A multi-disciplinary approach towards elucidating the genetics of multiple sclerosis

JNP DE VILLIERS

Dissertation presented for the degree of Doctor of Philosophy in the Medical Sciences at the Faculty of Health Sciences, University of Stellenbosch, Tygerberg.

Supervisor: Dr MJ Kotze
Co-supervisors: Prof L Warnich
Dr G de Jong

University of Stellenbosch
April 2003

DECLARATION

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

Signature:

Date:....14/03/2003.....

SUMMARY

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. Current knowledge suggests that MS is associated with autoimmunity and that infectious agents and hereditary factors may be involved. The demonstration of a higher recurrence risk of MS in families (4-5%) compared with the general population (0.1%) provides strong evidence for a genetic basis. Extensive analyses of the entire human genome to identify new genes that may underlie MS have indicated that several genes may contribute to disease susceptibility, but these remain largely unidentified.

In this study candidate genes involved in iron metabolism and immunology have been analysed for the first time within the context of both autoimmune and infectious disease susceptibility, in order to investigate the role of genetic and viral factors implicated in the pathogenesis of MS.

The Z-DNA forming repeat polymorphism in the promoter region of the solute carrier family 11 (proton-coupled divalent ion transporters) member 1 (*SLC11A1*) gene was found to be significantly associated with MS ($P<0.01$) in the genetically homogeneous Afrikaner population of South Africa, but not in the German and French populations using a case-control study and transmission linkage disequilibrium approach, respectively. However, significant differences were observed in genotype distribution between German MS patients with a primary- and secondary progressive disease course ($P<0.05$), and between the German patients with relapsing remitting and primary progressive MS ($P<0.05$). These findings provide further evidence that the *SLC11A1* gene is associated with MS, most likely due to its role in iron homeostasis.

In order to investigate the influence of viruses in the apparent multi-step aetiology of MS, serum and peripheral blood mononuclear cells (PBMCs) of MS patients, close relatives and unrelated controls were screened for the presence of MS-associated retrovirus (MSRV) and two herpes virus (HHV-6 and EBV) sequences. Detection of the *pol* gene expression of MSRV in the serum RNA of 69% of South African MS patients and in 70%

of their unaffected close relatives, whilst absent in the serum of 39 unrelated healthy control individuals ($P<0.001$), indicated that virus infections affect the population risk but not the familial risk in MS. HHV-6 sequences were also present at a significantly lower frequency ($P<0.04$) in the PBMCs of unrelated controls (5%) compared to MS patients (22.5%).

A point mutation (77C→G) in the gene encoding protein-tyrosine phosphatase, receptor-type C (*PTPRC*), which is essential for activation of T and B cells, was found to be associated with MS in the German population. Analysis of the Afrikaner and German study populations included in our study did not indicate a causative role for the *PTPRC* gene in MS. However, it seems likely that this mutation may contribute to disease expression, since in one of the South African families with two MS affected sibs, the most severely affected sister was heterozygous for the 77C→G mutation. The *PTPRC* mutation may therefore be of significance in disease prognosis.

The multidisciplinary study approach has led to a stepwise accumulation of scientific information, which forever changed our understanding of the disease process underlying MS.

OPSOMMING

Veelvoudige sklerose (VS) is 'n kroniese inflammatoriese siekte van die sentrale senuweestelsel. Oor die algemeen word aanvaar dat VS geassosieerd is met outoimmuniteit en dat infektiewe agente en oorerflike faktore 'n rol speel. Die hoër herhalingsrisiko van VS in families (4-5%) in vergelyking met die voorkoms in die algemene populasie (0.1%) dui op 'n genetiese basis. Alhoewel volledige analise van die mensgenoom om gene onderliggend aan VS te identifiseer aangedui het dat verskeie gene waarskynlik bydra tot vatbaarheid vir die siekte, is die aard van die gene wat betrokke is grootliks onbekend.

In hierdie studie is kandidaatgene betrokke by ystermetabolisme en immunulogie vir die eerste keer geanaliseer binne die konteks van beide outoimmuun en infektiewe siekte vatbaarheid, ten einde die rol van genetiese en virale faktore in die patogenese van VS te ondersoek.

Die Z-DNS herhalingsvolgorde polimorfisme in die promotor area van die *SLC11A1* geen was betekenisvol geassosieerd met VS ($P<0.01$) in die geneties homogene Afrikaner populasie van Suid-Afrika. 'n Soortgelyke assosiasie kon egter nie aangetoon word in die Duitse en Franse populasies deur gebruik te maak van onderskeidelik 'n gevalle-kontrole studie en transmissie-koppelings-disekwilibrium benadering nie. Betekenisvolle verskille in die genotipe verspreiding is egter tussen Duitse VS pasiënte met 'n sekondêr- en primêr progressiewe verloop van die siekte ($P<0.05$), en tussen die Duitse pasiënte met terugvallende en primêre progressiewe VS aangetoon ($P<0.05$). Hierdie bevinding verskaf verdere bewyse dat die *SLC11A1* geen geassosieerd is met VS, heel waarskynlik weens die rol van die geen in yster-homeostase.

Ten einde die invloed van virusse in die etiologie van VS te ondersoek is serum en witbloedselle van VS pasiënte, naby-verwante familielede en nie-verwante kontroles getoets vir die teenwoordigheid van die VS-geassosieerde retrovirus (MSRV) en twee herpesvirus (HHV-6 en EBV) geenvolgordes. Die *pol* geen uitdrukking van MSRV was

teenwoordig in die serum RNA van 69% van die Suid-Afrikaanse VS pasiënte en in 70% van hul ongeaffekteerde naby-verwante familielede, terwyl dit afwesig was in 39 nie-verwante kontrole individue ($P<0.001$). Dit dui daarop dat virusse waarskynlik die risiko vir VS meer in die populasie verhoog as in families. HHV-6 was ook teenwoordig teen 'n beduidende laer frekwensie ($P<0.04$) in nie-verwante kontroles (5%) in vergeleke met VS pasiënte.

'n Puntmutasie (77C-G) in die geen wat kodeer vir die proteïen tirosien fosfatase reseptor tipe C (PTPRC), wat belangrik is vir aktivering van T- en B-helperselle, is vroeër gevind om geassosieerd te wees met VS in die Duitse populasie. Analise van die Afrikaner en Duitse populasies in ons studie het egter geen bewyse gelewer dat die *PTPRC* geen 'n rol speel in VS nie. Dit egter is moontlik dat hierdie mutasie bydra tot die uitdrukking van VS, aangesien die mees geaffekteerde VS pasiënt in een van die Suid-Afrikaanse families met twee geaffekteerde susters positief getoets het vir die mutasie. Dié mutasie mag dus van belang wees in die prognose van VS.

Die multidissiplinêre studie-benadering en stapsgewyse insameling van wetenskaplike inligting het gelei tot 'n nuwe perspektief ten opsigte van die siekteproses onderliggend aan VS.

DEDICATION

This thesis is dedicated to all the people suffering from autoimmune diseases including my mother who overcame her disease through research. I do hope that this study will help other people in the same way, if not now then hopefully in the future by improving our understanding of complex diseases.

