Iron and Multiple Sclerosis

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DECLARATION

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

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SUMMARY

Multiple sclerosis (MS) is a disease that causes neurological dysfunction. Studies attempting to elucidate the role of genes in MS development may aid efforts to control the damage caused by the disease that affects two million people worldwide, e.g. improved diagnosis and treatment. Although the association of MS and genes has not been fully characterized the proposed genetic etiology has been supported by the observed association of MS with the Major Histocompatibility Complex (MHC), haplotype HLA-DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602. Iron, or rather the dysregulation thereof, has also been implicated as a precipitating factor in MS development.

Considering the factors of iron dysregulation and the genes involved in iron regulation, this study aims to identify variation within genes involved in iron metabolism namely the high iron gene (*HFE)*, solute-carrier family 40 (iron regulated transporter) member 1 gene (*SLC40A1*), hepcidin anti-microbial peptide (*HAMP*), cytochrome b reductase 1 (*CYBRD1*) and hemojuvelin (*HJV*). Screening of 40 patients (33 female, seven male; 33 Caucasian, seven Coloured) for each of the five genes was achieved by the Heteroduplex Single-Stranded Conformation Polymorphism (HEX-SSCP) technique. Semi-automated DNA sequencing allowed for verification and characterization of the variants detected. Results included identification of four novel variants present in only the Caucasian patient group, characterized as IVS4-53G→A (*HFE*) (one of 33 patients; 3%), IVS2-65delA (*CYBRD1*) (two of 32 patients; 6.3%), 3'UTR+26delACGTCACGTTTCAAAACTA (*CYBRD1*) (one of 31 patients; 3.2%) and 219delG (*HJV*) (two of 33 patients; 6%). In addition, a total of 15 previously described variants were identified (seven intronic and eight exonic) of which three were also prevalent in only the Caucasian patient group. This study aimed to investigate the differences

between patient and control group variant frequencies, gene-gene interaction and genotype-phenotype relationships. Analysis did not indicate statistically significant associations. However, these investigations were limited because of the small cohort size and lack of control serum iron and ferritin levels.

This pilot study detected variants within each of the five genes that were screened allowing for identification of potential markers and/or contributors to the disease, MS. Although statistical analysis, to elucidate the role of each/all of the variants identified, did not show significance, future studies of a larger cohort may indicate otherwise. This exploration has highlighted the potential role of iron and the iron metabolism related genes in the development of this disease. In doing so it has enriched the limited knowledge of the disease and the development of MS specifically within the South African population. It thus provides insight as to the direction that future genetic studies relating to MS and the role of iron in the development of the disease, should take.

OPSOMMING

Veelvuldige sklerose (VS) is ‘n siekte wat neurologiese disfunksie veroorsaak. Studies wat poog om die rol van gene in die ontwikkeling van VS te wys, mag hulp verleen aan pogings om die skade veroorsaak deur die siekte wat twee miljoen mense wêreldwyd affekteer, te beheer bv. deur verbeterde diagnose en behandeling. Alhoewel die assosiasie met VS en gene nog nie ten volle gekarakteriseer is nie, word die voorgestelde genetiese etiologie ondersteun deur die waargenome assosiasie van VS met die hoof histokombineerbaarheidskompleks, haplotipe HLA-DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602. Yster, of eerder die wanbalans daarvan, is ook geïmpliseer as ‘n presipiterende faktor in VS se ontwikkeling.

Deur in agname van yster-wanbalans en die gene betrokke by yster-regulering, beoog die studie om variasie te identifiseer in gene betrokke by yster-metabolisme, naamlik die hoë yster geen (*HFE*), oplosbare-draer familie 40 (yster gereguleerde vervoerder) lid 1 geen (*SLC40A1*), sitochroom b reduktase 1 (*CYBRD1*), hepsidien anti-mikrobe peptied (*HAMP*) en hemojuvelien (*HJV*). Die toets/sifting van 40 pasiënte (33 vroulik, sewe manlik; 33 Kaukasiër, sewe Kleurling) vir elk van die vyf gene was behaal deur middel van die heteroduplekse enkel-string konformasie polimorfisme (HEX-SSCP) tegniek. Deels-geoutomatiseerde DNS volgordebepaling het die bevestiging en karakterisering van die waargenome variante toegelaat. Resultate het ingesluit die indentifikasie van vier nuwe variante teenwoordig in slegs die Kaukasiër pasiënt groep, gekarakteriseer as IVS4-53G→A (*HFE*) (een van 33 pasiënte; 3%), IVS2-65delA (*CYBRD1*) (twee van 32 pasiënte; 6.3%), 3’UTR+26delACGTCACGTTTCAAAACTA (*CYBRD1*) (een van 31 pasiënte; 3.2%) en 219delG (*HJV*) (twee van 33 pasiënte; 6%). Bykomend was ‘n totaal van 15 reeds beskryfde variante geïdentifiseer (sewe intronies en agt eksonies) waarvan drie ook slegs in die Kaukasiër pasiënt groep voorkom. Die studie het ook die verskille tussen pasiënt- en kontrole-

groep variant frekwensies, geen-geen interaksie en genotipe-fenotipe verhoudings, ondersoek. Analise het nie gedui op statisties betekenisvolle assosiasies nie. Hierdie ondersoek was wel beperk deur klein studie kohort groottes en gebrek aan kontrole serum yster en ferritien vlakke.

Hierdie loodsondersoek het variante gevind in elkeen van die vyf gene wat ondersoek was en dit het toegelaat vir identifisering van moontlike merkers en/of bydraers tot die siekte, VS. Alhoewel statistiese analise om die rol van elk/almal van die variante geïdentifiseer te verduidelik, nie betekenisvol beduidend was nie, mag toekomstige studies in ‘n groter kohort groep anders aandui. Hierdie studie beklemtoon die potensiële rol van yster en die yster-metabolisme verwante gene in die ontwikkeling van dié siekte. Sodoende het dit die beperkte kennis van beide die genetiese etiologie van die siekte asook die ontwikkeling van VS spesifiek in die Suid-Afrikaanse bevolking, verryk. Dit voorsien dus insig oor die rigting wat toekomstige genetiese studies, verwant aan VS en die rol van yster metabolisme in dié siekte, moet inneem.

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‘The things that we simply cannot describe in words, they are the most important.’
- Anonymous

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LIST OF ABBREVIATIONS AND SYMBOLS

~	Average/mean
α	Alpha
β	Beta
β2m	β_2 microglobulin
χ^2	Chi-square
ºC	Degrees Celsius
=	Equal
µl	Microlitre
µl/ml	Microlitre per millilitre
µmol/L	Micro-moles per litre
-	Minus
%	Percentage
%C	Percentage crosslinking
+	Plus
±	Plus-minus
3’	3-prime
5’	5-prime
®	Registered trademark
™	Trademark
2n	Total alleles

A	Adenosine
A (ala)	Alanine
AA	Acrylamide
$AgNO_3$	Silver nitrate
Ann	Annealing temperature
APS	Ammonium persulphate
ASSP	Alternative Splice Site Predictor
ATP	Adenosine tri-phosphate

bp	Base-pair
BAA	Bisacrylamide
BBB	Blood brain barrier
BSA	Bovine serum albumin
C	Cytidine
C (Cys)	Cysteine
C2	Complement component 2
C4A	Complement component 4a
C4B	Complement component 4b
CBf	Complement factor B
CC	Caucasian control group
CNS	Central nervous system
CSF	Cerebrospinal fluid
CP	Ceruloplasmin
CPMS	Caucasian control patient group
CYBRD1	Cytochrome b reductase 1
D (Asp)	Aspartic acid
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
ddH_2O	Double distilled water
del	Deletion
dGTP	2'-deoxy-guanosine-5'-triphosphate
dHPLC	Denaturing high performance liquid chromatography
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
dNTP	2'-deoxy-nucleotide-5'-triphosphate
DTT	Dithiothreitol
dTTP	2'-deoxy-thymidine-5'-triphosphate
EAE	Experimental allergic encephalomyelitis
EBV	Epstein-Barr virus
EDSS	Expanded Disability Status Scale

EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
ESE	Exonic splicing enhancers
EtBr	Ethidium bromide
EtOH	Ethanol
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
Fe-Tf	Iron-transferrin
FLAIR	Fluid-attenuated inversion recovery
g	Gram
G	Guanosine
G (Gly)	Glycine
GAMES	The Genetic Analysis of Multiple sclerosis in EuropeanS (GAMES) collaborative
GIT	Guanidine isothiocyanate
H (His)	Histidine
HAMP	Hepcidin anti-microbial peptide
He	Heterozygous
HEPC	Hepcidin
HEX-SSCP	Heteroduplex Single-Stranded Conformation Polymorphism
HFE	High-iron
HGMD	Human Gene Mutation Database
HHV-6	Human Herpes 6
HJV	Hemojuvelin
HLA	Human leukocyte antigen
Ho	Homozygous
HWE	Hardy-Weinberg equilibrium
I (Ile)	Isoleucine
IgG	Immunoglobin G
IREG1	Iron-regulated transporter 1

IRP	Iron regulatory proteins
IVS	Intervening sequence
JAG1	Jagged 1
KAc	Potassium acetate
kb	Kilobases
KCl	Potassium chloride
$KHCO_3$	Potassium hydrogen carbonate
KH_2PO_4	Potassium phosphate dibasic
LEAP	Liver-expressed antimicrobial peptide
LMP	Large multifunctional protease
LT	Lymphotoxin
MC	Coloured control group
ml	Millilitre
mg/ml	Milligram per millilitre
MgAc	Magnesium acetate
$MgCl_2$	Magnesium chloride
MHC	Major Histocompatibility Complex
mM	Milli-moles per litre
MOG	Myelin-oligodendrocyte glycoprotein
MPMS	Coloured multiple sclerosis patients
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis
MSRV	Multiple sclerosis-associated retrovirus
MTP1	Metal transporter 1
N (Asn)	Asparagine
N	Homozygous wild-type
NaCl	Sodium chloride
Na_2HPO_4	di-sodium hydrogen phosphate

NaOH	Sodium hydroxide
ng	Nanogram
ng/µl	Nanogram per microlitre
$(NH_4)_2SO_4$	Ammonium sulphate
NRAMP2	Natural resistance-associated macrophage protein 2
NS	Not significant
OMIM	Online Mendelian Inheritance in Man
p	Short arm of chromosome
P	Probability
PAA	Polyacrylamide
PBS	Phosphate buffered saline
PD	Proton-density
pH	Potential of hydrogen
PML	Progressive multifocal leucoencephalopathy
pmol	Picomole
poly(A)	Poly adenosine
POU2AF1	Pou Domain, Class 2, Associating Factor 1
PPMS	Primary progressive multiple sclerosis
q	Long arm of chromosome
R (Arg)	Arginine
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediate
rpm	Revolutions per minute
RRMS	Relapsing-remitting multiple sclerosis
RT-PCR	Reverse transcriptase, polymerase chain reaction
S (Ser)	Serine
SDS	Sodium dodecyl sulphate
SLC11A1	Solute carrier family 11 (proton-coupled divalent metal ion transporter)

SLC40A1	Solute-carrier family 40 (iron regulated transporter) member 1
SPMS	Secondary progressive multiple sclerosis
T	Thymidine
T (Thr)	Threonine
TA	Tris-acetate
TAE	Tris-acetate-EDTA
TAP1	Transporter, ATP-binding cassette, major histocompatibilty complex, 1
TAP2	Transporter, ATP-binding cassette, major histocompatibilty complex, 2
Taq	Thermus aquaticus
TBE	Tris-borate/EDTA
Tf	Transferrin
TFR1	Transferrin receptor 1
TFR2	Transferrin receptor 2
Tm	Melting temperature
TNFα	Tumor necrosis factor-alpha
TNFβ	Tumor necrosis factor-beta
Tris-HCl	Tris hydrochloride [2-Amino-2-(hydroxymethyl)-1,3-propanediol-hydrochloride]
U	Units
UK	United Kingdom
USA	United States of America
UTR	Untranslated region
v	Version
V	Volt
V (Val)	Valine
vs	Versus
v/v	Volume per volume
VEP	Visually evoked potential

w/v	Weight per volume
x	Times
X	Stop codon/ termination codon
Y (Tyr)	Tyrosine

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CHAPTER ONE

1. LITERATURE REVIEW

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) (OMIM #126200) is known as an inflammatory disease of the central nervous system (CNS) (Compston *et al.* 1998, Ebers and Dyment 1998, Noseworthy 1999, Kotze *et al.* 2001, reviewed by Reipert 2004). Areas of damage or the formation of lesions (demyelination) mainly occurs in the white matter of the CNS. This damage is formed in response to inflammation and it is at these specific points that demyelination occurs (reviewed by Reipert 2004). This process is characterized by the loss of the myelin sheath surrounding the axon of the neuron and it is accompanied by the 'slowing down' or complete loss of nerve impulse transmission (reviewed by Reipert 2004).

Secondary symptoms include sensory disturbances, gait ataxia, limb weakness and fatigue (reviewed by Noseworthy *et al.* 2000, reviewed by Reipert 2004). Recurring attacks (relapses) may bring about damage to axons, formation of gliotic scar tissue and depletion of oligodendrocyte precursors. This, in turn, leads to loss of neurological function (Trapp *et al.* 1998, Lucchinetti *et al.* 1999, Bitsch *et al.* 2000, Noseworthy *et al.* 2000).

1.2 DISEASE CLASSIFICATION

MS presents earlier in females (18-30 years of age), compared to males (30-40 years of age) (reviewed by Reipert 2004). The onset and development of the disease is capricious, affecting over two million people globally (Al-Omaishi *et al.* 1999, Javed and Reder 2006). MS can be divided into three clinically distinct groups; namely i) relapsing-remitting (RRMS), ii) primary progressive (PPMS) and iii), secondary progressive (SPMS) MS (reviewed Reipert 2004). Differences concerning pathological features, clinical course and diagnosis, exists between PPMS, RRMS and SPMS and will be discussed further.

1.2.1 RELAPSING-REMITTING MS (RRMS)

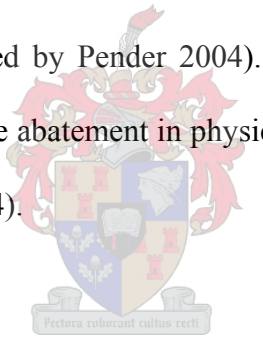
Relapsing-remitting MS is observed in 80% of patients (reviewed by Noseworthy *et al.* 2000) and its clinical course can be described as recurring acute attacks (relapses) during which neurological dysfunction and symptoms become apparent. This is followed by a period of remission that is characterized by 'neurological stability' as well as symptom stabilization or even improvement, until the next relapse occurs (reviewed by Noseworthy *et al.* 2000, Goodin *et al.* 2002, reviewed by Reipert 2004).

The occurrence, duration and recovery of relapses are highly variable. Attacks (relapses) can persist for days to months and periods of remission can continue for weeks up to years. Recovery can prove to be either an immediate or gradual process (reviewed by Reipert 2004). Relapsing-remitting MS affects predominantly females [female to male ratio of 2:1]. Symptoms of relapsing-remitting MS include sensory disturbances, gait ataxia, trunk and limb

parethesias, clumsiness, unilateral optic neuritis, sexual dysfunction and diplopia (Compston *et al.* 1998, reviewed by Noseworthy *et al.* 2000, Goodin *et al.* 2002). The clinical course of RRMS is seen in a minority of other neurological diseases and the abundance of symptoms aid the diagnosis of this MS subgroup (reviewed by Pender 2004).

1.2.2 PRIMARY PROGRESSIVE MS (PPMS)

Primary progressive MS presents in an estimated 20% of affected patients (mean age of onset approximately 39 years) and it has an almost similar incidence in both males and females (1.3:1.0) (Weinshenker 1994, McDonnell and Hawkins 1998, Cottrell *et al.* 1999, reviewed by Noseworthy *et al.* 2000, reviewed by Pender 2004). The clinical course is found to be steadily progressing with a noticeable abatement in physical ability (reviewed by Noseworthy *et al.* 2000, reviewed by Reipert 2004).



It is furthermore characterized by the absence of acute attacks. Frequently, primary progressive MS presents as a gradually developing 'chronic progressive myelopathy', also known as upper motor-neuron syndrome of the legs and paraparesis (reviewed by Noseworthy *et al.* 2000, reviewed by Reipert 2004, reviewed by Pender 2004).

The diagnosis of primary progressive MS proves to be difficult due to its clinical course being characteristic of other neurological diseases where symptoms similarly develop over years. Also, the presenting symptoms are few, reducing the distinctiveness of PPMS. An example is that of the magnetic resonance imaging (MRI) focal lesions present in fewer amounts

compared to RRMS and SPMS (Thompson *et al.* 1990, Kidd 1993, reviewed by Pender 2004).

1.2.3 SECONDARY PROGRESSIVE MS (SPMS)

Secondary progressive MS presents as RRMS. It will, however, similarly to PPMS, take on a pattern of 'steadily progressing' CNS dysfunction. This may still be accompanied by relapses, but at a reduced rate. It is best understood as the progression of neurological damage even between, or with the complete absence of relapses (reviewed by Noseworthy *et al.* 2000, reviewed by Reipert 2004). Within ten years of an initial diagnosis of relapsing-remitting MS, 50% of these patients develop secondary progressive MS, affecting more females than males (2:1) (reviewed by Noseworthy *et al.* 2000, reviewed by Reipert 2004).

1.3 MS IN CHILDREN

MS in children has also been reported, with 2.7-5% of all reported cases presenting prior to the age of 15 years. Individuals affected by MS in their early childhood and infant stages of life, account for 0.2-0.7% of the recorded cases (Duquette *et al.* 1987, Compston *et al.* 1998, Eraksoy *et al.* 1998, Ruggieri *et al.* 1999, reviewed by Gadoth 2003). Childhood MS onset is often associated with symptoms such as seizures and nausea, vomiting, headaches, brainstem and cerebellar dysfunction and fever (reviewed by Gadoth 2003). RRMS occurs in approximately 64% of the reported childhood MS cases, whereas SPMS is the second most common (24%) and PPMS the least common form seen in children (12%) (Sevon *et al.* 2001, reviewed by Gadoth 2003).

1.4 DIAGNOSIS

MS is currently diagnosed based on clinical information combined with visually evoked potential tests (VEP), cerebrospinal fluid (CSF) analysis and magnetic resonance imaging (MRI) visualization of the spinal cord and brain.

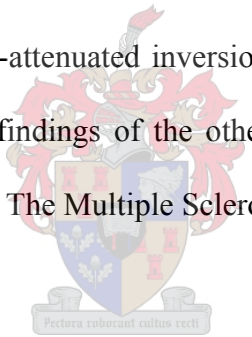
Previous criteria relied solely on clinical data. This required the incidence of at least two clinically identified episodes and at least two formed lesions that differ in time of occurrence and the CNS location affected. It was furthermore imperative that the observed symptoms be explained only by the presence of MS. Revision of the diagnostic criteria allowed for incorporation of paraclinical evidence as credible. Diagnosis thus still requires incidence of at least two distinct lesions but only one need to be clinically supported by the other(s) based on paraclinical findings. The latter includes abnormal VEP, positive CSF and MRI evidence and these will be discussed further (seminar by Compston and Coles 2002, Keegan and Noseworthy 2002).

