DEVELOPMENT OF A PATHOLOGY-SUPPORTED GENETIC TEST FOR IMPROVED CLINICAL MANAGEMENT OF PATIENTS DIAGNOSED WITH MULTIPLE SCLEROSIS

BY

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Declaration

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

Signature:.....

Date:.....

Summary

The aetiology of multiple sclerosis (MS) remains largely unknown, due to its multifactorial nature with environmental and genetic factors contributing to the risk. Several investigations highlighted the important role of the genetic component influencing disease susceptibility and progression.

In the present study genetic variations in the *MTHFR* (1298 A>C and 677 C>T) and *HFE* (845 G>A) genes previously, shown to affect folate and iron metabolism respectively, were studied in the context of MS. The aim of the study was to contribute the laboratory component of a pathology supported genetic testing approach used to identify a subgroup of MS patients with altered nutritional requirements due to genetic susceptibilities. The study population included 90 patients with a clinical diagnosis of MS and 49 control individuals, without any signs or symptoms of the disease, drawn from the same age- and population group.

Three mutation detection systems were compared in terms of accuracy, sensitivity, cost effectiveness and ease of operation in relation to the *MTHFR* and *HFE* gene mutations analysed. Analytical validity of the genetic assays was an important consideration; therefore the respective real-time polymerase chain reaction (RT-PCR) methods were compared with direct DNA sequencing as the gold standard. The methodology included use of the ABI™ 7900HT, the Roche LightCycler® 480 II system and the Corbett Rotor-Gene™ 6000 5-plex HRM. The same genotype results were obtained for the DNA samples tested with the three RT-PCR methods. In terms of cost effectiveness, ease of operation and optimization, the Corbett Rotor-Gene™ 6000 5-plex HRM thermal cycler, with use of the ABI™ *Taq*Man Genotyping assays was found to be the most efficient for mutation detection using relatively small sample batches.

Following successful standardization of the RT-PCR assays, genotype-phenotype correlation studies was performed in a subset of 43 MS patients with available data. Biochemical tests were previously done on blood samples at the National Health Laboratory Service (NHLS) chemical pathology laboratory at Tygerberg Academic Hospital. A novel finding of this study was that heterozygotes and homozygotes for mutation 1298 A>C in the *MTHFR* gene presented with lower serum iron levels (12.37 ± 5.91 µmol/l) in comparison to subjects without the C-allele (18.64 ± 7.15 µmol/l; P = 0.02). Furthermore, C-reactive protein (CRP) levels were found to be marginally significantly higher (P = 0.07) in the MTHFR 1298 A>C mutation-positive heterozygotes compared

to subjects without the C-allele (6.65 ± 4.96 mg/l vs 2.93 ± 2.31 mg/l), linking inflammation to the presence of the MTHFR 1298 A>C mutation. In comparison, the MTHFR 677 C>T as well as the HFE 845 G>A mutation showed no correlation with transferrin saturation, ferritin, haemoglobin or CRP levels. The absence of increased iron status in *HFE* mutation carriers was in accordance previous findings suggesting altered iron metabolism in MS patients with this mutation.

For the first time, high-throughput assays for functional polymorphisms in the *MTHFR* and *HFE* genes can now be offered as a routine service at the Tygerberg Academic Hospital. This application is used in combination with blood biochemistry tests as part of a comprehensive gene-based, pathology supported screening and intervention program aimed at improved quality of life in patients diagnosed with MS.

Opsomming

Die etiologie van meervoudige sklerose (MS) is nog grootendeels onbekend, as gevolg van die multifaktoriale aard van die siekte, met omgewings- en genetiese faktore wat bydra tot die risiko. 'n Aantal ondersoeke het reeds die belangrikheid van die genetiese komponent vir die vatbaarheid vir die siekte en die progressie daarvan beklemtoon.

In die huidige studie was genetiese variasies in die *MTHFR* (1298 A>C en 677 C>T) en *HFE* (845 G>A) gene bestudeer wat voorheen getoon het dat dit foliensuur- enystermetabolisme respektiewelik in die konteks van MS affekteer. Die doel van die studie was om die laboratorium komponent van 'n patologie-ondersteunde genetiese toets daar te stel wat gebruik kan word om 'n subgroep van MS pasiënte te identifiseer wat veranderderde voedingsbehoeftes het as gevolg van genetiese vatbaarheid. Die studiepopulasie het bestaan uit 90 pasiënte met 'n kliniese diagnose van MS en 49 kontroles sonder enige tekens of simptome van die siekte, wat ingesluit is vanuit dieselfde ouderdoms- en populasiegroep .

Drie mutasie analise sisteme was vergelyk in terme van akkuraatheid, sensitwiteit, kostedoeltreffendheid en gemak van gebruik met betrekking tot die *MTHFR* en *HFE* geen mutasies. Analitiese geldigheid van die genetiese toetse was 'n belangrike oorweging; daarom was die onderskeie rieëltyd polimerase kettingreaksie (RT-PKR) metodes vergelyk met direkte DNA volgordebepaling as die goue standaard. Die metodologie het die ABI™ 7900HT, die Roche LightCycler® 480 II sisteem en die Corbett Rotor-Gene™ 6000 5-plex HRM ingesluit. Dieselfde genotipe resultate was met die verskillende metodes verkry vir die DNA monsters wat getoets is met die drie RT-PKR metodes. Wat betref kostedoeltreffendheid, gemak van gebruik en optimisering, was die gebruik van die Corbett Rotor-Gene™ 6000 5-plex HRM Thermal Cycler, met die ABI™ *Taq*Man Genotyping essays die mees effektief vir mutasie opsporing van relatief klein getalle monsters.

Nadat die RT-PKR toetse suksesvol gestandardiseer was, was genotipe-fenotipe korrelasies uitgevoer in 'n subgroep van 43 MS pasiënte met die beskikbare data. Biochemiese toetse was voorheen gedoen op die betrokke bloedmonsters by die Nationale Gesondheid Laboratorium Diens (NHLS) se chemiese patologie laboratorium by Tygerberg Akademiese Hospitaal. 'n Nuwe bevinding van hierdie studie was dat heterosigote en homosigote vir die MTHFR 1298 A>C

mutasie gepresenteer het met laer serum yster vlakke (12.37 ± 5.91 μmol/l) in vergelyking met individue sonder die C-alleel (18.64 ± 7.15 μmol/l; P = 0.02). Verder was die C-reaktiewe proteien (CRP) marginaal betekenisvol hoër (P = 0.07) in die MTHFR 1298 A>C heterosigote in vergelyking met individue sonder die C alleel (6.65 ± 4.96 mg/l vs 2.93 ± 2.31 mg/l), wat aandui dat inflammasie verhoog mag wees in die teenwoordigheid van die MTHFR 1298 A>C mutasie. In vergelyking hiermee het die MTHFR 677 C>T sowel as die HFE 845 G>A mutasies geen korrelasie met transferrien versadiging, ferritien, hemoglobien of CRP-vlakke getoon nie. Die afwesigheid van verhoogde yster status in MS pasiënte met die *HFE* mutasie was in ooreenstemming met vorige bevindinge wat veranderde ystermetabolisme in MS pasiënte met hierdie mutasie aangedui het.

Vir die eerste keer is hoë deurvoer genetiese toetse nou vir funksionele polimorfismes in die *MTHFR* en *HFE* gene beskikbaar as 'n roetiene diens by die Tygerberg Akademiese Hospitaal. Dit kan gebruik word saam met bloed biochemiese toetse as deel van 'n omvattende geen-gebaseerde, patologie ondersteunde intervensie program wat daarop gemik is om die kwaliteit van lewe van pasiënte gediagnoseer met MS te verbeter.

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List of Abbreviations and Symbols

5’	5-prime
3’	3-prime
α	alpha
β	beta
©	copyright sign
°C	degrees Celsius
=	equal to
>	larger than
µg/L	microgram per litre
µl	micro litre
-	minus
%	percentage
+	plus
±	plus-minus
®	registered trademark
<	smaller than
A	adenine
A (Ala)	alanine
ATP	adenosine 5’-triphosphate
bp	base pair
BLAST	basic local alignment search tool
C (Cys)	cysteine
C	cytosine
CNS	Cerebro-Spinal Fluid
D (Asp)	aspartic acid
dATP	2’deoxy-adenosine-5’triphosphate
dCTP	2’deoxy-cytosine-5’triphosphate
ddATP	2’,3’-dideoxy-adenosine-5’triphosphate

ddCTP	2’,3’-dideoxy-cytosine-5’triphosphate
ddGTP	2’,3’-dideoxy-guanosine-5’triphosphate
ddH_2O	double distilled water
ddTTP	2’,3’-dideoxy-thymidine-5’triphosphate
dGTP	2’-deoxy-guanosine-5’-triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DMT1	divalent metal transporter 1
dsDNA	double stranded DNA
dTTP	2’-deoxy-thymidine-5’-triphosphate
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
FPN	ferroportin gene
FRET	fluorescence resonance energy transfer
g	gram
G (Gly)	glycine
G	guanine
H (His)	histidine
H_2O	water
HAMP	hepcidin antimicrobial peptide gene
H_3BO_3	boric acid
HFE	high iron gene
HH	hereditary haemochromatosis
HJV	hemojuvelin gene
HLA-A*3	major histocompatibility complex class I A3
HRM	high resolution melt
I (Ile)	isoleucine
IFNβ	interferon beta
IVS	intervening sequence
IRP1	iron regulatory binding protien 1

IRP2	iron regulatory binding protein 2
JH	juvenile haemochromatosis
L (Leu)	leucine
LiPA	reverse hybridization line-probe assay
M (Met)	methionine
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
MGB	minor groove binder
ml	millilitre
mM	milli-molar
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
N (Asn)	asparagine
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steato-hepatitis
ng	nanogram
ng/µl	nanogram per micro litre
NCBI	national centre for biotechnology innovation
NSAID	non-steroidal anti-inflammatory drug
NTC	non-template control
OMIM	online mendelian inheritance in man
p	short arm of chromosome
P (Pro)	proline
PCR	polymerase chain reaction

pmol	picomole
q	long arm of chromosome
Q (Glu)	glutamine
R (Arg)	arginine
RefSeq	reference sequence
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolution per minute
RT-PCR	real-time polymerase chain reaction
rxn	reaction
S (Ser)	serine
SLC40A1	solute carrier family 40 (iron regulated transporter) member 1
SNP(s)	single nucleotide polymorphism(s)
SOP	standard operating procedure
T (Thr)	threonine
T	thymine
T_A	annealing temperature
Taq	*Thermus aquaticus* polymerase enzyme
TBE	tris-borate-EDTA buffer
TE	tris-EDTA buffer
Tf	transferrin
TFR1	transferrin receptor 1
TFR2	transferrin receptor 2
T_M	melting temperature
TM	trademark
U	units
UTR	untranslated region
UV	ultraviolet
V (Val)	valine

V	volts
v/v	volume per volume
w/v	weight per volume
x	times
x g	times gravity
Y (Tyr)	tyrosine

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DEDICATIONS

I would like to dedicate this thesis to my parents, who raised me to believe, *When you believe anything is possible*.

Chapter 1

Literature Review

1.1. MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic neurological disorder which causes demyelination of axons in the brain and Central Nervous System (CNS) resulting from multifocal inflammatory events (Byun *et al.* 2008; Chilcott *et al.* 2003). MS is more prevalent in women and the onset of disease takes place mostly in young adults between 18 and 50 years of age (Weinshenker BG, 1998). Lesions formed due to MS are heterogeneous and are differentiated by inflammation, demyelination, and variable extent of axonal and oligodendrocyte damage (Bjartmar *et al.* 2003; Lassmann, 1998; Trapp *et al.* 1999). Several aspects play a role in the development of the disease, including environmental factors, such as decreased sunlight exposure, smoking, and the body's resistance to certain viruses and severe stress. Genetic factors and gene-gene interactions have also been proposed to have an effect on the disease process (Debouverie *et al.* 2008; Helen and David, 1998; Review by Marrie 2004; Remington, 1995; Robertson and Compston, 1995).

1.2. NEUROPATHOLOGY OF MS

The myelin sheath is an electrically insulating layer that forms the extended membrane of oligodendrocytes, enfolds the axons and allows for impulses to propagate through the neurons at high speed. Inflammation resulting from MS ruptures the myelin sheath protecting the axons causing demyelination and the formation of lesions (De Stefano *et al.* 2001; Ferguson *et al.* 1997; Trapp *et al.* 1999). MS lesions are found in the normal white matter and infrequently in the gray matter of the CNS (Lassmann, 1998; Narayana, 2005; Peterson *et al.* 2007; Trapp *et al.* 1999). Progressive demyelination and oligodendrocyte death results in either decreased nerve conductivity or complete loss of nerve impulse transmission, leading to impaired neurological function (Hickey *et al.* 1999; Trapp *et al.* 1999; reviewed by Reipert, 2004). Axonal injury is mostly irreversible (Davie *et al.* 1999) and the spheroids (swellings) can be detected within the lesions during later stages of the disease (McGavern *et al.* 1999; McGavern *et al.* 2000).

1.3. SIGNS AND CLINICAL SYMPTOMS OF MS

MS symptoms differ in severity from person to person and episodes are irregular after which the patient may go into remission (Byun *et al.* 2008). This is dependent on the level of axonal damage and demyelination in the brain and spinal cord (Kanda, 2003). Symptoms also depend on the location of the lesions formed. Clinical signs and symptoms may include chronic disabling fatigue that results in co-ordination problems or ataxia (Burnfield, 1996), weakness of the limbs (Beatty,

1995; Rasova *et al.* 2010; Rao *et al.* 1991), burning sensations and heat intolerance, which may exaggerate symptoms (Tandon *et al.* 1989). In addition, patients may also suffer from uncoordinated speech, urinary incontinence and constipation affecting 90% of patients (Langford, 1994). Spasticity, abnormal eye movements, optic neuritis (blurred and painful vision) and depression is found in one third of patients. Cognitive impairment (Bruce and Simon, 1999) is thought to be present in 40-70% of patients suffering from MS and is associated with loss of memory (Charil *et al.* 2003; Stapler and Lincoln, 1979). Patients may also suffer from cognitive dysfunction relating to sustained attention, conceptional reasoning and verbal fluency (Rao *et al.* 1991; Stapler and Lincoln, 1999).

1.4. DISEASE CLASSIFICATION

Classification and detection of MS was first reported by Dr Jean-Martin Charcot in 1868 at Salpêtrière Hospital, were he stated the disease as "presence of multiple plaques in the CNS" (Murray, 2005: 6).

Several types of MS have been classified as the disease affects each individual with a different combination of symptoms and severity. The four clinically distinct types are: relapsing remitting, secondary progressive, progressive relapsing and primary progressive MS (Lublin and Reingold, 1996).

1.4.1. Relapsing Remitting Multiple Sclerosis

In Relapsing Remitting MS (RR-MS), patients experience alternating periods of relapse and remission of the disease. Each relapse episode may set off new symptoms or exagerate existing symptoms. Patients may also undergo remission, which fluctuates greatly in time period and level of severity (Millefiorini *et al.* 1997). During this phase, which may proceed days to months, the symptoms often decrease or become absent (Lublin and Reingold, 1996; Pittock and Rodriquez, 2008). RR-MS accounts for 85-90% of MS cases (Mendes *et al.* 2003).

1.4.2. Secondary Progressive Multiple Sclerosis

This is the second phase of relapsing remitting MS seen in 90% of patients (Annapurna *et al.* 2002). The disease exacerbates as remissions take place and the number of relapses decreases. Approximately 40% of RR-MS patients enter this phase after 10 years and 60% within 25 years after disease onset (Lublin and Reingold, 1996; Rovaris *et al.* 2006).

1.4.3. Progressive Relapsing Multiple Sclerosis

Progressive Relapsing MS involves a steady neurologic decline with minimum recovery from acute attacks (Trojano and Paolicelli, 2001). This subtype is rare and found only in 5% of individuals suffering from MS (Lublin and Reingold, 1996).

1.4.4. Primary Progressive Multiple Sclerosis

Primary Progressive MS (PP-MS) is characterized by gradual disability in patients with no remissions (Lublin and Reingold, 1996; Trojano and Paolicelli, 2001). This type arises in 10-15% of MS patients. The onset of PP-MS typically commences in the late thirties or early forties and is often the most severe form of the disease (Cottrell *et al.* 1999; McDonnell and Hawkins, 2002; Miller and Leary, 2007).

1.5. POSSIBLE CAUSES OF MS

MS is a complex disease driven by several factors rather than a single cause. Genetics and the role of environmental factors are the primary focus of present studies. Understanding the genetic roots of MS could uncover the basic mechanism of disease, thereby leading to discoveries of treatments and possible prophylactic measures. Other theories concerning the cause of MS include viral infection and autoimmunity.

1.5.1. MS and Autoimmunity

For many years it has been proposed that MS is an autoimmune disease (Baranzini and Hauser, 2002; Peterson *et al.* 2007). Autoimmunity results from the immune system failing to recognize the constituents of an individual's body as "self", proceeding into an immune response against cells and tissues (Chilcott *et al.* 2003; Stefanova *et al.* 2002; Jerne, 1974). Several mechanisms have been proposed which may play a role in autoimmune diseases. These include Molecular Mimicry, where a viral antigen shares structural similarities with the native proteins (Hafler, 2004). This results in antibodies binding to both the pathogenic antigen as well as a self-antigen, amplifying the immune responses, which consequently gives rise to autoimmunity. The other is cytokines which are the main factors involved in immune regulation and initiation of autoimmunity. Specialized immunoregulatory cells such as regulatory T cells exert their effect either through suppressing or enhancing the immune response. This depends on their production via cytokines, which may ultimately result in autoimmunity (Vanderlugt and Miller, 2002; Von Herrath *et al.* 2003).

The CNS is often thought to be an immuno-privileged site. However, T cells have been detected within the CNS as inspectors for any injury or infection. The entry of T cells into the CNS is made possible through penetration of the blood brain barrier (BBB) (Hickey *et al.* 1991; Wekerle *et al.* 1987). T and B cells have auto-reactive characteristics and in some cases, healthy individuals have also been shown to stimulate T cell activation via myelin elements with no signs of pathogenesis.

The autoimmunity theory states that the activation of T cells only occurs when tolerance is lost, i.e. ability of an individual's immune system in differentiating 'self' from 'non-self' (Diaz-Villoslada *et al.* 1999; Sprent and Kishimoto, 2001). On activation of myelin-specific T cells, penetration of immune cells into the CNS across the blood-brain barrier (BBB) is allowed. Once in the CNS, secretion of pro-inflammatory cytokines and B Cells takes place (Hogquish *et al.* 2005). Myelin-specific CD4+ T cells are considered to be the initiators of disease in MS patients (Babbé *et al.* 2000; Jacobsen *et al.* 2002). This is done through their interaction with the antigens presented on the myelin sheets, resulting in inflammation and damage to myelin, oligodendrocytes and the axons (Zamvil and Steinman, 2003). However, this theory is currently considered controvertial (Chaudhuri and Behan, 2005), since this model originated from an animal model, experimental allergic encephalomyelitis (EAE) presenting brain inflammation, which is not similar to MS in all respects (Sriram and Steiner, 2005).

1.5.2. Infections and Viral Factors Concerning MS

There have been many theories and hypotheses concerning the actual initiation of MS in people apart from genetic factors, for example pathogenic infections.

The hygiene hypothesis states that autoimmune diseases result from a lack of exposure to multiple infective microorganisms in childhood due to excessive hygiene and advanced medication (Ascherio and Munger, 2007; Fleming and Fabry, 2007), whereas the prevalence hypothesis proposes that MS is caused by a single pathogen found in regions where MS prevalence is much higher. Once the pathogen reaches the desired site it causes persistent infection but asymptomatically. Therefore, no signs or symptoms are detected for many years until infection has spread far enough to cause demyelination (Ascherio *et al.* 2007; Kurtzke, 1993). A number of viruses have been linked to MS including Human herpes viruses, Varicella zoster virus and endogenous retroviruses such as the human endogenous retrovirus (HERV) (Christensen, 2007; Perron and Lang, 2010, Sotelo *et al.* 2008).

1.5.3. Oligodendrocyte Apoptosis

An alternative theory for the aetiology of MS is the apoptotic cell death of oligodendrocytes, the cells that produce myelin. In this paradigm, the immune system is not the primary cause of the disease, but functions to remove dead or damaged tissue. This is illustrated by the work of Barnett and Prineas (2004). When they examined newly-forming lesions, they found no peripheral immune cells. Instead, they observed fields of apoptotic oligodendrocytes. These cells were then phagocytised by microglia, which also stripped away myelin from the axons. Subsequently, peripheral immune cells penetrated the BBB. The most recent work from the laboratory of Prineas indicates that asctrocytes, the cells surrounding blood vessels and which are responsible for nutrient uptake into the brain, are also damaged in MS (Parratt and Prineas, 2010).

1.6. DIAGNOSIS

Early diagnosis of MS is important to minimize disease progression and demyelination of axons. The disease is often diagnosed in RR-MS patients between 15 and 60 years of age as they reveal symptoms indicative of CNS lesions, as opposed to other types of MS, which are often devoid of early symptoms (Thompson and Donald, 1996). Upon clinical examination, the involvement of two or more areas of CNS and related abnormalities should be found. Diagnosis is made possible through the use of Magnetic Resonance Imaging (MRI, Figure 1.1) and other electrophysiological tests such as Visual Evoked Potentials (VEP) used in clinical practice (Halliday *et al.* 1973; Offenbacher *et al.* 1993) which reveal abnormalities in anterior optic pathways (Annapurna *et al.* 2002) and Brainstem Auditory Evoked Potentials (BAEP) (Lublin and Reingold, 1996; Weinstock-Guttman *et al.* 1995).

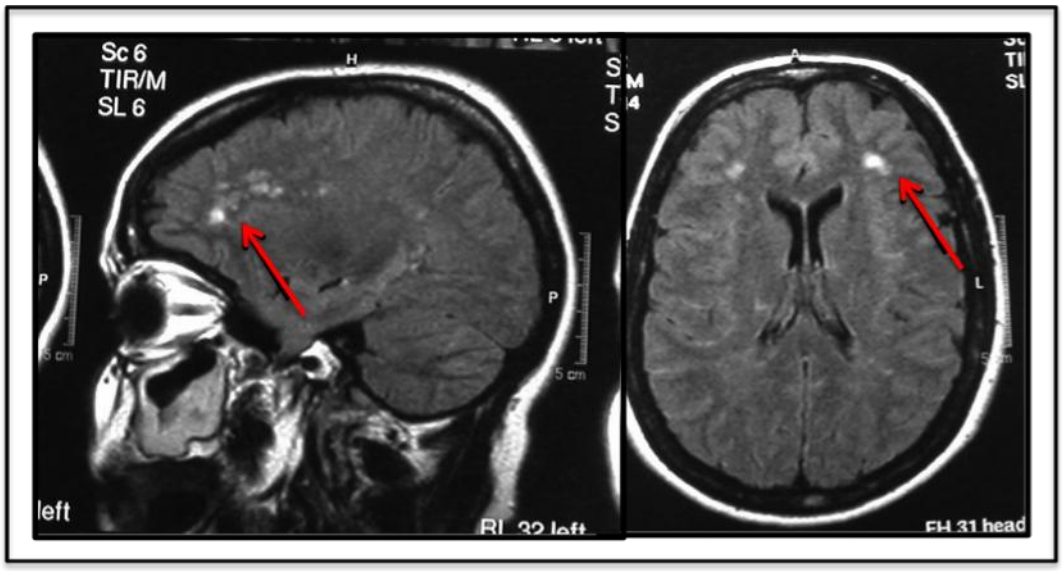

Figure 1.1. Image obtained through application of MRI of a relapsing remitting MS patient. Red arrows indicate lesions in the white matter of the brain caused by demyelination. (Source: Prof SJ van Rensburg, with permission)

In approximately 90% of patients, increased oligoclonal immunoglobin G (IgG) bands are found in the CSF. IgG is evoked through inflammation, however, oligoclonal bands are not a diagnostic tool for MS.

1.7. THERAPY MANAGEMENT

The management of MS is a complex and intricate issue, due to the vast diversity of symptoms in affected patients and the lack of a well-defined cause of the disorder. This has lead to the involvement of a wide variety of different clinical specialists in the therapeutic process, from neurologists and physiotherapists to occupational and speech therapists. The elusive nature of the pathogenic process underlying MS has resulted in a number of therapies strictly designed to improve the quality of life of the affected patients, as no universal cure for all forms of the disorder has yet been discovered. The treatments are focused on reducing the number of relapses, preventing or reducing the resulting neural damage and delaying the onset or progression of the disease.

Therapies used in MS have been categorized according to severity and disease type being treated. Current practice involves disease-modifying therapies aimed at suppression of the immune system.

1.7.1. Acute Corticosteroid Treatment

During the acute exacerbation stage of MS, intravenous methylprednisolone is given for 3 to 5 days (Tremlett *et al.* 1998), which has been proven to speed up the recovery (Beutler *et al.* 1996). This is due to the anti-inflammatory effect, as well as immunosuppressive characteristics of corticosteroids (Beutler *et al.* 1996; Romine *et al.* 1999; Sipe *et al.* 1994; Tremlett *et al.* 1998).

1.7.2. Interferons

One of the therapies modifying the disease status focuses on Beta Interferon (IFNβ) (Bidot *et al.* 2007; Chilcott *et al.* 2003). Interferons are cytokines that are triggered upon viral infection and thus prevent cell infection. The autoimmune response of interferons can be seen via the action of Gamma interferons secreted by T cells, which act upon macrophages, aggravating the disease state and destroying the myelin sheath. IFNβ influences this process by inhibiting leukocyte proliferation and antigen presentation. It also restrains T-cells from migrating across the BBB and alters cytokines for production of an anti-inflammatory environment (Yong *et al.* 1998). This effect of IFNβ, mainly IFNβ-1a and IFNβ-1b, is referred to as immunomodulatory action (Tandon *et al.* 1989; Weinstock-Guttman *et al.* 1995). Some of the drugs in use include Avonex (IFNβ-1a), given via injection at doses of 30 μg (6 million IU) once a week, Rebif (IFNβ-1a) is used three times a week at a dose of 22 μg (6 million IU) and Betaferon (IFNβ-1β) injected on alternative days at a dosage of 0.25 mg (8 million IU) (Annapurna *et al.* 2002). One of the main side effects known when using any of these treatments is flu-like symptoms, which can be reduced via a non-steroidal anti-inflammatory drug (NSAID) prior to interferon usage.

1.7.3. Glatiramer acetate (Copolymer-1)

One of the myelin analogues is glatiramer acetate, which is a synthetic polypeptide (Bidot *et al.* 2007; Chilcott *et al.* 2003). Although Glatiramer acetate's mode of action is not yet known, studies suggest that it suppresses T cell activation and inhibits lymphocyte migration (Miller *et al.* 1998; Prat *et al.* 1999). This is achieved when Glatiramer acetate competes with myelin basic protein and other myelin auto-antigens for binding sites on Major Histocompatibility Complex (MHC) Class II found on antigen presenting cells (Arnon *et al.* 1995). In a double-blind placebo trial conducted by Johnson *et al*, it was found that a dosage of 20 mg of glatiramer acetate in RR-MS patients decreased the relapse rate by up to 29% (Johnson *et al.* 1995; Johnson *et al.* 1998).

1.7.4. Intravenous Immunoglobin G (IgG)

Apart form the use of IgG as a marker in diagnosing MS, it is now known that this immunoglobin (Ig) molecule can play a role in disease treatment (Fazekas *et al.* 1997). Its mode of action imitates that of the binding of the Fc (Fragment crystallizable) Receptor portion of Ig molecules to the B cells, thus generating negative signals which ultimately cause B cell down-regulation (Bidot *et al.* 2007; Yan *et al.* 1990). Treatment involves intravenous delivery of 0.15 - 2g/kg of immunoglobin per month (Fazekas *et al.* 1997). This has a direct effect on relapse-rate though side effects have been identified in some cases which may include malaise, fever, headache, rash, and thrombo-embolism (Sorensen *et al.* 1998).

1.7.5. Methotrexate

Methotrexate has been shown to suppress the progression of chronic progressive MS when used in low doses. This requires the use of 7.5mg of the drug once every week (Goodkin *et al.* 1995). The use of this drug would however be contra-indicated in patients who have mutations in 1-carbon metabolism (Van Rensburg *et al.* 2010).

1.7.6. New MS Drugs

Several new drugs, including 2 emerging oral agents for MS, have been submitted to the US Food and Drug Administration (FDA) for approval. The advantage of the newer generation drugs is that they are more effective than IFNβ and they do not have to be injected. However, they are much more expensive and they may have serious side effects in some patients.

1.7.6.1. Natalizumab (Tysabri), marketed by Biogen Idec/Élan, is a humanised monoclonal antibody against the cellular adhesion molecule α4-integrin. After its approval by the FDA in 2004, it was subsequently withdrawn after it was shown to cause progressive multifocal leukoencephalopathy (PML) in some of the patients. It was however reapproved in 2006 under a special prescription program. However, by January 2010, 31 cases of PML cases had been reported. It is currently not recommended as first-line treatment in MS (Foley, 2010).

1.7.6.2. Leustatin (Cladribine; 2-chlorodeoxyadenosine triphosphate, trade name Leustatin) was originally developed as an anti-cancer drug, but was subsequently investigated as an oral agent for MS since it selectively targets CD4+ and CD8+ cells (Giovannoni *et al.* 2010). However, CD4+ cell counts remained low after treatment. The benefits were that cladribine significantly decreased the annualized relapse rate by almost 60% and decreased mean brain lesion count by 88% from baseline. However, the adverse events included 4 cases of carcinoma (Giovannoni *et al.* 2010).

Cladribine was approved for marketing in Australia and Russia, but on September 24, 2010 the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA), refused an application by Merck to market Cladribine in Europe, since they ruled that the benefits of Cladribine did not outweigh its risks. It is not known whether the FDA will approve the drug. (Source: downloaded 25/09/2010 http://www.medscape.com/viewarticle/729387).

1.7.6.3. Fingolimod

On September 22, 2010 the FDA announced Fingolimod (Gilenya, Novartis), to be the first approved oral treatment for MS. Fingolimod acts as a superagonist to sphingosine-1-phosphate receptors on lymphocytes, reducing their overall number in circulation. Over 2 years, Fingolimod showed decreased relapses (annualised relapse rate of 0.18 compared to 0.40 with placebo) and delayed disability progression (regression of 17.7% compared to 24.1% with placebo) in patients with relapsing forms of MS. However, some patients discontinued the study due to bradycardia/atrioventricular conduction block, elevated liver-enzyme levels, macular edema or hypertension (Kappos *et al.* 2010).

1.8. Genetic and Environmental Aspects of MS

MS is considered to be a multi-factorial disease with both genetic and environmental components. MS affects 1 million individuals worldwide, that is approximately one in every thousand (0.1%) individuals in a given population, but the prevalence varies between different countries (Anderson *et al.* 1992; Courtney *et al.* 2009).