ACKNOWLEDGEMENTS

My supervisor, Dr Maritha Kotze is thanked for her guidance, effort, focus and enthusiasm throughout this study.

My co-supervisors, Prof Louise Warnich and Dr Greetje de Jong for their valuable input.

The staff and fellow students at the Division of Human Genetics, Tygerberg for their support in and outside of the laboratory.

My colleagues at Genecare Molecular Genetics (Pty) Ltd, Dr Charlotte Scholtz and Dr Rochelle Thiart for all their support and understanding, I thank you.

Dr Lana du Plessis for her ability to put things in perspective, listening and giving support.

Dr Susan van Rensburg and Prof Erna Mansvelt are thanked for determination of iron parameters.

Dr Jonathan Carr is acknowledged for clinical assessment of South African patients.

I thank the University of Stellenbosch and the Harry and Doris Crossley Foundation for financial support.

The Freda and David Becker Trust and the Poliomyelitis Research Foundation are thanked for student bursaries.

My family for the unreserved support and love.

Finally I acknowledge my partner, Maggie for supporting me and keeping a steady head when it felt like everything around me was crumbling.

LIST OF ABBREVIATIONS

Bam H1	restriction endonuclease enzyme
CD4+	MHC class II restricted T-cells
CD8+	MHC class I restricted T-cells
CD4:CD8	ratio between CD4 and CD8
CD45	alias for PTPRC
CD45RA	isoform of CD45
cDNA	complementary DNA
χ^2	Chi-Square
CNS	central nervous system
CSF	cerebrospinal fluid
CVID	common variable immunodeficiency
DIG	digoxigenin
DNA	deoxyribonucleic acid
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DNAse	modifying enzyme, deoxyribonuclease
Df	degrees of freedom
EBV	epstein barr virus
EDSS	expanded disability status scale
EP	evoked potential
g/dl	grap per decilitre
HAM/TSP	myelopathy/tropical spastic paraparesis
HFE	human haemochromatosis gene
HH	hereditary haemochromatosis
HHV	human herpes virus
HHV-6	human herpes virus 6

HLA	human leukocyte antigen
HLA-A3	human leukocyte antigen A3
HLA-B7	human leukocyte antigen B7
HLA-Dw2	human leukocyte antigen Dw2
HSV	human simplex virus
HTVL-1	human T-lymphotropic virus type 1
IFN	interferon
ig	immunoglobulin
igG	immunoglobulin G
IgAD	IgA deficiency
IL	interleukin
LD	linkage disequilibrium
LOD	logarithm of odds
M	molar, moles per litre
MCP-3	monocyte chemotactic protein 3
MDV	marek's disease virus
MHC	major histocompatibility complex
µg/l	microgram per litre
µl	microlitre
µmol/l	micro mole per litre
µM	micro molar
mM	milli molar
MRI	magnetic resonance imaging
MS	multiple sclerosis
Mse 1	restriction endonuclease enzyme
Msp 1	restriction endonuclease enzyme
MSRV	multiple sclerosis-associated retrovirus
ng	nanogram
n.d.	not determined
NPL	nonparametric linkage
NRAMP1	natural resistance-associated macrophage protein 1

OCB	oligoclonal band
%C	percent crosslink
PAA	polyacrylamide
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PGE2	prostaglandin E
pmol	pico mole
PP	primary progressive
PPOX	protoporphyrin oxidase
PTPRC	protein-tyrosine phosphatase, receptor-type C
RNA	ribonucleic acid
RNAse	modifying enzyme, ribonuclease
RR	relapse remitting
Rsa 1	restriction endonuclease enzyme
RT-PCR	reverse transcriptase PCR
SLC11A1	solute carrier family 11 member 1
SP	secondary progressive
TDT	transmission/disequilibrium test
TNF	tumor necrosis factor alpha or alpha and beta
U	units
yrs	age in years

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

INTRODUCTION

MULTIPLE SCLEROSIS: A DISEASE OF THOUSAND FACES

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) characterised by multifocal damage of myelin and axonal loss resulting in various neurological symptoms. This disease does not follow a predictable pattern of inheritance and cannot yet be explained through an effect of a definable gene product or functional abnormalities (Chataway et al., 1998). The geographic heterogeneity of the disease and the varying prevalence rates in different population and ethnic groups suggest interplay between environmental and genetic factors. This calls for a multi-disciplinary approach to elucidate the aetiology of MS.

1.1 Clinical presentation, diagnosis, pathology and treatment

1.1.1 Clinical features

A wide range of clinical symptoms and signs, few of which are considered particularly specific to the disease, are exhibited by MS. Strikingly uniform however is the long-term outcome in disease severity (Ebers, 2000). Depending on the part of the CNS affected, the signs and symptoms of the patients vary. Some tend to appear early in the course of the disease and some later. Sensory impairment was the most common symptom in the initial stages, followed by optic neuritis, insidious motor functional impairment, limb ataxia, diplopia and/or vertigo and acute motor functional loss (Weinshenker et al., 1989). Abnormal reflexes, impairment of bowel and bladder control and sexual dysfunction are other observed signs. MS patients are more frequently affected by memory impairment and affective disorders as compared to the general populations (Miller, 1998).

1.1.2 Clinical categories and severity classification

MS may be categorised into several different types according to clinical course. There was clear consensus and preferences on the meaning of the terms relapsing remitting (RR), primary progressive (PP) and secondary progressive (SP) forms of MS as revealed by an international survey of clinicians involved with MS. According to the authors (Lublin and Reinhold, 1996) it was suggested that the PR form deserves a separate definition, as it was not included in the other definitions. As PR represents only a small fraction of MS patients, this suggestion has received very little support. The consensus definitions are as follows:

RR: Clearly defined disease relapses with full recovery or with sequelae and residua deficit upon recovery. Periods between disease relapses characterised by a lack o disease progression.
SP: Initial RR disease course followed by progression with or without occasional relapses. Minor remissions and plateaus may be accepted.
PP: Insidious onset and disease progression from onset with occasional plateaus an temporary minor improvements allowed.
PR: Progressive disease from onset, with clear acute relapses, with or without full recovery; periods between relapses characterised by continuing progression.

An increasing disability is displayed by PP and SP patients. A disability status scale, a scoring system for disability, was developed by Kurtzke (1983) and was later expande to include more subtle changes. The expanded disability status scale (EDSS) is used t measure MS-related impairment of various functional systems. These are pyramida cerebellar, brain-stem, sensory, bowel/ bladder, visual and cerebral functions as well a other neurological findings attributable to MS. Impairment is graded in 20 steps startin from zero (normal neurological examination) increasing to 1 (a single sign only) and the in half-point steps ranging to 10 (demise by MS). A median time of 15 years to EDSS 6 20 years for EDSS 7 and 25 years for EDSS 8 in RR MS was indicated by Ebers (2000) In the PP MS group it was 8 years to EDSS 6, 12 years to EDSS 7 and 15 years t EDSS 8. Definitions of benign and malignant MS, concerning severity of disability, hav

also been suggested (Lublin and Reinhold, 1996). If a sufferer remains fully functional in all neurologic systems after 15 years of disease onset, it is diagnosed as benign MS. A speedy progressive course, leading to significant disability in multiple neurologic systems or death in a relative short time after onset of disease, is categorized as malignant MS. Benign MS is normally considered a sub-category of RR MS. RR MS attacks are more frequent and some patients have a stepwise increase in neurological deficit as compared with benign MS.