The VEP test refers to evaluation of afferent CNS pathway conduction in reaction to sensory receptor stimulation. If conduction proves to be atypical this could be indicative of lesion presence with conduction affected by a demyelination event.

The CSF of a MS patient may show an increase in oligoclonal immunoglobin G (IgG) bands and their potential presence can be determined with protein electrophoresis of the CSF. The fact that oligoclonal IgG bands can be identified in the majority of MS patients (>90% of cases) strengthens its role as a diagnostic tool. As an indicator of inflammation, these bands

thus narrow the field of potential pathogenesis to that of an inflammatory disease. Analysis of both the VEP and CSF is of specific importance when it comes to diagnosing an individual free of acute attacks, showing progressive deterioration suggestive of PPMS (seminar by Compston and Coles 2002, Keegan and Noseworthy 2002).

The MRI scan aids in establishing the location, relative age, degree of damage and demyelination activity of lesions (McDonald 2001, seminar by Compston and Coles 2002, Keegan and Noseworthy 2002). The visual images obtained with MRI are achieved by scanning with radiowave pulses that recognize the relative increase of total water within lesions. The pulses can be manipulated to deliver different images, e.g. T1- and T2-weighted scans, proton-density (PD) and fluid-attenuated inversion recovery (FLAIR) (refer to Figure 1.1) and each may supplement the findings of the other (seminar by Compston and Coles 2002, Keegan and Noseworthy 2002, The Multiple Sclerosis gateway).



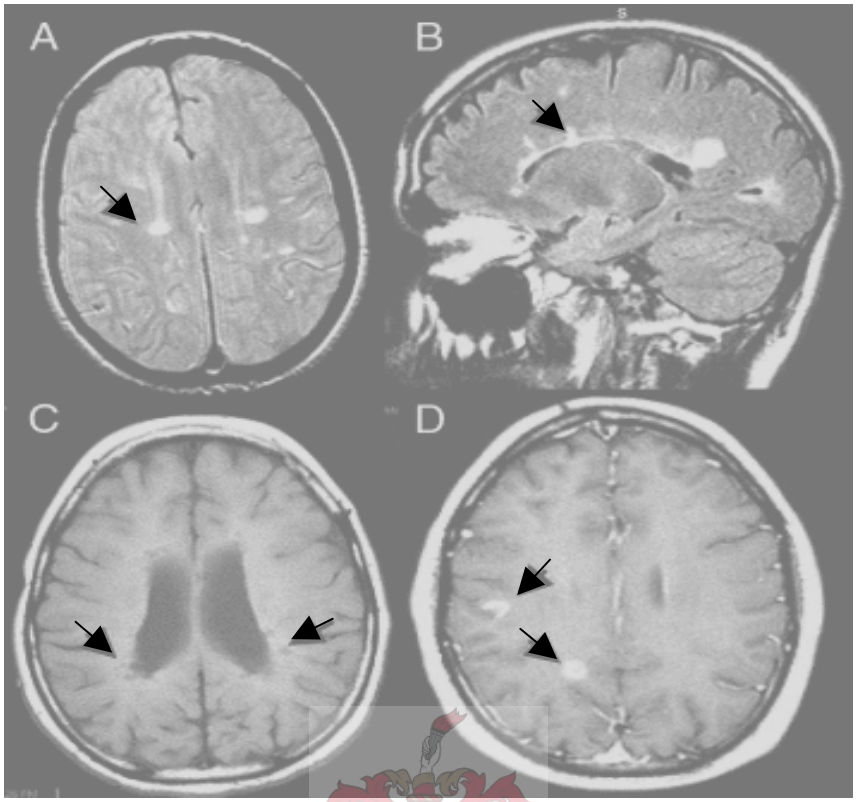


Figure 1.1 MRI scans of patients with RRMS. Images **A** and **B** (arrows indicate) show inflammation characteristic of MS. The inflammation can be seen as 'hyperintensities' with detection thereof allowed for by the fluid-attenuated inversion recovery (FLAIR); Figure **C** shows 'black holes'/ 'hypointensities' (arrows indicate) that mark the presence of 'chronic, inactive lesions' whereas **D** illustrates the presence of 'acute enhancing lesions' (arrows indicate) both achieved by T1 scans (Adapted from Javed and Reder 2006).

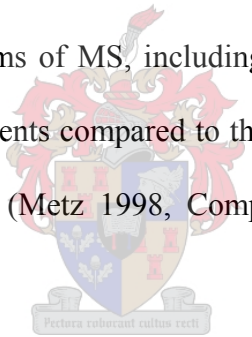
1.5 THERAPY

As described, MS pathogenesis could be characterized by relapses and remissions (RRMS), progression (PPMS) or a combination of both (SPMS). MS therapies are focused at: 1) reducing the incidence of relapses, 2) preventing and treating the damage due to these episodes and treatment of progression that is once more accompanied by prevention and

management of impairment secondary to this. It should be emphasized that the choice of treatment is dependent upon the diagnosed MS subtype.

Therapies include administration of corticosteroids or immunomodulators and plasma exchange. Corticosteroid therapy, e.g. intravenous allocation of methylprednisolone, is used to treat acute relapses accompanied by functional impairment with the aim of accelerating recovery. If patients prove unresponsive to corticosteroid treatment, a process of plasma exchange may be employed. Immunomodulatory treatment with glatiramer acetate, interferon β 1-b or interferon β 1-a, is known to reduce the frequency of relapse occurrence.

Treatments that address the symptoms of MS, including gabapentin and ondansitron, prove beneficial for a larger portion of patients compared to the aforementioned treatment, directed specifically at disease amelioration (Metz 1998, Compston and Coles 2002, Keegan and Noseworthy 2002).



A MS study, including a South African cohort, addressed the issue of low blood iron parameters observed within certain patients (van Rensburg *et al.* 2006). Analysis of iron, folate and homocysteine levels were perfomed and in the case of deficiency, the patients were advised to augment their diet with the following supplements in Recommended Daily Allowance amounts: iron (if their iron status was low), amino acids, essential fatty acids, vitamins and minerals with the aim of myelin regeneration, by compensating for deficiency in the nutrients needed for proper myelinogenesis. The exact amount and combination of nutrients was dependent upon the clinical levels established for each patient. The results obtained suggested a plausible influence of the regimen upon myelin regeneration.

1.6 SUGGESTED CAUSES OF MS

Although the disease is referred to as an inflammatory disease of the CNS, the exact cause of MS is still unidentified (Noseworthy 1999, Kotze *et al.* 2001). Current research has highlighted the potential contribution(s) of genes (Oksenberg *et al.* 1996, Oksenberg *et al.* 2001, Keegan and Noseworthy 2002), autoimmunity (Oksenberg *et al.* 2001, Keegan and Noseworthy 2002) and/or viral infection (Oksenberg *et al.* 2001, Keegan and Noseworthy 2002, Miller *et al.* 2002).

1.6.1 MS AND GENETICS

Adoption studies provide evidence suggestive of a genetic basis for familial aggregation of MS. A single study determined the MS occurrence rate in non-biological first-degree relatives, living with an index case, and compared it to both the general population and biologically related individuals sharing the same environment. A frequency similar to that of the general population was found, indicating a contribution of genes to the development of MS (Ebers *et al.* 1995).

A Canadian twin study has shown a higher concordance rate for monozygotic twins (25.9%) compared to dizygotic pairs (2.3%) and non-twin siblings (1.9%) (Ebers *et al.* 1986). A further study based on a British population similarly indicated higher concordance rate in monozygotic twins (25%) compared to dizygotic pairs (3%). The difference in concordance rate highlights the role of a genetic factor in MS development (Mumford *et al.* 1994). The high discordance within the monozygotic groups, however, indicates that a risk factor other than the genetic background may also be involved.

Sibling studies allow for comparison of full-siblings to half-siblings living together as well as half-siblings living apart. A Canadian sibling study showed a higher MS risk in full-siblings (3.46%) when compared to the entire half-sibling group (1.32%) (both paternal and maternal sibs included). If considered together with the more specific comparison of full-sibling (3.46%) to half-sibling in the same environment (1.17%), the definite role of genes becomes apparent. A final comparison of half-siblings of shared environment (1.17%) to half siblings living apart (1.47%) minimizes the potential involvement of an environmental factor and further emphasizes the role of genes in MS pathogenesis (Sadovnick *et al.* 1996).

1.6.1.1 GENES ASSOCIATED WITH MS

Various studies have emphasized the association existing between the Major Histocompatibility Complex (MHC) and MS (Hillert 1994, Kalman and Lublin 1999, Hillert 2006). Research indicated an association between the human leukocyte antigen haplotype (HLA)-DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602, more specifically represented as HLA allele Dw2/DR2/DR15/DR15, DQ6, and an increased risk of MS (Hillert 1994, Kalman and Lublin 1999, Hillert 2006).

Early evidence to support this association was found in a study comparing T-cell line production of lymphotoxin (LT) and tumor necrosis factor-alpha (TNFα). HLA-DR2-positive lines showed a greater production than the HLA-DR2-negative cases and both LT and TNFα are known to contribute to MS development (Zipp *et al.* 1995). The more recent studies have identified association with genes Jagged 1 (*JAG1*) (OMIM +601920) and Pou Domain, Class 2, Associating Factor 1 (*POU2AF1*) (OMIM *601206). Utilizing meta-analysis, The Genetic

Analysis of Multiple Sclerosis in EuropeanS (GAMES) collaborative, detected a total of 12 potential MS-associated markers outside of the MHC region. Genotyping narrowed the group to three markers denoted as D11S1986, D19S552 and D20S894 and these, in turn, implicated *JAG1* and *POU2AF1* as candidate genes (GAMES Collaborative 2006).

Investigation of *JAG1*, a ligand of the Notch receptor, suggested a relationship thereof with oligodendrocyte precursors and the process of myelin formation. It entailed 'downregulation' of *JAG1* expression and a resulting increase in both 'precursor maturation' and 'myelination' (Wang *et al.* 1998, John *et al.* 2002, GAMES Collaborative 2006). Studies have proposed that, in MS, the myelin sheath becomes vulnerable to attack due to antibody production (intrathecal). The *POU2AF1* gene is believed to act as regulator of this IgG gene expression (in the B-cells). In light of their respective functions, variation within *JAG1* and *POU2AF1*, may contribute to the MS pathogenesis (GAMES Collaborative 2006). Studies investigating various regions of interest, proved contradictory and a brief outline is given in Table 1.1 (Kalman and Lublin 1999).

Table 1.1 Overview of studies investigating various genetic regions of interest

Region of interest	Potential involvement	References
Major histocompatibilty complex, class II, DP β-1 (HLA DP)	Membrane protein, antigen presentation	(OMIM *142858, Chataway *et al.* 1998, Dekker *et al.* 1993, Howell *et al.* 1991,
Complement components 4a (C4A) and 4b (C4B) Complement factor B (CBf) Complement component 2 (C2)	Complement components	(OMIM +120810 (C4A), OMIM *12080 (C4B), OMIM *138470 (CBf), OMIM +21700 (C2), Francis *et al.* 1987, Hauser *et al.* 1989, Papiha *et al.* 1991)
Large multifunctional protease (LMP) Transporter , ABC, MHC, 1 (TAP1) Transporter, ABC, MHC, 2 (TAP2)	Transporter proteins	(OMIM *170260 (TAP1), OMIM *170261 (TAP2), Bell and Ramachandran 1995, Bennets *et al.* 1995, Liblau *et al.*1993, Spurkland *et al.* 1994, Vandevyver *et al.* 1994)
Tumor necrosis factors TNFα, TNFβ	Proinflammatory cytokine	(OMIM *191160, Braun *et al.* 1996, Garcia-Merino *et al.* 1996, Mycko *et al.* 1998, Roth *et al.* 1994, Sumner *et al.* 1993, Weinshenker *et al.* 1997)
Myelin-oligodendrocyte glycoprotein (MOG)	Related to myelin production	(OMIM *159465, Malfroy *et al.* 1995, Roth *et al.* 1995

1.6.2 MS AND AUTOIMMUNITY

An autoimmune disease can be described as triggering of an immunological response aimed at an individual's own bodily constituents. Such a response is suggested to occur secondary to the loss of tolerance of the T and B lymphocytes. T and B lymphocytes react to the presence of antigens as part of a normal immune response. However, this response is in some instances regulated, so as to inhibit a subsequent reaction, and this is termed tolerance. Loss of this control/tolerance could potentially allow for T and B lymphocyte response to self-antigens. Loss of tolerance may be attributed to one or more of the following factors: cytokines, immunoregulatory pathways, molecular mimicry and self-antigens.

The first factor, namely cytokines, could potentially contribute to initiation of an autoimmune reaction *via* the role they play in recruiting and further regulating immune cell function. The second factor, immunoregulatory pathways, constitutes T cells that either suppress or enhance immune function *via* their respective cytokine production patterns. It is then speculated that over-suppression/activation may result in an autoimmune response. The third factor of 'molecular mimicry' refers to the structural similarity between viral antigens and native proteins. Immune cells directed at both are thus produced and autoimmune damage initiated (refer to Figure 1.2).

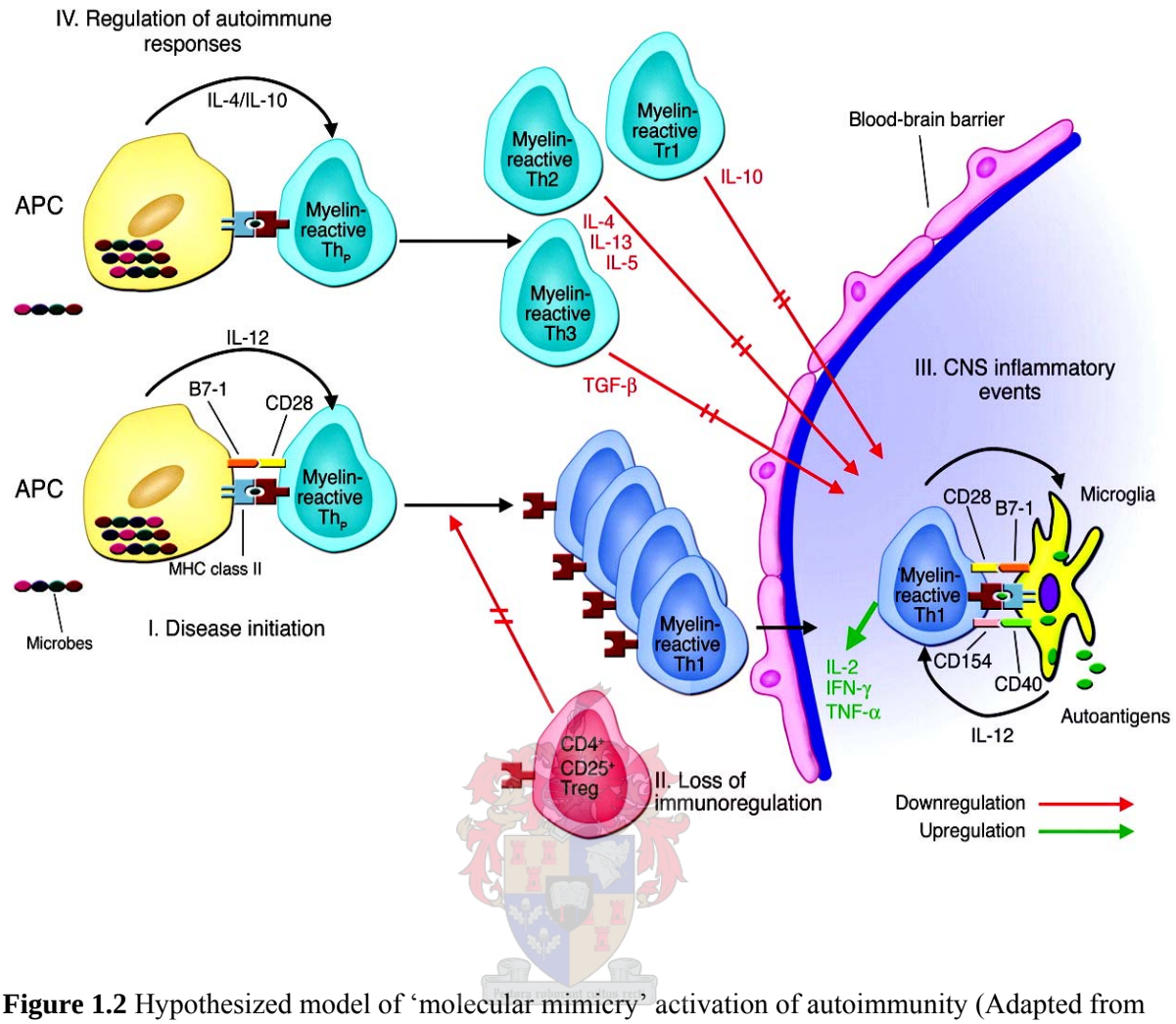


Figure 1.2 Hypothesized model of 'molecular mimicry' activation of autoimmunity (Adapted from Hafler 2004).

Tissue damage e.g. ischaemic injury, results in release of proteins from the site of damage. These proteins, referred to as 'hidden self-antigens', may in turn cause formation of antibodies directed at themselves. These proteins share similarity with the remainder of undamaged tissue and, this tissue too may be identified as foreign and immunologically attacked.

The proposed autoimmune pathogenesis of MS entails activation of T cells that are specific for myelin, perhaps due to loss of tolerance. Once activated, the cells move across the blood

brain barrier (BBB) from the peripheral circulation into the CNS. This is followed by the interaction of myelin antigens and CD4+ cells and the subsequent initiation of inflammation. Inflammation is characterized by the presence of cytokines, both nitrogen and oxygen radicals as well as macrophages which all contribute to the destruction of myelin.

Evidence for the role of the immune system in MS pathogenesis has frequently been illustrated in different studies. This would include studies showing the presence of various inflammatory cells including T cells, B cells and macrophages within and around lesions. Their cytokine secretions are also present and investigations have established their damaging effects e.g. cell culture research proving TNF-α cytotoxic to oligodendrocytes. Studies focusing on the animal model of MS, namely experimental allergic encephalomyelitis (EAE), have shown induction thereof by addition of autoreactive T cells to healthy animals. Further EAE studies have also established a macrophage-deduction, EAE prevention relationship again highlighting the immune component of the disease (Peakman and Vergani 1997, Kamradt and Mitchison 2001).