1.8.1. Genetic Factors

The potential role of genetics implicated in MS pathology has been increasingly recognized and research studies have thus focused on finding the series of genes involved as well as the inheritance pattern of the disease. It has also been indicated that one in every five people with MS has a familial history of the disease and the risk of inheriting MS in closely related relatives (including first, second and third degree relatives) is about 5% (Compston and Coles, 2002). In addition, twin studies provide an estimation of the contribution of the role of genetic and environmental factors through comparison of concordance rates in monozygotic twins (identical) and dizygotic twins (fraternal). The genetic effect is proven to have a higher concordance rate in monozygotic twins, where the risk of one twin increases to 300 per 1000 (30%) when the other twin is affected by the disease (Bobowick *et al.* 1978; Ebers and Sadovnick, 1994; Kalman and Lublin, 1999; Mackay and Myrianthopoulos, 1966; Weinshenker *et al.* 1989).

Extensive studies have been done to discover the genes responsible or contributing to the development and progression of MS. Recent genetic findings have indicated that MS is caused by defects in a series of genes that directly or indirectly play a role within pathways associated with MS development (Hoffjan and Akkad, 2010). Several vitamin D-related proteins have been identified that may influence the pathology of MS, with conflicting results (Fukazawa *et al.* 1999; Niino *et al.* 2000; Niino *et al.* 2002; Partridge *et al.* 2004; Smolders *et al.* 2009; Steckley *et al.* 2000; Tajouri *et al.* 2005; Yeo *et al.* 2004). Most recent findings have indicated a link between Vitamin D and gene polymorphisms in HLA alleles in MS (Niino *et al.* 2000; Ramagopalan *et al.* 2009). These include HLA-DR2 haplotype DRB1*1501, DQB1*0602, HLA-C5 and HLA-DRB1*11 susceptibility loci (Dean *et al.* 2008; Ramagopalan *et al.* 2007; Review by Compston and Coles, 2008). The HLA-DRB1*1501 haplotype contributes the highest genetic risk of MS development in those of Northern European ancestry (Ramagopalan *et al.* 2009; Review by Ascherio *et al.* 2010).

1.8.2. Environmental Factors

The role of environmental factors in the development of MS has gained greater support since epidemiological data revealed an unequal geographical distribution of the disease (Courtney *et al.* 2009). Increasing evidence support the notion that, similar to other multi-factorial diseases, different combinations of environmental and lifestyle risk factors could trigger the disease in genetically susceptible individuals.

The prevalence rate of MS ranges from 2 to 150 per 100,000 individuals (0,002% to 0,15%) (Granieri *et al.* 1993). In the study done by Kurtzke in 1985, geographical patterns of MS were identified in relation to latitude suggesting that the regions further away from the equator (both north and south of the equator), were at higher risk of developing the disease. Residents in the northern hemisphere were predominantly found to have a higher risk of developing MS than those in the southern hemisphere. The risk is greater in females than in males with the ratios between 1.5 and 2.5 in most populations. In high-risk populations the risk for MS in women is 1 in 200 (Henriksen and Hyllesred, 1988; Henriksen, 1999; Herna *et al.* 1999; Mayer *et al.* 2003; Orton *et al.* 2006). MS prevalence decreases to 5 cases per 100 000 people in Asia as well as in tropical and subtropical regions (Ascherio *et al.* 2007; Compston *et al.* 2006; Kurtzke, 1995; Rosati, 2001).

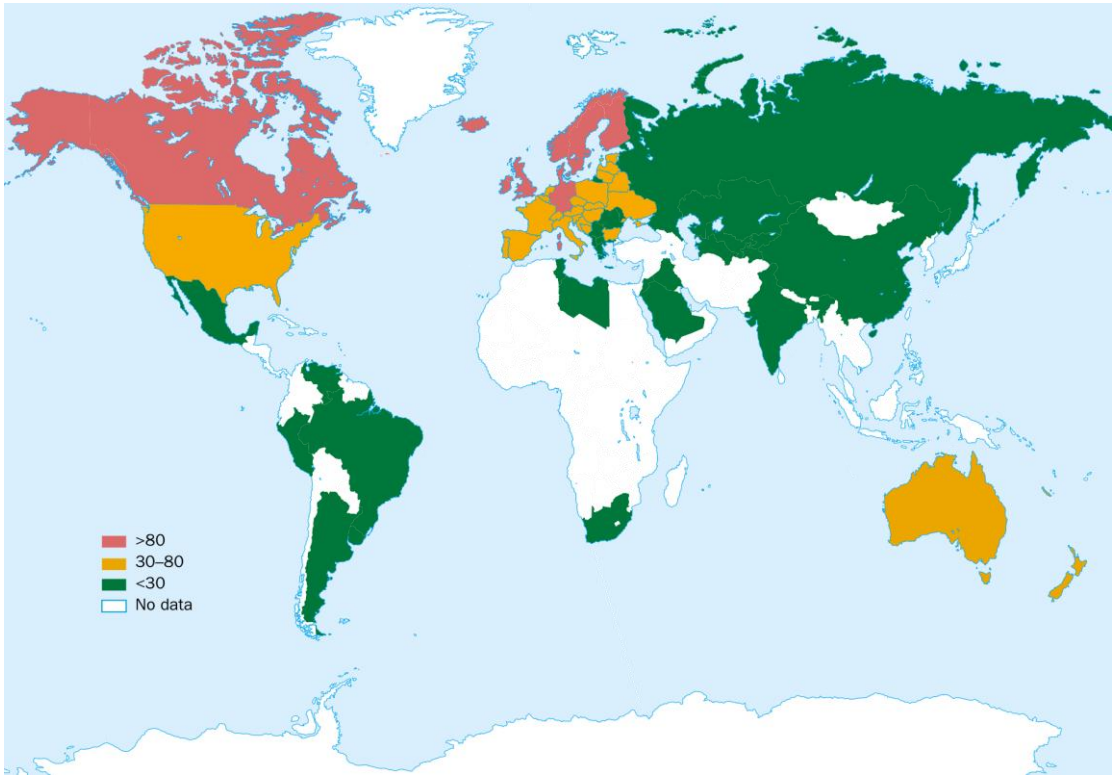

Figure 1.2. World Wide Prevalence of MS per 100 000 population (Adapted from Review by Marrie 2004).

Moreover, the risk of developing the disease after immigration to lower risk areas – such as South Africa, Hawaii or Dutch Antilles – past the age of 15 remains unchanged, while immigration under the age of puberty acquired the frequency of the indigenous population of the country of destination (Alter *et al.* 1971; Dean, 1967; Gale and Martin, 1995; Hammond *et al.* 2000; Kurtzke, 1985; Moffie, 1966). This may be due to the link between childhood infections and MS as the age of migration is a modifying factor in the disease process (Alter *et al.* 1962; Alter *et al.* 1966).

Environmental factors contribute greatly to MS. Infection is one of the factors that has received significant attention from MS researchers in South Africa (de Villiers *et al.* 2006) and elsewhere (Reviewed by Ebers 2008). It has been proposed that viral infection is one of the contributing factors in the aetiology of MS (Cosby *et al.* 1989; Haase *et al.* 1981; Hammerschlag *et al.* 2000; Stevens *et al.* 1980), but no conclusion has been reached regarding the time of infection, the severity or presence of clinical symptoms (Reviewed by Marrie 2004; Weiner, 1998).

A great deal of research has been focused on unravelling the apparent link between viral infection and MS, resulting in the discovery of an increased prevalence of antibodies to several viruses in the serum and CSF of patients suffering from MS when compared to that of the control population (Cook and Dowling, 1980; Haire, 1977). However, these finding were less evident when compared

to siblings, patients with the same HLA type or patients with neurological or non-neurological inflammation (Shirodaria *et al.* 1987; Visscher *et al.* 1981). Epstein-Barr virus (EBV) is one of the factors that is thought to have a primary role in the pathogenesis of MS (Compston *et al.* 1986; Shirodaria *et al.* 1987; Bray *et al.* 1983). Infection with EBV is mild in children, while in adults it causes infectious mononucleosis. Acute infection may lead to latent infection of B-lymphocytes (Straus, 1993). Several case and cohort studies have indicated an increased level in the EBV antibody serum titres in the MS population (Ascherio and Munch, 2007; Haahr *et al.* 1995; Hernan *et al.* 2001), whereas some did not (Casetta *et al.* 1994; Gusev *et al.* 1996; Lenman and Peters, 1969; Poskanzer *et al.* 1980; Souberbielle *et al.* 1990; Italian Multiple Sclerosis study group, 1989). Thus current evidence is insufficient to prove that EBV causes MS.

Vitamin D is one of the environmental factors that have been strongly associated with development of MS (Ascherio and Munger 2007; Burton *et al.* 2008; Cantorna *et al.* 1996; Smolders *et al.* 2008; Soilu *et al.* 2005; van der Mei *et al.* 2007). Extensive studies have been done throughout the world in order to link Vitamin D exposure and latitude in correlation to MS (Acheson *et al.* 1960; Kurtzke, 1967; var der Mei *et al.* 2001). It has been indicated that the rate of mortality from MS is less in the residence in high sunlight exposure regions (Freedman *et al.* 2000). In a study done in Australia, high sunlight exposure during childhood has been shown to have lower risk in MS development (van der Mei *et al.* 2003). Also, higher outdoor activity during childhood in Northern Norway has been illustrated to reduce the risk of MS (Kampman *et al.* 2007). In addition, a study done amongst American nurses reported that high dosage intake of dietary vitamin D (≥ 400 IU/day) resulted in higher serum 25(OH)D i.e. the major circulating form of vitamin D in the blood via Vitamin D binding protein (Myhr, 2009), which led to a 40% reduction in risk of developing MS (Munger *et al.* 2006). This correlates to studies that have demonstrated low serum vitamin D levels in 50 to 70% of MS patients (Nieves *et al.* 1994; Mahon *et al.* 2003; Soilu *et al.* 2008; Ozgocmen *et al.* 2005). Additionally, this corresponds to evidence that has indicated lower vitamin D levels in patients suffering from RR-MS during relapses (Soilu *et al.* 2005; Soilu *et al.* 2008; Smolders *et al.* 2008). Also, the rate of relapse occurrence has been associated with the level of vitamin D and sunlight exposure (Tremlett *et al.* 2008).

The effect of smoking as an environmental risk factor for early conversion of MS to clinically defined disease and risk for progression in MS has been studied extensively. In a follow-up study of 36 months performed in 129 patients with a clinically isolated syndrome, disseminated white-matter lesions on brain MRI resonance imaging, and positive oligoclonal bands in the cerebrospinal fluid, it was found that smoking is an independent but modifiable risk factor for disease progression. The

recommendation from this study was that this aspect should be considered in the counselling of patients with a clinically isolated syndrome (Pauli *et al.* 2008). The negative effect from smoking is most obvious in smokers who started early as it clearly worsens the prognosis of MS even in past smokers (Sundstrom and Nystrom 2008).

1.9. FOLATE AND MYELINATION

Due to the neurodegenerative characteristics of MS, pathways involved in myelin production have been researched for many years. One of the pathways involved in myelin production is the folate - vitamin B12 - methyl transfer pathway. Continuous availability of nutrients that are for example required as co-factors of enzymes involved in myelin production or maintenance needs to be emphasised (Selzer *et al.* 2003). Methylation is of particular importance for myelin production and maintenance, as may be reflected by the increased risk of MS associated with adolescent obesity (Munger *et al.* 2009). While this finding highlights the importance of a healthy diet for weight management in general, it also provided valuable insight on how susceptibility factors for the development or the severity of chronic diseases may be further compromised by the additional burden of obesity as a reflection of an inappropriate diet. Both obesity and smoking, implicated as important environmental / lifestyle risk factors for MS, are associated with raised homocysteiene levels. This could reflect nutrient deficiencies (e.g. folate, vitamin B12, zinc) that are essential for optimal myelin production and maintenance.

In absence or deficiency of nutrients required as co-factors for optimal activity of enzymes involved in the folate-Vitamin B12 pathway, a genetic defect(s) can be triggered and lead to disease development. Additionally, inhibition of these pathways through mutation, nitrous oxide and increase in the level of homocysteine may contribute to demyelination and neurodegeneration (van Rensburg *et al.* 2006).

1.9.1. Methylenetetrahydrofolate reductase

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in regulation of folate and homocysteine (Hcy) metabolism. The enzyme reduces the 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF), (Klotz *et al.* 2010; Goyette *et al.* 1994). Folate is a cofactor in the remethylation of homocysteine and thus absence or deficiency of it results in high homocysteine levels in the plasma. During homocysteine metabolism, the neurotoxic intermediate homocysteine, is remethylated into methionine through the catalytic action of activated methionine

synthase by methylcobalamin (Vitamin B_{12} coenzyme) (Kocer *et al.* 2009). Methionine, also found in dietary protein, then acts as a precursor of S-adenosylmethionine (SAM), which has an anti-inflammatory function and is crucial in the synthesis of phospholipids and myelin generation in the CNS (Rosenblatt, 1995; Surtees *et al.* 1991). Additionally 5,10-MTHF is necessary for nucleic acid synthesis by conversion of deoxyuracilmonophosphate (dUMP) to deoxythyminemonophosphate (dTMP) for *de novo* thymidine synthesis (Klotz *et al.* 2010).

Thus the importance of MTHFR activity in these pathways makes it an attractive candidate for analysis of functional genetic variants in diseases involving myelin pathology. It has been reported that MS patients have elevated concentrations of homocysteine in their plasma and CSF (Goyette *et al.* 1994; Besler and Comoglu, 2003; Ramsaransing *et al.* 2006; Reynolds *et al.* 1992). This may be due to deficiency in vitamin B12, folate or destruction in the enzymatic activity responsible for the metabolism of homocysteine (Vrethem *et al.* 2003). The gene that codes for the MTHFR enzyme, *MTHFR*, is located on chromosome one at position p36.3 (Goyette *et al.* 1994) and contains 11 exons (Frosst *et al.* 1995).

Reduced enzyme activity resulting in hyperhomocysteinemia has been linked to variation in the MTHFR gene. Two functional polymorphisms at nucleotide positions 677 (C>T, rs1801133) and 1298 (A>C, rs1801131) are the most ectensively studies variations in the *MTHFR* gene.

The point mutation 1298 A>C in the MTHFR gene (rs1801131) results in the substitution of alanine for glutamine at amino acid position 429 (Glu-429-Ala or E429A) of the regulatory domain of the enzyme (Rozen, 1997, van der Put *et al.* 1998). It causes increased concentrations of 5,10-MTHF during nucleic acid synthesis and cell proliferation in the course of inflammation (Linnebank *et al.* 2004). This reduces the levels of SAM, which diminishes the regeneration of phospholipids and myelination, a pathophysiological effect implicated in MS (Surtees *et al.* 1991). In the German case-control study conducted by Klotz *et al.* in (2009), *MTHFR* 1298 A>C was found to influence neurodegeneration and the incidence rate of MS. This association has received further support from an American study, which concluded that defects in the folate sensitivity of the homocysteine metabolism may affect disease status (Susser *et al.* 1998).

The *MTHFR* mutation designated 677 C>T (rs1801133) results from a cytosine to thymine change at position 677, which causes a substitution of valine for alanine at amino acid position 222 (Ala-222-Val or A222V) of the enzyme (Frosst *et al.* 1995). This mutation has been linked to a reduction in activity and increase in thermolability of the MTHFR enzyme (Jacques *et al.* 1996; Schwahn and

Rozen 2001; Yamada *et al.* 2001). The mean activity in the Ala-Val heterozygote is 65% with 30% reduced activity in the Val/Val homozygous state (Tajouri *et al.* 2006). The frequency of the polymorphism varies significantly between ethnic groups (Schneider *et al.* 1998). The MTHFR 677TT genotype occurs in approximately 10% of individuals in the North American population, which is similar to the frequency reported in the South African Caucasian population (Scholtz *et al.* 2002). The 677TT individuals are predisposed to mild hyperhomocysteinemia due to a decrease in the MTHFR enzymatic activity (Frosst *et al.* 1995).

The effect of the 677TT genotype on neurodegeneration was examined in a Japanese study consisting of 1 721 subjects with no history of stroke but diagnosed with white matter lesions via MRI assessment. The study reported an association of the MTHFR 677TT genotype with silent brain infarcts and advanced white matter lesions (Kohara *et al.* 2003). The study by Klotz *et al.* (2010) on the effect of the 677C>T mutation demonstrated elevated enzymatic activity in cultured fibroblasts and peripheral lymphocytes (Klotz *et al.* 2010). Some studies have found elevated levels of homocysteine in the CSF of MS patients (Baige *et al.* 1995; Besler *et al.* 2003; Klotz *et al.* 2010; Kolesar, 2000; Tajouri *et al.* 2006; Vrethem *et al.* 2003). This suggests that it may be possible to regulate the disease state, including the course and extent of myelin damage, by manipulation of the essential nutrients relevant to the myelination process (van Rensburg *et al.* 2006).

1.10. IRON AND MYELINATION

1.10.1 Myelination

Oligodendrocytes need iron to produce myelin (Connor and Menzies, 1996). Iron is essential for the functioning of several prosthetic groups in the cells. This includes heme and iron-sulfur clusters (Galy *et al.* 2005). The brain has been identified as one of the most iron-rich organs (Hallgren and Sourander, 1958). Oxidative metabolism requires iron as its basic component and the brain has been found to carry out oxidative metabolism at a very high rate, thus resulting in high levels of iron usage (Ikeda and Long, 1990).

Oligodendrocytes, illustrated in figure 1.2, are one of the types of cells found in the brain that are rich in iron (Connor *et al.* 1993; Levine, 1991). Oligodendrocytes are located within the white matter regions of the brain, near neural cell bodies and along blood vessels (Connor, 1992; Curnes *et al.* 1988; Rajan *et al.* 1976). Their primary function is the production and regeneration of myelin, which requires iron both directly and indirectly (Connor and Menzies, 1996)

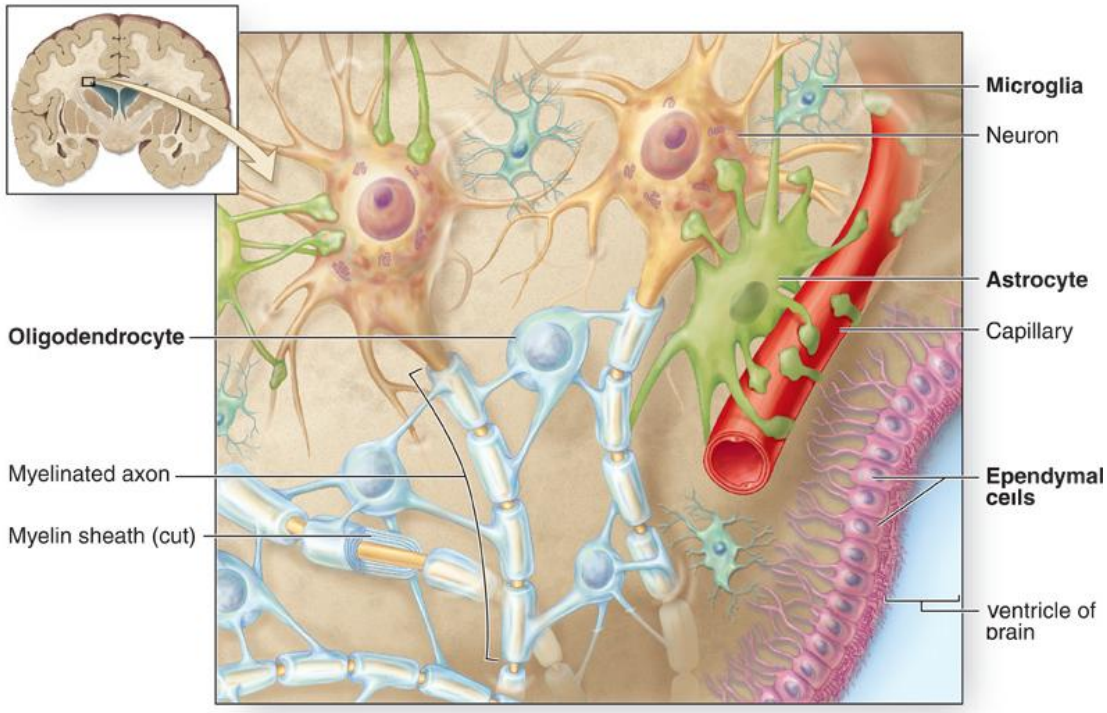

Figure 1.3. A diagram of the oligodendrocytes responsible for myelination (Adapted from McGraw Hill online learning centre).

Oligodendrocytes require iron to act as cofactors for cholesterol and lipid biosynthesis, which are key components of the myelin sheath (Larkin and Rao, 1990). Iron-requiring enzymes and coenzymes, which catalyze the two pathways both for synthesis and degradation of lipids and cholesterol, are enriched in oligodendrocytes (Bourre *et al.* 1984; Cammer, 1984; Tansey and Cammer, 1988). Moreover, oligodendrocytes carry out cholesterol and lipid biosynthesis at a higher rate than any other cell types found in the brain (Cammer, 1984; Pleasure *et al.* 1984).

Iron is indirectly involved in myelin production via its role in oxidative metabolism (Cammer, 1984; Hyden and Pigon, 1960). Enzymes such as glucose-6-phosphate dehydrogenase, succinic dehydrogenase, Nicotinamide Adenine Dinucleotide (NADH) and cytochrome oxidase require iron for their catalytic action in oxidative metabolism (Cammer, 1984). This highlights the importance of iron in the myelination process, as its deficiency affects activities carried out in the oligodendrocytes and thus myelin production. Neurodegenerative diseases that cause demyelination, in particular MS, may thus be affected by the deficiency and/or deregulation of iron (Adams 1988; Craelius *et al.* 1982; Drayer *et al.* 1987; Jensen *et al.* 2007; Kotze *et al.* 2001; LeVine and Chakrabarty 2004; LeVine *et al.* 1999; Zamboni, 2006). This is in contrast to disorders such as hereditary haemochromatosis (HH), in which the pathogenesis and associated tissue damage result from iron overload (Sheldon, 1935; Von Recklinghausen, 1889).

1.10.2. Iron Regulation

Cells absorb iron and heme from the diet via Fe^{2+} transporters such as the divalent metal transporter 1 (DMT1), divalent cation transporter 1 (Mims *et al.* 2005), macrophage protein 2 or heme carrier protein 1 (Shayeghi *et al.* 2005). Iron is then transported to plasma from the enterocytes through ferroportin (Donovan *et al.* 2005), which results in oxidation of ferrous to ferric iron by the multi-copper oxidase protein, haephestin. Serum transferrin (Tf) binds one or two Fe^{3+} atoms. Iron bound to Tf is then circulated in the serum and extra-vascular spaces, acting as iron transport protein for cells and tissues such as liver, heart and bone marrow (Aisen *et al.* 1998). The excess intracellular iron is stored in ferritin, which has a structure that is composed of 24 H and L subunits allowing for iron storage (Harrison and Arosio, 1996). When cells in the body require more iron it is released from ferritin (Kidane *et al.* 2006).

The ability of iron to take part in many reduction-oxidation (redox) reactions such as oxidative metabolism, oxygen delivery and storage, DNA replication and repair, lipid metabolism and many more, makes it one of the essential cofactors utilized in the body. Hence its deficiency may be involved in a wide range of diseases. Given its importance in health and disease, iron regulation and its mechanism of action has been studied extensively (Dunn *et al.* 2007; Escolar *et al.* 1999; Hentze *et al.* 2004; Kaplan *et al.* 2006; Philpott and Protchenko, 2008; Rouault, 2006).

Cellular iron concentration is regulated via the iron regulatory proteins, IRP1 and IRP2, which are ubiquitous regulators of cellular iron homeostasis (Galy *et al.* 2005; Puig *et al.* 2008). IRP1 and IRP2 are cytosolic RNA-binding proteins that post-transcriptionally modulate the translation of proteins involved in iron storage (ferritin), transport (transferrin receptor 1) and use via many enzymes (Dycke *et al.* 2007).

In a study done by Rouault, loss-of-function mutations (-/-) of IRP1 and IRP2 mice were generated through homologous recombination in embryonic cell lines, in order to illustrate the underlying mechanisms of iron regulation (Rouault *et al.* 2006). Loss of IRP1 function showed no abnormalities or dysregulation in iron metabolism, whereas IRP2 -/- mice developed a progressive neurologic syndrome associated with abnormalities and myelin degeneration such as microcytic anaemia, erythropoietic protoporphyria and neurodegeneration (Cooperman *et al.* 2005; LaVaute *et al.* 2001). Furthermore, ferritin over-expression was detected in affected neurons and in oligodendrocytes due to axonal damage. The experiment with IRP1 -/- and IRP2 -/- mice revealed that IRP2 exerts a dominant regulatory function in iron metabolism *in vivo* (Rouault, 2006).

The role of iron-related genes has also been investigated in South African patients with MS (Kotze *et al.* 2001, 2006). In 1999, Rooney *et al.* described MS, porphyria-like symptoms and a history of iron deficiency anaemia in a family of Scottish descent. In addition, van Rensburg *et al.* (2006) found low serum iron concentrations in approximately 50% of patients with MS. The hypothesis that mutations in the IRP2 gene may be associated with iron dysregulation in MS patients has been investigated in an previous study (Jalali 2008, BSc Hons thesis).

1.10.3. HFE

The *HFE* gene is located at chromosome position 6p21.3 with a length of 9.5 kilobases (kb) (OMIM +235200). The structure of *HFE* resembles that of the major histocompatibility complex (MHC) class 1 gene group. The protein encoded by this gene is a type 1 transmembrane glycoprotein (Lebron *et al.* 1998). The *HFE* gene has been associated with haemochromatosis (HH), an iron overload disorder (Feder *et al.* 1996) presenting a multigenic nature (Review by Piertangelo, 2006).

The HFE protein consists of three extracellular domains (α1-3), an untranslated cytoplasmic-3' tail and a final transmembrane domain with each component encoded by an individual exon (Bahram *et al.* 1999; Feder *et al.* 1996; Parkkila *et al.* 1997; Riegert *et al.* 1998). The α1 and α2 globular domains form an eight-stranded anti-parallel β-sheet platform, which is topped by two α helices and maintained on the surface of an immunoglobulin constant-like α3 domain. Another key feature is the cysteine residues within the α2 and α3 domains, responsible for disulfide bridge formation, which are analogous to those of MHC class 1 proteins. This similarity suggests involvement in the secondary and tertiary structure of the predicted 343 amino acid glycoprotein that *HFE* codes for (Bjorkman and Parham 1990; Feder *et al.* 1996; Feder *et al.* 1997; Lebron *et al.* 1998; Riegert *et al.* 1998). The cell surface expression of the protein is facilitated through the interaction between its α3 domain and β2 microglobulin, forming a heterodimer (Bahram *et al.* 1999; Feder *et al.* 1997). In the case of MHC proteins, the α1 and α2 helices create a groove for peptide binding, contrary to HFE, which does not bind proteins. Crystallographic studies have proven that the HFE α1 helix is located close to the α2 helix, forming a shallower and narrower groove than the MHC peptide-binding groove. The differences between these proteins regarding their physical structure imply that each one has a unique role in the intracellular transferrin-mediated iron uptake (Feder *et al.* 1998). A cluster of four histidine residues, resembling the structure of iron-binding sites found in many proteins, has been identified on the surface of the α1 domain (Lebron *et al.* 1998). It has been suggested that a complex is formed between the HFE protein and the transferrin receptor 1 (TFR1), which influences intracellular iron delivery (Feder *et al.* 1998; Parkkila *et al.* 1997). The binding

affinity of TFR1 for transferrin is greatly reduced through the complex formation with HFE (Feder *et al.* 1998, Gross *et al.* 1998, Ikutu *et al.* 2000). Due to the role of HFE in iron regulation and the importance of iron in the myelination process via oligodendrocytes, mutations in *HFE*, which alter the protein structure, may indirectly influence the maintenance of axons.

The most common mutation found in *HFE* is a guanine to adenine single nucleotide polymorphism (SNP) in exon 4 (845 G > A, rs1800562) resulting in a substitution of tyrosine for cysteine at amino acid position 282 (Cys-282-Tyr, C282Y) (Feder *et al.* 1996). The carrier frequency of this mutation has been reported as approximately one in ten among individuals of Northern European decent and one in six in the Caucasian population of South Africa (de Villiers *et al.*1999b). Homozygosity (two copies) for the C282Y mutation occurs in approximately 1 in 100 individuals in the South African population compared with 1 in 200 among Europeans. Several studies have also found significant differences in iron status in heterozygous individuals (Adams *et al.* 2005; Beutler *et al.* 2000; Beutler *et al.* 2003; Jackson *et al.* 2001). Inheritance of two copies of the C282Y mutation has been found to result in elevated transferrin saturation levels in approximately 50% of women and 80% of men (Delatycki *et al.* 2005).

It has been reported that among 1700 MS patients, no subjects with clinical HH were found (Valberg *et al.* 1989). In the study by Kotze *et al.* (2006), 118 patients with MS in the South African population were screened for the C282Y mutation. Seventeen of the MS patients included in this South African study were found to be heterozygous for the mutation with three having below and none above the reference values for transferrin saturation. One of the patients, as well as her sister following family screening, were furthermore found to be homozygous for the C282Y mutation in the presence of high iron stores, although iron deficiency was reported at an early age that required iron supplementation in the index case. They had no clinical manifestation of haemochromatosis and liver function enzymes were normal in both. These data raised the possibility that defective iron regulation in MS patients may be as a result of a metabolic blockage in the absorption of iron (Kotze *et al.* 2006). Most studies have found no difference in frequency of HFE between MS patients and the general population as also confirmed by Kotze *et al.* (2006).

Iron metabolism is influenced by chronic inflammation, a common feature of MS. HFE mutations are are not considered as as a cause of MS but may serve as a marker for iron dysregulation, either in the presence of high or low iron stores. Similarly, mutations in the MTHFR gene do not directly cause MS but represent an important biomarker to assess increased need for folate and other B-vitamins required as co-factors in the folate-homocysteine pathway. In this study the focus has

shifted from a one-gene-one-disease genetic testing approach to a pathology-supported genetic testing approach recently introduced at the Department of Pathology (Kotze *et al.* 2009; Schneider, 2009), to evaluate the genetic contribution in complex, multi-factorial diseases such as MS.

Pathology supported genetic testing requires that the patient's genetic profile be correlated with his/her clinical history and pathology (biochemical measurements of serum iron, homocysteine, etc.) to assess gene expression and response to treatment and/or lifestyle changes. Such a nutrigenetics approach forms an important part of personalized medicine to ensure continuous availability of nutrients pivotal for the biochemical pathways implicated in myelin production and maintenance.

1.11. AIMS AND OBJECTIVES OF THIS STUDY

The overall objectives of this study were to investigate the role of mutations in *MTHFR* and *HFE* in folate and iron metabolism, respectively, implicated in the development and/or progression of MS. To enable the incorporation of genetic testing as part of a comprehensive gene-based, pathology supported screening and intervention program aimed at improved quality of life in patients diagnosed with multiple sclerosis (MS), it was necessary to compare different mutation detection systems in terms of accuracy, sensitivity, cost effectiveness and ease of operation.

The specific aims were:

1. Standardization and optimization of Real Time Polymerase Chain Reaction (RT-PCR) assays to demonstrate the analytical validity of high-throughput genotyping in comparison with DNA sequencing as the gold standard.

