 3.8: Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *MTHFR* rs1801133 (677 C>T) primer set. The highlighted area indicates the *MTHFR* polymorphism position (nucleotide 677). The 'C' represents the CC Wild Type genotype.

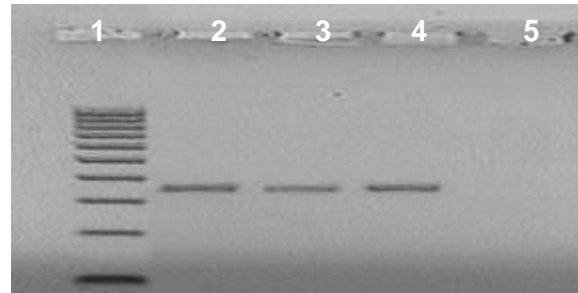


Figure 3.9: A 2.5% (w/v) agarose gel depicting the PCR amplicons synthesised with the *MTHFR* rs1801131 (1298 A>C) primer set visualised with 0.0001% (v/v) Ethidium Bromide. Lane 1 contains a 100-bp DNA ladder. Lanes 2 to 4 contains amplicons of 383bp and lane 5 contains the NTC. (bp = base pairs)

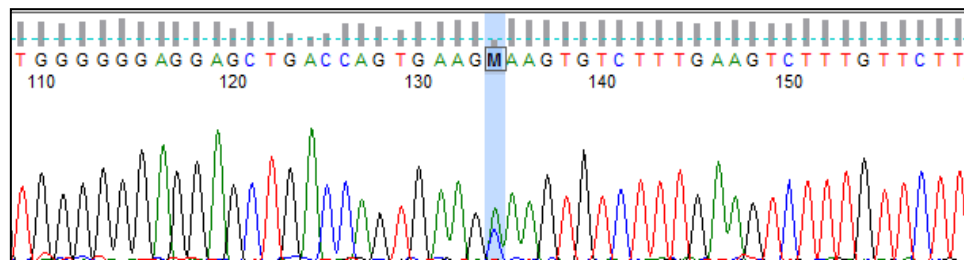


Figure 3.10: Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *MTHFR* rs1801131 (1298 A>C) primer set. The highlighted area indicates the *MTHFR* polymorphism position (nucleotide 1298). The 'M' represents the AC Heterozygous genotype.

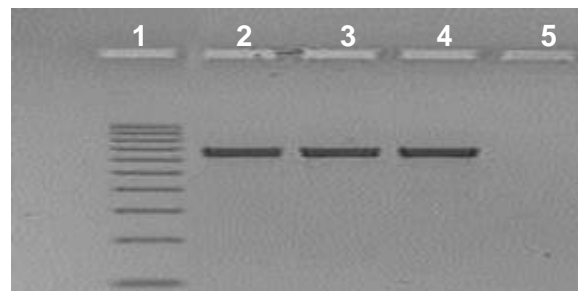


Figure 3.11: A 2.5% (w/v) agarose gel depicting the PCR amplicons synthesised with the *COMT* rs4680 (472 G>A) primer set visualised with 0.0001% (v/v) Ethidium Bromide. Lane 1 contains a 100-bp DNA ladder. Lanes 2 to 4 contains amplicons of 647bp and lane 5 contains the NTC. (bp = base pairs)

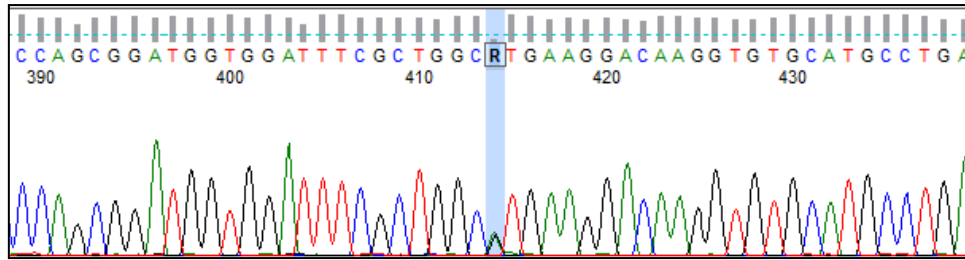


Figure 3.12: Electropherogram of the forward (sense) sequencing reaction of a PCR amplicon obtained with the *COMT* rs4680 (472 G>A) primer set. The highlighted area indicates the *COMT* polymorphism position (nucleotide 472). The 'R' represents the GA Heterozygous genotype.

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

For high-throughput genotyping, the Corbett Rotor-Gene™ 6000 series multiplexing system (5-Plex HRM model) with the ABI™ *TaqMan*® SNP genotyping assays were employed using allelic discrimination and scatterplot analysis for genotyping of the patient and control samples. Allelic discrimination analysis was applied which uses real time kinetic data from two or more channels simultaneously to allow genotype determination of the samples. The ABI™ *TaqMan*® SNP genotyping assays consisted of unlabelled primers specifically designed to amplify the target sequence and dual-labelled *TaqMan*® MGB (Minor Groove Binding) probes, FAM™ and VIC® used for allelic discrimination detection.

These dual-labelled probes have unique fluorophores acting as fluorescent dyes (FAM™ and VIC®) which are allele specific to ultimately differentiate between the different alleles of each SNP under investigation. The green sensor channel was employed for the detection of the FAM™ fluorescence probe with excitation wavelength 470 nm and emission wavelength 510 nm and the yellow channel sensor was engaged for detection of the VIC® fluorescence probe with excitation wavelength of 530 nm and emission wavelength of 555 nm by the Rotor-Gene™ fluorometer.

A scatterplot analysis was performed using multiple channels (green and yellow) for both the patient and control samples. Fluorescence values obtained from the FAM™ and VIC® dyes were normalized by the Rotor-Gene™ software and through a multi-component analysis, each sample was plotted as a single data point on the scatterplot. The genotypes were determined on the basis of regions defined on the scatterplot and the relative position of each sample to said regions.

All six assays (*FTO* rs9939609, Intron 1 T>A; *MTR* rs1805087, 2756 A>G; *MTRR* rs1801394, 66 A>G; *MTHFR* rs1801133, 677 C>T and rs1801131, 1298 A>C; *COMT* rs4680, 472 G>A) employed on the Corbett Rotor-Gene™ 6000 were performed successfully yielding clear amplification of the polymorphic target sequence and precise genotyping of each individual sample. Internal control samples were included in each run to verify the DNA sequence data obtained. To quantify the data generated during each run, a threshold was set to exclude small changes of fluorescence, due to probe degradation or other effects which do not show true amplification but rather a steady line. No amplification was detected with the non-template controls, corresponding to the absence of contamination during the analytical procedure and ensuring the credibility of the results generated during the study.

Due to the large amount of genotyping results generated from this study, only one sample batch of each assay is illustrated in this section to exemplify the results obtained through the Corbett Rotor-Gene™ 6000 series multiplexing system.

The allelic discrimination analysis for the *FTO* rs9939609 (intron 1 T>A) assay is presented in figure 3.13 with the legend and genotypes illustrated by table 3.2. The lines without intermitted circles represent the (T) allele, which is detected by FAM™-labelled probes, while the lines with the intermitted circles signifies the mutant (A) allele detected by the VIC®-labelled probes. Amplification of both alleles simultaneously, indicated by significant and roughly equal fluorescence from both dyes, represents the heterozygous (T/A) genotype. A threshold level (>0.1) was established as a discriminatory parameter during analysis to indicate the presence of allele-specific fluorescence when above the threshold setting.

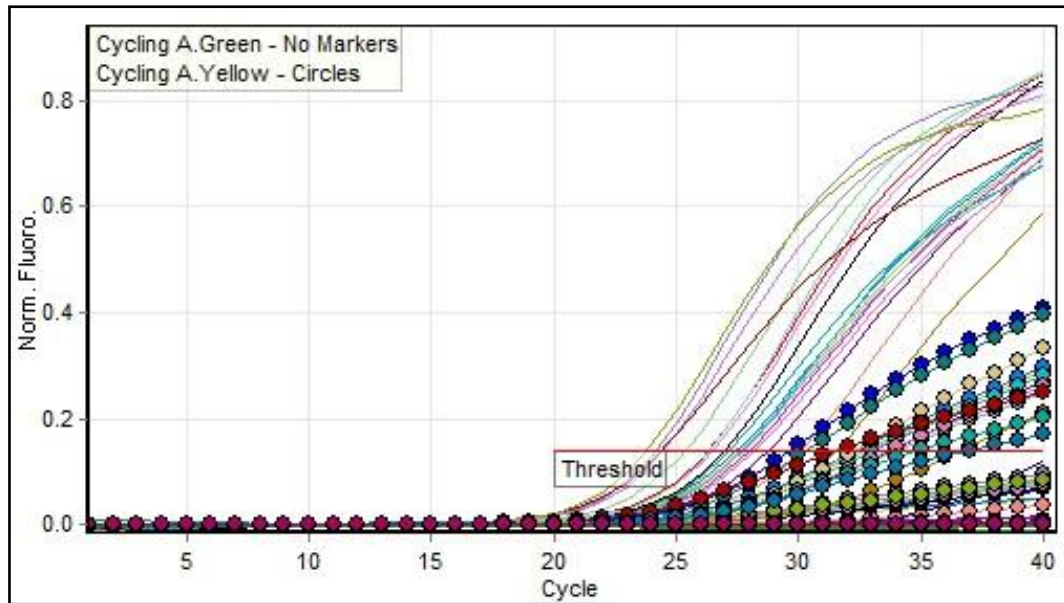


Figure 3.13: Allelic discrimination analysis of *FTO* rs9939609 (intron 1 T>A) using the ABI™ TaqMan® (C_30090620_10) genotyping assay. (Normalized fluorescence vs. number of cycles)

In addition to allelic discrimination analysis, scatterplot analysis was also performed to further verify the results obtained and to validate the methods used. Scatterplot analysis was applied using two channels simultaneously. Genotype acquisition is based on specific regions found on the scatterplot as well as relative expression of amplification of the green (FAM™) and yellow (VIC®) channels respectively. Upon normalization the different fold increases of each channel and log transformation accentuated the differences in expression of each individual sample.

The scatterplot analysis for the *FTO* rs9939609 (intron 1 T>A) assay is presented in figure 3.14 with the legend and genotypes depicted in table 3.2.

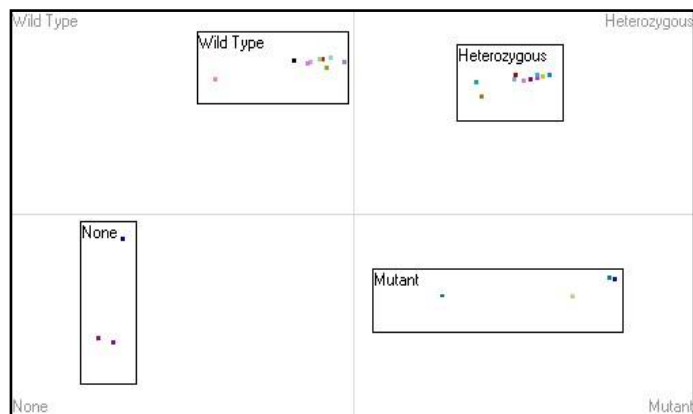


Figure 3.14: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC® fluorescence) of the ABI™ TaqMan® (C_30090620_10) *FTO* rs9939609 (intron 1 T>A) genotyping assay. The legend is presented in table 3.2.

Right: **Table 3.2:** Legend for figure 3.13 and 3.14, specifying genotypes of the samples based on Allelic discrimination data and Scatterplot analysis.

No.	Colour	Name	Genotype	Cycling A.Green	Cycling A.Yellow
1	Red	sample	Wild Type	Reaction	No Reaction
2	Yellow-Green	sample	Heterozygous	Reaction	Reaction
3	Blue	sample	Mutant	No Reaction	Reaction
4	Purple	sample	Heterozygous	Reaction	Reaction
5	Pink	sample	Wild Type	Reaction	No Reaction
6	Light Blue	sample	Heterozygous	Reaction	Reaction
7	Teal	sample	Mutant	No Reaction	Reaction
8	Light Red	sample	Wild Type	Reaction	No Reaction
9	Bright Pink	sample	Heterozygous	Reaction	Reaction
10	Black	sample	Wild Type	Reaction	No Reaction
11	Cyan	sample	Heterozygous	Reaction	Reaction
12	Gold	sample	Mutant	No Reaction	Reaction
13	Light Green	sample	Wild Type	Reaction	No Reaction
14	Light Cyan	sample	Wild Type	Reaction	No Reaction
15	Light Blue	sample	Heterozygous	Reaction	Reaction
16	Light Purple	sample	Wild Type	Reaction	No Reaction
17	Light Pink	sample	Wild Type	Reaction	No Reaction
18	Pink	sample	Heterozygous	Reaction	Reaction
19	Red	sample	Heterozygous	Reaction	Reaction
20	Gold	sample	Heterozygous	Reaction	Reaction
21	Yellow-Green	WT	Wild Type	Reaction	No Reaction
22	Teal	HET	Heterozygous	Reaction	Reaction
23	Blue	HOM	Mutant	No Reaction	Reaction
24	Blue	NTC1		No Reaction	No Reaction
25	Purple	NTC2		No Reaction	No Reaction
26	Pink	NTC3		No Reaction	No Reaction

The allelic discrimination analysis for the *MTR* rs1805087 (2756 A>G) assay is presented in figure 3.15 with the legend and genotypes illustrated by table 3.3. The lines with intermitted circles represent the (A) allele, which is detected by VIC[®]-labelled probes, while the lines without the intermitted circles signifies the mutant (G) allele detected by the FAM[™]-labelled probes. Amplification of both alleles simultaneously indicative of significant fluorescence of both dyes represents the heterozygous (A/G) genotype. A threshold level (>0.1) was established as a discriminatory parameter during analysis to indicate the presence of allele specific fluorescence above the threshold setting.

Due to the large number of samples tested and data generated during the genotyping for each of the assays employed in this study, Fig 3.15 shows only a representation of the results obtained.

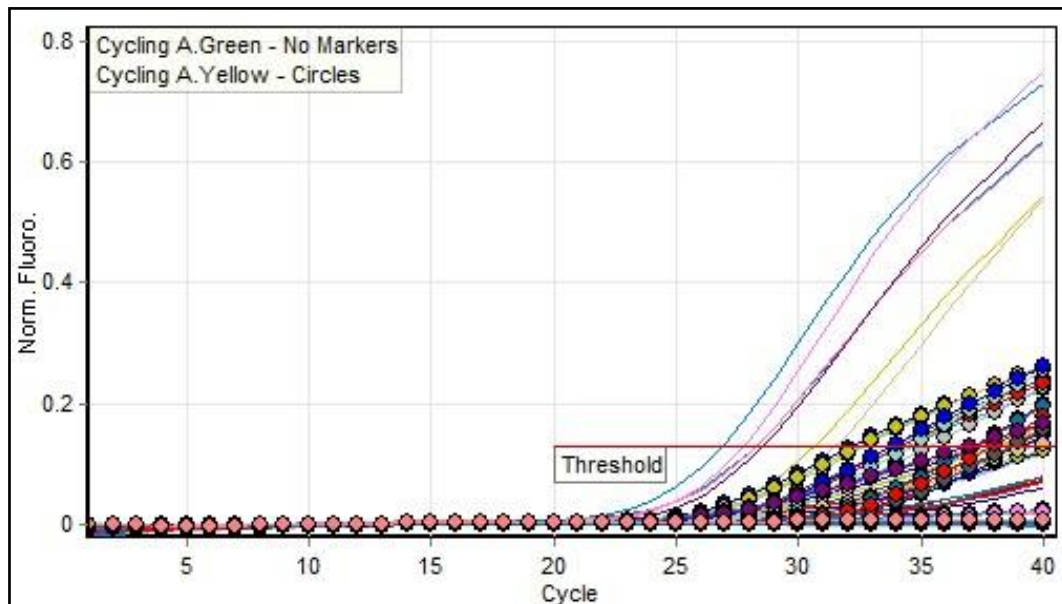


Figure 3.15: Allelic discrimination analysis of *MTR* rs1805087 (2756 A>G) using the ABI[™] TaqMan[®] (C_12005959_10) genotyping assay. (Normalized fluorescence vs. number of cycles).