1.6.3 VIRAL FACTORS IMPLICATED IN MS

The hypothesized involvement of a viral factor in the pathogenesis of MS is motivated by the following a) studies establishing a link between viral infection and chronic neurological diseases (Connolly *et al.* 1967, Padgett *et al.* 1971, Gilden 2005), b) the presence of high concentrations of IgG in MS patients (Gilden *et al.* 1996), c) the variable clinical course of both viral infections and MS (Gilden *et al.* 1996, Al-Omaishi *et al.* 1999, Steinman 2001) and d) research showing association between a number of micro-organisms and MS (Murray *et al.*

1992, Stewart *et al.* 1992, Boerman *et al.* 1993, Gilden *et al.* 1996, Soldan *et al.* 1997, Ferrante *et al.* 1998, Sririam *et al.* 1999, Friedman *et al.* 1999, Mirandola *et al.* 1999, Ascherio and Munch 2000, Dessau *et al.* 2001, Tsai and Gilden 2001, Rodriguez *et al.* 2001, Ascherio *et al.* 2001, Ascherio and Munch 2003, de Villiers *et al.* 2006).

The link between viral infection and chronic neurological disease dates back to the 1960s (Gilden 2005). One case is the subacute sclerosing panencephalitis, a chronic inflammatory disease that affects both the white and grey matter of the CNS. *Paramyxovirus* nucleocapsids were found in the brain matter of patients with the disease (Gilden 2005). Later research on subacute sclerosing panencephalitis further identified high serum and CSF concentrations of measles-specific antibodies in patients (Connolly *et al.* 1967, Gilden 2005). Progressive multifocal leucoencephalopathy (PML), a disease characterized by dementia and motor loss, is caused by human *papovavirus* (*JC virus*). Presence of the virus was observed in the oligodendrocytes of a single patient (Padgett *et al.* 1971).

Disease relating to the CNS is seldom characterized by a high IgG concentration. All diseases showing IgG presence have clinically manifested inflammation and the majority is caused by infection. A high IgG concentration is seen in 90% of MS patients and it is localized to the CSF and the brain (Gilden *et al.* 1996).

The clinical course of MS can vary with regards to degree of damage and inflammation and similarly a single infectious agent can elicit various pathologies (Al-Omaishi *et al.* 1999, Steinman 2001). *Treponema pallidun* causes neurosyphilis and is an example of a disease

displaying a number of different pathologies. It varies according to area affected and characteristics of the lesions formed (Gilden *et al.* 1996).

Micro-organisms potentially associated with MS, include the *JC virus*, *Coronavirus*, *Herpesviruses* and *Chlamydia pneumoniae* (Murray *et al.* 1992, Ferrante *et al.* 1998, Friedman *et al.* 1999, Sririam *et al.* 1999, Ascherio and Munch 2003). Studies investigating the potential association, have delivered controversial results but this could be due to the inability to detect a virus whilst in its latent period (Boerman *et al.* 1993, Gilden *et al.* 1996, Mirandola *et al.* 1999, Dessau *et al.* 2001, Tsai and Gilden 2001, Rodriguez *et al.* 2001).

An example of research proving viral detection involves the *JC virus* identified in the CSF of MS patients (9%). The virus was not observed in either the control group or patients with other neurological diseases (Ferrante *et al.* 1998). Similarly detection of certain Coronaviruses' ribonucleic acid (RNA) was found only in MS patients (Stewart *et al.* 1992). Two herpesviruses, i.e. Epstein-Barr virus (EBV) and Human herpes 6 (HHV-6) have shown association with MS (Friedman *et al.* 1999, Ascherio and Munch 2003). Findings include increase in anti-EBV in serum titres prior to onset of MS as well as elevated HHV-6 antibodies in relapsing-remitting MS patients (Soldan *et al.* 1997, Ascherio *et al.* 2001).

Further research investigating potential viral contributors has identified the presence of MS-associated retrovirus (MSRV) (retroviral elements) within chromosomal regions which in turn have shown association with MS (Perron *et al.* 1997, Perron *et al.* 2000). A study investigating MS within the South African population (de Villiers *et al.* 2006), identified the presence of this virus and furthermore allowed for genotyping of the solute carrier family 11

(proton-coupled divalent metal ion transporter), member 1 (*SLC11A1*) gene with regards to the promoter region in which a) allele two contributes to infection resistance and b) alleles three and five promote autoimmunity (Searle and Blackwell 1999, Kotze *et al.* 2001, de Villiers *et al.* 2006). Of specific interest was the identification of two related patients, both with the *SLC11A1* gene alleles three and five, of which one showed presence of MSRV. The virus positive individual was characterized by early onset of MS and this finding highlights both the suggested viral etiology and the interaction of genetics and the environment in MS development (de Villiers *et al.* 2006).

1.7 MS AND IRON

Iron plays a crucial role in processes of myelinogenesis, immunity and infection resistance. Dysregulation thereof can thus disrupt myelin formation, impair immune system function and increase the success of pathogen infection. Myelin damage, autoimmunity and viral infection are all proposed contributors to MS and the link of iron to each, in turn, highlights its role in MS development.

Iron is an important key factor for the synthesis of myelin. It serves as part of the catalytic centre of various enzymes involved in lipid synthesis for which the oligodendrocytes are responsible (LeVine and Makclin 1990, Connor *et al.* 1995, LeVine and Chakrabarty 2004). Lipid is the main constituent of myelin (Morell *et al.* 1993). This function of iron may thus account for its high concentration established in oligodendrocytes, and further observed in the myelin, of healthy individuals (Dwork *et al.* 1988, Gerber and Connor 1989, Connor and Menzies 1990, Connor *et al.* 1990, LeVine and Makclin 1990, Levine 1991, LeVine and

Chakrabarty 2004). The presence of ferritin receptors located on the oligodendrocytes, as established by Hulet *et al.* (1999), provides additional evidence supporting the role of iron in the myelinogenesis process. The ferritin receptors allow for the tranfer of iron to the oligodendrocytes. Irregular mental and motor function and myelin production have been linked to iron deficiency within the CNS (Connor *et al.* 2001).

However, if not homeostatically controlled, the high iron concentrations are suggested to contribute to MS development (LeVine and Chakrabarty 2004). Tissues from MS and EAE cases have shown locationally uncharacteristic iron deposits within e.g. macrophages and neurons (LeVine 1997, Forge *et al.* 1998, LeVine and Chakrabarty 2004). This iron dysregulation may show release of iron from the proteins they characteristically bind to after which the iron may remain unbound or form associations with surrounding molecules. Whether unbound or weakly associated, both forms have the potential to catalyze reactions causing reactive oxygen intermediate (ROI) formation (LeVine and Chakrabarty 2004). ROI is responsible for oxidative tissue damage that includes impairment of molecules such as deoxyribonucleic acid (DNA), lipids and proteins. Collectively a) the presence of lipid peroxidation products in both MS and EAE tissues and b) the beneficial use of treatment focused on oxidative damage interruption, provides evidence suggesting that iron dysregulation contributes to MS development (Hunter *et al.* 1985, Brett and Rumbsy 1993, LeVine and Chakrabarty 2004). The damage caused to DNA molecules, due to the oxidative tissue damage, may include base modification and single-strand breaks (Stohs and Bagchi 1995, Lloyd *et al.* 1997, Ahsan *et al.* 2003). Research suggests that the altered DNA becomes immunogenic and that the resulting autoimmune response entails autoantibody formation directed against self-antigens e.g. 'ROI-modified' DNA molecules. The response may

furthermore include autoantibody binding to native (ROI-unaffected) DNA molecules (Blount *et al.* 1989, Ahsan *et al.* 2003).

The described potential for a pathogenic etiology in MS, in turn, emphasizes the possible contribution of iron. The growth and thus survival of these pathogens, is in part dependent upon the availability of iron. Infection is met by the reduction in plasma iron levels referred to as the 'iron-withholding defence system' that includes iron binding by transferrin and suppressed iron efflux from macrophages (Brock 2000, Kotze *et al.* 2001, Ong *et al.* 2006).

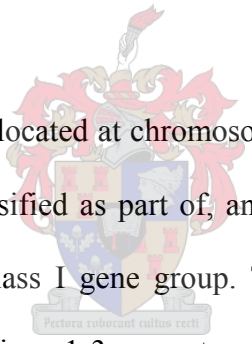
Iron contributes to the proper functioning of the immune system. Studies of iron deficiency have illustrated a) failure of T-cells to proliferate normally, b) decreased number of circulating T lymphocytes and c) reduction of cytotoxic activity of lymphocytes (Brock 2000). During infection, normal iron regulation is therefore crucial to concomitantly achieve a) viral resistance and b) proper immune function. Homeostatic disruptions of iron metabolism may allow for infectious success of the proposed pathogenic factors in MS (Brock 2000, Kotze *et al.* 2001, Ong *et al.* 2006).

In summary, the processes of myelinogenesis and immune system development (autoimmunity) are dependent upon iron availability. Accurate homeostatic control of iron, in turn, is needed to guard against ROI formation and infection (viral).

1.8 GENES INVOLVED IN IRON HOMEOSTASIS

The importance of iron homeostasis within the body highlights the potential role of factors involved in iron transport and metabolism. These factors include genetics and more specifically, the genes involved in the molecular control of the metal. The high-iron gene (*HFE*), solute-carrier family 40 (iron regulated transporter) member 1 gene (*SLC40A1*), hepcidin anti-microbial peptide gene (*HAMP*), cytochrome b reductase 1 gene (*CYBRD1*) and hemojuvelin gene (*HJV*) will subsequently be discussed.

1.8.1 *HFE*



The *HFE* gene (OMIM +235200) is located at chromosome position 6p21.3-22.1 and spans a total of 9.5 kilobases (kb). It is classified as part of, and shares similarities with, the major histocompatibility complex MHC class I gene group. The first structural similitude is the presence of three extracellular domains α1-3, an untranslated cytoplasmic-3' tail and a final transmembrane domain, each encoded for by an individual exon (Feder *et al.* 1996, Parkkila *et al.* 1997, Riegert *et al.* 1998, Bahram *et al.* 1999). A further similarity is the presence of cysteine residues within the α2 and α3 domains. These residues are responsible for disulfide bridge formation, which in turn has a suggested involvement in the secondary and tertiary structure of the predicted 343 amino acid glycoprotein that *HFE* encodes for (Bjorkman and Parham 1990, Feder *et al.* 1996, Feder *et al.* 1997, Lebrón *et al.* 1998, Riegert *et al.* 1998). The protein interacts with β_2 microglobulin (β2m) and this association allows for cell surface expression (Feder *et al.* 1997, Bahram *et al.* 1999).

HFE does, however, differ from the MHC class I molecules with regards to expression and function. Areas of *HFE* expression is limited e.g. epithelia, crypt cells and the function is not related to antigen-binding, due to narrowing of the cleft needed for such associations (Parkkila *et al.* 1997, Bastin *et al.* 1998, Bahram *et al.* 1999). Its function is rather related to binding of transferrin receptor 1 (TfR1), which is accompanied by a decrease in TfR 1 affinity for transferrin (Tf) binding. The resulting effect is decreased iron absorption (Parkkila *et al.* 1997, Feder *et al.* 1998, Lebron *et al.* 1998, Drakesmith *et al.* 2002). An animal study, characterized by the disruption of the *HFE* gene homolog in mice, showed a remarkable increase in liver iron concentration suggesting *HFE* as a key factor in iron regulation (Zhou *et al.* 1998).

Development of the iron-overload disease, haemochromatosis, has been accredited to the presence of variants within the *HFE* gene. The variants described include the C282Y missense mutation and the H63D polymorphism (Feder *et al.* 1997, Bahram *et al.* 1999).

1.8.2 *SLC40A1*

The solute-carrier family 40 (iron regulated transporter) member 1 gene, *SLC40A1*, is located at locus 2q32 where it comprises a length of 20,18 kb (OMIM *604653, Haile 2000, Njajou *et al.* 2001, GENATLAS database). Also known as ferroportin 1, iron-regulated transporter 1 gene (*IREG1*) or metal transporter 1 gene (*MTP1*), the gene has a total of eight exons that allows for the coding of a highly conserved, 571 amino acid, iron exporting protein characterized by the presence of ten transmembrane domains. The 5'untranslated region (UTR) of the mRNA comprises an iron responsive element shown to bind iron regulatory

proteins (IRP). Sites of protein expression include the syncytiotrophoblasts of the placenta, Kupfler cells of the liver, duodenal enterocytes and reticuloendothelial macrophages (Leibold and Munro 1988, McKie *et al.* 2000, Abboud and Haile 2000, Donovan *et al.* 2000, Njajou *et al.* 2001, Lymboussaki *et al.* 2003, Pietrangelo 2004). Functional studies, in which the *SLC40A1* gene was deleted from mice intestines, showed iron deficiency anemia, highlighting iron homeostasis (Donovan *et al.* 2005).

Variants identified within the *SLC40A1* gene, including the single nucleotide transversions, A144C and A77D, have shown association with the autosomal dominantly inherited haemochromatosis type 4 (Njajou *et al.* 2001, Montosi *et al.* 2001).

1.8.4 *HAMP*

The hepcidin anti-microbial peptide (*HAMP)* gene is positioned at locus 19q13 where it encompasses a 2.5 kb region (OMIM *606464). Alternatively named liver-expressed antimicrobial peptide (*LEAP*) and hepcidin (*HEPC*), the gene includes three exons and it allows for synthesis of a prepropeptide that is 84 amino acids in length (Krause *et al.* 2000, Park *et al.* 2001, Pigeon *et al.* 2001, reviewed by Ganz 2003).

A 24-residue N-terminal signal sequence, a pentaaginyl proteolysis site and an active C-terminal peptide consisting of 25 amino acids characterize the protein. A total of eight cysteines allow for the formation of four disulfide bridges and this, in turn, ensures the stabilization of the active peptide, beta-sheet structure (Krause *et al.* 2000, Park *et al.* 2001, reviewed by Ganz 2003). The positional separation of the hydrophobic and hydrophilic side

chains observed in *HAMP* is characteristic of antimicrobial peptides (reviewed by Ganz 2003). Protein expression sites identified *via* reverse transcriptase polymerase chain reaction (RT-PCR) analysis included the liver, heart, brain and lungs (Krause *et al.* 2000). The role of *HAMP* as iron regulator is evidenced by murine studies in which transgenic mice developed microcytic hypchromic anemia due to *HAMP* overexpression (Nicolas *et al.* 2002).

The *HAMP* gene has been associated with the development of juvenile haemochromatosis. Described variants include R56X, responsible for protein truncation, and 1 bp deletion, 93delG, resulting in an elongated propeptide (Roetto *et al.* 2003).

1.8.3 *CYBRD1*

Cytochrome b reductase 1 (*CYBRD1*), alias *DCYTB*, is located at chromosome position 2q31 (OMIM *605745). The gene includes five exons within its 35.6 kb length and it codes for a 4 254 bp long mRNA that, when spliced, gives rise to three alternative transcripts. The 286 amino acid protein contains six transmembrane domains and four conserved Histidine residues. The *CYBRD1* gene was mapped to chromosome two by the International Radiation Hybrid Mapping Consortium (McKie *et al.* 2001).

Functionally, the protein has a suggested involvement in transport of iron across the epithelial cells of the intestine, with its expression observed in the brush-border membrane of duodenal enterocytes. It may be responsible for the reduction of iron from ferric to ferrous ($Fe^{3+} \rightarrow Fe^{2+}$), the latter form allowing for transport into villus cells *via* the divalent metal transporter 1 (*DMT1* also known as natural resistance-associated macrophage protein 2 (*NRAMP2*))

(McKie *et al.* 2001, Lee *et al.* 2002, Hentze *et al.* 2004). Evidence from animal studies (mice) showed contradictory findings as to the suggested role of the *DCYTB* gene in iron homeostasis. Early findings show murine haemochromatosis resulting in increased *DCYTB* expression whilst a more recent study, involving loss of gene function *via* gene targeting, demonstrated normal dietary iron absorption (Muckenthaler *et al.* 2003, Herrmann *et al.* 2004, Gunshin *et al.* 2005).

1.8.5 *HJV*

The hemojuvelin (*HJV*) gene spans 4 265 base pairs and is positioned at chromosome region 1q21 (OMIM *608374). The gene includes four exons coding for a 2.2 kb mRNA transcript with the various spliced isoforms encoding for proteins proposed to be 200, 313 and 423 amino acids in length (GENATLAS database, Papanikolaou *et al.* 2004, Celec 2005).

The suggested structure is that of a 'von Willebrand factor type D domain' containing transmembrane protein furthermore characterized by the presence of an 'Arginine-Glycine-Asparagine' (Arg-Gly-Asp) motif. Sites of protein expression include the heart, skeletal muscle, liver and pancreas (Celec 2005). Murine studies that entailed mutation of the *HJV* gene were achieved by the integration of a targeting construct. The loss of gene function was met with iron overload and these animal models thus suggest that the *HJV* gene is involved in iron homeostasis (Niederkofler *et al.* 2005).

The development of haemochromatosis type 2a has shown association with variants identified in the *HJV* gene. The mutations include the single nucleotide variants, I218T and G320V (Papanikolaou *et al.* 2004).

1.9 OBJECTIVES OF THIS STUDY

Objectives of this study:

i Mutation analysis of *HFE, SLC40A1, HAMP, CYBRD1* and *HJV* was performed to investigate these genes as potential modifier loci in MS the pathogenesis.

ii Statistical analysis of the variants identified to:

a) test for significant differences in variant prevalence between the patient and control groups

b) investigate potential gene-gene interaction

c) establish genotype-phenotype correlations with the determined serum iron and ferritin levels

CHAPTER TWO

2. DETAILED EXPERIMENTAL PROCEDURES

2.1 SUBJECTS

A total of 40 blood samples were obtained from patients presenting with MS. The patient cohort comprises of 33 females and seven males (mean age: females ~ 43; males ~ 39). Ethnically the group consists of seven Coloured cases (six females and one male) and 33 Caucasian individuals (27 females and six males). The population termed 'Caucasian' consists of individuals of European origin (Dutch, German, British and French). The 'Coloured' individuals are descendant of the San, Khoi, Javanese, African Negro and Western European populations (Loubser *et al.* 1999). Patients were referred to Tygerberg hospital, South Africa and diagnosed with relapsing-remitting type MS. Ethical approval for the project has been obtained from the Research Committee of theUniversity of Stellenbosch, no: 96/099. Written informed consent was obtained from both the patient and control groups.

An additional 70 whole blood samples were obtained as population-matched controls, these included 20 individuals of Coloured (17 females and three males) and 50 Caucasian individuals (38 female and 12 males) (mean age: females ~ 42; males ~ 45).

2.2 METHODS

2.2.1 DNA extraction

A modification of the Miller *et al.* (1988) technique was used to extract DNA from a 15 ml whole blood sample. The initial extraction step required the transfer of each whole blood sample to a 50 ml Falcon tube (Merck), allowing for the addition of 30 ml of cold lysis buffer (155 mM ammonium chloride (NH_4Cl), 10 mM potassium hydrogen carbonate ($KHCO_3$) and 0.1 mM ethylene diamine tetra-acetic acid (EDTA) – pH 7.4). The solution was placed on ice for 15 minutes and mixed by means of inversion at 5-minute intervals. This step allowed for complete lysis of the red blood cells and was followed by centrifugation for 10 minutes at 1500 revolutions per minute (rpm) (Hermle Z 200 A, Labnet).