1.1.3 Disease subtypes

Immunogenetic studies of human leukocyte antigen (HLA) have indicated that there may be two distinct sub-types of MS: Asian-type and Western-type. The clinical differences between Western and Asian types of MS based on the natural history of the disease as well as magnetic resonance imaging scanning of the brain and spinal cord of a group of Japanese patients, was reported by Kira et al. (1996). The patients, all residing in Kyushu, were diagnosed as having a Western or Asian type MS. Asian type MS was characterized by a RR course and a selective involvement of the optic nerve and spinal cord. This disease pattern is known to occur in some Asian populations. Disseminated central nervous system (CNS) disease was more prevalent in patients with Western type MS. Western type MS sufferers showed a DR2 association, which Asian type MS patients did not. This led to Kira at al. (1996) to suggest the presence of two aetiologically distinct diseases in Asians. A study done by Dean et al. (1994) indicated that MS in the black South African populace, albeit rare, has more similarities with the disease as occurring among the oriental people, than among the white people in Southern Africa or black people of North America or the Caribbean.

1.1.4 Pathology

The oligodendrocyte, a principal target of immune attack in MS patients, synthesises and maintains the myelin sheath of up to 40 neighbouring nerve axons in the CNS. Myelin consists of a condensed membrane, spiraled around axons to form the insulating segmented sheath needed for salutatory axonal conduction. A cluster of voltage-gated sodium channels can be found at the unmyelinated nodes of Ranvier between the myelin segments from where the action potential is propagated and spreads passively down the myelinated nerve segment to trigger another action potential at the next node. The clinical and biochemical features associated with MS can thus be explained by the consequences of demyelination. For example, partially demyelinated axons conduct impulses at reduced velocity, explaining the characteristic delays in conduction of evoked potentials. Demyelinated axons can discharge spontaneously and show increased mechanical sensitivity, accounting for the flashes of light on eye movement and electrical sensation running down the spine or limbs on neck flexion (Lhermitte's symptom and sign). Partially demyelinated axons whose conduction is compromised cannot sustain the fall in the membrane capacity due to a rise in temperature leading to the appearance of symptoms after a hot bath or exercise (Uhthoff's phenomenon).

1.1.5 MS diagnostic criteria

There is no definitive diagnostic test for MS, but the emerging modern paraclinical techniques such as magnetic resonance imaging (MRI), oligoclonal bands (OCB) and evoked potentials (EPs) can facilitate diagnosis. The diagnosis of MS is fundamentally clinical and requires that a patient at the appropriate age had two distinct episodes of neurological disturbances implicating distinct sites in the white matter of the CNS. Other possible causes for the clinical symptoms need to be excluded before a diagnosis of MS can be made (Compston, 1998). The diagnostic criteria as suggested by Poser et al. (1983) form the basis of the diagnostic procedure, although an international panel on MS diagnosis recently presented revised diagnostic criteria (McDonald et al., 2001). These authors defined attacks as symptoms of neurological dysfunction, with or without objective confirmation, lasting for more than 24 hours. Clinical evidence of lesions

indicates signs of neurological dysfunction demonstrable by neurological examination. Paraclinical or subclinical evidence indicates lesions that are only demonstrable by various tests, such as MRI and EPs, and not by clinical neurological examination, laboratory support applies only to examination of cerebral spinal fluid (CSF) for oligoclonal bands and increased production of immunoglobulin G (IgG).

The Poser criteria have been most commonly used for the identification of patients to be included in epidemiology studies or therapeutic trails in recent years. In the revised criteria, the focus remains on the objective demonstration of dissemination of lesions in both time and space. These new MS criteria accept the diagnosis of patients with a variety of presentations, including monosymptomatic disease suggestive of MS, disease with a typical RR course and disease with insidious progression, without clear attacks and remissions. This reflects an improved understanding of the disease and usefulness of new technology.

1.1.6 Treatment

Since different immunopathological pathways appear to be involved in different subgroups of MS patients, the treatment of this disease may be more complex than previously anticipated (Lassmann, 1999). Available treatments for (relapsing forms of) MS are only partially effective, and therefore steps need to be taken to identify more effective treatments for this disease (Noseworthy, 1999). Future treatment options include strategies to interfere with disease-relevant, specific or nonspecific immune mechanisms and drugs that might promote remyelination (van Oosten et al., 1998). In a recent study the potential benefits of antioxidant administration together with an appropriate diet has also been highlighted (Syburra and Passi, 1999). Choosing specific treatment for a given patient and using specific drugs in different disease stages could potentially improve the outcome. Genetic determination is hypothesised to be responsible for diverse therapeutic responses. Thus, identification of genetic polymorphisms influencing drug metabolism may significantly facilitate the development of effective therapies. Compston and Coles (2002) discussed at length the five aims of treatment, namely:

1. reduce relapse rates
2. prevent fixed disability linked to relapse
3. provide symptomatic management of fixed neurological deficits
4. prevent disability acquired through progression
5. treat established progression

Current treatments involve either steroids, which fight inflammation caused by the immune system reaction, or immuno-suppressant drugs, which depress immune system function. Both these approaches create serious side effects and may slow, but not stop, the progress of the disease. They are also effective mostly at very early stages of disease.

1.2 **Natural history of Multiple Sclerosis**

1.2.1 Prevalence and disease onset

MS affects approximately 2,5 million people across the globe (Homes et al., 1995). Although more females are affected (2:1), there is no difference in disease severity between the sexes. This disease has an incidence of about 7 per 100 000 every year, a prevalence of about 120 per 100 000 and a lifetime risk of 1 in 400. Disease onset is usually in the third or fourth decade, but 2% of patients with MS present before the age of 10 years and 5% before the age of 16 years. Eighty percent of patients present with RR MS, and in a quarter of these patients, MS never affects daily activities, while up to 15% become severely disabled within a short period of time. In the remaining 20% the disease is progressive, affecting the spinal cord and less frequently the optic nerve, cerebrum or cerebellum. Life expectancy is at least 25 years from disease onset and most patient's die from unrelated causes (Compston and Coles, 2002).

1.2.2 Geographic Distribution

In a recent report on the worldwide prevalence of MS (Pugliatti et al., 2002) the difficulty in defining the geographical distribution of MS (figure 1) was pointed out. Prevalence studies from different areas assessed at different times had to be compared and the problems encountered were as follows:

1. Variability of surveyed population (size, age structure and ethnic origin and composition) (Rosati, 1994).
2. Difference when determining the numerator (early cases or benign MS) (Sadovnick and Ebers, 1993)
3. Ascertainment of cases i.e. geographic and time variables, access to medical care, local medical expertise, number of neurologists and accessibility to new diagnostic procedures (Sadovnick and Ebers, 1993; Noseworthy et al., 2000).
4. Use of different diagnostic criteria and the interobserver variability in application.