2. Comparison of mutation detection using three RT-PCR instruments for quality assurance purposes:
   - The ABI™ 7900HT (Applied Biosystems®, Foster City, California, USA),
   - The Roche LightCycler® 480 II system (Roche Applied Science, Germany),
   - The Corbett Rotor-Gene™ 6000 5-plex HRM (originally Corbett Research, Australia; now Rotor-Gene™ Q, QIAGEN®, Germany).
3. Screening of MS patients and controls for *MTHFR* and *HFE* mutations, and comparison with DNA sequencing of controls for quality assurance purposes:
   - MTHFR 1298 A>C,
   - MTHFR 677 C>T,
   - HFE 845 G>A.

Ultimately, the detection of genetic variation influencing serum iron status and homocysteine levels would provide a scientific basis for long-term nutritional intervention in a subgroup of MS patients with altered nutritional requirements (nutrigenetics application).

Chapter 2

Detailed Experimental Procedures

2.1. Study population

The study population consisted of 90 patients diagnosed with MS according to MRI scans and clinical examination. In addition, 49 blood and saliva samples were obtained from subjects drawn from the same population and age group, without any signs or symptoms of MS as control subjects. A subset of 43 MS patients were subjected to biochemical determinations including serum iron, transferrin, transferrin saturation, ferritin, haemoglobin and the generally used inflammatory marker, C-reactive protein. Serum iron, transferrin, transferrin saturation, haemoglobin and CRP concentrations were determined on Siemens Advia 1800 autoanalyser and ferritin was done on Seimens Centaur analyser. These pathology assays were performed at the department of Chemical Pathology (NHLS) using standard techniques.

The MS patient samples were obtained from studies previously done by Kotze *et al.* (2001) and an extention of the pilot study of van Rensburg *et al.* (2006), after obtaining informed consent (Appendix A). Therefore no further details on the clinical characteristics of the study population are provided as the focus of the study was to use these DNA samples to standardize the genotype assays required to implement the pathology supported genetic testing approach for MS (performed under research protocol NO7/09/203 that includes both a research and service component).

Ethical approval has been obtained from the Human Research Committee of the University of Stellenbosch (NO7/09/203) (Appendices B, C, D, E).

2.2. DNA Extraction

2.2.1. DNA Extraction from Whole Blood Using the QIAGEN Mini Kit

For this protocol the buffy coat was used from each patient. This consisted of an initial centrifugation step of whole blood at 3000 rpm (revolution per minute) for 5 minutes. Subsequent to this step the buffy coat (middle layer consisting of white blood cells) was removed and transferred to a new eppendorf tube. Into a 1.5 ml microcentrifuge tube, 20 µl QIAGEN Proteinase K was added. Thereafter 200 µl of blood sample (buffy coat) was pipetted into the tube. From buffer A 200 µl was added to the mixture and was vortexed for 15 seconds to ensure efficient lysis. This was then incubated for 10 minutes at 56ºC, for the DNA to reach its maximum lysis. The tube was then centrifuged briefly for removal of drops from the inside of the lid. Addition of 200 µl of 100% ethanol was carried out, followed by pulse-vortexing for 15 seconds. The mixture was then carefully applied to a QIAamp Mini spin column (2 ml collection tube) and was centrifuged at 8000

rpm for 1 minute. The QIAamp Mini spin column was then placed into a 2 ml collection tube. Into the QIAamp Mini spin column 500 µl wash buffer 1 was added which was followed by centrifugation at 8000 rpm for 1 minute. QIAamp Mini spin column was once more placed into another collection tube, which was followed by addition of 500 µl of wash buffer 2. The tube was then centrifuged for 3 minutes at 14000 rpm. The QIAamp Mini spin column was placed into a clean 1.5 ml microcentrifuge tube with addition of 100 µl buffer AE. It was then incubated at room temperature for 5 minutes and further centrifugation at 8000 rpm. The final solution contained purified DNA solution, which was put on a shaker over night. The obtained DNA solution was stored at 4ºC and at -20ºC for long-term use.

2.2.2. DNA Extraction from Buccal Swabs Using QIAGEN Mini Kit

The Buccal swabs were placed into a 2 ml microcentrifuge tube and to it 600 µl PBS solution was added. To each tube 20 µl QIAGEN Proteinase and 400 µl buffer AL was added and mixed for 15 seconds, by means of vortexing. The tubes were then incubated for 10 minutes at 56ºC. Brief centrifugation was done for removal of drops from the lid. Into a QIAamp Mini spin column in a 2 ml collection tube, 700 µl of the swab mixture was added and centrifuged at 8000 rpm for 1 minute. The QIAamp Mini spin column containing the unpurified DNA was then placed into a clean 2 ml collection tube and the previous step was repeated. Addition of 500 µl wash buffer 1 to the QIAamp Mini spin column was completed. The mixture was centrifuged for 1 minute at 8000 rpm. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and 500 µl wash buffer 2 was added to it which was followed by centrifugation at 14000 rpm for 3 minutes. In a 1.5 ml microcentrifuge tube the QIAamp Mini spin column was placed in and 150 µl of AE buffer was added. The solution was incubated at room temperature for 5 minutes and centrifuged at 8000 rpm for 2 minutes. The final solution consisted of purified DNA, which was left on the shaker overnight and was then stored at 4ºC and -20ºC for long-term use.

2.2.3. DNA Extraction from Whole Blood Using the QIAamp Blood Midi Kit (Spin Protocol)

This protocol was used for purification of genomic DNA from 2 ml of whole blood.

Into a 15 ml centrifuge tube, 200 µl of GIAGEN Protease was inserted followed by addition of 2 ml blood, which was mixed with the enzyme through vortexing. Buffer AL (2.4 ml) provided by the kit was then combined with the mixture. The vials were then inverted several times and vortexed for one minute. To ensure lysis the samples were incubated at 70ºC for ten minutes. Thereafter 2 ml of 96-100% ethanol were added to the tubes and were mixed through inversion and vigorous shaking. Half of the mixture was then transferred onto the QIAamp Midi column placed in a 15 ml centrifuge tube. Centrifugation was done at 3000 rpm (1850 x g) for three minutes. The filtrates

were then discarded and the remainder of the solutions was loaded on the column and was centrifuged for three minutes at 3000 rpm (1850 x g (times gravitational acceleration)). Wash steps of the genomic DNA were then carried out through addition of 2 ml Buffer AW1 to the column and centrifugation for 5000 rpm (4500 x g) for one minute. AW2 (2 ml) was then added and centrifuged for 15 minutes at 5000 rpm (4500 x g). The column was then placed in a clean 15 ml centrifuge tube and the collection tube containing the filtrate was discarded. Nuclease free water (300 µl) was then added directly onto the membrane of the column, followed by five minutes incubation at room temperature and two minutes centrifugation at 5000 rpm (4500 x g). In order to obtain maximum concentration the eluate containing the DNA was reloaded onto the column and was incubated at room temperature for five to ten minutes. Additional centrifugation was carried out for two minutes at 5000 rpm (4500 x g). The solution containing purified genomic DNA was then incubated on a shaker at room temperature overnight to ensure even distribution of the DNA in the buffer.

2.2.4. DNA Extraction from saliva using Oragene DNA / Saliva Kit

The saliva samples were collected in the Oragene DNA vial containing Oragene DNA solution, which allowed for stabilization of the collected DNA samples (2 ml).

The Oragene DNA/saliva samples in the Oragene vial were then mixed thoroughly by inversion for a few seconds. The samples were incubated at 50ºC for two hours in an air incubator. Thereafter 500 µl of the mixture was transferred to a 1.5 ml microcentrifuge tube. Oragene DNA purifier (OG-L2P, supplied) was added to the microcentrifuge tube and was mixed by vortexing for a few seconds that followed ten minutes incubation on ice. The centrifugation step was carried out at room temperature at 13000 rpm (15000 x g) for five minutes, which resulted in the separation of the supernatant from the pellet containing impurities, and was then transferred into a new microcentrifuge tube. To the tube, 500 µl of 95-100% ethanol was added which was mixed thoroughly by inversion. The samples were incubated at room temperature for ten minutes to allow for DNA precipitation. The tubes were placed in the centrifuge in a known orientation (in order to position the pellet at the tip of the tube below the hinge as the DNA pellet will be invisible) and were spun for two minutes at 13000 rpm (15000 x g). The supernatant was carefully removed and discarded from the acquired pellet containing the DNA. An ethanol wash step was carried out by addition of 250 µl of 70% ethanol and incubation at room temperature for one minute, after which the ethanol was removed completely without disturbing the pellet. Nuclease-free water (100 µl) was then used to dissolve the DNA pellet through five seconds of vortexing. Additional vigorous pipetting and vortexing as well as overnight incubation on a shaker (room temperature) followed, to ensure that the obtained DNA sample is evenly distributed in the buffer.

2.3. DNA Quantification

Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, USA) using v 3.5.2 software package was applied for detection of DNA quality and quantity. All Genomic DNA samples were diluted to final concentration of 10 ng/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. Polymerase Chain Reaction (PCR) Amplification

2.4.1. Oligonucleotide Primers

Oligonucleotide primers and probes were designed to screen specific exonic regions of *MTHFR* (see figure 2.1 MTHFR 677 C>T and figure 2.2 for MTHFR 1298 A>C) and *HFE* (see figure 2.3) using the LightCycler® Probe Design Software 2.0 (Version 1.0. R.36). The reference sequences were obtained from the National Centre of Biotechnology (NCBI, www.ncbi.nlm.nih.gov) for *MTHFR* (NG_013351.1) and *HFE* (NM_000410). The primers and probes used in the RT-PCR experiments with the Roche LightCycler® 480 II system are specified in table 2.1.

5' 10814 – 15120 3'

GGAGGCTTCAACTACGCAGTGGACCTGGTGAAGCACATC**CGAAGTGAGTTTGGTGAC**
TACTT...tccctgtggtctcttcatccctcgccttgaacaggtggaggccagcctctcctgactgtcatccctattggcagGTTACCCC
AAAGGCCACCCCGAAGCAGGGAGCTTTGAGGCTGACCTGAAGCACTTGAAGGAGAAG
GTGTCTGCGG**GAGCCGATTTCATCATCACGGCAGC**TT**TTCTTTGAGGCTGACACATT**
CTTCCGCTTTGTGAAGGCATGCAC**CGACATGGGCATCACTTG**CCCCATCGTCCCCGG
GATCTTTCCCATCCAGgtgaggggcccaggagagcccataagctccctccaccccactctcaccgcaccgtcctcgcacaggc
tgggggctctgggtggagtgctgagttcgctgagttcttcccagatctcctctcaggtccagaacttgcacagcgttgcttggccaccccattttgg
ttacctctaattttccccccaaaacccagcaacagtgtctgttgaggggtttgttgta

Figure 2.1. *MTHFR* reference sequence with the forward and reverse primers highlighted in blue for MTHFR 677 C>T. Fluorescein-bound Probe 1 is highlighted in green and the LC-Red-460-bound Probe 2 highlighted in red. The red "C" indicates the position of the SNP. The bases in uppercase indicate exonic regions with the lower case representative of intronic regions.

5' 16381 – 17010 3'

ggcctgcagaccttccttgcaaatacatctttgttcttgggagcgggagggcagaagaagtttgcatgcttgtggttgacctgggaggagtcagg
ggcagaatttacaggaatggcctcctgggcatgtggtggcactgccctctgtcaggagtgtgccctgacctctgggcacccctctgccagGG
GCAATTCCTCTTCCCCTGCCTTTGGGGAGCTGAAGGACTACTACCTCTTCTACCTGAA
GAGCAAGTCCCCCAAGGAGGAGCTGCTGAAGATGTGGGGGGAGGAGCTGACCAGTGA
AGAAAGTGTCTTTGAAGTCTTCGTTCTTTACCTCTCGGGAGAACCAAACCGGAAT
GGTCACAAAgtgagtgatgctggagtggggaccctggttcatcccctgcccctggcctgaccccagctgcaggccaggctgcgggg
ctgtgacttccccatcctgtgccctcccctccatgctgtggacatggcaaagggagaagggtaagttgggagacctccacctggaagggcttag
ggaggcaaagacaggctgggtctttgttgggggccgtgagagggactcagggtgccaaacctgatggtcgccccagccagctcaccgtctcc

Figure 2.2. *MTHFR* reference sequence with the forward and reverse primers for MTHFR 1298 A>C highlighted in blue. Fluorescein-bound Probe 1 and LC-Red-460-bound Probe 2 highlighted in green and red, respectively. The red "A" indicates the position of the SNP.The bases in uppercase indicate exonic regions with the lower case representing intronic regions.

5' 10361 – 10990 3'

GgtaaacagatcccctctcctcatccttcctctttcctgtcaagtgcctcctttggtgaaggtgacacatcatgTGACCTCTTCAGTGA
CCACTCTACGGTGTCGGGCCTTGAACTACTACCCCCAGAACATCACCATGAAGTGGCG
AAGGATAAGCAGCCAATGGATGCCAAGGAGTTCGAACCTAAAGACGTATTGCCCAATG
GGATGGGACCTACCAGGGCTGGATAACCTTGGCTGTACCCCCTGGGGAAGAGCAGA
GATATACGTGCCAGGTGGAGCACCCAGGCCTGGATCAGCCCCTCATTGTGATCTGG
GgtatgtgactgatgagagccaggagctgagaaaatctattggggGTTGAGAGGAGTGCCTGAGgaggtaattatggcagtgagatgaggat
ctgctctttgttaggggtgggctgagggtggcaatcaaaggctttaacttgctttttctgttttagAGCCCTCACCGTCTGGCAC
CCTAGTCATTGGAGTCATCAGTGGAATTGCTGTTTTTGTCGTCATCTTGTTCATTGGAAT
TTTGTTCATAATATTAAGGAAGAGGCAGGGTTCAAgtgagtaggaacaaggggaagtctcttagtacctctg

Figure 2.3. *HFE* reference sequence illustrating the alignment of the forward and reverse primers for HFE 845 G>A highlighted in blue. LC-Red-640-bound Probe 1 is highlighted in red and Fluorescein-bound Probe 2 highlighted in green. The yellow "G" indicates the position of the SNP.The bases in uppercase indicate exonic regions with the lower case representing intronic regions.

Table 2.1. Details of the custom-designed Roche primers and HybProbe probes used in the RT-PCR.

MTHFR 677 C>T						
Forward Primer	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
CGAAGTGAGTTTGGTGACTAC	731	751	21	47.6	59.1	0.5
Reverse Primer	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
CAAGTGATGCCCATGTCG	933	916	18	55.6	59.2	0.5
Probe 1	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
GAGCCGATTTCATCATCACGCAGC-Fluorescein	846	869	24	54.2	65.2	0.2
Probe 2	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
LC Red640-TTCTTTGAGGCTGACACATTCTTCCGCTTTG-Phosphate	872	902	31	45.2	68.1	0.2
MTHFR 1298 A>C						
Forward Primer	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
GCTGAAGGACTACTACCTCTTCTA	1381	1404	24	45.8	60.1	0.5
Reverse Primer	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
CACTTTGTGACCATTCCG	1534	1517	18	50	59.8	0.5
Probe 1	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
CCAGTGAAGCAAGTGTCTTTGAAGTC-Fluorescein	1461	1486	26	46.2	64.8	0.2
Probe 2	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
LC Red 640-CGTTCTTTACCTCTCGGGAGAACCAAAC-Phosphate	1489	1516	28	50	68.3	0.2
HFE 845 G>A						
Forward Primer	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
GGCTGGATAACCTTGGCTGTA	10584	10604	21	52.4	60	0.5
Reverse Primer	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
CTCAGGCACTCCTCTCAAC	10743	10725	19	57.9	60.1	0.5
Probe 1	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
LC Red 640-CTGGCACGTATATCTCTGCTCTTCCC-Phosphate	10637	10612	26	53.8	65.2	0.2
Probe 2	**5' Pos**	**3' Pos**	**Length**	**GC%**	**Tm**	**Conc**
CTGATCCAGGCCTGGGTGCTCC-Fluorescein	10661	10640	22	68.2	68.3	0.2

2.4.2. PCR Reaction Mixture and Thermal Cycling Parameters

PCR was performed in 50 µl reactions, using a commercially available Kit from Promega (GoTaq® Flexi DNA Polymerase PCR). Each reaction mixture contained 1 µl of ~200 ng template DNA, 0.2 mM of each deoxyribonucleotide triphosphate (dNTP) (Fermentas), 10 µl GoTaq Flexi Buffer, 1.5 mM magnesium chloride ($MgCl_2$), 60 pmol of each primers, 1.25 U GoTaq® polymerase.

The amplification of target sequences was performed using Applied Biosystems® 2720 PCR instruments. The PCR procedure exemplified an initial denaturation step at 95ºC for 5 minutes. Followed by 40 cycles of denaturation at 94ºC for 30 seconds, annealing of 55.5ºC for 30 seconds and a 2 minutes extension at 72ºC, which continued with a final extension period at 72ºC for 10 minutes.

2.5. Gel Electrophoresis

Electrophoresis was carried out using a 2% (w/v) agarose gel consisting of 2.0 g agarose and 100 ml 1x TBE Buffer (90 mM Tris-HCl, 90 mM boric acid [H_3BO_3] and 2.2 mM EDTA [pH 8.0]).

Each PCR product (3 µl) was mixed with 5 µl blue Loading Dye (consisting of 15% Ficoll® 400, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA) and was loaded onto the gel. Gel electrophoresis was then run at 100 V for 1 hour in electrophoresis buffer (80 ul EtBr in 800 ml 1x TBE). A 1 Kb DNA Ladder (Promega) was used to determine the fragment size of each PCR fragment. Each fragment on the gel was visualized using an ultraviolet light transilluminator (TFX-35M Life Technologies, GIBRO BRL).

2.6. DNA Sequencing

PCR products obtained for the HFE 845 G>A and MTHFR 677 C>T SNPs were sent for post-PCR clean-up as well as automated sequencing at the DNA Sequencing Central facility of Stellenbosch University.

Sequencing of the control samples for the MTHFR 1298 A>C SNP were sent to Inqaba Biotechnical Industries (Pty) Ltd for post PCR clean up for sequencing and automated sequencing.

2.7. Bioinformatics and Sequence Analysis

In order to analyze the sequences obtained using conventional PCR, Staden package was used, which consisted of wide variety of functions for sequence manipulation and interpretation. The software consists of a series of tools that are particularly flexible for DNA sequence preparation (pregap4), assembly (gap4), editing (gap4) and DNA/protein sequence analysis (spin). In this study Pregap4 was used which is a tool applied for pre-processing sequencing chromatogram files (such as ABI files). The Pregap4 allowed for sequence format conversions, quality assessment, repeat and

contaminant screening and interfaces. The Gap4 program was used for sequence assembly and editing. This program also presented Bayesian consensus algorithm. Several graphical display links was offered by this software, which facilitated the assembly to be viewed at different levels of detail. Estimate base accuracies, which use phred base quality values, aided in calculating the quality of the consensus sequence. Other configuring modules such as trace format conversion, quality clip and Gap4 shot-gun assembly was used. Window length average confidence of 3' and 5' numbers of uncalled bases, as well as minimum exact match, maximum percentage mismatch, maximum consensus length and database size could be configured. This assisted in parts of assembly that required editing or extra data to be easily found and as a result rapid finishing and consensus of known accuracy could be achieved.

The application of this software assigned for viewing the nucleotide sequence of the subjects. In addition, the target sequence could be directly compared to the reference sequence (www-bimas.cit.nih.gov/molbio/readseq), for detection of mutations viewed in different colour presentation of that specific nucleotide base change. Bayesian consensus algorithm offered a graphical representation of the nucleotides peaks and allocation of the mutations found.

2.8. Real-Time Polymerase Chain Reaction (RT-PCR)

2.8.1. Applied Biosystems® *Taq*Man® SNP Genotyping with the 7900HT

ABI™ *Taq*Man® SNP Genotyping assays are standardised mixtures that consist of unlabelled primers and *Taq*Man® MGB probes (FAM™ and VIC® dye-labelled). The pre-designed assays are suitable for end-point genotyping by allelic discrimination analysis for SNPs. The assays employed in this study were MTHFR 677 C>T (C_1202883_20, rs1801133), MTHFR 1298 A>C (C_850486_20, rs1801131) and HFE 845 G>A (C_1085595_10, rs1800562).

The 40X *Taq*Man® assay was diluted to 20X in sterile SABAX water (double distilled water). The components of a 20 µl reaction were: 20 ng/µl of template DNA (2.0 µl), 10 µl of *Taq*Man® Universal PCR Master Mix (P/N 4304437), 20X *Taq*Man® SNP Genotyping Assay (1.0 µl) and 7.0 µl SABAX double distilled water (table 2.2). The amplification was carried out with a three-step thermal cycling program consisting of an initial hold step at 95°C for 10 minutes, 45 cycles of denaturation at 92°C for 15 seconds and annealing/extension at 60°C for 1 minute (table 2.3), followed by allelic discrimination run.

Table 2.2. ABI™ Allelic Discrimination PCR Reaction

Reaction Components	Volume/Well (5 µl Vol rxn)	Volume/Well (20 µl Vol rxn)	Final Concentration
TaqMan Universal PCR Master Mix (10X)	2.5	10	1X
20X TaqMan SNP Genotyping Assay Mix	0.25	1	1X
Nuclease Free Water	0.25	7	
Genomic DNA	2	2	
Total	5	20	

Table 2.3. ABI™ Thermal Cycling Conditions

Times and Temperatures		
Initial Steps	**Denaturation**	**Anneal/Extend**
Hold	40 cycles	
10 min 95˚C	15 sec 92˚C	1 min 60˚C

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

Rotor-Gene™ 6000 series Multiplexing System, 5-Plex HRM model (Corbett Research, Australia) was used with ABI™ *Taq*Man® SNP Genotyping Assays. Mutation detection using this system is achieved by allelic discrimination and scatterplot analysis of the fluorescence data obtained from the RT PCR run. Individual reactions with a total volume of 10 ul consisted of *Taq*Man® Universal PCR Master Mix (5 µl), 20X *Taq*Man® SNP Genotyping assay (0.5 µl), double-distilled water (3.5 µl) and 1 µl of template DNA (10 ng/µl). This reaction mixture was used for all the samples analysed with this instrument. Thermal Cycling parameters used included a 45 cycle repeat of initial hold step for 10 minutes at 95°C, denaturation at 92°C for 15 seconds and an annealing/final extension step for 1 minute at 60°C with the set channel (Green / FAM™ dye and Yellow / VIC® dye) to acquire at the end of the cycling step.

2.8.3. Roche LightCycler® 480 II

LightCycler® 480 mix, consisting of custom designed primers and probes (HybProbes probes) and LightCycler® 480 Genotyping Master was applied for amplification of target DNA as well as SNP analysis using melting curves. The 20 µl reaction mixture was prepared containing 5 µl of 10 ng/µl template DNA, in addition to 9 µl PCR grade water (nuclease free), 1 µl of the primer set (0.5 µM each), 1µl HybProbe probes (0.2 µM each) and 4 µl of 5x concentration LightCycler® 480 Genotyping Master. LightCycler® 480 II, 96 well plate was set up with the total of 20 µl reaction volume per well accordingly. The thermal cycling program applied for genotyping with mono-colour HybProbe probe is listed in table 2.4.

Table 2.4. RT-PCR protocol for the Roche LightCycler® 480 II system using 96 well plates.

Programs				
Program Name	**Cycles**	**Analysis Mode**		
Pre-Incubation	1	None		
Amplification	45	Quantification		
Melting Curve	1	Melting Curves		
Cooling	1	None		
Target °C	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Acquisitions (per °C)**
PreIncubation				
95	None	00:10:00	4.4	-
Amplification				
95	None	00:00:10	4.4	-
Primer Dependent*	Single	00:00:10	2.2	-
72	None	00:00:10	4.4	-
Melting Curve				
95	None	00:01:00	4.4	-
40	None	00:02:00	1.5	-
75	Continuous	-	-	6
Cooling				
40	None	00:00:30	1.5	-

* Annealing temperature: MTHFR 1298 A>C = 52ºC, MTHFR 677 C>T = Variable, HFE 845 G>A = Variable.

2.9 Statistical Analysis

All data were analysed by a professional statistician affiliated with the Department of Pathology, using StatSoft® STATISTICA version 6. One-Way ANOVA, Bonferroni was used to analyse for statistically significant differences between dependent numerical variables of iron (transferrin, percentage transferrin saturation, ferritin, serum iron levels) and the independent genotype variables for MTHFR 677 C>T, MTHFR 1298 A>C and HFE 845 G>A. This test uses mean and standard deviation to calculate significant differences and was selected due to its ability to analyse designs with a single categorical independent variable.

Chapter 3

Results

The procedures described below were performed in 90 patients diagnosed with MS according to MRI scans and clinical examination and 49 control samples. The MS patient samples were obtained from studies previously done by Kotze *et al.* (2001) and van Rensburg *et al.* (2006), after obtaining informed consent (Appendix A). No further details on the clinical characteristics of the study population are provided as the focus of the study was to use these DNA samples to standardize the genotype assays required to implement the pathology supported genetic testing approach for MS (performed under research protocol NO7/09/203 that includes both a research and service component). Conventional sequencing was perfomed for MTHFR 677 C>T, MTHFR 1298 A>C and HFE 845 G>A, using random selection of samples. This was done to verify the results obtained from the RT-PCR runs. Due to the random nature of the selection process, not all genotypes are represented in the conventional sequencing results.

3.1. Conventional Sequencing – Gels and Electropherograms

The PCR products obtained with the MTHFR 677 C>T conventional primers were visualized with ethidium bromide in a 2% agarose gel, presented in figure 3.1.1. The sequencing results are presented as electropherograms for each control sample in figures 3.1.2, 3.1.3 and 3.1.4. Forward and reverse sequencing reactions were carried out, with either included here in a randomized manner to further reduce selection bias and emphasize the analytic validity of the genotyping.

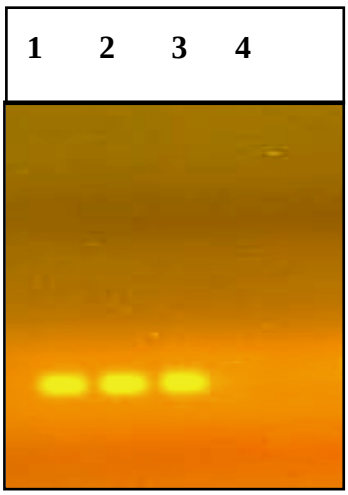

Figure 3.1.1. A 2% (w/v) agarose gel visualized with 0.0001% (v/v) ethidium bromide (EtBr). Lanes 1, 2 and 3 contain amplicons of 256 bp, using *MTHFR* 677 C>T-primer set. Lane 4 contains the NTC (Non-Template Control) PCR reaction product.
Abbreviations: bp = base pairs.

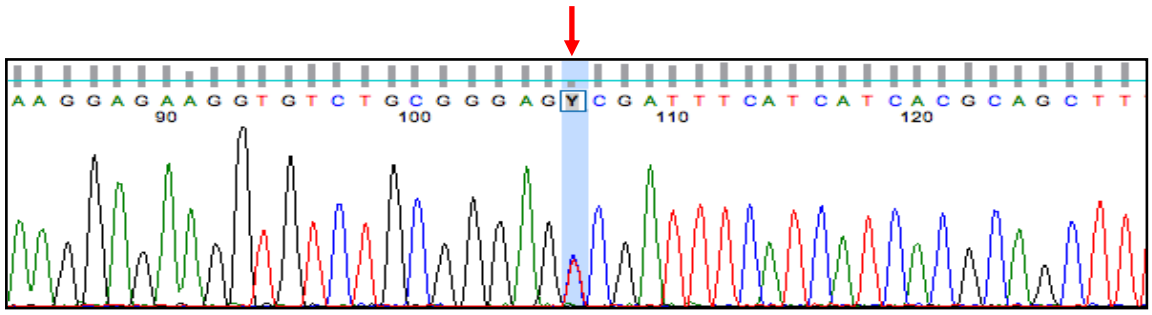

Figure 3.1.3. Electropherogram depicting the forward (sense) sequencing reaction of an amplified PCR product obtained with the MTHFR 677 C>T primer set. The arrow indicates the SNP position. The *Y* at the indicated position corresponds to a genotype of CT, which signifies a Heterozygote.

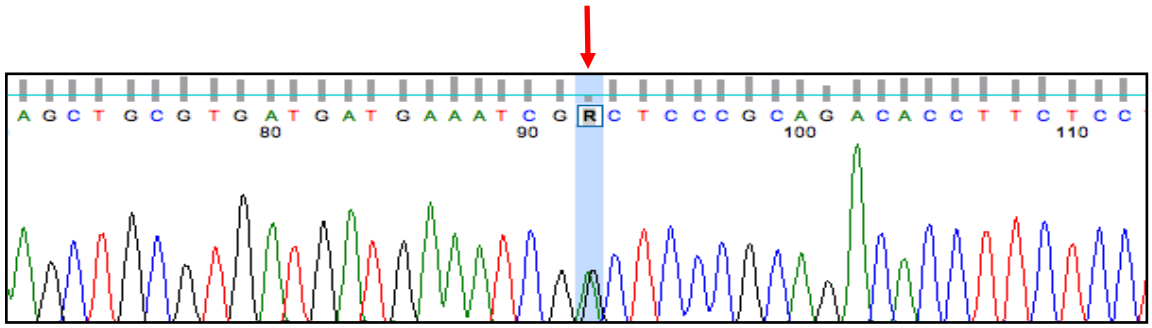

Figure 3.1.2. Electropherogram depicting the reverse (anti-sense) sequencing reaction of an amplified PCR product obtained with the MTHFR 677 C>T primer set. The arrow indicates the SNP position. The *R* at the indicated position corresponds to a genotype of GA, which signifies a Heterozygote.

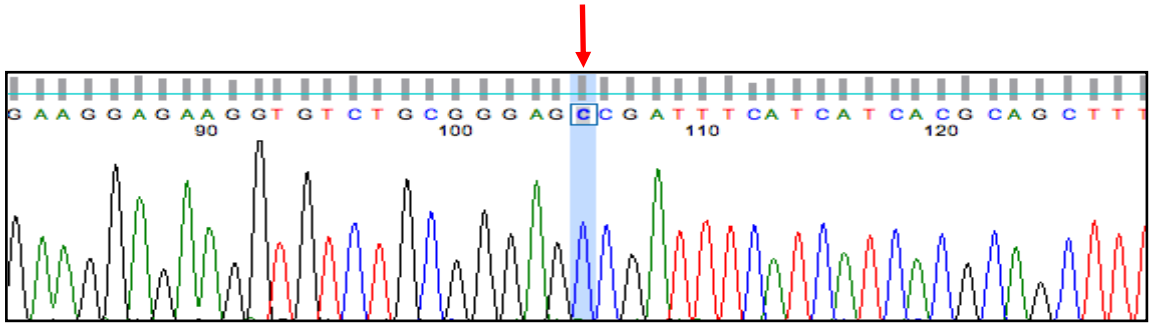

Figure 3.1.4. Electropherogram depicting the forward (sense) sequencing reaction of an amplified PCR product obtained with the MTHFR 677 C>T primer set. The arrow indicates the SNP position. The *C* at the indicated position corresponds to a genotype of CC, which signifies a Wild Type genotype.