The supernatant was removed and the washing of the pellet with 10 ml cold phosphate buffered saline (PBS) (27 mM potassium chloride (KCl), 137 mM sodium chloride (NaCl), 8 mM di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and 1.5 mM potassium dihydrogen orthophosphate (KH_2PO_4)), followed. The solution was centrifuged for 10 minutes at 1500 rpm (Hermle Z 200 A, Labnet) and the supernatant was subsequently discarded. Addition of 3 ml nucleic lysis buffer (10 mM Tris(hydroxymethyl)aminomethane (Tris-HCl) ($(CH_2OH)_3CNH_2$-Cl), 400 mM NaCl and 2 mM EDTA – pH 8.2), 1% (w/v) sodium dodecyl sulphate (SDS) and 1.5 mg/ml proteinase K (Roche Diagnostics), aided the resuspension of the pellet. It was then mixed and placed in a water bath at 55ºC for overnight incubation.

The incubation step was followed by the addition of 1 ml saturated 6 mM NaCl and vigorous shaking of the solution for 1 minute. The sample was then centrifuged for 30 minutes at 3500 rpm (Hermle Z 200 A, Labnet) and the supernatant placed in a new Falcon tube. The supernatant was shaken for 15 seconds, centrifuged for 15 minutes at 2500 rpm (Hermle Z 200 A, Labnet) and transferred to a clean tube. Two volumes of ice-cold (±99.9%) (v/v) ethanol (EtOH) was added to the solution to allow for precipitation of the DNA at room temperature (30 minutes).

The DNA was transferred to a new 1.5 ml tube (Eppendorf). This was followed by the addition of 1 ml 70% (v/v) EtOH for the removal of excess salt. The solution was centrifuged for 15 minutes at 14 000 rpm (4ºC) (Avanti^{TM} 30 Centrifuge, Beckman), the EtOH was removed and the pellet air-dried at room temperature. The DNA pellet was dissolved in 200-800 µl double distilled water (ddH_2O) (dependent on the pellet size), shaken at room temperature overnight and then stored at 4ºC. Spectrophotometry allowed for determination of DNA quantity and quality (Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies,USA)).

2.2.2 Polymerase chain reaction (PCR) amplification

Polymerase chain reaction amplification of the various exons under investigation was performed in 25 µl reactions, consisting of 50 ng DNA, 0.25 mM of each 2'-deoxynucleotide (dNTP) (dATP, dCTP, dGTP, dTTP) (Fermentas), 10 pmol of each primer (Inqaba Biotech), 0.5U *Taq* polymerase (Fermentas), 1 x ammonium sulphate buffer ($(NH_4)_2SO_4$) (Fermentas) and magnesium chloride ($MgCl_2$) (Fermentas) as specified in Table 2.1. Primer design was

achieved using the Primer3 programme (Rozen and Skaletsky 2000) and the reference gene sequences as listed in Table 2.1.

Amplification was achieved using an Applied Biosystems PCR cycler (GeneAmp®PCR system 2700). Four different PCR programs were used to amplify the exons under investigation and they have been designated programs A to D.

PCR program A was characterized by an initial denaturation step at 94ºC for 5 minutes. This was followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing for 30 seconds (as specified for each exon and listed in Table 2.1), and a 30 second extension at 72ºC. A final extension step was performed at 72ºC for 10 minutes.

Program B was initiated by a 2 minute, 95ºC denaturation step. This was ensued by 35 cycles of denaturation at 95ºC for 30 seconds, annealing for 45 seconds (as specified for each exon and listed in Table 2.1) and an extension at 72ºC for 30 seconds. Program completion was characterized by an extension step at 72ºC for 10 minutes.

Program C was as follows: denaturation for 5 minutes at 95ºC. The subsequent 35 cycles of denaturation at 95ºC for 1 minute and annealing for 2 minutes (as specified for each exon and listed in Table 2.1) preceded a final 72ºC, 10 minute, extension step.

PCR conditions for program D consisted of denaturation at 95ºC for 2 minutes. This was followed by 10 cycles of denaturation for 30 seconds at 95ºC, annealing for 45 seconds (according to the specified annealing temperature listed in Table 2.1 (Ann 1)) and a 30 second

extension at 72ºC. The same conditions were then repeated for a total of 30 cycles (according to the specified annealing temperature listed in Table 2.1 (Ann 2)). An extension step was achieved at 72ºC for 5 minutes.

Table 2.1 Oligonucleotide primers designed for amplification of PCR products subjected to HEX-SSCP analysis

EXON	FORWARD PRIMER (5'-3')	*Tm (ºC)	REVERSE PRIMER (5'-3')	*Tm (ºC)	PRODUCT SIZE (bp)	$MgCl_2$	PCR PROGRAM	ANN 1	ANN 2	REFERENCE SEQUENCE
			***HFE* gene**							NM_000410 (GENATLAS)
[a] 1	TTACTGGGCATCTCCTGAGC	62.45	CTAGTTTCGATTTTTCCACCCC	60.81	256	1.5 µl	C	55		
2A	[b] ACATGGTTAAGGCCTGTTGC	60.40	[a] TACCCTTGCTGTGGTTGTGA	60.40	298	1.5 µl	C	55		
2B	[a] TGACCAGCTGTTCGTGTTCT	60.40	[b] CAGCTGTTTCCTTCAAGATGCA	60.81	257	1.5 µl	C	55		
[a] 3A	CTTGGGGATGGTGGAAATAG	60.40	CTCCAGGTAGGCCCTGTTCT	64.50	279	1.5 µl	C	57		
[a] 3B	CGAGGGCTACTGGAAGTACG	64.50	CTGCAACCTCCTCCACTCTG	64.50	280	1.5 µl	C	57		
4A	[b] TGGCAAGGGTAAACAGATCC	60.40	[a] TCCACCTGGCACGTATATCTC	62.57	289	1.5 µl	C	57		
4B	[a] TACCCCCAGAACATCACCAT	60.40	[b] CTCAGGCACTCCTCTCAACC	64.50	265	1.5 µl	C	57		
5	[a] GAGAGCCAGGAGCTGAGAAA	62.45	[b] CAGAGGTACTAAGAGACTTC	58.35	297	1.5 µl	C	55		
6	[b] TAGTGCCCAGGTCTAAATTG	58.35	[b] TGAGTCTCTAGTTTTGTCTCC	58.66	202	1.5 µl	C	57		
			***SLC40A1* gene**							NM_014585 (GENATLAS)
[c] 1A	CCAGTCGGAGGTCGCAGG	66.73	CAGGAGTGCAAGGAACTGG	62.32	318	0.75 µl	D	60		
[c] 1B	CCAAAGTCGTCGTTGTAGTC	60.4	TTCCTCCAGAACTCGTGTAG	60.4	276	2 µl	B	55		
[d] 2	TGGATAAGCATTCTGCCCTC	60	AAAGCATGTGTACTTGGATG	56	275	2 µl	B	55		
[c] 3	GATAAGGAAGCAACTTCCTG	58.35	CCTGGTTGTTTCTCTCCTAG	60.4	339	2 µl	E	60	55	
[d] 4	GGATAAGAACAGTCTCACTG	58	TTCATCCTTTACCACTACCAG	60	243	2 µl	E	60	55	
[d] 5	TTAAACTGCCTTGTTTAGTG	54	GCCTCATTTATCACCACCG	58	278	2 µl	E	60	55	
[c] 6	TTGTGTAAATGGGCAGTCTC	58.35	CATTTAAGGTCTGAACATGAG	56.71	368	3 µl	D	60		
[c] 7A	GCTTTTATTTCTACATGTCC	54.25	CCAGTTATAGCTGATGCTC	58.01	352	2 µl	D	60		
[c] 7B	GGGTACGCCTACACTCAG	62.18	CAGTTGTAATTTCAGGTATC	54.25	298	2 µl	E	60	55	
[c] 7C	GAAGATATCCGATCAAGGTTC	58.66	TTAATGGATTCTCTGAACCTAC	57.08	259	2 µl	B	55		
[c] 8A	TTGAAATGTATGCCTGTAAAC	54.76	TTCCTTCCTAACTTCTTTTGC	56.71	343	3 µl	D	60		
[c] 8B	CCGATTTGCCCAAAATACTC	58.35	TTTCCATGCCTCAACATAAGG	58.66	297	2 µl	B	55		
[c] 8C	GTTTTTACCACAGCTGTGCC	60.4	GTCTTCATACTTGAAGAATTTG	55.22	359	2 µl	B	55		

Key: *Tm = 2(nA+nT) + 4(nG+nC) (Thein and Wallace 1986), Abbreviations: Tm – melting temperature, Ann – annealing temperature, bp – base pairs
References: [a]VR Human, [b]Prof C Camaschella, [c]This study, [d]Njajou *et al.* 2001

Table 2.1 Oligonucleotide primers designed for amplification of PCR products subjected to HEX-SSCP analysis **(continued)**

EXON	FORWARD PRIMER (5'-3')	*Tm (ºC)	REVERSE PRIMER (5'-3')	*Tm (ºC)	PRODUCT SIZE (bp)	$MgCl_2$	PCR PROGRAM	ANN 1	REFERENCE SEQUENCE
			***HAMP* gene**						NM_021175.2 (GENATLAS)
[e] 1	AGCAAAGGGGAGGGGGCTCAGACC	71.40	TCCCATCCCTGCTGCCCTGCTAAG	69.69	262	1.5 µl	D	60	
[a] 2	AAACCACTTGGAGAGGAGCA	60.40	GAAGGAAGGGAATGTGAGCA	60.40	235	1.5 µl	D	55	
[a] 3	GCAACAGTGATGCCTTTCCT	60.40	CCAGCCATTTTATTCCAAGACC	60.81	272	1.5 µl	D	55	
			***CYBRD1* gene**						NM_024843 (GENATLAS)
[f] 1	GAGACAGCCCCAAGAAGTCG	64.5	TTCACGGAGGACCCTCTGCC	66.55	378	2 µl	A	60.5	
[f] 2	CCAGTGTGTCAAACTGTTC	58.01	CATTTACAGTCTGAATTG	54.25	346	2 µl	A	51.1	
[f] 3	TTGTCATACACATATTGC	52.8	CATTTTCCCAGTGAACAAGTA	56.71	318	2 µl	A	53.8	
[f] 4A	GCATGTTGCTGTATCATCCTGT	60.81	AGAGTAGGCTGGCATGGAAC	62.45	254	2 µl	A	57	
[f] 4B	AAATGGAGGCACTGAACAGG	60.4	AGGAGAAGCAAAACTGTAGAGC	60.81	217	2 µl	A	57	
			***HJV* gene**						ENS00000168509 (ENSEMBL)
[f] 1	TCTGGCCAGCCATATACTCC	62.45	CAGCATTTGGACGAGACA	57.62	293	1.5 µl	A	58	
[f] 2	CACTCCACATTATCCTTACC	58.35	ATGCCCACCCCTACATAGC	62.32	284	2 µl	A	56	
[f] 3A	ACACTCCGATAGAGCAGAGG	62.45	TCTTCGATGCCATGTACCG	60.16	298	2 µl	A	56	
[f] 3B	TAGAGGTGGGGGTTCATCAG	62.45	CGGCCTTCATAGTCACAAGG	62.45	300	2 µl	A	58	
[f] 3C	GACCTGATGATCCAGCACAA	60.4	TGGCTTGGACAAAGAGGAAG	60.4	287	2 µl	A	56	
[f] 3D	CCGGACCCTTGTGACTATGA	62.45	GTGCCGTGGAAGAATCCTC	62.32	279	2 µl	A	58	
[f] 4A	TCAAGGATTGAGGGCCATAG	60.4	TGGATCTCCACATGGTTCC	60.16	300	2 µl	A	56	
[f] 4B	GGTGGATAATCTTCCTGTAGC	60.61	CGACGATTGCGCTCTGAT	59.9	288	2 µl	A	56	
[f] 4C	GCTCTCCTTCTCCATCAAGG	62.45	CTGAGCTGCCACGGTAAAGT	62.45	256	2 µl	A	58	
[f] 4D	GGGCTTCCAGTGGAAGATGC	64.5	CCCCTTACTGAATGCAAAGC	60.4	238	2 µl	A	58	
[f] 4E	CATCTCTTCCCCTCAGATGC	62.45	GATCCGGAATGCAGTAACCT	60.4	300	2 µl	A	56	
[f] 4F	AAGCAGGGCCTAGGAGACAC	64.5	TGCTTTCAGCTCTTGCCTCT	60.4	283	2 µl	A	58	
[f] 4G	CTGCATTCCGGATCTCTGTG	62.45	TTTTGAATCAAGAAAGCAGAACA	55.64	291	2 µl	A	56	
[f] 4H	TGTGTGTGTAAGGTATGTTCTGC	60.99	CTGATACTTCCGAGCCCTCTTTC	64.55	261	2 µl	A	58	

Key: *Tm = 2(nA+nT) + 4(nG+nC) (Thein and Wallace 1986), Abbreviations: Tm – melting temperature, Ann – annealing temperature, bp – base pairs
References: [a]VR Human, [e]Merryweather-Clarke, [f]F Booley

2.2.3 Agarose gel electrophoresis

PCR products were electrophoresed on a 2% (w/v) horizontal agarose gel [consisting of 4 g agarose, 200 ml Tris-borate/EDTA (TBE) (90 mM Tris-HCl (pH 8.0), 90 mM boric acid (H_3BO_3) and 1 mM EDTA) and 0.01% ethidium bromide (EtBr))] to determine successful amplification. The PCR product (5 µl) and Cresol red loading buffer (5 µl) [consisting of 0.02% (w/v) cresol red and 0.34% (w/v) sucrose] was mixed and loaded in the wells of the agarose gel. Electrophoresis was performed at 120 V for an hour in 1 x TBE buffer solution. A 100 base pair (bp) ladder (Fermentas) verified amplification of the correct fragment size. Visualization was achieved by ultraviolet light transillumination on the Multigenius Bio Imaging System (Syngene, Cambridge,UK).

2.2.4 Heteroduplex Single-Stranded Conformation Polymorphism analysis (HEX-SSCP)

The PCR amplified products were subjected to Heteroduplex Single-Stranded Conformation Polymorphism (HEX-SSCP) analysis (Kotze *et al.* 1995) performed on a Hoefer vertical gel apparatus. Electrophoresis was achieved on 12% (w/v) polyacrylamide (PAA) gels supplemented with urea ($(NH_2)_2CO$) [consisting of 7.5% (w/v) Urea, 1.5 x TBE (135 mM Tris-HCl (pH 8.0), 135 mM boric acid and 1.5 mM EDTA), 12% (w/v) PAA (1%C of a 40% stock [99 acrylamide (AA):1 bisacrylamide (BAA)], 0.1% (w/v) ammonium persulfate (APS) and 0.1% (v/v) TEMED.

Gels were cast, allowed to completely polymerise and then placed into the electrophoresis tank filled with 1 x TBE buffer. The upper chamber was subsequently filled with 1.5 x TBE

buffer (135 mM Tris-HCl (pH 8.0), 135 mM boric acid and 1.5 mM EDTA). Bromophenol blue loading buffer (15 µl) [consisting of 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 95% (v/v) formamide (de-ionised) and 20 mM EDTA] was added to 20 µl of the PCR products. The solution was then denatured for 10 minutes at 95ºC and immediately placed on ice. The denatured PCR product (15 µl) was loaded and electrophoresed at 4˚C at 300V for 16 hours.

Further HEX-SSCP analysis was performed using 10% polyacrylamide gels supplemented with glycerol [gel consisting of 10% (v/v) glycerol, 0.5 x TBE (45 mM Tris-HCl (pH 8.0), 45 mM boric acid and 0.5 mM EDTA), 600 µl APS and 60 µl TEMED]. This additional analysis was done only for exons showing limited visualization on the original urea supplemented gels. The protocol used was similar to the one described for urea with differences pertaining to TBE concentration and temperature. The upper chamber of the electrophoresis tank was filled with 0.5 x TBE buffer and electrophoresis was done at room temperature.

Staining of gels was achieved using both silver staining and EtBr staining. The silver staining method (Beidler *et al.* 1982) entailed disassembling of gels and fixing of the DNA in 300 ml fixing solution (10% (v/v) ethanol and 0.5% (v/v) acetic acid) for 10 minutes. Washing with dH_2O for 1 minute, followed. The gels were stained in 300 ml staining solution (consisting of 0.1% (w/v) silver nitrate ($AgNO_3$)) and rinsed in dH_2O for 5 seconds. The final developing step was marked by addition of formaldehyde to the developing solution (1.5% (w/v) sodium hydroxide (NaOH) and 0.155% (v/v) formaldehyde) and the subsequent developing of the gels therewith. The gel developing continued until clear bands were visually identified whereafter the gels were rinsed with dH_2O and sealed between 2 transparencies. Washing of

the gels with the silver staining solutions was achieved using an orbital shaker (Stoball Life Sciences Inc, NC,USA).

Staining with EtBr was achieved by placing the gels within the EtBr solution (0.01% (v/v)) for 10 minutes, followed by 3 minutes of destaining in ddH_2O. DNA fragments were visualized (ultraviolet light transillumination) and photographed with the Multigenius Bio Imaging System (Syngene, Cambridge,UK).

2.2.5 Restriction enzyme digestion

Restriction enzyme digestion was performed for the *HFE* gene variant, IVS2+4T→C, and the *CYBRD1* gene variant, S266N, because successful scoring of the variants could not be achieved using HEX-SSCP images. Genotyping of the variants was achieved by using the enzymes *RsaI* (IVS2+4T→C) (Fermentas) and *TspRI* (S266N) (New England Biolabs).

Digestion with *RsaI* was performed in 20 µl reactions consisting of 10 µl PCR product, 1 x Tango^{TM} buffer [consisting of 33mM Tris-acetate (TA) (pH 7.9 at 37°C), 10 mM magnesium acetate (MgAc), 66 mM potassium acetate (KAc), 0.1 mg/ml bovine serum albumin (BSA)] and 2U enzyme. The digestion reactions were placed in a water bath for 16 hours at 37ºC.

TspRI digestion was similarly achieved in 20 µl reactions. This included 10 µl PCR product, 1 x NEBuffer 4 (consisting of 50 mM KAc, 20 mM TA, 10 mM MgAc, 1 mM dithiothreitol (DTT)), 100 µg/ml BSA and 10U enzyme. Water bath incubation was performed for 16 hours at 65ºC.

Subsequently, 10 µl of the digested product was mixed with 10 µl cresol loading buffer [consisting of 0.02% (w/v) cresol red and 0.34% (w/v) sucrose] and loaded on a 2% (w/v) agarose horizontal agarose gel [consisting of 4 g agarose, 200 ml 1 x TBE (90 mM Tris-HCl (pH 8.0), 90 mM boric acid and 1 mM EDTA) and 0.01% (v/v) ethidium bromide (EtBr))]. Loading of a 100 bp O'GeneRuler^{TM} (5 µl) (0.1 µg/µl, Fermentas) allowed for identification of digested fragment sizes. Samples were then electrophoresed at 80 V for 90 minutes in 1 x TBE buffer solution (90 mM Tris-HCl (pH 8.0), 90 mM boric acid and 1 mM EDTA). Visualization of the gels using the ultraviolet light transillumination on the Multigenius Bio Imaging System (Syngene, Cambridge,UK), allowed for genotype determination.