Taking all this into account it is better to describe the global prevalence of MS in three frequency zones (Kurtzke, 2000):

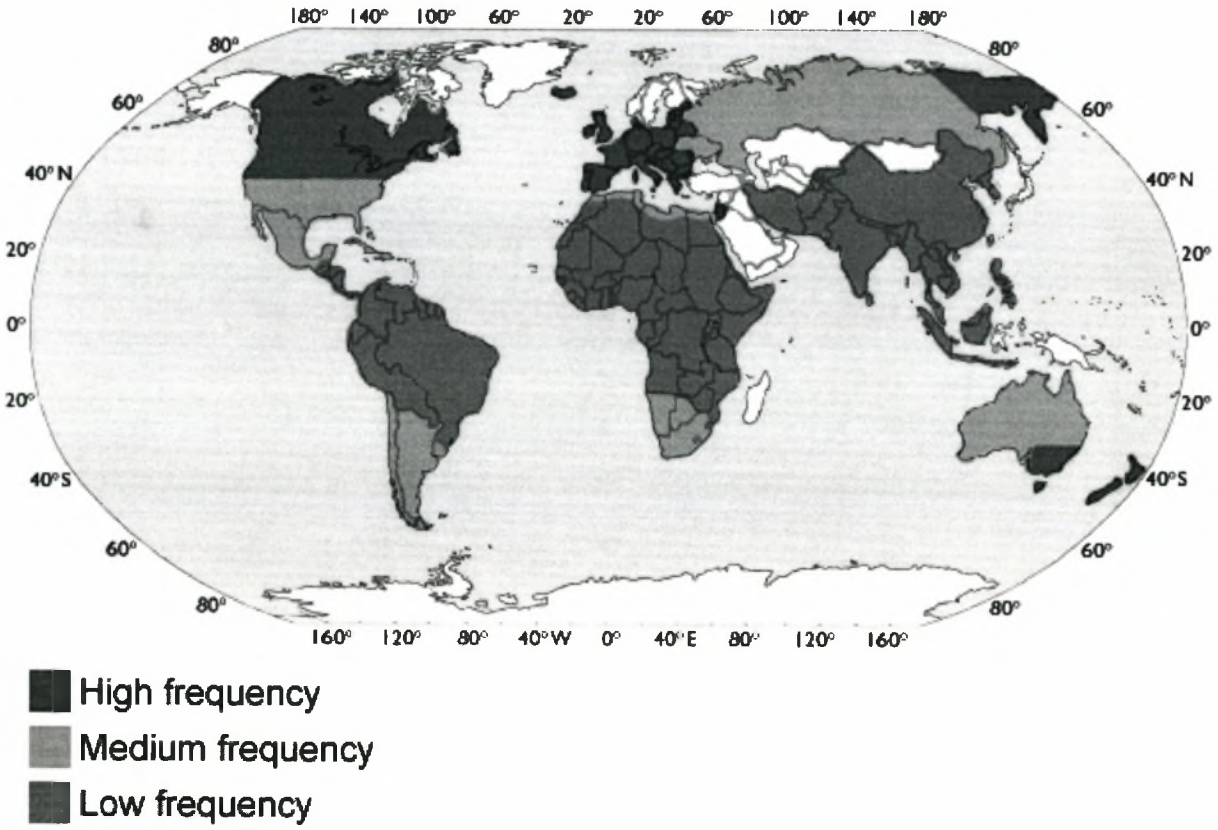
- High frequency areas (more than 30 cases per 100 000) include Europe, Israel, Canada, northern US, southeastern Australia, New Zealand, and easternmost Russia.
- Medium frequency areas include southern US, rest of Australia, South Africa, the southern Mediterranean basin, Russia into Siberia, the Ukraine and parts of Latin America.
- Low frequency areas (less than 5 cases per 100 000) include the rest of Asia, Africa and northern South America.

The data from migration studies suggest that if the exposure to a higher risk environment occurs during adolescence (before 15 years of age), then the migrant assumes the higher risk of the environment. This concept was illustrated in studies of the native-born South African white population with low incidence of the disease versus high incidence of MS among white immigrants from Great Britain, where the disease is much more prevalent (Dean and Elian, 1993).

"Epidemics" of MS have been reported and these provide further evidence of the importance of environmental factors in MS. The most notable "epidemic" was described on the Faroe Islands after they were occupied by British troops in World War II. Similar

increases in incidence of the disease were seen on Shetland and Orkney Islands, in Iceland, and in Sardinia. A specific "point agent" for these "epidemics" was never identified (Kurtzke, 2000).

Figure 1: World distribution of multiple sclerosis (adapted from Kurtzke, 2000).



There are also population studies that show difference in susceptibility to MS between different populations. Lapps in Scandinavia appear to be resistant to the disease, contrary to the expectations based on their geographic distribution. Native Americans and Hutterites very infrequently suffer from MS, as opposed to other residents of North America. MS is uncommon in Japan, China and South America. It is practically unknown among the indigenous people of equatorial Africa and among native Inuit in Alaska. When the racial differences are correlated, White populations are at greater risk than Asian or African populations. We cannot yet explain these obvious inconsistencies in disease distribution, but the knowledge of them may be helpful in assessing specific patients (Weinshenker et al., 1989).

1.3 **Aetiology**

1.3.1 Autoimmunity

The autoimmune nature of MS has long been suspected. It is known that patients with MS have inflammation and demyelination in their CNS and oligoclonal bands in their cerebrospinal fluid (CSF). These abnormal immunoglobulins are identified in a high percentage of patients with clinically definite MS during exacerbations of RR disease, or persistently in a significant proportion of chronic-progressive patients. The composition of the inflammatory infiltrate together with the local expression of various cytokines and other immune-associated molecules suggest that the basis of the inflammatory response is a T-cell mediated immunological process (Cannella and Raine, 1995; Sorensen et al., 1999; Lassmann, 2002). The T-cell mediated inflammation leads to proliferation, activation, and entry into the circulation of autoreactive T cells; they express adhesion molecules and induce reciprocal change in endothelia, allowing access across the blood-brain barrier into the CNS mediating damage associated with the disease (Compston and Coles, 2002). In MS immune dysfunction can be detected locally in CNS and CSF as well as systemically in peripheral circulation (table 1).

Table 1: Immunologic abnormalities in CSF, whole blood and serum in MS patients.

CSF	Serum	Blood
▲ IFN-gamma	▲ IFN-gamma	▲ IFN-gamma
▲IgG and oligoclonal bands	▲ TNF	▲ IL-2
▲ TNF	▲ IL-2	▲ IL-4
▲ activated CD4+ cells	▲ IL-2 receptors	▲ IL-1
	▼ PGE-2 release by macrophages	
	▼ CD8+	

IFN-gamma, Interferon gamma; IgG, immunoglobin G; TNF, Tumor necrosis factor alpha or alpha and beta; CD4+, MHC class II restricted T-cells; CD8+, MHC class I restricted T-cells; IL-1,2,4, interleukins; PGE2, prostaglandin E

1.3.2 Viral factors

The clinical and pathological features of MS implicate viral infections as either cofactors in its aetiology or triggers of relapses, although no specific environmental factors have been identified. Special focus has befallen several herpes viruses because of their ability to cause latent infections that periodically reactivate very similar to the RR course of MS. Furthermore, most herpes viruses can be readily found within the CNS and several are known to induce demyelination, both in humans and in experimentally infected animals (Simmons, 2001; Stohlman and Hinton, 2001).