The scatterplot analysis for the *MTR* rs1805087 (2756 A>G) assay is presented in figure 3.16 with the legend and genotypes depicted in table 3.3.

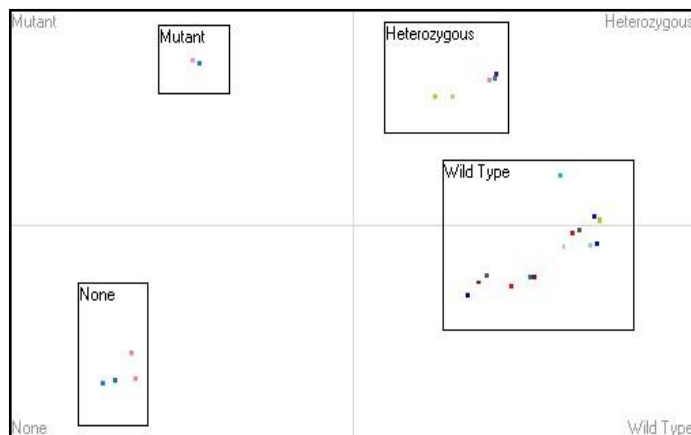


Figure 3.16: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC® fluorescence) of the ABI™ TaqMan® (C_12005959_10) MTR rs1805087 (2756 A>G) assay. The legend is presented in table 3.3.

Right: **Table 3.3:** Legend for figure 3.15 and 3.16, specifying genotypes of the samples based on Allelic discrimination data and Scatterplot analysis.

No.	Colour	Name	Genotype	Cycling A.Green	Cycling A.Yellow
1	Yellow	sample	Mutant	Reaction	No Reaction
2	Blue	sample	Heterozygous	Reaction	Reaction
3	Light Blue	sample	Mutant	Reaction	No Reaction
4	Cyan	sample	Heterozygous	Reaction	Reaction
5	Light Green	sample	Heterozygous	Reaction	Reaction
6	Red	sample	Wild Type	No Reaction	Reaction
7	Dark Blue	sample	Wild Type	No Reaction	Reaction
8	Grey	sample	Wild Type	No Reaction	Reaction
9	Dark Grey	sample	Heterozygous	Reaction	Reaction
10	Red	sample	Heterozygous	Reaction	Reaction
11	Yellow	sample	Heterozygous	Reaction	Reaction
12	Blue	sample	Heterozygous	Reaction	Reaction
13	Light Red	sample	Heterozygous	Reaction	Reaction
14	Light Yellow	sample	Heterozygous	Reaction	Reaction
15	Light Cyan	sample	Wild Type	No Reaction	Reaction
16	Red	sample	Wild Type	No Reaction	Reaction
17	Dark Blue	sample	Wild Type	No Reaction	Reaction
18	Dark Grey	sample	Wild Type	No Reaction	Reaction
19	Red	sample	Wild Type	No Reaction	Reaction
20	Blue	WT	Wild Type	No Reaction	Reaction
21	Purple	HET	Heterozygous	Reaction	Reaction
22	Pink	HOM	Mutant	Reaction	No Reaction
23	Light Blue	NTC1		No Reaction	No Reaction
24	Teal	NTC2		No Reaction	No Reaction
25	Light Red	NTC3		No Reaction	No Reaction

The allelic discrimination analysis for the *MTRR* rs1801394 (66 A>G) assay is presented in figure 3.17 with the legend and genotypes provided in table 3.4. The lines with intermitted circles represent the (A) allele, which is detected by VIC[®]-labelled probes, while the lines without the intermitted circles signifies the mutant (G) allele detected by the FAM[™]-labelled probes. Amplification of both alleles simultaneously indicative of significant fluorescence of both dyes represents the heterozygous (A/G) genotype. A threshold level (>0.1) was established as a discriminatory parameter during analysis to indicate the presence of allele specific fluorescence above the threshold setting.

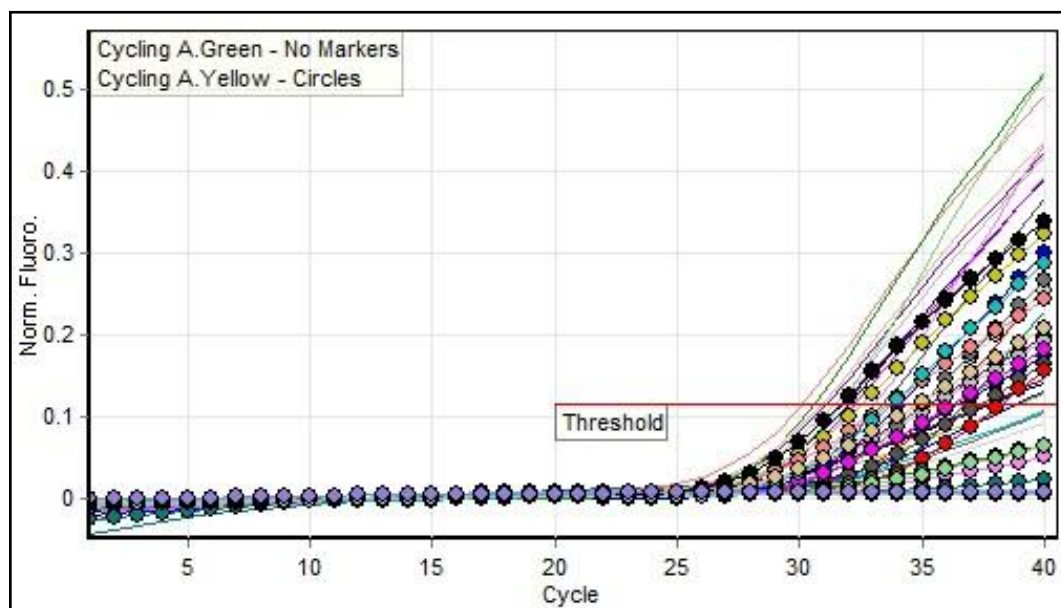


Figure 3.17: Allelic discrimination analysis of *MTRR* rs1801394 (66 A>G) using the ABI[™] TaqMan[®] (C_3068176_10) genotyping assay. (Normalized fluorescence vs. number of cycles).

The scatterplot analysis for the *MTRR* rs1801394 (66 A>G) assay is presented in figure 3.18 with the legend and genotypes depicted in table 3.4.

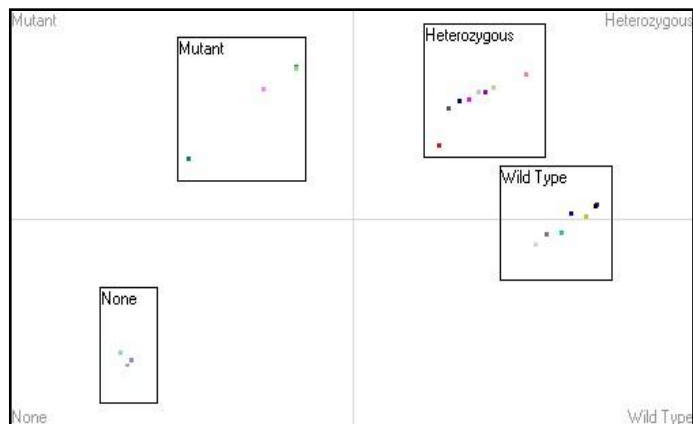


Figure 3.18: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC® fluorescence) of the ABI™ TaqMan® (C_3068176_10) MTRR rs1801394 (66 A>G) assay. The legend is presented in table 3.4.

Table 3.4: Legend for figure 3.17 and 3.18, specifying genotypes of the samples based on Allelic discrimination data and Scatterplot analysis.

No.	Colour	Name	Genotype	Cycling A.Green	Cycling A.Yellow
1	Blue	sample	Heterozygous	Reaction	Reaction
2	Purple	sample	Heterozygous	Reaction	Reaction
3	Grey	sample	Wild Type	No Reaction	Reaction
4	Light Grey	sample	Heterozygous	Reaction	Reaction
5	Dark Grey	sample	Wild Type	No Reaction	Reaction
6	Black	sample	Heterozygous	Reaction	Reaction
7	Red	sample	Heterozygous	Reaction	Reaction
8	Yellow	sample	Heterozygous	Reaction	Reaction
9	Blue	sample	Heterozygous	Reaction	Reaction
10	Purple	sample	Heterozygous	Reaction	Reaction
11	Pink	sample	Mutant	Reaction	No Reaction
12	Teal	sample	Mutant	Reaction	No Reaction
13	Red	sample	Heterozygous	Reaction	Reaction
14	Green	sample	Mutant	Reaction	No Reaction
15	Magenta	sample	Heterozygous	Reaction	Reaction
16	Black	sample	Heterozygous	Reaction	Reaction
17	Cyan	WT	Wild Type	No Reaction	Reaction
18	Tan	HET	Heterozygous	Reaction	Reaction
19	Light Green	HOM	Mutant	Reaction	No Reaction
20	Light Cyan	NTC1		No Reaction	No Reaction
21	Light Blue	NTC2		No Reaction	No Reaction
22	Light Purple	NTC3		No Reaction	No Reaction

The allelic discrimination analysis for the *MTHFR* rs1801133 (677 C>T) assay is presented in figure 3.19 with the legend and genotypes illustrated by table 3.5. The lines with intermitted circles represent the (C) allele, which is detected by VIC[®]-labelled probes, while the lines without the intermitted circles signifies the mutant (T) allele detected by the FAM[™]-labelled probes. Amplification of both alleles simultaneously indicative of significant fluorescence of both dyes represents the heterozygous (C/T) genotype. A threshold level (>0.1) was established as a discriminatory parameter during analysis to indicate the presence of allele specific fluorescence above the threshold setting.

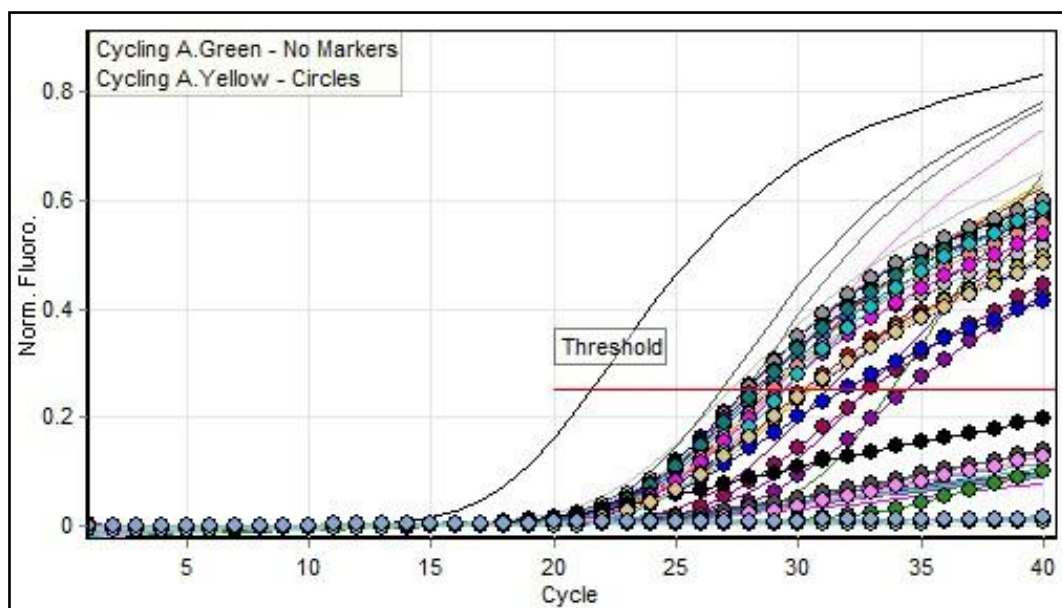


Figure 3.19: Allelic discrimination analysis of *MTHFR* rs1801133 (677 C>T) using the ABI[™] TaqMan[®] (C_1202889_20) genotyping assay. (Normalized fluorescence vs. number of cycles).

The scatterplot analysis for the *MTHFR* rs1801133 (677 C>T) assay is presented in figure 3.20 with the legend and genotypes depicted in table 3.5.

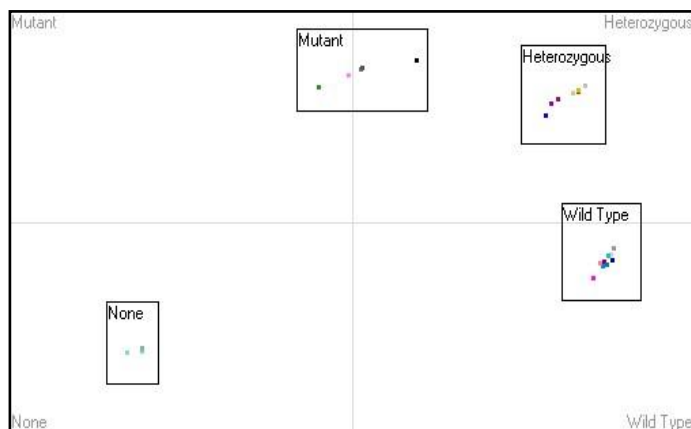


Figure 3.20: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC® fluorescence) of the ABI™ TaqMan® (C_1202889_20) *MTHFR* rs1801133 (677 C>T) assay. The legend is presented in table 3.5.

Table 3.5: Legend for figure 3.19 and 3.20, specifying genotypes of the samples based on Allelic discrimination data and Scatterplot analysis

No.	Colour	Name	Genotype	Cycling A.Green	Cycling A.Yellow
1	Blue	Sample	Wild Type	No Reaction	Reaction
2	Purple	Sample	Heterozygous	Reaction	Reaction
3	Magenta	Sample	Heterozygous	Reaction	Reaction
4	Grey	Sample	Wild Type	No Reaction	Reaction
5	Light Grey	Sample	Heterozygous	Reaction	Reaction
6	Dark Grey	Sample	Wild Type	No Reaction	Reaction
7	Black	Sample	Mutant	Reaction	No Reaction
8	Dark Grey	Sample	Mutant	Reaction	No Reaction
9	Red	Sample	Heterozygous	Reaction	Reaction
10	Yellow	Sample	Heterozygous	Reaction	Reaction
11	Blue	Sample	Heterozygous	Reaction	Reaction
12	Purple	Sample	Wild Type	No Reaction	Reaction
13	Pink	Sample	Mutant	Reaction	No Reaction
14	Blue	Sample	Wild Type	No Reaction	Reaction
15	Teal	Sample	Wild Type	No Reaction	Reaction
16	Red	Sample	Wild Type	No Reaction	Reaction
17	Green	Sample	Mutant	Reaction	No Reaction
18	Magenta	Sample	Wild Type	No Reaction	Reaction
19	Black	HOM	Mutant	Reaction	No Reaction
20	Cyan	WT	Wild Type	No Reaction	Reaction
21	Gold	HET	Heterozygous	Reaction	Reaction
22	Light Green	NTC 1		No Reaction	No Reaction
23	Light Cyan	NTC 2		No Reaction	No Reaction
24	Blue	NTC 3		No Reaction	No Reaction

The allelic discrimination analysis for the *MTHFR* rs1801131 (1298 A>C) assay is presented in figure 3.21 with the legend and genotypes provided in table 3.6. The lines without intermitted circles represent the (A) allele, which is detected by FAM™-labelled probes, while the lines with the intermitted circles signifies the mutant (C) allele detected by the VIC®-labelled probes. Amplification of both alleles simultaneously indicative of significant fluorescence of both dyes represents the heterozygous (A/C) genotype. A threshold level (>0.1) was established as a discriminatory parameter during analysis to indicate the presence of allele specific fluorescence above the threshold setting.