2.2.6 Semi-automated DNA sequencing

Samples showing conformational variation upon HEX-SSCP analysis were subjected to semi-automated DNA sequencing. The PCR products were purified prior to sequencing using the Wizard SV Gel and PCR Clean-Up System (Promega). Briefly, the purification technique included addition of an equal volume of membrane binding solution (4500 mM guanidine isothiocyanate (GIT), 500 mM KAc (pH 5.0)) to the PCR product. The solution was then transferred to a Spin Column assembly and incubated for 1 minute at room temperature. Centrifugation of the column was done at 14 000 rpm for 1 minute (Eppendorf centrifuge 5415 D) and the filtered liquid discarded.

Subsequently, membrane washing solution (10 mM KAc (pH 5.0), 80% (v/v) ethanol, 0.017 mM EDTA) (700 µl) was added to the Spin Column assembly and centrifuged at 14 000 rpm for 1 minute (Eppendorf centrifuge 5415 D). This was followed by a further washing step that

entailed addition of 500 µl membrane washing solution and centrifugation at 14 000 rpm for 1 minute (Eppendorf centrifuge 5415 D). The filtered solution/ flow-through was discarded and a final centrifugation step was performed to allow for evaporation of the residual EtOH (14 000 rpm, 1 minute) (Eppendorf centrifuge 5415 D). The Minicolumn was placed in a clean 1.5 µl microcentrifuge tube and 50 µl nuclease free water applied directly to the column centre. The final step incorporated incubation for 1 minute at room temperature and centrifugation (14 000 rpm, 1 minute) (Eppendorf centrifuge 5415 D). Eluted DNA was stored at 4ºC.

Cycle sequencing was performed on a GeneAmp®PCR system 2700 thermocycler. Each reaction consisted addition of 3.3 ng/µl primer (Table 2.1), 1 µl termination ready reaction mix [BigDye® Terminator v3.1 cycle sequence kit (Applied Biosystems)] and 9.9 ng of the purified PCR product. The cycle program was initiated by a denaturation step for 10 seconds at 96ºC. This was followed by 25 cycles of denaturation for 10 seconds at 96ºC, annealing for 10 seconds at 96ºC and an extension for 4 minutes at 60ºC. The products were loaded onto an ABI Prism 3130Xl Genetic Analyzer (Applied Biosystems) automated sequencer and subsequently electrophoresed. Analysis of sequencing results entailed a) visual examination of the electropherograms and b) alignment of sequences with the reference (wild-type) sequence (reference sequences for primer design: listed in Table 2.1) using BioEdit Sequence Alignment Editor v7.0.1 (Hall 1999).

2.2.7 Statistical analysis

Allele and genotype frequencies were calculated for both the patient and control populations. The Hardy-Weinberg equilibrium (HWE) test was performed for the respective populations with regards to each variant identified ($p>0.05$). The two groups were then compared statistically and differences tested for significance by means of chi-squared (χ^2) analysis with Yates' correction and/or Fischer's exact test (applicable to smaller population sizes) (Elston and Forthofer 1977). Statistical significance was defined as a probability value smaller than 0.05. Analysis was achieved using the, Microsoft Excel 2000 software, allowing for determination of frequencies and probability values. Results were verified with both STATISTICA (StatSoft, Inc. (2003) STAT (data analysis software system), version 6 and the Epi Info computer software (Epi Info™ (utilities StatCalc) v3.3.2, release date: 9 February 2005; Division of Public Health Surveillance). Investigation of gene-gene interaction and genotype-phenotype correlations were done according to Butt *et al.* (2003).

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Table 2.2 Alphabetic list of chemicals and reagents

Chemical/Reagent	Supplier
AA (acrylamide)	Sigma-Aldrich
Acetic acid	Associated Chemical Enterprises
Agarose	Bio-basic
APS (ammonium persulfate)	Associated Chemical Enterprises
BAA (bisacrylamide)	Sigma-Aldrich
BigDye® Terminator v3.1 cycle sequence kit	Applied Biosystems
Boric acid	Sigma-Aldrich
Bromophenol blue	Sigma-Aldrich
BSA (bovine serum albumin for TspR I digestion)	New England Biolabs
Cresol red	Sigma-Aldrich
ddH_2O (double distilled water)	Adcock Ingram
2'-deoxynucleotide (dNTPs) (dATP, dCTP, dGTP, dTTP)	Fermentas
EDTA (ethylene diamine tetra-acetic acid)	Sigma-Aldrich
EtBr (ethidium bromide)	Sigma-Aldrich
EtOH (ethanol)	Sigma-Aldrich
Formaldehyde	Associated Chemical Enterprises
Formamide	Sigma-Aldrich
Gliserol	Merck
Glucose	Associated Chemical Enterprises
KCl (potassium chloride)	Sigma-Aldrich
$KHCO_3$ (potassium hydrogen carbonate)	Sigma-Aldrich
KH_2PO_4 (potassium dihydrogen orthophosphate)	Sigma-Aldrich
O'GeneRuler™ (Ladder 100 bp)	Fermentas
$MgCl_2$ (magnesium chloride)	Fermentas
NaCl (sodium chloride)	Sigma-Aldrich
Na_2HPO_4 (di-sodium hydrogen orthophosphate anhydrous)	Sigma-Aldrich
NaOH (sodium hydroxide)	Sigma-Aldrich
NEBuffer 4	New England Biolabs
NH_4Cl (ammonium chloride)	Sigma-Aldrich
$(NH_4)_2SO_4$ (ammonium sulfate buffer)	Fermentas
PBS (phophate buffered saline)	Oxoid
Proteinase K	Roche diagnostics
Rsa I	Fermentas
SDS (sodium dodecyl sulphate)	Sigma-Aldrich
Silver nitrate ($AgNO_3$)	Sigma-Aldrich
Tango™ buffer	Fermentas
***Taq* polymerase**	Fermentas
TEMED	Sigma-Aldrich
Tris-HCl (Tris(hydroxymethyl)aminomethane)	Sigma-Aldrich
TspR I	New England Biolabs
Urea	Sigma-Aldrich
Wizard SV Gel and PCR Clean-Up System	Promega
Xylene cyanol	Sigma-Aldrich

CHAPTER THREE

The potential involvement of genes related to iron metabolism in the development of multiple sclerosis

3. ABSTRACT

Iron has been implicated in the development of multiple sclerosis (MS) due to the various roles of iron in neurodegeneration, myolinogenesis, immunity, autoimmunity and infection resistance. Our study population included unrelated MS patients from South Africa, with individuals mainly from the Caucasian and Coloured populations. Genes involved in iron homeostasis/metabolism have been screened for variants possibly involved in the pathogenesis of the disease. These genes include *HFE*, *SLC40A1, HAMP, CYBRD1* and *HJV*. Mutation detection was performed by screening with the Heteroduplex Single-Stranded Conformation Polymorphism (HEX-SSCP) technique. This allowed for detection of 19 variants. Four of the variants were novel, including IVS4-53G→A (*HFE*), IVS2-65delA (*CYBRD1*), 3'UTR+26delACGTCACGTTTCAAAACTA (*CYBRD1*) and 219delG (*HJV*). The known variants identified, included seven intronic (*HFE*: IVS2+4T→C, IVS4+48G→A, IVS4-44T→C, IVS5-47G→A; *SLC40A1*: IVS1-24G→C; *CYBRD1*: IVS1-4C→G, IVS2+8T→C) and eight exonic variants (*HFE*: H63D, C282Y; *SLC40A1*: I109, V221; *HAMP*: G71D; *CYBRD1*: S266N; *HJV*: A310G, S264).

The statistical analysis performed involved testing for significant differences in variant prevalence between the patient and control groups, potential gene-gene interaction and genotype-phenotype correlations. These investigations, however, revealed no statistically significant associations. The study succeeded in identifying variants and further investigation in a larger cohort is needed to establish the potential contribution of these variants in the development of MS.

3.1 INTRODUCTION

Multiple sclerosis is a disease that incapacitates the proper functioning of the central nervous system. It is characterized by inflammation that causes demyelination of axons and as a result the ability of the CNS to conduct nerve impulses is diminished (Compston *et al.* 1998, Ebers and Dyment 1998, Noseworthy 1999, reviewed by Reipert 2004). Symptomatic manifestation of this loss of neurological function may include gait ataxia and sensory disturbances (Trapp *et al.* 1998, Lucchinetti *et al.* 1999, Bitsch *et al.* 2000, Noseworthy *et al.* 2000).

The disease predominately affects females and has been categorized into three sub-types that vary according to the pathogenesis [relapsing-remitting multiple sclerosis (RRMS), primary progressive (PPMS) and secondary progressive (SPMS)] (Weinshenker 1994, McDonnell and Hawkins 1998, Cottrell *et al.* 1999, reviewed by Noseworthy *et al.* 2000, reviewed by Pender 2004, reviewed Reipert 2004). RRMS is characterized by an ongoing cycle of acute attack (neurological dysfunction) followed by remission (stabilizing) (reviewed by Noseworthy *et al.* 2000, Goodin *et al.* 2002, reviewed by Reipert 2004). PPMS shows a more gradual development of disease (reviewed by Noseworthy *et al.* 2000, reviewed by Reipert 2004)

whilst SPMS could be explained as a combination of the first two sub-types with neurological deterioration observed both during and between acute attacks (reviewed by Noseworthy *et al.* 2000, reviewed by Reipert 2004).

Several factors have been implicated in the etiology of MS, including a genetic contribution (Oksenberg *et al.* 1996, Oksenberg *et al.* 2001, Keegan and Noseworthy 2002), autoimmunity (Oksenberg *et al.* 2001, Keegan and Noseworthy 2002) and viral infection (Oksenberg *et al.* 2001, Keegan and Noseworthy 2002, Miller *et al.* 2002). Iron, in turn, has a suggested role in each of these factors (Blount *et al.* 1989, LeVine and Makclin 1990, Connor *et al.* 1995, Connor *et al.* 2001, Ahsan *et al.* 2003, LeVine and Chakrabarty 2004).

The majority of studies addressing the genetics of MS have delivered contradictory or ambiguous findings (Kalman and Lublin 1999, Kotze *et al.* 2001). The research aimed at investigating MS and genetics in the South African population is limited. One such study involved screening of a Caucasian cohort for the *HFE* gene mutations H63D and C282Y. Comparison of patient variant frequencies to those obtained for the controls did not reveal statistically significant associations (Kotze *et al.* 2005).

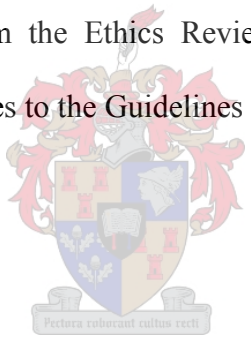
Iron availability is essential for both the proper synthesis of myelin and normal immune system function (Blount *et al.* 1989, LeVine and Makclin 1990, Connor *et al.* 1995, Brock 2000, Connor *et al.* 2001, Ahsan *et al.* 2003, LeVine and Chakrabarty 2004). However, iron is involved in the formation of reactive oxygen intermediates (ROI), molecules that are responsible for oxidative tissue damage. The molecules attacked by these intermediates have been implicated in the initiation of an autoimmune response (Blount *et al.* 1989, Stohs and Bagchi 1995, Lloyd *et al.* 1997, Ahsan *et al.* 2003). Additionally, iron is needed for survival

of pathogens and if not withheld from host-infecting agents, it may contribute to the infectious success of pathogens (Brock 2000, Kotze *et al.* 2001, Ong *et al.* 2006).

This study aims to elucidate the potential role of iron in MS development, by investigating genes involved in iron homeostasis and/or metabolism, including *HFE*, *SLC40A1, HAMP, CYBRD1* and *HJV*, in the South African Caucasian and Coloured populations.

3.2 METHODS

Ethical approval was obtained from the Ethics Review Committee of the University of Stellenbosch (ref: 96/099) that adheres to the Guidelines of Helsinki.



3.2.1 Subjects

The study cohort included 40 unrelated MS patients and 70 healthy population-matched controls from the South African population. The patient group included 33 Caucasian (27 females and six males) and seven Coloured (six females and one male) individuals all diagnosed with relapsing-remitting type MS. Serum iron and ferritin levels were determined for the majority of the patient cohort. The control group included 60 females (43 Caucasian, 17 Coloured) and ten males (seven Caucasian, three Coloured). Iron parameters were not established for the control cohort. In this study, Caucasian refers to individuals from European descent and "Coloured" refers to individuals with Mixed Ancestry (Loubser *et al.* 1999). Written informed consent was obtained from all the participants.

3.2.2 Experimental procedures

A modification of the Miller *et al.* (1988) technique was used to extract DNA from the whole blood samples obtained. Polymerase chain reaction (PCR) amplification was performed using the primers as listed in Table 2.1 (see Chapter Two) to amplify the various exons of the genes investigated. Heteroduplex Single-Stranded Conformation Polymorphism (HEX-SSCP) analysis (Kotze *et al.* 1995) was achieved by resolving the PCR products on 12% polyacrylamide gels supplemented with 7.5% urea. Samples were denatured at 95˚C for 10 min and electrophoresis was done at 4˚C for 16 hours (300V). The exons characterized by limited visualization on the 12% gels were further analyzed using 10% polyacrylamide gels supplemented with 10% glycerol. The gels were subsequently stained with ethidium bromide or by means of the silver staining method (Beidler *et al.* 1982). DNA on ethidium bromide stained gels were visualized by ultraviolet light transillumination. Samples indicating mobility shifts or conformational variation were subjected to semi-automated DNA sequencing (ABI Prism 3130Xl Genetic Analyzer) (refer to Chapter Two for the detailed experimental procedures).

3.2.3 Statistical analysis

Allele and genotype frequencies were calculated for both the patient and control populations. Upon determination of the Hardy-Weinberg equilibrium (HWE) ($p>0.05$), the groups were compared statistically and analysis was achieved by chi-squared (χ^2) analysis with Yates' correction and/or Fischer's exact (applicable to smaller population sizes) (Elston and Forthofer 1977). A probability value smaller than 0.05, was defined as statistically significant.

Analysis performed to determine potential gene-gene interaction and genotype-phenotype correlations, was as according to Butt *et al.* (2003).

3.4 RESULTS

The variants identified in the genes investigated as well as the allele frequencies thereof for patient and control groups are shown in Table 3.1. All the variants studied were in Hardy-Weinberg equilibrium for the various population groups, except for the IVS1-4C→G (*CYBRD1*) and IVS4+48G→A (*HFE*) variants due to an uneven distribution of genotypes (IVS1-4C→G, Caucasian: 12 heterozygous individuals present in the patient group compared to two heterozygous and three homozygous control individuals; IVS4+48G→A, Caucasian: 19 heterozygous patient individuals compared to 48 heterozygous individuals in the control group; Coloured: five heterozygous patients compared to 14 heterozygous individuals in the control group). Statistical analysis was performed using the chi-squared (χ^2) analysis with Yates' correction and/or Fischer's exact test. However, no statistically significant associations were observed upon investigation of the variants identified. The uncorrected chi-squared and probability values for the different variants are provided in the text following.

Table 3.1 Allele frequencies of the variants identified within this study

Gene	Exon/ Intron	Variant	Allele frequencies[a]		Allele frequencies[a]	
			CPMS	CC	MPMS	MC
			(2n=66)	*(2n=100)*	*(2n=14)*	*(2n=38)*
HFE	2	H63D[c] (Feder *et al.* 1996)	0.14	0.09	0.07	0.21
			(2n=64)	*(2n=98)*	*(2n=12)*	*(2n=38)*
	2	IVS2+4T→C[b] (Beutler and West 1997)	0.25	0.37	0.3	0.45
			(2n=60)	*(2n=86)*		
	4	C282Y[b] (Feder *et al.* 1996)	0.06	0.06	-	-
			(2n=66)	*(2n=98)*	*(2n=14)*	*(2n=38)*
	4	IVS4+48G→A[c] (Totaro *et al.* 1997)	0.44	0.49	0.36	0.37
			(2n=66)	*(2n=98)*		
	4	IVS4-53G→A[c, d] (This study)	0.02	-	-	-
			(2n=66)	*(2n=98)*	*(2n=14)*	*(2n=38)*
	4	IVS4-44T→C[c] (Beutler and West 1997)	0.05	0.01	0.14	0.13
			(2n=66)	*(2n=98)*	*(2n=14)*	*(2n=34)*
	5	IVS5-47G→A[b] (Beutler and West 1997)	0.41	0.48	0.64	0.56
			(2n=64)	*(2n=78)*	*(2n=12)*	*(2n=18)*
SLC40A1	1	IVS1-24G→C[b] (Devalia *et al.* 2002)	0.80	0.82	1.00	0.72
					(2n=14)	*(2n=26)*
	4	I109[c] (Zaahl *et al.* 2004)	-	-	0.07	0.04
			(2n=48)	*(2n=82)*	*(2n=14)*	*(2n=24)*
	6	V221[b] (Devalia *et al.* 2002)	0.75	0.68	0.93	0.66
			(2n=58)			
HAMP	3	G71D[c, d] (Merryweather-Clarke *et al.* 2003)	0.03	-	-	-
			(2n=66)	*(2n=86)*	*(2n=14)*	*(2n=24)*
CYBRD1	1	IVS1-4C→G[b] (Zaahl *et al.* 2004)	0.18	0.10	0.21	0.04
			(2n=66)	*(2n=86)*	*(2n=14)*	*(2n=24)*
	2	IVS2+8T→C[b] (Zaahl *et al.* 2004)	0.81	0.86	0.90	0.83
			(2n=64)			
	2	IVS2-65delA[c, d] (This study)	0.03	-	-	-
			(2n=62)	*(2n=78)*	*(2n=14)*	*(2n=36)*
	4	S266N[b] (McKie *et al.* 2001)	0.69	0.79	0.86	0.69
			(2n=62)			
	4	3'UTR+26delACGTCACGTTTCAAAACTA[c] (This study)	0.02	-	-	-
			(2n=66)			
HJV	3	219delG[c, d] (This study)	0.03	-	-	-
			(2n=64)			
	4	S264[c, d] (Lee *et al.* 2004)	0.02	-	-	-
			(2n=64)			
	4	A310G[c, d] (Lee *et al.* 2004)	0.02	-	-	-

Abbreviations: *CPMS* Caucasian MS patient group, *CC* Caucasian control group, *MPMS* Coloured MS patient group, *MC* Coloured control group, *2n* total alleles (variation amongst variants according to the sample total successfully amplified), [a]Allele frequency of polymorphic allele denoted, [b]Identified in both heterozygous and homozygous state, [c]Identified only in heterozygous state, [d]Variants identified only in the patient group, *Probablity values determined for the allele frequencies

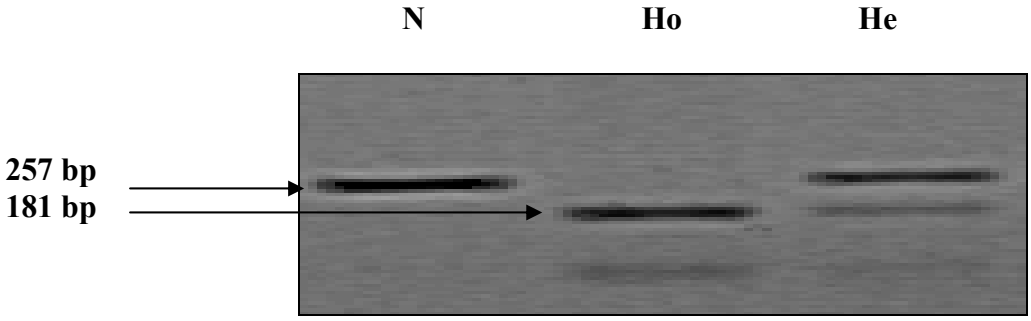
***HFE* gene**

Analysis of the *HFE* gene allowed for detection of six previously characterized variants. These included four intronic variants namely IVS2+4T→C, IVS4+48G→A, IVS4-44T→C and IVS5-47G→A. The remainder, H63D and C282Y, were positioned within exons two and four, respectively. In addition, a novel intronic variant was detected and characterized as IVS4-53G→A.