There is a considerable interest in a theory that exposure to a virus may lead to immunopathologic condition resulting in MS. One possible explanation for this is molecular mimicry between viral and CNS proteins so that antiviral response is mediated against myelin. Molecular mimicry is characterised by an immune response to an environmental agent that cross-reacts with a host antigen resulting in disease (Levin et al., 2002). Another possibility is that autoimmunity results from super antigenic stimulation of T-cells by viral or bacterial proteins. Super antigens may bind to specific T-cell receptor proteins, producing non-specific stimulation of a large number of T-cells. This may result in clonal expansion of T-cells reactive to myelin or oligodendrogliocyte antigens. Levin et al. (2002) showed a clear link between virus infection, autoimmunity and neurological disease in humans by studying patients with human T-lymphotropic

virus type 1 (HTVL-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a disease that may be indistinguishable from MS.

Perron et al. (1997) described a novel retrovirus that was dubbed MS-associated retrovirus (MSRV). This virus was detected in patients with MS, but not in control individuals. Dolei and co-workers (2002) analysed the blood and CSF of Sardinian MS patients and detected the MSRV in 50% and in 40% of control CSF. In the blood samples the MSRV was detected in all the samples and in some patients with inflammatory neurologic disease, but rarely in healthy blood donors (Dolei et al., 2002). The author concluded that MSRV might represent a marker of neurologic disease of inflammatory origin. In a recent review Simmons (2001) summarised the role of herpes virusses in MS (table 2).

Table 2: Summary of the potential role played by herpes viruses in MS (adapted from Simmons, 2001).

Relapsing remitting epidemiology	Geographical distribution consistent with MS	Circumstantial serological support	Virus or viral nucleic acid in blood or brain during acute MS	Plaque-associated viral antigen or DNA sequences	Viruses known to be neurotropic
HSV EBV	VZV MDV	EBV HHV-6	HSV HHV-6	HHV-6	HSV-1 and -2 VZV HHV-6 and -8

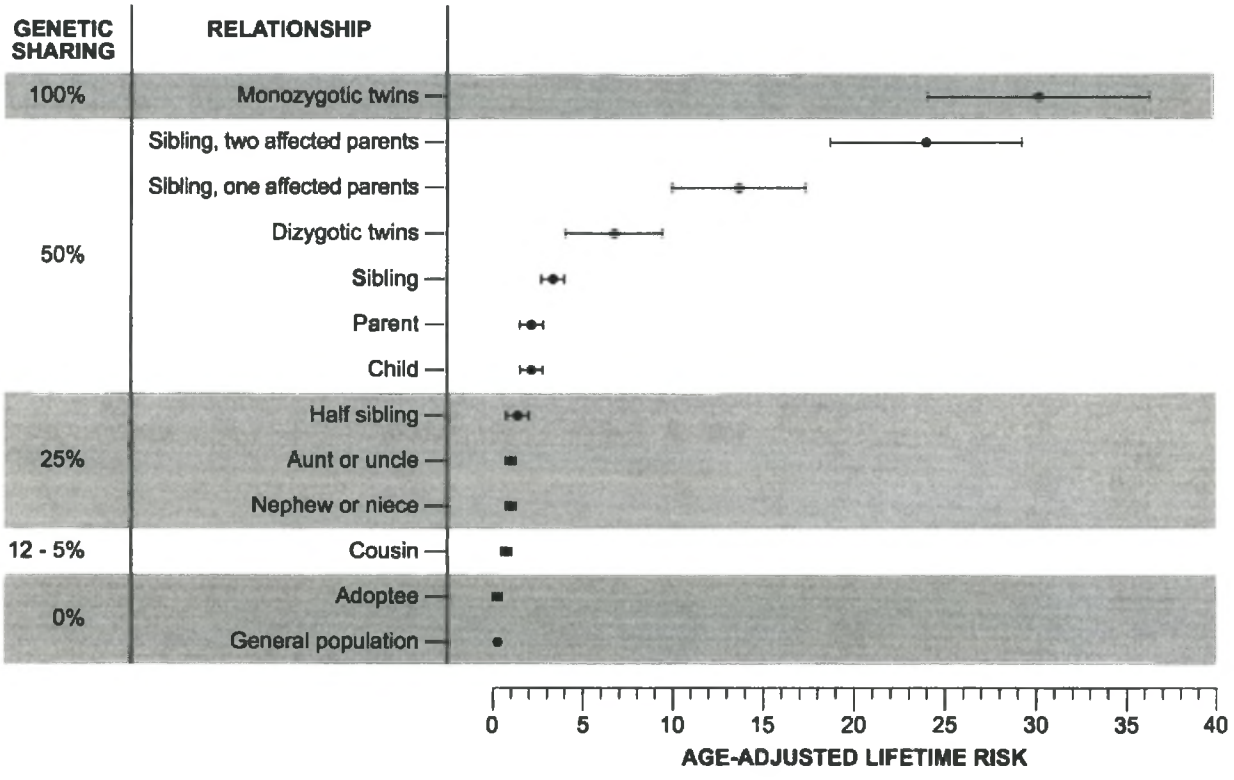
HSV, herpes simplex virus; VZV, varicella zoster virus; EBV, Epstein-Barr virus; HHV, human herpesvirus; MDV, Marek's disease virus

1.3.3 Genetic factors

The estimated familial occurrence in Caucasian MS populations is about 15%. The age-adjusted risk is higher for siblings (3%), parents (2%), and children (2%) than for second-and third-degree relatives (figure 2). Recurrence in monozygotic and dizygotic twins is 35% and 6% respectively, and is higher in children of conjugal pairs with MS (20%) than in the offspring with a single affected parent (2%) (Ebers et al., 1995; Sadovnick et al., 2000; Compston and Coles, 2002). Genetic epidemiology has strongly suggested the involvement of genetic determinants in MS and the following statements surmise the results of genetic epidemiology and molecular genetic studies to date (Sadovnick, 2002).

a. MS results from an interaction of genetic and environmental factors
b. The familial aggregation of MS is due to the genetic material these individuals share with the index patient
c. MS appears to be oligogenic (more than one gene involved)
d. HLA does not appear to be a "deterministic" gene for MS
e. Genetic susceptibility factors may overlap, at least to some extent, between the general population and individuals with MS

Figure 2: Lifetime risk of multiple sclerosis (adapted from Compston and Coles, 2002).



1.4 The search for genetic factors underlying MS

While a relatively large number of genes may underlie the MS phenotype, interactions between different genes could result in a dramatic increase in disease susceptibility (synergistic gene effects). The genes that have attracted most interest as possible susceptibility factors in MS can be divided into three main groups: genes affecting immune functions, myelin structural genes and genes implicated in genome wide screening studies. Most of the gene associations described in a specific population could not be confirmed in other populations, suggesting that the genetic susceptibility factors of MS may vary between different populations. The following approaches have been sought by researchers in an attempt to explain the complexity of MS.

