Analysis of single nucleotide polymorphisms with opposite effects on serum iron parameters in South African patients with multiple sclerosis

By

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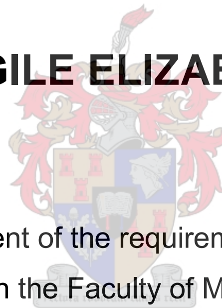

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Division of Chemical Pathology
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DECLARATION

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ABSTRACT

There is growing interest in how genetic and environmental risk factors interact to confer risk for dysregulated iron homeostasis, which is considered a possible pathogenic mechanism in multiple sclerosis (MS). While iron deficiency has been associated with greater disability and disease progression, cerebral accumulation and overload of insoluble iron has also been reported in MS patients. Variation in the matriptase-2 (TMPRSS6) gene has recently been described that may lead to reduced iron levels, which raised the question of whether it may be involved in dysfunctional iron regulation as a pathogenic mechanism in MS.

The aims of the study were as follows: 1)) comparison of the allele frequencies and genotype distribution for TMPRSS6 A736V (rs855791, c.2207C>T) and HFE C282Y (rs1800562, c.845G>A) between patients diagnosed with MS and unaffected controls; 2) determination of the effects of clinical characteristics, relevant lifestyle factors and genotype on serum iron parameters in MS patients compared to population matched controls; and 3) determination of clinical outcome in relation to age of onset and degree of disability in MS patients.

The study population included 121 Caucasian MS patients and 286 population-matched controls. Serum iron, transferrin, ferritin and transferrin saturation levels were available from previous studies and lifestyle factors were subsequently documented in a subgroup of 68 MS patients and 143 controls using the study questionnaire. Genotyping of TMPRSS6 A736V and HFE C282Y were performed using allele-specific TaqMan technology.

The genotype distribution and allele frequencies of TMPRSS6 A736V and HFE C282Y did not differ between MS patients and controls. MS patients homozygous for the iron-lowering minor T-allele of TMPRSS6 A736V had significantly lower serum iron levels ($p=0.03$) and transferrin saturation levels ($p=0.03$) compared to CC homozygotes. In MS patients the iron-loading minor A-allele of HFE C282Y was also associated with a paradoxical decrease in serum ferritin ($p<0.01$) compared to GG homozygotes. When considering the combined effect of the minor alleles of TMPRSS6 A736V and HFE C282Y with opposite effects on iron levels, we found a significant reduction in serum ferritin levels ($p<0.05$), independent of age, sex, body mass index (BMI) or dietary red meat intake in MS patients. A similar effect was not observed in the population- and age-matched controls. Higher dietary red meat intake correlated significantly with increased ferritin only in controls ($p=0.01$ vs. 0.21 for MS patients). In the presence of the minor allele of HFE C282Y, the TMPRSS6 A736V CT and TT genotypes were associated with a significantly earlier age of onset of MS when the post hoc test was applied ($p=0.04$).

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All the study aims were successfully accomplished. Our results support the possibility of an epistatic effect between TMPRSS6 A736V and HFE C282Y associated with reduced ferritin levels in MS patients. Pathology-supported genetic testing (PSGT) applied in this study as a new concept for analysis of complex diseases with a genetic component, is well placed to optimise clinical management in patients with MS.

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OPSOMMING

Daar heers toenemende belangstelling in hoe die wisselwerking tussen genetiese en omgewingsfaktore die risiko tot wanregulering van yster-homeostase beïnvloed. Laasgenoemde is ‘n moontlike patogeniese meganisme vir meervoudige sklerose (MS). Alhoewel verhoogde gestremdheid en siekteprogressie met ystertekort geassosieer is, is ysterophoping in die serebrum asook ‘n oormaat onoplosbare yster al by MS-pasiënte gevind. Variasie in die matriptase-2 (TMPRSS6) geen wat tot verlaging in ystervlakke kan lei, is onlangs beskryf en laat die vraag ontstaan of dit betrokke is by wanregulering van yster-homeostase as patogeniese meganisme in MS.

Die doelwitte van die studie was as volg: 1) vergelyking van alleelfrekwensies en genotipeverspreiding vir TMPRSS6 A736V (rs855791, c.2207C>T) en HFE C282Y (rs1800562, c.845G>A) tussen MS-pasiënte en ongeaffekteerde kontroles; 3) bepaling van die effekte van kliniese indikators, relevante leefstylfaktore en genotipe op serum yster parameters in MS-pasiënte in vergelyking met populasie-ooreenstemmende kontroles; en 4) bepaling van kliniese uitkoms ten opsigte van aanvangsouderdom en graad van MS-aantasting.

Die studiepopulasie het uit 121 kaukasiese MS-pasiënte en 286 kontroles van dieselfde populasie, wat nie die siekte het nie, bestaan. Serum yster, transferrin, ferritien en transferrien-versadigingsvlakke was beskikbaar vanaf vorige studies. Leefstylfaktore is in ‘n subgroep van 68 MS-pasiënte en 143 kontroles gedokumenteer met behulp van die studie-vraelys. TMPRSS6 A736V en HFE C282Y genotipering is met alleel-spesifieke TaqMan-tegnologie uitgevoer.

Beide pasiënte en kontroles het dieselfde genotipeverspreiding en alleelfrekwensies getoon. Die A-alleel van HFE C282Y is met ‘n paradoksale verlaging in serum ferritien geassosieer ($p<0.01$) in MS-pasiënte met TMPRSS6 A736V, moontlik weens geen-geen interaksie wat nie deur ouderdom, liggaamsmassa-indeks of inname van rooivleis in die dieet beïnvloed is nie ($p<0.05$) en nie by kontroles gevind is nie. MS-pasiënte wat homosigoties is vir die T-alleel van TMPRSS6 A736V, het statisties betekenisvolle laer serum ystervlakke ($p=0.03$) en transferrienversadiging ($p=0.03$) getoon in vergelyking met CC-homosigote. In MS-pasiënte was die yster-oorlading A-alleel van HFE C282Y ook geassosieer met ‘n paradoksale afname in serum ferritien ($p<0.01$) in vergelyking met GG-homosigote. Wanneer die gekombineerde effek van die risiko-geassosieerde allele van TMPRSS6 A736V en HFE C282Y met teenoorgestelde effekte op ystervlakke geanaliseer word, is daar ‘n statisties

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beteknisvolle afname in serum ferritienvlakke ($p<0.05$), onafhanklik van ouderdom, geslag, liggaamsmassa-indeks of rooivleisinname in MS-pasiënte. 'n Soortgelyke effek is nie waargeneem in populasie- en geslag-gelyke kontroles nie. Die inname van rooivleis in die dieet was betekenisvol minder by MS-pasiënte teenoor kontroles ($p=0.03$) en dit het slegs betekenisvol met verhoogde ferritien by kontroles gekorreleer ($p=0.01$ teenoor 0.21 by MS-pasiënte). In die teenwoordigheid van die risiko-geassosieerde alleel van HFE C282Y, is die TMPRSS6 A736V CT en TT genotipes geassosieer met 'n statisties-betekenisvolle vroeër aanvangsouderdom van MS soos bepaal met die post hoc-toets ($p=0.04$).

Al die doelwitte van die studie is suksesvol uitgevoer. Die resultate ondersteun die moontlikheid van 'n epistatiese effek tussen TMPRSS6 A736V en HFE C282Y wat geassosieer is met 'n verlaging in ferritienvlakke in MS-pasiënte. Patologie-gesteunde genetiese toetsing soos toegepas in hierdie studie as 'n nuwe konsep vir analise van komplekse siektes met 'n genetiese komponent, is goed geplaas om kliniese hantering van MS-pasiënte te optimaliseer.

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DEDICATIONS

In Loving Memory

Of

UTata

Rolihlahla Nelson Madiba Mandela

1918 July – 2013 December

Your ability to: Abandon your own Will, Lead with Integrity, Forgive, Love Selflessly and Unconditionally!!!

Will forever be cherished!!!

Education is indeed a greatest Asset: Thank you for paving the way for me to reach greatest heights I never thought possible!!!

Tata Ulale ngoxolo

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LIST OF SYMBOLS AND ABBREVIATIONS

List of symbols and abbreviations, their full scientific names

Abbreviations and symbols	Definitions
α	Alpha
β	Beta
©	Copyrights
°C	Degrees celsius
=	Equals to
≥	Greater than or equals to
<	Less than
≤	Less than or equals to
H_2O	Water
ddH_2O	Double distilled water
g	Gram
g/L	Gram per liter
m^2	Square meter
µg/L	Micrograms per liter
µmol/L	Micromole per liter
µL	Microliter
v	Volts
-	Minus
%	Percentage
+	Plus
±	Plus or minus
®	Registered trademark
™	Trademark
3´	3-prime
5´	5-prime
A	
ABI	Applied biosystems instrument
ADEM	Acute disseminated encephalomyelitis
APR	Acute phase reactants
B	
BBB	Blood-brain barrier

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BMI	Body mass Index
BMP	Bone morphogenetic protein
C	
C	Coding region
CAFSU	Central analytical facility of stellenbosch university
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
D	
DMD	Disease-modifying drugs
DMT	Disease-modifying treatments
DMT-1	Divalent metal transporter 1
DNA	Deoxynecleic acid
dH_2O	Distilled water
dNTPs	Deoxyribonucleoside triphosphates
DSS	Disability status scale
E	
EDTA	Ethylenediaminetetraacetic acid
EDSS	Expanded Disability Status Scale
ENV	
F	
FDA	Food and drug administration
Fe	Iron iron
FPN	Ferroportin
FSS	Functional System Score
FS	Functional System
G	
GWAS	Genome-wide association study
H	
H	Heavy chain
HERV	Human endogenous retrovirus
HIV	Human immune virus
HH	Hereditary hemochromatosis
HREC	Human Research Ethics Committee
HFE	Human hemochromatosis gene
HWE	Hardy-Weinberg equilibrium

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IBRO	International Brain Research Organization
ID	Iron deficiency
IDA	Iron deficiency anemia
IDT	Integrated DNA technologies
IFN	Interferon
IL	Interlukin
IRIDA	Iron refractory iron deficiency anemia
K	
Kg	Kilogram
L	
LD	Linkage disequilibrium
L	Light chain
L	Litre
LSD	Least significant difference
M	
MGB	Minor groove binder
$MgCl_2$	Magnesium Chloride
MHC	Major histocompatibility complex
MRC	Medical Research Council
MRI	magnetic resonance image
MS	Multiple sclerosis
N	
NCBI	National center for biotechnology information
NCD	Non-communicable disorders
NTC	None template control
P	
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PPMS	Primary progressive multiple sclerosis
PRMS	Progressive relapses multiple sclerosis
PSGT	Pathology supported genetic testing
rpm	Revolution per minute
RRMS	Relapsing remitting multiple sclerosis
RT-PCR	Reverse transcriptase polymerase chain reaction
S	
SNP	Single nucleotide polymorphism

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SPMS	Secondary progressive multiple sclerosis
SOP	Standard operating procedures
T	
Taq	Thermus aquaticus
Tf	Transferrin
TfR	Transferrin receptor
TMPRSS6	Transmembrane protease serine 6
U	
UTR	Untranslated region

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CHAPTER 1

LITERATURE REVIEW

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1.1 INTRODUCTION

Multiple Sclerosis (MS) is a chronic neurological disease first described by the French neurologist Jeane-Martin Charcot in 1868. Pathologically MS is characterised by the presence of diffuse inflammation and plaques of demyelination throughout the central nervous system (CNS), leading to functional impairment, reduced quality of life, morbidity and early mortality due to MS (Dutta and Trapp 2007; Zwibel, 2009).

Despite more than 50 years of concerted investigative effort, the underlying pathophysiology of MS known to involve a series of complex interactions between environmental factors acting upon a susceptible genotype, remains incompletely understood. There is also growing appreciation for its heterogeneity in geographic and ethnic distribution, clinical presentation, prognosis and treatment response (Taylor et al. 2010). Furthermore, mounting evidence suggests that clinical and genetic sub-types of this disease exist; raising the possibility that individualized treatment of these groups could be therapeutically beneficial. Several studies confirmed the marked inter-patient variance in clinical presentation and therapeutic response to disease-modifying treatments (DMTs) in MS patients (Axtell et al. 2010; Stewart and Vu Tran, 2012). A homogeneous system of classification based on diagnostic hierarchy could therefore obscure subtle differences in presentation that may require individualised treatment.

Consistent with the notion that certain sub-groups of MS exist, previous studies performed in South Africa highlighted the role of deranged iron homeostasis as a pathogenic mechanism in MS, dependent on poorly understood and complex interactions between genetic and environmental risk factors (Kotze et al. 2001; Kotze et al. 2006, van Rensburg et al. 2012). On the one hand, myelin production and maintenance requires a continuous supply of iron and other micronutrients to oligodendrocytes in the central nervous system (the cells that synthesise myelin); a process which is tightly controlled and dependent on the concerted and balanced activity of many regulatory proteins such as ferritin and transferrin (Todorich et al. 2009). Conversely, the presence of insoluble iron deposits demonstrated via magnetic resonance imaging (MRI) in demyelinating plaques and their constituents in MS has been linked to its pathogenesis, while these do not contribute towards cerebral iron bioavailability. This may result in iron deficiency and suboptimal myelination capacity by oligodendrocytes (Rouault and Cooperman 2006). Clinical implications of the apparent “iron paradox” in MS posing a significant challenge regarding our understanding of the pathogenesis of this disease, has recently been addressed in an editorial by van Rensburg and van Toorn (2010) and a review by van Rensburg et al. (2012).

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The specific research question leading to the current study was whether genotyping for low-penetrance mutations or functional single nucleotide polymorphisms (SNPs) involved in iron homeostasis would be clinically useful to identify a subgroup of MS patients with altered requirements of iron intake in the diet. Clinical relevance of SNPs depends on the environment and interaction with other genes; therefore lifestyle factors and relevant biochemical parameters also need to be considered in addition to the medical and family history of patients when genetic testing is performed. The limitations of genetic testing related to many iron-related disorders may be overcome when performed within a pre-defined clinical profile, obtained using biochemical testing, to assess the phenotype expression of a gene and to monitor response to treatment (Kotze et al. 2009). The ultimate aim is to effectively reduce the cumulative effect of multiple risk factors associated with the onset and progression of the disease process.

1.2. **EPIDEMIOLOGY OF MS**

Both neurodegenerative and autoimmune processes have been implicated in the pathogenesis of MS (Roach, 2004; Compston and Coles, 2008), involving multiple complex interactions between genetic and environmental risk factors (Ramagopalan et al. 2008; Healy et al. 2009). The primary pathological characteristics of MS include diffuse inflammation and plaques of axonal demyelination throughout the cerebrum, cerebellum, brainstem, optic nerves and spinal cord. The latter may be triggered when the capacity for compensatory remyelination in the CNS is exceeded (Bo et al. 2006; Trapp and Nave, 2008; Dutta and Trapp, 2011; Antony et al. 2011; Van Horssen et al. 2011). Growing interest in the possible role of iron deficiency as a contributory factor in disease onset and/or progression (Rooney et al. 1999; Van Rensburg et al. 2006, 2012) is supported by predominance of MS in females compared with males (Byun et al. 2008; Koch-Henriksen, 2010).

MS affects more than 2.5 million people globally and places a severe emotional and financial burden on families, caregivers and society at large (Paty et al. 1997; Zwibel, 2009; Gandhi et al. 2010; Taylor et al. 2010; Dutta and Trapp, 2011). MS is more prevalent in high latitude regions, with higher disease prevalence rates reported in Europe, Canada, the United States of America, New Zealand as well as several parts of Australia (Modi et al. 2008; Kakalacheva et al. 2011). Lower prevalence rate has been favouring countries such as Japan and South Africa that are closer to the equator (Ross, 1998; Ascherio and Munger, 2007; Kakalacheva et al. 2011). However, in South Africa the prevalence of MS has increased since previously reported (Dean, 1967; Bird and Satoyoshi, 1975; Naing et al. 2006), and approximately 23000 people may be affected by MS (Du Toit, 2006).

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The frequency of MS is unevenly distributed among racial groupings (Byun et al. 2008; Ebers, 2008; Simpson et al. 2011), predominantly affecting Caucasians (mainly those of the European origin) compared to the Asian population while it is very rare in African subpopulations (Modi et al. 2008). Several prevalence studies in South Africa have shown that MS occurs in all ethnic subgroups, but with very wide frequency distributions amongst African blacks, mixed-ancestry, Indians and Caucasians (English and Afrikaans speaking), with observed prevalence rates per 100 000 people of 0.23, 1.72, 7.15 and 25.64, respectively (Bhigjee et al. 2007). This shows that MS prevalence has increased in South Africa since it was first documented by Dean (1967). Unevenness in frequency distributions of MS between the diverse racial groupings (Caucasians versus Africans), together with the high prevalence of other chronic neurological disorders such as acute disseminated encephalomyelitis (ADEM) amongst African populations, may partly reflect differences in genetic background between populations (Weinstock-Guttman et al. 2003).

1.3. **DIAGNOSIS**

MS is difficult to diagnose due to the heterogeneity of the disease and other diseases mimicking MS (Polman et al. 2005). There are at present no symptoms, physical findings or laboratory tests that can be used to diagnose MS. The diagnosis is therefore made by neurologists using a combination of tests, by assessing personal history, clinical symptoms and MRI, evoked potentials and analysis of spinal fluid. The severity of disability is measured using a neurological exam called the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke, 1983).

1.3.1. **The McDonald Diagnostic Criteria**

Patients are diagnosed according to the McDonald criteria by their respective neurologists so that when they join the MS research programme at Tygerberg, they were already diagnosed. The McDonald diagnostic criteria, updated and revised in 2005 by an international panel on diagnosis of MS (Polman et al. 2005), contain guidelines used for the clinical diagnosis of MS (McDonald et al. 2001). These include the detection of multiple foci of demyelination in the CNS, and evidence of dissemination in time and space of lesions typical of MS, i.e. evidence of damage in at least two separate areas of the CNS, which includes the brain, spinal cord and optic nerves as well as evidence that the damage occurred at least one month apart. The guidelines are used to confirm a definite MS diagnosis with a recommendation list including clinical presention with two or more episodes of neurological disturbance and objective clinical evidence for more than two lesions. For clinical presentation with two or more attacks and objective clinical evidence of one lesion,

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recommended additional tests include two or more MRI-detected lesions and positive CSF analysis (detection of oligoclonal bands), or dissemination in space demonstrated by MRI with the fulfilment of at least three lesion appearances (Polman et al. 2005). The panel recommendations are summarised in appendix B.

1.3.2. **Kurtzke EDSS**

The Expanded Disability Status Scale (EDSS; Kurtzke 1983; Appendix A) is a test that is used to determine disability in MS. It is considered to be the “gold standard” for disability measurement and it is used in all clinical trials – it is an objective test and can be done by any clinician, not necessarily a neurologist. The EDSS value varies over time – people can become better or worse over time and as a result of treatment. The scale ranges from 0 (no neurological symptoms) to 10 (death due to MS), so the higher the score the greater the disability.

The neurological impairment is measured by the total score derived from clinical assessment of eight functional systems (FS) primarily affected in this disease, namely pyramidal, sensory, visual, cerebral, cerebellar, brainstem and bladder/bowel functioning as well as mobility. The original score was expanded and modified to increase its sensitivity for detecting changes in disease progression (Kurtzke, 1965). Scores ranging from 0 to 3.5 indicate impairment in isolated FS, while those ranging from 4.0 to 7.0 indicate difficulty in ambulation. A score of greater than 8.0 indicates more severe disability requiring assistance in walking and communication (Kurtzke, 1965; Amato and Ponziani, 1999).

Since the EDSS requires a visit of the patient to a clinician, putting constraints on the follow-up of patients, especially those who have difficulty with ambulation, some studies have evaluated the use of novel alternative approaches to disease monitoring, including self-reported questionnaires and telephonic interviews (Cheng et al. 2001; Lechner-Scott et al. 2003). An internet-based method has recently been validated (Leddy et al. 2013).