The IVS2+4T→C variant characterized by a T to C transition at nucleotide position four at the 5' end (donor site) of intron two, was identified in both the Caucasian and Coloured populations using restriction enzyme digestion (Figure 3.1). A total of 12 Caucasian patients were heterozygous for the variant and two were homozygous. The variant representation within the Coloured patient group included two heterozygous individuals and one homozygous patient. Similarly, hetero- and homozygosity for the variant was observed within the Caucasian (28 heterozygous and four homozygous) and Coloured (11 heterozygous, three homozygous) control individuals (statistical analysis: Caucasian: $\chi^2 = 2.45$, $P = 0.12$; Coloured: $\chi^2 = 0.49$, $P = 0.49$).

Figure 3.1

A) IVS2+4T→C*



B) Variant creates a recognition site

Banding pattern	Restriction digest sizes
N	257 bp
He	257 bp, 181 bp
Ho	181 bp

C) *RsaI* recognition site 5' GT↓AC 3'

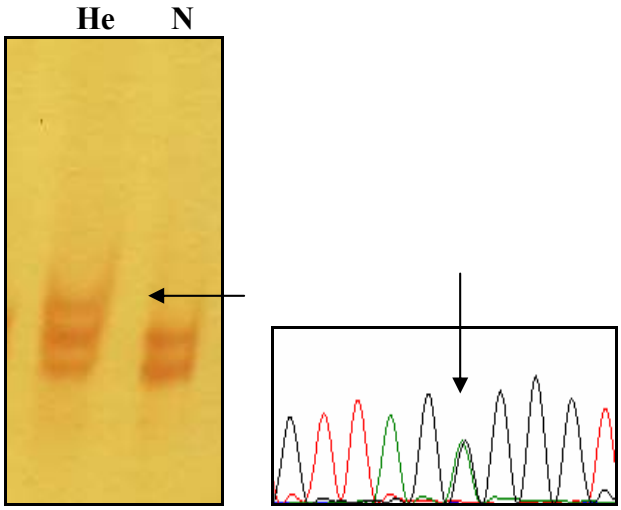
5' tgaccagctgttcgtgttctatgatcatgagagtcgccgtgtggagccccgaactccatgggtttccagtagaatttcaagccaga gtggctgcagctgagtcagagtctgaaagggtgggatcacatgttcactgttgacttctggactattatggaaaatcacaaccacagcaa ggg**gt ac**gtggagaggggcctcaccttcctgaggttgtcagagcttttcatcttttca**tgcatcttgaaggaaacagctg 3'**

Legend to figure 3.1 A) Schematic representation of restriction enzyme digestion using *RsaI* of the IVS2+4T→C variant detected in the *HFE* gene [N = homozygous wild-type, Ho = homozygous variant, He = heterozygous banding pattern], Arrows indicate: varying sized DNA fragments generated using 2% horizontal agarose gel electrophoresis stained with EtBr, **B)** Size differences indicated when variant is present and fragment cut with *RsaI*, **C)** Partial sequence of exon two of the *HFE* gene, Arrow indicates: enzyme recognition site, [*green*, primer binding sites, *blue* enzyme recognition sequence], *100 bp DNA ladder was loaded (not shown here) to verify the size of the fragments generated

The intronic variant IVS4+48G→A caused a G to A transition at nucleotide position 48 at the 5' end of intron four. The variant presented only in its heterozygous form upon analysis of this study cohort. The variant was observed in 29 patients and 48 controls within the Caucasian group whilst the Coloured population showed prevalence of the variant in five patient and 14 control individuals. The presence of IVS4-44T→C, causing a T to C transition at nucleotide position 44 at the 3' end (acceptor site) of intron four, within the study cohort, was identified only in a heterozygous state. The variant was observed in three Caucasian patients and in one population-matched control individual. This variant was also shown to be present in the Coloured population, and was observed in two patients and five control individuals (statistical analysis: Caucasian: $\chi^2 = 2.06$, $P = 0.15$; Coloured: $\chi^2 = 0.01$, $P = 0.92$). The known intronic variant identified, IVS5-47G→A (G to A transition 47 nucleotides from the 3' end of intron 5), was detected in 22 Caucasian patients (17 heterozygous, five homozygous) and in 35 Caucasian controls (23 heterozygous, 12 homozygous). The Coloured group was characterized by three heterozygous and three homozygous patients. Control screening revealed presence of the variant in a total of 13 individuals (seven heterozygous, six homozygous) (statistical analysis: Caucasian: $\chi^2 = 0.79$, $P = 0.37$; Coloured: $\chi^2 = 0.29$, $P = 0.59$). Identification of the novel, single nucleotide base pair variant, IVS4-53G→A, causing a G to A transition at nucleotide position 53 at the 3' end of intron four, was limited to the Caucasian patient group and was identified only in one of 33 patients (refer to Figure 3.2). The variant was present in a heterozygous state.

Figure 3.2

A) IVS4-53G→A



Legend to figure 3.2 Schematic representation of **A)** heteroduplex $AgNO_3$ visualization (12% polyacrylamide gel supplemented with 7.5% urea) and electropherogram of the novel variant, IVS2-65delA, identified in the *CYBRD1* gene [electropherograms: *red* thymidine (T), *blue* cytidine (C), *green* adenosine (A), *black* guanosine (G)] Arrows indicate: aberrant bands (HEX-SSCP); point of variation (electropherograms), N = homozygous wild-type, He = heterozygous banding pattern

The exonic variant, H63D characterized by a nucleotide change from C to G at nucleotide position 187 (g.187C→G, changing the amino acid from Histidine to Aspartic acid) was identified only in its heterozygous state in nine Caucasian MS patients and in one Coloured MS patient. Analysis of the ethnically-matched control individuals recognized nine Caucasian and six Coloured individuals heterozygous for the variant (statistical analysis: Caucasian: χ^2 = 0.88, P = 0.35; Coloured: χ^2 = 1.38, P = 0.24). Screening for the C282Y missense mutation that results in a change at nucleotide position 845 from G to A (g.845G→A, changing the amino acid from Cysteine to Tyrosine), identified two homozygous Caucasian MS patients

and its absence from the Coloured MS patient group. Investigation of the Caucasian control population was subsequently performed, with heterozygosity for the variant established in a total of five control individuals (statistical analysis: Caucasian: $\chi^2 = 0.04$, $P = 0.83$).

***SLC40A1* gene**

Investigation of the *SLC40A1* gene revealed the presence of variation within intron 1 (IVS1-24G→C) and exons four (I109) and six (V221). These are all previously described variants.

The IVS1-24G→C variant caused a G to C transversion at nucleotide position 24 at the 3' end of intron one. Analysis of the Caucasian patient group allowed for detection of this variant in 21 homozygous and nine heterozygous patients. The Caucasian control was characterized by a total of 28 homozygous and eight heterozygous individuals. Screening of the Coloured population allowed for detection of the variant in six homozygous patients. The Coloured control group was characterized by five heterozygous and four homozygous individuals. The statistical analysis of the variant within the Coloured population indicated a marginally significant uncorrected probability value (uncorrected: $\chi^2 = 4.00$, $P = 0.05$; Yates' corrected: $\chi^2 = 2.25$, $P = 0.13$; Fischer's exact: $P = 0.06$ (one-tailed) and $P = 0.07$ (two-tailed)).

I109, a synonymous amino acid substitution (Isoleucine) changing a C to T at nucleotide position 327 (g.327C→T), was identified in one Coloured patient and in one population-matched control individual (statistical analysis: Coloured: $\chi^2 = 0.21$, $P = 0.65$). The V221 variant, changing a T to C at nucleotide position 663 (g.663T→C), causing a synonymous substitution (Valine), was observed in both the Caucasian and Coloured population groups.

Homozygosity for this variant was observed in six Coloured patients whilst only one patient was heterozygous for the variant. A total of 12 heterozygous and 12 homozygous individuals were identified in the Caucasian patient group. Screening of the control group allowed for detection of the variant within both the Caucasian (22 heterozygous and 17 homozygous control individuals) and Coloured (eight heterozygous and four homozygous individuals) population groups (statistical analysis: Caucasian: $\chi^2 = 0.66$, $P = 0.42$; Coloured: $\chi^2 = 3.36$, $P = 0.07$).

HAMP gene

Investigation of the *HAMP* gene, allowed for detection of the previously characterized G71D mutation in exon 3, in two Caucasian patients. This variant, changing a G to an A at nucleotide position 212 (g.212G→A), causing a Glycine to Aspartic acid amino acid substitution, was absent in the population-matched control individuals and was observed only in the heterozygous state.

CYBRD1 gene

HEX-SSCP analysis of the *CYBRD1* gene identified three previously described variants, including two intronic, [IVS1-4C→G (intron one) and IVS2+8T→C (intron two)] and one exonic [S266N (exon four)]. Mutation analysis further revealed two novel variants, including IVS2-65delA (intron two) and 3'UTR+26delACGTCACGTTTCAAAACTA (3' untranslated region) and a previously described single nucleotide variant, S266N (McKie *et al.* 2001)

within exon four. Analysis of *CYBRD1* gene, exons one, two and three showed absence of variation.

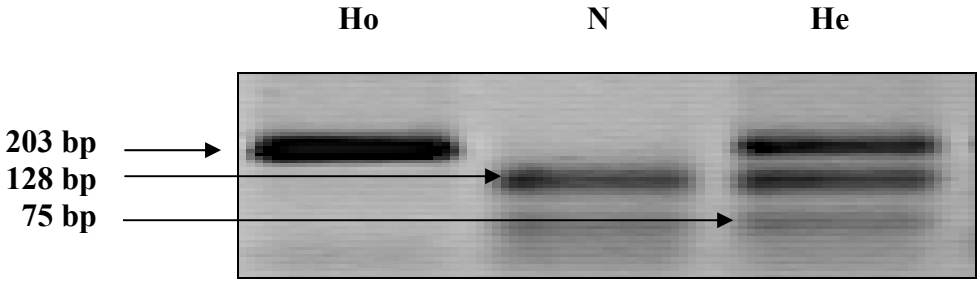
The intronic variant, IVS1-4C→G, causing a C to G transversion at nucleotide position four at the 3' end of intron one, was identified in its heterozygous state in 12 Caucasian and three Coloured patients. Upon analysis of the Caucasian control group, two heterozygous and three homozygous individuals were identified. Investigation of the Coloured control population revealed one heterozygous individual for this variant (statistical analysis: Coloured: $\chi^2 = 2.80$, $P = 0.10$). Variant, IVS2+8T→C, causing a T to C transition at nucleotide position eight from the 5' end of intron two, was present in both the Caucasian and Coloured patient and control groups. The Caucasian group included 12 heterozygous and 21 homozygous patients and an additional 12 heterozygous and 31 homozygous control individuals. The Coloured patient group included two heterozygous and five homozygous individuals whilst the control group comprised of four heterozygous and eight homozygous cases (statistical analysis: Caucasian: $\chi^2 = 0.50$, $P = 0.46$; Coloured: $\chi^2 = 0.04$, $P = 0.85$).

The exonic variant S266N, causing a transversion at nucleotide position 797 (g.797G→A) that results in the amino acid substitution of Serine with Asparagine, was present in both the ethnic groups studied. The variant was also identified in both the heterozygous and homozygous state. A total of 15 heterozygous and 14 homozygous Caucasian patients were identified and the Caucasian control group included 14 heterozygous and 24 homozygous individuals. The Coloured patient group showed two heterozygous and five homozygous individuals. Screening of the population-matched control individuals identified seven

heterozygous cases and nine homozygous individuals (statistical analysis: Caucasian: χ^2 = 1.89, P = 0.17; Coloured: χ^2 = 1.39, P = 0.24) (refer to Figure 3.3).

Figure 3.3

A) S266N



B) Variant abolishes a recognition site

Banding pattern	Restriction digest sizes
N	128 bp, 75bp
He	203 bp, 128 bp, 75 bp
Ho	203 bp

C) *TspRI* recognition site 5' CAG↓TG 3'

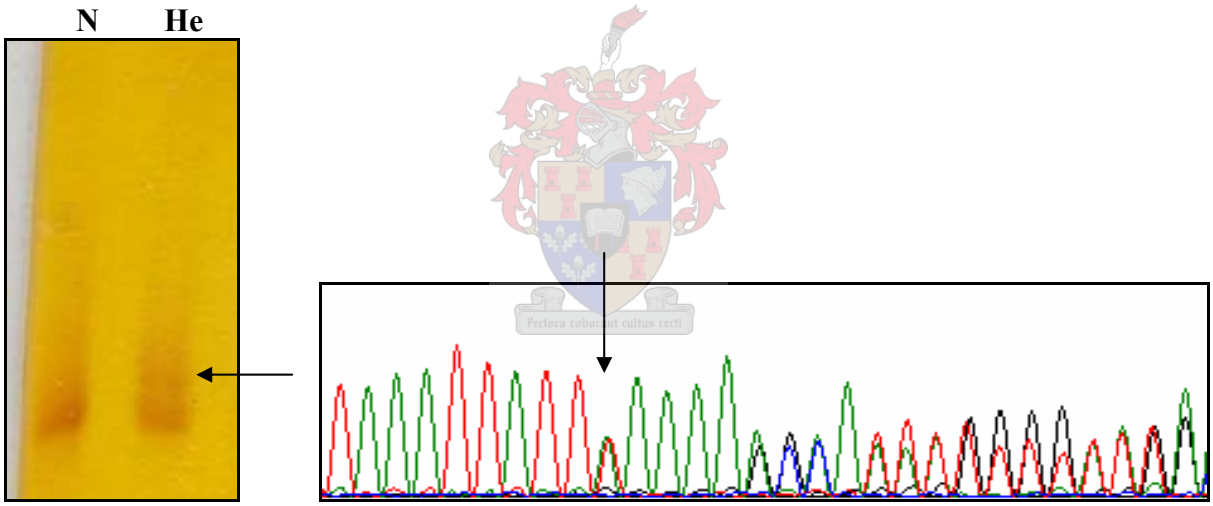
5' aaatggaggcactgaacagggagcaagaggttccatgccagcctactctggcaacaacatggacaaatcagattcagagttaaa
cag tgaagtagcagcaaggaaaagaaacttagctctggatgaggctgggcagagatctaccatgtaaaatgttgtagagatagagcc
atataacgtcacgtttcaaaacta**gctctacagttttgcttctcct 3'**

Legend to figure 3.3 A) Schematic representation of restriction enzyme digestion using *TspRI* of the S266N variant detected in the *CYBRD1* gene [N = homozygous wild-type, Ho = homozygous variant, He = heterozygous banding pattern], Arrows indicate: varying sized DNA fragments generated using 2% horizontal agarose gel electrophoresis stained with EtBr, **B)** Size differences indicated when variant is absent and fragment cut with *TspRI*, **C)** Partial sequence of exon four of the *CYBRD1* gene, Arrow indicates: enzyme recognition site, [*green*, primer binding sites, *blue* enzyme recognition sequence], *100 bp DNA ladder was loaded (not shown here) to verify the size of the fragments generated

The novel *CYBRD1* gene variant IVS2-65delA caused a deletion of an A at nucleotide position 65 from the 3' end of the intron (refer to Figure 3.4). The variant was identified in two Caucasian patients, both being heterozygous and was not identified in the control group. A novel 19 bp deletion in the 3' untranslated region of the gene (3'UTR+26delACGTCACGTTTCAAAACTA') was identified in one Caucasian patient in a heterozygous state and was absent from the population-matched control individuals (refer to Figure 3.5).