The PCR products obtained with the MTHFR 1298 A>C conventional primers were visualized with ethidium bromide in a 2% agarose gel, presented in figure 3.1.5. The sequencing results are presented as electropherograms for each control sample in figures 3.1.6, 3.1.7 and 3.1.8. Forward and reverse sequencing reactions were carried out, with either included here in a randomized manner to further reduce selection bias and emphasize the analytical validity of the genotyping.

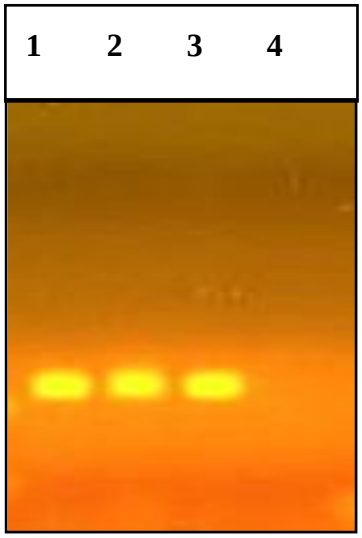

Figure 3.1.5. A 2% (w/v) agarose gel visualized with 0.0001% (v/v) ethidium bromide (EtBr). Lanes 1, 2 and 3 contain amplicons of 465 bp, using *MTHFR* 1298 A>C-primer set. Lane 4 contains the NTC (Non-Template Control) PCR reaction product.
Abbreviations: bp = base pairs.

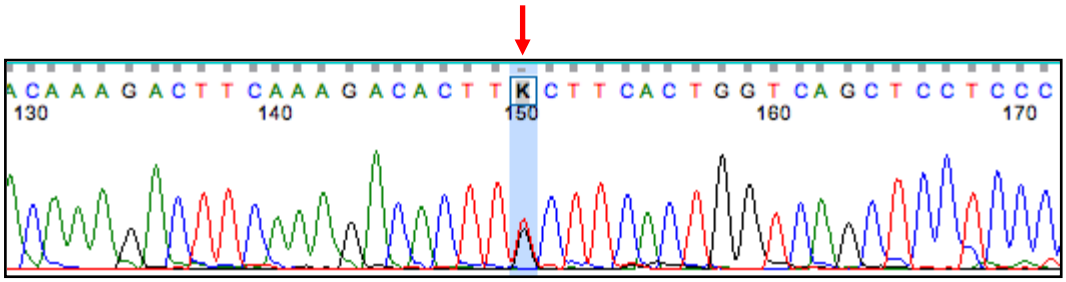

Figure 3.1.6. Electropherogram depicting the reverse (anti-sense) sequencing reaction of an amplified PCR product obtained with the MTHFR 1298 A>C primer set. The arrow indicates the SNP position. The *K* at the indicated position corresponds to a genotype of AC, which signifies a Heterozygous genotype.

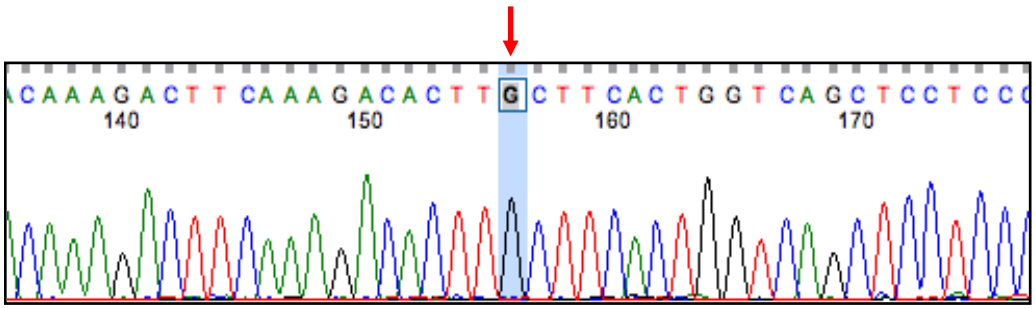

Figure 3.1.7. Electropherogram depicting the reverse (anti-sense) sequencing reaction of an amplified PCR product obtained with the MTHFR 1298 A>C primer set. The arrow indicates the SNP position. The *G* at the indicated position corresponds to a genotype of GG, which signifies a Homozygous genotype.

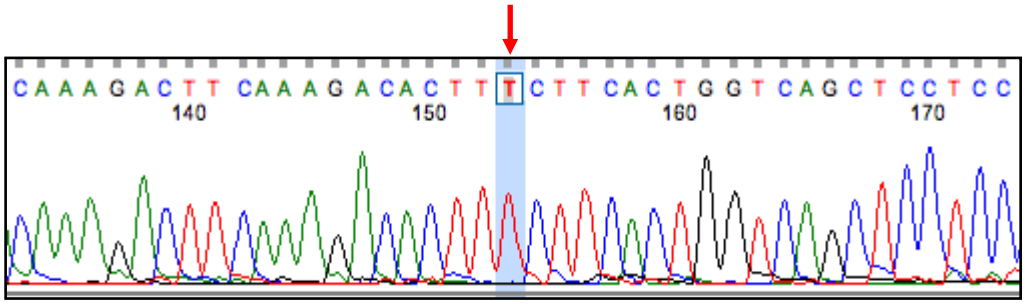

Figure 3.1.8. Electropherogram depicting the reverse (anti-sense) sequencing reaction of an amplified PCR product obtained with the MTHFR 1298 A>C primer set. The arrow indicates the SNP position. The *T* at the indicated position corresponds to a genotype of TT, which signifies a Wild Type genotype.

The PCR products obtained with the HFE 845 G>A conventional primers were visualized with ethidium bromide in a 2% agarose gel, presented in figure 3.1.9. The sequencing results are presented as electropherograms for each control sample in figures 3.1.10, 3.1.11 and 3.1.12. Forward and reverse sequencing reactions were carried out, with either included here in a randomized manner to further reduce selection bias and emphasize the validity of the genotyping.

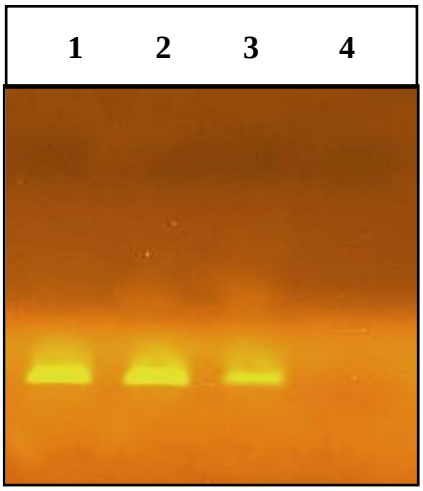

Figure 3.1.9. A 2% (w/v) agarose gel visualized with 0.0001% (v/v) ethidium bromide (EtBr). Lanes 1, 2 and 3 contain amplicons of 465 bp, using *HFE* 845 G>A-primer set. Lane 4 contains the NTC (Non-Template Control) PCR reaction product.
Abbreviations: bp = base pairs.

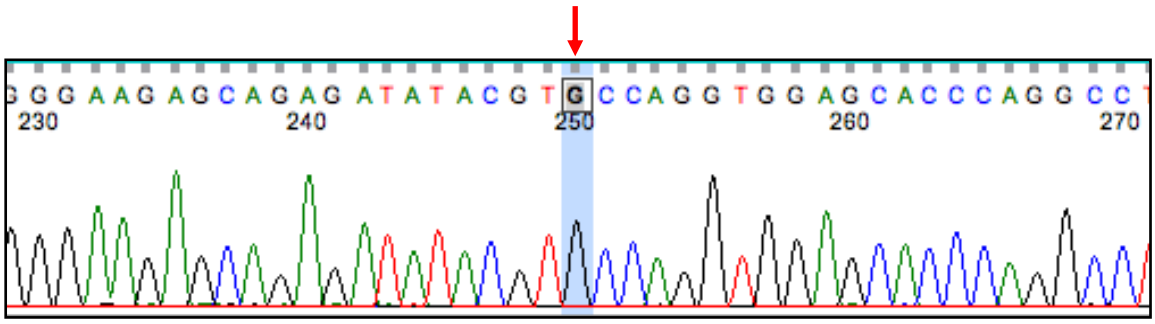

Figure 3.1.10. Electropherogram depicting the forward (sense) sequencing reaction of an amplified PCR product obtained with the *HFE* 845 G>A primer set. The arrow indicates the SNP position. The *G* at the indicated position corresponds to a genotype of GG, which signifies a Wild Type genotype.

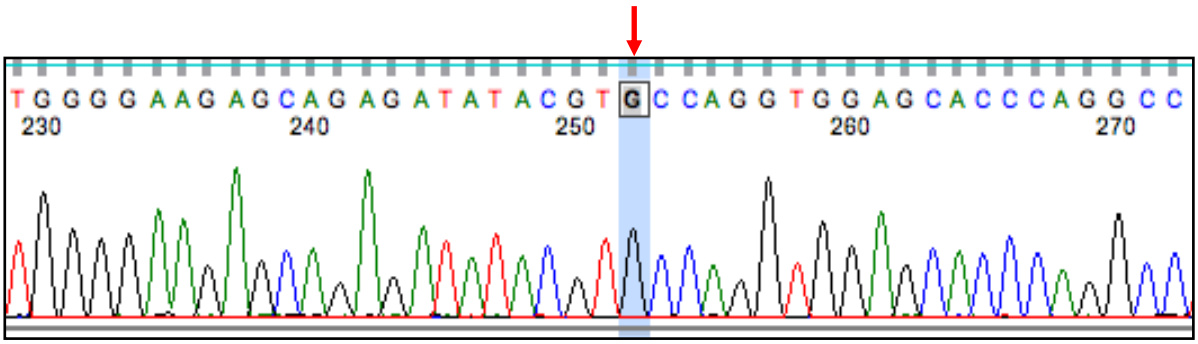

Figure 3.1.11. Electropherogram depicting the forward (sense) sequencing reaction of an amplified PCR product obtained with the *HFE* 845 G>A primer set. The arrow indicates the SNP position. The *G* at the indicated position corresponds to a genotype of GG, which signifies a Wild Type genotype.

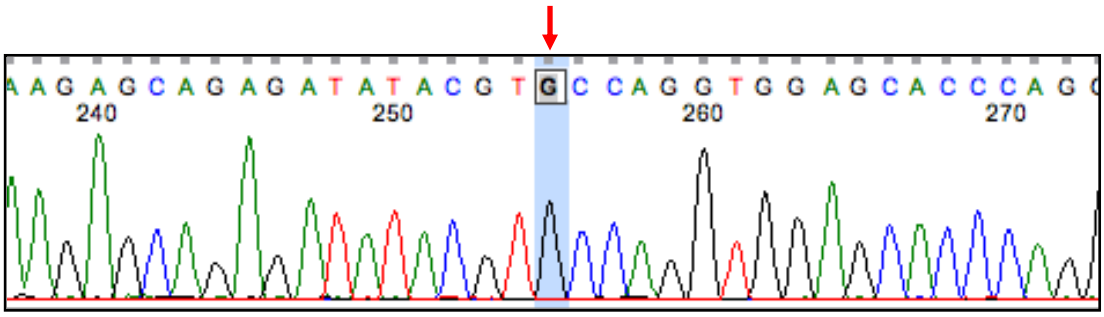

Figure 3.1.12. Electropherogram depicting the forward (sense) sequencing reaction of an amplified PCR product obtained with the *HFE* 845 G>A primer set. The arrow indicates the SNP position. The *G* at the indicated position corresponds to a genotype of GG, which signifies a Wild Type genotype.

3.2. ABI™ 7900HT

The ABI™ 7900HT was used with ABI™ *Taq*Man® SNP Genotyping assays in this investigation. Each 96 well plate contained MS DNA samples (10 ng/μl) with unknown genotypes, 12 NTCs with nuclease free water (strongly recommended by ABI™) and four DNA controls with known genotypes determined by conventional PCR and DNA sequencing prior to the RT PCR run. An amplification run using the Standard Curve (AQ) setting was performed, during which amplicons are exponentially synthesized and the associated fluorescence plotted as a graph by the ABI™ SDS software package (version 2.3) displaying "ΔRn (unit of fluorescence) versus cycles." Thereafter, a post-amplification scan with the allelic discrimination setting was done and the SDS software presents the results on an allelic discrimination scatterplot by contrasting the reporter fluorescence. After signal normalization and multi-component analysis, the software plots the data obtained from a well as a single data-point on the scatterplot. The allelic discrimination analysis displays the results as an "Allele Y (Al 2) versus Allele X (Al 1)" graph.

All three assays (MTHFR 677 C>T, MTHFR 1298 A>C and HFE 845 G>A) ran on the ABI 7900HT were successful, yielding clear amplification of the polymorphic target sequence as well as precise genotype calling for each sample. This was further verified by the internal controls loaded on each plate, matching the DNA sequencing data. In addition, the NTCs revealed clear clustering per plate with absence of both alleles in the amplification and allelic discrimination analysis, corresponding to the absence of contamination in the procedure. The results obtained were then used in the statistical analysis.

The investigation of MTHFR 677 C>T in two sample batches yielded the amplification graphs presented in figures 3.2.1 (sample batch one) and 3.2.3 (sample batch two). These graphs illustrate successful amplification and fluorescence by the ABI™ *Taq*Man® SNP Genotyping assay. The allelic discrimination scatterplots depicted in figures 3.2.2 (sample batch one) and 3.2.4 (sample batch two) clearly indicate the different genotype groups with wild types represented as red dots, heterozygotes in green, homozygotes in blue and NTCs in black.

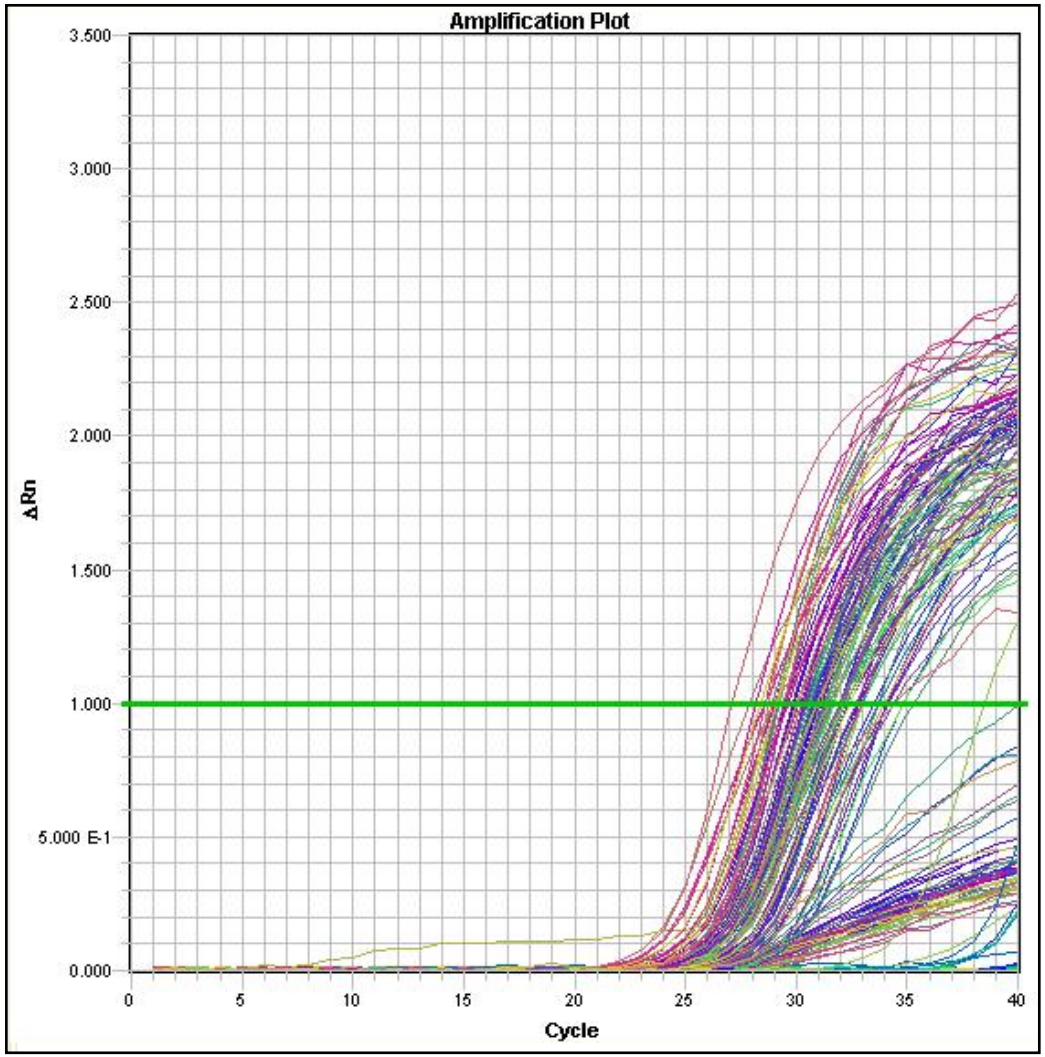

Figure 3.2.1. Amplification achieved in sample batch one using the ABI™ *Taq*Man® MTHFR 677 C>T assay (ΔRn vs number of cycles). The threshold level is set at 1. ΔRn = unit of fluorescence.

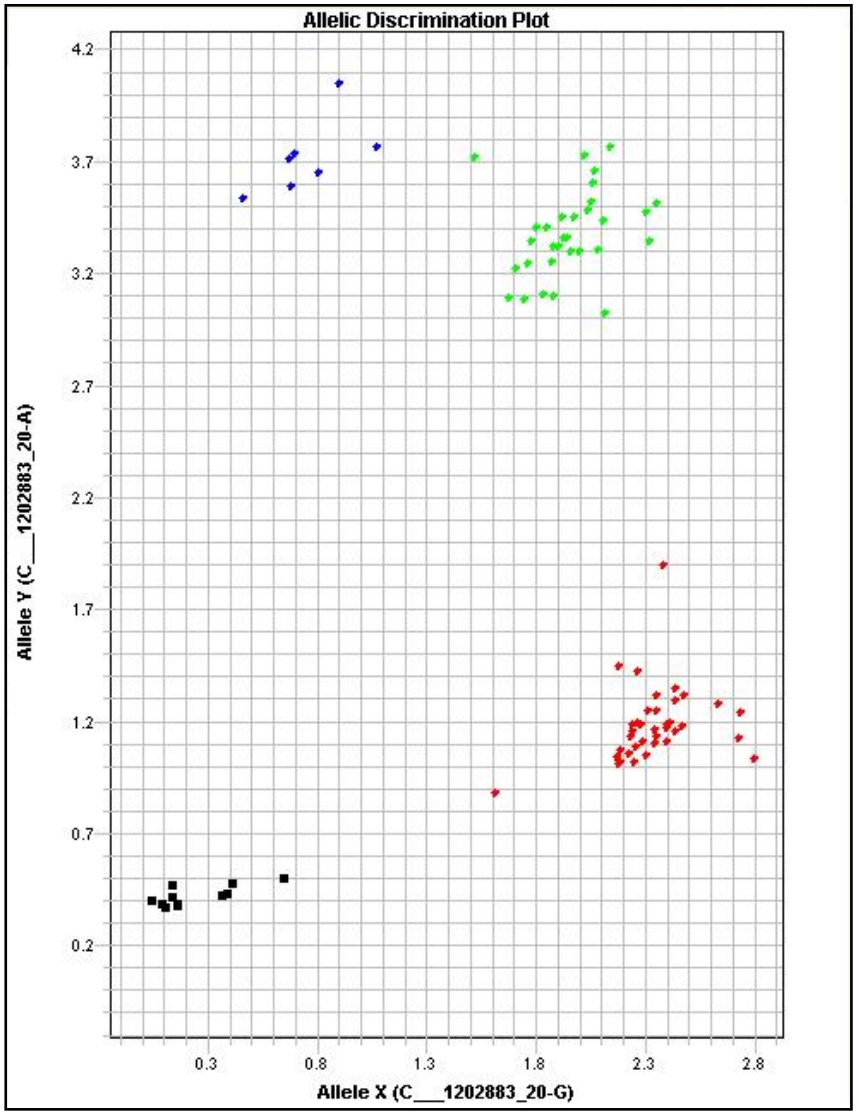

Figure 3.2.2. Allelic Discrimination Analysis for sample batch one using the ABI™ *Taq*Man® MTHFR 677 C>T assay (Y (C_1202883_20-A) vs Allele X (C_1202883_20-G)). Black = NTC, Red = Wild type, Green = Heterozygous, Blue = Homozygous.

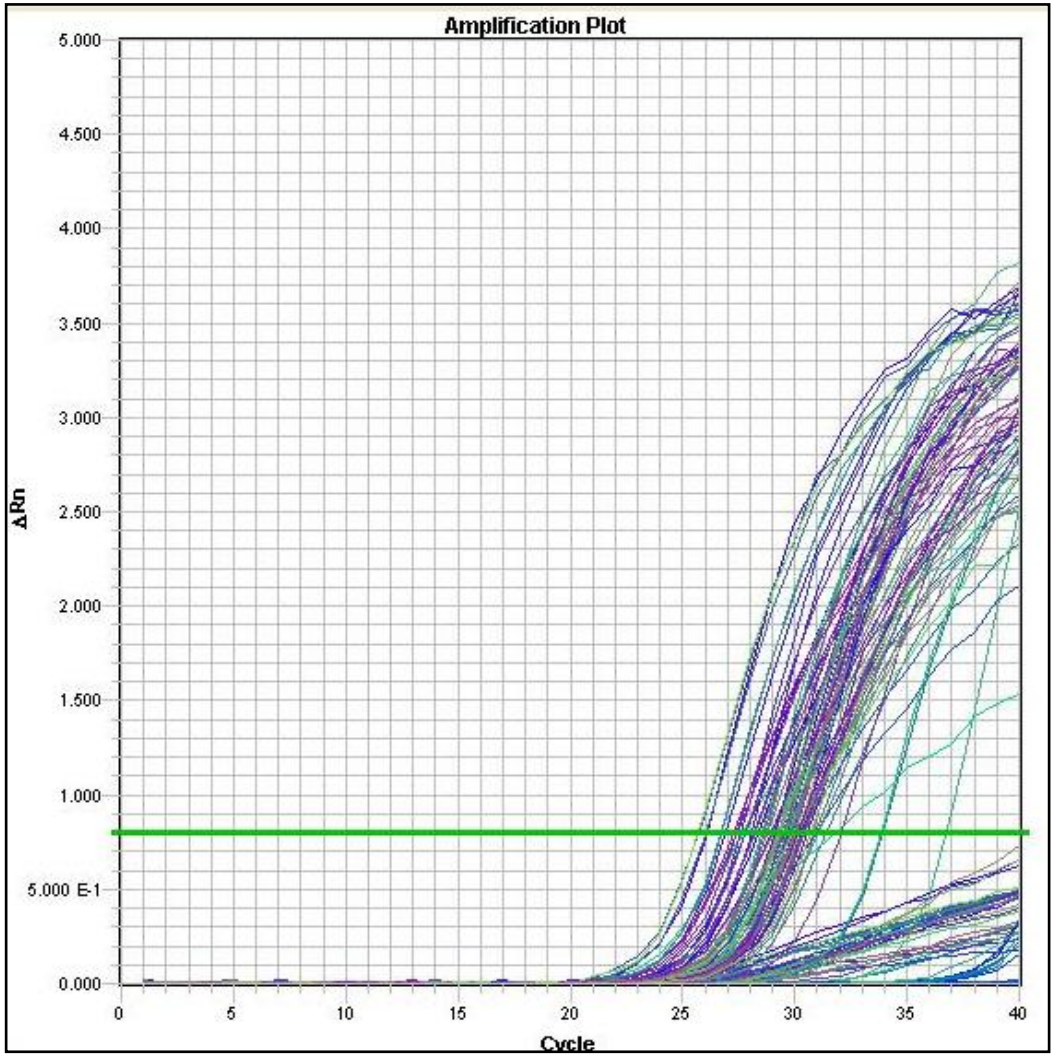

Figure 3.2.3. Amplification achieved in sample batch two using the ABI™ *Taq*Man® MTHFR 677 C>T assay (ΔRn vs number of cycles). The threshold level is set at 1. ΔRn = unit of fluorescence.

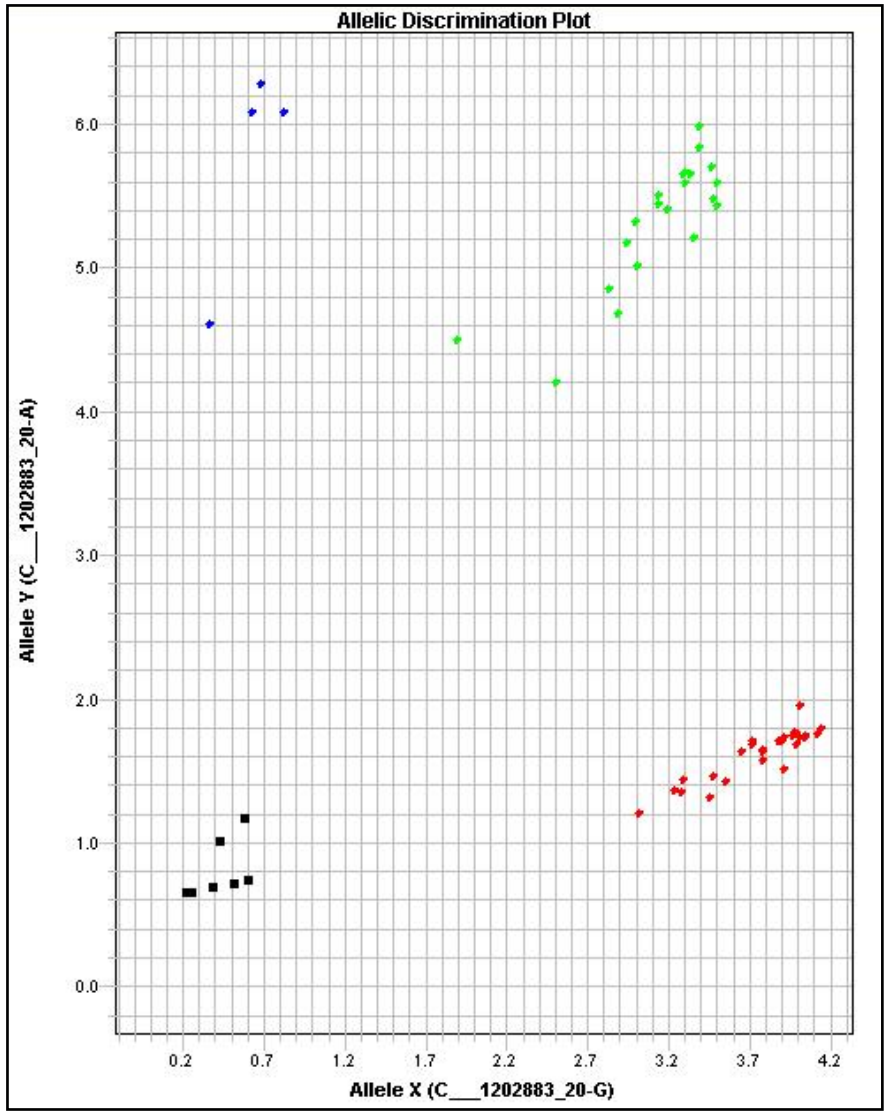

Figure 3.2.4. Allelic Discrimination Analysis for sample batch two using the ABI™ *Taq*Man® MTHFR 677 C>T assay (Allele Y (C_1202883_20-A) vs Allele X (C_1202883_20-G)). Black = NTC, Red = Wild type, Green = Heterozygous, Blue = Homozygous.

The genotype distribution observed for MTHFR 677 C>T achieved through application of the ABI™ 7900HT system is presented in figure 3.2.5 and represents the combined frequencies for both sample batches. The total study population, which includes MS samples and controls, was found to consist of 51.82% wild type, 32.85% heterozygotes, 8.76% homozygotes and 6.57% were undetermined.

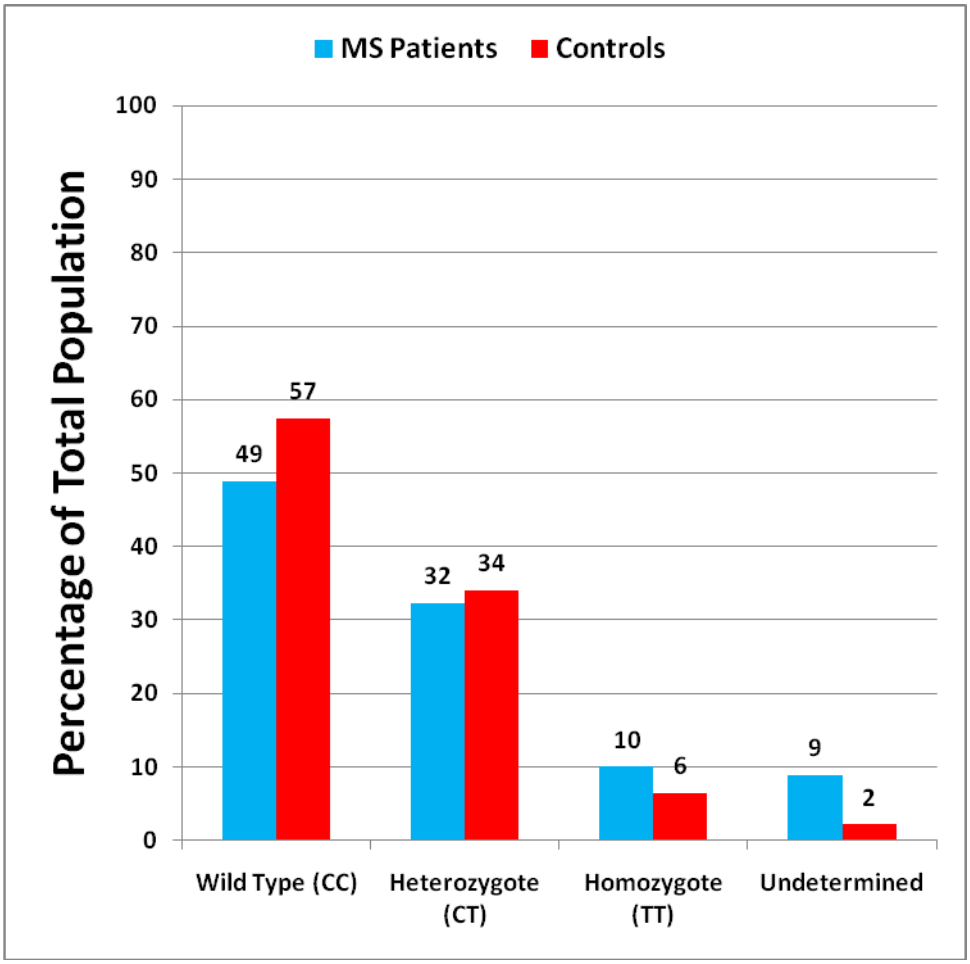

Figure 3.2.5. Genotype distribution of 139 samples obtained using the ABI™ *Taq*Man® MTHFR 677 C>T assay. 90 MS patient samples (in blue) presented with 44 wild type (49%), 29 heterozygotes (32%), 9 (10%) homozygotes and 8 (9%) undetermined genotypes. Among the 49 control samples (in red), 27 (57%) were wild type, 16 (34%) heterozygotes, 3 (6%) homozygotes and 1 (2%) was undetermined.