1.3.3. Magnetic Resonance Imaging

MRI is a sensitive, non-invasive imaging method that can detect lesions in the brain and spinal cord (Lassmann et al. 2001, Dutta and Trapp, 2007). It is used for diagnosis and to monitor the course of the disease as well as the clinical management of patients with MS (Bakshi et al. 2008). During diagnosis, MRI shows lesion dissemination in space and time, and can evaluate conditions that can clinically mimic MS (Polman et al. 2005; Charil et al. 2006). MRI involves assessing lesions using non-contrast longitudinal spin-lattice (T1)- and

transverse spin-spin (T2)- weighted images, as well as gadolinium enhanced T1-weighted images (Schenck, 2003; Neema et al. 2007). Gadolinium is a contrast agent that is injected intravenously to show places where the blood-brain barrier has degraded due to inflammation. The enhancement of lesions allows detection of contrast agent accumulated in interstitial space (Bakshi et al. 2008). Changes in T1 give an indication of chronic lesions which are accompanied by severe axonal loss and swelling, whilst that of T-2 indicates disruption of the BBB with or without acute demyelination (Fisher et al. 2007).

1.4. CLINICAL CHARACTERISTICS AND SUBTYPES OF MS

There can be few diseases with as much variation in clinical outcome as seen in MS (DeLuca et al. 2007). This heterogeneity makes it very difficult to diagnose and treat patients. A classification of subtypes was suggested by Lublin and Reingold (1996), which included relapsing-remitting (RRMS), secondary progressive (SPMS) and primary progressive (PPMS) forms and is still adhered to in order to facilitate the evaluation of patients taking part in clinical trials.

However, patients are heterogeneous in their clinical course and progression, differing with regards to age of onset, episode duration and character, symptom severity, risk of irreversible neurological damage and functional disability, as well as associated neuropathology and capacity for axonal remyelination, and significant interpersonal variance (Victor and Ropper, 2001; Byun et al. 2008; Koch-Henriksen and Sorensen, 2010). Furthermore, there is no immunological or biochemical test that can reliably be used either to diagnose MS or to place patients into disease categories.

The clinical categories were designed to range from less severe disease (RRMS) to greater severity (SPMS) to patients with a rapidly progressing course (PPMS). The implication of the classification is that patients show progression from less severe to more severe over time, and that a reversal of disability is not to be expected; i.e. that the majority of patients with RRMS will undergo transition to SPMS, a chronic and progressive disease sub-type characterized by minimal periods of remission, while PPMS, a further sub-type, is characterized by on-going neurological injury and a progressive accumulation of functional disability (Lublin and Reingold, 1996; Vollmer, 2007). However, relapses of acute or sub-acute onset, during which neurological dysfunction is clinically evident, are often offset by periods of partial or complete remission, both being highly variable in frequency and duration (Hauser and Oksenberg, 2006; Trapp and Nave, 2008).

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A recent study by Tremlett et al. (2012) has cast doubt on the assumption of a steady progression of disability in MS. They examined 16,132 EDSS scores of patients who were not taking immuno-modulatory drugs over one and two years, and found that up to 30 per cent of patients experienced *improvements* in disability as measured by the EDSS. There were even improvements in some people with primary progressive MS. Although a benign form of MS was not included in the original classification of disease subtypes, several authors have pointed out that some patients do not follow the general rule of steady progression, but were stable at an EDSS of less than 3 for more than 10 or even 20 years, and have suggested that "benign MS" should form a separate category (Pittock et al. 2004). However, another study has questioned the validity of "benign MS", reporting that deterioration of cognitive function, fatigue, pain, and depression were detected in some patients with benign MS, which had a negative impact on their work and social activities. Although newer quantitative MRI techniques show less tissue damage, as well as greater repair and compensatory efficiency following MS injury, the authors suggest that currently accepted criteria for benign MS diagnosis may cause an overestimation of true prevalence (Correale et al. 2012).

The confusion that exists in the literature concerning the classification of MS subtypes based on clinical criteria, as well as deterioration or improvement may possibly be addressed by regarding MS from a gene-diet perspective. Davis et al. (2013, 2014) demonstrated that identification of low-penetrance genetic risk factors may reinforce the importance of adequate fruit, vegetable and folate intake and restriction of saturated/trans fat intake in the diet. Intake of at least five fruits and vegetables per day resulted in a favourable lower EDSS with a significant reduction (28%) noted for each extra day at least five portions were consumed (Davis et al. 2014). This finding concurs with the findings of a recent study by van Rensburg et al. (2013), showing that the EDSS scores of 12 patients following a nutrition support programme improved significantly (29.9% decrease in EDSS from 3.50 to 2.45; $p=0.021$), while patients who did not stay on the regimen had a worse EDSS score after 6 months (13.9% increase from 4.83 to 5.50). After 7 years, the patients who stayed on the programme improved even more to a mean EDSS of 1.4, compared to patients not on the programme who had a worse outcome of 8.4 ($p < 0.0001$). It may therefore be that "benign MS" is not a category, but may indicate the outcome of lifestyle behaviour. South African MS patients who smoked had a significantly greater EDSS score than non-smokers ($p < 0.001$), which confirms the deleterious effect of smoking on MS (Hernan et al. 2005; Di Pauli et al. 2008).

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Further evidence that clinical categories may be less useful in the genomics era came from a study by Kotze et al. (2001), who investigated the relevance of serum iron status in RRMS, SPMS and PPMS. While genetic variation in the promoter region of the NRAMP1 gene was identified as a risk factor for MS, it was not associated with any of these disease subtypes. However, ferritin was significantly lower in RRMS than in PPMS, but after males known to have higher iron status compared to females were excluded, the difference between these clinical MS subtypes was no longer significant. The relevance of iron deficiency in MS was further investigated by van Rensburg et al. (2006), who showed that iron deficiency was significantly associated with an earlier age of diagnosis, although the reason for the iron deficiency in about 30% of patients was not apparent from their diet. It therefore seems possible that genetic subgroups could be identified in that may benefit from altered iron intake irrespective of clinical subgroups that are difficult to define and may change over time.

Prolonged disruption of the blood-brain barrier (BBB) is an important trigger for relapse, and has been associated with a decreased remission frequency, progressive neurological impairment and decline, a reduced capacity for axonal remyelination, and a decreased potential for functional recovery (Vollmer, 2007; Trapp and Nave, 2008; Mowry et al. 2009). RRMS and progressive disease are characterized by focal and diffuse demyelination respectively, with the location of lesions in the central nervous system (CNS) considered a partial determinant of disability risk (Compston and Coles, 2008; Mowry et al. 2009; Pithadia et al. 2009). MS may present with a wide variety of symptoms occurring either in isolation or various combinations, with no single symptom considered pathogenic of the disease state. Initial presenting symptoms may include mild weakness, sensory and visual loss, impaired co-ordination, fatigue and cognitive dysfunction. As the disease progresses, these may extend to complete paralysis, blindness and involuntary loss of bladder and bowel control (Lublin and Reingold, 1996; Berti et al. 2000; Gandhi et al. 2010).

1.5. **THERAPEUTIC INTERVENTION STRATEGIES FOR MS**

A major limitation in the treatment of MS is that currently available therapies, while reducing relapse rate, are ineffective in attenuating the eventual risk of irreversible neurological disability (Wingerchuk, 2008; Tullman, 2013). As such, none of the approved MS medications are licensed by the FDA for attenuation of disability progression (Tremlett et al. 2012). Furthermore, many drugs currently used for MS are expensive, of limited availability, and associated with a range of adverse side-effects, which has prompted a growing interest in the benefits of non-pharmacological measures to improve treatment outcomes in this disease (Tullman, 2013). The development of effective and affordable treatment strategies

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for MS remains an important area of research, consistent with the ultimate aim of the present study to formulate individualised harm-reduction strategies for genetically susceptible individuals, designated Pathology Supported Gene-based Intervention (van Rensburg et al. 2012; Kotze et al. 2013).

1.5.1. **Disease Modifying Drugs**

Disease-modifying drugs (DMDs) are most commonly used in the context of MS, while the related term disease-modifying anti-rheumatic drugs (DMARDs) are used in reference to a variety of autoimmune conditions. DMDs work by modulating the immune response and limiting BBB permeability and CNS invasion by T lymphocytes. This results in attenuated neural tissue injury and demyelination stemming from the increased expression and activity of various pro-inflammatory mediators, including cytokines, matrix metalloproteinases and cellular adhesion molecules (Wingerchuk et al. 2001). DMDs currently approved by the US Food and Drug Administration (FDA) for use in MS include Interferon beta-1 (IFN-β -1a and b), Glatiramer Acetate (Copolymer-I/Copaxone) and Mitoxantrone (Novantrone) (Confavreux et al. 2003; Pithadia et al. 2009). These drugs have been shown to significantly reduce the number of CNS lesions evident on MRI as well as decrease the frequency of relapses and reduce the rate of disease progression (Tullman, 2013).

Interferon beta-1 (IFN-β -1a and b)

The use of Interferon beta is mainly considered for patients who experience frequent relapses and show incomplete recovery or accumulation of CNS lesions on MRI (Clanet et al. 2002; Panitch et al. 2002). Interferon works by decreasing BBB permeability and therefore limiting neural tissue damage by restricting the entry of T lymphocytes into the CNS (Comi, 2009; Pithadia et al. 2009; Tullman, 2013). Early initiation of treatment is associated with a delayed disability onset as well as reduced rate of disease progression in MS, while a delay therein may lead to irreversible neurological impairment (Comi et al. 2001; Kappos et al. 2006; Baumhack, 2008; Milo and Panitch, 2010; Tullman, 2013). However, such beneficial effects are primarily confined to patients with mono- as compared to multi-focal CNS lesions, while interferon use is also associated with a host of serious side-effects, including myalgia, fatigue, fever, anorexia and insomnia (Comi et al. 2001; Kappos et al. 2006; Pithadia et al. 2009).

Glatiramer Acetate (Copolymer-I/Copaxone)

Synthetic polypeptide compounds such as Glatiramer Acetate (Copolymer-I/Copaxone) have been shown to reduce the relapse rate in RRMS by almost a third (Steinman, 2007;

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Baumhack, 2008; Pithadia et al. 2009). Common side-effects associated with the use of glatiramer acetate include redness, swelling flushing, shortness of breath, chest pain, anxiety and rapid heartbeat (Johnson et al. 2005; Weber et al. 2008).

Methylprednisolone

Methylprednisolone is a commonly used corticosteroid generally used for the treatment of acute relapses, while chronic treatment is considered in progressive MS, with SPMS in particular showing clinically favorable outcomes (Hohol et al. 1999; Pithadia et al. 2009). While short-course intravenous (IV) methylprednisolone is generally well-tolerated, prolonged use is associated with various side-effects, including hypertension, impaired glucose tolerance, osteoporosis, increased infection risk, gastric ulcers and oedema (Pithadia et al. 2009; Tullman, 2013).

Mitoxantrone (Novantrone)

Although the use of Mitoxantrone, an anthracenedione with antineoplastic properties, has been shown to reduce the number and severity of relapses in chronic and advanced MS by up to 67%, its administration is restricted to three years due to the cardiotoxicity associated with prolonged use (Hartung et al. 2002; Pithadia et al. 2009).

1.5.2. **Dietary and lifestyle modifications / Non-pharmacological Interventions**

Multiple studies have suggested that a well-balanced diet may have a positive impact on symptom severity in MS (Frederick, 1973; Payne, 2001; Pithadia et al. 2009; Habek et al. 2010, Davis et al. 2014). Adequate intake of micronutrients such as iron and vitamin B12 is essential for maintenance and optimisation of myelin synthesis and repair, while the antioxidant properties of vitamin C and E may effectively limit free radical – mediated neuronal damage (Zhang et al. 2001; van Rensburg et al. 2006; Pithadia et al. 2009). Lifestyle risk factors such as cigarette smoking have been associated with numerous health concerns (Franklin and Nelson, 2003; Riise and Nortvelt, 2003; Ascherio and Munger, 2007). In MS, the effect of cigarette smoke has been associated with increased disease risk and progression (Healy et al. 2009; Shirani and Tremlett, 2010).

Recent studies performed in South Africa confirmed the deleterious effect of smoking on MS disability as assessed by EDSS, with multiple vascular risk factors found to be significantly affected by lifestyle factors such as diet and physical activity (Davis et al. 2014). These findings may offer a solution to counteract the detrimental effects of the genetic risk factors contributing to the development of high homocysteine levels and obesity in MS patients. This

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is of particular relevance in view of the findings of Sternberg et al. (2013), performed in nearly 300 MS patients, which demonstrated higher cardiovascular risk factors with use of three major DMDs (IFN-β, Glatiramer acetate, Natalizumab). The use of drugs acting on the cardiovascular system (including antihypertensive, hypolipidaemic, antiplatelets) furthermore correlated significantly with MS disease severity. These findings support the implementation of a non-pharmacological intervention strategy in the resource-poor South African context, focused on the iron metabolic pathway in the present study.

1.6. **IRON IN MS**

Iron dysregulation is considered a possible pathogenic mechanism involved in the inflammatory process underlying MS. However, it is uncertain whether iron deficiency or iron overload has a deleterious effect on disease development and/or progression (Toshdiwal and Zarling, 1992; van Rensburg et al. 2012; LeVine et al. 2013). In MS patients a gender effect may contribute towards disease susceptibility found to affect more women than men (Byun et al. 2008; Koch-Henriksen, 2010). Higher iron levels in blood could have a protective effect in men, as opposed to lower iron levels in women which could be predisposing factor for increased risk of developing MS.

Biochemical indicators of iron status include serum iron, transferrin, transferrin saturation and ferritin (Custer et al. 1995; Benyamin et al. 2009). Glycosylated transferrin binds 2 Fe^{3+} iron atoms and maintains them in a soluble form. Iron saturated transferrin delivers iron to the blood stream for distribution to iron requiring pathways for metabolic processes (Aisen, 2004).

Transferrin saturation (%) is a good indicator of circulating iron in the blood stream, where disrupted/imbalanced homeostasis is indicated by transferrin saturation <16 % or > 45 % which are clinical indicators of iron deficiency and iron overload, respectively (Hentze et al. 2010).

The brain cells that produce myelin, the oligodendrocytes, have a very high requirement for iron (van Rensburg et al. 2012; Levine and Chakrabarty, 2004; Bruck, 2005; Compston and Coles, 2008), leading to the hypothesis that an increased iron intake could improve myelin synthesis and repair in patients with iron deficiency. (Bruck, 2005; Trapp and Nave, 2008; Taylor et al. 2010; Van Horseen et al. 2011). About 30% of patients with MS have low iron parameters (van Rensburg et al. 2006); however, the reasons for the iron deficiency have not been established. These findings have presented researchers and clinicians with the

responsibility to develop tests and treatment options which could benefit patients with altered dietary requirements due to their genetic background.

1.6.1. **Physiology of iron**

Iron is an essential micronutrient required by almost all living organisms and abundant in nature, with sources ranging from plants to animal products (Lieu et al. 2001; Cairo et al. 2006). It is required as a co-factor for numerous enzymes and almost all the biological systems in the body require iron for their optimal function. This includes the respiratory systems, energy production system, immune system, normal red blood cell synthesis, DNA synthesis and cell replication and proliferation (Andrews et al. 1999; Conrad et al. 1999; Le and Richardson, 2002). Red blood cells (erythrocytes) require an adequate supply of iron in order to enhance oxygen binding to haemoglobin for transportation, while mitochondria require a complete iron-sulphur-haem cluster for energy production through oxidative phosphorylation (Beard, 2001; Rouault, 2013). Futhermore, iron is an important nutritional element for brain and CSF functions, where it is required for oligodendrocyte maturation (Abo-Krysha and Rashed, 2008; Todorich et al. 2009).

Dietary iron exists in two forms, namely heme and non-heme iron, with each form determining the rate of its absorption into the body (Conrad and Umbreit, 2000; Lieu et al. 2001). Heme iron is derived from animal proteins such as hemoglobin and myoglobin as well as iron-containing enzymes (Conrad and Umbreit, 2000). This form is more readily absorbable as it is taken up directly by the mucosal cells, and its sources include fish, poultry and red meat (Lynch et al. 1989; Conrad et al. 1991; Lieu et al. 2001).

Iron status and dietary components such as calcium, casein, phosphates, phytates, polyphenols and soybeans, do not influence the absorption rate of heme iron (Lieu et al. 2001). Several vegetables (beans, lentils, spinach) and fruit (apricots, peanuts, prunes) are sources of non-heme iron which has a poorer absorption rate than heme iron, hence requiring enhancers (ascorbic acid, citric acid, cysteine glutathione, etc) that promote iron absorption (Conrad and Umbreit, 2000). However, the absorption rate of non-heme iron becomes three times higher when taken together with heme iron sources in the same meal (Geissler and Singh, 2011). Due to its ability to readily exchange electrons, iron exits in three distinct oxidation states, namely ferrous (Fe^{2+}), Ferric (Fe^{3+}) and Ferryl (Fe^{4+}) which allows reversible binding to several atoms including oxygen, nitrogen and sulphur, depending on the biological redox potential (Beard, 2001; Lieu et al. 2001).

The body's main routes for excreting iron include sweating, shedding of skin cells and gastrointestinal excretion. These are unregulated mechanisms that lead to daily iron loss in humans, of approximately 1.0 mg/day in adult males versus adult females who lose an additional 0.5 mg due to menses (Green et al. 1968; Cairo et al. 2006). Optimal iron levels are therefore maintained through tightly regulated iron absorption pathways to ensure iron homeostasis at both systemic and cellular levels (Hentze et al. 2004; Donovan et al. 2006; Bleackley et al. 2009). Some of the regulatory proteins involved are listed in Table 1.1. Iron homeostasis is the body's main mechanism to prevent iron toxicity (Hentze et al 2004) and disruption may result in iron deficiency or overload (Hallberg and Hulthen, 2000; Cairo et al. 2006; McLaren et al. 2011).

Table 1.1. **Regulatory proteins involved in the iron absorption pathway**

Regulatory Protein	Function	Cell-Site	References
Divalent Metal Iron transporter1 (DMT1)	Iron import	Duodenal mucosa	Andrews, 2000
Ferroportin (FPN)	Iron export	Enterocyte, hepatocyte, Macrophage	Pietrangelo, 2004
Transferrin (Tf)	Iron transport	Plasma	Aisen, 2004
Transferrin receptors (TfRs)	Tf iron uptake	BBB and CSF endothelial	Aisen, 2004, Hentze et al. 2004
Ferritin (H- and L chains)	Iron store, antioxidant	Cytosolic , Mitochondrial	Harrison and Arosio, 1996; Levi et al. 2001
Duodenal cytochrome b (Dcytb)	Ferric Reductase	Luminal Duodenum	McKie et al. 2001
Ceruloplasmin (Cp), Copper-containing Hephaestin (Cu-Heph)	Ferrous Oxidase	Duodenum, Serum	Hellman and Gitlin, 2002; Vulpe et al. 1999
Hepcidin	Systemic iron regulator	Hepatocytes, Liver	Ganz and Nemeth, 2006, 2012
Hereditary hemochromatosis (HFE)	Hepcidin expression	Hepatocytes, Liver	Feder et al. 1996; Nemeth et al.2004
Matriptase-2 (TMPRSS6)	Hepcidin expression	Hepatocytes, Liver	Du et al. 2008;

1.6.2. **Systemic and Cellular iron metabolism**

Iron is metabolised and regulated at both the cellular- and systemic levels that are tightly controlled by several proteins involved with iron absorption and release (Anderson et al. 2009; Finberg et al. 2010; Ganz and Nemeth, 2012). Regulatory proteins required for absorption of iron by proximal intestinal (small) enterocytes include divalent metal transporter 1 (DMT-1/SLC11A2) and Ferroportin 1 (FPN1/SLC40A1) protein. DMT-1, an

importer of dietary non-heme iron (Andrews, 2000; Mackenzie and Garrick, 2005) with the help of reductase duodenal cytochrome b, converts Fe^{3+} to Fe^{2+} iron (Mckie et al 2001). The Fe^{2+} iron undergoes exportation from the duodenal mucosa into the blood-circulation by ferroportin FPN1 protein (Anderson et al. 2009; Fuqua et al. 2012). In the systemic circulation, hephaestin and ceruloplasmin oxidases help transferrin transporter protein to access iron by converting Fe^{2+} back to the Fe^{3+} form, which can be loaded onto an apo-transferrin for distribution to cells (Vulpe et la. 1999; Hellman and Gitlin, 2002; Wessling-Resnick, 2006; Steere et al. 2012).