Figure 3.4

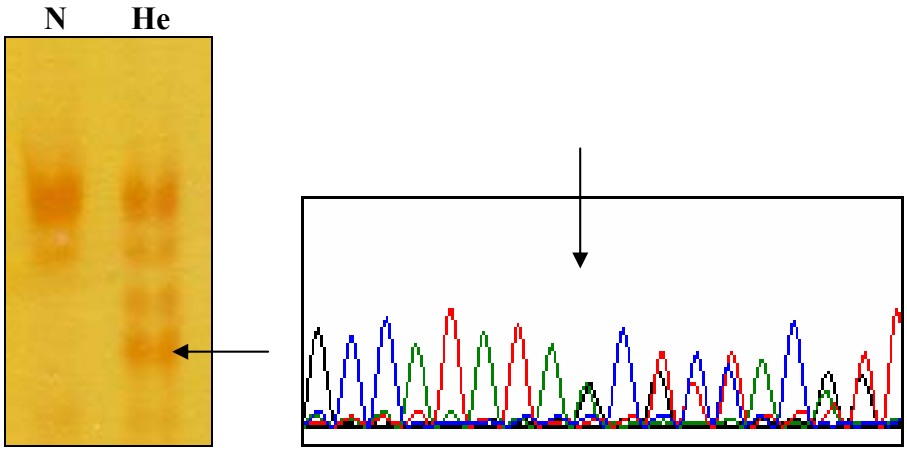
A) IVS2-65delA



Legend to figure 3.4 Schematic representation of **A)** heteroduplex $AgNO_3$ visualization (12% polyacrylamide gel supplemented with 7.5% urea) and electropherogram of the novel variant, IVS2-65delA, identified in the *CYBRD1* gene [electropherograms: *red* thymidine (T), *blue* cytidine (C), *green* adenosine (A), *black* guanosine (G)] Arrows indicate: aberrant bands (HEX-SSCP); point of variation (electropherograms), N = homozygous wild-type, He = heterozygous banding pattern

Figure 3.5

A) 3'UTR+26delACGTCACGTTTCAAAACTA



Legend to figure 3.5 Schematic representation of **A)** single-stranded conformation polymorphism, $AgNO_3$ visualization (12% polyacrylamide gel supplemented with 7.5% urea) and electropherogram of the novel variant, 3'UTR+26delACGTCACGTTTCAAAACTA, identified in the 3' untranslated region of the *CYBRD1* gene [electropherograms: *red* thymidine (T), *blue* cytidine (C), *green* adenosine (A), *black* guanosine (G)] Arrows indicate: aberrant bands (HEX-SSCP); point of variation (electropherograms), N = homozygous wild-type, He = heterozygous banding pattern

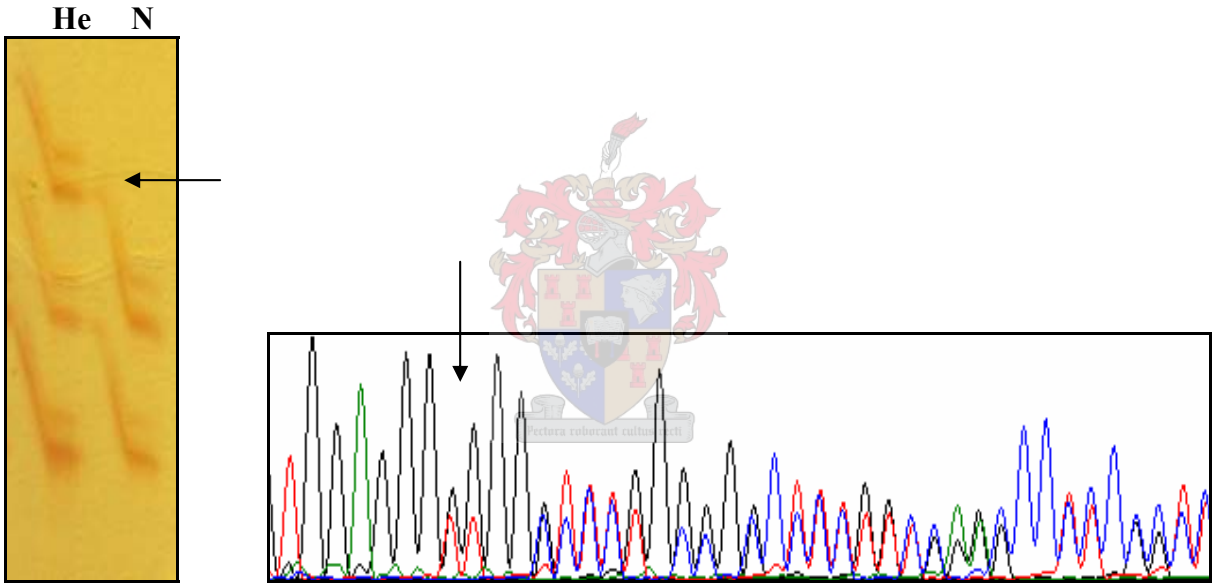
***HJV* gene**

Analysis of the *HJV* gene identified variants within exons three and four. This included a novel deletion, 219delG (exon three) (refer to Figure 3.6), and two previously identified single base pair variants namely S264 and A310G (exon four). The single base pair deletion 219delG, that results in the substitution of Valine with Tryptophan (amino acid codon 73), was observed in two heterozygous Caucasian patients whilst the S264 and A310G variants

were observed in one patient of the Caucasian population, heterozygous for both variants. The S264 variant changes a G to C at nucleotide position 663 (g.663G→C), causing a synonymous substitution (Serine). The A310G variant shows a transition from C to G at nucleotide position 928 (g.928C→G) resulting in the amino acid substitution of Alanine with Glycine.

Figure 3.6

A) 219delG



Legend to figure 3.6 Schematic representation of **A)** single-stranded conformation polymorphism, $AgNO_3$ visualization (12% polyacrylamide gel supplemented with 7.5% urea) and electropherogram of the novel deletion, 219delG, in the *HJV* gene [electropherograms: *red* thymidine (T), *blue* cytidine (C), *green* adenosine (A), *black* guanosine (G)] Arrows indicate: aberrant bands (HEX-SSCP); point of variation (electropherograms), He = heterozygous banding pattern, N = homozygous wild-type

Gene-gene interaction and genotype-phenotype correlations

Individual variants were statistically compared to allow for identification of potential gene-gene interaction (Butt *et al.* 2003). However, the analysis performed showed no statistically significant associations (data not shown).

Genotype-phenotype analysis involved stratification of patients according to both ethnicity and their specific serum iron and ferritin levels. This clinical data (iron parameters) could not be obtained for the control samples and comparison was performed between the stratified patient groups. The serum iron reference range of 6.60-30.40 µmol/L (as determined by the routine diagnostic lab where the blood analysis was done) was considered. Subsequently, the investigation of genotypes and iron entailed the comparison of patients with relatively lower serum iron levels (6.60-14.5 µmol/L) to patients with relatively average (14.6-22.44 µmol/L) and relatively higher (22.45-30.40 µmol/L) serum iron levels, respectively (individual patient serum iron levels not shown). There were no Coloured patients showing relatively higher iron serum levels and only the remaining two groups were compared (relatively lower serum iron compared to relatively average serum iron levels). Statistical analysis did not reveal significant differences in genotype/allele frequency representation within one group relative to the other.

The ferritin reference range of 10-290 µmol/L (as determined by the routine diagnostic lab where the blood analysis was done) served as guideline for the placing of patients (ethnicity considered) in groups showing relatively lower (12-105 µmol/L), relatively average (106-198 µmol/L) and relatively higher (199-291 µmol/L) ferritin levels (individual patient ferritin

levels not shown). The majority of the patient group showed relatively lower levels, with the exception of six cases (109 µmol/L, 129 µmol/L, 137 µmol/L, 169 µmol/L, 190 µmol/L, above the reference range: 321 µmol/L). No variant, present in only one of the two groups (patients showing either relatively average or higher ferritin levels) or within the extreme 'above the reference range' case, explaining the exclusion of the six patients from the majority, was observed. Patient groups were not statistically compared due to the minority representation of relatively average and relatively higher ferritin levels.

The final analysis entailed examination of the iron range represented by each genotype of a single variant. Observed differences in iron levels with respect to variant status (heterozygous, homozygous mutant or homozygous wild-type) would potentially indicate variant association with relatively lower, average or higher iron levels. This, in turn, could suggest or discard variant contribution to disease. However, stratification of patients delivered groups represented by only few individuals and the small values were not further compared. Also, statistical analysis for all the abovementioned stratifications did not reveal any statistically significant associations.

3.5 DISCUSSION

The genetic variation identified within this study included both intronic and exonic single nucleotide substitutions and deletions. The total of 19 variants identified included four novel, variants: IVS4-53G→A (*HFE*), IVS2-65delA (*CYBRD1*), 3'UTR+26delACGTCACGTTTCAAAACTA (*CYBRD1*) and 219delG (*HJV*). The remainder of the group consisted of seven intronic and eight exonic known single nucleotide variants

(*HFE*: IVS2+4T→C, IVS4+48G→A, IVS4-44T→C, IVS5-47G→A; *SLC40A1*: IVS1-24G→C; *CYBRD1*: IVS1-4C→G, IVS2+8T→C; *HFE*: H63D, C282Y; *SLC40A1*: I109, V221; *HAMP*: G71D; *CYBRD1*: S266N; *HJV*: A310G, S264).

Detection of seven of the variants was restricted to only the patient group of the Caucasian population (IVS4-53G→A (*HFE*), G71D (*HAMP*), IVS2-65delA (*CYBRD1*), 3'UTR+26delACGTCACGTTTCAAAACTA (*CYBRD1*), 219delG (*HJV*), A310G (*HJV*) and S264 (*HJV*). These possible disease-causing mutations (potentially contributing to the dysregulation of iron and consequently the development of MS) were identified in eight of 33 (24%) Caucasian patients and it included all the novel variants detected in this study. The variants will subsequently be discussed as either novel or previously characterized.

A total of four novel variants were characterized (only in the Caucasian population) in this investigation including an intronic variant in the *HFE* gene, IVS4-53G→A (one of 33 Caucasian patients), two intronic deletions in the *CYBRD1* gene, IVS2-65delA (two of 32 Caucasian patients) and 3'UTR+26delACGTCACGTTTCAAAACTA (one of 31 Caucasian patients), and an exonic deletion in the *HJV* gene, 219delG (two of 33 Caucasian patients).

The novel variant, IVS4-53G→A (*HFE*), is suggested to alter a constitutive acceptor site, according to information generated with the Alternative Splice Site Predictor (ASSP). Although the variant frequency did not show significance within this study population, screening of a larger cohort and functional analysis will allow for elucidation of its exact, if any, contribution to disease development.

ASSP was further used to analyse the intronic deletion, IVS2-65delA (*CYBRD1*). All the donor and acceptor sites present within the normal intronic sequence were also present upon analysis of the variant sequence. However, the donor and acceptor sites succeeding the deleted position are located at a base pair later compared to their positions within the normal sequence and this could alter splicing of the mRNA.

The 3'UTR+26delACGTCACGTTTCAAAACTA (*CYBRD1*) deletion is positioned in the 3'-untranslated (3'-UTR) region of the *CYBRD1* gene. Polyadenylation, addition of a poly(A) tail to mRNA, is dependent on signal sequences located within the 3'-untranslated region (5'-AAUAAA-3', 'GU' rich region). The deletion is thus suggested to affect the polyadenylation process and failure to add the poly(A) tail may affect the mRNA stability and initiation of translation (Brown 2002). Future studies should be done to determine the functional significance of this deletion.

The HJV gene, 219delG variant causes a frameshift resulting in synthesis of a protein with the incorrect amino acid sequence (Brown 2002). Also, the variant is suggested to create a premature stop codon resulting in truncation of the protein. Investigation with the ESE finder (Cartegni *et al.* 2003) showed altering of all SF2/ASF sites, indicating that normal splicing could also be disrupted. The suggested result is a mutated protein and analysis to determine the functional consequence of the deletion should include the protein truncation test (PTT) (Den Dunnen and Van Ommen 1999).

The analysis of gene-gene interaction and genotype-phenotype correlations did not prove statistically significant. These interactions warrant further investigation in a larger cohort that will ensure improved representation of the variants identified.

Limitations as to the number of MS studies that have focused on the South African population and more specifically the genes examined in this study, proves comparison thereof exceedingly difficult. In keeping with this study's hypothesis of a potential iron involvement in MS development, comparisons can be made a) with research focused on other diseases related to iron disorder e.g. haemochromatosis and b) to the genotype frequencies within other populations, by utilizing the HAPMAP database. The variation in allele frequencies amongst studies can be attributed to randomness of cohort choice, differences in cohort stratification including size, ethnicity and gender, and varying degrees of sensitivity of the techniques used for screening. Alternatively, differences in allele frequencies, established upon comparison with studies similar in cohort demographics, may be indicative of variant contribution to disease development. This comparative analysis will be discussed briefly within the subsequent description of known variants, identified within this study. In some instances, the literature provided limited or no Coloured population studies. Those variant frequencies were then compared to those found in Caucasian-based research. African Negro is included in the mixed ancestry of the Coloured population and the latter was compared to the HAPMAP population of 'Sub-Saharan Africa' (de Villiers *et al.* 1999).

This study identified a total of seven known intronic variants, present within the genes *HFE* (IVS2+4T→C, IVS4+48G→A, IVS4-44T→C, IVS5-47G→A), *SLC40A1* (IVS1-24G→C) and *CYBRD1* (IVS1-4C→G, IVS2+8T→C) (Beutler and West 1997, Totaro *et al.* 1997,

Devalia *et al.* 2002, Zaahl *et al.* 2004). Intronic variants have the potential to affect the splicing of mRNA (Pagani and Baralle 2004, Krawzack *et al.* 2006). Development of human inherited disease due to splicing abnormalities, can be attributed to a range of variants and according to the Human Gene Mutation Database (HGMD), single base-pair substitutions, specifically located within splice junctions, represent an estimated 9.5% thereof (Stenson *et al.* 2003, Krawczak *et al.* 2006).

The IVS2+4T→C variant in the HFE gene has been described as a polymorphism with reverse transcriptase-PCR analysis thereof indicating that mRNA splicing is unaffected by its presence (Christiansen *et al.* 1999). This variant has been observed in various studies investigating haemochromatosis including analysis of the South African population. Allele frequencies of one such a Caucasian based study, established the variant allele frequency at 0.38 in the patient group and 0.25 in the controls (de Villiers *et al.* 1999). An additional Caucasian, haemochromatosis study established allele frequencies of 0.39 in their patients and 0.31 in their controls (Zaahl *et al.* 2004). Findings within this study proved contradictory, with lower allele frequencies observed in the patient groups and higher frequencies determined for the control groups (Caucasian: patient group = 0.25; control group = 0.37 and Coloured: patient group = 0.3; control group = 0.45). The observed dissimilarity in patient frequencies could be attributed to the studies' investigating different diseases. The comparison of Coloured to Caucasian, as performed here, is not ideal and may further explain the variation. Alternatively, the findings in this study of lower frequency within the patient group compared to the controls, may suggest the non-significant role of this variant in the MS population.

The known IVS4+48G→A variant (Totaro *et al.* 1997) does not affect a splice-site consensus sequence within intron four of the *HFE* gene. Combined heterozygosity for the polymorphism and the C282Y allele, shows transferrin saturation levels that fall within the normal range, indicating that it may hold no significance at a functional level (Jeffrey *et al.* 1999). The C282Y variant has been associated with the iron overload disease, haemochromatosis and is described in subsequent paragraphs as one of the single nucleotide variants identified in this study. The Caucasian patient group was characterized by variant allele frequency of 0.44 whilst the control group frequency was an estimated 0.49. The Coloured patient (0.36) and control (0.37) groups showed similar allele frequencies. The frequencies determined in this study proved higher than those observed for HAPMAP (absent in European population, Sub-Saharan Africa: 0.029).

Intronic variants IVS4-44T→C, IVS5-47G→A, IVS1-24G→C, IVS1-4C→G and IVS2+8T→C were subjected to Alternative Splice Site Predictor (ASSP) analysis. The data generated, did not identify functional significance for any of the known intronic variants with regards to splice-site recognition sequence alteration. However, variants affecting the splicing process are not positionally limited to splice sites but may affect the regulatory elements such as ‘enhancers’ and ‘silencers’. The regulatory elements may be positioned within exons or introns at varying distances from splice sites (Pagani and Baralle 2004).

The allele frequencies for the IVS4-44T→C variant within the Caucasian population were lower (Caucasian: patients = 0.05, controls = 0.01) and in the Coloured group, conversely, higher (Coloured: patients = 0.14, controls = 0.13) compared to the frequencies of a South African population based hemocromatosis study (Zaahl *et al.* 2004) (Caucasian: patients = 0.10, controls = 0.06).

The IVS5-47G→A variant allele was observed at frequencies of 0.41 and 0.64 within the Caucasian and Coloured patient groups, respectively. The control groups showed frequencies of 0.48 (Caucasian) and 0.56 (Coloured). Frequencies were compared to HAPMAP (Caucasian = 0.47) and a South African haemochromatosis study (Zaahl *et al.* 2004) (Caucasian: patient group = 0.50; control group = 0.46). This study indicated relatively higher Coloured and lower Caucasian variant frequencies. The lower Caucasian value of this study may indicate that the variant is not contributive to MS however the differences could be explained by comparison to a haemochromatosis study. The higher Coloured cohort frequencies may stem from its comparison to the Caucasian group.

Modest variation was observed upon comparison of allele frequencies, for the IVS1-24G→C variant, between this investigation (Caucasian: patients = 0.80, controls = 0.82; Coloured: patients = 1.00, controls = 0.72) and the South African haemochromatosis study (Zaahl *et al.* 2004) (Caucasian: patients = 0.84, controls = 0.88). Both the studies show relatively high allele frequencies in both the patient and control groups and this may indicate that the variant is common in all populations, not contributing to disease.

The allele frequencies of IVS1-4C→G and IVS2+8T→C, were compared to the frequencies observed in the haemochromatosis study in which these variants were characterized as novel. The allele frequencies determined in this study were higher for all populations (IVS1-4C→G (Caucasian: patients = 0.18, controls = 0.10; Coloured: patients = 0.21, controls = 0.04); IVS2+8T→C (Caucasian: patients = 0.81, controls = 0.86; Coloured: patients = 0.90, controls = 0.83). The Zaahl *et al.* (2004) study showed a) presence of IVS1-4C→G in only the Caucasian patient group (0.03), and b) IVS2+8T→C in both the Caucasian patient (0.67) and

control group (0.64). The frequencies identified for the variants were relatively low but still higher than those observed in the comparative study suggesting it be researched further. The presence of the IVS1-4C→G in the Coloured group of this study but absence thereof from the Zaahl *et al.* (2004) study should also be considered.

The entire group of single nucleotide polymorphisms detected in this study have been characterized previously. It includes the *HFE* gene variants H63D (Feder *et al.* 1996) and C282Y (Feder *et al.* 1996), the *SLC40A1* gene variants I109 (Zaahl *et al.* 2004) and V221 (Devalia *et al.* 2002), a single variant in the *HAMP* gene namely G71D (Merryweather-Clarke *et al.* 2003), the *CYBRD1* gene variant S266N (McKie *et al.* 2001) and two variants within the *HJV* gene: A310G and S264 (Lee *et al.* 2004).

Variant H63D is characterized by an amino acid change from Histidine to Aspartate. The residue affected by the nucleotide change is located in a loop within the α1 domain. Histidine normally interacts and forms a salt bridge with Aspartate (Asp-73) but the substitution from basic to acidic is suggested to disrupt the side by side positioning of the two residues. It is proposed to disturb bridge formation and affect the loop arrangement locally without altering the protein fold. The normal function of the *HFE* gene is related to binding of transferrin receptor 1 (*TFR1*) and an accompanying decrease in *TFR1* affinity for transferrin (Tf) binding. The H63D variant still allows for the reduction in affinity, but to a lesser degree than seen in the case of the normal *HFE* protein (Feder *et al.* 1996, Parkkila *et al.* 1997, Feder *et al.* 1998, Lebron *et al.* 1998, Drakesmith *et al.* 2002, OMIM +235200). The calculated variant allele frequencies (Caucasian: patients = 0.14, controls = 0.09; Coloured: patients = 0.07, controls = 0.21) were all marginally higher, with the exception of the Coloured patient group,

than those observed in the Kotze *et al.* (2005) study that investigated MS within a group of South African Caucasians (Caucasian: patients = 0.12, controls = 0.08). Similar to this study, their patient variant frequency did not indicate significance when compared to their control value.

The C282Y mutation shows the amino acid change from Cysteine to Tyrosine. The sulphur-containing residue affected by the mutation is located in the α3 domain where it forms disulfide bonds with a further Cysteine (Cys-203) residue. *HFE* normally interacts with the β2m light chain to allow for expression at the cell surface. Replacement of the Cysteine with the aromatic ring containing Tyrosine hinders this interaction and the eventual cell surface expression. When C282Y is present, *HFE* cannot bind TFR and bring about the reduction in affinity between the latter and the Fe-Tf complex (The Medical Biochemistry page, Feder *et al.* 1996, Feder *et al.* 1997, Feder *et al.* 1998, Lebron *et al.* 1998, OMIM +235200).