1.4.1 Whole genome screens

There are two methods to map susceptibility loci, namely linkage and association studies. Linkage studies entail the search for markers that co-segregate with a disease in a family whereas association studies compare allele frequencies at certain markers between clinically affected individuals and unaffected controls to identify possible statistically significant differences. The principal requirements for genome screening in complex traits are a sizeable and well-validated clinical resource, a map of highly polymorphic markers covering the whole genome and the technology to complete the large number of genotypings required (Chataway et al., 1998). The discovery of short sequence repeats (Nakamura et al., 1987), CA repeats (Weber and May, 1989), fluorescent labelling, sizing (Diehl et al., 1990; Ziegle et al., 1992) and mapping within the human genome (Dib et al., 1996) enabled genome screening to develop. Commercial companies market extensive genome kits in combination with high throughput technology and high-powered software for analysis. Since 1994 when the first genome screen was attempted (Davies et al., 1994), several studies of complex diseases such as MS have been performed. Table 3 provides a summary and overlap comparison of four genome screens (Haines et al., 1996; Sawcer et al., 1996; Ebers et al., 1996; Kuokkanen et al., 1997), two meta-analysis studies of the data (The Transatlantic Multiple Sclerosis Genetics Cooperative 2001; Wise et al., 1999) and a

new genome wide screen on a Nordic population (Akesson et al., 2002). In none of these studies a single locus with formal significant linkage could be identified, but a number of chromosomal regions of importance were highlighted. In a meta-analysis of these four genome screens, Wise and co-workers (1999) identified 5 regions with a *P*-value less than 0.05. A meta-analysis performed by three of the original genome screen studies (Haines et al., 1996; Sawcer et al., 1996; Ebers et al., 1996) revealed a total of eight regions that had non-parametric linkage (NPL) scores greater than 2.0. The authors pointed out that overall, their linkage results suggest that MS is likely to be multigenic in its genetic susceptibility (The Transatlantic Multiple Sclerosis Genetics Cooperative 2001). In a recent study Akesson et al. (2002) identified 17 regions that exceeded the 5% significance lod score threshold, although no genome wide significance were observed. Using a DNA pooling strategy, Sawcer et al. (2002) confirmed previous linkage candidate areas at positions 1p, 6p, 17q and 19q and stated that their pooling genome protocol is currently used in 18 additional European studies in order to search for susceptibility genes shared between populations of common ancestry, as well as ethnically diverse populations.

Table 3: Summary and overlap comparison of genome screen results in different populations.

American (Haines et al., 1996)	British (Sawcer et al., 1996)	Canadian (Ebers et al., 1996)	Finnish (Kuokkanen et al., (1997)	Meta-analysis (Wise et al., 1999)	Meta analysis (Transatlantic cooperation 2001)	Nordic (Akesson et al., 2002)
	1p36-p33	1p36-p33				
2p23	2p23-p21	2p23-p21		2p		
	3p14-p13	3p14-p13			3p	
3q22-q24		3q22-q24	3q			3q21.1
4q31-qter	4q31-qter					
5q13-q23	5q12-q13	5q12-q13			5q	
6p21	6p21	6p21	6p	6p	6p	6p21
6q27	6q22-q27				6q	
7q11-q22		7q21-q22				
			17q22	17q	17q11; 17q22	17q25
18p11		18p11				
19q13	19q12-q13	19q13		19q		

Adapted from (Dyment et al., 1997)

There have been several follow-up studies concerning genomic regions of interest (Broadley et al., 2001; Chataway et al., 1998; Chataway et al., 1999; Coraddu et al., 2001; D'Alfonso et al., 1999; Dai et al., 2001; Larsen et al., 2000; Oturai et al., 1999; Sawcer et al., 2002; Vandenbroeck et al., 2002; Vitale et al., 2002; Xu et al., 1999). Although the findings in genome screens revealed potentially interesting chromosomal regions, it failed to detect one major gene. This supports the notion that MS is indeed a polygenic disease where many genes are involved, each having a minor effect.

1.4.2 Candidate gene studies

Since MS is thought to be an autoimmune disease mediated by autoreactive T cells directed against myelin antigens, various cytokines, chemokines and their receptors and autoantigens etc. are attractive candidates for MS susceptibility. Thus far, studies trying

to identify disease-modifying effects in MS have identified four genes of probable importance: *HLA class II, apoE, IL-1ra and IL-1β*, Kantarci et al. (2002) gives a very comprehensive summery of association studies performed of non-MHC candidate genes with disease severity in MS (Kantarci et al., 2002). Known genetic factors considered to be of importance in MS susceptibility following a literature survey are discussed in the following sections.

1.4.2.1 HLA

Jersild and co-workers have reported in 1972 that MS is associated with HLA-A3, -B7 and -Dw2. Since 1972, advances in HLA typing techniques, from cellular typing to serology and DNA full identification of HLA polymorphism, have shown that cellularly defined specificity Dw2, which has a confirmed role in MS, corresponds to DR15 and DQ6 in serologic nomenclature and to the haplotype DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602 by genomic nomenclature (Hillert, 1994).

Haines et al. (1998) in a collaborative study, analysed a data set of 98 multiplex MS families to test for an association with the HLA-DR2 allele in familial MS and to determine whether genetic linkage to the major histocompatibility complex (MHC) was caused by such an association. The authors used three highly polymorphic markers (HLA-DR, D6S273, and TNF-beta) in MHC demonstrated strong genetic linkage (parametric lod scores of 4.60, 2.20, and 1.24, respectively) and a specific association with the HLA-DR2 allele was confirmed; the transmission disequilibrium test (TDT) yielded a *P* value of less than 0.001. Stratifying the results by HLA-DR2 status showed that the linkage results were limited to families segregating HLA-DR2 alleles. These results demonstrated that the HLA-DR2 allelic association could explain genetic linkage to the MHC. This study also indicated that sporadic and familial MS share a common genetic susceptibility, in addition, preliminary calculations suggested that the MHC explains between 17% and 62% of the genetic aetiology of MS (Haines et al., 1998). A large number of studies have confirmed the association of MS with a haplotype carrying a MHC class II HLA-DR15 and HLA-DQ6 alleles, however, since the original association this finding has contributed relatively little to the understanding of disease mechanisms

(Olerup and Hillert, 1991). Association studies of HLA genes is complicated by the presence of highly polymorphic genes and strong linkage disequilibrium, requiring large numbers of individuals to be analysed. Masterman et al. (2000) confirmed the importance of DRB1*15 susceptibility to MS in 948 Swedish patients, but failed to show any influence on either disease course or disease severity. Ligers et al. (2001) showed that HLA-DRB1*15 is not the sole MHC determinant of MS susceptibility in northern-European populations by finding a similar sharing of linkage in families with and without the HLA-DRB1*15 allele. It was pointed out that there remains a possibility that the association of MS with HLA-DRB1*15 is due to linkage disequilibrium with a nearby locus and/or to the presence of disease-influencing allele(s) in DRB1*15 negative haplotypes. Two other studies performed in large pedigrees with a strong family history indicated the importance of the HLA-DRB1*15 in conjunction with other haplotypes as a modifier in MS susceptibility (Dyment et al., 2001; Vitale et al., 2002). In a study by Rubio and co-workers (2002) using log-linear modelling analysis of constituent haplotypes that present genomic regions containing HLA class I, II and III genes, it was shown that having class I and II susceptibility variants on the same haplotype provides an additive risk for MS. It was suggested that by using the approach as outlined in the article the contribution of HLA to MS might be defined more accurately.