Cellular iron uptake is a receptor-mediated process and virtually all bodily cells express transferrin receptor 1 (TfR1) protein, which binds and internalises the diferric transferrin known as a holo-transferrin complex by endocytosis (Aisen, 2004; Hentze et al. 2004).

When the body's demand for iron supply has been met, excess iron is stored in a number of protein stores. This includes ferritin protein composed of 24-subunits of heavy (H) and light (L) chains. This globular protein has the capacity to store up to 4 500 iron atoms (Harrison and Arosio, 1996; Arosio et al. 2009). Approximately 20% of absorbed iron is stored in ferritin protein in a non-toxic form (Harrison and Arosio, 1996; Cairo et al. 2006). The H- (21 kDA) and L (19 kDA) subunits differ metabolically and functionally, which allows ferritin to be tissue specific (Harrison and Arosio, 1996; Hentze et al. 2004). The former, has a high capacity to take up and make iron readily available for cellular use, and is located in iron requiring cells. The L- subunit retains iron for prolonged periods and hence is found in iron storing tissues (Harrison and Arosio, 1996; Hentze et al. 2004). Apart from its function as storage protein, ferritin has antioxidant capability to counteract iron toxicity in the body (Torti and Torti, 2002; Hintze and Theil, 2005). Storage proteins such as haemoglobin and myoglobin consist of a heme structure that allows storage of up to 70% of absorbed iron (Zhang and Enns, 2009). Storage sites include the bone marrow, liver and spleen (Green et al. 1968; Cairo et al. 2006). Macrophages recycle approximately 0.66 % iron daily from damaged red blood cells (Finch et al. 1970; Cairo et al. 2006).

Ferritin serves as a major intracellular iron storage protein (450 kDA) that is found in virtually all cell types and stores iron atoms that are released in a very controlled manner depending on the body's demand (Harrison and Arosio, 1996). Apart from its storage function, ferritin also serves as body's natural buffering system against both iron deficiency and overload, by keeping iron soluble and in a non-toxic form (Torti and Torti, 2002; Hintze and Theil, 2005). In clinical practice serum ferritin concentrations are used for the assessment of iron status,

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with lower levels (SF ≤ 20 µg/l) indicating iron store depletion while elevated levels (women: SF > 200 µg/l, men: SF > 300 µg/l) may indicate iron overload (Liu et al. 2003; Guggenbughl et al. 2011). Increased iron stores are associated with increased iron absorption (Cairo and Pietrangelo, 2000; Hentze et al. 2004; Geissler and Singh, 2011).

Iron regulation is influenced by several inflammatory conditions (Torti and Torti, 2002; Hentze et al. 2010) involving regulatory genes encoding proteins that facilitate the expression of hepcidin, a 25-amino acid peptide known as the central iron regulator (Lee, 2009; Ganz, 2011; Ganz and Nemeth, 2012). TMPRSS6 and HFE serve as the negative and positive modulators for hepcidin expression, respectively (Pietrangelo, 2002; Ramsay et al. 2009; Ganz, 2011; Ganz and Nemeth, 2012). Down regulation of hepcidin expression by TMPRSS6, suppresses its production to increase ferroportin activity with a subsequent increase in iron uptake (Du et al. 2008; Ramsay et al. 2009; Ganz, 2011). This is achieved by proteolytic activity of TMPRSS6 to cleave hemojuvelin, a key regulator protein in the hepcidin transcription pathway that regulates hepcidin release via bone morphogenetic protein (BMP) (Barnett et al. 2006; Ramsay et al. 2009).

A primary function of hepcidin is to protect against iron overload and its deleterious consequences. This is accomplished by binding to the sole iron exporter protein ferroportin, initiating its cellular internalisation and subsequent degradation, resulting in inhibited absorption of iron from duodenal enterocytes as well as decreased release of iron from macrophages into the bloodstream for erythropoiesis (Nemeth et al. 2004; Ganz, 2011).

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Regulation of the iron absorption pathway by hepcidin and ferroportin interaction is illustrated in Figure1.1

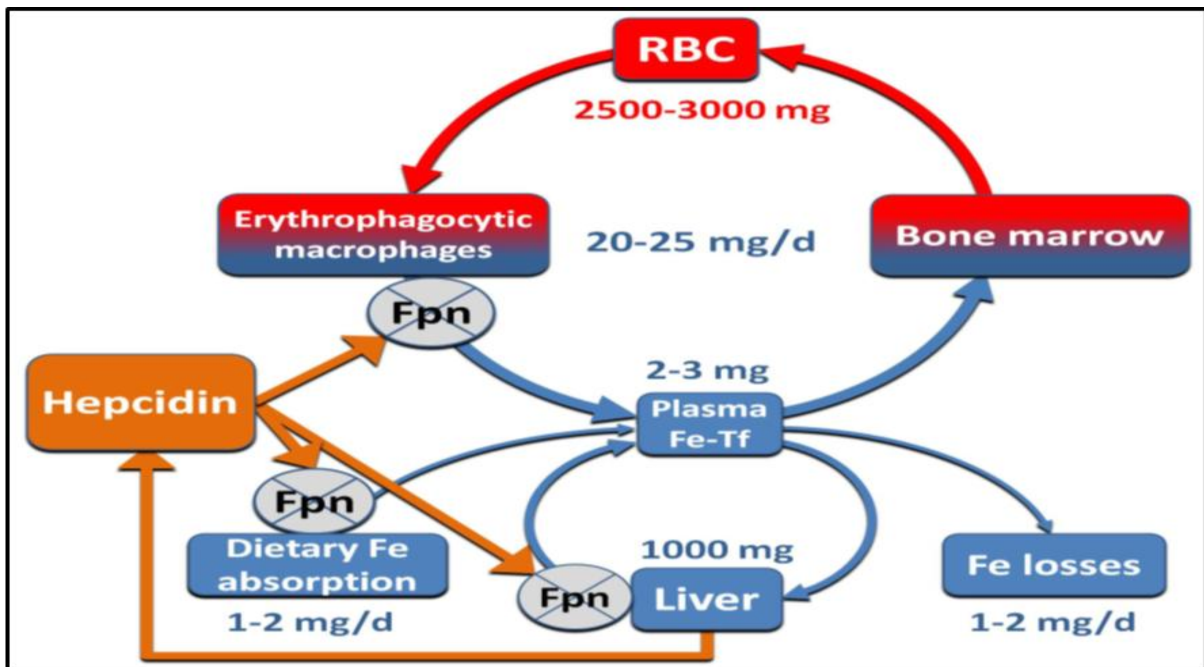

Figure 1.1. Hepcidin interaction with ferroportin controls the main iron flow into plasma. . [Used with the permission of Dr. Ganz from Ganz, 2011]

1.6.3. **Iron-related disorders**

The two most commonly reported iron-related disorders of opposite phenotypes are hereditary hemochromatosis (HH) and iron deficiency anaemia (IDA) (Morse et al. 1999; Leiu et al. 2001; McLaren et al. 2010).

Iron accumulation is the causal factor for HH, which manifests as a wide spectrum of diseases including liver disease, diabetes mellitus and skin hyperpigmentation. Conversely, several non-genetic factors predispose towards iron deficiency, including frequent blood loss, low body weight, insufficient dietary intake, age and gender (Andrews et al. 1999; Andrews, 2000; Melis et al. 2008). The iron content in the body differs by gender with higher amounts in adult men (4.0 g) than women (3.5 g), which could mainly be due to frequent blood loss through menses in females (Andrews et al. 1999; Geissler and Singh, 2011).

1.6.3.1. **Iron deposition in the brain versus iron deficiency**

In addition to the use of MRI in the diagnostic work-up of MS patients, specialised MRI sequences used exclusively for research purposes provides an iron tracking tool to evaluate

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the pathogenesis of MS (Bakshi et al. 2008; Khalil et al. 2011). In the gray and white matter of the human brain parenchyma, this specialised MRI technique is used to detect iron which is stored within ferritin in the basal glangia, globus pallidus, red nucleus and substantia nigra (Bagnato et al. 2011, 2013). These MRI sequences can also reveal the presence of insoluble iron deposits such as hemosiderin in MS lesions derived from microbleeds as well as hemoglobin in blood vessels (Rouault, 2013).

Insoluble iron deposits identified on MRI have been documented as an iron accumulation in the brains of patients with MS, and reported as a potential contributing risk factor in the disease pathogenesis through oxidative stress (LeVine et al. 2013). However, this 'excess iron' hypothesis should be revisited before considering iron chelation therapy for all patients with MS (LeVine et al. 2013), as the effect of iron deficiency has been a subject of interest in MS as well, where lower concentrations in a subset of patients have been strongly suspected to worsen disease symptoms (van Rensburg et al. 2006). The assumption that brain iron overload detected by MRI techniques is a reflection of abnormal iron accumulation in neurons (Weinreb et al. 2010), has led to chelation therapies in diseases such as Friedreich's and Parkinson's disease (Boddaert et al. 2007; Sian-Hulsmann et al. 2011). This assumption however, may not be correct for all neurodegenerative diseases and has been questioned in a most recent report (Rouault, 2013).

A possible mode of iron accumulation in the brain has previously been proposed that "*the pathology associated with iron accumulations may result from functional iron deficiency in some disease*" (Rouault and Cooperman, 2006). Whether this is the case in MS requires further investigation.

Iron may accumulate in certain brain regions such as the globus pallidus, putamen, substantia nigra and caudate nucleus (Bradbury, 1997). These regions contain tyrosine hydroxylase, which requires iron for optimum function and is involved with dopamine production. Its enzymatic activity is vulnerable to iron deficiency which leads to impaired motor co-ordination (Beard et al. 2002; Levenson et al. 2004; Frantom et al. 2006). In the brain, iron exists in several forms such as protein-bound, non-heme and ferric (Fe^{3+}), which are non-reactive (Koeppen, 1995). Brain cells requiring a constant supply of iron include oligodendrocytes, neurons, microglia and astrocytes, which have distinct metabolic features. Sufficient iron supply to these cells is limited by the blood BBB and blood-CFS barrier in the CNS (Bradbury, 1997; Ballabh et al. 2004). Oligodendrocytes are the principal cells staining for iron in the brain under normal conditions (Todorich et al. 2009) and are known as the

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factory for production of myelin proteins and lipids that are essential for nerve function (LeVine and Chakrabarty, 2004; Van Horssen et al. 2011). The abnormality of iron metabolism in oligodendrocytes has been associated with reduced myelin production (Todorich et al. 2009) and subsequent axonal damage/loss (Trapp et al. 1999; Bruck, 2005; Taylor et al. 2010).

Myelin production and maintenance requires continuous delivery of iron to oligodendrocytes, a mechanism dependent on the availability of functional iron through regulation by transferrin and ferritin (Abo-Krysha and Rashed, 2008; Todorich et al. 2009). Of concern is that increased iron stores have been detected in MS lesions, including macrophages, myelin and oligodendrocytes (Toshdiwal and Zarling, 1992; Barnett et al. 2006; Gemmati et al 2012). In particular, H-Ferritin has been identified as major iron source with readily deliverable iron to oligodendrocytes (Todorich et al. 2011), whereas iron in the L-Ferritin molecule is stored for prolonged period, and may be reflected as excess when detected by MRI (Hentze et al. 2004*)*. When L-Ferritin that is unavailable iron for cell use is detected on MRI, this may mean that sequestered iron deposits contain insoluble iron that is not bioavailable to oligodendrocytes, resulting in functional iron deficiency states (Rouault and Cooperman, 2006; van Rensburg and van Toorn 2010; van Rensburg et al. 2012). Similarly, iron deposits could reflect the iron contained within erythrocyte haemoglobin, deposited due to microbleeds as a consequence of inflammation, blocked venules and damaged endothelial cells. When the trapped red blood cells are disintegrated, haemoglobin undergoes precipitation as insoluble hemosiderin (Rouault, 2013).

1.6.3.2. **Genetic factors and iron status**: **TMPRSS6 and HFE**

Low penetrance mutations (also referred to as functional SNPs) in the TMPRSS6 and HFE genes have been implicated in the pathogenesis of iron-related as well as immune-mediated disorders (Cairo et al. 2006; Milet et al. 2007; Benyamin et al. 2009; An et al. 2012).

More than 40 polymorphisms in the TMPRSS6 gene have been identified to date; of these the relatively common TMPRSS6 A736V SNP (rs855791, c.2207 C>T) located in the serine protease domain of the TMPRSS6 gene has been strongly associated with increased risk for developing iron deficiency in the general population (Finberg et al. 2008; Guillem et al. 2008; Melis et al. 2008). This non-synonymous genetic variant causes a nucleotide substitution of C to T on chromosome 22, at nucleotide position 2207, within the coding region of exon 17 (Finberg, 2008; Ramsay et al. 2009; An et al. 2012). In the presence of the TMPRSS6

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A736V SNP, hepcidin expression is upregulated and leads to raised hepcidin concentrations, which in turn inhibits intestinal iron absorption and macrophage release (Benyamin et al. 2009; Tranglia et al. 2011). In particular, the TMPRSS6 A736V T-allele has been significantly associated with a reduction in iron parameters such as serum iron, ferritin and transferrin saturation (An et al. 2012; Gan et al. 2012).

Conversely, low-penetrance mutations in the HFE gene have been associated with hereditary iron overload (hemochromatosis). This includes the most extensively studied HFE C282Y mutation (rs1800562, c.845G>A) causing a cysteine to tyrosine change at amino acid position 282, and HFE H63D (rs1799945, c.187C>G) caused by a histidine to aspartate amino acid substitution at position 63 (Feder et al. 1996; McLaren et al. 2010). These mutations result in a defective HFE protein which down-regulates hepcidin expression, with subsequent increased iron absorption causing iron overload observed in patients with HH (Feder et al. 1996; Nemeth and Ganz, 2006; Pietrangelo, 2007). The effect of HFE C282Y accounts for nearly 85% of hemochromatosis cases, where homozygosity for the risk-associated A-allele is associated with elevated transferrin saturation and ferritin levels, while elevated transferrin saturation levels in heterozygotes (GA) is but rarely accompanied by a clinically significant increase in body iron stores (McLaren et al. 2010; McLaren et al. 2011). Compound heterozygous HFE C282Y/H63D has been associated with elevated iron stores, while a less significant effect is observed in the absence of the HFE C282Y (McLaren et al. 2010, 2011).

Iron overload

Genetic variations underlying iron overload have been studied extensively in MS patients from different population groups. Gemmati et al. (2012) reported an association between disability progression rates and the low-penetrance C282Y mutation in the iron-overload gene HFE in MS subjects, but serum iron parameters were not determined to support their findings. Comparably, Ramagopalan et al. (2008) found no such gene effect on clinical outcome in MS. Notably, Kotze et al. (2006) was the first to report that South African MS patients homozygous for HFE C282Y lack clinical manifestation of haemochromatosis, leading to the hypothesis that the effect of HFE may be masked by other genetic variant(s) with an opposite effect on iron metabolism. These and similar discrepancies in the literature have led to the development of a pathology-supported genetic testing (PSGT) strategy for complex, multifactorial conditions including those involving iron dysregulation (Kotze et al. 2009). This test concept requires that the detection of a low-penetrance mutation such as HFE C282Y is correlated with relevant biochemical parameters where possible as the

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genetics alone is not sufficient to explain the phenotypic expression of the gene known to be influenced by the environment. PSGT is focused on the development of diagnostic and therapeutic algorithms (Kotze et al. 2013), which may serve to identify genetic sub-groups of MS patients requiring different treatment strategies (van Rensburg et al. 2012). Due to the inter-individual variability of iron status known to be dependent upon multiple genetic and environmental influences, it seems possible that an individualized approach to correction of deranged iron metabolism in MS patients might be therapeutically beneficial. This realisation led to the identification of a subgroup of iron-deficient MS adult patients and children who benefited clinically from a systematic approach and development of a tailored management plan incorporating controlled iron supplementation to target iron deficiency (Rooney et al. 1999, van Rensburg et al. 2006; van Toorn et al. 2010).

Iron deficiency

Regulation of iron homeostasis is intricately related to that of the inflammatory response via the hepatic secretion and activity of acute phase reactants (APR) such as ferritin and the recently discovered antimicrobial peptide hormone hepcidin. This is in keeping with the growing appreciation that the liver acts as a master regulator of iron homeostasis in humans and other mammals (Meynard et al. 2013). We hypothesise that variation in the TMPRSS6 gene on expression and activity of hepcidin, which is viewed as a biochemical nexus point in various iron metabolism pathways, may be an important mechanism underlying the development of iron deficiency anaemia in MS, as well as an inducer of chronic inflammation or immune hyper-activation characteristic of this disease. Iron deficiency may be an initial trigger of MS when occurring in the context of inflammation and other relevant genetic or environmental factors which decrease iron uptake and release (Kotze et al. 2003; de Villiers et al. 2006), contributing towards demyelination and reducing the remyelination capacity of oligodendrocytes. This is consistent with the view that genetic sub-types of MS exist that differs in their predisposition towards an iron deficiency phenotype (Rooney et al. 1999; van Rensburg et al. 2012). However, it should be emphasised that many environmental factors influence iron status and contribute towards inflammation by mechanisms that are either dependent or independent of hepcidin function. Many other genetic factors also influence iron homeostasis. Specifically, mutation or knockout of the TMPRSS6 gene (which up-regulates hepcidin expression secondary to decreased TMPRSS6 activity) influences iron parameters such as ferritin, and may result in a severe, atypical and treatment-resistant form of iron deficiency anaemia (IDA); iron-refractory IDA.

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Recent studies showed that common variation in the TMPRSS6 gene influences the interaction between hepcidin and iron parameters in patients without clinically evident IDA and modulates the risk of defective erythropoiesis in genetically susceptible individuals (Pichler et al. 2011; Pelusi et al. 2013).

Inflammation

Results from recent animal studies provide further evidence in support of the shared relationship between iron deficiency and inflammation. It was previously shown that mice with IDA associated with low hepcidin levels manifested a pro-inflammatory state that was reduced in TMPRSS6 knock-out mice in IDA associated with high hepcidin levels. However, factors influencing the transcriptional response associated with chronic hepcidin over-expression resulting from inactivation of the TMPRSS6 gene remained unknown (Pagani et al. 2011). Riba and colleagues (2013) recently showed that inactivation of this gene results n down-regulation of hepatic pathways connected to the immune and inflammatory response related to macrophage activation and inflammatory cytokine production. The induction of hepatic hepcidin secretion by pro-inflammatory mediators such as Interleukin-6 (IL-6) results from the binding of a phosphorylated Stat3 protein to the promoter region of the hepcidin gene. In turn, hepcidin suppresses the transcription and expression of IL-6 *in vivo* (Nemeth et al. 2004; Maliken et al. 2011; Ganz, 2013).

Iron metabolism is closely related to functioning of the immune system and it is widely appreciated that both increased and decreased serum iron levels predispose towards infection (Riba et al. 2013). For example, it is now known that many molecules encoded by genes associated with the major histocompatibility complex (MHC) such as HFE (hemochromatosis) protein influence serum levels of various iron parameters. Also, not only is transferrin a necessary growth factor for T lymphocytes, but cytokines released from these cells could reciprocally influence transferrin expression and therefore iron status (Lieu et al. 2001). Furthermore, the solute carrier 11a1 (SLC11A1, previously NRAMP1) gene that encodes an iron transporter located on the lysosomal membrane of immune cells, has been associated with MS as it may confer either resistance to infection or promote immunity (Kotze et al. 2001; de Villiers et al. 2006). This finding reported for the first time in the genetically homogenous population of South Africa providing a restricted gene pool facilitating association studies, has been confirmed in some but not all subsequent studies (Comabella et al. 2004; Gazouli et al. 2008). Common and rare variants in the TMPRSS6 gene have been implicated in the pathogenesis of many disorders which involve impaired iron and immune regulation (Benyamin et al. 2009; An et al. 2012). About 40 genetic

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variations have been detected in this gene (De Falco et al. 2013). The most extensively studied TMPRSS6 A736V non-synonymous genetic variant characterised by a nucleotide substitution of C to T at position 2207 in exon 17 on chromosome 22, has been strongly associated with an increased risk of developing iron deficiency in the general population (Finberg, 2008; Ramsay et al. 2009; An et al. 2012). TMPRSS6 A736V is associated with decreased serum iron and ferritin levels, while a similar effect on transferrin saturation has recently been confirmed (An et al. 2012; Gan et al. 2012). However, the effect of TMPRSS6 A736V has not been previously analysed in MS patients.