A more recent study describes analysis of the *HFE* gene variants C282Y and H63D within MS patients from both Croatia and Slovenia (Ristić *et al.* 2005). Genotype comparison of patients with ethnically matched controls revealed no significance. Further genotype comparisons, made with respect to the different MS subtypes a) between the different types within the patient group and b) between the patient and control population for the respective sub-groups, also proved insignificant. However, the genotype combination C282Y/wild-type, showed a significant relationship with earlier onset of the disease compared to the other genotypes studied, with the authors suggested further analysis thereof within a larger patient group. This current investigation determined variant allele frequencies of 0.06 (patients) and 0.06 (controls) less than the frequency obtained from HAPMAP (European population =

0.958). Similar to this study, Zaahl *et al.* (2004) detected the variant solely within the Caucasian group and this was established at relatively higher frequencies (patients = 0.08 and controls = 0.10). An MS study (South African Caucasian population) also detected higher frequencies compared to the values identified in this study (patients = 0.09, controls = 0.10) but similarly their statistical analysis thereof did not prove significant (Kotze *et al.* 2005). Contrary to this study, Rubio *et al.* (2004) indicated a higher frequency of the C282Y variant within their patient group compared to the controls and suggested that the variant could be in linkage disequilibrium with factors involved in the development of MS.

HAMP gene variant, G71D, is characterized by the amino acid change from Glycine to Aspartic acid. The variant is localized between two Cysteine residues within the first β pleated sheet of the protein molecule. It is suggested to affect the protein structure and hence activity level due to the amino acid change from a neutral residue to an acidic, charged one (Merryweather-Clarke *et al.* 2003). The variant G71D was identified at a low frequency in only the Caucasian patient group (0.03), however, the variant allele prevalence was notably higher compared to the general population frequency (0.001) established in the Merryweather-Clarke *et al.* (2003) study.

The *CYBRD1* gene variant, S266N, shows the change from the polar, hydrophilic Serine to the polar, uncharged Asparagine. The allele frequencies of the variant, within this study, includes Caucasian: patients = 0.69; controls = 0.79 and Coloured: patients = 0.86; controls = 0.69. Zaahl *et al.* (2004) identified higher frequencies (Caucasian: patients = 0.76, controls = 0.86) with the exception of this study's Coloured patient group. HAPMAP values included a European population frequency of 0.271 and Sub-Saharan African population frequency of

0.042. Comparison to the HAPMAP values showed remarkably higher frequencies in this study. The higher variant prevalence within the Coloured patient group of this study, suggests that it could increase risk of MS although statistical evaluation did not prove significant. The higher Caucasian frequencies of the Zaahl *et al.* (2004) study could be because of the difference in disease investigated and techniques used for detection. Alternatively, this suggests the variants to be insignificant in MS development. However, the value is higher than the HAPMAP population and further research in a larger cohort is needed to clarify these contradictory findings.

The *HJV* gene variant A310G results in the amino acid change from Alanine to Glycine (Lee *et al.* 2004). Both the amino acids are non-polar and hydrophobic, suggesting that the variant may have no effect at the structural level (The Medical Biochemistry page). The allele frequency determined for the A310G within this study (0.02) was equal to the low frequency of 0.02 established for the variant within an African American population (Barton *et al.* 2004).

This study identified a total of three known synonymous single base variants in which the identified change does not result in an amino acid substitution. It includes variants, I109 (Zaahl *et al.* 2004) and V221 (Devalia *et al.* 2002) detected in the *SLC40A1* gene and S264 identified in the *HJV* gene. These polymorphisms may nonetheless contribute to disease development with an estimated half of all single nucleotide polymorphisms, causing human disease, resulting in splicing defects (Cartegni *et al.* 2003).

The allele frequencies determined for the I109 variant in this study included 0.07 in the Coloured patient group and 0.04 in the ethnically matched, control population. Variant V221 was detected within both the Caucasian and Coloured groups (Caucasian: patients = 0.75, Caucasian: controls = 0.68; Coloured: patients = 0.93, controls: 0.67). S264 was detected at a frequency 0.02 within only the Caucasian patient group. Zaahl *et al.* (2004) identified I109, in the Caucasian patient group (0.008) and V221 in both the Caucasian patient (0.21) and control groups (0.08). This study thus detected these polymorphisms at higher frequencies and this suggests that further research be done.

The cohort was sufficient in size (patients: 40, controls: 70) for the projected aims of this pilot study. It delivered insight as to a) the identification of variants potentially involved in MS development and b) further provided baseline data with regards to variant frequency. A larger sample size will allow for more informative statistical analysis, providing a more specific value of variant frequency within the South African population and subsequent elucidation of variant contribution.

Representation of ethnicity seen within the South African population was partly achieved with inclusion of 33 Caucasian and seven Coloured patients. Individual statistical analysis was performed for the Caucasian and Coloured groups respectively. The values obtained provided a general impression of variant presence and frequency within the relevant South African ethnic groups. Obtaining more descriptive data would require improvements as to population representation. It would entail a) a more equal distribution of each group e.g. 50 Caucasian, 50 Coloured and b) inclusion of the other ethnic groups within South Africa.

The variants potentially associated with MS could a) be associated with a specific type of the disease or b) may contribute equally to all the classified subtypes. These variables could be addressed by studies with large sample sizes that include all the subtypes or alternatively the variables could be reduced by the choice of a homogenized patient group including a single MS sub-type. This pilot study investigated a smaller sample size of only relapsing-remitting multiple sclerosis (RRMS) patients.

Relapsing-remitting MS predominately affects females [female to male ratio of 2:1]. Similarly this study included a gender majority of females within both the patient and control groups (patient gender ratio of 5:1; control gender ratio of 4:1).

A total of three techniques, including Heteroduplex Single-Stranded Conformation Polymorphism (HEX-SSCP), restriction enzyme digestion and semi-automated DNA sequencing analysis, were used to allow for detection and verification of variants.

The HEX-SSCP technique allowed for visual detection of conformational variation. The technique, however, does not provide 100% sensitivity and additional restriction enzyme digestion was subsequently done for the variants identified in the *HFE* gene, exon two b (IVS2+4T→C) and the *CYBRD1* gene, exon four b (S266N) (refer to Figure 3.3). HEX-SSCP analysis of the remaining exons delivered satisfactory visualization. All the variants detected in this study were subjected to semi-automated DNA sequencing analysis to allow for verification and characterization. However, the technique accuracy may be reduced due to human error and *Taq* polymerase misincorporation mistakes (Clarke and Whittam 1992).

Successful PCR amplification and subsequent screening of 22 control samples for the IVS2-65delA variant, proved challenging and troubleshooting thereof was ineffective. The binding site of the primer (forward) is located in close proximity to the deleted position, identified within the patient group and this could account for the incomplete amplification of the control population.

The clinical data collected for this study included the two parameters namely iron and ferritin serum levels. Iron serum is characterized by diurnal fluctuations and is affected by factors such as menstruation and pregnancy. The levels are further known to decrease in response to inflammation and infection that may be unrelated to the disease under investigation (Borch-Iohnsen 1995). Similarly, ferritin is affected by factors such as inflammation, infection, gender and age (Cook *et al.* 1992, Borch-Iohnsen 1995). Transferrin receptor level estimates however are not influenced by infection and inflammation (Cook *et al.* 1992, Borch-Iohnsen 1995). Inclusion of more than the two parameters collected in this study may provide a more accurate estimation of iron status and so deliver improved insights as to the full extent of the iron imbalance. A limitation imposed on this investigation was the unavailability of control individuals' iron serum and ferritin levels, required for statistical comparison. Financial constraints and the hampered recall of control individuals did not allow for the collection thereof.

CHAPTER FOUR

4. CONCLUSIONS AND FUTURE PROSPECTS

Multiple sclerosis is a debilitating disease characterized by the loss of neurological function. The symptoms of the disease, that mainly affects young adults, include sensory disturbances, gait ataxia and fatigue. Individuals affected by multiple sclerosis thus experience a marked decrease in their quality of life. Research attempting to elucidate the complex etiology of this multifactorial disorder, has addressed the potential contributions of iron, autoimmunity, pathogenic infection and genetics. MS research focused on the South African population and more specifically, the involvement of iron and genetics in the occurrence of MS within this population is relatively limited. This study attempted to investigate the role of iron and the genes related to iron metabolism within a South African cohort.

The first aim of this study was to identify variants that could prove to be contributors to and/or markers of disease. The genes screened included *HFE*, solute-carrier family 40 (iron regulated transporter) member 1 gene (*SLC40A1*), hepcidin anti-microbial peptide (*HAMP*), cytochrome b reductase 1 (*CYBRD1*) and hemojuvelin (*HJV*). A total of 19 variants of which four were novel: IVS4-53G→A (*HFE*), IVS2-65delA (*CYBRD1*), 3'UTR+26delACGTCACGTTTCAAAACTA (*CYBRD1*) and 219delG (*HJV*), were identified. The remainder comprised of seven intronic variants and eight exonic single nucleotide variants. Seven of the variants (including all the novel variants identified) namely

IVS4-53G→A (*HFE*), G71D (*HAMP*), IVS2-65delA (*CYBRD1*), 3'UTR+26delACGTCACGTTTCAAAACTA (*CYBRD1*), 219delG (*HJV*), A310G (*HJV*) and S264 (*HJV*), were identified only in the Caucasian patient group and in no control individuals. These findings did not prove statistically significant, however, the presence thereof in the patient population alone, highlights its potential contribution to the development of MS and thus warrants further investigation.

The second aim of this investigation included statistical analysis of variant frequency within the patient group compared to the control population. Additional analysis attempted to identify linkage disequilibrium and/or gene-gene interaction between the variants identified. The final examination was aimed at establishing genotype-phenotype correlations with the serum iron and ferritin levels determined for the patient population.

Statistical analysis for both a) individual variants and b) variant combinations (gene-gene interaction and linkage disequilibrium) (data not shown) did not prove significant. This could be attributed to small sample size or alternatively the variants identified in this study could be in linkage disequilibrium or show gene-gene interaction with other disease-causing loci. Some of the genotype-phenotype investigations were restricted due to small sample size whilst the remainder did not deliver statistically significant results. Future studies should thus aim to analyse these variants in a larger cohort. This could be done in conjunction with screening both a) the promoter regions (*HFE*, *SLC40A1*, *HAMP*, *CYBRD1* and *HJV*) and additional genes related to iron metabolism e.g. Transferrin receptor 2 (*TFR2*) and Ceruloplasmin (*CP*).

The four novel IVS4-53G→A (*HFE*), IVS2-65delA (*CYBRD1*), 3'UTR+26delACGTCACGTTTCAAAACTA (*CYBRD1*) and 219delG (*HJV*) variants have suggested functional significance and although only seen in a minority of cases, it may contribute to the MS etiology. The single patient heterozygous for the IVS4-53G→A variant showed iron and ferritin levels categorized as relatively 'low' according to the respective reference ranges chosen for this study (van Rensburg *et al.* 2006). The two individuals presenting as heterozygous for the IVS2-65delA variant were also characterized as having relatively 'low' ferritin whilst one individual showed 'low' and the second 'average' iron levels. The deletion, IVS2-65delA, was detected in two patients of whom one was classified as 'low ferritin level'. The second deletion 3'UTR+26delACGTCACGTTTCAAAACTA was identified in a single Caucasian patient showing 'average' iron and low 'ferritin'. This study did not prove these observations to be significant but a larger cohort may deliver insight as to the contribution of these novel variants. Future aims should include elucidation of variant effect by means of functional analysis of the novel variants identified within this study e.g. PTT, murine 'knockout' studies, protein expression comparisons (wild-type vs. variant) and RNA studies (reverse transcriptase-PCR analysis).

Several of the known variants identified have functional significance in haemochromatosis. This suggests that these variants may contribute to MS development seeing as it too is suggested to be a disease of iron disorder. Comparisons with a number of studies indicated higher frequency within this investigation of the following variants: IVS4+48G→A (Coloured patient group), IVS4-44T→C (Coloured patient group), IVS5-47A→G (Coloured patient group), IVS1-24G→C (Coloured patient group), IVS1-4C→G (both Caucasian and Coloured patient groups), IVS2+8T→C (both Caucasian and Coloured patient groups), H63D

(Caucasian patient group), S266N (both Caucasian and Coloured patient groups), I109 (Coloured patient group) and V221 (both Caucasian and Coloured patient groups). These observations may be the result of the small Coloured cohort size and comparison of the Coloured population to other Caucasian studies. Conversely, study of a larger cohort could indicate these variants to be significant as individual or combined contributors to MS disease and future research should aim to investigate these observations further.

The study thus succeeded in identification of variants but failed to determine their significance in the development of MS. However, as a pilot study, with a relatively small cohort, the variant non-significance seen here may be disproved and further characterized by means of further research in larger populations.

The differences observed upon comparison of the identified variants to the frequencies within other studies could be attributed to failure of the techniques utilized, to detect all the relevant variation and future aims should include screening for novel and/or known variants by means of denaturing high performance liquid chromatography (dHPLC) or a similar sensitive technique. The technique provides a sensitivity of 92-100% and allows for screening of larger fragments (198-732 bp) than the HEX-SSCP technique utilized in this study. The SSCP component is characterized by sensitivity of 70-100% (130-250 bp sized fragments) (Xiao and Oefner 1992, Bonner and Ballard 1999), although the variant detection rate of this study was improved by the additional analysis of the heteroduplexes (HEX-SSCP). Furthermore, restriction enzymes could be used (where possible) to screen for the known variants identified in this study's cohort within a larger sample size. The digestion technique is less time consuming compared to HEX-SSCP screening method.

A further limitation pertaining to techniques used in this study included unsuccessful PCR amplification of the control group upon screening of the IVS2-65delA (*CYBRD1*) variant. The forward primer binds close to the deletion site and may explain the failure of control sample amplification. Future studies should aim to redesign a primer with a binding site further from, but still including, the variant position.

General improvements as to choice of cohort should be addressed in future studies. A larger cohort size would potentially a) ensure that all control groups conform to the requirements of Hardy-Weinberg equilibrium, b) generate values sufficient in size for further statistical analysis and c) provide a more accurate estimation of variant frequency within the South African population. The control group should be fully characterized in terms of iron, ferritin and additional clinical evidence e.g. transferrin receptor levels, to allow for statistical comparison to the patient groups. Patient information relating to age of onset, neurological evaluation according to a disability scale e.g. Expanded Disability Status Scale (the EDSS; Kurtzke 1983) and relevant personal information could be included in future investigations.

Stratification of the cohort according to sub-type of MS disease investigated, ethnicity and gender ratio will contribute to identification of meaningful results.

The primary shortcomings of this investigation included small cohort size and failure to determine iron and ferritin levels for the control population. However, the study enriches our knowledge of MS and its possible underlying genetic component within the South African population. This investigation allowed for identification of variants that could potentially,

when investigated in a larger, stratified study, prove to be contributors to and/or valuable markers of MS disease.

CHAPTER FIVE

5. REFERENCES

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ELECTRONIC-DATABASE INFORMATION

Alternative Splice Site Predictor (ASSP) analysis, (http://es.embnet.org/~mwang/assp.html)

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STATISTICA (StatSoft, Inc. (2003) STAT (data analysis software system), version 6 and the Epi Info computer software (Epi Info™ (utilities StatCalc) v3.3.2, release date: 9 February 2005; Division of Public Health Surveillance, http://www.cdc.gov/epiinfo/

The Medical Biochemistry page, http://www.indstate.edu/thcme/mwking/amino-acids.html

The Multiple Sclerosis gateway, http://www.ms-gateway.com

Table 3.1 Allele frequencies of the variants identified within this study

Gene	Exon/ Intron	Variant	Allele frequencies[a]		Allele frequencies[a]	
			CPMS	**CC**	**MPMS**	**MC**
			(2n=66)	*(2n=100)*	*(2n=14)*	*(2n=38)*
HFE	2	H63D[c] (Feder *et al.* 1996)	0.14	0.09	0.07	0.21
			(2n=64)	*(2n=98)*	*(2n=12)*	*(2n=38)*
	2	IVS2+4T→C[b] (Beutler and West 1997)	0.25	0.37	0.3	0.45
			(2n=60)	*(2n=86)*		
	4	C282Y[b] (Feder *et al.* 1996)	0.06	0.06	-	-
			(2n=66)	*(2n=98)*	*(2n=14)*	*(2n=38)*
	4	IVS4+48G→A[c] (Totaro *et al.* 1997)	0.44	0.49	0.36	0.37
			(2n=66)	*(2n=98)*		
	4	IVS4-53G→A[c, d] (This study)	0.02	-	-	-
			(2n=66)	*(2n=98)*	*(2n=14)*	*(2n=38)*
	4	IVS4-44T→C[c] (Beutler and West 1997)	0.05	0.01	0.14	0.13
			(2n=66)	*(2n=98)*	*(2n=14)*	*(2n=34)*
	5	IVS5-47G→A[b] (Beutler and West 1997)	0.41	0.48	0.64	0.56
			(2n=64)	*(2n=78)*	*(2n=12)*	*(2n=18)*
SLC40A1	1	IVS1-24G→C[b] (Devalia *et al.* 2002)	0.80	0.82	1.00	0.72
					(2n=14)	*(2n=26)*
	4	I109[c] (Zaahl *et al.* 2004)	-	-	0.07	0.04
			(2n=48)	*(2n=82)*	*(2n=14)*	*(2n=24)*
	6	V221[b] (Devalia *et al.* 2002)	0.75	0.68	0.93	0.66
			(2n=58)			
HAMP	3	G71D[c, d] (Merryweather-Clarke *et al.* 2003)	0.03	-	-	-
			(2n=66)	*(2n=86)*	*(2n=14)*	*(2n=24)*
CYBRD1	1	IVS1-4C→G[b] (Zaahl *et al.* 2004)	0.18	0.10	0.21	0.04
			(2n=66)	*(2n=86)*	*(2n=14)*	*(2n=24)*
	2	IVS2+8T→C[b] (Zaahl *et al.* 2004)	0.81	0.86	0.90	0.83
			(2n=64)			
	2	IVS2-65delA[c, d] (This study)	0.03	-	-	-
			(2n=62)	*(2n=78)*	*(2n=14)*	*(2n=36)*
	4	S266N[b] (McKie *et al.* 2001)	0.69	0.79	0.86	0.69
			(2n=62)			
	4	3'UTR+26delACGTCACGTTTCAAAACTA[c] (This study)	0.02	-	-	-
			(2n=66)			
HJV	3	219delG[c, d] (This study)	0.03	-	-	-
			(2n=64)			
	4	S264[c, d]	0.02	-	-	-
			(2n=64)			
	4	A310G[c, d] (Lee *et al.* 2004)	0.02	-	-	-

Abbreviations: *CPMS* Caucasian MS patient group, *CC* Caucasian control group, *MPMS* Coloured MS patient group, *MC* Coloured control group, *2n* total alleles (variation amongst variants according to the sample total successfully amplified), [a]Allele frequency of polymorphic allele denoted, [b]Identified in both heterozygous and homozygous state, [c]Identified only in heterozygous state, [d]Variants identified only in the patient group, *****Probablity values determined for the allele frequencies