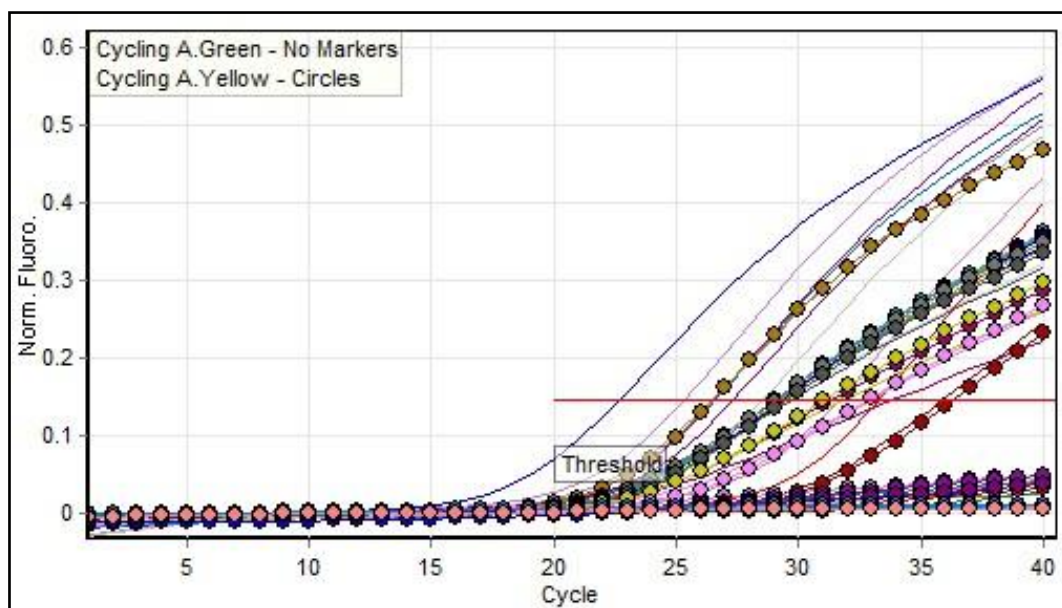


Figure 3.21: Allelic discrimination analysis of *MTHFR* rs1801131 (1298 A>C) using the ABI™ TaqMan® (C_850486_20) genotyping assay. (Normalized fluorescence vs. number of cycles).

The scatterplot analysis for the *MTHFR* rs1801131 (1298 A>C) assay is presented in figure 3.22 with the legend and genotypes depicted in table 3.6.

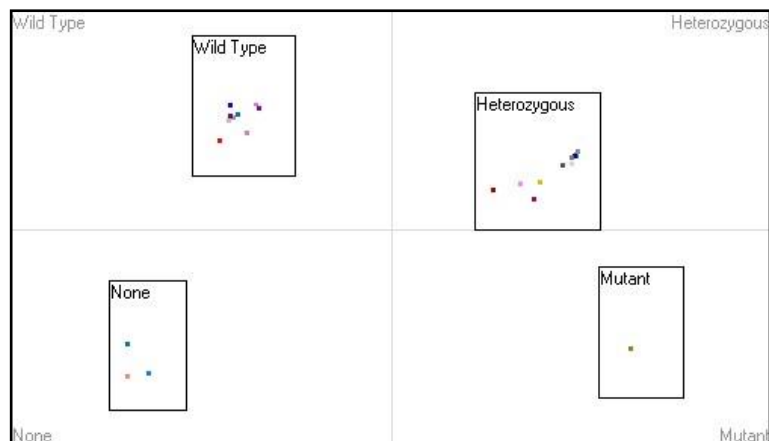


Figure 3.22: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC® fluorescence) of the ABI™ TaqMan® (C_850486_20) MTHFR rs1801131 (1298 A>C) assay. The legend is presented in table 3.6.

Table 3.6: Legend for figure 3.21 and 3.22, specifying genotypes of the samples based on Allelic discrimination data and Scatterplot analysis

No.	Colour	Name	Genotype	Cycling A.Green	Cycling A.Yellow
1	Light Purple	sample	Heterozygous	Reaction	Reaction
2	Light Purple	sample	Wild Type	Reaction	No Reaction
3	Pink	sample	Wild Type	Reaction	No Reaction
4	Red	sample	Heterozygous	Reaction	Reaction
5	Blue	sample	Wild Type	Reaction	No Reaction
6	Light Green	sample	Heterozygous	Reaction	Reaction
7	Teal	sample	Heterozygous	Reaction	Reaction
8	Dark Blue	sample	Wild Type	Reaction	No Reaction
9	Blue	sample	Heterozygous	Reaction	Reaction
10	Purple	sample	Wild Type	Reaction	No Reaction
11	Magenta	sample	Heterozygous	Reaction	Reaction
12	Grey	sample	Heterozygous	Reaction	Reaction
13	Grey	sample	Wild Type	Reaction	No Reaction
14	Grey	sample	Wild Type	Reaction	No Reaction
15	Dark Grey	sample	Heterozygous	Reaction	Reaction
16	Dark Grey	sample	Heterozygous	Reaction	Reaction
17	Red	sample	Wild Type	Reaction	No Reaction
18	Yellow-Green	sample	Heterozygous	Reaction	Reaction
19	Brown	HOM	Mutant	No Reaction	Reaction
20	Purple	WT	Wild Type	Reaction	No Reaction
21	Pink	HET	Heterozygous	Reaction	Reaction
22	Blue	NTC 1		No Reaction	No Reaction
23	Teal	NTC 2		No Reaction	No Reaction
24	Red	NTC 3		No Reaction	No Reaction

The allelic discrimination analysis for the *COMT* rs4680 (472 G>A) assay is presented in figure 3.23 with the legend and genotypes illustrated by table 3.7. The lines without intermitted circles represent the (G) allele, which is detected by FAM™-labelled probes, while the lines with the intermitted circles signifies the mutant (A) allele detected by the VIC®-labelled probes. Amplification of both alleles simultaneously indicative of significant fluorescence of both dyes represents the heterozygous (G/A) genotype. A threshold level (>0.1) was established as a discriminatory parameter during analysis to indicate the presence of allele specific fluorescence above the threshold setting.

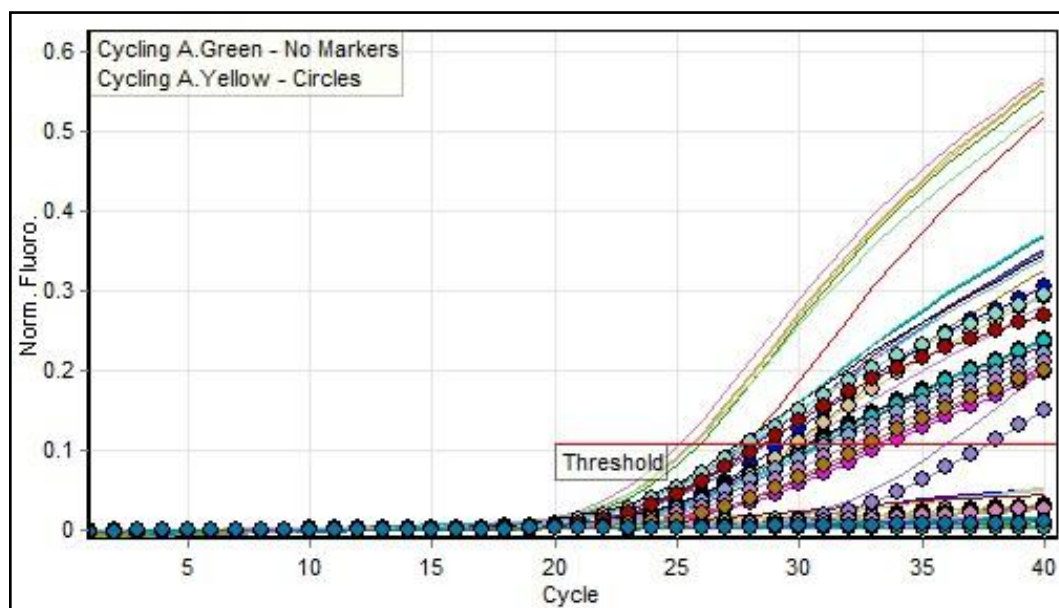


Figure 3.23: Allelic discrimination analysis of *COMT*rs4680 (472 G>A) using the ABI™ TaqMan® (C_25746809_50) genotyping assay. (Normalized fluorescence vs. number of cycles).

The scatterplot analysis for the *COMT* rs4680 (472 G>A) assay is presented in figure 3.24 with the legend and genotypes depicted in table 3.7.

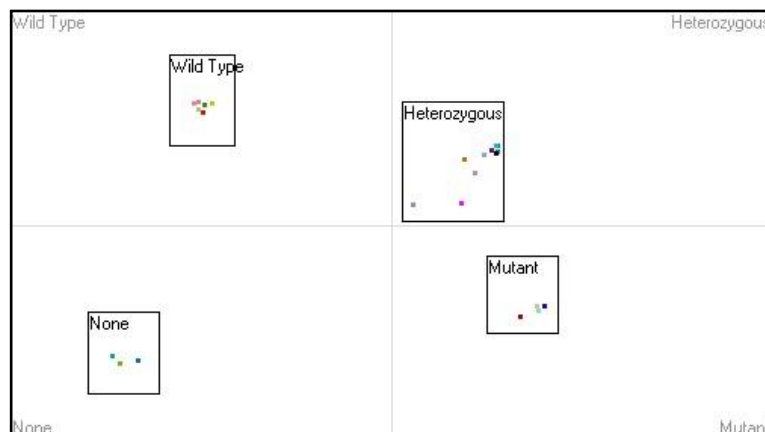


Figure 3.24: Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs. VIC® fluorescence) of the ABI™ TaqMan® (C_25746809_50) COMT rs4680 (472 G>A) assay. The legend is presented in table 3.7.

Table 3.7: Legend for figure 3.23 and 3.24, specifying genotypes of the samples based on Allelic discrimination data and Scatterplot analysis

No.	Colour	Name	Genotype	Cycling A.Green	Cycling A.Yellow
1	Red	sample	Wild Type	Reaction	No Reaction
2	Yellow	sample	Wild Type	Reaction	No Reaction
3	Blue	sample	Mutant	No Reaction	Reaction
4	Purple	sample	Heterozygous	Reaction	Reaction
5	Pink	sample		No Reaction	No Reaction
6	Light Blue	sample	Heterozygous	Reaction	Reaction
7	Teal	sample	Heterozygous	Reaction	Reaction
8	Light Red	sample	Wild Type	Reaction	No Reaction
9	Green	sample	Wild Type	Reaction	No Reaction
10	Magenta	sample	Heterozygous	Reaction	Reaction
11	Black	sample	Heterozygous	Reaction	Reaction
12	Cyan	sample	Heterozygous	Reaction	Reaction
13	Gold	sample	Mutant	No Reaction	Reaction
14	Light Green	sample	Wild Type	Reaction	No Reaction
15	Light Cyan	sample	Mutant	No Reaction	Reaction
16	Light Blue	sample	Heterozygous	Reaction	Reaction
17	Light Purple	sample	Heterozygous	Reaction	Reaction
18	Light Purple	sample	Heterozygous	Reaction	Reaction
19	Pink	WT	Wild Type	Reaction	No Reaction
20	Red	HOM	Mutant	No Reaction	Reaction
21	Gold	HET	Heterozygous	Reaction	Reaction
22	Yellow	NTC 1		No Reaction	No Reaction
23	Teal	NTC 2		No Reaction	No Reaction
24	Blue	NTC 3		No Reaction	No Reaction

3.3 Comparison of Genotype Distribution and Allele Frequencies in the control and patient study groups

The genotype distribution observed for *FTO* rs9939609 (Intron 1 T>A) among the 108 patients and 195 control samples achieved through application of RT-PCR is summarized in figure 3.25.

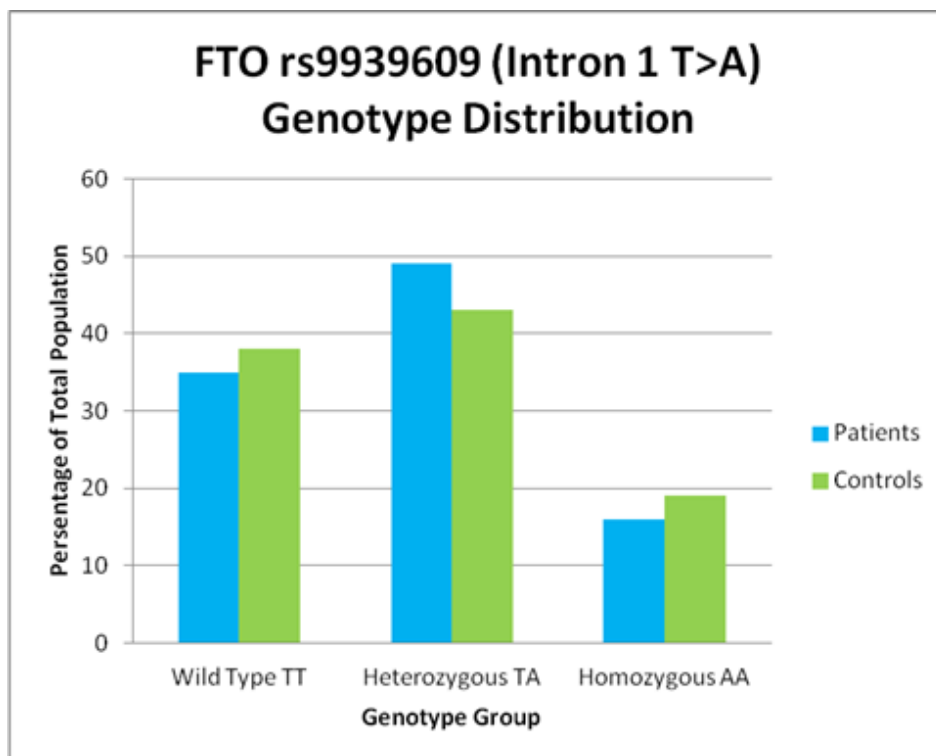


Figure 3.25: Genotype distribution of 303 samples included in this study obtained using the ABI™ TaqMan® (C_30090620_10) *FTO* rs9939609 (intron 1 T>A) assay. 108 MS patients presented with 38 Wild Type AA (35%), 53 Heterozygous AG (49%) and 17 Homozygous GG (16%). Among the 195 control samples, 75 were Wild Type AA (38%), 84 Heterozygous AG (43%) and 36 Homozygous GG (19%).

The genotype distribution observed for *MTR* rs1805087 (2756 A>G) among the 111 patients and 194 control samples achieved through application of RT-PCR is depicted in figure 3.26.

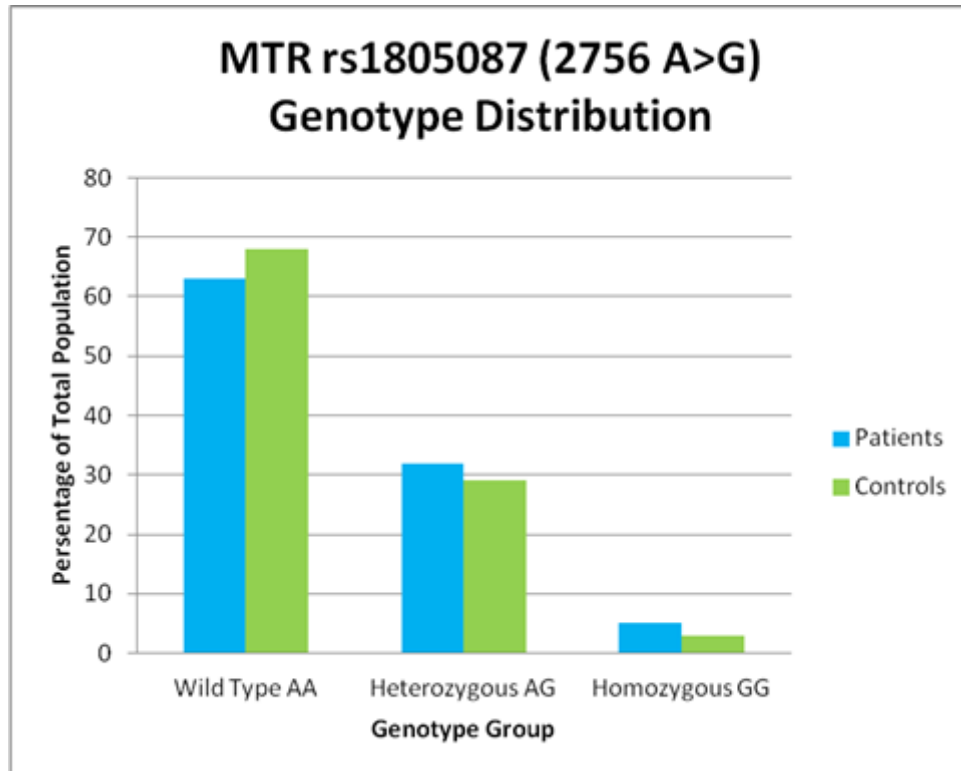


Figure 3.26: Genotype distribution of 305 samples included in this study obtained using the ABI™ TaqMan® (C_12005959_10) *MTR* rs1805087 (2756 A>G) assay. 111 MS patients presented with 70 Wild Type AA (63%), 35 Heterozygous AG (32%) and 6 Homozygous GG (5%). Among the 194 control samples 132 were Wild Type AA (68%), 56 Heterozygous AG (29%) and 6 Homozygous GG (3%).