1.7. **Aims**

The present study aimed to determine the individual and combined effects of TMPRSS6 A736V and HFE C282Y on serum iron parameters in relation to lifestyle factors, age of disease onset and clinical outcome as assessed by the EDSS in South African MS patients.

The specific objectives were as follows:

- Comparison of the allele frequencies and genotype distribution of TMPRSS6 A736V (rs855791, c.2207C>T) and HFE C282Y (rs1800562, c.845G>A) between patients diagnosed with MS and unaffected controls.
- Determination of the effects of clinical characteristics, lifestyle factors and genotype on serum iron, transferrin, transferrin saturation and ferritin levels in MS patients compared to population matched controls.
- Determination of clinical outcome in relation to age of onset and degree of disability in MS patients.

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CHAPTER 2

MATERIALS AND METHODS

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2.1. **RESEARCH MATERIALS**

2.1.1. **Ethics Approval and Clearance**

Overall ethics approval for the project has been granted (N07/09/203 and N09/08/224) by the Human Research Ethics Committee of Stellenbosch University, at the Faculty of Medicine and Health Sciences.

2.1.2. **Selection of study population**

The study cohort consisted of 426 unrelated subjects: 307 Caucasian females and 100 males, 11 Coloured (Mixed ancestry) females, 3 Asian females, 4 African females and 1 male. All non-Caucasian individuals (Africans, Coloureds and Asians) were excluded from comparative analyses in order to exclude race as a confounder. Full data sets were not available on all study participants; therefore the sample numbers included per analysis were indicated where appropriate.

2.1.2.1. **Cases**

A total of 126 unrelated patients previously diagnosed with MS were included in the present study. This included 49 DNA samples of Caucasian patients from a study previously performed (Kotze et al. 2001), their age of MS onset (diagnosis/symptoms) was previously recorded but no additional information on clinical and lifestyle profiles were available. The EDSS scores of MS patients were determined as previously described (van Rensburg et al. 2006). For the purpose of this study, only baseline EDSS values available for 48 MS patients were used.

2.1.2.2. **Controls**

A total of 300 unrelated controls individuals without a history or clinical presentation of neurological illness were identified from a secure database (access at www.gknowmix.org) of individuals enrolled in a chronic disease risk screening program.

The Caucasian study population consisted of 121 MS patients (16 males, 105 females) and 286 controls (84 males, 202 females), between the ages of 19 and 98 years, used in comparative studies. Subgroup analysis was performed in the event that a full data set was not available for all individuals.

2.1.3. **Inclusion and Exclusion Criteria**

Inclusion criteria were: patients diagnosed with definite MS by their respective neurologists, and informed consent provided.

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Exclusion criteria were: individuals with other demyelinating diseases such as neuromyelitis optica, and informed consent for laboratory testing not provided.

2.2. **DATA CAPTURING METHODS**

The medical and lifestyle profiles of a subset of study participants (70 patients and 59 controls) were collected through a questionnaire (Gknowmix™ nutrition questionnaire) available on the website (www.gknowmix.com), which contained questions on dietary intake, medical history (family and own), exposure to lifestyle risk factors and daily exercise (physical and/or daytime activity). Scores were assigned for dietary portions of several foods containing carbohydrate, fat (saturated/trans), folate, fruit/vegetable and fibre. Under medical history, the following were recorded: MS medication, age of disease diagnosis, age of first symptom presentation and disability status which was indicated by EDSS scores ranging from 0.0 to 10.0 (Kurtzke, 1983) (Appendix B). Lifestyle risk factors such as alcohol consumption was calculated as units and risk scores (0 to 4) were generated accordingly (abstain = 0, occasionally/1-2 Units = 1, week/1 -13 Units = 2, week/13-21 Units = 3 and 22 Units or more = 4), while exposure to cigarette smoke was recorded as current smoker or previous (less than a year). Scores (1 to 4) were also assigned for physical activities as follows: none/occasionally = 1, once per week = 2, 2-3 times = 3, and a score of 4 was assigned for exercising 4 or more times per week. Daytime activity recorded as any day activity that lasted more 30 minutes including walking and occupational activities, and scored (1 to 3) as 1 = sedentary, 2 = moderate and a score of 3 was noted as intense day activity. Body mass index (BMI) which has direct correlation to the measures of body fat was here assessed as it marks one of the major contributing health risk factors in the general population. The weight categories include 18.5-24.9 kg/m^2 for a normal weight, BMI below 18.5 kg/m^2 indicates underweight and overweight between 25.0- and 29.9 kg/m^2, while obesity is categorised by BMI above 30.0 kg/m^2.

2.2.1. **Blood status parameters analysis**

Peripheral venous blood samples were collected from all eligible subjects by a suitably experienced clinician according to standard practices. Serum iron, ferritin, transferrin levels as well as transferrin saturation were determined as previously reported (Kotze et al. 2006; van Rensburg et al. 2006) and results were entered into a secure database.

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2.2.2. **Research methods**

Saliva and whole blood samples were obtained from study participants with informed consent and DNA was extracted from saliva and whole blood for genotyping of TMPRSS6 A736V and HFE C282Y.

2.2.3. **Extraction of DNA from saliva and whole blood samples**

Genomic DNA from saliva samples were accomplished using the Oragene® –DNA/Saliva Kit (protocol), and extractions from whole blood was performed using the QIAGEN® QIAamp DNA Blood Midi Kit as described in the protocol (Spin Protocol) provided. The extraction procedures were as follows:

2.2.3.1. **DNA extraction from Saliva**: Oragene® –DNA/Saliva Kit

The Oragene® DNA/Saliva sample (viscous) was mixed in the Oragene® –DNA vial by inverting several times and gentle shaking for a few seconds. The sample was incubated at 50°C in a water bath for 1 hour, in order to release adequate DNA into the solution. Under these conditions nucleases are permanently inactivated. After incubation, 500 µl of the sample was transferred to a microcentrifuge tube (1.5 ml), and a 20 µl Oragene®-DNA purifier (OG-L2P) solution was added and vortexed for 10 seconds. The mixed sample was incubated on ice for 10 minutes, in order to effectively remove the impurities. Following incubation, the vial was centrifuged at 13,000 rpm (15,000 X *g*) at room temperature (22 – 25°C) for 10 minutes, allowing proper separation of DNA from the precipitated impurities. Glycogen solution (5 µl) was added into the supernatant containing DNA, which made the pellet more visible.

A pipet tip was used to carefully transfer the supernatant into a fresh microcentrifuge tube, and the remaining pellet containing impurities was discarded. Then 95 – 100% ethanol solution (500 µl), at room temperature, was added to the supernatant and gently mixed by inversion 10 times. The sample was incubated at room temperature (22 – 25°C) for 10 minutes, to allow the DNA to fully precipitate. The tube was then placed in the microcentrifuge in a known orientation (e.g, with the hinge portion of the cap pointing away from the centre of the rotor, to keep track of the DNA pellet, which was expected to be located at the tip of the tube below the hinge), and centrifuged at room temperature (22 – 25°C) at 13,000 rpm (15,000 X *g*) for 2 minutes.

The supernatant containing impurities was carefully discarded using a pipet tip without disturbing the DNA pellet, which was washed with 70% ethanol (250 µl) and incubated for 1

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minute at room temperature to remove residual inhibitors. Thereafter the ethanol was completely removed. This was followed by the addition of a DNA buffer (100 µl) (Nuclease Free Water) and mixing by vortexing for at least 5 seconds, to completely dissolve the DNA pellet. The purified DNA samples were incubated at room temperature on a shaker overnight to allow complete homogenization, and were stored at 4°C for frequent use.

2.2.3.2. DNA extraction from Whole Blood: QIAamp® DNA Blood Midi Kit (Spin Protocol)

From each whole blood sample, 2 ml blood containing Ethylenediaminetetraacetic acid (EDTA) was treated with several QIAGEN solutions in order to extract and purify the DNA. The extractions were performed using the QIAamp DNA whole blood extraction protocol (QIAGEN®) as follows:

The first step of cell lysis in order to release DNA from the nucleus was achieved by adding QIAGEN protease stock solution (200 µl) into a 15 ml centrifuge tube containing whole blood samples (2 ml), and vigorous shaking the contents on a vortex apparatus to increase the proteolysis reaction. A second lysing solution, buffer AL (2.4 ml) was added into the centrifuge tube mix, followed by thorough mixing by inverting the tube at least 15 times for adequate lysis. One extra minute of brief vortexing was added to ensure that the lysed solution was homogenous. The homogenous solution was incubated on a dry block (70°C) for 10 minutes, to increase DNA yield.

After incubation and removal of the 15 ml centrifuge tube with homogenised sample from the dry block, 96 - 100% ethanol (2 ml) was added. The solution was thoroughly mixed by inverting the tube 10 X with additional vigorous shaking (vortexing). Thereafter approximately 3 ml of the solution was transferred into the QIAamp® Midi spin column, and placed back into the 15 ml centrifuge tube, then centrifuged for 3 minutes at 3000 rpm (1850 x *g*). The filtrate was discarded and the remaining solution (~ 3 ml) was transferred into the QIAamp® Midi spin column and centrifuged at 3000 rpm (1850 x *g*) for 3 minutes.

The QIAamp® Midi spin column was transferred into a fresh 15 ml collection tube, a volume (2 ml) of wash buffer AW1 was added to the column membrane, then centrifuged for 1 minute at 5000 rpm (4500 x *g*). A second wash buffer AW2 (2 ml) was added to the QIAamp® Midi spin column, then centrifuged (5000 rpm/4500 x *g*) for 15 minutes, to ensure complete removal of the AW2 from the QIAamp® Midi spin column. The column was placed into a clean 15 ml collection tube, and the filtrate was discarded. The DNA was eluted from

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the QIAamp® Midi spin column by adding 300 µl of nuclease-free water which was equilibrated to room temperature (22 – 25°C), directly onto the column membrane. The column was incubated at room temperature for 5 minutes, and then centrifuged at 5000 rpm (4500 x *g*) for 2 minutes. The eluted contents (~300 µl) were reloaded onto the QIAamp® Midi spin column and the previous incubation and centrifugation steps were repeated to maximize the DNA concentrations obtained. The DNA samples were stored at 4°C for frequent use, while aliquots were stored at - 20°C for long term usage.

2.3. **DNA QUANTIFICATION AND AMPLIFICATION**

2.3.1. **The NanoDrop instrument**

A NanoDrop® ND-1000 Spectrophometer was used to measure the concentration and purity of the DNA samples. The results were subsequently captured on a connected v3.5.2 software package. The software was switched on, and then the Nucleic Acid analysis was selected. The pedestal surfaces were cleaned with ethanol (70%) and double distilled water (dd-H_2O). Upon completion the instrument was initialized by loading 2 µl of nuclease free water, followed by blanking (0.0 ng/µl) the instrument by loading 2 µl of RNase free water onto the pedestal. After blanking was completed, 2 µl DNA sample was pipetted onto the pedestal and measurements (DNA concentration, purity ratios) were captured and stored for each sample. The pedestal was cleaned between each measurement. DNA purity was assessed by absorbance readings at ratios of 260/280 (1.6 – 1.9) and 260/230 (> 2.0), which indicated the absence of proteins and RNA contaminants, respectively. All the DNA samples of known concentrations were diluted with nuclease free water to give a final 10 ng/µl DNA concentration, which was suitable for RT-PCR genotyping.

2.3.2. **Oligonucleotide primer design**

The polymorphisms studied included the rs855791 TMPRSS6, which is a polymorphism that causes a pyrimidine nucleotide base change from cytosine (C) to thymine (T) at nucleotide position 2207 (c. 2207 C>T), resulting in an alanine (A) to (V) valine substitution at amino acid position 736 (A736V). The rs1800562 HFE polymorphism which results in a purine nucleotide base change from guanine (G) to adenine (A) at position 845 (c. 845 G>A), leads to a substitution of cysteine (C) by tyrosine (Y) at amino acid position 282 (C282Y).

Oligonucleotide primer sequences for the selected polymorphisms (TMPRSS6 c.2207C>T and HFE c.845 G>A) were designed using available online primer design software packages (Table 2.1). The genomic reference sequences were extracted from the National Center for Biotechnology Information (NCBI), an online database (www.ncbi.nlm.nih.gov). The primer

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sets were then ordered from Whitehead Scientific Pty (Ltd) and synthesised by Integrated DNA Technologies (IDT). Conventional PCR methodology and direct DNA sequencing were employed using these primers to identify genotyping controls for use in the high-throughput real-time PCR (RT-PCR) screening.

Primers designed were specific for the selected SNPs, (rs855791 / c.2207 C>T) TMPRSS6 A736V and (rs1800562 / c.845 G>A) HFE C282Y, using appropriate software programs available online.

- NCBI database was used to retrieve SNP sequences using available reference numbers (rs numbers) as assigned for each SNP (www.ncbi.nlm.nih.gov).
- Primer3Plus tool was used to design oligonucleotide primers (forward and reverse) flanking the SNP of interest (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/).
- SNPCheck v3-Manchester tool was used to identify the presence of other SNPs that may be situated within the primers. This was done to avoid result discrepancies during genotyping analysis (https://ngrl.manchester.ac.uk/SNPCheckV3/snpcheck).
- The IDT Oligoanalyzer tool was used to evaluate the primer parameters including primer melting temperature, GC content, hairpin structure, dimers (self/homo and hetero) (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/).

The PCR products were visualized by agarose gel electrophoresis. The unpurified PCR products were sent for sequencing analysis at the Central Analytical Facility of Stellenbosch University.

Table 2.1 Oligonucleotide primer sequences designed for conventional PCR methodology

Gene	SNP ID	SNP position	Primer	Oligonucleotide Sequence (5' - 3')	G-C Content (%)	Tm (°C)	TA (°C)	Amplicon Size (bp)
TMPRSS6	rs855791	c.2207 C>T V736A	Forward	TGA CCT CAG GTG TTC CGT C	57.8	56.7	59	534
			Reverse	AGG CTT CAG CAG GCT GAT G	57.8	57.8		
HFE	rs1800562	c.845 G>A C282Y	Forward	TGG CAA GGG TAA ACA GAT CC	50.0	54.8	56	396
			Reverse	TAC CTC CTC AGG CAC TCC TC	60.0	57.9		

Abbreviations: SNP, single nucleotide polymorphism; ID, identification; Tm, melting temperature; TA, annealing temperature; bp, base pairs; TMPRSS6, transmembrane protease, serine-6 gene; HFE, hemochromatosis gene.

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2.3.3. **DNA amplification by conventional PCR methodology**

Amplification of the targeted genetic variations in the TMPRSS6 and HFE genes enabled the identification of the respective genotypes through conventional DNA sequencing. Conventional PCR methodology was performed using a Promega Go*Taq*® Flexi DNA Polymerase PCR kit, for amplification of the targeted sequence region of the TMPRSS6 gene on chromosome 22 flanking the c2207 C>T (rs855791) polymorphism, and that of the HFE gene on chromosome 6 flanking the HFE c.845 G>A (rs1800562) polymorphism.

Applied Biosystems® thermal cyclers (2700 and 9700) were used for targeted regions amplification. The reaction mixture contained a colourless Go*Taq*® Flexi Buffer (1 X) (Promega), $MgCl_2$ (1.5 mM) (Promega), Forward and Reverse primers (0.24 µM each) (IDT), Go*Taq*® DNA polymerase (1.25 U) (Promega), template DNA (100 ng), dNTPs {dATP, dTTP, dGTP and dCTP (0.2 mM each)} (Applied Biosystems) and nuclease-free water. The amplification was performed on the Applied Biosystems® GeneAmp® PCR System 2700 thermal cycler, in 25 µl reactions under specific thermal cycle conditions for the individual genes TMPRSS6 (Table 2.2) and HFE (Table 2.3).

Table 2.2 Thermal Cycling conditions of conventional PCR for TMPRSS6 c.2207C>T

Steps	Cycles (X)	Temperature (°C)	Time (minute)
Initial Denaturation	1	95	02:00
Denaturation	28	95	00:30
Annealing		59	00:30
Extention		72	00:30
Final Extention	1	72	05:00
Cooling	1	22	01:00

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Table 2.3 Thermal Cycling conditions of conventional PCR for HFE C282Y

Steps	Cycles (X)	Temperature (°C)	Time (minute)
Initial Denaturation	1	94	05:00
Denaturation	35	94	00:30
Annealing		56	00:30
Extention		72	02:00
Final Extention	1	72	10:00
Cooling	1	22	01:00

Conventional PCR amplification of targeted sequence regions flanking the TMPRSS6 A736V and HFE C282Y polymorphisms, yielded different sized amplicon fragments that were separated by gel electrophoresis on a 2 % (w/v) agarose gel and visualized by staining with ethidium bromide.

2.3.4. **Gel Electrophoresis analysis**

The success of the PCR amplification reactions was evaluated by gel electrophoresis. PCR products were separated on a 2 % (w/v) agarose gel to ensure that the expected fragment sizes were obtained as compared with a size marker loaded on the same gel.

Two grams (g) of agarose powder (Seakem® LE agarose) was weighed and added into a 100 ml of 1 X TBE (90 mM Tris-HCl (pH 8.0), 90 mM Boric acid, 1mM EDTA (pH 8.0) and ddH$_2$O) buffer solution in an Erlenmeyer flask. The mixture-containing flask was placed in a microwave for 3 minutes on highest setting, and then equilibrated at room temperature (22 – 25°C) for 10 to 15 minutes. The gel was poured into a casting tray with a comb inserted to make wells on the gel, and was allowed to solidify at room temperature for 30 minutes. The comb was carefully removed from a solid gel, and the casting tray was transferred to the electrophoresis tank containing running buffer (700 ml 1 X TBE buffer and 70 µl 0.01 % (v/v) Ethidium Bromide).

Each labelled well was loaded with a mixture of 5 µl of loading dye (Ficoll Orange G) and either 3 µl of PCR product or 3 µl of the 100bp DNA Ladder (Promega®). The electrophoresis was performed at 100V for 45 minutes to allow separation of the amplicons. The gel was visualised under ultra-violet light using a transilluminator (GibcoBRL Life Technologies, California, USA).

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2.3.5. **Conventional DNA sequencing analysis**

The unpurified PCR products obtained for the TMPRSS6 c.2207 C>T (rs855791) and HFE c.845 G>A (rs1800562) polymorphisms were sent to the Central Analytical Facility of Stellenbosch University (CAFSU), for Post PCR Clean-Up and Direct DNA Sequencing. The automated DNA sequencing analyses enabled identification of three genotypes (homozygous Wild Type, heterozygous and homozygous Mutant) for each SNP (Table 2.4). The DNA sequencing results from CAFSU were analysed using the FinchTV Version 1.4.0 software (www.geospiza.com), which generated the electropherograms depicting nucleotide sequences of each gene (TMPRSS6 and HFE). The four ddNTP (ddATP, ddCTP, ddGTP and ddTTP) molecules are distinguished by four distinct fluorescence colours and are represented as colour peaks on the electropherogram. The position of the SNP was detected by comparing these nucleotide sequences to the gene reference sequences obtained from the NCBI database (www.ncbi.nlm.nih.gov).

Table 2.4 Identified genotypes by conventional DNA sequencing

Genotypes	**TMPRSS6 c.2207 C>T (A736V)**	**HFE c.845 G>A (C282Y)**
Homozygous Wild Type	CC	GG
Heterozygous	CT	GA
Homozygous Mutant	TT	AA

Abbreviations: SNP, single nucleotide polymorphism; TMPRSS6, transmembrane protease, serine-6 gene; HFE, hemochromatosis gene

2.4. **GENOTYPING ANALYSIS**

Control samples for TMPRSS6 A736V and HFE C282Y were identified via DNA sequencing of internal control samples (K1, K2, K3, K4 and K6), for the identification of the wild-type, heterozygous and homozygous genotypes. These were included as controls in subsequent genotyping of 426 DNA samples from study participants: 126 MS patients (121 Caucasian, 5 Coloured) and 300 controls (286 Caucasian, 6 Coloured, 5 African, 3 Asians).