1.4.2.2 CD45, Protein-Tyrosine Phosphatase, Receptor-Type C

In 3 of 4 independent case-control studies, Jacobsen et al. (2000) demonstrated an association between MS and a C-to-G transition at nucleotide 77 in exon 4 of the protein-tyrosine phosphatase, receptor-type C (*PTPRC*) gene. Although the mutation did not change the encoded amino acid, it prevented splicing of exon 4 pre-mRNA. Furthermore, it was found that the PTPRC mutation was linked to and associated with the disease in 3 MS nuclear families. Vorechovsky et al. (2001) determined allele frequencies for the 77C-G polymorphism in large numbers of MS patients and patients with common variable immunodeficiency (CVID), IgA deficiency (IgAD) and over 1,000 controls to assess whether aberrant splicing of PTPRC caused by this polymorphism results in increased susceptibility to these diseases. They could detect no difference in the frequency of the 77G allele in patients and controls in these disorders with a strong

autoimmune component in aetiology. Likewise, Barcellos et al. (2001), Tchilian et al. (2002) and Miterski et al. (2002) found no evidence of genetic association between the PTPRC polymorphism and MS susceptibility or disease course. Ballerini et al. (2002) detected the 77C-G mutation in a small number of Italian MS patients but not in a matched group of healthy controls (Fisher exact test, *P* value=0.02). This finding suggests a role, in at least a group of patients, for the *PTPRC* mutation in genetic susceptibility to MS. Jacobsen et al. (2002) furthermore detected a novel mutation C to A at position 59. The mutation interferes with alternative splicing and with antibody binding to the CD45RA domain, as previously detected in MS patients (Jacobsen et al., 2000).

1.4.2.3 Monocyte chemotactic protein 3 (MCP-3)

Monocyte chemotactic protein 3 (MCP-3) is a chemokine that attracts mononuclear cells, including monocytes and lymphocytes, the inflammatory cell types that predominate in MS lesions. Fitten et al. (1999) have studied the possible association between the presence of a CA/GA microsatellite repeat polymorphism in the promoter-enhancer region of the MCP-3 gene and the occurrence of MS in the Swedish population. They could not determine any significant associations. Nelissen et al. (2002) did a follow-up study in the Belgium population and detected a positive association with the A3 and a negative association with the A2 allele detected in the promotor area of the gene. Further studies are warranted to investigate this association.

1.5 **The role of iron metabolism in MS**

There are several lines of support for a role of iron dysregulation in MS, which appear to be compatible with many processes involved in the pathogenesis of MS. Iron overload may predispose individuals to virus infection while inadequate supply of iron may impair immune function, thereby influencing the course of infection. The frequency distribution of transferrin (an iron- and zinc-binding protein) phenotypes was found to differ in the cohort of MS patients and controls studied by Schiffer et al. (1994). Zeman et al. (2000)

highlighted the potential role of transferrin determination in cerebrospinal fluid (CSF) as a means to distinguish between RR, SP and PP MS, and noted that transferrin is also a growth factor of importance in proliferation of activated T lymphocytes. Differences in iron parameters measured in CSF of MS patients have furthermore been reported by several other groups (LeVine et al., 1999; Weller et al., 1999).

Increased mean serum ferritin levels were reported by Valberg et al. (1989) in MS patients, although no subjects with clinically manifested hereditary hemochromatosis (HH) were recognised by these authors among 1700 patients with MS. Since MS and HH affects the same ethnic group, these findings may indicate interaction of the HH gene with iron-related genetic and/or environmental factors involved in the MS phenotype.

The high demand of iron in the brain and CNS and their sensitivity to iron-induced peroxidative damage, suggests the need for stringent regulation of iron availability in these organs. Dysregulation of iron homeostasis in the brain may influence the myeliniation process, particularly during the early stages of development. The importance of sufficient and timely iron delivery to the brain has been repeatedly demonstrated (Walter, 1990; Connor and Menzies, 1996), such as the phenomenon of hypomyelination in iron-deficient rats (Larkin et al., 1990). Hulet et al. (1999) demonstrated that the normal pattern of transferrin and ferritin binding is disrupted in the brain tissue of MS patients. This finding provided evidence of ferritin binding in human brain and suggests that loss of ferritin binding is involved in or is a consequence of demyelination associated with MS. Data on iron concentration in the brains of MS patients have been conflicting and, to our knowledge, the possible role of iron has not previously been studied at the DNA level. Low or high levels of other heavy metal divalent cations have also been associated with the pathogenesis of MS in the past. Specifically a report published by Downey (1992) raised the possibility that copper may be an environmental risk factor for MS via disturbance of the haem biosynthesis pathway.

1.6 Objectives of the study

The overall objective of the study is to improve our understanding of MS by studying genes involved in iron metabolism and immune function within the context of autoimmune and infectious disease susceptibility, in order to investigate the complex interaction between genetic and viral factors implicated in the pathogenesis of MS.

The specific aims were as follows:

1. Screen MS patients and population-matched controls for genetic variation in candidate genes involved in iron metabolism and immune function, in order to define a possible link between genetic and environmental factors.
2. Compare frequencies of specific viral sequences among MS subgroups, close relatives and unrelated controls to better define possible MS subgroups and disease expression within families.

The ultimate goal is to develop a comprehensive molecular diagnostic screening approach for detection of a genetic predisposition for MS at an early age, so that relevant environmental triggers of the disease can be avoided if possible.

1.7 References

Akesson E, Oturai A, Berg J, Fredrikson S, Andersen O, Harbo HF, Laaksonen M, Myhr KM, Nyland HI, Ryder LP, Sandberg-Wollheim M, Sorensen PS, Spurkland A, Svejgaard A, Holmans P, Compston A, Hillert J and Sawcer S. A genome-wide screen for linkage in Nordic sib-pairs with multiple sclerosis. Genes and Immunity 2002; 3: 279-285.

Ballerini C, Rosati E, Salvetti M, Ristori G, Cannoni S, Biagioli T, Massacesi L, Sorbi S and Vergelli M. Protein tyrosine phosphatase receptor-type C exon 4 gene mutation distribution in an Italian multiple sclerosis population. Neuroscience Letters 2002; 328: 325-327.

Barcellos LF, Caillier S, Dragone L, Elder M, Vittinghoff E, Bucher P, Lincoln RR, Pericak-Vance M, Haines JL, Weiss A, Hauser SL and Oksenberg JR. PTPRC (CD45) is not associated with the development of multiple sclerosis in U.S. patients. Nature Genetics 2001; 29: 23-24.