The genotype distribution observed for *MTRR* rs1801394 (66 A>G) among the 111 patients and 195 control samples achieved through application of RT-PCR is summarized in figure 3.27.

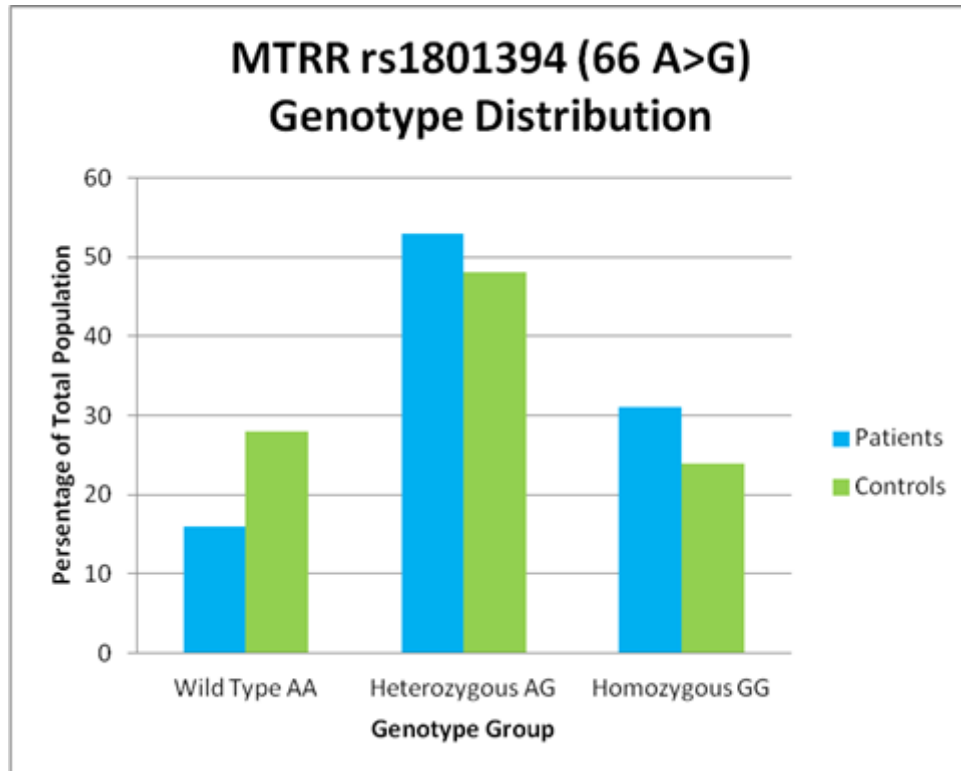


Figure 3.27: Genotype distribution of 306 samples included in this study obtained using the ABI™ TaqMan® (C_3068176_10) *MTRR* rs1801394 (66 A>G) assay. 111 MS patients presented with 18 Wild Type AA (16%), 59 Heterozygous AG (53%) and 34 Homozygous GG (31%). Among the 195 control samples 54 were Wild Type AA (28%), 95 Heterozygous AG (48%) and 46 Homozygous GG (24%).

The genotype distribution observed for *MTHFR* rs1801133 (677 C>T) among the 114 patients and 195 control samples achieved through application of RT-PCR is summarized in figure 3.28.

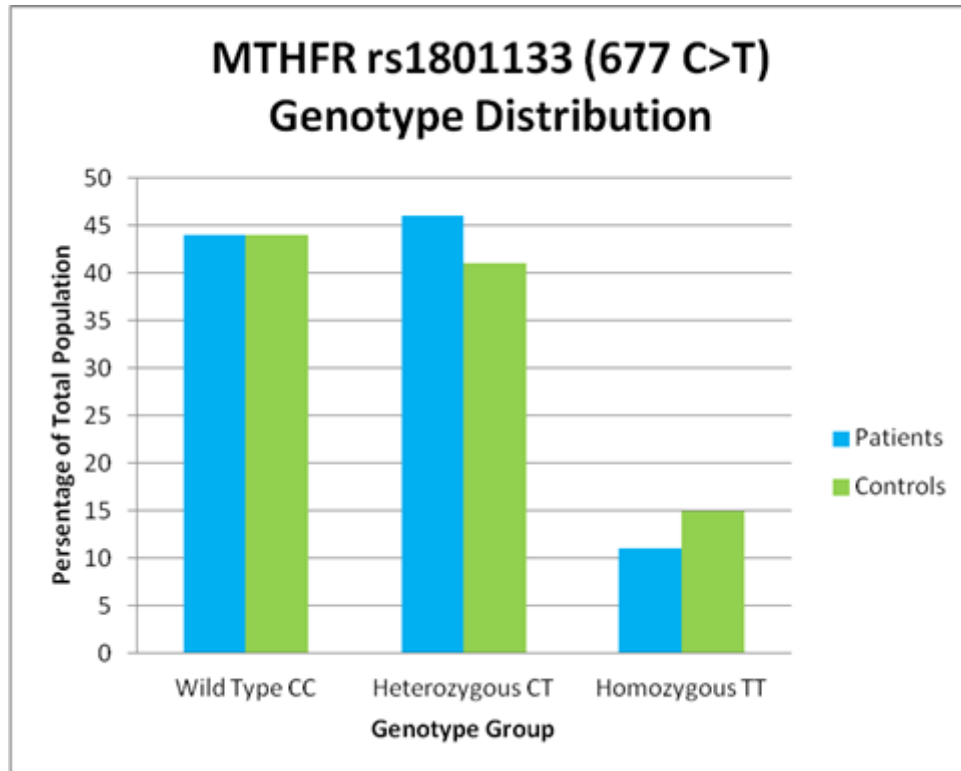


Figure 3.28: Genotype distribution of 309 samples included in this study obtained using the ABI™ TaqMan® (C_1202889_20) *MTHFR* rs1801133 (677 C>T) assay. 114 MS patients presented with 50 Wild Type CC (44%), 52 Heterozygous CT (46%) and 12 Homozygous TT (11%). Among the 195 control samples 86 were Wild Type CC (44%), 80 Heterozygous CT (41%) and 29 Homozygous TT (15%).

The genotype distribution observed for *MTHFR* rs1801131 (1298 A>C) among the 114 patients and 195 control samples achieved through application of RT-PCR is summarized in figure 3.29.

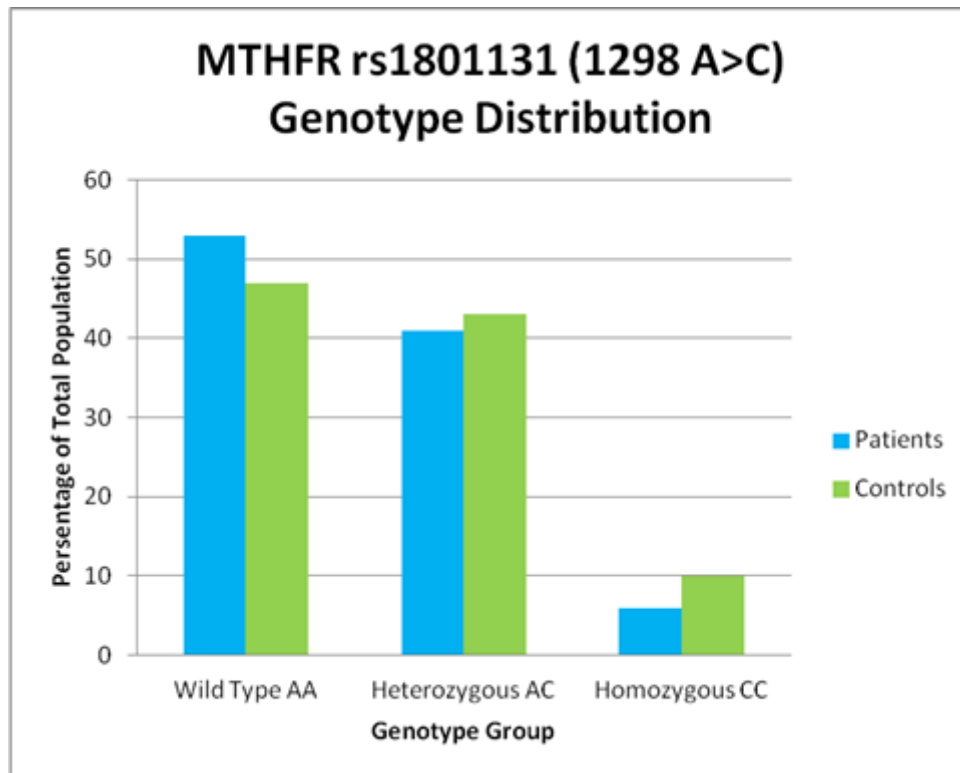


Figure 3.29: Genotype distribution of 309 samples included in this study obtained using the ABI™ TaqMan® (C_850486_20) *MTHFR* rs1801131 (1298 A>C) assay. 114 MS patients presented with 60 Wild Type AA (53%), 47 Heterozygous AC (41%) and 7 Homozygous CC (6%). Among the 195 control samples 92 were Wild Type AA (47%), 83 Heterozygous AC (43%) and 20 Homozygous CC (10%).

The genotype distribution observed for *COMT* rs4680 (472 G>A) among the 110 patients and 195 control samples achieved through application of RT-PCR is summarized in figure 3.30.

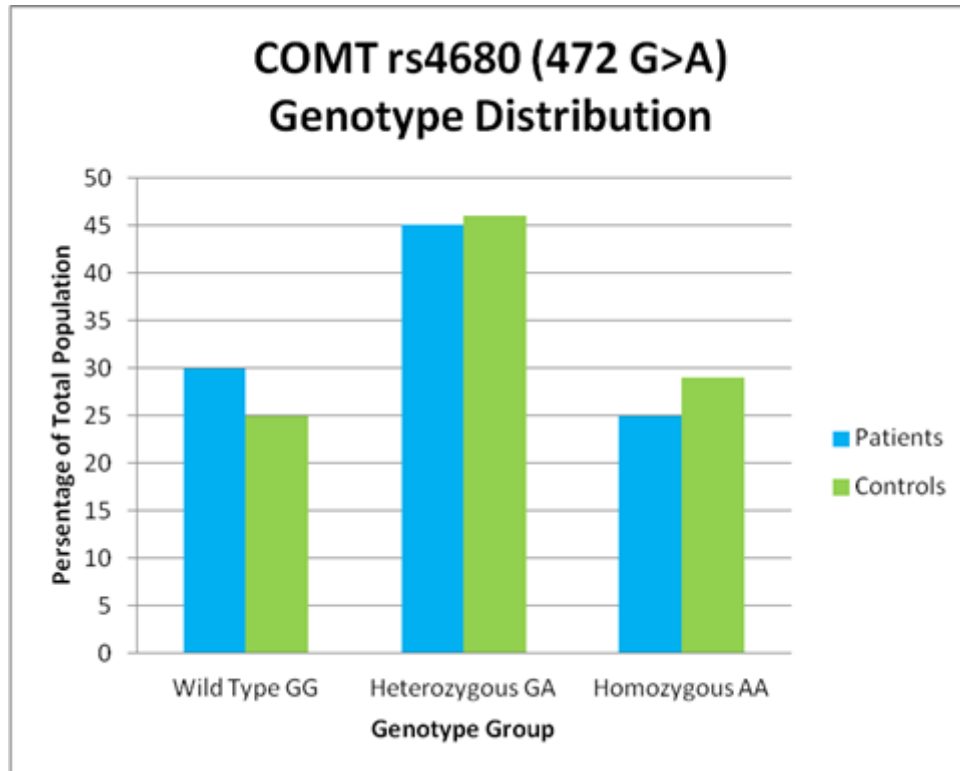


Figure 3.30: Genotype distribution of 305 samples included in this study obtained using the ABI™ TaqMan® (C_25746809_50) *COMT* rs4680 (472 G>A) assay. 110 MS patients presented with 33 Wild Type GG (30%), 50 Heterozygous GA (45%) and 27 Homozygous AA (25%). Among the 195 control samples 50 were Wild Type GG (25%), 89 Heterozygous GA (46%) and 56 Homozygous AA (29%).

On completion of *FTO* rs9939609 (intron 1 T>A), *MTR* rs1805087 (2756 A>G), *MTRR* rs1801394 (66 A>G), *MTHFR* rs1801133 (677 C>T) and rs1801131 (1298 A>C) and *COMT* rs4680 (472 G>A) SNP analysis, the observed genotype distribution and minor allele frequencies were summarised in table 3.8 for the 195 control samples and 114 MS patients. All six SNPs were found to be in Hardy-Weinberg equilibrium (HWE). The genotype distribution and allele frequencies did not differ significantly between males and females and patients and controls. The p-values provided are from a 2 degree of freedom test for genotype categories and from an additive model for allele frequencies.

Table 3.8: Genotype distribution and minor allele frequencies of *FTO* rs9939609, *MTR* rs1805087, *MTRR* rs1801394, *MTHFR* rs1801133 and rs1801131 and *COMT* rs4680 polymorphisms shown to be in Hardy Weinberg equilibrium in both patients and controls. Number of genotypes/alleles given with percentages in brackets.