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2.4.1. **RT-PCR amplification**

High-throughput RT-PCR methodology was applied for genotyping using Applied Biosystems (ABI™) TaqMan® SNP Genotyping assays for TMPRSS6 A736V and HFE C282Y SNPs. This was performed on the ABI™ 7900HT Fast Real-Time PCR System using assays TMPRSS6 rs855791 C___3289902_10 and HFE rs1800562 C___1085595_10. These assays are standardized and consist of allele-specific primers and TaqMan® Minor Groove Binder (MGB) probes (dye-labelled FAM™ and VIC® denoting either mutant or wild type alleles) for the detection of the SNP of interest. Sterile double-diluted SABAX water was used to perform a 20 X dilution series on the 40 X TaqMan® SNP Genotyping assays, as required for the RT-PCR instrument used for end-point genotyping by allelic discrimination.

Genotyping of DNA samples was performed on an ABI™ 7900HT Fast RT-PCR system using ABI™ TaqMan® SNP Genotyping assays. The reaction mixture contained: 2.75 µl of SABAX water (nuclease free water), 5 µl of TaqMan® genomic master mix, 0,25 µl of 20 X TaqMan® SNP Genotyping assay and 4 µl of 10ng/ µl template specific DNA made up to a 12 µl final reaction volume. The thermal cycling conditions for RT-PCR amplification runs is summarised in table 2.5.

Table 2.5 Thermal Cycling conditions of RT-PCR

Steps	Cycles (X)	Temperature (°C)	Time (minute)
Initial Denaturation	1	95	10:00
Denaturation	40	92	00:15
Annealing/Extention		60	01:00

2.5. **Statistical analysis**

Genotype distribution and allele frequencies of TMPRSS6 A736V and HFE C282Y were evaluated from allele counts and Hardy-Weinberg equilibrium was tested using an online Court-lab Hardy Weinberg calculator (Michael H. 2005-2008). Analysis of variance (ANOVA) was used to determine the individual and combined effects of TMPRSS6 A736V and HFE C282Y on clinical outcomes (age of symptom onset, age of diagnosis and EDSS score) as well as iron parameters (serum iron, ferritin, transferrin and transferrin saturation). Prior to performing genotype-phenotype association studies, possible demographic factors and lifestyle habits were identified as potential confounders that were subsequently adjusted for. These included age, sex, BMI, dietary red meat intake, alcohol intake, smoking status and

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physical activity. A log transformation model was used to correct normality for non-symmetric distributions as was the case for serum ferritin. Fisher least significant difference (LSD) post-hoc tests were performed to determine possible differential effects of TMPRSS6 A736V and HFE C282Y, single or combined, on serum iron parameters and clinical outcome. Significance threshold was set at $p \leq 0.05$.

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CHAPTER 3

RESULTS

3.1. **Study population**

The Caucasian study population consisted of 121 MS patients (16 males, 105 females) and 286 controls (84 males, 202 females) between the ages of 19 and 98 years. All non-Caucasian individuals (5 MS patients and 14 controls) were excluded in comparative studies to exclude race as possible confounder. Analysis of the TMPRSS6 rs855791 (c.2207C>T, A736V) and HFE rs1800562 (c.845G>A, C282Y) polymorphisms (Table 3.1) was conducted with conventional PCR and direct DNA sequencing (as the gold standard) for the identification of the respective wild-type, heterozygous and homozygous genotypes.

Table 3.1 Low-penetrance mutations studied in relation to iron metabolism

Gene	Genetic Variations	Metabolic Associations
TMPRSS6	rs855791, c.2207 C>T, (A736V)	Iron deficiency anaemia
HFE	rs1800562, c.845 G>A, (C282Y)	Iron overload, Hereditary hemochromatosis

Abbreviations: TMPRSS6, transmembrane protease, serine-6 gene; HFE, hemochromatosis gene

3.1.1. *Optimisation of high throughput genotyping*

The accuracy of the RT-PCR screening method used was verified by comparing pre-sequenced internal control samples and including them as positive controls during each allelic specific PCR run to corroborate the results generated during the screening process. In the present study, the initial optimisation / assay standardisation phase involved genotyping of 300 DNA samples from unrelated controls, which was performed by a high-throughput RT-PCR genotyping methodology as described in sections 2.4.1 and 2.3.5. The standardised RT-PCR assays for each selected SNP were used for genotyping of 126 MS patients whose genotyping results were subsequently compared with that of the control subjects.

3.1.2. *Conventional PCR and gel electrophoresis*

The DNA samples were successfully amplified by conventional PCR and yielded PCR products as visualized on an agarose gel (Fig.3.1 A-B) using the techniques described in sections 2.3.3 and 2.3.4. Amplification of the internal control samples yielded the expected DNA fragment sizes as compared against a 100 bp DNA ladder (lane 1), for the TMPRSS6

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(534 bp: lane 2-5) and HFE (396 bp: lane 2-4) gene sequences. No amplification occurred with the non-template control (NTC) samples that did not contain any DNA, shown by the blank lanes which confirm the absence of contamination in each PCR reaction.

Figure 3.1 illustrates separation of PCR amplicons (534 bp) synthesised with the TMPRSS6 (A736V) primer set visualized with 0.0001 % (v/v) Ethidium Bromide solution. Lane 1 contains a 100 bp DNA ladder with the amplicons in lane 2 through 5 and NTC in lane 6 indicating the absence of contamination. PCR amplicons (396 bp) synthesised with the HFE (C282Y) primer set is visualized with 0.0001 % (v/v) Ethidium Bromide solution. Lane 1 contains a 100 bp DNA ladder, with the amplicons in lane 2 through 4 and NTC in lane 5 indicating the absence of contamination.

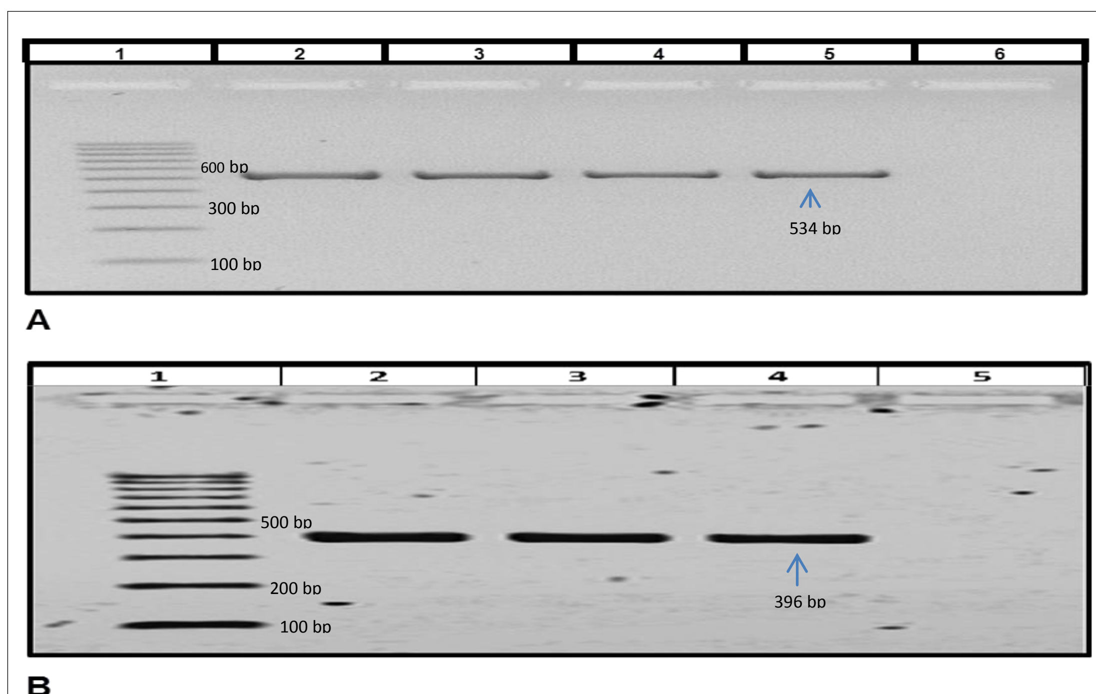

Figure 3.1. A) A 2 % (w/v) agarose gel depicting PCR amplicons synthesised with TMPRSS6 (A736V) primer set. **B)** A 2 % (w/v) agarose gel depicting PCR amplicons synthesised with the HFE (C282Y) primer set.

3.1.3. *Genotype identification by Direct DNA sequencing*

The PCR products were sequenced to detect the presence of the TMPRSS6 A736V and HFE C282Y SNPs, and this was achieved by direct DNA sequencing (section 2.3.5). In Figure 3.2 A-C through 3.3 A-C, the analysed data is presented as electropherograms showing the position of the SNP.

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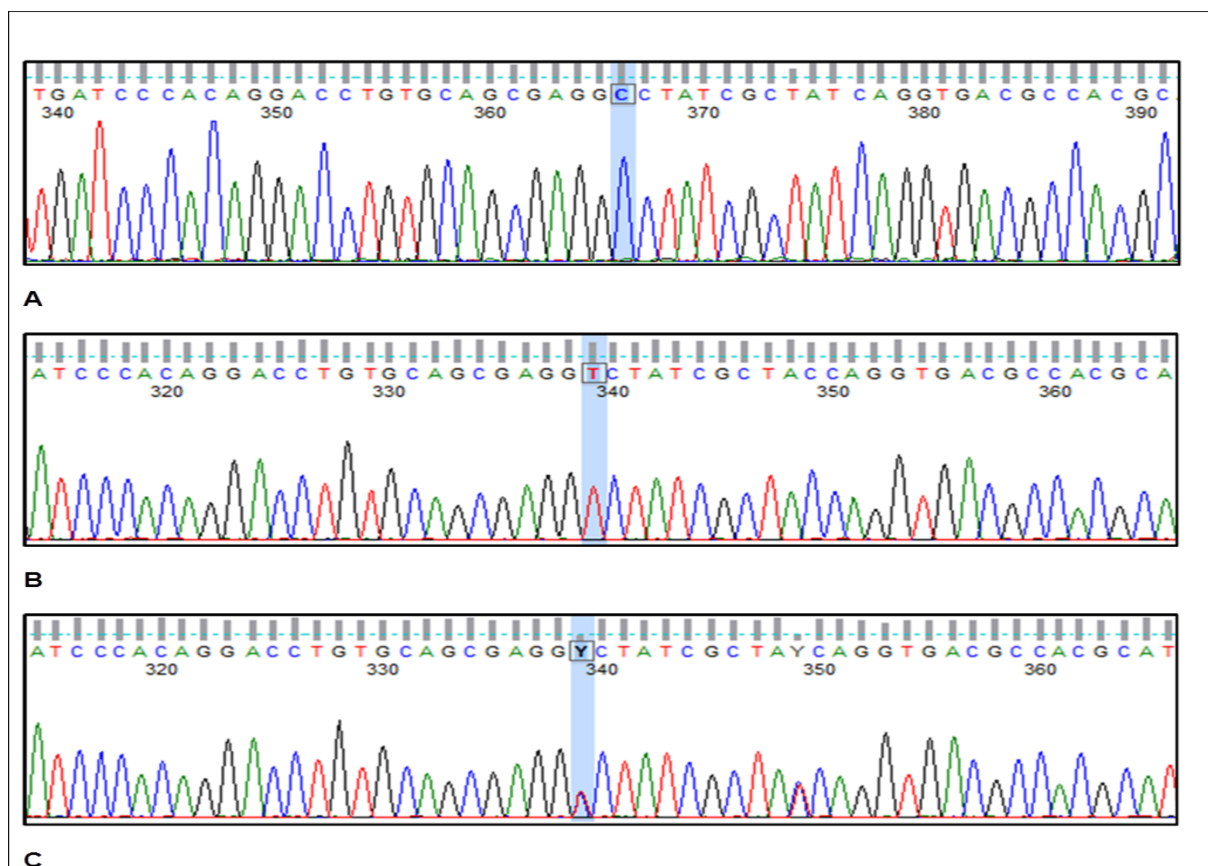
3.1.3.1. TMPRSS6 A736V

DNA sequencing reactions confirmed the presence of TMPRSS6 A736V through the identification of three genotypic states; Wild-Type (mutation not detected), homozygous and heterozygous genotypes. The Wild-Type (Fig.3.2 A), Homozygous (Fig.3.2 B) and heterozygous (Fig.3.2 C) genotypes for TMPRSS6 A736V, were identified from the forward (sense) and reverse (anti-sense) sequencing reactions. The SNP position is shown by electropherograms and is highlighted in light blue colour, Fig.3.2 A–C, which gives a representation of some of the results.

Figure 3.2 shows the electropherogram generated from the TMPRSS6 (A736V) primer set. The SNP position indicated by C (blue) represents a Wild-Type (CC) genotype. The electropherogram generated from the TMPRSS6 (A736V) primer set shows the SNP position indicated by T (blue), representing a homozygous (TT) genotype. The electropherogram generated from the TMPRSS6 (A736V) primer set for the SNP position indicated by Y (blue) represents a heterozygous (CT) genotype.



3.2. A) Electropherogram depicting the reverse (anti-sense) sequencing reaction of PCR amplicon of TMPRSS6 (A736V) primer set yielded a Wild-Type (CC) genotype. **B**) Electropherogram depicting the forward (sense) sequencing reaction of PCR amplicon of TMPRSS6 (A736V) primer set yielded

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homozygous (TT) genotype. **C)** Electropherogram depicting the forward (sense) sequencing reaction of PCR amplicon of TMPRSS6 (A736V) primer set yielded heterozygous (CT) genotype.

3.1.3.2. HFE C282Y

The DNA sequencing reactions confirmed the presence of HFE C282Y through the identification of three genotypic states; Wild Type (mutation not detected), homozygous and heterozygous genotypes. The Wild Type (Fig.3.3 A), Homozygous (Fig.3.3 B) and heterozygous (Fig.3.3 C) genotypes for HFE C282Y amplicon, were identified from the forward (sense) and reverse (anti-sense) sequencing reactions. The SNP position is shown by electropherograms, and is highlighted in light blue colour.

Figure 3.3 shows the electropherogram generated from the HFE (C282Y) primer set. The SNP position is indicated by G (blue) representing the Wild Type (GG) genotype. The electropherogram generated from the HFE (C282Y) primer set for the SNP position indicated by A (blue), represents the homozygous (AA) genotype. The electropherogram generated from the HFE (C282Y) primer set for the SNP position indicated by R (blue) represents a heterozygous (GA) genotype.

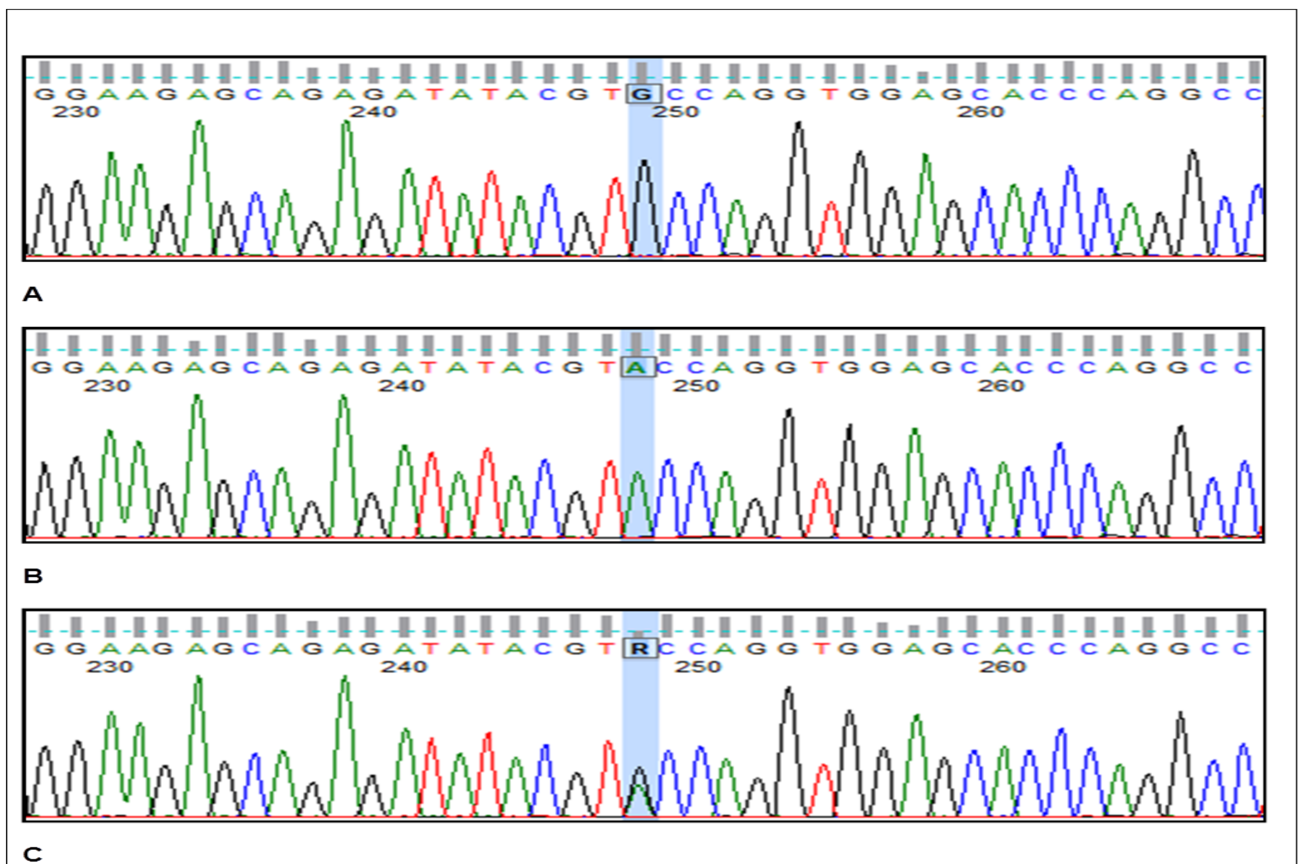

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Figure 3.3. A) Electropherogram depicting the forward (sense) sequencing reaction of PCR amplicon of HFE (C282Y) primer set, yielded a Wild Type (GG) genotype. **B**) Electropherogram depicting the forward (sense) sequencing reaction of PCR amplicon of HFE (C282Y) primer set, yielded a homozygous (AA) genotype. **C**) Electropherogram depicting the forward (sense) sequencing reaction of PCR amplicon of HFE (C282Y) primer set, yielded a heterozygous (GA) genotype.

3.1.4. *High-throughput RT-PCR*

Genotyping of 426 DNA samples (126 MS patients and 300 controls) was performed by a high-throughput RT-PCR assay on the ABI™ 7900HT instrument, using the TaqMan® SNP genotyping assays for the two selected SNPs as described in section 2.4.1. Allele specific RT-PCR amplification was successfully achieved for both the TMPRSS6 A736V and HFE C282Y SNPs, with discriminated allele pairs in three different combinations of wild-type (non-mutant) and carrier (mutant) forming groups according to fluorescence colours (blue, green and red) as depicted in the amplification and allelic discrimination plots (Fig.3.4 A-B and 3.5 A-B).

Figures 3.4 A and B show amplification and allelic discrimination plots depicting successful genotyping reactions and genotype clustering of TMPRSS6 A736V.