The investigation of MTHFR 1298 A>C in two sample batches yielded the amplification graphs presented in figures 3.2.6 (sample batch one) and 3.2.8 (sample batch two). These graphs illustrate successful amplification and fluorescence by the ABI™ *Taq*Man® SNP Genotyping assay. The allelic discrimination scatterplots depicted in figures 3.2.7 (sample batch one) and 3.2.9 (sample batch two) clearly indicate the different genotype groups with wild type represented as red dots, heterozygotes in green, homozygotes in blue and NTCs in black.

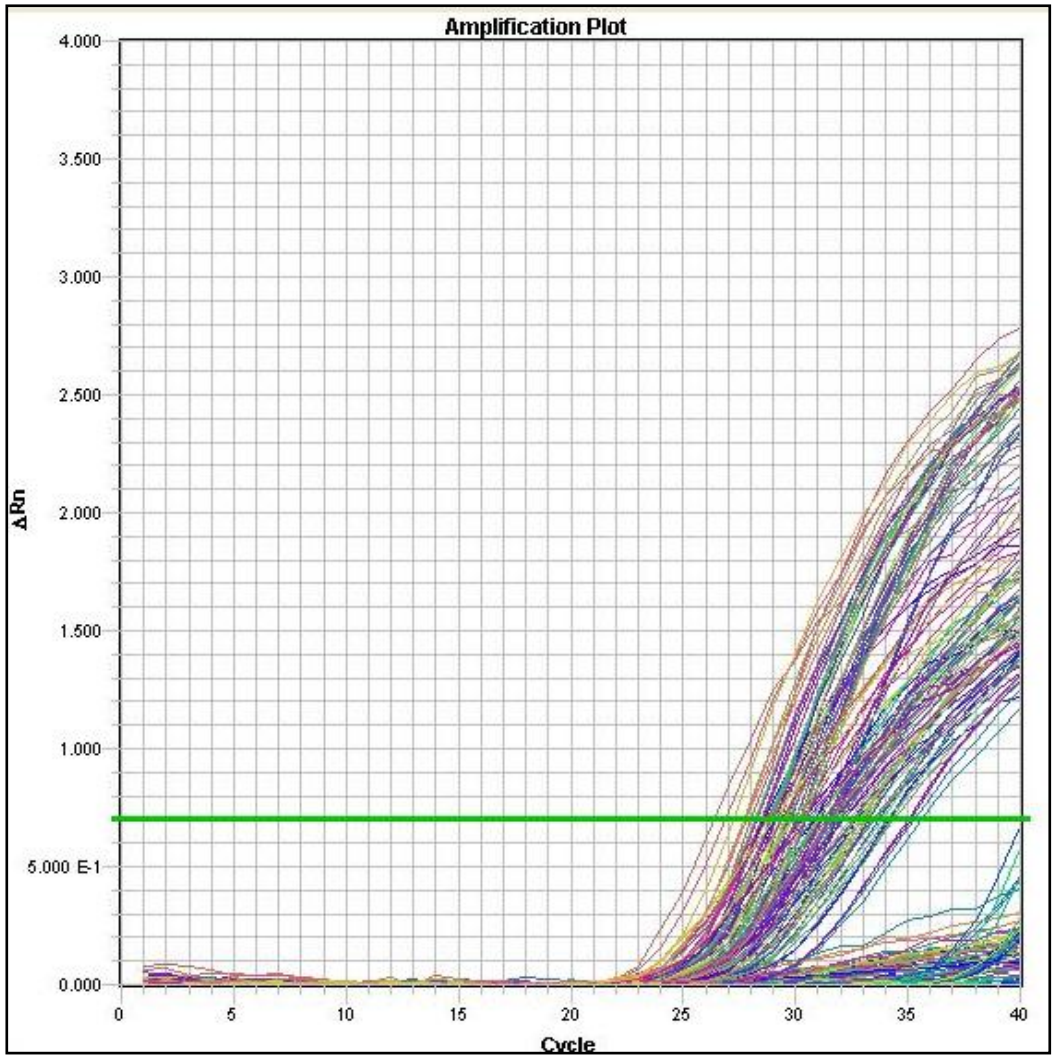

Figure 3.2.6. Amplification achieved in sample batch one using the ABI™ *Taq*Man® MTHFR 1298 A>C assay (ΔRn vs number of cycles). The threshold level is set at 0,7. ΔRn = unit of fluorescence.

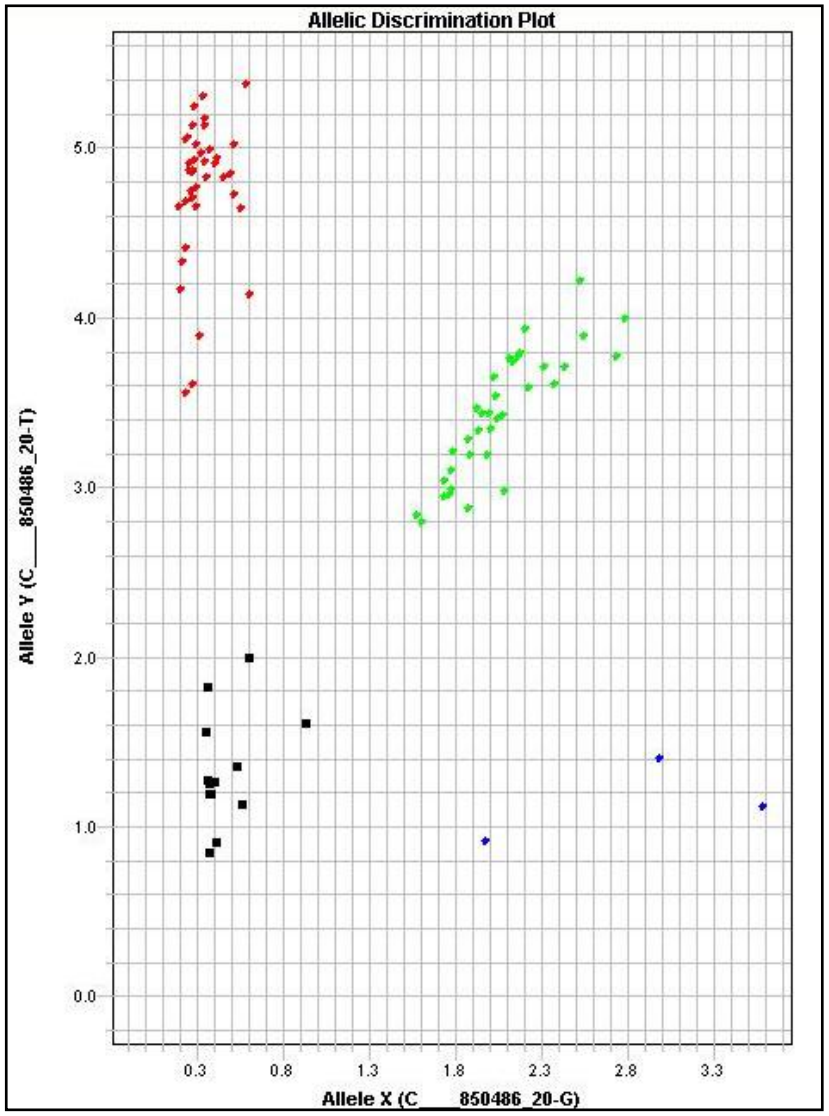

Figure 3.2.7. Allelic Discrimination Analysis for sample batch one using the ABI™ *Taq*Man® MTHFR 1298 A>C assay (Allele Y (C_850486_20-T) vs Allele X (C_850486_20-G)). Black = NTC, Red = Wild type, Green = Heterozygous, Blue = Homozygous.

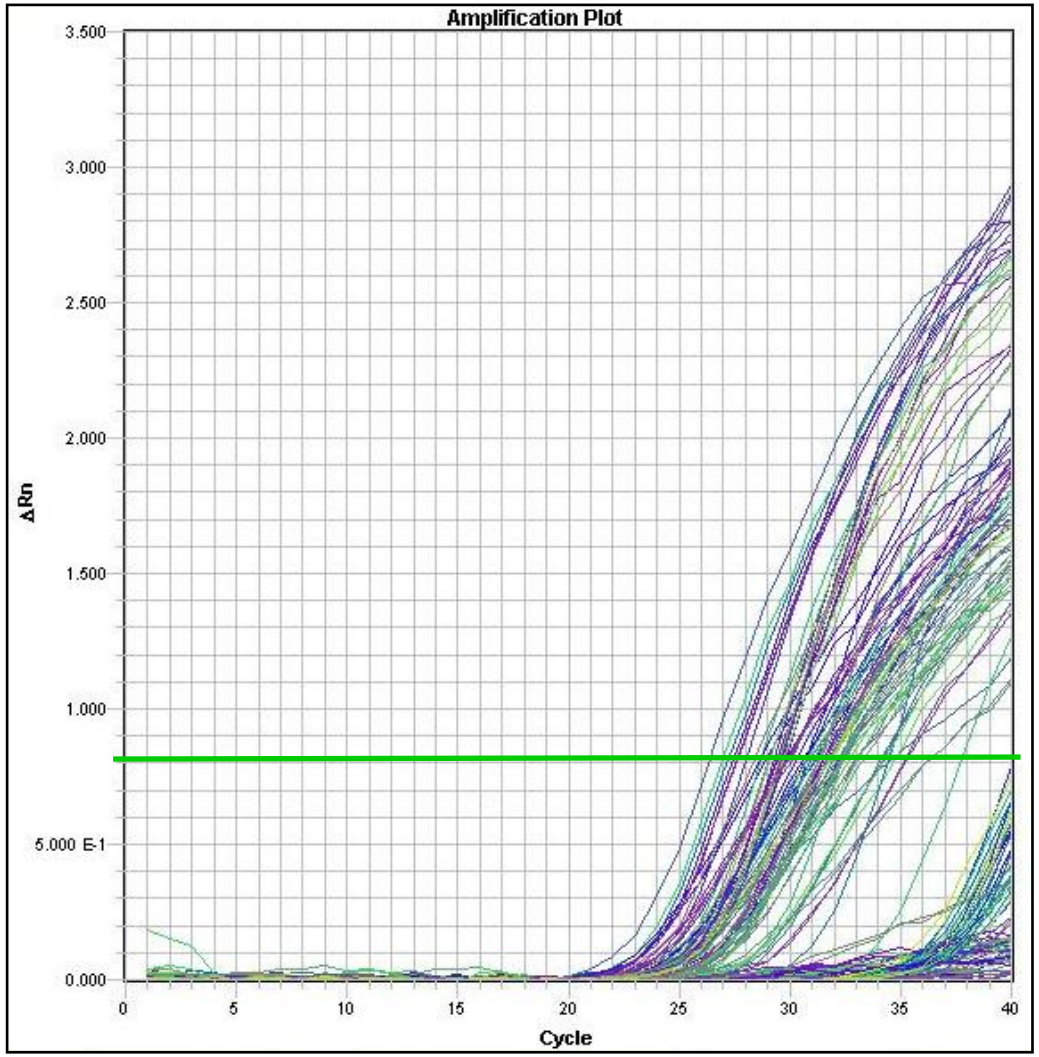

Figure 3.2.8. Amplification achieved in sample batch two using the ABI™ *Taq*Man® MTHFR 1298 A>C assay (ΔRn vs number of cycles). The threshold level is set at 0,7. ΔRn = unit of fluorescence.

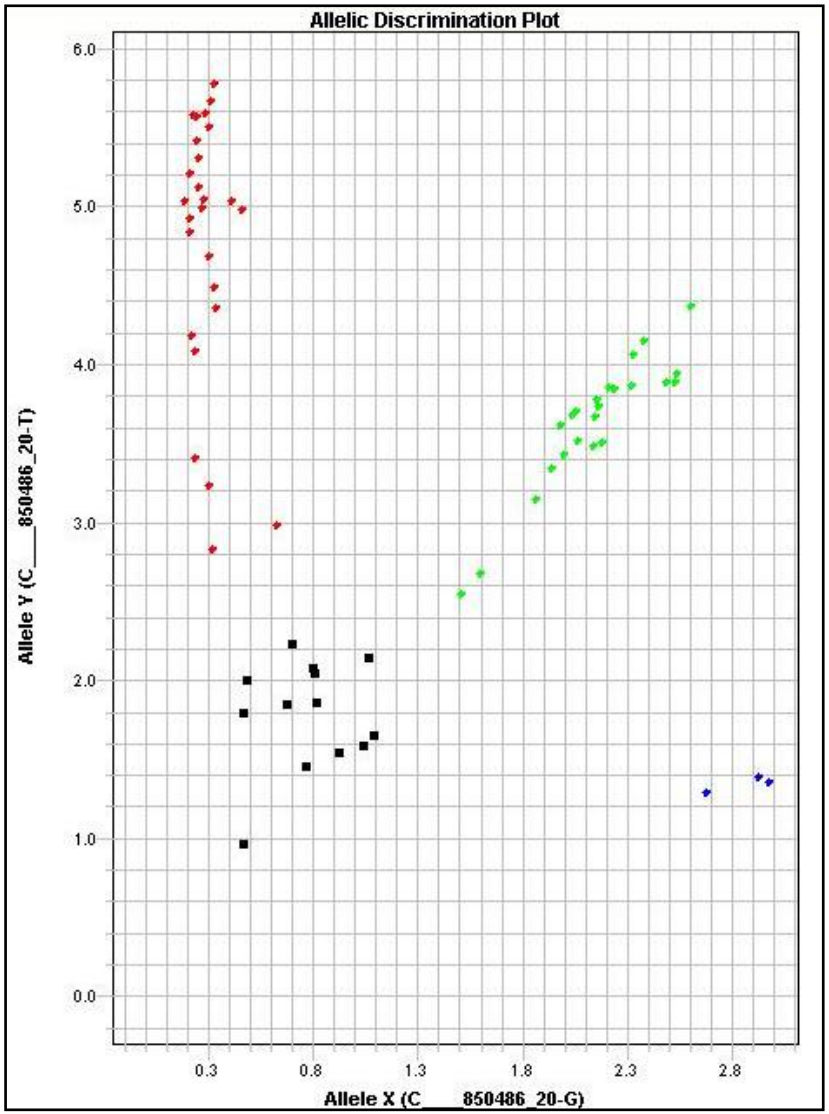

Figure 3.2.9. Allelic Discrimination Analysis for sample batch two using the ABI™ *Taq*Man® MTHFR 1298 A>C assay (Allele Y (C_850486_20-T) vs Allele X (C_850486_20-G)). Black = NTC, Red = Wild type, Green = Heterozygous, Blue = Homozygous.

The genotype distribution observed for MTHFR 1298 A>C achieved through application of the ABI™ 7900HT system is presented in figure 3.2.10 and represents the combined frequencies for both sample batches. The total study population, which includes MS samples and controls, was found to consist of 45.32% wild type, 41.01% heterozygotes, 4.32% homozygotes and 9.35% were undetermined.

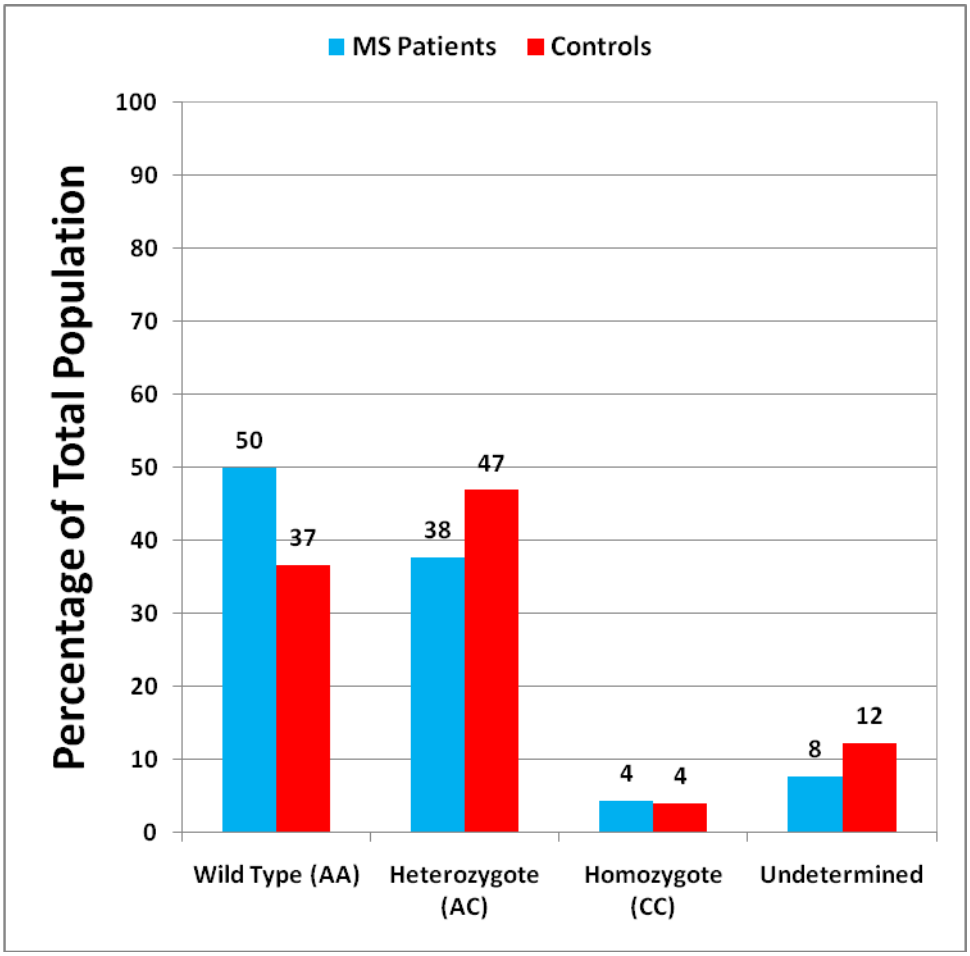

Figure 3.2.10. Genotype distribution of 139 samples obtained using the ABI™ *Taq*Man® MTHFR 1298 A>C assay. 90 MS patient samples (in blue) presented with 45 (50%) wild type, 34 (37.78%) heterozygotes and 4 (4.44%) homozygotes and 7 (7.78%) undetermined genotypes. Among the 49 control samples (in red), 18 (36.73%) were wild type, 23 (46.94%) heterozygotes, 2 (4.08%) homozygotes and 6 (12.24%) were undetermined.

The investigation of HFE 845 G>A in two sample batches yielded the amplification graphs presented in figures 3.2.11 (sample batch one) and 3.2.13 (sample batch two). These graphs illustrate successful amplification and fluorescence by the ABI™ *Taq*Man® SNP Genotyping assay. The allelic discrimination scatterplots depicted in figures 3.2.12 (sample batch one) and 3.2.14 (sample batch two) clearly indicate the different genotype groups with wild types represented as red dots, heterozygotes in green, homozygotes in blue and NTCs in black.

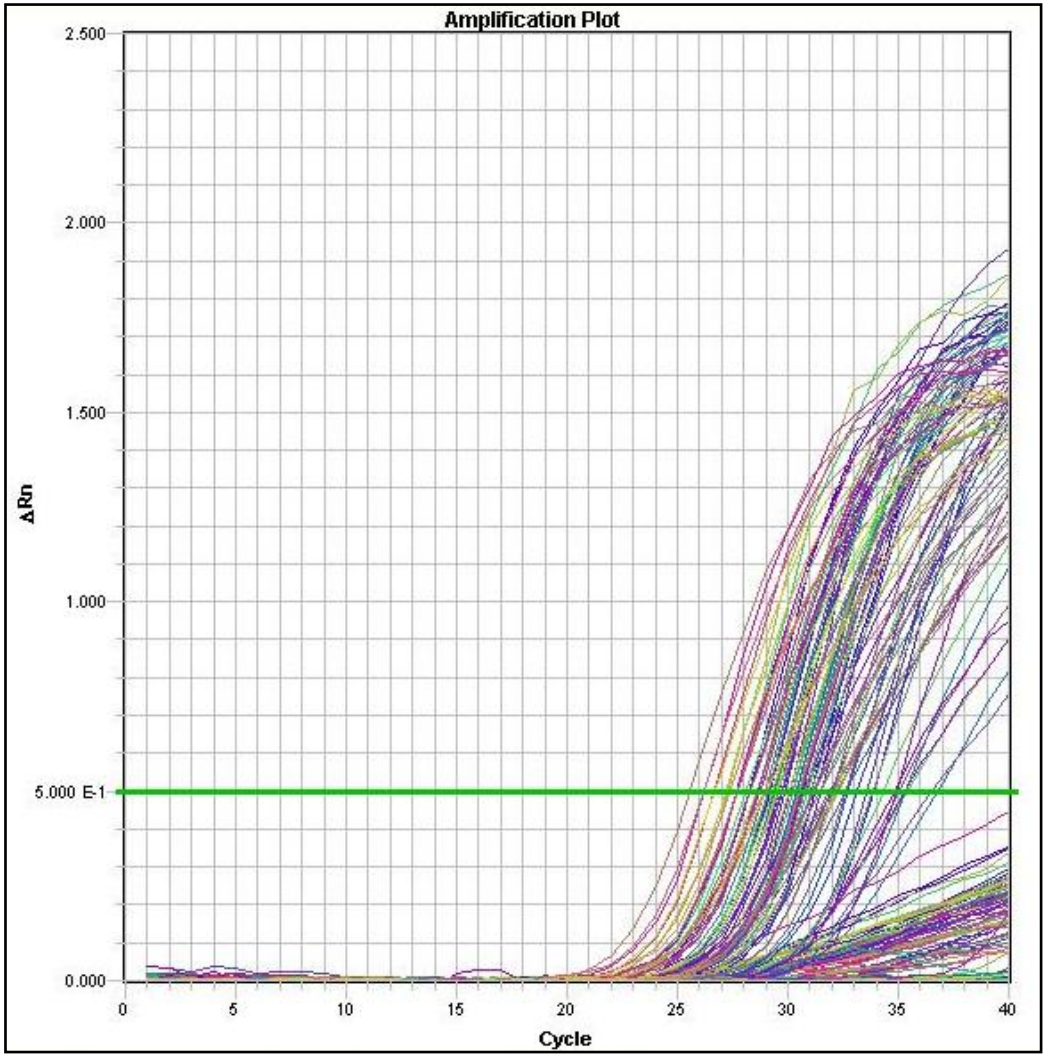

Figure 3.2.11. Amplification achieved in sample batch one using the ABI™ *Taq*Man® HFE 845 G>A assay (ΔRn vs number of cycles). The threshold level is set at 0,5. ΔRn = unit of fluorescence.

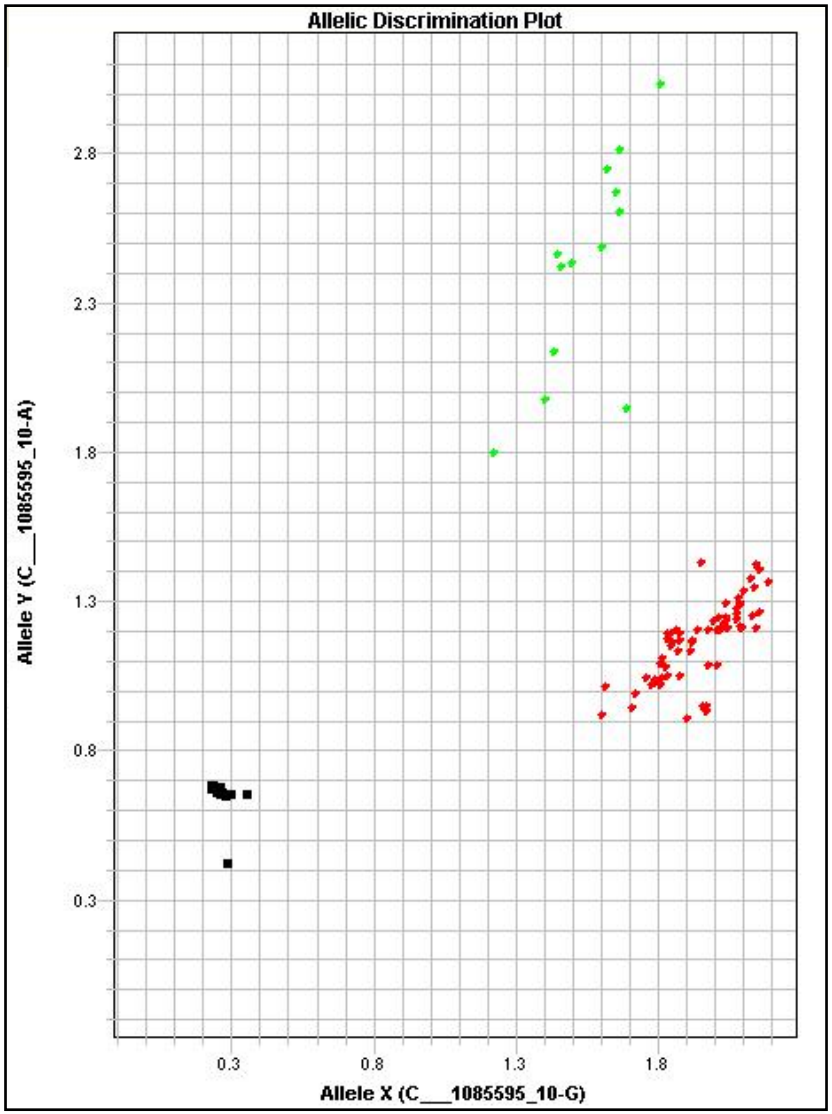

Figure 3.2.12. Allelic Discrimination Analysis for sample batch one using the ABI™ *Taq*Man® HFE 845 G>A assay (Allele Y (C_1085595_10-A) vs Allele X (C_1085595_10-G)). Black = NTC, Red = Wild type, Green = Heterozygous.

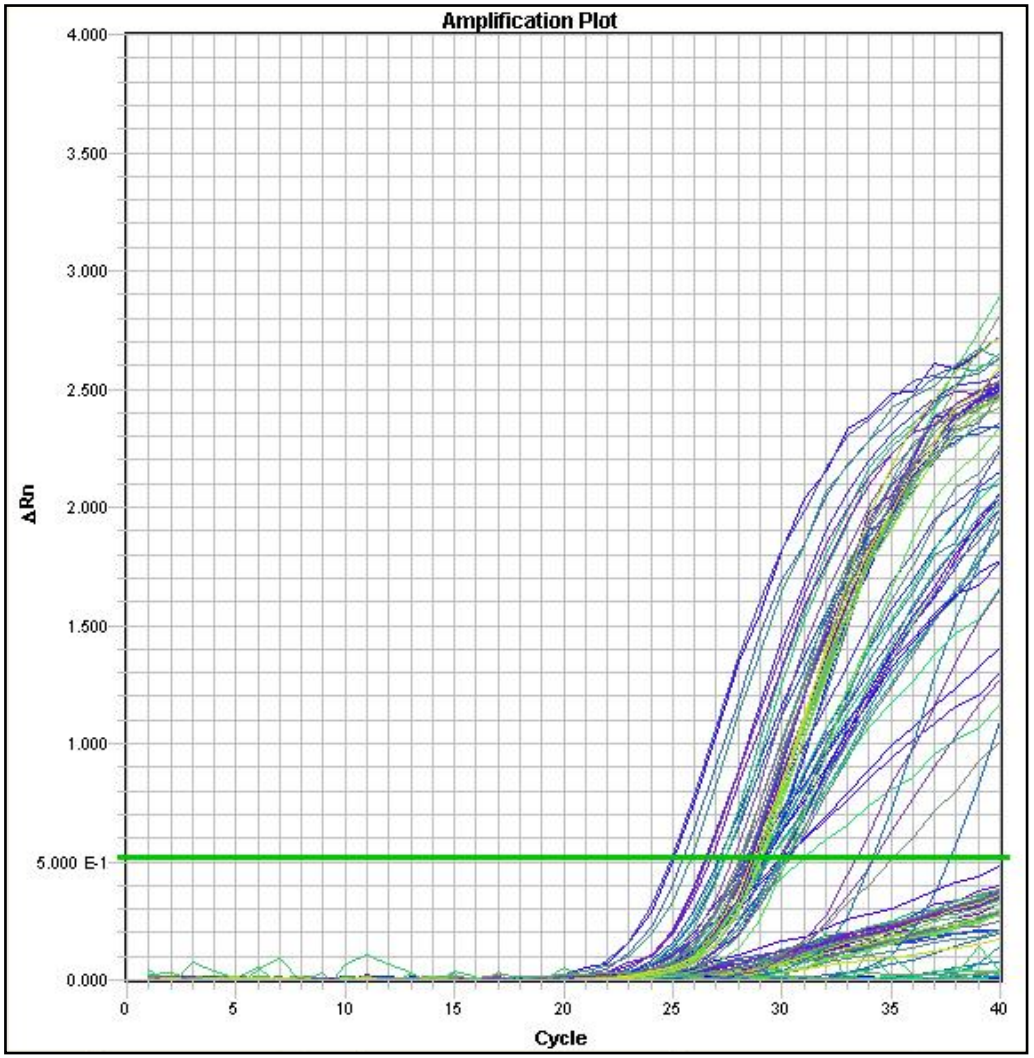

Figure 3.2.13. Amplification achieved in sample batch one using the ABI™ *Taq*Man® HFE 845 G>A assay (ΔRn vs number of cycles). The threshold level is set at 0,5. ΔRn = unit of fluorescence.

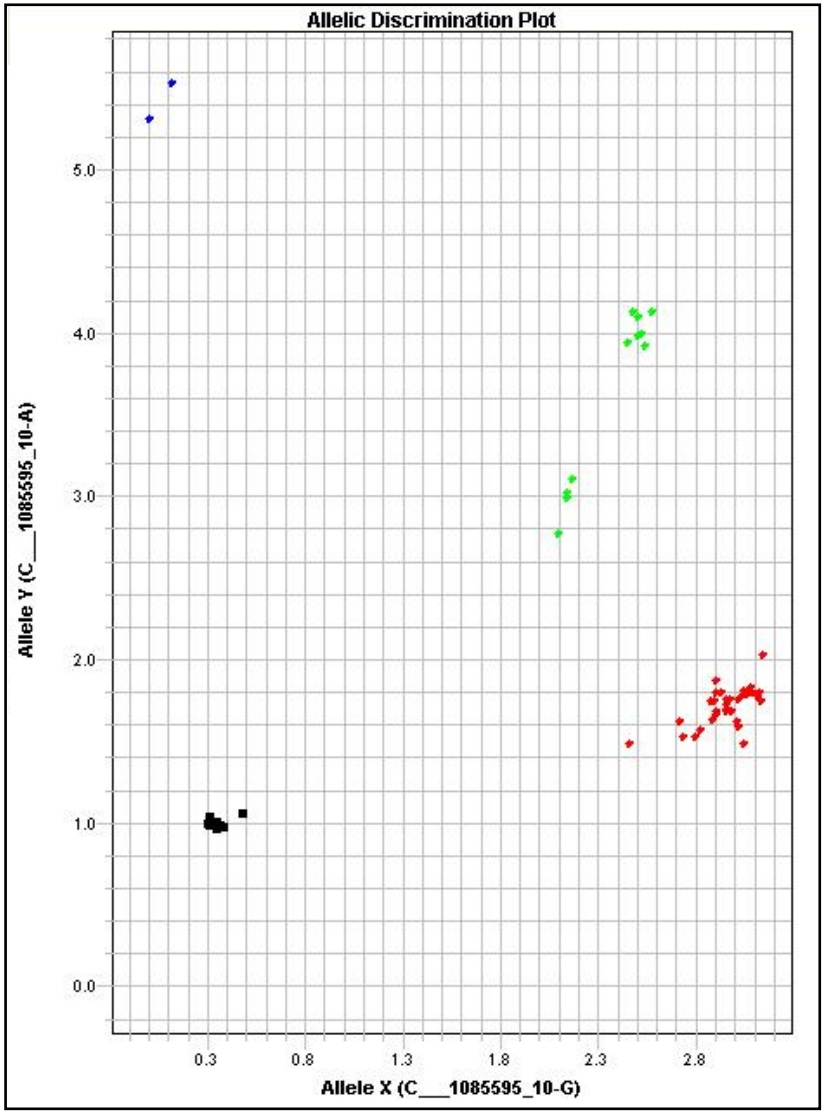

Figure 3.2.14. Allelic discrimination analysis for sample batch two using the ABI™ *Taq*Man® HFE 845 G>A assay (Allele Y (C_1085595_10-A) vs Allele X (C_1085595_10-G)). Black = NTC, Red = Wild type, Green = Heterozygous, Blue = Homozygous.