	Patients			Controls			P-values	
	All	Male	Female	All	Male	Female	Males vs. Females	Patients vs. Controls
<i>FTO</i> rs9939609 (intron 1 T>A)								
N	108	15	93	194	68	127		
T/T	38 (35)	5 (33)	33 (35)	75 (38)	21 (30)	54 (43)	0.189	0.316
T/A	53 (49)	10 (67)	43 (46)	84 (43)	34 (50)	50 (39)		
A/A	17 (16)	0.00	17 (18)	36 (19)	13 (20)	23 (18)		
A	87 (40)	10 (33)	77 (41)	156 (40)	60 (46)	96 (37)	0.339	0.796
HWE	1.000	0.175	0.810	0.180	1.000	0.080		
<i>MTR</i> rs1805087 (2756 A>G)								
N	111	15	96	194	68	126		
A/A	70 (62)	10 (67)	60 (62)	132 (68)	48 (71)	84 (67)	0.563	0.640
A/G	35 (32)	5 (33)	30 (31)	56 (29)	18 (26)	38 (30)		
G/G	6 (6)	0.00	6 (6)	6 (3)	2 (3)	4 (3)		
G	47 (22)	5 (17)	42 (22)	68 (18)	22 (16)	46 (18)	0.318	0.379
HWE	0.575	1.000	0.588	1.000	1.000	1.000		
<i>MTRR</i> rs1801394 (66 A>G)								
N	111	15	96	195	67	128		
A/A	18 (17)	3 (20)	15 (16)	54 (28)	23 (34)	31 (24)	0.131	0.122
A/G	59 (53)	11 (73)	48 (50)	95 (48)	25 (37)	70 (55)		
G/G	34 (30)	1 (7)	33 (34)	46 (24)	19 (28)	27 (21)		
G	127 (57)	13 (43)	114 (59)	187 (48)	63 (47)	124 (48)	0.135	0.051
HWE	0.438	0.166	0.884	0.667	0.070	0.370		
<i>MTHFR</i> rs1801133 (677 C>T)								
N	114	16	98	195	67	128		
C/C	50 (44)	6 (38)	44 (45)	86 (43)	27 (40)	59 (46)	0.598	0.358
C/T	52 (46)	8 (50)	44 (45)	80 (41)	31 (46)	49 (38)		
T/T	12 (11)	2 (12)	10 (10)	30 (15)	9 (13)	20 (16)		
T	76 (33)	12 (38)	64 (33)	140 (36)	49 (37)	89 (35)	0.537	0.679
HWE	1.000	1.000	1.000	0.161	1.000	0.078		
<i>MTHFR</i> rs1801131 (1298 A>C)								
N	114	16	98	195	67	128		
A/A	61 (54)	11 (69)	50 (51)	92 (47)	33 (49)	59 (46)	0.218	0.354
A/C	46 (40)	4 (25)	42 (43)	83 (42)	25 (37)	58 (45)		
C/C	7 (6)	1 (6)	6 (6)	20 (10)	9 (19)	11 (9)		
C	60 (26)	6 (19)	54 (28)	123 (31)	43 (32)	80 (31)	0.991	0.151
HWE	0.811	1.000	0.594	0.868	0.370	0.648		
<i>COMT</i> rs4680 (472 G>A)								
N	110	16	94	195	67	128		
G/G	33 (25)	3 (19)	30 (32)	50 (29)	15 (22)	35 (27)	0.257	0.658
G/A	50 (45)	8 (50)	42 (45)	89 (46)	36 (54)	53 (42)		
A/A	27 (30)	5 (31)	22 (23)	56 (29)	16 (24)	40 (31)		
A	104 (53)	18 (56)	86 (46)	201 (51)	68 (21)	133 (52)	0.600	0.382
HWE	0.337	1.000	0.447	0.249	0.713	0.097		

A genotype risk score (1-10) was calculated for each individual by counting the minor alleles for *FTO* rs9939609 (intron 1 T>A), *MTR* rs1805087 (2756 A>G), *MTRR* rs1801394 (66 A>G), *MTHFR* rs1801133 (677 C>T) and *MTHFR* rs1801131 (1298 A>C). A minimum risk score of 0 and a maximum of 7 were obtained in both the control and patient study groups. None of the patients or controls was homozygotes for all 5 SNPs, which would correspond to the highest risk score of 10. No significant association with the risk score, or difference between the controls and MS patients, were detected regarding MS susceptibility. No difference was also detected when *COMT* rs4680 (472 G>A) was considered as part of the risk score, either including G or A as the risk-associated allele (data not shown). This SNP was not included in the results presented in table 3.9 since it is uncertain whether the minor/low-activity or common/high-activity allele of *COMT* rs4680 (472 G>A) should be considered the risk allele in the context of MS.

Table 3.9: Distribution and frequencies of calculated genotype risk score (number of risk alleles) of *FTO* rs9939609 (intron 1 T>A), *MTR* rs1805087 (2756 A>G), *MTRR* rs1801394 (66 A>G), *MTHFR* rs1801133 (677 C>T), *MTHFR* rs1801131 (1298 A>C) in controls and MS patients.

Risk Score	Controls	MS Patients	Controls (n=195)		MS Patients (n=114)	
			Female	Male	Female	Male
0	2 (1)	1 (1)	2 (2)	0 (0)	1 (1)	0 (0)
1	14 (7)	5 (4)	9 (7)	5 (7)	3 (3)	2 (12)
2	32 (16)	20 (18)	22 (17)	10 (15)	18 (18)	2 (12)
3	55 (28)	38 (33)	37 (29)	18 (27)	30 (31)	8 (50)
4	52 (27)	26 (23)	33 (26)	19 (28)	22 (22)	4 (25)
5	25 (13)	16 (14)	15 (12)	10 (15)	16 (16)	0 (0)
6	13 (7)	7 (6)	10 (8)	3 (4)	7 (7)	0 (0)
7	2 (1)	1 (1)	0 (0)	2 (3)	1 (1)	0 (0)
8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
9	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

3.4 Clinical Studies

Since all six SNPs analysed in this study may cause biochemical/metabolic abnormalities only when a high-risk environment is entered, the effect of lifestyle factors relevant to the genes and biochemical parameters were also assessed.

Genotype-phenotype association studies were performed with due consideration of the potential confounding effects of gender, age and lifestyle risk factors, including smoking status, alcohol consumption, physical activity and diet composition as may also be reflected by body mass index (BMI). Clinical outcomes were evaluated in relation to biochemical parameters and EDSS score at baseline.

Baseline comparisons

The clinical characteristics are summarised in table 2.1. Due to the known high female-to-male ratio in MS patients, also evident in this study, the data are presented separately according to gender in the 114 patients and 195 control individuals. MS patients were on average 4 years younger than controls ($p=0.006$), while no age differences were observed between males and females either in the control or patient subgroups. As expected, more patients compared with controls had a family history of MS ($p=0.045$).

No significant differences were observed between the groups in relation to several vascular risk factors evaluated, as listed in table 2.1. Homocysteine levels were found to be significantly higher in MS patients compared to controls in males, but not in females (interaction $p=0.017$). Homocysteine was significantly higher (29%) in males compared with females ($p<0.001$) in both the MS patients and controls, after adjusting for age. Homocysteine was shown to increase with age, by 0.56% per year, resulting in an increase of 5.6% every 10 years.

Alcohol consumption was significantly higher in control individuals ($p<0.001$), while more MS patients were found to be current smokers ($p<0.001$). As may be expected, physical activity levels were significantly lower in MS patients than controls (<0.001). No significant effects of these three variables were observed on homocysteine levels in our study cohort, therefore adjustments were made for age and gender only in the genotype-phenotype association studies related to homocysteine levels.

Genotype effect on homocysteine levels

Possible genotype association with homocysteine levels was assessed in a subset of 60 MS patients and 87 controls. High homocysteine levels were significantly associated with the *FTO* rs9939609 in an additive allelic model in the MS study population, but not in controls, after adjustment for age and gender ($p=0.003$). The risk-associated A-allele was significantly associated with increased homocysteine levels in both the gender adjusted (figure 3.31) and female MS patients ($p=0.006$). In

the presence of each A allele, homocysteine increased with 15% in MS patients. This translated into a 30% increase in homocysteine levels among MS patients homozygous for *FTO* rs9939609. When males were excluded from this analysis the presence of each A-allele increased the homocysteine levels by 14% in female MS patients. None of the other SNPs tested or the combined risk score showed a similar association with homocysteine levels (data not shown).

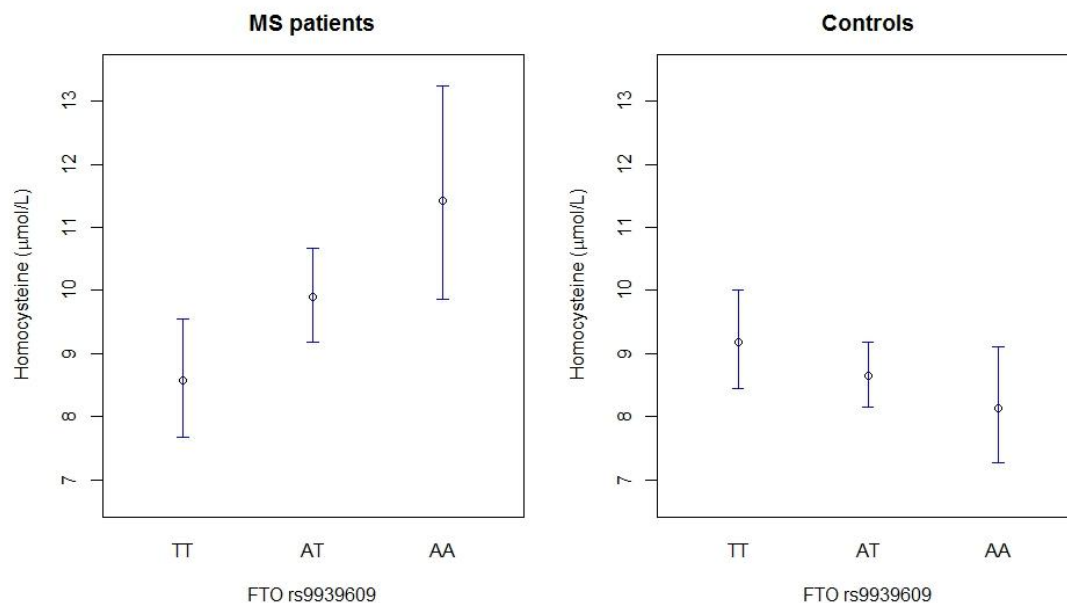


Figure 3.31: Graph depicting the estimated effect with 95% confidence interval of the *FTO* rs9939609 (intron 1 T>A) risk-associated A-allele on homocysteine levels in MS patients (left) and controls (right) after adjustment for age and gender ($p=0.003$).

Correlation between homocysteine and BMI

A significant correlation was observed between BMI and homocysteine levels in relation to various vascular risk factors and lifestyle factors analysed (Table 3.10). These findings were similar in MS patients and controls as no differences in association were detected between these study groups, after adjustment for age and gender, and in females adjusted for age only (data not shown).

Table 3.10: P-values for testing of association between clinical characteristics, BMI and homocysteine levels in 60 MS patients and 87 controls combined, adjusted for MS status, and age and gender where relevant.

Clinical and lifestyle Characteristics	Males and females		Females only		Males and females		Females only	
	Homocysteine	MS Patients vs. Controls	Homocysteine	MS Patients vs. Controls	BMI	MS Patients vs. Controls	BMI	MS Patients vs. Controls
Homocysteine	-	-	-	-	0.074	0.456	0.045	0.665
Body mass index (BMI)	0.055	0.912	0.045	0.798	-	-	-	-
Cholesterol, total	0.048	0.409	0.081	0.547	0.095	0.342	0.314	0.319
Serum Folate	0.689	0.051	0.460	0.252	0.631	0.203	0.826	0.934
Vitamin B12	0.057	0.051	0.212	0.602	0.652	0.264	0.364	0.768
C-reactive protein	0.662	0.322	0.453	0.147	0.001	0.394	0.001	0.413
Smoking	0.076	0.946	0.236	0.947	0.571	0.162	0.086	0.806
Alcohol intake	0.512	0.069	0.950	0.133	0.232	0.981	0.442	0.928
Physical activity	0.387	0.362	0.123	0.500	0.006	0.227	0.062	0.402
Fat Score	0.920	0.078	0.112	0.325	0.006	0.168	0.001	0.085
Folate Score	0.063	0.071	0.011	0.217	0.017	0.788	0.078	0.747
Fruit/veg/fibre Score	0.717	0.496	0.885	0.393	0.095	0.802	0.741	0.424
≥ 5 fruits/vegetables daily	0.401	0.367	0.179	0.372	0.109	0.202	0.462	0.678

Homocysteine and BMI were log-transformed for modelling.

BMI correlated significantly with higher homocysteine levels ($p < 0.05$) in females, after adjusting for age with no difference in correlation noted between MS patients and controls (Figure 3.32). For every one unit increase in BMI a 1.3% increase in homocysteine levels was found. BMI also correlated significantly with CRP levels ($p < 0.001$). BMI was increased with 1.8% (95% CI: 0.8 to 2.8%) for each unit increase in CRP, in the combined group, adjusted for MS status and age and gender; as well as in the females, adjusted for MS status and age.

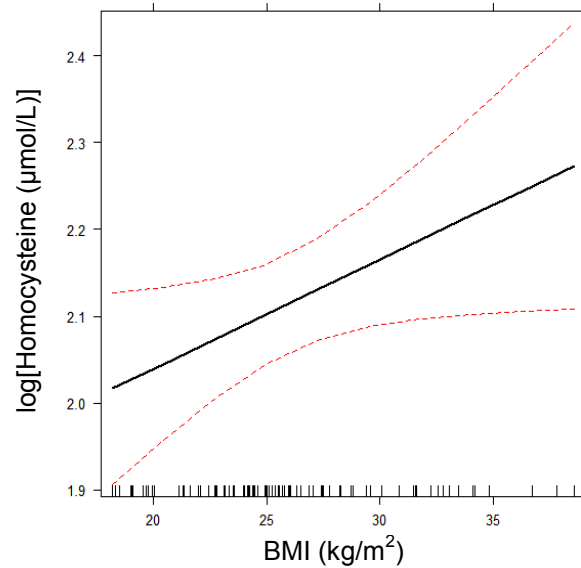


Figure 3.32: Graph depicting the significant correlation between BMI and homocysteine levels ($p = 0.046$) in females after adjusting for age and MS status. The solid line shows modelled effect and broken lines are 95% confidence levels for estimates.

Homocysteine levels also correlated significantly with total cholesterol levels ($p < 0.05$) after adjustment for diagnosis of MS, age and gender, with no difference noted between MS patients and controls (Figure 3.33). Every one unit increase in cholesterol correlated with a 5% increase in homocysteine levels, irrespective of age, gender and MS diagnosis.

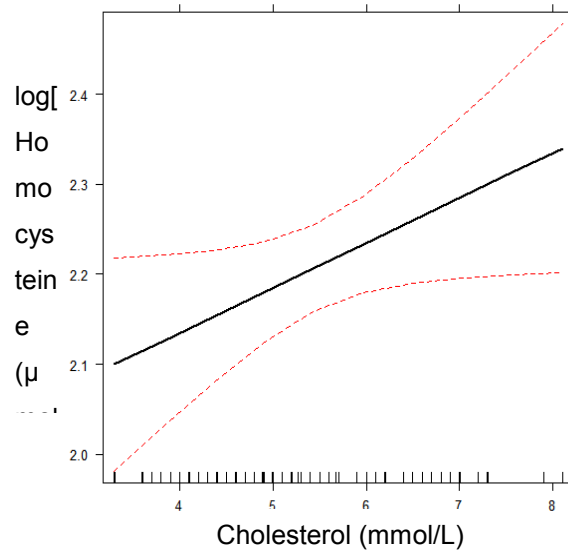


Figure 3.33: Graph depicting the significant correlation between cholesterol and homocysteine levels ($p < 0.05$), after adjusting for age, gender and MS diagnosis. The solid line shows modelled effect and broken lines are 95% confidence levels for estimates.

For every 1 unit increase in the saturated/trans fat score the BMI increased with 0.13 kg/m², ($p < 0.001$) while BMI decreased with 0.20 with every 1 unit increase in the folate score ($p < 0.02$). Intake of folate in the diet also had a statistically significant effect on homocysteine levels in females ($p < 0.02$). For every 1 unit increase in the folate score, homocysteine levels decreased with 1.5%. These differences were similar in MS patients and controls (Figure 3.34).