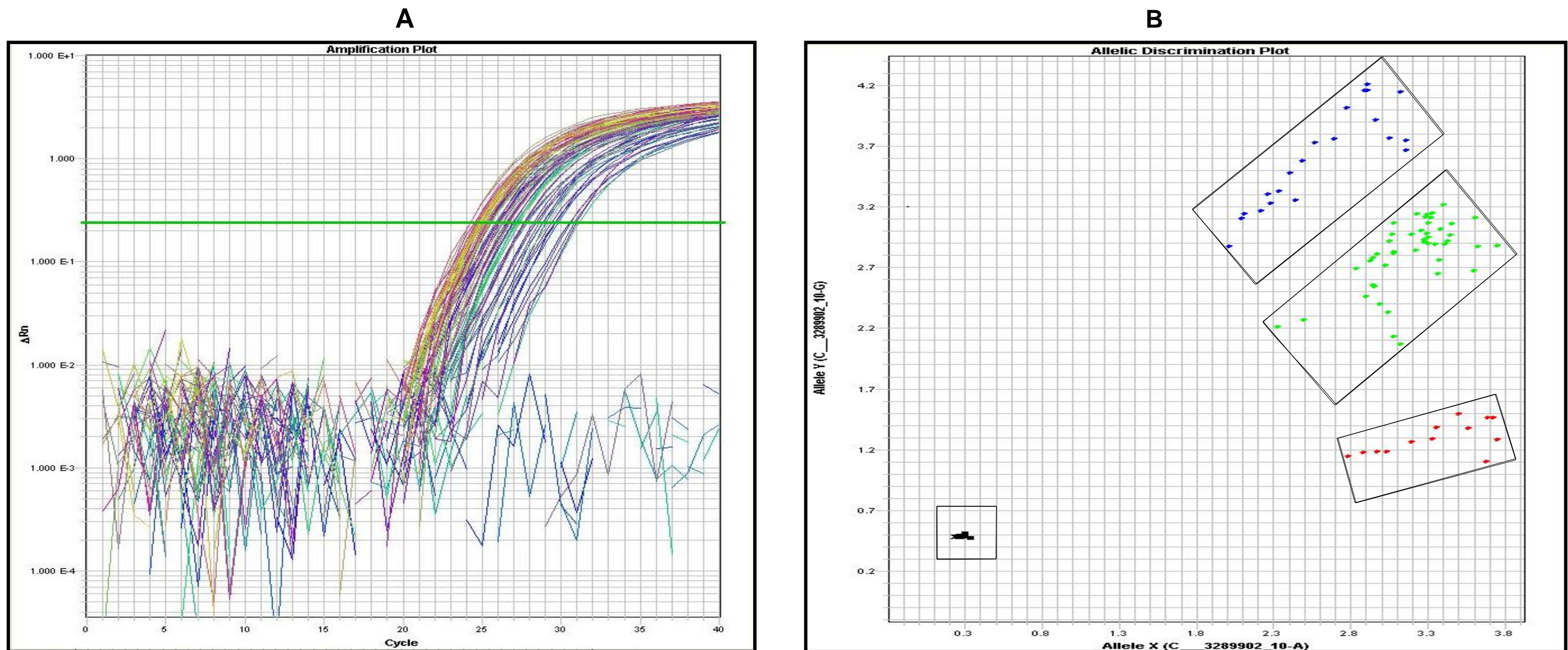

Figure 3.4: **A**) Amplification plot obtained using the ABI™ *Taq*Man® assay for TMPRSS6 (A736V). (**B**) Representation of an allelic discrimination analysis using ABI™ *Taq*Man® assay for TMPRSS6 (A736V) [Allele Y (C_3209902_10-G) vs Allele X (C_3209902_10-A)]. Blue dots = Wild type (CC), red dots = homozygous (TT), green dots = heterozygous, black dots = NTCs (controls for the assay).

Figures 3.5 A and B show amplification and allelic discrimination plots depicting successful genotyping reactions and genotype clustering of HFE C282Y.

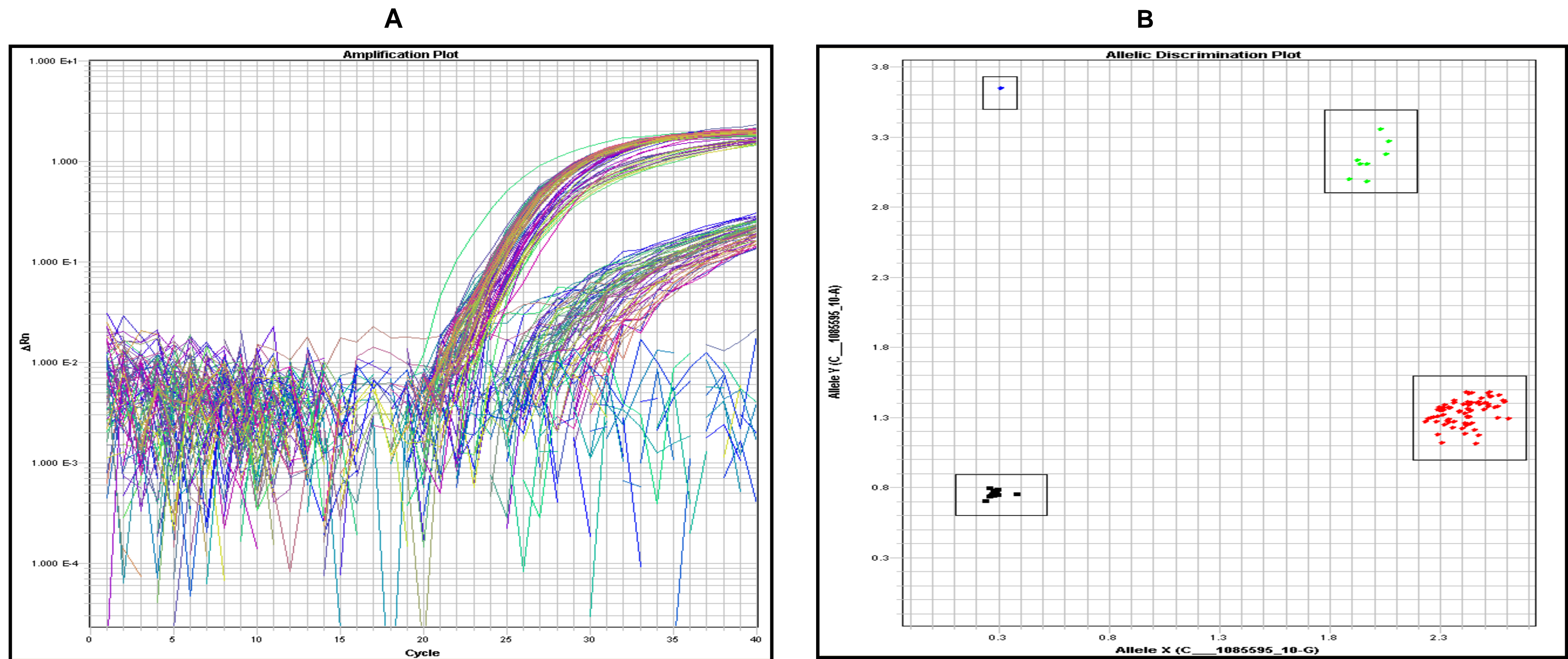

Figure 3.5: **A**) Amplification plot obtained using the ABI™ *Taq*Man® assay for HFE (C282Y). **B**) Representation of an allelic discrimination analysis using ABI™ *Taq*Man® assay for HFE (C282Y) [Allele Y (C_1005595_10-A) vs Allele X (C_1005595_10-G)]. Blue dots = homozygous (AA), red dots = Wild-type (GG), green dots = heterozygous (AG), black dots = NTCs (controls for the assay).

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3.2. **Genotype distribution and allele frequency comparison of TMPRSS6 A736V and HFE C282Y SNP between MS patients and controls from the same population group**

Genotype distribution of TMPRSS6 A736V as observed in 407 MS patients and controls was achieved by ABI™ TaqMan® TMPRSS6 rs855791 genotyping assay is shown in Figure 3.6. No significant difference in TMPRSS6 A736V distribution was found between MS and control population (p = 0.419).

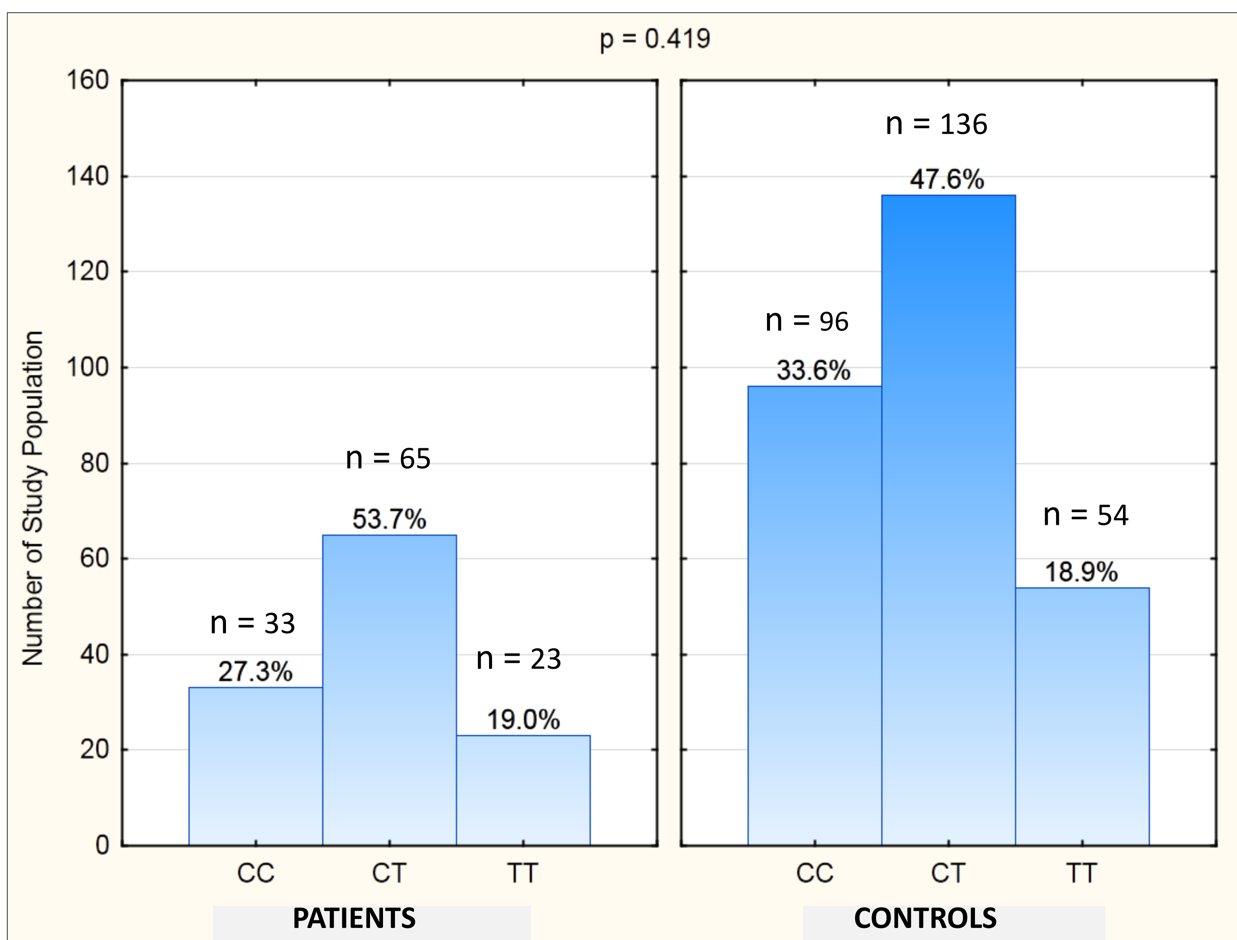

Figure 3.6. Comparison of genotype distribution of TMPRSS6 A736V between 121 MS patients and 286 controls: genotypes present as Wild Type (CC), heterozygous (CT) and homozygous (TT).

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Determination of the genotype distribution of HFE C282Y observed in 306 MS patients and controls was achieved by ABI™ TaqMan® HFE rs1800562 genotyping, shown in Fig. 3.7. We found no significant difference in the HFE C282Y genotype distribution between MS patients and the control group (p = 0.706). Due to the small number of HFE homozygotes (AA) these individuals (2 patients and 3 controls) were excluded from subsequent genotype-phenotype association studies. This was considered appropriate due to the autosomal recessive inheritance pattern of the type of haemochromatosis characterised by this genotype, while the effect on iron loading in heterozygotes depends on gene-gene and gene-environment interaction similar the low-penetrance TMPRSS6 A736V mutation/SNP.

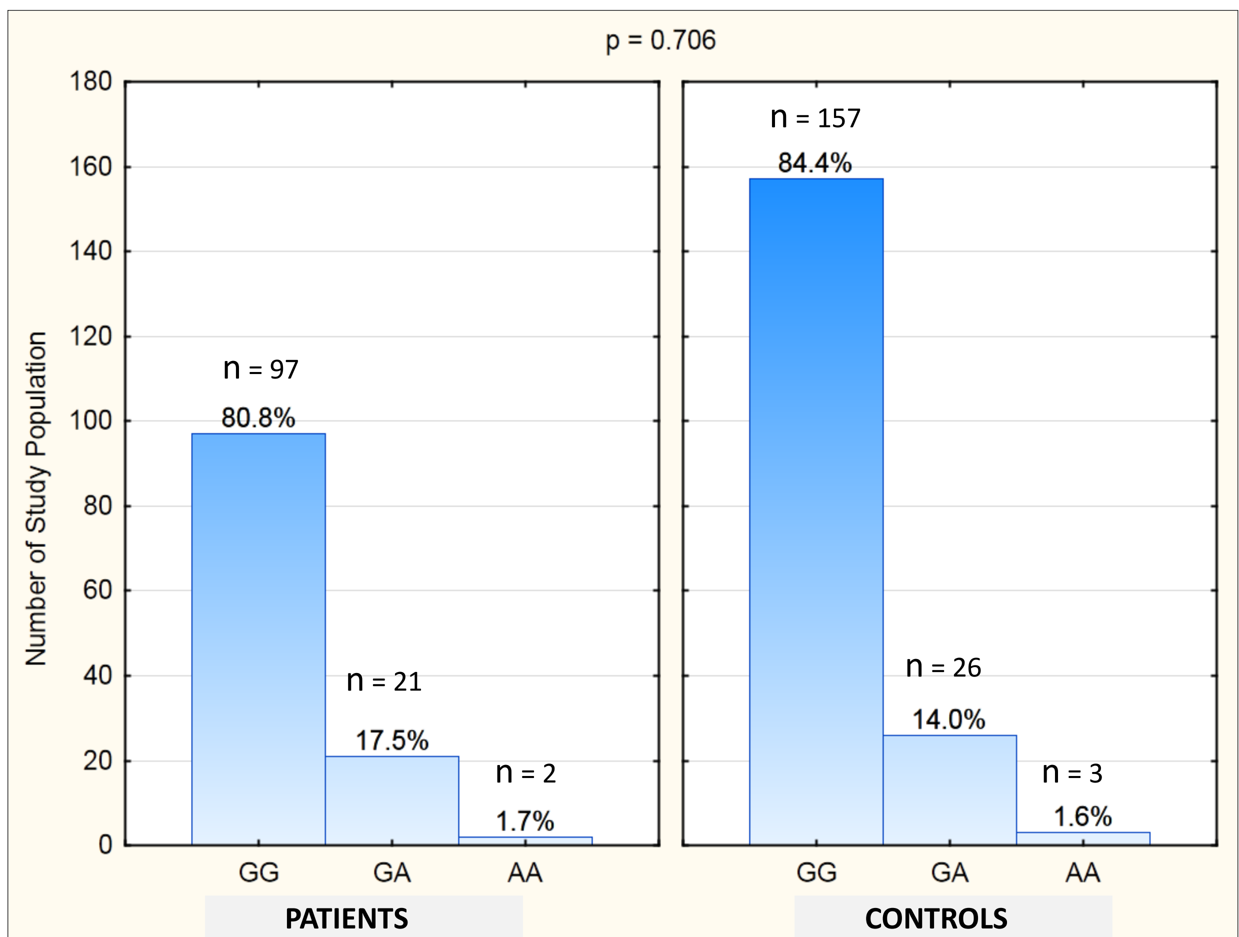

Figure 3.7. Comparison of genotype distribution of HFE c.845 G>A (C282Y) between 120 MS patients and 186 control subjects: genotypes present as Wild Type (GG), heterozygous (GA) homozygous (AA).

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In both the patient and control groups the polymorphisms analysed were in Hardy-Weinberg equilibrium (HWE): TMPRSS6 c.2207 C>T (HWE: p = 0.368 in MS patients and p = 0.635 in controls) and HFE c.845 G>A (HWE: p = 0.494 in MS patients and p = 0.129 in controls). Genotype distribution did not differ significantly between MS patients and controls (Fig. 3.6 and 3.7), with p-values of 0.419 and 0.706 for TMPRSS6 c.2207 C>T and HFE c.845 G>A, respectively. Notably, genotyping results for the TMPRSS6 A736V are shown here for the first time in a South African population - the distribution of the risk-associated T-allele was 45 % in MS patients and 43 % in controls. Similarly, distribution of the HFE c.845 G>A risk-associated A-allele was not significantly different between MS patients and controls, with frequencies of 10 % and 9 %, respectively.

3.3. Genotype-phenotype association

Clinical characteristics

The baseline clinical and biochemical characteristics of 121 Caucasian MS patients and 286 Caucasian controls are compared in Table 3.2. Due to an increased prevalence of both MS and iron deficiency in females, a gender-based comparison was also performed in both groups, which confirmed a significant difference in serum iron parameters between males and females. A significant difference in mean age was noted between MS patients and controls (p=0.002). Therefore, comparison of the biochemical parameters were repeated in 59 controls within the same age group as the MS patients to exclude the possibility that any differences detected could be ascribed to older age of the control group. MS patients were more likely to be current smokers (p=0.04) and ate less red meat as part of their diet (p=0.03) when compared to controls. Heritability of MS was confirmed by a significantly higher family history of this condition in MS patients compared with controls (p=0.04).

Table 3.2. Baseline clinical and biochemical characteristics of MS patients compared to controls including gender-based comparison in both study groups. The number of samples analysed in each group is provided in brackets.

	MS Patients (n=121) Means			**Controls (n=286) Means**			
Gender	Female (105)	Male (16)	Gender comparison (P-value)	Female (202)	Male (84)	Gender comparison (P-value)	Patients vs controls (P-value)
Age (years)	44.95	44.00	0.85	53.53	52.24	0.59	**0.002**

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	MS Patients (n=121) Means			Controls (n=286) Means			
Gender	Female (105)	Male (16)	Gender comparison (P-value)	Female (202)	Male (84)	Gender comparison (P-value)	Patients vs controls (P-value)
	(104)	(16)		(201)	(83)		
Family history of MS (yes)	4	0		1	1		**0.04**
Clinical indicators							
Body Mass Index (kg/m^2)	25.65(57)	26.06 (5)	0.88	26.74(196)	26.44 (78)	0.71	0.61
Age of Symptom Onset (years)	34.4 (88)	36.07(15)	0.62	N/A	N/A	N/A	N/A
Age of Diagnosis (years)	37.69(83)	39.93(14)	0.48	N/A	N/A	N/A	N/A
EDSS score	3.78 (43)	4.30 (5)	0.58	N/A	N/A	N/A	N/A
Biochemical indicators							
Serum iron (µmol/L)	16.90(89)	18.94(15)	0.31	16.1 (39)	20.5 (20)	**0.02**	0.77
Serum transferrin (g/L)	2.65 (88)	2.56 (15)	0.52	2.71 (39)	2.51 (20)	**0.005**	0.94
Transferrin saturation (%)	26.43(91)	29.65(15)	0.38	24.72 (79)	33.47 (35)	**0.0002**	0.62
Serum ferritin (µg/L)	89.21 (105)	250.93 (15)	**0.01**	105.63 (131)	274.67 (55)	**<0.0001**	0.57
Lifestyle habits							
≥5 fruit and/vegetables daily (score)	4.77 (61)	5.00 (7)	0.79	4.61 (102)	4.76 (41)	0.71	0.67
Red meat intake (score)	1.85 (61)	1.86 (7)	0.99	2.38 (102)	2.85 (41)	0.10	**0.03**
Current cigarette smoker (yes)	13	3		18	11		**0.04**
Alcohol intake (score)	0.77 (57)	1.29 (7)	0.14	1.26 (102)	1.50 (40)	0.15	0.07
Physical activity (score)	1.90 (61)	2.29 (7)	0.40	2.41 (103)	2.68 (41)	0.19	0.07

Abbreviations: MS, multiple sclerosis. For the 48 MS patients from a previous study (Kotze *et al.* 2001) the lifestyle factors were not documented at the time of sample collection.

The influence of non-genetic factors on iron parameters in MS patients and controls are shown in Table 3.3 for study participants where these were documented at entry into the study. Significant correlation between red meat intake and physical activity levels with serum ferritin and transferrin saturation were observed in controls (p≤0.01), but not in the MS patients (p>0.05), while BMI was associated with several iron parameters in both MS patients and controls. These modifiable lifestyle factors were therefore also considered as potential confounders during subsequent statistical analysis, where appropriate.