The genotype distribution observed for HFE 845 G>A achieved through application of the ABI™ 7900HT system is presented in figure 3.2.15 and represents the combined frequencies for both sample batches. The total study population, which includes MS samples and controls, was found to consist of 71.94% wild type, 17.99% heterozygotes, 1.44% homozygotes and 8.63% were undetermined.

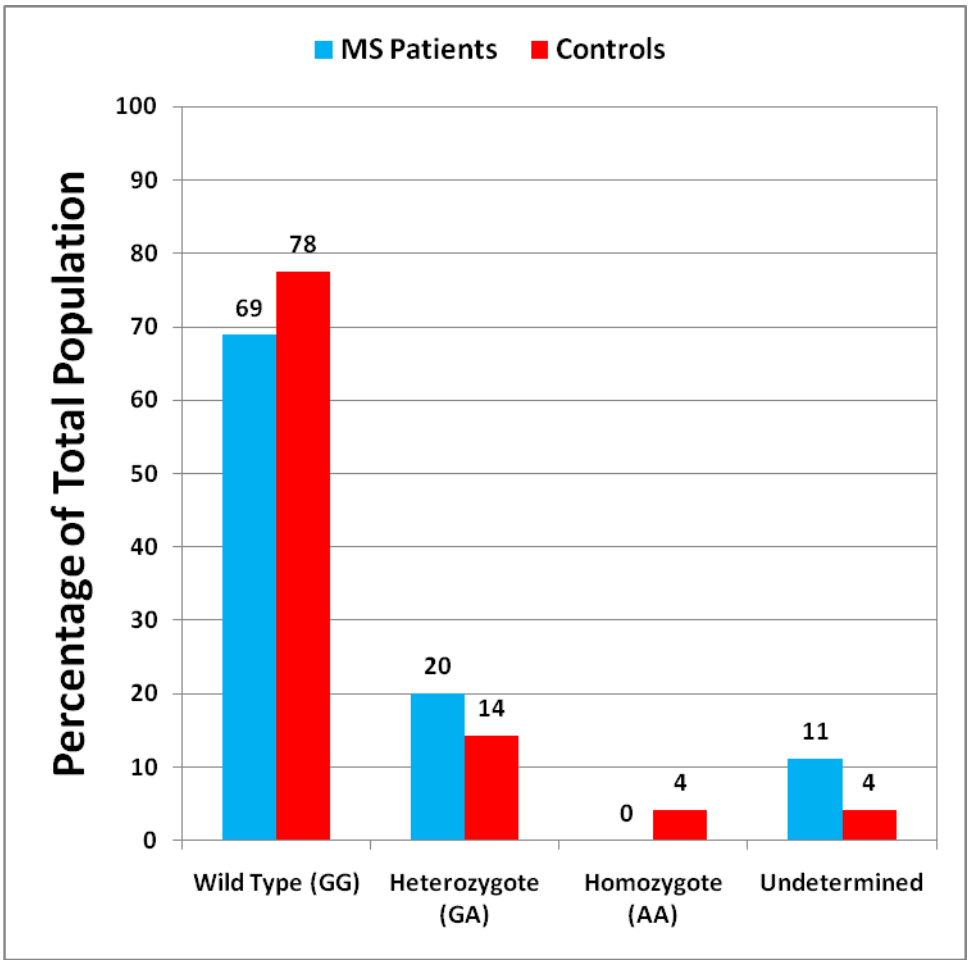

Figure 3.2.15. Genotype distribution of 139 samples obtained using the ABI™ *Taq*Man® HFE 845 G>A assay. 90 MS patient samples (in blue) presented with 62 (69%) wild type, 18 (20%) heterozygotes, no homozygotes and 10 (11%) undetermined genotypes. 38 (78%) wild type were observed among the 49 control samples (in red), 7 (14%) heterozygotes, 2 (4%) homozygotes and 2 (4%) were undetermined.

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

In this study, the Corbett Research Rotor-Gene™ 6000 Series Multiplexing System (5-Plex HRM model) and the ABI TaqMan® SNP Genotyping assays were applied using Allelic Discrimination and Scatterplot analysis for amplification and genotyping. A total of 17 MS samples, 6 controls and 1 None Template Control (NTC) were used for each assay. This test run was to allow for the optimization of the assays, ensure specific binding of the primers and probes, verify amplification of the target sequence, evaluates fluorescence detection of the probes and precision in data acquired by the instrument.

Allelic Discrimination analysis was applied which uses real-time kinetic data from two or more channels to allow genotyping of the samples. ABI™ *Taq*Man® Genotyping Assays consists of unlabeled primers used for amplification of the target sequence and *Taq*Man® MGB Probes, FAM™ and VIC® dyes used in allelic discrimination detection. These probes are labelled with different fluorophores acting as a reporter dye. FAM™ and VIC® fluorescence dyes are allele specific employed to differentiate between wild type and mutant form of each SNPs under investigation. For this reason, the green sensor channel was employed for detection of the FAM™ fluorescence with excitation wavelength of 470 nm and emission wavelength of 510 nm by the Rotor-Gene™ fluorometer and the yellow channel for detection of the VIC® fluorescence with excitation wavelength of 530 nm and emission wavelength of 555 nm, respectively.

The allelic discrimination analysis for the MTHFR 677 C>T assay is presented in figure 3.3.1 with the legend and genotypes illustrated by table 3.3.1. The lines with intermittent circles represent the wild type (C) allele, which is detected by VIC®-labelled probes, while the lines without intermittent circles signify the homozygous (T) allele detected by FAM™-labelled probes. Amplification of both alleles indicated through significant (i.e. above the threshold level) fluorescence of both dyes represents the heterozygous (CT) state. The indicated threshold level (0.1) is a discriminatory parameter employed during analysis which determines the difference between a positive and negative amplification reading. Fluorescence readings above the threshold indicate the presence of a specific allele.

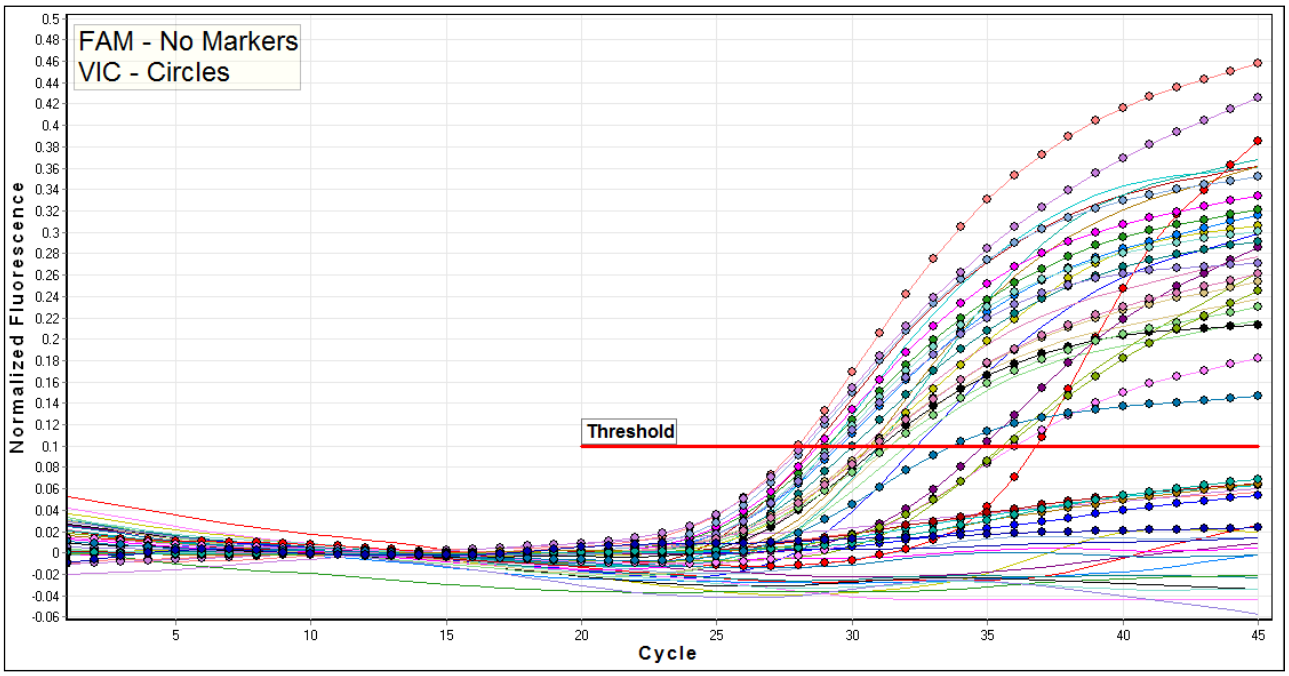

Figure 3.3.1. Allelic discrimination analysis using the ABI™ *Taq*Man® MTHFR 677 C>T assay (normalized fluorescence vs number of cycles).

In addition to allelic discrimination analysis, scatterplot analysis was applied in this study. Scatterplot analysis is performed using two channels simultaneously. The genotype determination is based on regions found on the scatterplot, as well as relative expression of amplification across two channels. The plot accounts for different fold increase in each channel, and log transformed to highlight the differences in expression between samples.

The scatterplot analysis for the MTHFR 677 C>T assay is presented in figure 3.3.2 with the legend and genotypes illustrated by table 3.3.1. Genotype determinations are made according to the FAM™ / VIC® fluorescence, as indicated in the aforementioned table. The genotype distribution observed in the patient screening was: 62,5% wild type (CC), 16,67% heterozygous (CT) and 20,83% homozygous/mutant (TT). The control group consisted of two (33%) wild type (CC), two (33%) homozygous/mutant (TT) and two (33%) heterozygous (CT) samples.

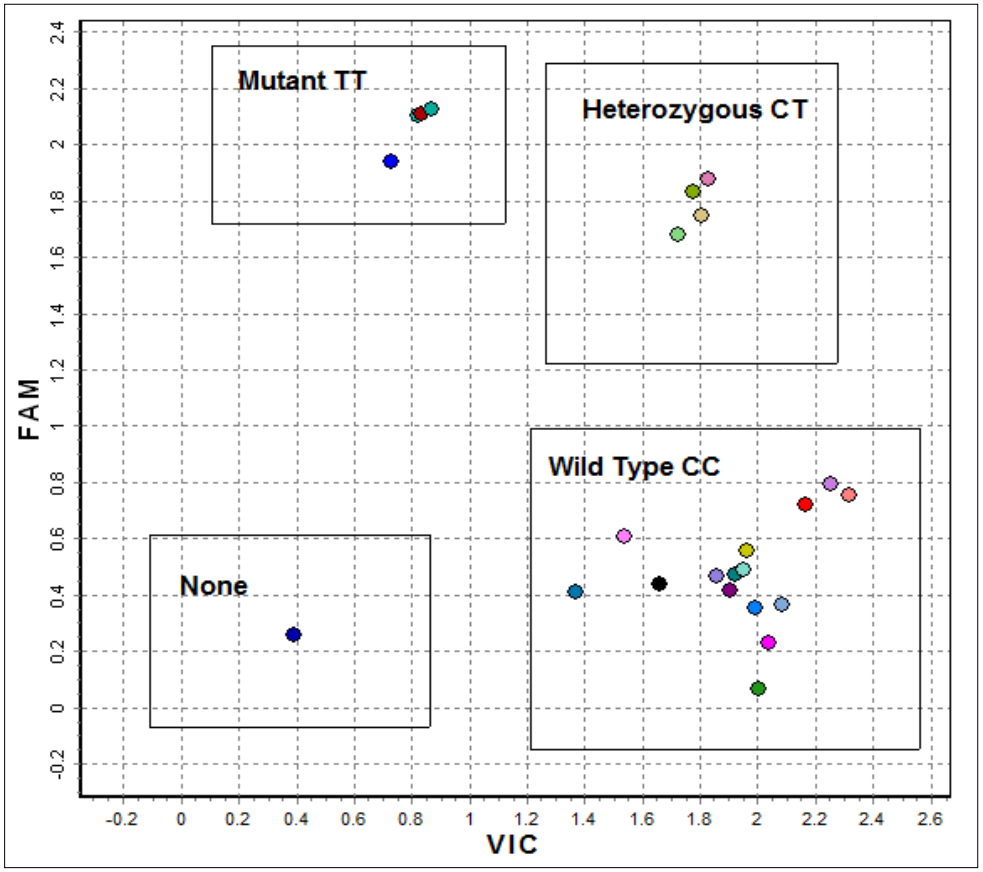

Figure 3.3.2. Genotypes grouped by scatterplot analysis (FAM™ fluorescence vs VIC® fluorescence) of the ABI™ *Taq*Man® MTHFR 677 C>T assay. The legend is presented in table 3.3.1.

Table 3.3.1. Legend for figure 3.3.1 and 3.3.2, indicating genotypes of the samples based on Allelic Discrimination data and Scatterplot analysis.

No.	Colour	Name	Genotype	FAM™	VIC®
1		TvR	Wild Type CC	No Reaction	Reaction
2		KN	Wild Type CC	No Reaction	Reaction
3		PS	Mutant TT	Reaction	No Reaction
4		PJvR	Wild Type CC	No Reaction	Reaction
5		MH	Wild Type CC	No Reaction	Reaction
6		RLR	Wild Type CC	No Reaction	Reaction
7		NvDB	Wild Type CC	No Reaction	Reaction
8		NN	Wild Type CC	No Reaction	Reaction
9		FP	Wild Type CC	No Reaction	Reaction
10		RS	Wild Type CC	No Reaction	Reaction
11		SDS	Wild Type CC	No Reaction	Reaction
12		TFS	Mutant TT	Reaction	No Reaction
13		SJvR	Heterozygous CT	Reaction	Reaction
14		LDJ	Heterozygous CT	Reaction	Reaction
15		ES	Wild Type CC	No Reaction	Reaction
16		NA	Wild Type CC	No Reaction	Reaction
17		MB	Wild Type CC	No Reaction	Reaction
18		CC	Wild Type CC	No Reaction	Reaction
19		MN	Heterozygous CT	Reaction	Reaction
20		HC	Mutant TT	Reaction	No Reaction
21		S1	Mutant TT	Reaction	No Reaction
22		K1	Heterozygous CT	Reaction	Reaction
23		K2	Mutant TT	Reaction	No Reaction
24		K3	Wild Type CC	No Reaction	Reaction
25		NTC	None	No Reaction	No Reaction

The allelic discrimination analysis for the MTHFR 1298 A>C assay is presented in figure 3.3.3 with the legend and genotypes illustrated by table 3.3.2. The lines with intermittent circles represent the homozygous (C) allele, which is detected by VIC®-labelled probes, and lines without intermittent circles correspond to the wild type (A) allele detected by FAM™-labelled probes. Amplification of both alleles indicated through significant (i.e. above the threshold level) fluorescence of both dyes represents the heterozygous (AC) state. The threshold level is set at 0.29558.

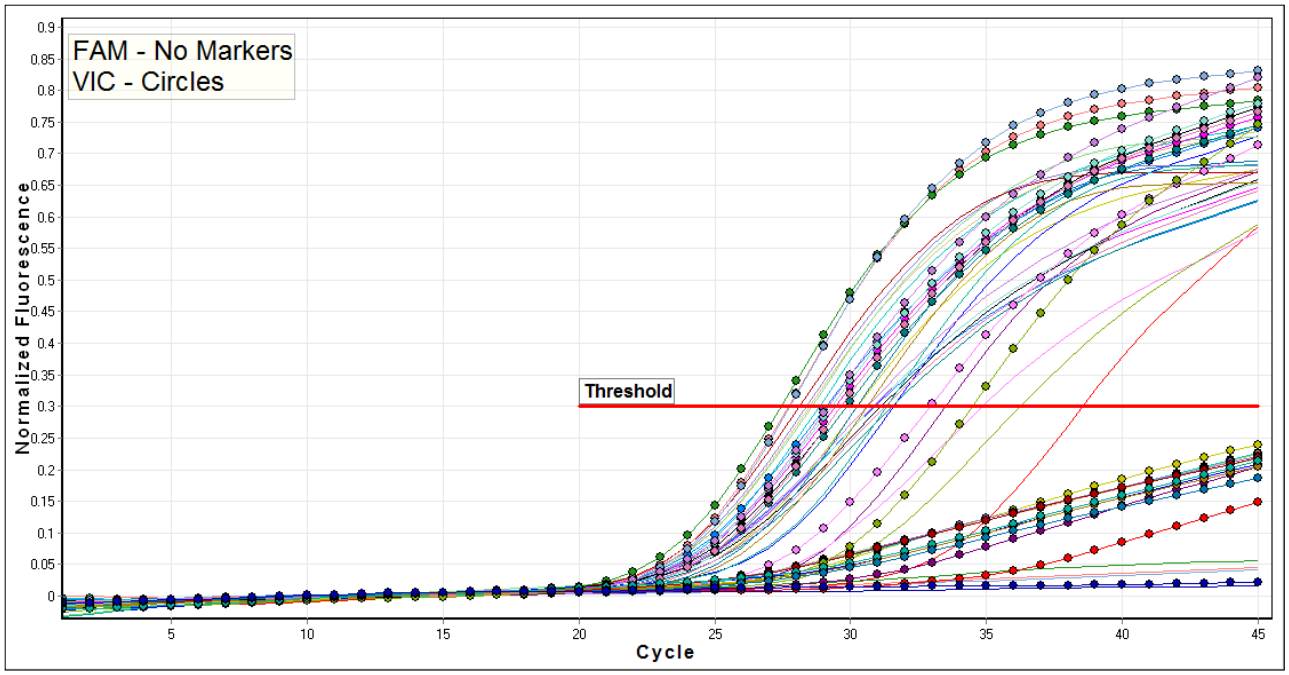

Figure 3.3.3. Allelic Discrimination analysis using the ABI™ *Taq*Man® MTHFR 1298 A>C assay (normalized fluorescence vs number of cycles).

The scatterplot analysis for the MTHFR 1298 A>C assay is presented in figure 3.3.2 with the legend and genotypes illustrated by table 3.3.4. The genotype distribution observed was: 50% wild type (AA), 37,5% heterozygous (CT) and 12,5% homozygous/mutant (TT). The control group consisted of two wild type (CC), two homozygous/mutant (TT) and two heterozygous (CT) samples.

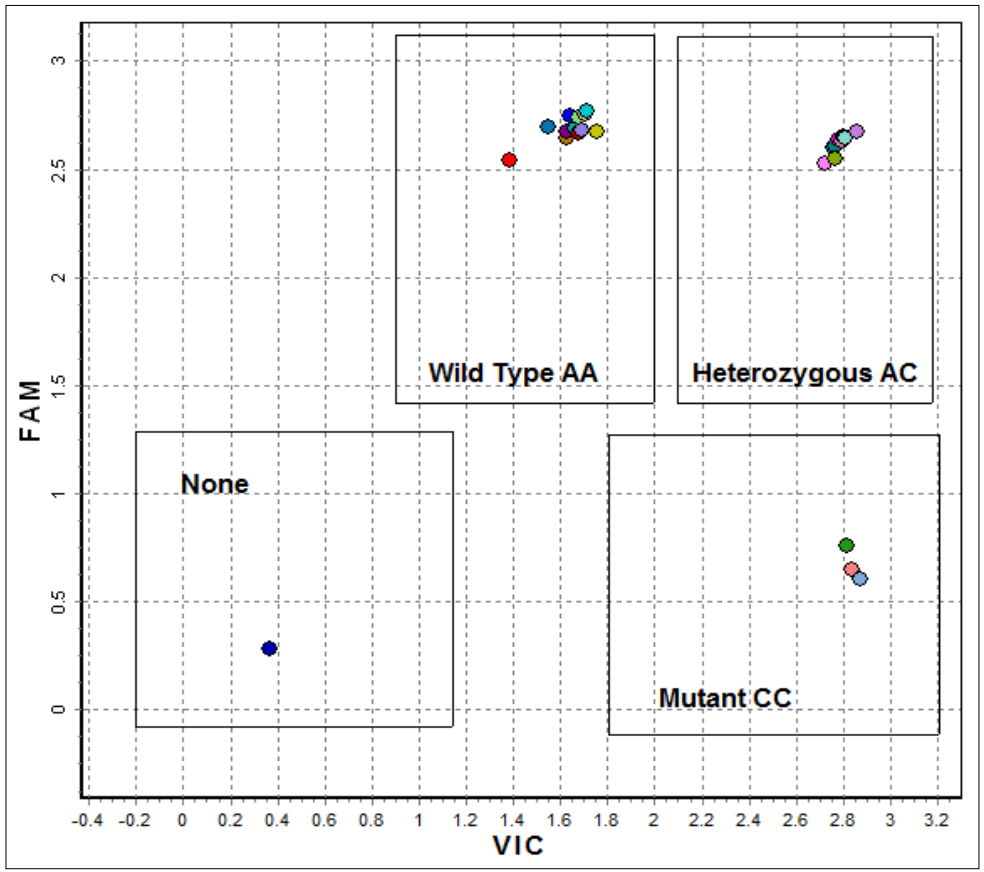

Figure 3.3.4. Genotypes grouped by scatter analysis (FAM™ fluorescence vs VIC® fluorescence) of the ABI™ *Taq*Man® MTHFR 1298 A>C assay. The legend is presented in table 3.3.2.

Table 3.3.2. Legend for figure 3.3.3 and 3.3.4, indicating genotypes of the samples based on Allelic Discrimination data and ScatterPlot analysis.

No.	Colour	Name	Genotype	FAM™	VIC®
1		TvR	Wild Type AA	Reaction	No Reaction
2		KN	Wild Type AA	Reaction	No Reaction
3		PS	Wild Type AA	Reaction	No Reaction
4		PJvR	Wild Type AA	Reaction	No Reaction
5		MH	Heterozygous AC	Reaction	Reaction
6		RLR	Heterozygous AC	Reaction	Reaction
7		NvDB	Heterozygous AC	Reaction	Reaction
8		NN	Mutant CC	No Reaction	Reaction
9		FP	Mutant CC	No Reaction	Reaction
10		RS	Heterozygous AC	Reaction	Reaction
11		SDS	Heterozygous AC	Reaction	Reaction
12		TFS	Wild Type AA	Reaction	No Reaction
13		SJvR	Wild Type AA	Reaction	No Reaction
14		LDJ	Wild Type AA	Reaction	No Reaction
15		ES	Heterozygous AC	Reaction	Reaction
16		NA	Mutant CC	No Reaction	Reaction
17		MB	Wild Type AA	Reaction	No Reaction
18		CC	Heterozygous AC	Reaction	Reaction
19		MN	Heterozygous AC	Reaction	Reaction
20		HC	Wild Type AA	Reaction	No Reaction
21		BR	Wild Type AA	Reaction	No Reaction
22		MJ	Heterozygous AC	Reaction	Reaction
23		EB	Wild Type AA	Reaction	No Reaction
24		MK	Wild Type AA	Reaction	No Reaction
25		NTC	None	No Reaction	No Reaction

The allelic discrimination analysis for the HFE 845 G>A assay is presented in figure 3.3.5 with the legend and genotypes illustrated by table 3.3.3. Lines with intermittent circles represent the wild type (G) allele, which is detected by VIC®-labelled probes, while the lines without the intermittent circles signify the homozygous (A) allele detected by FAM™-labelled probes. Amplification of both alleles indicated through significant (i.e. above the threshold level) fluorescence of both dyes represents the heterozygous (GA) state. The threshold level is set at 0.1.

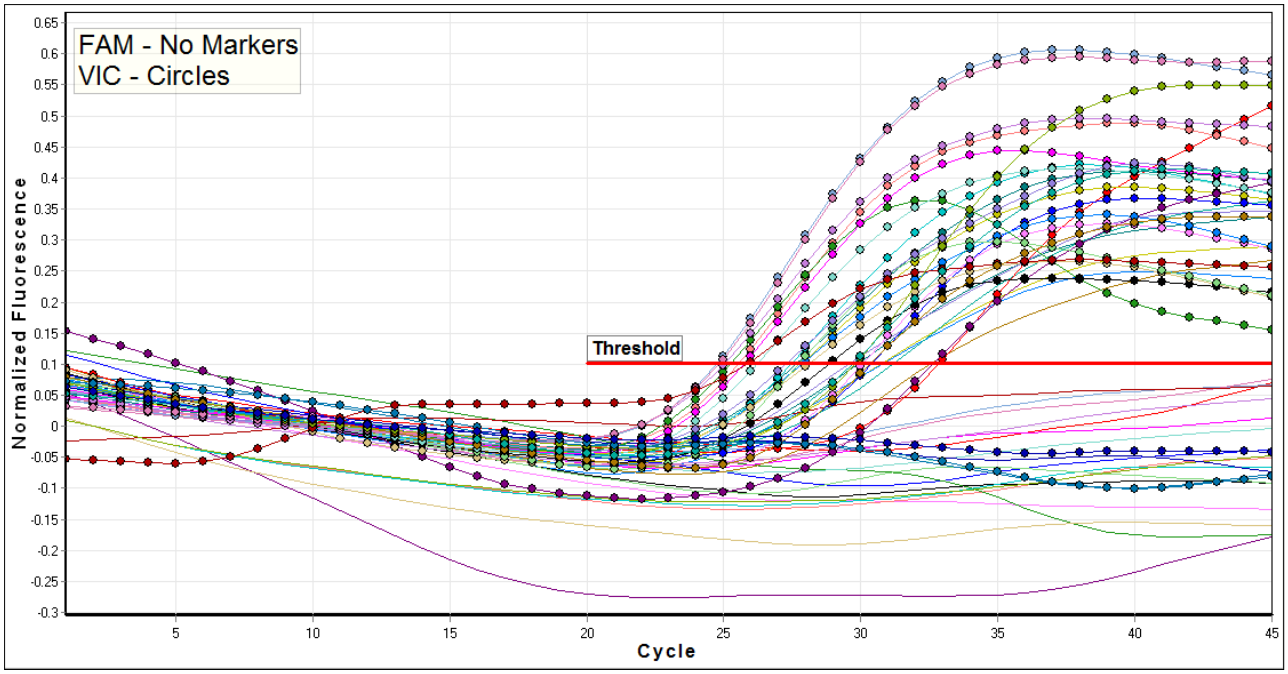

Figure 3.3.5. Allelic Discrimination analysis using the ABI™ *Taq*Man® HFE 845 G>A assay (normalized fluorescence vs number of cycles).

The scatterplot analysis for the HFE 845 G>A assay is presented in figure 3.3.6 with the legend and genotypes illustrated by table 3.3.3. The genotype distribution observed was: 70.83% wild type (GG), 25% heterozygous (GA), 0% homozygous/mutant (AA) and 4.17% undetermined. The potential causes for the failed reaction are numerous and include defects in the sample tube, pipetting error during reagent addition, erroneous tube position on the rotor of the instrument, ambient fluorescence of nearby sample tubes on the rotor may have interfered with the optical detector unit or the sample DNA may be degraded in the *HFE* region of interest thus preventing primer/probe binding and amplification/fluorescence. The control group consisted of five wild type (CC) and one heterozygous (CT) sample.

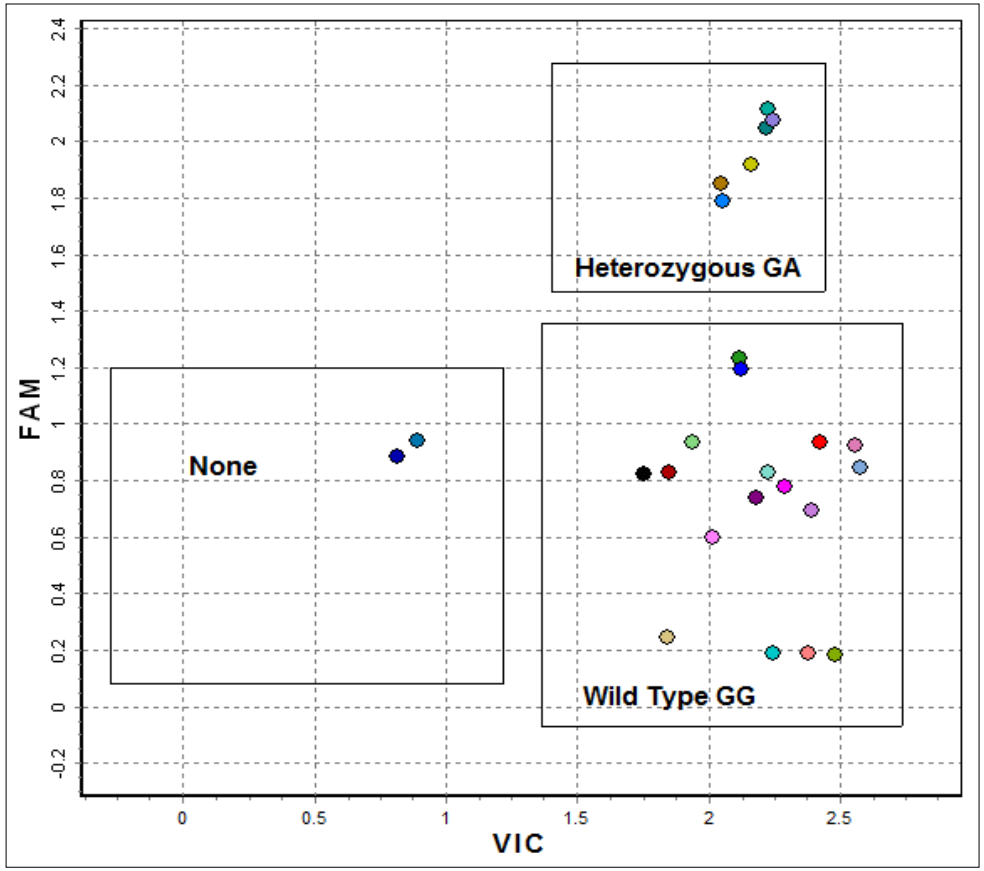

Figure 3.3.6. Genotypes grouped by scatter analysis (FAM™ fluorescence vs VIC® fluorescence) of the ABI™ *Taq*Man® HFE 845 G>A assay. The legend is presented in table 3.3.3.