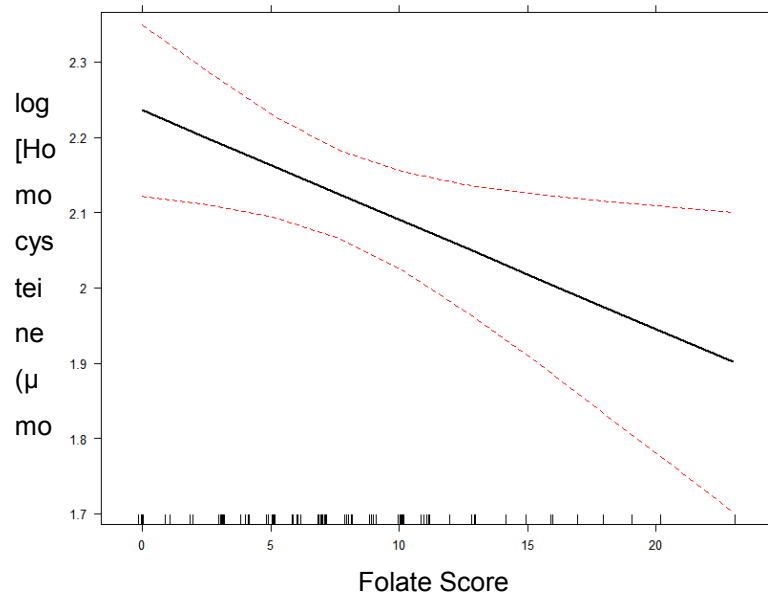


Figure 3.34: Graph depicting homocysteine levels in females reducing as the dietary folate score increases ($p < 0.02$). The black line shows a modelled effect for a female of average age and the red broken lines are 95% confidence levels for estimates

Genotype and lifestyle factors in relation to BMI

Control individuals with two copies (AA homozygote) of the obesity-associated minor allele of *FTO* rs9939609 were more likely (41%) to be obese (BMI ≥ 30 kg/m²) compared with heterozygotes (21.5%) or those individuals with two copies of the common T-allele (28.8%). The average BMI was the highest (35.1 kg/m²) in obese control individuals homozygous for the *FTO* rs9939609 A-allele compared to all other genotype groups evaluated. Similar observations were not made in the MS patient study group. The likelihood that the known deleterious effect of the risk-associated *FTO* rs9939609 A-allele on weight gain in the general population (Figure 3.35) may be eliminated by a healthy diet and/or high physical activity was therefore assessed further.

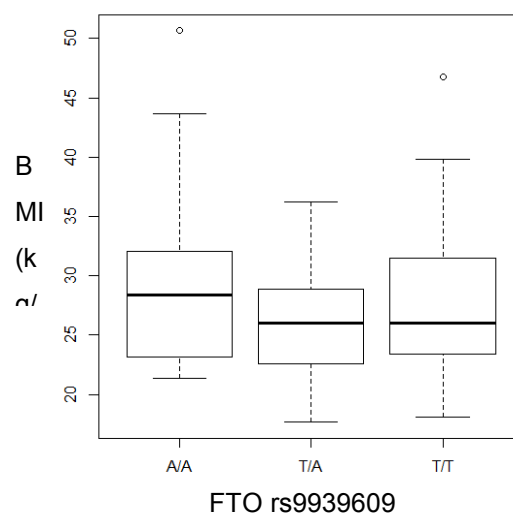


Figure 3.35: Effect of the *FTO* rs9939609 polymorphism on increased body mass index (BMI) in the control population.

Diet

Of the 13 obese *FTO* rs9939609 AA homozygotes, 11 (85%) had a low folate intake compared with only 2 (15%) with a moderate-to-high folate score. The proportion of the study population with a low folate score was markedly higher in obese subjects with the *FTO* rs9939609 AA genotype compared with all other genotype groups. In the 12 normal-weight individuals with the *FTO* AA genotype, 4 (33%) had a favourable high folate score. The interaction effect of *FTO* rs9939609 with increased dietary folate intake on BMI is shown in Figure 3.36.

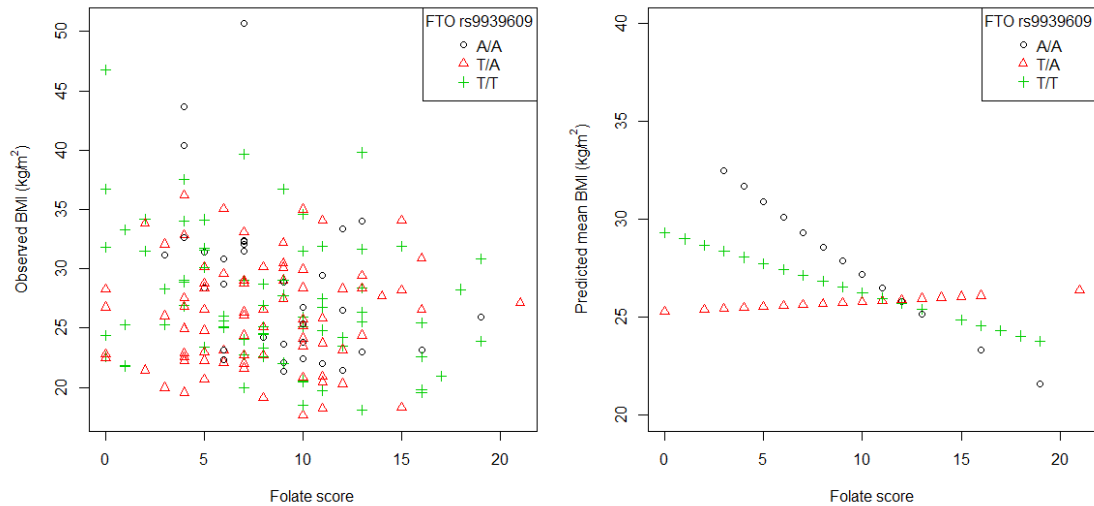


Figure 3.36: Effect of the interaction between *FTO* rs9939609 and folate score on BMI in the control group. Left: Observed BMI. Right: Predicted mean BMI with increased folate intake.

In addition to the favourable effect of a high folate score on BMI, evident in 4 of the 12 normal-weight control individuals homozygous for the risk-associated *FTO* A-allele, a low saturated/trans fat score correlated with a normal BMI in 10 (83.3%) individuals predisposed to obesity. The interaction effect of *FTO* rs9939609 with increased intake of saturated/trans fats on BMI is shown in Figure 3.37.

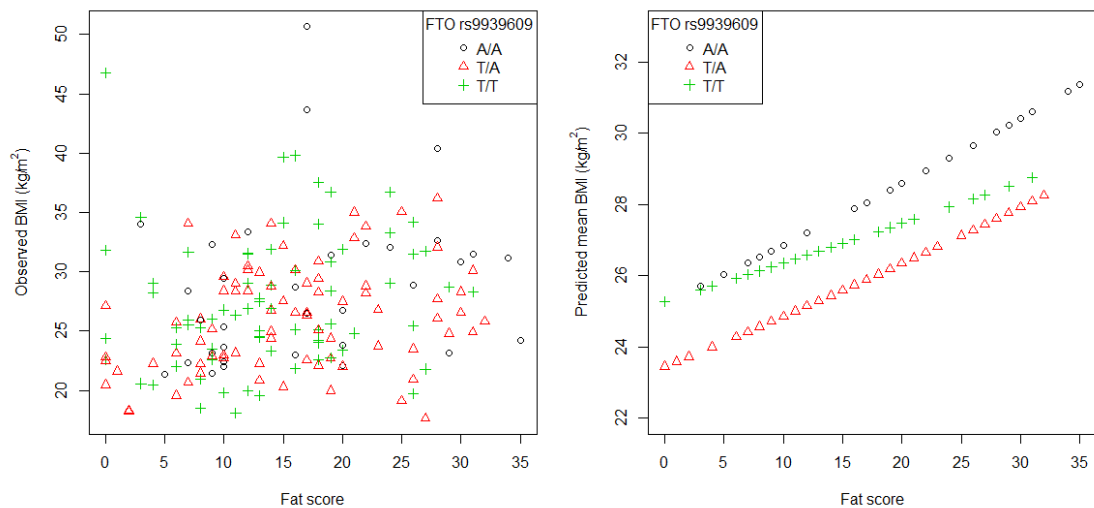


Figure 3.37: Effect of the interaction between *FTO* rs9939609 and saturated/trans fat score on BMI. Left: Observed BMI in the control group. Right: Predicted mean BMI with increased intake of saturated/trans fats.

Physical activity

Moderate physical activity resulted in a reduction in BMI, but the effect was not statistically significant. However, in individuals with a high physical activity score, BMI reduced by 9.9% (95% CI: 4.0 to 15.4%) compared to those with a low physical activity score, after adjusting for age, gender and MS diagnosis, in the combined group ($p < 0.006$).

Several *FTO* rs9939609 AA homozygotes reported a high physical activity level consistent with their normal BMI, despite this genetic predisposition for obesity. The interaction effect of *FTO* rs9939609 with different physical activity levels is shown in Figure 3.38.

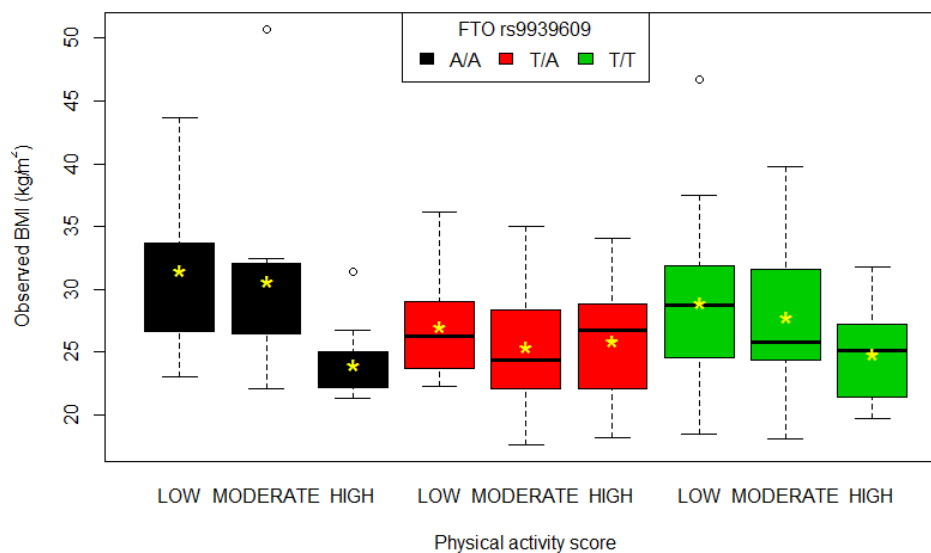


Figure 3.38: Effect of the interaction between *FTO* rs9939609 and level of physical activity on BMI. The yellow stars show the modelled effects.

EDSS in MS patients

Smoking was associated with worse clinical outcome as assessed by EDSS in MS patients ($p < 0.001$) (figure 3.39). Among the non-smokers, 32 (86%) scored lower than 5 (favourable) on EDSS, while only 2 (15%) smokers did.

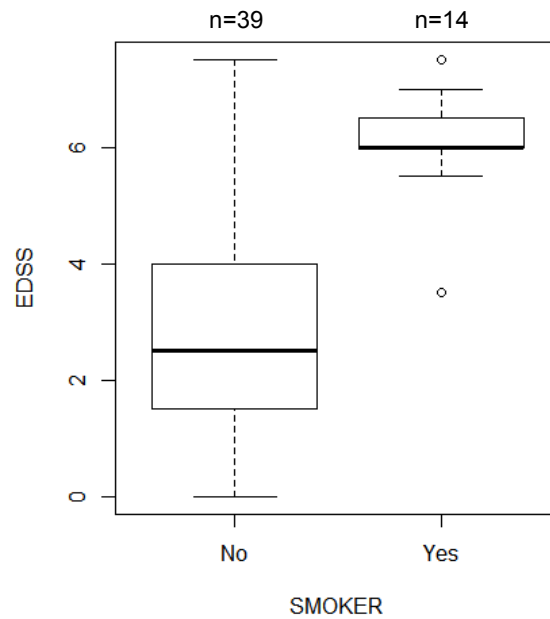


Figure 3.39: Box plot depicting the significant deleterious effect ($p < 0.001$) of smoking on MS disability as assessed by EDSS.

In a sub-analysis of the fruit/vegetable and fibre score, intake of at least five fruits and vegetables per day resulted in a favourable lower EDSS ($p=0.035$) as a measure of MS disability. A reduction of 28% in EDSS was noted for each extra day in which at least 5 portions of fruits/vegetables were consumed (Figure 3.40).

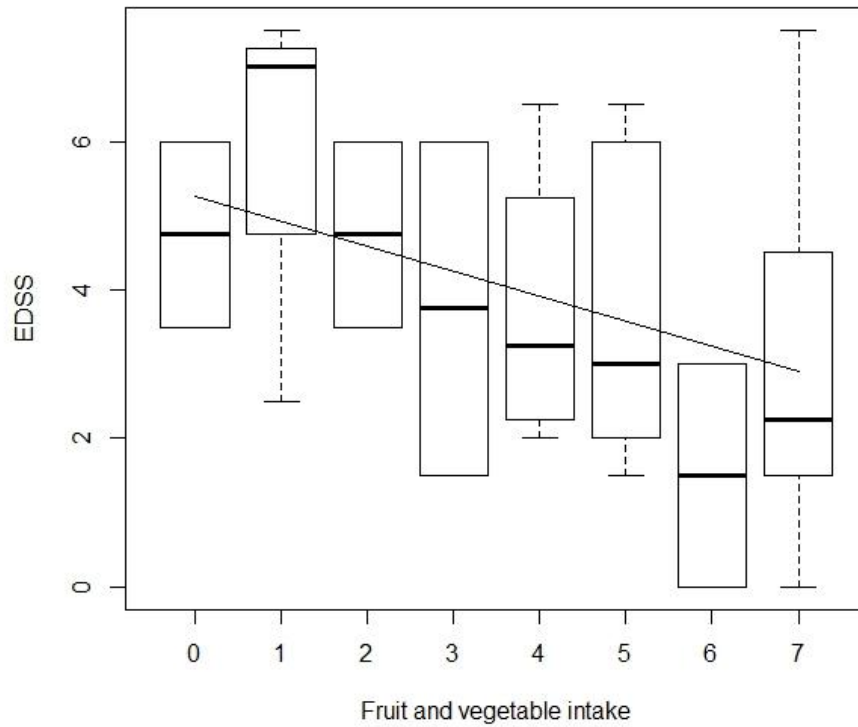


Figure 3.40: Box plots depicting the significant association ($p=0.035$) between EDSS and number of days per week of eating at least five fruits and vegetables per day in MS patients over the previous three months.

The minor low-activity *COMT* rs4680 (472 G>A) allele was associated with MS less disability after adjusting for gender ($p=0.040$). Patients with the *COMT* rs4680 (472 G>A) AA genotype scored on average 1.9 points lower on EDSS than patients with the common G allele (Figure 3.41).

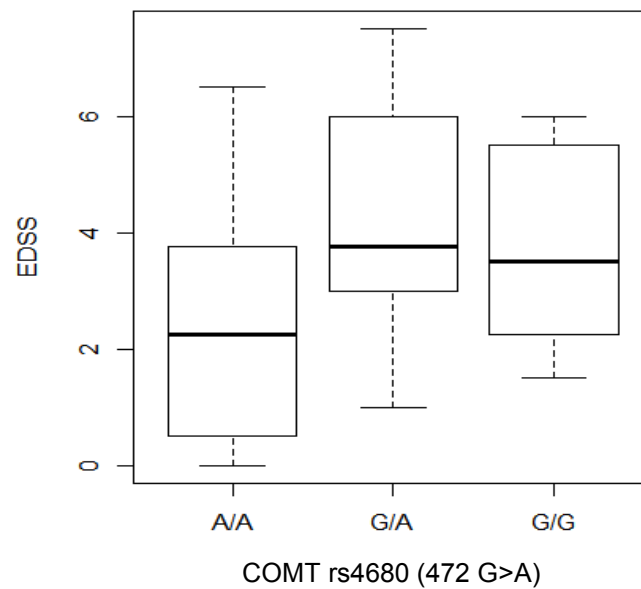


Figure 3.41: Box plot depicting the significant association ($p=0.044$) between the low-activity *COMT* A-allele and reduced EDSS indicating a favourable recessive gene effect.

The deleterious effect of the major COMT rs4680 G-allele may be modified in MS patients eating at least five fruits and vegetables per day and/or those who smoke. Table 3.11 shows the number of MS patients according to smoking status and daily intake of at least five fruits and vegetables in relation to the COMT rs4680 genotype and the EDSS score. A joint statistical model consisting of a weighted average of genetic and lifestyle factors evaluated in relation to EDSS could explain approximately 50% of the variation in EDSS (data not shown) and will be further refined in future studies.