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Table 3.3. P-values for Spearman rank-correlations determined to evaluate the relationship between non-genetic factors and iron parameters as assessed in a subset of 68 MS patients and 143 controls.

	MS patients				**Controls**			
	Serum iron	**Serum transferrin**	**Transferrin saturation**	**Serum ferritin**	**Serum iron**	**Serum transferrin**	**Transferrin saturation**	**Serum ferritin**
BMI	**0.02**	0.09	**<0.01**	0.73	**0.02**	0.32	**0.02**	**<0.01**
Dietary red meat intake	0.32	0.69	0.45	0.21	0.84	0.32	0.69	**0.01**
Weekly alcohol intake	0.47	0.96	0.61	0.48	0.90	0.73	0.97	0.42
Physical activity level	1.00	0.95	0.86	0.20	0.24	0.72	**<0.01**	0.71

Abbreviation: BMI, body mass index

Genetic analysis

The genotypic distribution and allele frequencies of TMPRSS6 A736V (Figure 3.6) and HFE C282Y (Figure 3.7) did not differ significantly between patients and controls. There was also no significant difference in the combined genotype distribution of these mutations between patients and controls (data not shown). Due to the small number of individuals with the genotype combination TMPRSS6 CC/HFE GA, these 4 patients and 7 controls were excluded in Figure 3.8, showing the study groups used for the genotype-phenotype association study presented in Figure 3.9. The combined effect of TMPRSS6 A736V and HFE C282Y is shown in relation to serum ferritin levels, demonstrating a significantly lower concentration in MS patients with the TMPRSS6 CT/HFE GA and TMPRSS6 TT/HFE GA genotype combinations compared with the other genotype combinations ($P<0.01$). This significant reduction in log-ferritin was only observed in MS patients ($p=0.03$) but not controls ($p>0.05$), and remained significant after adjustment for age ($p=0.01$), BMI ($p=0.04$), and dietary red meat intake ($p=0.04$). This significant effect was also confirmed in females only, which excluded sex as a possible confounder.

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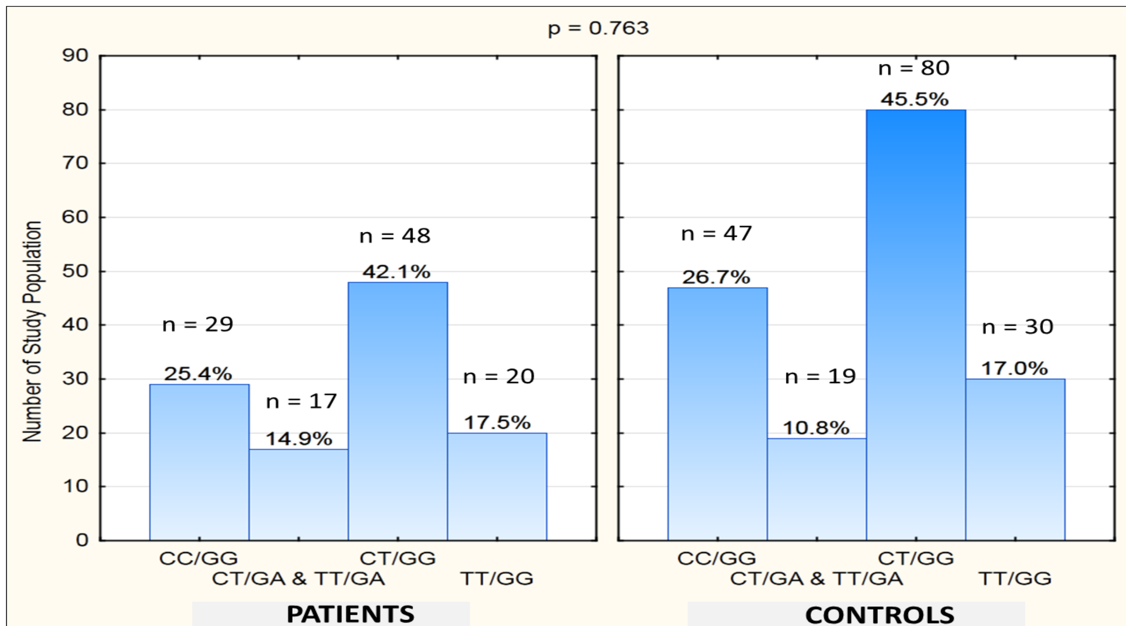

Figure 3.8 Distribution of TMPRSS6 A736V / HFE C282Y combined genotypes determined in 114 MS patients and 176 controls.

As shown in Figure 3.9 the TMPRSS6 CT/HFE GA and TMPRSS6 TT/HFE GA genotype combinations were associated with a significant decrease in log-ferritin level when compared to wild-type TMPRSS6 CC homozygotes for MS patients but not controls (p=0.027). The initial question which prompted the investigation of the combined effects of the TMPRSS6 and HFE SNPs was apparently reduced penetrance of HFE C282Y in MS patients (Kotze et al. 2006). In this study it was found that in the presence of the TMPRSS6 T-allele (CT/TT genotype) in patients carrying the risk-associated A-allele of the HFE C282Y mutation (which is more likely expected to result in increased ferritin), ferritin levels were reduced.

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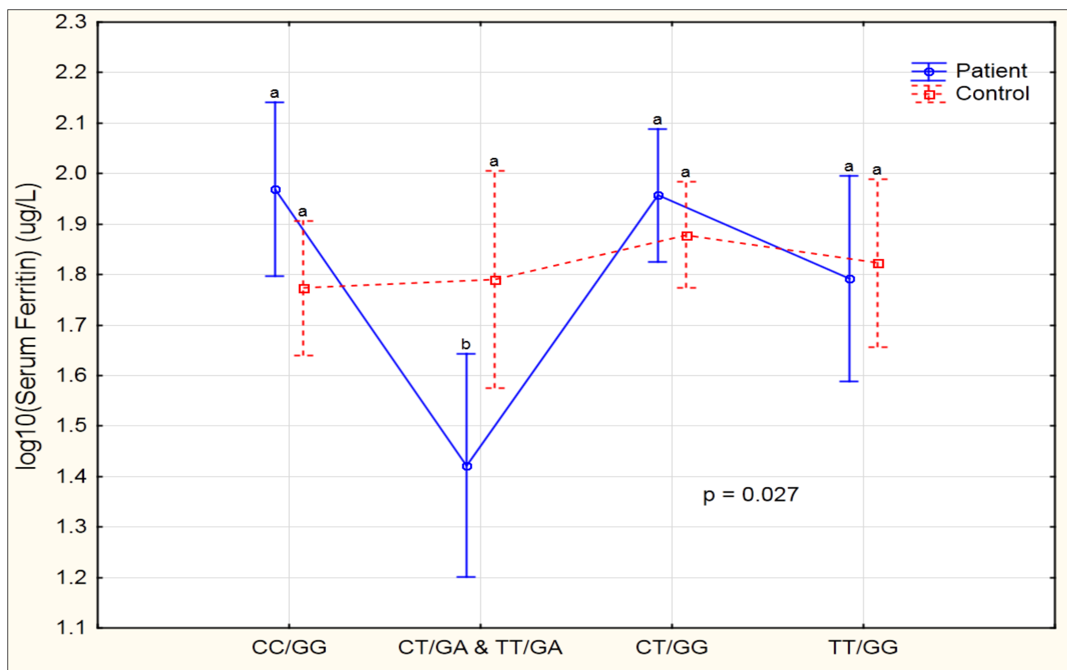

Figure 3.9. Effect of combined TMPRSS6 and HFE genotypes on log-ferritin levels in MS patients compared with controls. The a and b labels indicates post-hoc test differences between genotypes.

The same iron lowering trend was also seen for transferrin saturation in TMPRSS6 TT/HFE GG (21.45 %; 95% CI 16.24-26.6) vs the TMPRSS6 CC/HFE GG wild type genotype combination (29.26 %; 95% CI 24.66-33.88. This association became significant when the LSD Post Hoc Test was applied ($p = 0.028$).

HFE C282Y is expected to increase serum ferritin and transferrin saturation levels (Allen et al. 2008), rather than reducing it as found for ferritin in MS patients in this study. In the HFE C282Y heterozygous MS patients serum iron and transferrin saturation were not increased compared to the wildtype GG genotype, while serum ferritin was significantly lower in GA heterozygous MS patients (44.6 µg/L; 95% CI 27.9-61.3) than in MS patients with the GG wildtype genotype of HFE C282Y (119.8 µg/L; 95% CI 99.7-139.9; $p < 0.01$). In this comparison the HFE C282Y homozygotes (AA genotype) were excluded due to small sample size (2 patients only) and association of this genotype with the most common form of hereditary haemochromatosis showing an autosomal recessive inheritance pattern as confirmed in South African patients (de Villiers et al. 1999) and many other studies worldwide.

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Similar to the gene effect in the general population reported in the literature and our control samples (data not shown), serum ferritin was lower in patients with the TT genotype of TMPRSS6 A736V (86.6 µg/L; 95% CI 53.9-119.4), but not significantly different from the total group of MS patients with the CC wild type genotype (122.2 µg/L; 95% CI 82.3-162.1). Similarly, the serum iron concentration of MS patients with the TT genotype of TMPRSS6 A736V was lower (14.28 µmol/L, 95% CI 11.95-16.60 µmoles/L) than the serum iron concentration of patients with the wild-type CC genotype (18.59 µmol/L, 95% CI 16.04-21.14 µmoles/L), at a significance of p = 0.081 for the trend, as calculated by the Kruskal-Wallis test. This difference between the CC and TT genotypes of TMPRSS6 A736V became significant when the p value was calculated by the LSD Post Hoc Test (p=0.029). When the female patients (n=89) were considered separately to exclude the effect of gender on serum iron levels, the Post Hoc Test remained significant for the difference between TT and CC. The percentage transferrin saturation of patients with the TT genotype of TMPRSS6 A736V was also lower (22.4 % 95% CI 18.4-26.4 %) than that of patients with the wild-type CC genotype (29.7 %, 95% CI 25.1 – 34.2 %), at p = 0.102, as calculated by the Kruskal-Wallis test. The difference between the CC and TT genotypes of TMPRSS6 A736V became significant when the p- value was calculated by the LSD Post Hoc (p = 0.034). When determined in female patients only, although the patients with the TT variant still had lower transferrin saturation than those with the CC genotype, this difference was no longer significant. When the serum iron concentrations in MS patients were investigated differentially, a trend was found that patients with the TMPRSS6 TT/HFE GG genotype combination had lower serum iron concentrations (14.3 µmol/L; 95% CI 11.3-17.3) than patients with both the TMPRSS6 and HFE wild type CC/GG genotype combination (18.2 µmol/L; 95% CI 15.6-20.9; p = 0.28). This became marginally significant when the LSD Post Hoc Test was applied (p = 0.05), possibly indicating that the iron-lowering effect of TMPRSS6 A736V (C>T) is stronger than the iron-increasing effect of HFE C282Y (G>A) in MS patients.

A non-significant trend towards an earlier age of symptom presentation was noted in 103 MS patients homozygous for the risk-associated T-allele of TMPRSS6 A736V or heterozygous for the HFE C282Y (data not shown). The influences of combined TMPRSS6 A736V and HFE C282Y genotypes on age of onset and degree of MS disability are summarized in Table 3.4 in a subset of MS patients who provided this information at entry into the study. When considered together, MS patients with the TMPRSS6 CT/HFE GA and TMPRSS6 TT/HFE GA genotype combinations had a significantly lower age of symptom onset (30 years, 95% CI 24 – 37 years) than patients the wild type TMPRSS6 CC/HFE GG genotype combination

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(39 years 95% CI 34 – 44 years); p = 0.04 after applying the LSD post hoc test. A non-significant trend-association was also found between decreased ferritin levels and an earlier age of symptom onset (p=0.08) after exclusion of the two HFE AA homozygotes.

Table 3.4. Influence of TMPRSS6 A736V and HFE C282Y genotypes on age of onset and degree of MS disability in MS patients.

(n)	**MS patients**				**P-value**	**Post-hoc CC/GG vs. CT/GA & TT/GA**
	CC/GG	**CT/GA & TT/GA**	**CT/GG**	**TT/GG**		
Age of symptom onset (years) (103)	38.78	30.17	34.16	33.17	0.177	**0.040**
Age of diagnosis (years) (97)	41.27	38.85	36.51	36.69	0.373	0.519
EDSS (score) (48)	4.33	4.88	3.37	3.25	0.224	0.577

Abbreviation: EDSS; expanded disability status scale

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CHAPTER 4

DISCUSSION AND CONCLUSION

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The focus of the present study was to evaluate combined effects of the low-penetrance TMPRSS6 A736V and HFE C282Y variants with opposite effects on iron metabolism in patients with MS, in an attempt to clarify the role of iron dysregulation in the disease process. The study population included 121 Caucasian MS patients and 286 population-matched controls. In order to exclude race as a confounder, 19 non-Caucasian individuals were excluded from the initial MS study cohort. Subgroup analyses were performed in relation to variables where full data sets were not available for all individuals. Serum iron, transferrin, ferritin and transferrin saturation levels were available from previous studies and lifestyle factors were subsequently documented in a subgroup of 68 MS patients and 143 controls using the study questionnaire. Genotyping of TMPRSS6 A736V and HFE C282Y were performed using allele-specific TaqMan technology after successful optimisation of this high throughput genotyping method in our laboratory.

When genetic tests are performed for variants with low penetrance, such as TMPRSS6 A736V and HFE C282Y, it is imperative that the tests or genetic markers are clinically validated and also comply with clinical utility and laboratory validation or optimisation requirements. Clinical validity refers to how well the test results detect or predict an association with the phenotype or disease, while clinical utility refers to the ability to prevent disease or administer therapies to remove or reduce the risk of the associated phenotype or disease. These were important considerations for justification of this study as neurologists are increasingly being made aware that they need to test for iron deficiency at diagnosis of MS. However, mostly only haemoglobin, and infrequently, ferritin determinations are requested. Therefore, application of a PSGT strategy aimed at early detection of a genetic predisposition for iron deficiency in a subgroup of MS patients (Kotze et al. 2009; van Rensburg et al. 2012) may provide an added incentive for clinicians to determine the need for iron supplementation in MS patients. Correcting iron deficiency at first disease presentation and monitoring of iron parameters over time may prevent future relapses and prevent disease progression, as evidenced in children with MS (van Toorn et al. 2010).

MS is a severely debilitating and progressive neurological disorder associated with significant morbidity and early mortality worldwide. None of the DMDs for MS has to date obtained licensed approval for improving or reducing disability in MS (Tremlett et al. 2012). Based on these observations, there currently exists a growing need to identify genetic risk factors which may be triggered by environmental exposures or lifestyle habits that could aid the development of diagnostic or therapeutic algorithms which could potentially decrease symptom severity and increase quality of life in MS patients. The genotype and allele

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frequencies of TMPRSS6 A736V are described here for the first time in South African MS patients and unaffected controls, while the prevalence of HFE C282Y has been described previously (de Villiers et al. 1999; Kotze et al. 2006) and confirmed in this study. TMPRSS6 A736V is common in the general population, with previous studies indicating a prevalence of 45% for the risk-associated T-allele (Nai et al. 2011). This correlates with the estimated 45% minor allele frequency of TMPRSS6 A736V in MS patients and 43% in the South African Caucasian controls studied, with genotype distributions also found to be similar in both groups.

Multiple previous studies have demonstrated and subsequently confirmed associations between the minor allele of TMPRSS6 A736V and reduced iron parameters, ineffective erythropoiesis and an increased risk of developing iron deficiency (Benyamin et al. 2009; Tanaka et al. 2010; An et al. 2012). These effects are particularly relevant in light of the significant influence of iron regulation on remyelination capacity in MS and its relationship with disease and disability progression (Rooney et al. 1999; van Rensburg et al. 2006; van Rensburg et al. 2012). Significant correlations between homozygosity for the minor allele (TT genotype) of TMPRSS6 A736V and reduced iron levels and transferrin saturation are therefore in accordance with these previous results. Associations determined between this variant and a trend for earlier age of symptom presentation, which became significant in the presence of HFE C282Y ($p<0.04$), are also indicative of an accelerated disease process underlying earlier onset and clinical detection of MS in this genetic sub-group of patients. These findings also correlate with previous studies which suggest that reduced iron levels correlate with earlier clinical onset in MS (Van Rensburg et al. 2006).

The results of the present study confirmed that TMPRSS6 A736V is associated with decreased serum concentrations of iron and transferrin saturation in MS patients and controls, similar to previous findings in the general population. Unexpectedly, however, genetic variation in the HFE gene produced a significant decrease in serum ferritin, only in the MS patients but not in the controls. HFE C282Y is usually associated with increased ferritin, while this gene effect appears to be modified in MS patients (Kotze et al. 2006). About 87% of patients who have hemochromatosis with high transferrin saturation and high serum ferritin have genetic variations in the HFE gene, while being homozygous for HFE C282Y was shown to have a greater effect towards documented iron-overload-related disease in men (28.4%) than in women (1.2%) (Allen et al. 2008). Since this genotype is associated with an autosomal recessive type of haemochromatosis that may cause organ damage, if left untreated in patients diagnosed with this condition, individuals with two copies

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of the faulty gene were excluded from comparative studies involving serum iron parameters in relation to TMPRSS6 A736V. Although this SNP is associated with iron deficiency in the general population it has not been associated with a specific clinical diagnosis of disease similar to the wide spectrum of clinical manifestation associated with homozygosity for HFE C282Y in the presence of high iron stores (due to gene-gene and gene-environment interaction).

In the present study we found that both the TT genotype of TMPRSS6 A736V and the GA genotype of HFE C282Y are associated with earlier age of symptom onset in MS, and that when these two variants were simultaneously present, the effect became statistically significant (p=0.04). An explanation of these effects would have to be sought by considering the individual effects of the two variants. Although variation in the HFE and TMPRSS6 genes affect expression of hepcidin, the major iron regulatory protein, association of common variants in these genes with iron parameters may also occur independent of serum hepcidin in the general population (Galesloot et al. 2013). The results presented in the present study indicate that in combination, the minor alleles of TMPRSS6 A736V and C282Y are associated with decreased iron concentrations in MS patients; TMPRSS6 possibly by partly blocking iron absorption and HFE unexpectedly decreasing ferritin levels by an unknown mechanism that may involve the inflammatory response. All other iron parameters analysed in MS patients, including serum iron and transferrin saturation, were increased in MS patients with the HFE C282Y mutation as would be expected (data not shown), although these differences did not reach statistical significance.

Based on our findings it may be speculated that HFE C282Y may exert its effect in MS patients on the synthesis of serum ferritin, which is primarily derived from macrophages (Cohen et al. 2010). Drakesmith et al. (2005) showed that the expression of HFE (a MHC Ib protein) in macrophages may be downregulated by a protein associated with the HIV retrovirus. The association between HFE and inflammation has previously been highlighted (Kotze et al. 2003, 2006), while a connection between retroviral infection in MS has been shown as well in South African MS patients (de Villiers et al. 2006). Perron et al. (2012) detected antigen to the ENV protein of the Human Endogenous Retroviral family 'W' (HERV-W) in peripheral blood mononuclear cells (PBMCs) of 73% of patients with MS, but not in chronic infection, systemic lupus, most other neurological diseases and healthy donors. HERV-W was also present in macrophages within MS brain lesions and in macrophages in the vicinity of cerebral blood vessels. Whether HERV-W interacts with HFE to exert an effect on ferritin production in these macrophages as well remains to be established. Ferritin

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donates iron to myelin-producing oligodendrocytes (Hulet et al. 2000; Todorich et al. 2011); therefore it may be hypothesized that a decrease in ferritin would impact myelin production.

The combined effect of the HFE and TMPRSS6 genotypes on serum ferritin is of particular interest as previous studies have shown that South African MS patients with genetic HH i.e. C282Y homozygotes presenting with elevated transferrin saturation and ferritin levels, do not manifest organ damage such as hepatic dysfunction characteristic of the disease, as well as showing fewer clinical manifestations (Kotze et al. 2006). These results led us to speculate that unknown genetic variations implicated in iron, inflammatory and/or immune dysregulation might affect the penetrance of genetic HH or disease expression of MS separate from known environmental risk factors (Kotze et al. 2006). Subsequently, it was discovered that deleterious mutations in the TMPRSS6 gene cause iron-refractory iron deficiency anaemia (IRIDA) (Finberg et al. 2008). While it is possible that this may relate to increased expression of pro-inflammatory cytokines in MS, further evaluation thereof was beyond the scope of the current study.