Table 3.3.3. Legend for figure 3.3.5 and 3.3.6, indicating genotypes of the samples based on Allelic Discrimination data and ScatterPlot analysis.

No.	Colour	Name	Genotype	FAM™	VIC®
1		TvR	Wild Type GG	No Reaction	Reaction
2		KN	Heterozygous GA	Reaction	Reaction
3		PS	Wild Type GG	No Reaction	Reaction
4		PJvR	Wild Type GG	No Reaction	Reaction
5		MH	Wild Type GG	No Reaction	Reaction
6		RLR	Heterozygous GA	Reaction	Reaction
7		NvDB	Heterozygous GA	Reaction	Reaction
8		NN	Wild Type GG	No Reaction	Reaction
9		FP	Wild Type GG	No Reaction	Reaction
10		RS	Wild Type GG	No Reaction	Reaction
11		SDS	Wild Type GG	No Reaction	Reaction
12		TFS	Wild Type GG	No Reaction	Reaction
13		SJvR	Wild Type GG	No Reaction	Reaction
14		LDJ	Wild Type GG	No Reaction	Reaction
15		ES	Wild Type GG	No Reaction	Reaction
16		NA	Wild Type GG	No Reaction	Reaction
17		MB	Heterozygous GA	Reaction	Reaction
18		CC	Wild Type GG	No Reaction	Reaction
19		MN	Wild Type GG	No Reaction	Reaction
20		HC	Wild Type GG	No Reaction	Reaction
21		BR	Heterozygous GA	Reaction	Reaction
22		MJ	Wild Type GG	No Reaction	Reaction
23		EB	Heterozygous GA	Reaction	Reaction
24		MK	Undetermined	No Reaction	No Reaction
25		NTC	None	No Reaction	No Reaction

3.4. Roche LightCycler® 480 II

Custom designed Roche primers and HybProbe probes were used for the mutation detection experiments using melt curve genotyping with the Roche LC 480 II system. The investigation of MTHFR 1298 A>C in two sample batches at an annealing temperature of 52ºC yielded the amplification graphs presented in figures 3.4.1 (sample batch one) and 3.4.3 (sample batch two). These graphs illustrate successful amplification and fluorescence by the primers and HybProbe probes, respectively. The melting peaks depicted in figures 3.4.2 (sample batch one) and 3.4.4 (sample batch two) clearly indicate the different genotype groups with wild types represented by blue peaks, heterozygotes in red and homozygotes in pink. Baseline colours (cyan, green, brown, etc.) represent NTCs, failed reactions or empty wells on the plate. Genotypes obtained were verified by the internal controls loaded on each plate, matching the DNA sequencing data.

Application of an adapted version of the above protocol for mutation detection of MTHFR 677 C>T and HFE 845 G>A were unsuccessful. Optimization of the protocols for these SNPs included adjustments of the thermal cycling condition (specifically annealing temperature and melting curve parameters), $MgCl_2$ titration, HybProbe probe titration and testing of the primer efficacy (through conventional PCR). The above approach for the optimization of the protocol was unsuccessful in yielding the desired results (data not shown).

The difficulties encountered during this process resulted from the customized nature of the primers and HybProbe probes, as they were not standardized pre-designed assays, and constraints on available time for optimization and standardization of the LC 480 II system.

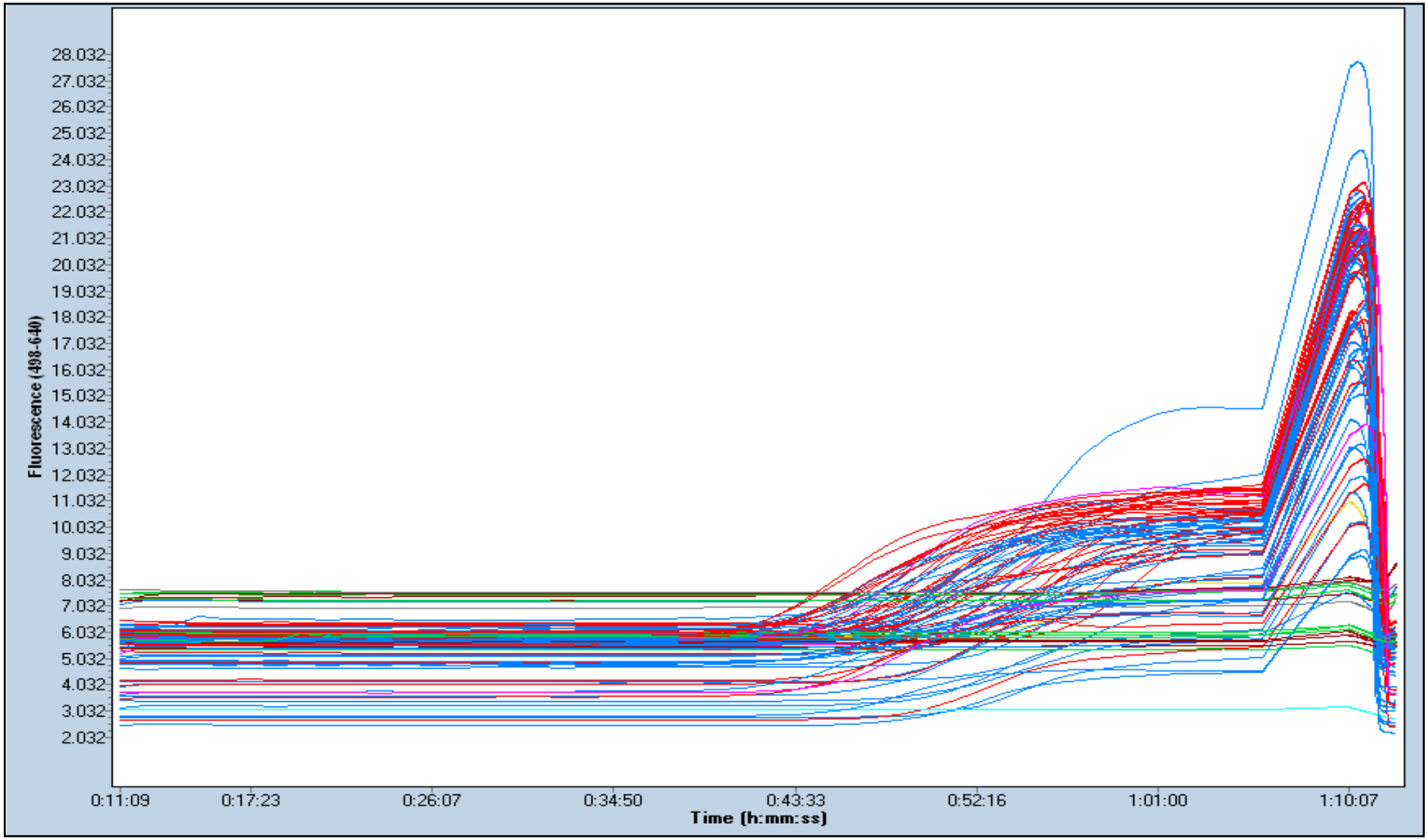

Figure 3.4.1. Amplification achieved in sample batch one using the Roche primers and HybProbe probes for MTHFR 1298 A>C (fluorescence at 498-640 nm vs time). The exponential phase was reached at ~00:43:33 and at ~1:05:00 the melting curve phase commenced.

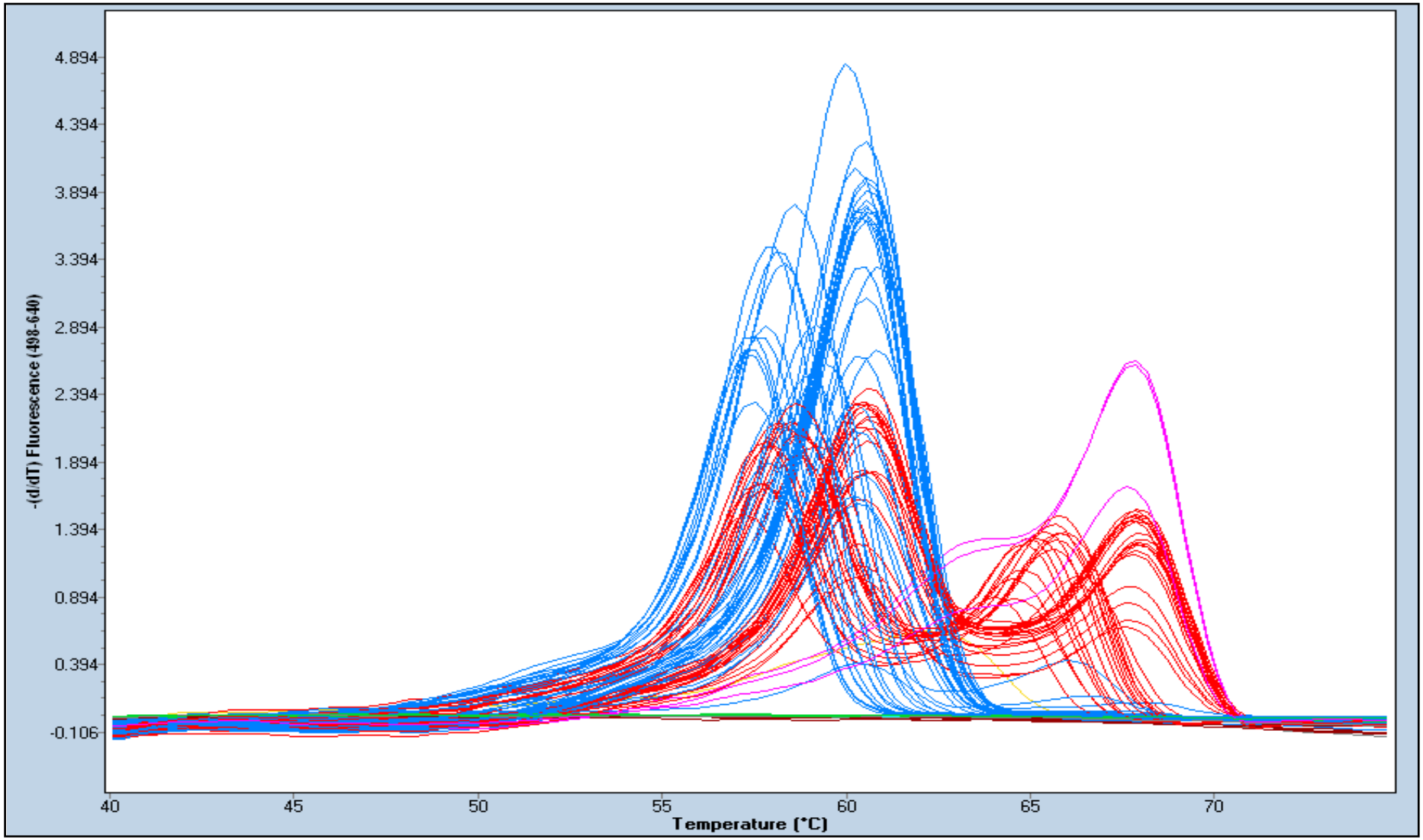

Figure 3.4.2. Melting peaks achieved in sample batch one using the Roche primers and HybProbe probes for MTHFR 1298 A>C (fluorescence at 498-640 nm vs temperature). Blue peaks represent wild type, red peaks represent heterozygotes and pink peaks represent homozygotes. Baseline colours represent NTCs, failed reactions or empty wells on the plate.

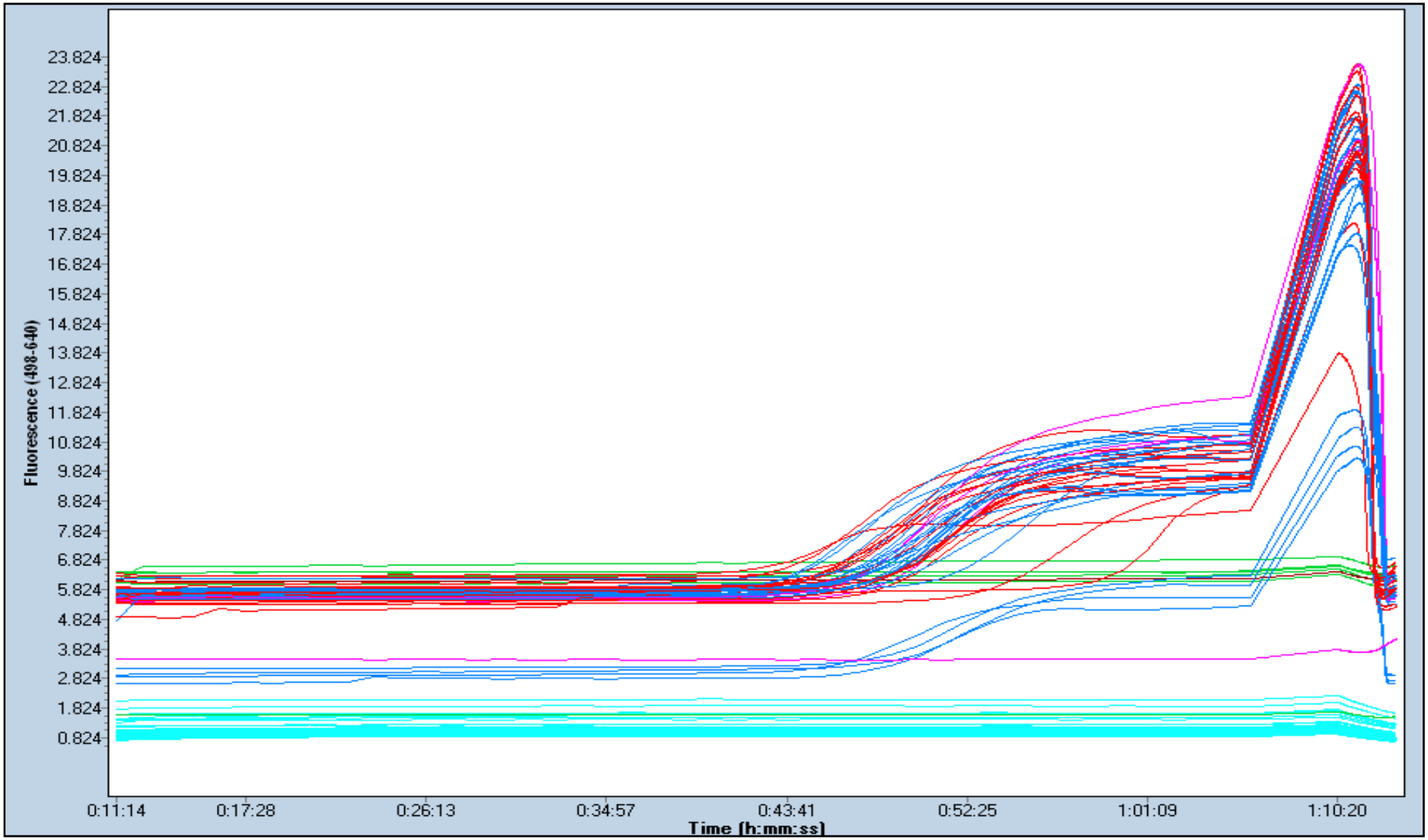

Figure 3.4.3. Amplification achieved in sample batch two using the Roche primers and HybProbe probes for MTHFR 1298 A>C (fluorescence at 498-640 nm vs time). The exponential phase was reached at ~00:43:41 and at ~1:05:00 the melting curve phase commenced.

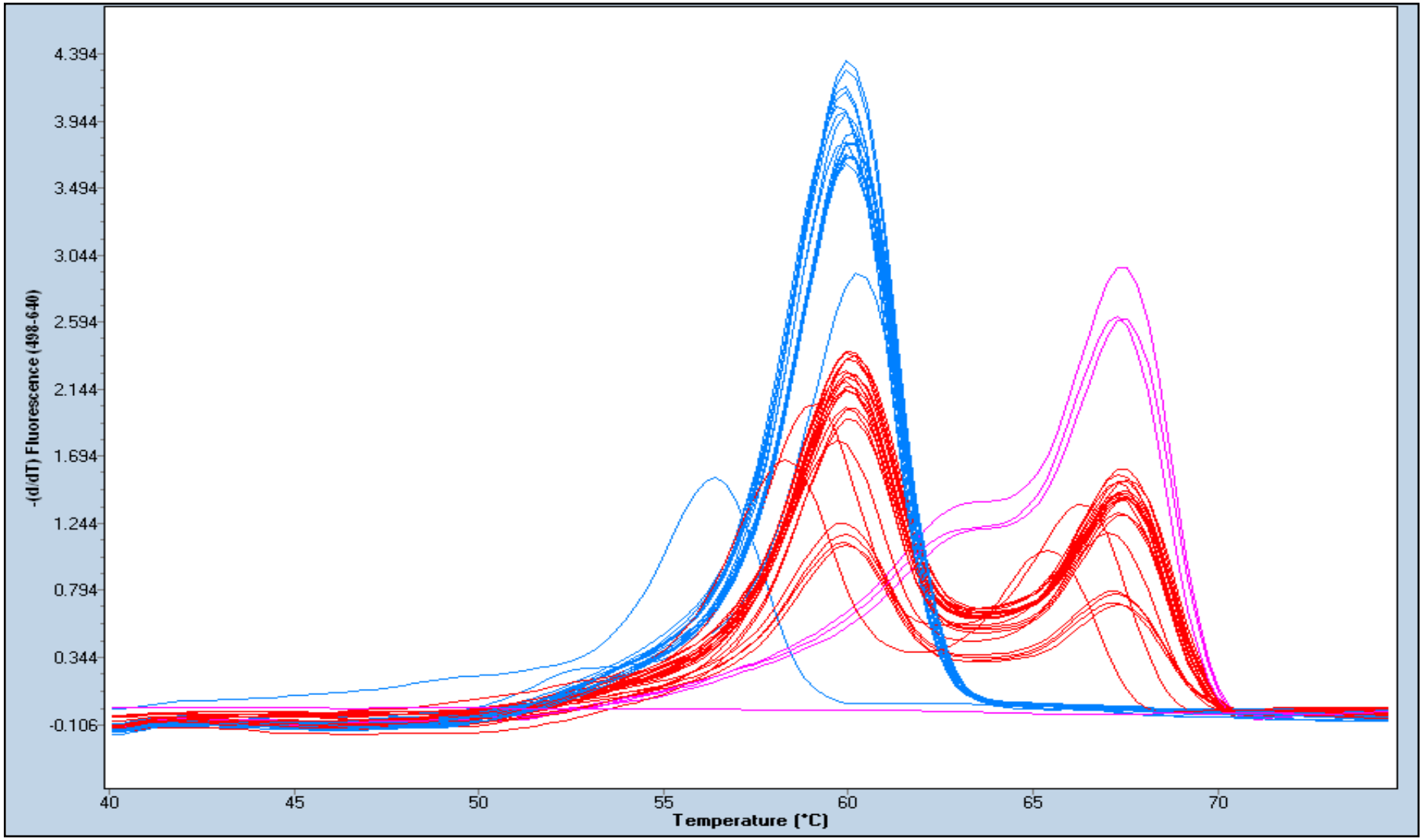

Figure 3.4.4. Melting peaks achieved in sample batch two using the Roche primers and HybProbe probes for MTHFR 1298 A>C (fluorescence at 498-640 nm vs temperature). Blue peaks represent wild type, red peaks represent heterozygotes and pink peaks represent homozygotes. Baseline colours represent NTCs, failed reactions or empty wells on the plate.

The genotype distribution observed for MTHFR 1298 A>C achieved through application of the Roche LC 480 II system is presented in figure 3.4.5 and represents the combined frequencies for both sample batches. The total study population, which includes MS samples and controls, was found to consist of 40.29% wild type, 39.57% heterozygotes, 4.32% homozygotes and 15.83% were undetermined.

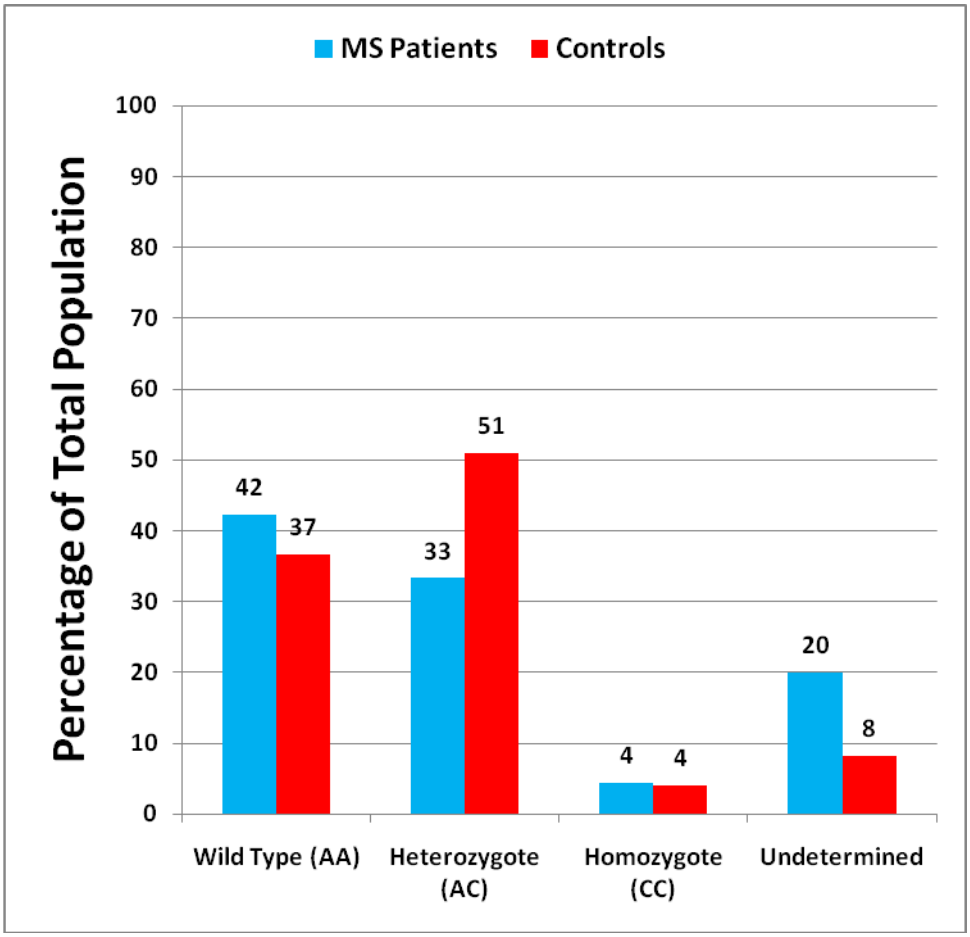

Figure 3.4.5. Genotype distribution of 139 samples obtained using the Roche LightCycler® 480 II system for MTHFR 1298 A>C. 90 MS patient samples (in blue) presented with 38 (42%) wild type, 30 (33%) heterozygotes, 4 (4%) homozygotes and 18 (20%) undetermined genotypes. 18 (37%) wild type were observed among the 49 control samples, 24 (51%) heterozygotes, 2 (4%) homozygotes and 4 (8%) were undetermined.

3.5. Genotype-Phenotype Correlation in MS patients

The genotype distribution for the two *MTHFR* gene mutations (Scholtz *et al.* 2002) and the *HFE* gene mutation (de Villiers *et al.* 1999) were similar to that previously described in the Caucasian population of South Africa. Further comparisons of allele frequency and genotype distribution were also not made between MS patient and control samples due to the small sample size of the control group.

Following the standardisation of *MTHFR* and *HFE* mutation detection using the different RT-PCR methods described above, mutation status was correlated with biochemical parameters. Categorical variables were applied for comparison of the three genotypes (wild type, heterozygous and homozygous) for each SNP (MTHFR 1298 A>C, MTHFR 677 C>T and HFE 845 G>A) to specific biochemical parameters. Only 43 female MS patients (with a complete data set) were subjected to genotype-phenotype correlation which included serum iron parameters, as these values are naturally higher in males than females (MS is also more common in females than males). The male MS patients and controls were excluded from this analysis as the numbers were too small to perform meaningful statistical comparisons.

Serum iron was found to be significantly different (P = 0.02) between the wild type and heterozygous MS patients with mutation 1298 A>C in the *MTHFR* gene. Figure 3.5.1 shows the low serum iron levels in heterozygotes and homozygotes relative to the wild type. The transferrin saturation presented with a similar pattern (data not shown) although the difference was not statistically significant (P = 0.10). CRP levels were found to be marginally higher (P = 0.07) in *MTHFR* 1298 A>C heterozygotes relative to the wild type, which is in accordance with increased inflammation in MS patients. No statistically significant differences were found in iron parameters or CRP levels in relation to the MTHFR 677 C>T and HFE 854 G>A mutations.

Table 3.5.1. Comparison of iron parameters among MTHFR 1298 A>C genotypes in a female study population.

MTHFR 1298 A>C						
	Wildtype[a] (n=18)	**He[b] (n=20)**	**Ho[c] (n=5)**	**P-value**		
	Mean ± STD	**Mean ± STD**	**Mean ± STD**	**ab**	**ac**	**bc**
S-Iron (µmol/l)*	18.64 ± 7.15	12.37 ± 5.91	13.72 ± 5.15	**0.02**	0.42	1.00
Tf (g/l)*	2.66 ± 0.40	2.58 ± 0.49	2.47 ± 0.54	1.00	1.00	1.00
% Tf Saturation*	28.53 ± 10.27	20.27 ± 11.47	20.31 ± 6.10	0.10	0.53	1.00
Ferritin (µg/l)*	86.17 ± 68.18	79.08 ± 97.43	62.64 ± 70.97	1.00	1.00	1.00
HGB (g/dl)*	13.61 ± 2.81	13.26 ± 1.13	12.93 ± 1.25	1.00	1.00	1.00
CRP (mg/l)*	2.93 ± 2.31	6.65 ± 4.96	3.90 ± 3.35	0.07	1.00	0.68

CRP = C-reactive protein, He = Heterozygote, HGB = haemoglobin, Ho = Homozygote, S-Iron = serum iron, STD = standard deviation, Tf = transferrin. * Reference Ranges: S-Iron(µmol/l)=10.0-30.0; Tf (g/l)=2.0-3.6; % Tf Saturation=20-50; Ferritin (µg/l)=10-291; HGB (g/dl)=13.0-17.0; CRP (mg/l)=0.0-10.0.

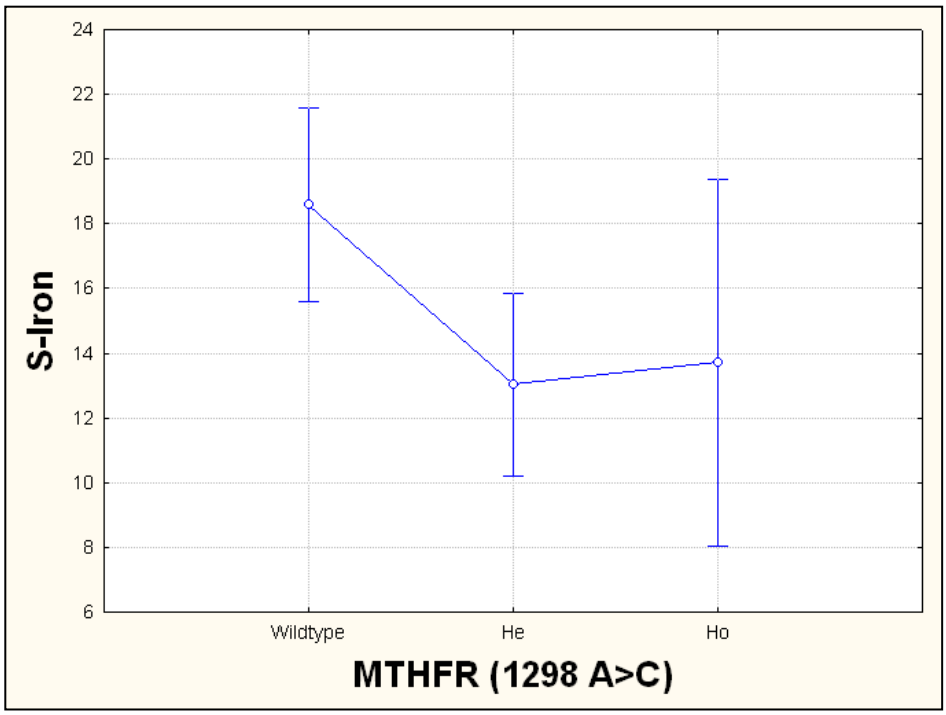

Figure 3.5.1. Comparison of serum iron concentrations (S-Iron) among MTHFR 1298 A>C genotypes in a female study population. Mean ± STD: Wildtype[a]: 18.64 ± 7.15; He[b]: 12.37 ± 5.91; Ho[c]: 13.72 ± 5.15; Significant difference: P–value: **0.02** [ab]

Chapter Four

Discussion

The aetiology of multiple sclerosis (MS) remains largely unknown, due to its multifactorial nature with poor nutrition and genetic factors contributing to the risk. Several investigations highlighted the important role of the genetic component influencing disease susceptibility and progression (Bell *et al.* 1996, Ebers *et al.* 1996). In the present study genetic variations that influence iron and folate metabolism was studied in the context of MS, aimed at the development of a method to apply a pathology supported genetic test. This requires that the genetic profile is correlated with the patient's biochemistry (including serum iron parameters) to assess gene expression and response to treatment and/or lifestyle changes.

The objective of this study was not to prove or disprove the clinical utility of nutrigenetic testing in MS patients, but rather to contribute the laboratory component of performing *MTHFR* and *HFE* gene mutation detection accurately in the Pathology Research Facility of the University of Stellenbosch using high-throughput RT-PCR analysis. The same DNA samples were therefore analysed using three different RT-PCR methodologies for comparison with direct DNA sequencing as the gold standard. For the first time, high-throughput assays for clinically useful mutations in the *MTHFR* and *HFE* genes can now be offered as a routine service at the Tygerberg Academic Hospital.

In the first part of the current study, comparison of three mutation detection systems, namely: Corbett Rotor-Gene™ 6000 5-plex HRM, ABI™ 7900HT and the Roche LightCycler® 480 II system was carried out. Control samples of known genotype using direct DNA sequencing as the gold standard were included in each run to prove analytical validity of the mutation detection methods used. The three instruments were compared on the basis of accuracy, sensitivity, cost-effectiveness and ease of operation. Three SNPs were investigated, two in the *MTHFR* and one in *HFE* gene, through the application of the ABI *Taq*Man® Genotyping Assays. In the second part of the study, statistical analysis was employed to correlate different genotypes with specific biochemical measurements performed in a subset of the study population. In these 43 MS (all female) patients included for genotype-phenotype analysis standard methodology was used for biochemical determinations as previously described (van Rensburg *et al.* 2006). These results were discussed in the context of a newly developed pathology supported genetic testing approach used as part of a health outcomes research project aimed at improved quality of life in MS patients.