Table 3.11 Summary of genotype distribution of COMT rs4680 (472 G>A) according to relevant lifestyle factors reported in MS patients with an EDSS score ≤ 3 indicative of benign MS as well as ≥ 6 indicative of disability that requires assistance to walk.

Genotype vs. EDSS score ≤ 3	COMT GG	COMT GA	COMT AA
Total (n=22)	6 (27%)	7 (32%)	9 (41%)
Smoker (n=0)	0	0	0
≥ 5 fruits & vegetables daily (n=16)	4 (25%)	4 (25%)	8 (50%)
Non-smokers with intake of ≥ 5 fruit/veg daily (n=16)	4 (25%)	4 (25%)	8 (25%)
Genotype vs. EDSS score ≥ 6	COMT GG	COMT GA	COMT AA
Total (n=12)	3 (25%)	7 (58%)	2 (17%)
Smoker (n=7)	1 (14%)	4 (57%)	2 (29%)
≥ 5 fruits & vegetables daily (n=6)	1 (17%)	5 (83%)	0
Non-smokers with intake of ≥ 5 fruit/veg daily (n=3)	1 (33%)	2 (67%)	0

Chapter 4

Discussion

Due to its multifactorial nature, the aetiology of MS remains unknown although it is generally accepted that both genetic and environmental factors, including poor nutrition and lifestyle factors, contribute to disease susceptibility, progression and quality of life. In this study functional SNPs in the *FTO* (rs9939609, intron 1 T>A), *MTR* (rs1805087, 2756 A>G), *MTRR* (rs1801394, 66 A>G), *MTHFR* (rs1801133, 677 C>T and rs1801131, 1298 A>C) and *COMT* (rs4680, 472G>A) genes were evaluated in 114 MS patients and 195 control individuals without neurological diseases to assess the relationship between genetic and environmental risk factors as may be reflected by biochemical abnormalities and other clinical indicators implicated in the disease process.

This study first focused on the analytical validation of high-throughput genotyping assays that can be applied in future 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. An important aim was to elucidate the mechanism underlying raised homocysteine levels in MS patients, previously shown in numerous studies including meta-analyses (Ramsaransing *et al.* 2006; Triantafyllou *et al.* 2008; Zhu *et al.* 2011; Zoccolella *et al.* 2012). The long-term objective of the MS research program is to improve the clinical management of MS patients based on a scientific understanding of the role of inflammation, oxidative stress, iron dysregulation and the methylation metabolic pathway in the disease process.

This study has shown for the first time to our knowledge that the underlying disease process of MS moderates the effect of the *FTO* rs9939609 polymorphism on homocysteine levels. Raised homocysteine was associated with *FTO* rs9939609 in an additive allelic model in MS patients ($p=0.003$), while a similar effect was not observed in the control group. This finding suggests that *FTO* is involved in the mechanism underlying the association between MS and raised homocysteine levels due to a cumulative effect exacerbated by the disease process and dietary factors.

This study represents an important step towards a global trend to develop cost-effective laboratory assays that can facilitate disease prevention, diagnosis and treatment in the era of personalised medicine. Demonstration of analytical validation, clinical validation and clinical utility are essential components of the test development process (Grosse and Khoury 2006).

4.1 Analytical validation of high-throughput real time (quantitative) PCR

Analytical validity refers to the provision of an accurate SNP detection system, based on the agreement between different genotyping methods. It also relates to the degree to which indirect SNP detection methods and genotyping of single nucleotide polymorphisms (SNPs) consistently produce the correct results. When DNA samples of known genotype are not available as positive and negative (internal) controls, typically provided in commercially available test kits, the test results need to be compared against direct sequencing considered to be the gold standard. This way such controls can be provided for future use in routine genetic tests and for research purposes.

DNA extracted from 195 unrelated Caucasian control individuals (67 male and 128 female) were used to perform analytical validation of the high-throughput real time polymerase chain reaction (RT-PCR) genotyping method, against direct sequencing as the gold standard. Conventional PCR, followed by direct sequencing of the PCR fragments, was performed on 5 internal control samples (k1-5) for detection of the selected SNP. This resulted in the identification of the three different genotypes (positive and negative controls) to be used for analytical validation of high throughput genotyping (RT-PCR). The internal control samples (k1-5) were included in each RT-PCR run and compared to the direct sequencing results to corroborate the results generated during RT-PCR genotyping. Such comparison of the RT-PCR results with direct sequencing has been proven to be a dependable approach for analytical validation of new genotyping methods.

In this study RT-PCR amplification, using fluorescence resonance energy transfer probes, was successfully used to genotype DNA samples of the controls for *FTO* (rs9939609, intron 1 T>A), *MTR* (rs1805087, 2756 A>G), *MTRR* (rs1801394, 66 A>G), *MTHFR* (rs1801133, 677 C>T and rs1801131, 1298 A>C) and *COMT* (rs4680, 472G>A). The results were corroborated through the inclusion of direct sequenced internal controls. This provided a method for simultaneously analysing large numbers of DNA samples for the present study.

RT-PCR is almost identical to conventional PCR. Conventional PCR is used to amplify and simultaneously quantify a specific DNA region, with detection of the PCR product when the reaction is completed. RT-PCR enables both quantification and detection in “real time” as the reaction progresses. Two common methods used for product detection in RT-PCR are non-specific fluorescent dyes that intercalate with

any double-stranded DNA and sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target. The latter was used in this study. ABI™ *TaqMan*® pre-designed SNP Genotyping Assays were used to detect the SNPs of interest and consisted of two primers for amplifying the polymorphic sequence of interest and two *TaqMan*® MGB probes for distinguishing between the two alleles. Each *TaqMan*® MGB probe contains a reporter dye at the 5' end of each probe, VIC® dye is linked to the 5' end of the allele 1 probe and FAM™ dye is linked to the 5' end of the allele 2 probe.

RT-PCR was analytically validated against direct sequencing as the gold standard for use in the genotyping analysis for all the SNPs used in this study.

4.2 Comparison of genotype distribution and allele frequencies between study groups

Following the successful optimisation of the RT-PCR assays for *FTO* (rs9939609, intron 1 T>A), *MTR* (rs1805087, 2756 A>G), *MTRR* (rs1801394, 66 A>G), *MTHFR* (rs1801133, 677 C>T and rs1801131, 1298 A>C) and *COMT* (rs4680, 472G>A) SNPs in the 195 Caucasian control individuals, 114 unrelated Caucasian MS patients were genotyped using the Applied Biosystems™ (ABI) *TaqMan*® SNP Genotyping Assays on the Corbett Rotor-Gene™ 6000. Only Caucasians were included in the present study as there were only 4 MS patients of Mixed Ancestry identified in the original study population. They were excluded from analysis as inclusion could have affected the power if the statistical analysis negatively.

All six SNPs were found to be in Hardy-Weinberg equilibrium (HWE). The genotype distribution and allele frequencies of the SNPs studied did not differ significantly between males and females, or between patients and controls. The allele frequencies and genotype distribution found in our study population were similar to that described previously in the Caucasian population of South Africa and elsewhere.

In this study 19% of the control and 16% of the MS study population had both *FTO* rs9939609 obesity risk alleles, which is similar to the 18% found in Western and Central Europeans in a study by Ho *et al.* (2010). The frequency of the risk associated A-allele was found to be 46%, very similar to the 40% found in our study, for both the patients and controls.

MTR rs1801394 genotype distribution in our control study population (68% AA, 29% AG, 3% GG) and MS patients (62% AA, 32% AG, 6% GG) was similar to a study of 805 individuals in Brazil (65% AA, 31% AG, 4%GG) (Galbiatti *et al.* 2010). They found the minor G allele frequency to be 23.6% compared to our 18% and 22% in the control and MS patients respectively.

The *MTRR* rs1801394 polymorphism showed similar genotype distribution and minor allele frequencies in our study compared to those reported in the literature. Only 28% of our control population and 17% of the MS patients presented with the wild type AA genotype compared to 23% observed in previous studies. The mutant G-allele was found to be 53% compared to 48% in our control and 57% in the South African MS population studied (ncbi.nlm.nih.gov; opensnp.org).

Scholtz *et al.* (2002) reported that the *MTHFR* rs1801133 polymorphism minor allele frequency differs significantly among the different South African population groups, with the lowest allele frequency detected in the Black population (4%), intermediate in the Coloured population (18%) and highest in the Caucasian population (36%). We found the same in our Caucasian control population with the minor T-allele frequency being 37%, with MS patients 33%. The homozygous mutant genotype was found to be approximately 10% in previous studies, which is very close to the 11% identified in our MS population and slightly lower than the 15% in the control population (Matthews 2001; Scholtz *et al.* 2002).

The genotype distribution of the *MTHFR* rs1801131 did not differ significantly from that found in previous studies. In a Swedish study the minor C-allele frequency was 33%, with 12% of the Caucasian control population being homozygous (CC) for the rs1801131 (1298 A>C) polymorphism and 46% wild type (AA) (Guelpen *et al.* 2006). We found the wild type genotype to be present in 47% of control individuals and 54% MS patients with the minor C-allele frequency 31% and 26% for the two groups respectively.

The *COMT* rs4680 genotype distribution in MS patients (25% GG, 45%GA, 30% AA) and control individuals (29% GG, 46% GA, 29% AA) in the present study was very similar to that described in the literature (24% GG, 49% GA, 27% AA). The minor allele frequency in MS patients (53%) and control individuals (51%) were also the same as previous findings in other studies (51%) (Martinez *et al.* 2009).

A genotype risk score (0-10) was calculated for each individual by counting the minor alleles for *FTO* rs9939609 (intron 1 T>A), *MTR* rs1805087 (2756 A>G), *MTRR*

rs1801394 (66 A>G), *MTHFR* rs1801133 (677 C>T) and *MTHFR* rs1801131 (1298 A>C). A minimum risk score of 0 and a maximum of 7 were obtained in both the control and patient study groups. None of the patients or controls was homozygous for all 5 SNPs, which would correspond to the highest risk score of 10. No significant association with the risk score, or difference between the controls and MS patients, was detected regarding MS susceptibility. No difference was also detected when *COMT* rs4680 (472 G>A) was considered as part of the risk score, either including G or A as the risk-associated allele. Both the minor/low-activity or common/high-activity allele of *COMT* rs4680 (472 G>A) may be considered a risk allele depending on the clinical context.

4.3 Evaluation of genotype and lifestyle factors influencing intermediate risk phenotypes

Genotype-phenotype association studies were performed after adjustment for potential confounding effects of gender, age and lifestyle risk factors where appropriate. Relevant variables documented were correlated with the observed genotypes and allele counts. Clinical outcomes were evaluated in relation to homocysteine levels, BMI and EDSS score at baseline. Adjustments were not made for medications known to influence homocysteine levels in the statistical models as none of the patients reported treatment with folate antagonists.

As was also evident in this study, MS is more common in females compared with males (Loren and Rolak, 2002). In the view of this and the fact that MS patients were on average 4 years younger than controls, all statistical analyses were performed after adjustment for age and gender. Homocysteine was significantly higher (29%) in males compared with females ($p < 0.001$) in both the MS patients and controls studied, after adjusting for age. This finding is in accordance with previous studies showing that homocysteine levels are affected by both age and gender (González-Gross *et al.* 2012; Zoccolella *et al.* 2012). Homocysteine was shown to increase with age, by 0.56% per year, resulting in an increase of 5.6% every 10 years in our study population.

While hypercholesterolaemia, hypertension, diabetes and peripheral vascular disease were previously identified as important risk factors for MS, the mechanism underlying elevated homocysteine levels in MS remains to be identified (Zhu *et al.* 2011). No significant differences were observed between the MS patients and controls analysed in the current study in relation to several vascular risk factors

evaluated, including total cholesterol and BMI. However, homocysteine levels were found to be significantly higher in MS patients compared to controls in males, but not in females (interaction $p=0.017$). Zoccolella *et al.* (2012) reported that plasma homocysteine levels are higher in male MS patients but not in females compared to neurological disease controls. This result was shown to be independent of possible confounding factors such as age and folate and cobalamin levels.

Females with MS presented with significantly higher vitamin B₁₂ levels compared to controls ($p=0.024$) in the present study. This observation may be ascribed to self-reported vitamin B₁₂ injections commonly administered to MS patients suffering from chronic fatigue. Defects in vitamin B₁₂ metabolism (whether due to low B₁₂ levels, oxidation of the cobalt atom or due to genetic variations) contribute to defective myelination as a result of inappropriate fatty acid synthesis, resulting in the incorporation of odd-chain and methyl branched fatty acids into myelin (Kishimoto *et al.*, 1973; Ramsay *et al.* 1977).

A six month pilot study performed in 18 South African MS patients highlighted the importance of a functional folate-vitamin B₁₂-methylation pathway and adequate availability of nutrients required as enzyme co-factors and for myelin production (van Rensburg *et al.* 2006). A nutritional regimen designed to promote myelin regeneration were shown to improve disability significantly in compliant patients, as measured by the EDSS. Notably, homocysteine accumulation in MS patients was also associated with cognitive impairment in the study performed by Russo *et al.* (2008), which supports measurement of homocysteine as a marker of oxidative stress and inflammation across clinical domains.

Genotype and lifestyle factors in relation to homocysteine

Possible genotype association with homocysteine levels was assessed in a subset of 60 MS patients and 87 controls. Neither *MTR* (rs1805087, 2756 A>G), *MTRR* (rs1801394, 66 A>G), *MTHFR* (rs1801133, 677 C>T and rs1801131, 1298 A>C) *COMT* (rs4680, 472G>A) nor the combined risk score showed any significant association with any of the biochemical parameters measured.

Homocysteine levels were associated with *FTO* rs9939609 in an additive allelic model in the MS study population, but not in controls, after adjustment for age and gender ($p=0.003$). This has shown for the first time to our knowledge that the underlying disease process of MS moderates the effect of the *FTO* rs9939609 polymorphism on homocysteine levels. The risk-associated A-allele was associated

with increased homocysteine levels in both the gender adjusted and female MS patients ($p=0.006$). In the presence of each A allele, homocysteine increased with 15% in MS patients. This translated into a 30% increase in homocysteine levels among MS patients homozygous for *FTO* rs9939609. When males were excluded from this analysis the presence of each A-allele increased the homocysteine levels by 14% in female MS patients.

This finding may be important as it has been suggested that homocysteine plays a role in MS neurodegenerative phases through different potential mechanisms of action (Zoccolella *et al.* 2011). Experimental *in vitro* studies have shown that homocysteine may determine mitochondrial dysfunction, enhance oxidative stress, activate apoptotic pathways, and increase cytosolic calcium. Excitotoxicity on neurons by means of overstimulation of N-methyl-D-aspartate receptors may also be induced by homocysteine (Mattson and Shea, 2003; Moore 2010; Obeid and Herrmann, 2006; Tjattas *et al.* 2004). Additionally, increased levels of homocysteine may lead to or induce neuro-inflammation (Obeid and Herrmann, 2006), interfere with the response of natural killer cells, adhesion molecules, and both B and T lymphocytes (Kim *et al.* 1997; Koga *et al.* 2002), and reduce SAM, the important methyl donor in various methylation reactions (Mattson and Shea, 2003). All of the above mentioned mechanisms have been described to be relevant in the pathogenesis and/or progression of MS.

Vascular risk factors and obesity have also been implicated in disease development and progression in MS. Significant correlations were observed between BMI and homocysteine levels as well as cholesterol and homocysteine levels in relation to various vascular risk factors and lifestyle factors. These findings were similar in MS patients and controls as no differences in association were detected between these study groups, after adjustment for age and gender, and in females adjusted for age only.

When adjusted for MS status, age and gender, homocysteine levels correlated significantly with total cholesterol levels ($p=0.048$). No difference was noted between MS patients and controls, suggesting that this correlation is not disease specific. For every one unit increase in cholesterol, homocysteine levels increased with 5% in the present study, irrespective of age, gender and MS status. This finding is similar to previous studies (Nygård *et al.* 1995; O *et al.* 1998; Olszowski *et al.* 1989), where a positive correlation between increased levels of cholesterol and high plasma levels of homocysteine has been observed in patients with homocysteinemia.