In light of the iron-lowering effect of the minor T-allele of TMPRSS6 A736V despite co-inheritance of the HFE G>A mutation, it may be hypothesized that the interaction between these genes could at least in part explain the previously noted lack of clinic manifestation of HH commonly observed in MS patients as reported by Kotze et al. (2006). The index case in this study was diagnosed with both MS and haemochromatosis, but showed a lack of clinical manifestation of inherited iron overload. In the present study it was found that this patient is homozygous for the TMPRSS6 polymorphism (data not shown).

Epistatic gene-gene interactions are increasingly recognised as primary drivers of the phenotypic expression of chronic, non-communicable diseases (NCD) such as MS and haemochromatosis. Despite the promise that new developments towards routine application of whole exome/genome sequencing techniques could overcome the limitations of targeted SNP genotyping (Kotze and Gamieldien 2013), the clinical relevance of the combined effect of TMPRSS6 A736V and HFE C282Y may have remained undetected without the pre-existing knowledge driven PSGT approach applied in this study (van Rensburg et al. 2012). This concept has been developed over the course of the preceding decade (Kotze et al. 2013) after careful consideration of ethical issues related to potential genetic discrimination (Kotze et al. 2004). The PSGT concept was developed based on our experience in patients suspected to have HH, which provides a fitting example of the use of genetics in a complex multifactorial disease in general (Kotze et al. 2009). By considering HFE genotyping in

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relation to iron parameters to confirm the diagnosis and aid the formulation of tailored management strategies, HFE genotyping translated into a rapid genetic test applied routinely in South Africa from 1999 (de Villiers et al. 1999). While implementation of HFE genotyping resulted in significant reduction of invasive liver biopsies during clinical work-up towards a diagnosis of HH, it remains unknown to what extent personalized genomics for other iron-related conditions could benefit existing management strategies either in the general population or MS patients with iron deficiency. A greater understanding of gene-gene and gene-environment interaction relevant to both MS and HH could benefit our understanding of iron regulatory pathways and may lead to the development of novel therapies for these conditions in future.

In the present study patients with MS displayed lower dietary intake of red meat compared to controls, which may impact on availability of iron in the diet, particularly in a subset of patients with increased requirements due to a genetic predisposition for iron deficiency. While genetic anaemia may be caused by a variety of missense mutations in the TMPRSS6 gene (De Falco et al. 2013), it is well known that many other pathological or environmental factors could contribute towards treatment-resistant IDA, including malabsorpsion states and gastrointestinal tumours. It is interesting to note that TMPRSS6 also plays a role in a microcytic anaemia associated with progressive iron overload and hepcidin deficiency, i.e. beta thalassemia intermedia (Guo et al. 2013). It cannot therefore be assumed that all cases of IDA resistant to oral iron supplementation are due to high impact mutations or functional SNPs in the TMPRSS6 gene. It also remains unknown how many patients with treatment-resistant IDA will have the low-penetrance mutation in the TMPRSS6 gene investigated here, since at least 40 mutations have been described to date in this gene (De Falco et al. 2013). The SNP studied here contributes significantly to iron deficiency in the general population, while complete disruption of TMPRSS6 has a dominant effect over that of HFE on the regulation of iron metabolism (Finberg et al. 2011, Valenti et al. 2012) that may be of more relevance in a family context.

Results presented in this study confirmed the serum iron and transferrin saturation lowering effect of TMPRSS6 A736V in the South African Caucasian population. Interaction between this functional polymorphism and HFE C282Y may account for the significant ferritin-lowering effect observed in MS patients and could at least partly explain the low penetrance and lack of phenotypic expression of genetic HH previously observed in MS patients (Kotze et al. 2006). Just as PSGT has shown considerable advantages in the formulation of a cost-effective and timely therapeutic strategy to HH, it seems likely that consideration of the

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TMPRSS6 and HFE genotypes in relation to serum iron parameters and diet could help stratify MS patients with regards to the need and predictive response to iron supplementation to limit disability progression and improve disease outcomes in these individuals, with potential applications for iron deficiency in the general population in future. Higher dietary red meat intake correlated significantly with increased ferritin only in controls ($p=0.01$ vs. 0.21 for MS patients). This result may be related to the significantly lower intake of red meat in MS patients compared with controls ($p=0.03$) and/or reflect a different response to dietary iron intake in MS patients due to the genetic background. The potential benefit of iron supplementation in MS patients with an apparent iron deficiency subtype of MS has recently been substantiated by exome sequencing (Jalali et al. 2012; Kotze and Gamieldien 2013) in the index case described by Rooney et al. (1999) as well as the children with MS studied by van Toorn et al. 2010). Various high-impact deleterious mutations involved in iron regulation and homeostasis were identified in these MS patients in addition to variation in the TMPRSS6 gene.

The above-mentioned results were obtained despite several limitations, which can be summarised as follows: 1) biochemical testing of iron parameters was not performed in the same laboratory for all samples as these were available from previous studies performed in different laboratories; 2) only two iron-related genes were investigated in this study, which could underestimate the risk of combined effects exerted by multiple functional polymorphisms; and 3) determining MS onset by definitive diagnosis or onset of first symptoms may be affected by various factors and therefore rigorous case definitions and consideration of differences between clinical subtypes are recommended for future studies.

We conclude that the results obtained in this study confirm that genotype associations cannot always predict biochemical phenotypes; therefore measurement of iron parameters in MS patients was important to determine the mechanism underlying the link between iron-related genes and earlier age of onset of MS. Gemmati et al. (2012) reported an association between disability progression rates and variation in the HFE gene in MS patients, leading them to speculate that iron overload may be involved in accelerated disease progression. However, these authors did not present data on serum iron parameters which is a limitation as the present study indicated that there may paradoxically be an inverse effect on iron status in MS patients with the HFE C282Y mutation. This mutation was found to be associated with earlier development of MS in this study when ferritin levels are reduced possibly as a consequence of co-inheritance with TMPRSS6 A736V in a subgroup of MS patients with a compromised immune system. The clinical usefulness of the PSGT concept

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for MS (van Rensburg et al. 2012) was confirmed as it would not have been possible to explain the results obtained in this study without the application of a multidisciplinary approach.

When a low penetrance mutation / functional polymorphism such as TMPRSS6 A736V or HFE C282Y are detected in a DNA sample, it does not mean that the individual will necessarily develop the associated disease. The individual may however be vulnerable to environmental triggers that can interact with the genetic risk factor and together contribute to disease development. It is therefore important to know whether environmental factors are involved in disease expression prior to genetic testing and to understand the mechanisms of action as a consequence of gene-gene and gene-environment interactions considered in this study in relation to iron metabolism. Although prolonged periods of exposure could worsen the severity of disease in genetically compromised individuals, most environmental risk factors can be modified or avoided. Genetic testing therefore provides an opportunity for early awareness and guidance, since it is only when people are well informed that they are able to take proper preventive measures or receive targeted treatment tailored to individual needs.

In summary, our results may indicate an epistatic effect between TMPRSS6 A736V and HFE C282Y associated with reduced ferritin levels in MS patients. This finding confirms the role of TMPRSS6 A736V as a genetic modifier of hereditary haemochromatosis, correlating with a lack of clinical manifestation of HFE C282Y in a subgroup of MS patients. It also highlights the value of pathology-supported genetic testing as a new concept that is well placed to optimise clinical benefit in patients with iron-related disorders.

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CHAPTER 5

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APPENDIX A

Expanded Disability Status Scale (EDSS)

AND

Functuctional System Scores (FSS)

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KURTZKE EXPANDED DISABILITY STATUS SCALE (EDSS) AND FUNCTIONAL SYSTEMS SCORES (FSS)

Patient……………………………………………………………………………………..
Date…………………………………
Examiner………………………………………………………………………….
Final score………………………………………

The Kurtzke Expanded Disability Status Scale (EDSS)

Disability score	Neurological status
□ 0.0	Normal neurological examination (all grade 0 in all Functional System (FS) scores) (Excludes cerebral function grade 1)
□ 1.0	No disability, minimal signs in one functional score (FS∗) (i.e. grade 1)
□ 1.5	No disability, minimal signs in more than one FS∗ (more than 1 FS grade 1)
□ 2.0	Minimal disability in one FS (one FS grade 2, others 0 or 1)
□ 2.5	Minimal disability in two FS (two FS grade 2, others 0 or 1)
□ 3.0	Moderate disability in one FS (one FS grade 3, others 1 or 0), or mild disability in three or four FS (three or four FS grade 2, others 0 or 1) though fully ambulatory
□ 3.5	Fully ambulatory but with moderate disability in one FS (one grade 3) and one or two FS grade 2, or two FS grade 3 (others 0 or 1) or five grade 2 (others 0 or 1)
□ 4.0	Fully ambulatory without aid, self-sufficient, up and about some 12 hours a day despite relatively severe disability consisting of one FS grade 4 (others 0 or 1), or combination of lesser grades exceeding limits of previous steps; able to walk without aid or rest some 500 meters
□ 4.5	Fully ambulatory without aid, up and about much of the day, able to work a full day, may otherwise have some limitation of full activity or require minimal assistance; characterized by relatively severe disability usually consisting of one FS grade 4 (others or 1) or combinations of lesser grades exceeding limits of previous steps; able to walk without aid or rest some 300 meters
□ 5.0	Ambulatory without aid or rest for about 200 meters; disability severe enough to impair full daily activities (e.g. to work a full day without special provisions): (Usual FS equivalents are one grade 5 alone, others 0 or 1; or combinations of lesser grades usually exceeding specifications for step 4.0)

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- ☐ 5.5 Ambulatory without aid for about 100 meters; disability severe enough to preclude full daily activities (Usual FS equivalents are one grade 5 alone, others 0 or 1; or combination of lesser grades usually exceeding those for step 4.0)
- ☐ 6.0 Intermittent or unilateral constant assistance (cane, crutch, brace) required to walk about 100 meters with or without resting; (Usual FS equivalents are combinations with more than two FS grade 3+)
- ☐ 6.5 Constant bilateral assistance (canes, crutches, braces) required to walk about 20 meters without resting; (Usual FS equivalents are combinations with more than two FS grade 3+)
- ☐ 7.0 Unable to walk beyond approximately five meters even with aid, essentially restricted to wheelchair; wheels self in standard wheelchair and transfers alone; up and about in wheelchair some 12 hours a day; (Usual FS equivalents are combinations with more than one FS grade 4+; very rarely pyramidal grade 5 alone)
- ☐ 7.5 Unable to take more than a few steps; restricted to wheelchair; may need aid in transfer; wheels self but cannot carry on in standard wheelchair a full day; May require motorized wheelchair; (Usual FS equivalents are combinations with more than one FS grade 4+)
- ☐ 8.0 Essentially restricted to bed or chair or perambulated in wheelchair, but may be out of bed itself much of the day; retains many self-care functions; generally has effective use of arms; (Usual FS equivalents are combinations, generally grade 4+ in several systems)
- ☐ 8.5 Essentially restricted to bed much of day; has some effective use of arm(s), retains some self care functions; (Usual FS equivalents are combinations, generally 4+ in several systems)
- ☐ 9.0 Helpless bed patient; can communicate and eat; (Usual FS equivalents are combinations, mostly grade 4+)
- ☐ 9.5 Totally helpless bed patient; unable to communicate effectively or eat/swallow; (Usual FS equivalents are combinations, almost all grade 4+)
- ☐ 10.0 Death due to MS

Note1: EDSS steps 1.0 to 4.5 refer to patients who are fully ambulatory and the precise step number is defined by the Functional System score(s). EDSS steps 5.0 to 9.5 are defined by the impairment to ambulation and usual equivalents in Functional Systems scores are provided.

Note 2: EDSS should not change by 1.0 step unless there is a change in the same direction of at least one step in at least one FS.

The Functional Systems Scores (FSS)

□ **Pyramidal Function**

0 - Normal

1 - Abnormal signs without disability

2 - Minimal disability

3 - Mild to moderate paraparesis of hemiparesis (detectable weakness but most function sustained for short periods, fatigue a problem); severe monoparesis almost no function)

4 - Marked paraparesis or hemiparesis (function is difficult); moderate quadriparesis (function is decreased but can be sustained for short periods) or monoplegia

5 - Paraplegia, hemiplegia, or marked quadriparesis

6 - Quadriplegia

9 - Unknown

□ **Cerebellar Function**

0 - Normal

1 - Abnormal signs without disability

2 - Mild ataxia (tremor or clumsy movements easily seen, minor interference with function)

3 - Moderate truncal or limb ataxia (tremor or clumsy movements interfere with function in all spheres)

4 - Severe ataxia in all limbs (most function is very difficult)

5 - Unable to perform coordinated movements due to ataxia

9 - Unknown

□ **-** Record no 1 in small box when weakness (grade 3 or worse on pyramidal) interferes with testing

□ **Brainstem Function**

0 - Normal

1 - Signs only

2 - Moderate Nystagmus or other mild disability

3 - Severe Nystagmus, marked extraocular weakness, or moderate disability of other cranial nerves

4 - Marked dysarthria or other marked disability

5 - Inability to speak or swallow

9 - Unknown

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Sensory Function

0 - Normal

1 - Vibration or Figure - writing decrease only in 1 or 2 limbs

2 - Mild decrease in touch or pain or position sense, and/or moderate decrease in vibration in 1 or 2 limbs, or vibratory (c/s Figure writing) decrease alone in 3 or 4 limbs

3 - Moderate decrease in touch or pain or position sense, and/or essentially lost vibration in 1 or 2 limbs; or mild decrease in touch or pain and/or moderate decrease in all proprioceptive tests in 3 or 4 limbs

4 - Marked decrease in touch or pain or loss of proprioception, alone or combined in 1 or 2 limbs; or moderate decrease in touch or pain and/or severe proprioceptive decrease in more than two limbs

5 - Loss (essentially) of sensation in 1 or 2 limbs; or moderate decrease in touch or pain and/or loss of proprioception for most of the body below the head

6 - Sensation essentially lost below the head

9 - Unknown

Bowel and Bladder Function

(Rate on the basis of the worse function, either bowel or bladder)

0 - Normal

1 - Mild urinary hesitancy, urgency, or retention

2 - Moderate hesitancy, urgency, or retention of bowel or bladder, or rare urinary incontinence (intermittent self-catheterization, manual compression to evacuate bladder, or finger evacuation of stool)

3 - Frequent urinary incontinence

4 - In need of almost constant catheterization (and constant use of measures to evacuate stool)

5 - Loss of bladder function

6 - Loss of bowel and bladder function

9 - Unknown

Visual Function

0 - Normal

1 - Scotoma with visual acuity (corrected) better than 20/30

2 - Worse eye with scotoma with maximal visual acuity (corrected) 20/30 to 20/59

3 - Worse eye with large scotoma, or moderate decrease in fields, but with maximal visual acuity (corrected) 20/60 to 20/99

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4 - Worse eye with marked decrease of fields and maximal visual acuity (corrected) of 20/100 to 20/200; grade 3 plus maximal acuity of better eye of 20/60 or less

5 - Worse eye with maximal visual acuity (corrected) less than 20/200; grade 4 plus maximal acuity of better eye of 20/60 or less

6 - Grade 5 plus maximal visual acuity of better eye of 20/60 or less

9 - Unknown

 Cerebral (or mental) Functions

0 - Normal

1 - Mood alteration only (does not affect EDSS score)

2 - Mild decrease in mentation

3 - Moderate decrease in mentation

4 - Marked decrease in mentation (chronic brain syndrome – moderate)

5 - Dementia or chronic brain syndrome – severe or incompetent

9 - Unknown

The Kurtzke Expanded Disability Status Scale (EDSS) is a method of quantifying disability in multiple sclerosis (Kurtzke, 1983). The EDSS quantifies disability in eight Functional Systems (FS) and allows neurologists to assign a Functional System Score (FSS) in each of these. The Functional Systems are: pyramidal, cerebellar, brainstem, sensory, bowel and bladder, visual, and cerebral.

Reference

Kurtzke, J.F. 1983. Rating neurological impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology,* 33:1444-1452.

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APPENDIX B

Guidelines To McDonald Criteria

TABLE: Recommendations List {adapted from the "Revisions to McDonald Diagnostic Criteria for Multiple sclerosis" (Polman et al. 2005)}

Clinical presentation	Additional data needed for MS diagnosis
-Two or more episode of neurological disturbance for which causative lesions are likely to be inflammatory or demyelinating in nature (attacks) -Objective clinical evidence of two or more lesions	No additional tests are recommended
-Two or more attacks -Objective clinical evidence of one lesion	Two or more MRI-detected lesions and positive CSF analysis (detection of oligoclonal bands) OR Dissemination in space demonstrated by MRI: Fulfilling at least three of: • one gadolinium enhanced lesion • one infratenotorial lesion • one juxtacortical lesion • three periventricular lesion OR Await further clinical attack
-One attack -Objective clinical evidence of two or more lesions	Dissemination in space demonstrated by MRI with previous criteria OR Await second clinical attack
-One attack -Objective clinical evidence of one lesion (monosymptomatic presentation; clinically isolated syndrome)	Dissemination in space demonstrated by MRI with previous criteria OR Two or more MRI-detected lesions consistent with positive CSF analysis And Dissemination in space demonstrated by MRI with previous criteria OR Second clinical attack
-Insidious neurological progression	One year disease progression and any two of the following: • positive brain MRI • positive spinal cord MRI (two focal T2 lesions) • positive CSF

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APPENDIX C

Ethics Approval Documents

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19 November 2007

Dr S Janse van Rensburg
Division of Chemical Pathology
Dept of Pathology

Dear Dr Janse van Rensburg

RESEARCH PROJECT: "THE DEVELOPMENT AND COMMERCIALISATION OF A COMPREHENSIVE GENE-BASED, PATHOLOGY SUPPORTED INTERVENTION PROGRAM FOR IMPROVED QUALITY OF LIFE IN PATIENTS DIAGNOSED WITH MULTIPLE SCLEROSIS (MS)"

PROJECT NUMBER : N07/09/203

At a meeting of the Committee for Human Research that was held on 3 October 2007 the above project was approved on condition that further information that was required, be submitted.

This information was supplied and the project was finally approved on 12 November 2007 **for a period of one year from this date**. This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in all further correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

Patients participating in a research project in Tygerberg Hospital will not be treated free of charge as the Provincial Government of the Western Cape does not support research financially.

Due to heavy workload the nursing corps of the Tygerberg Hospital cannot offer comprehensive nursing care in research projects. It may therefore be expected of a research worker to arrange for private nursing care.

Yours faithfully

CJ VAN TONDER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9207 / E-mail: cjvt@sun.ac.za
CJVT/pm

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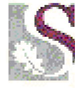

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Ethics Letter

18-Apr-2013

Ethics Reference #: N07/09/203
Title: The development and commercialisation of a comprehensive gene-based, pathology supported intervention program for improved quality of life in patients diagnosed with multiple sclerosos (MS).

Dear Professor Susan Janse Van Rensburg,

At a meeting of the Health Research Ethics Committee that was held on 17 April 2013, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 17 April 2013 Expiry Date: 17 April 2014

If you have any queries or need further help, please contact the REC Office 0219389207.

Sincerely,

REC Coordinator
Mertrude Davids
Health Research Ethics Committee 2

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27 June 2012

MAILED

Dr S Janse van Rensburg
Division of Chemical Pathology
NHLS, Tygerberg Hospital
Francie van Zijl Drive
Tygerberg
7500

Dear Dr Janse van Rensburg

"The development and commercialisation of a comprehensive gene-based,pathology supported intervention program for improved quality of life in patients diagnosed with multiple sclerosis (MS)."

ETHICS REFERENCE NO: N07/09/203

RE : AMENDMENT

Your letter dated 30 May 2012 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee.

The following amendments were approved:
1. Updated and amended protocol and participant informed consent form.
2. Addition of collaborators/ investigators: Dr R van Toorn, Dr F Cronje, Prof C Lochner, Dr J Gamieldien, Ms L Moremi.

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
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27 June 2012 13:18

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