Methodology comparison

Applied Biosystems® TaqMan® SNP Genotyping (7900HT)

The ABI™ 7900HT Fast Real-Time PCR System was used with pre-designed and optimized ABI™

*Taq*Man® SNP Genotyping assays in this study. The 7900HT can run 96 or 384 reactions simultaneously. The assays are multiplexed, designed for end-point analysis (which employs fluorophore-labelled probes) and have been standardized to work with *Taq*Man® Universal PCR Master Mix under one thermal cycling condition (table 2.3).

The assays consist of unlabelled sequence-specific forward and reverse primers for amplification of a polymorphic target sequence. Additionally, the assay contains two *Taq*Man® MGB probes with a reporter dye at the 5' end referred to as VIC® dye connected to the 5' end of allele 1 probe and FAM™ dye linked to the 5' end of the allele 2 probe. At the 3' end of each probe, a minor groove binder (MGB) is attached. This allows for increase in the melting temperature (Tm) of a given probe length (Afonina *et al.* 1997; Kutyavin *et al.* 1997). This modification allows for designing shorter probes with greater differences in Tm values between matched and mismatched probes resulting in precise allelic discrimination. The probe hybridization efficiency may be reduced with a single nucleotide mismatch between the probe and target sequence which results in reduction of reporter dye cleaved from a quenched probe. Moreover, the AmpliTaq Gold DNA polymerase found in the *Taq*Man® Universal PCR Master Mix dislocates the mismatched probe without cleaving it. All the above-mentioned factors minimize the production of the non-specific fluorescence signal and thus increase the accuracy of results.

ABI™ *Taq*Man® SNP Genotyping assays employ amplification of the target sequence through PCR and thereafter post-PCR read and analysis can be applied using allelic discrimination. During target amplification, the *Taq*Man® MGB probe annealing takes place between the forward and reverse primer sites on the complementary sequence. Upon annealing, the proximity of the reporter dye to the quencher dye coincide in quenching of the reporter fluorescence through Forster-type energy transfer (Fluorescence resonance energy transfer (FRET); Förster, 1948; Lakowicz, 1983). Extension of the primers bound to the target sequence takes place through AmpliTaq Gold DNA polymerase. This polymerase carries out cleavage of the probes hybridized to the target sequence, which results in the separation of reporter dye from the quencher dye and therefore increase in fluorescence by the reporter. The fluorescence signal corresponds to the presence of the specific allele in every sample. Fluorescence by both dyes signifies the presence of both alleles and thus heterozygosity of the sample.

In MTHFR 677 C>T genotyping, the ABI™ *Taq*Man® probes were designed on the reverse strand – hence the detection was reported as a change from a G>A. The VIC®-labelled probes only bound to alleles containing guanine (G) in the 677-nucleotide position of *MTHFR*, corresponding to the wild

type allele and therefore detection in the yellow channel. FAM™-labelled probes were assigned to the alleles containing adenine (A) in nucleotide position 677 of the *MTHFR* gene, corresponding to the presence of the SNP and therefore detection in the green channel. For the purposes of this study, all RT-PCR results for MTHFR 677 C>T were presented in the forward notation to comply with international standards. Therefore wild types are designated CC, heterozygotes CT and homozygotes TT.

The MTHFR 1298 A>C assay probes were also designed on the reverse strand, resulting in detection of a T>G nucleotide change. The VIC®-labelled probes were designed to bind to alleles containing guanine (G) in the 1298-nucleotide position of *MTHFR* gene, corresponding to the mutant/ homozygous allele, and therefore detection in the yellow channel. FAM™-labelled probes were appointed to the alleles containing thymine (T) in nucleotide position 1298 of *MTHFR*, representing wild type alleles and therefore detection in the green channel. As with the MTHFR 677 C>T SNP, all RT-PCR results for MTHFR 1298 are presented in the forward notation to comply with international standards. Therefore wild types are designated AA, heterozygotes AC and homozygotes CC.

The *HFE* G845A (C282Y) ABI™ *Taq*Man® assay consisted of two fluorophore-labelled probes: one for each allele. The VIC®-labelled probes were designed to specifically bind to the allele that contains guanine (G) in nucleotide position 845 of the gene, analogous to the wild type allele. The FAM™-labelled probes in this assay bound only to the allele containing adenine (A) in position 845, representing the presence of the C282Y mutation.

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

The Corbett Research Rotor-Gene™ 6000 Series is a Real-Time PCR instrument used for target amplification, endpoint genotyping, as well as High Resolution Melt (HRM) analysis. This system can run up to 36 or 72 reactions simultaneously, depending on the rotor type employed. The software provides simplicity as well as a superior open experimental platform for advanced users. The Rotor-Gene™ 6000 instrument can be applied in multiplex reactions with six different excitation sources (e.g. Blue, Green, Yellow, High Resolution Melt) and six detection filters combined with a fixed optical path. This allows for reduced calibration and compensation as well as minimizing the fluorescence variability between each sample. An electric current is conducted in one direction by a light emitting diode, which excites the samples from the bottom of the chamber. This allows for energy transmission at the base of clear, thin walled PCR tubes. A photomultiplier

then collects the fluorescence emitted from the samples. The fixed optical path ensures constant excitation for every sample as the rotation around the chamber takes place.

The Corbett Research Rotor-Gene™ 6000 Series Multiplexing System provides several analysis modes such as Quantitation analysis, two standard curve analysis, Delta Delta Ct Relative Quantitation, Melt Curve analysis, Comparative Quantitation, Allelic Discrimination, Scatter-Plot Analysis, Endpoint Analysis as well as High Resolution Melt analysis. In this study, ABI™ *Taq*Man® SNP Genotyping assays were also employed with the Rotor-Gene™ 6000 instrument.

Roche LightCycler® 480 II

The Roche LightCycler® 480 II system enables real-time performance of PCR with rapid cycling of either 96 or 384 samples. The instrument allows for several applications such as Relative Quantification, Endpoint Genotyping and Melt Curve Genotyping. The optical detection system can detect a broad range of sequence-dependent (e.g. HybProbe probes and hydrolysis probes) as well as sequence-independent probes (e.g SYBR Green 1). The detection unit consists of two parts. The lamp unit uses a Xenon reflector lamp as excitation light source with a wavelength ranging from 430 to 630nm, allowing a variety of different fluorophores to be used. The other is the optic unit consisting of several parts such as liquid light guide with light pipe, the emission filters wheel, the excitation filters wheel and the CCD camera with camera optics. The liquid light guide allows for the light emitted by the Xenon lamp to be passed to the optic unit, where the light pipe converts the round to rectangular profile shaping that of the PCR multiwall plate. The revolving filter wheel driven by a stepper motor positions six different filters, according to which the wavelength of the amplification reaction by the excitation fluorophores is determined. The light passed through the excitation filter is then projected on the PCR multiwall plate through a large field lens collecting the rays from the plate. The fluorescent light emitted by the excited fluorophores during the amplification reaction is passed on into the optics module in a vertical position. This limits the shading effects within the plate wells, as well as limiting distortion or variation in the signals being captured from the wells placed on the edges of the plate compared to the centered wells allowing homogeneous sensitivity of the complete plate. The CCD camera then detects the fluorescence signals. Parallel to this, a reference channel is used to measure the intensity of the Xenon lamp in order to compensate for possible intensity fluctuation that may affect the fluorescence signals, ensuring low intra- and inter-assay variation.

In the present research study Melt Curve Genotyping was applied using HybProbe probes suitable for SNP detection. During this detection format two custom designed sequence-specific

oligonucleotide probes were labeled with two different fluorophores, donor dye or fluorescein and an acceptor dye, LightCycler® Red 640. The probes hybridize to the polymorphic target sequence of the amplified DNA sequence from the 5' to the 3' orientation, resulting in close proximity of the dyes. Fluorescein found at the 3' end of the HybProbe is excited at a wavelength of 480nm, which results in energy discharge and therefore excitation of the acceptor dye/ LightCycler® Red 640 bound to the 5' end of the second HybProbe probe emitting light at a longer wavelength, resulting in signal detection. For the HybProbe probe SNP detection uses Melt Curve analysis. In this detection format, binding of the HybProbe probe to the complementary template takes place at a temperature below that of the *T*m of the oligonucleotide probe resulting in the close proximity of the dyes and production of fluorescence resonance energy transfer (FRET). As the temperature rises, the probes melt off at their analogous *T*ms and therefore inhibiting FRET production. This results in a decreased fluorescence signal. The temperature at which the sensor probe melts is dependent on the polymorphic target sequence. In the presence of the SNP in the target region with the hybridized sensor probe, the complex becomes destabilized and melts at a lower temperature in comparison to the wild type target region.

Comparison

The three above-mentioned RT-PCR mutation detection systems, Corbett Rotor-Gene™ 6000 5-plex HRM, ABI™ 7900HT and LightCycler® 480 II, are comparable in terms of accuracy, sensitivity and specificity since the exact same genotypes were observed for the individual samples analysed. However, it must be emphasised that it was a time consuming process to standardise the *MTHFR* and *HFE* gene assays on each individual instrument, with only the *MTHFR* A1298C assay analytically validated finally on all three instruments. Failure to successfully perform the *MTHFR* 677C>T and *HFE* 845G>A assays using the Roche LightCycler® 480 II (only available in the laboratory from June 2010) demonstrates the difficulty of providing consistent results due to the many variables that needs to be taken into account to obtain a reliable result. While standardised pre-designed assays were used on the Corbett Rotor-Gene™ 6000 5-plex HRM and ABI™ 7900HT instruments, genotyping on the Roche LightCycler® 480 II was performed with custom designed primers and probes. Although standardised assays are also available for melt-curve genotyping using the Roche LightCycler® 480 II, the cost in relation to the number of reactions that can be performed was restrictive.

In terms of cost effectiveness, ease of operation and optimization, the Corbett Rotor-Gene™ 6000 5-plex HRM thermal cycler, with use of the ABI™ TaqMan Genotyping assays was found to be the most efficient for mutation detection using relatively small sample batches (maximum of 36 or 72,

depending on the rotor type). For larger sample batches, e.g. 96 or 384, ABI™ *Taq*Man® SNP Genotyping assays with either the Roche LightCycler® 480 II or ABI™ 7900 HT is most convenient.

Genotype-Phenotype Correlation

The objective of this study was not to prove or disprove the clinical utility of nutrigenetic testing in MS patients, but rather to contribute the laboratory component for *MTHFR* and *HFE* gene mutation detection as part of an integrated pathology supported genetic testing approach. The limited genotype-phenotype correlation study preformed in a subset of 43 MS patients was in agreement with this concept.

In this study, a novel finding was that heterozygotes and homozygotes for MTHFR 1298 A>C, presented with lower serum iron in comparison to subjects without the C-allele (P = 0.02). This association supports a German case-control study conducted by Klotz *et al.* (2009), where *MTHFR* 1298 A>C was found to influence neurodegeneration and the incidence rate of MS. Furthermore, CRP levels were found to be marginally significantly higher (P = 0.07) in the MTHFR 1298 A>C mutation-positive subjects, possibly linking inflammation to the presence of the MTHFR 1298 A>C mutation. This supports the findings of Soilu-Hänninen *et al.* (2005) that reported that CRP values were similar in patients with MS and in healthy controls but higher during MS relapses than in remission (P = 0.01).

In comparison, the MTHFR 677 C>T mutation showed no correlation with transferrin saturation, ferritin, haemoglobin or CRP levels. Mutation 677 C>T is known to reduce MTHFR enzymatic activity in cultured fibroblasts and in peripheral lymphocytes (Klotz *et al.* 2010). However Linnebank *et al.* (2004) and Wullner *et al.* (2005) have previously reported that MTHFR 1298 A>C, influences neurodegeneration irrespective of the MTHFR 677 C>T variant. The results of the present study imply that this may be related to decreased iron concentrations, although the exact biochemical mechanism is not known.

The importance of studying biochemical parameters related to iron and folate metabolism in MS patients is supported by the fact that iron deficiency is linked to folate deficiency (Thoradeniya *et al.* 2006). Homocysteine is a marker of vitamin B deficiencies and inflammation. The connection between inflammation, iron and folate is considered to be of particular relevance in MS patients (Connor and Menzies, 1996; Kotze *et al.* 2001; van Rensburg *et al.* 2006; van Rensburg *et al.* 2009, van Rensburg and van Toorn, 2010). Higher homocysteine levels frequently reported in MS patients

imply impaired methylation. Low levels of vitamin B12 are associated with low iron uptake and atrophy of glands producing intrinsic factor in the stomach. Inhibition of vitamin B12 uptake leads to the development of pernicious anaemia and subacute degeneration of the spinal cord, a condition that may mimic MS (Coyle, 2006; Herndon, 2006; Surtees, 1993).

Optimal functioning of the folate-vitamin B12-methyl transfer pathway is a prerequisite for myelin production and maintenance (Selzer *et al.* 2003). It has been shown that while functional polymorphsisms in genes of the methyl transfer pathway may cause inadequate myelination and serious disability from childhood, supplementation with the chemical substrate following each metabolic block could restore the myelin as well as some of the functional deficiencies (Surtees *et al.* 1991). Demyelination may occur when the substrates or co-factors of the folate pathway (including B vitamins and zinc) are depleted (Selzer *et al.* 2003). The latter study furthermore showed that demyelination may also follow when this metabolic pathway is blocked by nitrous oxide anaesthesia, since nitrous oxide causes irreversible oxidation of the cobalt in vitamin B12.

Genetic variation in the MTHFR gene indicates an increased requirement of folate and other B-vitamins to prevent homocysteine accumulation, destruction and damage of the DNA integrity, dysregulation of gene expression, chromatin destruction and thus epigenetic dysregulation. In addition, folate is necessary for red blood cell formation and growth. Due to the water-soluble nature of this B-vitamin, folate is not stored in the body in large amounts, thus its continual supply through diet is essential to maintain its normal levels in the serum. Decreased levels of folate may lead to folate-deficiency anaemia presented as enlarged red blood cells known as megalocytes or megaloblasts (megaloblastic anemia) in the bone marrow. Homozygotes with the two copies of the 677 C>T mutations have only 30% of normal MTHFR enzyme activity. Compound heterozygotes with both the 677 C>T and 1298 A>C mutations have less than 50% of normal MTHFR enzyme activity. MTHR enzyme activity could be restored to normal with folate supplementation (nutrigenetics application). Caution should also be taken with administration of certain drugs that interfere with folate metabolism, especially in patients with deleterious mutations in the *MTHFR* gene that may be switched on in the presence of low folate status (pharmacogenetics application).

Variation in the *HFE* variant G>A, is usually associated with over-absorption of iron from the diet (nutrigenetics application) and may result in haemochromatosis. The risk of organ damage is significantly increased in homozygotes with high serum ferritin and transferrin saturation levels. However, the risk of organ damage is usually not increased in heterozygotes. In a study to

determine the effects of these genetic mutations in MS, Kotze *et al.* (2006) reported no organ damage in MS patients heterozygous or homozygous for mutation C282Y in the *HFE* gene.

The importance of iron deficiency in the aetiology of MS was emphasized by the finding that oligodendrocytes need iron to synthesize myelin (Connor and Menzies, 1996). A case study including two children with relapsing-remitting MS reported significantly lower iron concentrations in both subjects (van Toorn *et al.* 2010). Iron supplementation resulted in sustained remission and no further relapses in these children. The relevance of iron deficiency in MS may have been missed previously because iron is traditionally associated with oxidative damage, and iron chelation would rather be considered appropriate to prevent oxidation during demyelination (van Rensburg *et al.* 2009). The presence of iron is however stringently controlled under normal circumstances as intracellular iron is either bound up inside proteins such as the haem groups of cytochromes and catalase or sequestered by the iron transporters (transferrin and ferritin). Within the oligodendrocytes a highly functional antioxidant system is continually present to scavenge free radicals (Baud *et al.* 2004).

Pathology supported genetic testing

In April 2009, the Department of Pathology at Stellenbosch University initiated a process to develop pathology supported molecular genetic tests, which is based on an integrated service- and research approach (Schneider 2009). The aim is to match disease diagnosis and therapeutic design with the clinical picture, pathology, environmental risk factors and genetic profile of the patient. This new test concept requires that (1) genetic testing is performed within a specific clinical and pathology/biochemical profile, (2) the patient report contains both the biochemical and genetic test results for clinical application in the context of relevant documented environmental factors, and (3) gene expression and monitoring of response to treatment are assessed through the accompanying pathology and genetic test parameters (Kotze *et al.* 2009).

The limitations of DNA testing – where a positive test does not necessarily mean that the associated disease will develop and a negative test does not exclude other risk factors not included in the test – can be overcome with the application of pathology supported genetic testing. Furthermore, to ensure clinical usefulness of genetic variations evaluated as part of this process, careful review of the literature is necessary to prevent the use of SNPs of uncertain functional significance in genetic tests. In contrast, genome wide association studies may identify risk alleles in the absence of supporting data on relevant metabolic impairments. This process therefore involves the use of the

Gknowmix Software applied in conjunction with high-throughput real-time polymerase chain reaction (PCR) technologies that are benchmarked against direct DNA sequencing as the gold standard (Kotze *et al.* 2009). In order to address the important ethical and scientific issues pertaining to pathology supported gene-based intervention, the information must preferably be captured in a database preferably as part of properly designed and ethically approved research project that will advance evidence-based medicine (Kotze *et al.* 2009).

Combined service and research approach

The present investigation represents a sub-study of the project entitled "The development and commercialization of a comprehensive gene-based, pathology supported intervention program for improved quality of life in patients diagnosed with multiple sclerosis (MS)". This translational research project includes both a service and a research component, registered under number N07/09/203. Integration of data relating to the spectrum of risk factors influencing iron and folate metabolism provides a major healthcare opportunity to improve the quality of life for patients with demyelinating diseases such as MS (van Rensburg and van Toorn, 2010).

Autoimmunity is considered the major cause of chronic, inflammatory diseases such as MS. However, about one third of patients do not respond to immune modulatory therapy (Axtell *et al.* 2010; Byun *et al.* 2008). Failure to identify a major genetic component underlines the importance of a step-wise risk evaluation process that aims to support myelin production in patients at an early stage of demyelination, while stabilising the disease progression in more severely affected patients through a personalized treatment programme. The novel approach of the present study includes assessment of genetic susceptibilities and lifestyle risk factors in relation to the metabolic profile of each patient, as genetic differences may affect how drugs and nutrients are metabolised by the body, taking into account relevant gene-drug (pharmacogenetics) and gene-diet (nutrigenetics) interactions. The unique program includes the following (Kotze *et al.* 2010):

- Combines pathology, pharmacogenetics and nutrigenetics in one test application;
- Assesses biochemical levels and genes involved in iron dysregulation and methylation;
- Nutrigenetics is applied to develop a supplementation program;
- Pharmacogenetics is applied for contra-indication of anti-folate drugs (e.g. methotrexate);
- Blood levels of iron parameters, homocysteine, CRP and cholesterol are measured;
- Folate-Vit. B12, Vit. D and iron levels are monitored for optimal myelin production;
- Individualised reports are generated with an integrative software program.
- Offers an integrated, personalized treatment regimen for each patient;

- Inexpensive approach compared to conventional treatments.

The assessment of biochemical levels and genes involved in iron dysregulation and the methylation process forms the core element of this new healthcare approach applied for the first time in MS patients. Clinical management is improved through the concurrent application of diagnostic pathology tests in conjunction with predictive nutrigenetics and pharmacogenetics. The following steps are involved:

1. **Documentation of the patient's medical history and environmental risk:**
   - Medical status, including neurological examination, infections and family history,
   - Nutrition status, including nutrient intake, food allergies or intolerances,
   - Existing medication use and side-effects,
   - Lifestyle evaluation for risk factors, including smoking, body mass index (BMI).
2. **Laboratory testing to tailor the treatment strategy:**
   - Pathology tests for relevant biochemical and haematological determinations,
   - Genetic testing according to family history/iron status/homocysteine levels,
   - Immunological testing to avoid histamine production.
3. **Interpretation of test results provided in an integrated report, including:**
   - Disease status,
   - Environmental and lifestyle risk factors,
   - Treatment guidelines for implementation.
4. **Intervention according to combination of genetic and environmental factors:**
   - Target gene-environmental mismatches: genetic variations or mutations determine different responses to medication or environmental exposure,
   - Pharmacological or nutritional information is combined with the DNA and clinical profile for treatment aimed at the regeneration of myelin and avoidance of risk factors.
5. **Monitor treatment response and compliance management:**
   - Impact on disease status is monitored after implementation of a nutrigenetics diet support program and a Mediterranean style diet, including no smoking, supplements of iron if indicated, folate, Vit. B12, antioxidants, lecithin, Vit. D, mineral, essential fatty acids and amino acids required for myelin production and maintenance in oligodendrocytes (van Rensburg *et al.* 2006).

The therapeutic strategy combines gene testing, pharmacogenetics and nutrigenetics in one treatment where a healthy diet and supplement programme are used in addition to medication where appropriate. Particularly, in the approximate one-third of patients who are non-responders to

Interferon beta therapy, this approach may assist to improve the quality of life in patients who do not respond to drugs due to the fact that they may belong to a sub-group of MS patients with a metabolic form of demyelination.

Chapter Five

Conclusions

In this study, the three mutation detection systems (Corbett Rotor-Gene™ 6000 5-plex HRM, ABI™ 7900HT and Roche LightCycler® 480 II) investigated were found to be comparable in terms of accuracy, sensitivity and specificity. In terms of cost effectiveness, ease of operation and optimization, the Corbett Rotor-Gene™ 6000 5-plex HRM thermal cycler, with use of the ABI™ TaqMan Genotyping assays was found to be the most efficient for mutation detection using relatively small sample batches (maximum of 36 or 72, depending on the rotor type). For larger sample batches, e.g. 98 or 384, ABI™ *Taq*Man® SNP Genotyping assays with either the Roche LightCycler® 480 II or ABI™ 7900 HT is recommended.

Although considerable research into the causes of MS has resulted in large volumes of information, much of this information is contradictory and not generally or routinely applicable. MS provides an excellent example of a complex disease that is best addressed by pathology supported genetic testing approach. What this means, is that genetic testing is performed within a specific pathology/biochemical and clinical profile, that both the biochemical and genetic test results are provided in a patient report together, and that gene expression and/or response to treatment is monitored through these accompanying pathology and genetic test parameters. A specific and focused approach to the screening of MS is now applicable for the first time at the Pathology Research Facility of the University of Stellenbosch. This application aims to facilitate the support of myelin production in patients at an early stage, as well as stabilizing disease progression of demyelinating diseases in more developed patients, through a personalized nutrition support program. This approach is relatively less expensive when compared to more conventional treatment programs of MS, some of which may be contra-indicated due to genetic factors involved in drug response.

Application of the above approach to facilitate improved clinical management of MS patients requires careful ethical consideration. This was addressed in the research protocol (N07/09/203), which forms the foundation of our combined service and research approach as follows:

- 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 (e.g. hereditary haemochromatosis caused by mutations in the *HFE* gene) 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 by the pateints.
- 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.

All personal and medical information of patients must be and are kept confidential and not made available to others without permission of the tested individual. In the case of genetic testing the information obtained may not only relate to the tested individual, but also his/her close family members. Therefore it is preferable that both pre- and post-test counseling be provided by registered genetic counsellors to all patients referred for genetic testing. However, it is now accepted internationally that because of the small number of registered genetic counsellors available and the diversity of the types of genetic tests, other knowledgeable healthcare professionals will need to help educate their patients about the genetic basis of monogenic and multi-factorial diseases, the clinical/biochemical consequences, and the genetic testing options available. Accurate, full and unbiased information should be provided to individuals and families to enable them to make informed decisions about genetic testing and treatment/intervention options.

In conclusion, all the aims of the study have been accomplished. We demonstrated the optimization steps needed before genetic testing can be applied as part of a pathology supported gene-based intervention strategy using high-throughput RT PCR. These were included in the standard operating procedures (SOP's) developed for use in the Pathology Research Facility. The demonstration of reduced serum iron levels correlating with variation in the MTHFR gene in a subgroup of MS patients furthermore opened a new realm of research possibilities for future studies in the field of MS and other demyelinating diseases.

Chapter Six

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NanoDrop® users manual: http://www.nanodrop.com/techsupport/nd-1000-usersmanual.pdf

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APPENDIX A

PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH INVOLVING GENETIC STUDIES

TITLE OF RESEARCH PROJECT:
The development of a comprehensive gene-based, pathology supported intervention program for improved quality of life in patients diagnosed with multiple sclerosis (MS).

REFERENCE NUMBER: N07/09/203

PRINCIPAL INVESTIGATOR: Prof SJ van Rensburg

ADDRESS: Division of Chemical Pathology, Tygerberg Academic Hospital / University of Stellenbosch, Tygerberg 7505

CONTACT NUMBER: (021) 938 4611

We would like to invite you to participate in a research study that involves genetic analysis and possible long-term storage of blood or tissue specimens. Please take some time to read the information presented here which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part initially.

This research study has been approved by the ethics **Committee for Human Research at Stellenbosch University** and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, and the SA Department of Health's 2004 Guidelines: *Ethics in Health Research: Principles, Structures and Processes.*

What is Genetic research?

Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Occasionally genetic material is obtained from other sources such as saliva or a cheek swab. Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

What does this particular research study involve?

This study will search for hereditary factors that can influence the development of multiple sclerosis (MS) and also look for certain nutrition and lifestyle factors that may interact with genetic risk factors. The study will aim to provide further evidence that nutrition intervention can improve quality of life, at least in a subgroup of patients who have different requirements for certain nutrients (e.g. iron, folate) due to genetic alterations. Biochemical determinations of blood and urine will also be determined for correlation and to assess the influence of nutrition intervention on quality of life in MS patients. Individuals without MS will also be included in the study as controls, to compare the impact of genetic factors in relation to biochemical parameters and clinical outcome. You will be provided with a personalised lifestyle modification plan approved by a clinician based

on your medical and family history and the results of pathology and genetic tests. Although you may receive the lifestyle and diet recommendations for immediate implementation, details on any specific genetic alteration identified will not be provided without genetic counselling by a clinician or registered genetic counsellor.

Why have you been invited to participate?

You have been invited to participate as you have been diagnosed with MS, or as a control subject who may have expressed an interest in participating to draw comparisons. We would like to use the information obtained from you to determine whether certain risk factors occur more often in affected patients than individuals who do not have MS. We are also interested to find out how your genetic profile may influence your response to the intervention program that may be recommended.

What procedures will be involved in this research?

You will be asked to fill in forms with questions pertaining to your health status, family history, use of (chronic) medication, etc. and a number of lifestyle factors such as alcohol consumption, smoking etc. Your length and weight may be measured and five small blood bottles of 5 ml each (about four tablespoons) and/or a tissue biopsy (e.g. cheek swab) may be taken for laboratory determinations. You may also be requested to collect urine samples in containers that will be provided, for biochemical tests.

Are there any risks involved in genetic research?

You may experience minor pain or bruising at the site where specimen is taken for laboratory testing. 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. Based on your family history and personal health profile, genetic counselling/consultation may be advisable. Some insurance companies may mistakenly assume that taking part in genetic research indicates a higher risk for disease. Thus, no information about you or your family will be shared with such companies. Since the techniques to be used may be experimental and thus possibly unreliable, a quality control system will be implemented as part of this project whereby some of the specimens used for the study may be analysed by different laboratories, possibly using different mutation detection methods.

Are there any benefits to your taking part in this study and will you get told your results?

Your blood will be tested to develop an individualized nutrition intervention program based on the outcome of the laboratory results. The test results will be provided to a clinician who will explain this to you or refer you to a registered dietician who has been trained to interpret the genetic results in the context of relevant lifestyle risk factors and blood biochemistry that may reflect gene-environment interaction. Some of the tests that will not have an immediate effect on the intervention strategy may be done at a later time when batches of samples are available; this will be done to limit testing time and costs involved. This research is expected to benefit people with the same condition in the future as they might be in a position to get treatment earlier to improve quality of life.

Results of routine biochemical tests such as iron parameters and homocysteine levels will be made available to you but genetic results will only be made known to you if they indicate that you may:

- Have a predisposition or a genetic risk factor that can be minimised by nutrition and lifestyle changes
- Need genetic counselling

OR

- If you request a report.

It is important to understand that the individualised intervention program to be provided in your test report may or may not improve your quality of life, as it is experimental and clinical validity still needs to be evaluated in a double-blind, randomised clinical trial.

How long will your blood be stored and where will it be stored?
Your specimen will be stored at the University of Stellenbosch Medical School in a specifically dedicated fridge or freezer for at least 5 years or at the laboratory that performed the same test(s) as a routine service. A material transfer agreement will be signed by a representative of the university if/when your specimen is shipped from the University of Stellenbosch to another laboratory or country (e.g. for quality assurance, collaborative research).

If your blood is to be stored is there a chance that it will be used for other research?

Your specimen will only be used for the research related to the ethically approved protocol. Also if the researchers wish to use your stored specimen for **additional research in the field of MS** they will be required to apply for permission to do so from the Human Research Ethics Committee at Stellenbosch University that can be contacted at telephone number 021 938 9657. If you do not wish your specimens to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form without any effect on your treatment. If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

How will your confidentiality be protected?
The specimens will be given an ID number and only the researchers and clinicians involved in the study will have access to the original questionnaires and assessment forms with identifying information. Specimens sent to other laboratories local or abroad will be shipped only with the ID number attached to them and your name will not be disclosed to the collaborator. If ever information comes to light that could be important for the individual or her descendants, all possible attempts will be made to contact these participants and counsel them. The results of the study will be included in scientific articles and student theses, without revealing the identity of the study participants.

Will you or the researchers benefit financially from this research?

You will not be paid to take part in this study; however the results from this project may bring benefits to you, your family or community in the future.

Important information: In the event that this research leads to the development of a commercial application or patent, you or your family will not receive any profits or royalties although the researchers may benefit in this respect

Declaration by participant

By signing below, I ……..……. agree to take part in the research study entitled: The development of a comprehensive gene-based, pathology supported intervention program for improved quality of life in patients diagnosed with multiple sclerosis (MS)

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I have received a signed duplicate copy of this consent form for my records.

Tick the option you choose:

☐ I agree that my blood or tissue sample can be stored **indefinitely** but I can choose to request at any time time that my stored sample be destroyed. My sample will be identified with a special study code that will remain linked to my name and contact details. I have the right to receive confirmation that my request has been carried out. My sample may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field without revealing my identity.

OR

☐ I agree that my blood or tissue sample can be stored **indefinitely** after the project is completed but that it is anonymised with all possible links to my identity removed, and that the researchers may then use it for additional research in this or a related field. Once my sample is anonymised by deleting my name from the data base and destroying this consent form, my rights to the sample labelled with only a unique ID number, are waivered. My sample may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field.

OR

☐ Please destroy my blood sample as soon as the current research project has been completed.

Signed at (*place*) ..... on (*date*) .....

..... **.....**

Signature of participant Signature of witness

Declaration by investigator

I *(name)* …… declare that:

- I explained the information in this document to ……..……...
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research as discussed above.
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....….....…….. on (*date*) ……...……...

..... **.....**

Signature of investigator Signature of witness

Declaration By Interpreter

I *(name)* …… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) …….. using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....….....…….. on (*date*) ……...…….. 2005.

..... **.....**

Signature of interpreter Signature of witness

APPENDIX B

APPENDIX C

APPENDIX D

APPENDIX E