Recently Marrie *et al.* (2010) highlighted the connection between five vascular risk factors and MS disease progression which confirms the importance of the above mentioned findings. These included hypercholesterolemia, hypertension, diabetes, heart disease and peripheral vascular disease. Participants in the study who reported one or more vascular comorbidity at the time of diagnosis had a more than 1.5-fold increase in risk of ambulatory disability. This translated in up to 6 years in the time from diagnosis to needing a walking stick for ambulation. This can be equal to an EDSS score of around 5-6, indicating moderate disability.

In the present study it was found that every one unit increase in BMI resulted in a 1.3% increase in homocysteine levels. Conflicting results between BMI and homocysteine levels have been reported (Hultdin *et al.* 2005; Nakazato *et al.* 2011; Osganian *et al.* 1999) with some studies finding a weak association and other studies not. Similarly we found the statistical significant positive relationship between homocysteine and BMI only in the female population (patients and controls) and not in the whole study population.

In the female population (patients and controls), the intake of folate in the diet had a statistically significant effect on homocysteine levels ($p=0.011$). In our study we found that homocysteine decreased by 1.5% for every 1 unit increase in the folate score. Similar results were found by Zappacosta *et al.* (2013), who showed that homocysteine levels were reduced as a result of a folate enriched diet, or 5-MTHF or folic acid supplementation to a similar extent compared to baseline levels and significantly compared to placebo. As a consequence of the biochemical reactions in which homocysteine is involved, deficiencies of the vitamins folic acid (B_9), pyridoxine (B_6), or cobalamin (B_{12}) can also lead to high homocysteine levels (Selhub 1999; Miller 1994). The inverse relationship between folate and homocysteine found in the present study shows that by treating possible causes of elevated homocysteine through a folate rich diet, homocysteine levels can be lowered.

Genotype and lifestyle factors in relation to BMI

The *FTO* rs9939609 risk associated A-allele has been associated with a higher BMI and obesity risk in various studies and meta-analyses (Ewens *et al.* 2011; Frayling *et al.* 2007; Hardy *et al.* 2010; Huang *et al.* 2012; Loos *et al.* 2008; Peng *et al.* 2011; Sentinelli *et al.* 2012; Steemburgo *et al.* 2012; Tabara *et al.* 2009; Vasan *et al.* 2012). Ortega-Azorín *et al.* (2012) suggested that the traditional Mediterranean diet (MedDiet), rich in folate, fruits, vegetables, fish, legumes, olive oil and nuts and low in saturated fat, could modulates the effect of *FTO* rs9939609 on diabetes risk. They

found that if adherence to the MedDiet was low, individuals with the *FTO* rs9939609 obesity risk associated A-allele had a higher risk of developing type 2 diabetes (OR=1021, 95%CI: 1.03-1.40; p=0.019) than people with the wild type TT genotype. Interestingly, individuals with high adherence to the MedDiet showed no association of this polymorphism with type 2 diabetes risk (OR=0.97, 95%CI: 0.85-1.16; p=0.673). Further adjustment for BMI, tobacco smoking, alcohol consumption or education did not have an effect on the statistical significance of the results. They also found that when individuals' folate intake was low, those with the obesity risk associated allele had higher fasting plasma glucose concentration compared with TT genotype individuals. This interaction was once again not observed when folate intake was high. These findings suggest that the effect of the *FTO* rs9939609 polymorphism may be dependent on the dietary patterns.

Consistent with the finding that the known deleterious effect of the risk-associated *FTO* rs9939609 A-allele on weight gain in the general population may be modulated by a healthy diet and/or high physical activity (Ortega-Azorín *et al.* 2012), the present study confirmed an effect of folate intake on obesity in the control. For every 1 unit increase in the folate score BMI decreased with 0.20 kg/m² (p<0.02). Of the 13 obese *FTO* rs9939609 AA homozygotes, 11 (85%) had a low folate score compared with only 2 (15%) with a moderate-to-high folate score. The proportion of the study population with a low folate score was higher in obese subjects with the *FTO* rs9939609 AA genotype compared with all other genotype groups. In the 12 normal-weight individuals with the *FTO* AA genotype, 4 (33%) had a favourable high folate score.

In individuals with a folate score above 13, the effect of *FTO* rs9939609 on BMI was neutralised. When the folate score decreases, the deleterious effect of *FTO* rs9939609 became evident on increased BMI. This finding validates the folate score of Gknowmix™ medical history and lifestyle questionnaire used in our study cohort.

In addition to the favourable effect of a high folate score on BMI, evident in 4 of the 12 normal-weight control individuals homozygous for the risk-associated *FTO* A-allele, a low saturated/trans fat score correlated with a normal BMI in 10 (83.3%) individuals predisposed to obesity. BMI was shown to increase with 0.13 kg/m² for every 1 unit increase in the saturated/trans fat score (p<0.001). We found that the interaction between *FTO* rs9939609 and the saturated/trans fat score were most notable between the mutant AA-genotype and heterozygous TA-genotype. A Brazilian study by da Silva *et al.* (2012) investigating the association between

variants in the *FTO* gene and anthropometric phenotypes related to obesity found similar results. In their study they found significant differences regarding BMI between the TA and AA genotype, but not between TT and AA. This was asserted to the moderate sample size evaluated or some gene-gene or environmental interaction that could not have been addressed with their study approach.

In the present study the MS patients reported a significant lower level of physical activity than the control population ($p < 0.001$). This result may be expected due to the disabling effect of MS. BMI was found to be reduced by moderate physical activity, but not significantly. High physical activity was found to reduce BMI by 9.9% (95% CI: 4.0 to 15.4%) compared to low activity ($p < 0.006$), after adjusting for age, gender and MS diagnosis, in the combined group. A study investigating the effect of the *FTO* rs9939609 polymorphism on body fat accumulation found that the *FTO* A-allele was highly associated with obesity associated measures including BMI in a white Danish study group. ($p = 0.002$). They also found that low physical activity accentuates the effect of the *FTO* rs9939609 polymorphism on body fat accumulation and subsequently BMI (Andreasen *et al.* 2008). Several *FTO* rs9939609 AA homozygotes identified in this study reported a high physical activity level consistent with their normal BMI, despite this genetic predisposition for obesity.

BMI also correlated with CRP levels in the combined group, showing a significant ($p < 0.001$) increase of 1.8% (95% CI: 0.8 to 2.8%) in BMI for every unit increase in CRP. This result was found in both the MS and control population. The presence of a causal relationship between BMI and CRP is disputed, and a study investigating the direction of causality between BMI and CRP found that the strong association between the two is likely to be driven by BMI. CRP is a marker of elevated adiposity (Timpson *et al.* 2011). A study investigating CRP levels and disease progression in MS showed that CRP levels were similar in MS patients and healthy control individuals, but higher in MS patients during relapse than in remission. CRP is an acute-phase hepatic protein induced by tumour necrosis factor (TNF)- α , interleukin (IL)-1, IL-6, oncostatin and leukaemia inhibitory factor (Soilu-Hänninen *et al.* 2005)

4.4 Evaluation of genotype and lifestyle factors influencing MS disability status

The Expanded Disability Status Scale (EDSS) is a method of quantifying disability in MS (Kurzke 1983). Functional systems such as cerebellar, pyramidal, sensory, brainstem, bowel and bladder, cerebral, visual and other, are measured for disability,

allowing neurologists to assign a functional system score (FSS) in each category. People who are fully ambulatory fall in the EDSS range of 1.0 to 4.5 with impairment to ambulation defined as an EDSS score of 5.0 to 9.5 with 10 being death due to MS (Kurtzke 1983). Significant effects of lifestyle and diet were observed in relation to EDSS as a measure of MS disability in MS patients

Similar to Healy *et al.* (2009), we found a positive association between smoking and higher EDSS scores ($p < 0.001$) in MS. The Gknowmix™ medical history and lifestyle questionnaire data documented in our study population showed a worse clinical outcome as assessed by EDSS due to smoking in MS patients ($p < 0.001$). Among the non-smokers, 32 (86%) scored lower than 5 (favourable) on EDSS, while only 2 (15%) smokers did. MS patients were also more likely to be current smokers than control individuals ($p < 0.001$). These results confirm the findings of the United Kingdom cohort study (Manouchehrinia *et al.* 2013), that smoking is associated with a more severe disease and faster disability progression. Smoking cessation was also associated with a slower progression of disability. The survival of oligodendrocytes, the cells that synthesise myelin, and maturation of oligodendrocyte precursor cells (adult stem cells resident in the brain that have the capacity to remyelinate axons) would be especially vulnerable to cigarette smoke – even passive smoking – as a result of an oxidative effect (Van Rensburg *et al.* 2010).

As a possible counter measure to worsening EDSS in MS patients, the present study demonstrated that the intake of at least five fruits and vegetables per day have a favourable lowering effect on the EDSS score ($p = 0.035$). A reduction of 28% in EDSS was noted for each extra day in which at least 5 portions of fruits/vegetables were consumed. This result confirms that nutrition plays an important role in MS disease course. A recent study similarly found that people consuming a diet high in green leafy vegetables, hydrogenated fats, tomato, yellow vegetables, fruit juices, onion and other vegetables had a lower risk of developing MS ($p = 0.026$; OR=0.42; 95% CI: 0.19-0.90) (Jahromi *et al.* 2012). These findings supported our results and could in future be incorporated into a MS management programme.

A possible protective recessive effect of the minor low-activity *COMT* rs4680 A allele on EDSS was also associated with MS disability ($p = 0.040$), after adjusting for gender. Patients with the *COMT* rs4680 (472 G>A) AA genotype scored on average 1.9 points lower than patients with the minor GG allele. The *COMT* rs4680 polymorphism affects the thermolability of the enzyme, altering enzyme activity and protein abundance. The effect in enzyme activity is a three- to four-fold decrease in

COMT enzyme activity leading to a reduction of noradrenalin catabolism within synapses (Chen *et al.* 2004; Lachman *et al.* 1996; Lotta *et al.* 1995; Shield *et al.* 2004). This lowering of activity and the resulting reduction of noradrenalin catabolism within synapses is postulated to decrease inflammation occurring in neuro-inflammatory diseases. Noradrenalin may confer a natural immunosuppressive protection within the normal healthy brain environment by reducing the role of antigen-presenting cells (APCs), as brains from healthy individuals demonstrate a lower threshold of inflammation compared to other organs (Lassmann *et al.* 1991).

4.5 Study Limitations

Detailed information on clinical, biochemical and environmental factors relevant to the development of MS were not available for all study participants investigated in this study. This issue resulted in a significant reduced total study population for statistical analysis and as a result may explain some common correlations not found.

Not all genetic variations possibly implicated in homocysteine dysregulation and the genetic susceptibility of multiple sclerosis were analysed. Only Caucasians were included in the present study.

Chapter 5

Conclusion and Future Prospects

As the application of genetic testing expanded from single-gene disorders to multi-factorial disorders over recent years, the clinical utility of genetic tests became increasingly important. Such tests are most useful when improvement of adverse health outcomes such as disability, morbidity, and/or mortality are obvious or can be proven through adoption of effective interventions in health outcomes studies. Clinical utility is defined as the ability to prevent or administer therapies to remove, reduce or defer the risk of disease, while clinical validity refers to how well the test results detect or predict the associated disorder (e.g. sensitivity, specificity).

When a mutation with low penetrance, as analysed in this study, is detected in an individual, this person will not necessarily develop the associated disease, as the risk may depend on the co-existence of relevant environmental triggers. This means that genetic risk factors may determine disease susceptibility (increased risk), while environmental factors may determine disease development. By knowing which genes interact with which environmental factors it is possible to potentially prevent the disease or at least minimise cumulative risk. Diet and lifestyle changes are usually effective to reduce the risk associated with low-penetrance gene mutations as those demonstrated in this study. However, when the risk of disease has not been identified early enough before symptoms developed, more intensive treatment might be needed. Medical intervention is often necessary when someone inherited a high-penetrance mutation, which implies that the associated disease will develop with little or no influence from the environment, if left untreated. For this reason, it is important to always consider the medical and family history of a person when genetic testing is performed. The limitations of genetic testing can be overcome when performed within a pre-defined clinical profile in conjunction with relevant pathology tests to assess the phenotype expression of a gene and to monitor response to treatment (Kotze et al. 2013).

Ethical Considerations

While medical conditions are universal, any information obtained from genetic testing is highly personal; therefore a decision about genetic testing requires careful consideration. The following aspects are explained with use of the study medical history and lifestyle questionnaire, as part of the pre-test genetic counselling/consultation process, before specimens are collected for laboratory testing including genetics (reproduced with permission from www.Gknowmix.com):

- The genetic test will only screen for specific genetic alterations expected to provide useful information in relation to treatment/diet intervention.
- Detection of genetic alterations (positive test) implies that other family members may also have the genetic change(s).
- Failure to detect a specific genetic alteration (negative test result) does not exclude undefined gene mutations or other risk factors not tested for.
- Early detection or pre-clinical diagnosis of treatable or preventable genetic diseases is beneficial.
- Genetic testing may result in better motivation for lifestyle changes or targeted treatment, or possibly anxiety when genetic risk factors are identified in an individual without clinical symptoms of a disease.
- The genetic material is stored for reference purposes or to perform follow-up testing and may be stored and included in a genetic database for research related to the test requested, unless declined.
- The genetic material may be tested at different laboratories or using different mutation detection methods as part of a quality control process.
- 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.
- 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.
- 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.

Recommendations and Future Prospects

A web-based system has been established during the course of this study to simplify the follow-up process for assessment of the effectiveness of the intervention strategy that may in future be advised, based partly on the results obtained in this study. Monitoring of clinical outcome over time was beyond the scope of the present investigation. By using the automated data integration and reporting system, it is possible to combine clinical and lifestyle information with biochemistry and genetic test results for translation into a pathology supported gene-based intervention plan tailored to the needs of the individual (van Rensburg *et al.* 2012). Evaluation of

biochemical measurements and BMI relevant to genetic risk factors analysed, such as the *FTO* rs9939609 (intron 1 T>A), provides a means to assess phenotypic expression of the variant form of the gene and to monitor response to treatment, aimed at prevention of cumulative risk.

Detection of *FTO* rs9939609 using the genotyping method described in this study reinforces the importance of folate intake to reduce vascular risk as a consequence of homocysteine accumulation and obesity. It is possible that adequate intake of folate in the diet would diminish the effect of *FTO* rs9939609 on BMI. *FTO* rs9939609 as a single effect, or as part of the risk score, had no effect on BMI after adjustment for potential confounding factors including homocysteine and folate score. The nutrition and lifestyle assessment scores evaluated in this study provides a valuable clinical tool for identification of individuals at increased risk of obesity and associated medical conditions, including MS. This study not only confirmed the importance of homocysteine as a vascular risk factor in MS patients, but also provided a solution to counteract the effect of these levels on BMI and consequently MS risk. An implementation study is warranted to determine whether *FTO* rs9939609 and *COMT* rs4680 genotyping incorporated as part of a lifestyle modification program will translate into reduced incidence of MS and/or disease progression. While the minor low-activity *COMT* rs4680 allele was found to be associated with a lower MS disability as assessed by EDSS, intake of at least five fruits and vegetables per day resulted in a favourable lower EDSS with a reduction of 28% noted for each extra day at least five portions were consumed.

In conclusion, all the aims of the study have been accomplished. It was shown for the first time that the underlying disease process of MS moderates the effect of the *FTO* rs9939609 polymorphism on homocysteine levels. This finding is consistent with the role of *FTO* in demethylation and epigenetic changes. Identification of *FTO* rs9939609 reinforces the importance of adequate folate intake in the diet that can be assessed accurately with use of the study questionnaire applied in this study. This study therefore contributed to a practical application in future for improved clinical management of patients diagnosed with MS.

Chapter 6

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