Broadley S, Sawcer S, D'Alfonso S, Hensiek A, Coraddu F, Gray J, Roxburgh R, Clayton D, Buttinelli C, Quattrone A, Trojano M, Massacesi L and Compston A. A genome screen for multiple sclerosis in Italian families. Genes and Immunity 2001; 2: 205-210.

Cannella B and Raine CS. The adhesion molecule and cytokine profile of multiple sclerosis lesions. Annals of Neurology 1995; 37: 424-435.

Chataway J, Feakes R, Coraddu F, Gray J, Deans J, Fraser M, Robertson N, Broadley S, Jones H, Clayton D, Goodfellow P, Sawcer S and Compston A. The genetics of multiple sclerosis: principles, background and updated results of the United Kingdom systematic genome screen. Brain 1998; 121: 1869-1887.

Chataway J, Sawcer S, Feakes R, Coraddu F, Broadley S, Jones HB, Clayton D, Gray J, Goodfellow PN and Compston A. A screen of candidates from peaks of linkage: evidence for the involvement of myeloperoxidase in multiple sclerosis. Journal of Neuroimmunology 1999; 98: 208-213.

Compston A. Future options for therapies to limit damage and enhance recovery. Seminars in Neurology 1998; 18: 405-414.

Compston A and Coles A. Multiple sclerosis. Lancet 2002; 359: 1221-1231.

Connor JR and Menzies SL. Relationship of iron to oligodendrocytes and myelination. Glia 1996; 17: 83-93.

Coraddu F, Sawcer S, D'Alfonso S, Lai M, Hensiek A, Solla E, Broadley S, Mancosu C, Pugliatti M, Marrosu MG and Compston A. A genome screen for multiple sclerosis in Sardinian multiplex families. European Journal of Human Genetics 2001; 9: 621-626.

D'Alfonso S, Nistico L, Zavattari P, Marrosu MG, Murru R, Lai M, Massacesi L, Ballerini C, Gestri D, Salvetti M, Ristori G, Bomprezzi R, Trojano M, Liguori M, Gambi D, Quattrone A, Fruci D, Cucca F, Richiardi PM and Tosi R. Linkage analysis of multiple sclerosis with candidate region markers in Sardinian and Continental Italian families. European Journal of Human Genetics 1999; 7: 377-385.

Dai Y, Xu C, Holmberg M, Oturai A, Fredrikson S, Sandberg-Wollheim M, Laaksonen M, Spurkland A, Myhr KM, Ryder LP, Sorensen PS, Svejgaard A, and Hillert J. Linkage analysis suggests a region of importance for multiple sclerosis in 3p14-13. Genes and Immunology 2001; 2: 451-454.

Davies JL, Kawaguchi Y, Bennett ST, Copeman JB, Cordell HJ, Pritchard LE, Reed PW, Gough SC, Jenkins SC, Palmer SM, et al. A genome-wide search for human type 1 diabetes susceptibility genes. Nature 1994; 371: 130-136.

Dean G and Elian M. Motor neuron disease and multiple sclerosis mortality in Australia, New Zealand and South Africa compared with England and Wales. Journal of Neurology, Neurosurgery and Psychiatry 1993; 56: 633-637.

Dean G, Bhigjee AI, Bill PL, Fritz V, Chikanza IC, Thomas JE, Levy LF and Saffer D. Multiple sclerosis in black South Africans and Zimbabweans. Journal of Neurology, Neurosurgery and Psychiatry 1994; 57: 1064-1069.

Dib C, Faure S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, Marc S, Hazan J, Seboun E, Lathrop M, Gyapay G, Morissette J and Weissenbach J. A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature 1996; 380: 152-154.

Diehl SR, Ziegle J, Buck GA, Reynolds TR and Weber JL. Automated genotyping of human DNA polymorphisms. American Journal of Human Genetics 1990; 47 (supplement): 177.

Dolei A, Serra C, Mameli G, Pugliatti M, Sechi G, Cirotto MC, Rosati G and Sotgiu S. Multiple sclerosis-associated retrovirus (MSRV) in Sardinian MS patients. Neurology 2002; 58: 471-473.

Downey D. Porphyria induced by palladium-copper dental prostheses: a clinical report. Journal of Prosthetic Dentistry 1992; 67: 5-6.

Dyment DA, Sadovnick AD, Ebers GC and Sadovnich AD. Genetics of multiple sclerosis. Human Molecular Genetics 1997; 6: 1693-1698.

Dyment DA, Wilier CJ, Scott B, Armstrong H, Ligers A, Hillert J, Paty DW, Hashimoto S, Devonshire V, Hooge J, Kastrukoff L, Oger J, Metz L, Warren S, Hader W, Power C, Auty A, Nath A, Nelson R, Freedman M, Brunet D, Paulseth JE, Rice G, O'Connor P,

Duquette P, Lapierre Y, Francis G, Bouchard JP, Murray TJ, Bhan V, Maxner C, Pryse-Phillips W, Stefanelli M, Sadovnick AD, Risch N and Ebers GC. Genetic susceptibility to MS: a second stage analysis in Canadian MS families. Neurogenetics 2001; 3: 145-151.

Ebers GC, Sadovnick AD and Risch NJ. A genetic basis for familial aggregation in multiple sclerosis. Canadian Collaborative Study Group. Nature 1995; 377: 150-151.

Ebers GC, Kukay K, Bulman DE, Sadovnick AD, Rice G, Anderson C, Armstrong H, Cousin K, Bell RB, Hader W, Paty DW, Hashimoto S, Oger J, Duquette P, Warren S, Gray T, O'Connor P, Nath A, Auty A, Metz L, Francis G, Paulseth JE, Murray TJ, Pryse-Phillips W, Risch N, et al. A full genome search in multiple sclerosis. Nature Genetics 1996; 13: 472-476.

Ebers CG. The natural history of multiple sclerosis. Neurological Sciences 2000; 21 (supplement 2): 815-817.

Fiten P, Vandenbroeck K, Dubois B, Van Coillie E, Nelissen I, Van Damme J, Ligers A, Hillert J, Andersson M, Olsson T and Opdenakker G. Microsatellite polymorphisms in the gene promoter of monocyte chemotactic protein-3 and analysis of the association between monocyte chemotactic protein-3 alleles and multiple sclerosis development. Journal of Neuroimmunology 1999; 95: 195-201.

Haines JL, Ter-Minassian M, Bazyk A, Gusella JF, Kim DJ, Terwedow H, Pericak-Vance MA, Rimmler JB, Haynes CS, Roses AD, Lee A, Shaner B, Menold M, Seboun E, Fitoussi RP, Gartioux C, Reyes C, Ribierre F, Gyapay G, Weissenbach J, Hauser SL, Goodkin DE, Lincoln R, Usuku K, Oksenberg JR, et al. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. The Multiple Sclerosis Genetics Group. Nature Genetics 1996; 13: 469-471.

Haines JL, Terwedow HA, Burgess K, Pericak-Vance MA, Rimmler JB, Martin ER, Oksenberg JR, Lincoln R, Zhang DY, Banatao DR, Gatto N, Goodkin DE and Hauser

SL. Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group. Human Molecular Genetics 1998; 7: 1229-1234.

Hillert J. Human leukocyte antigen studies in multiple sclerosis. Annals of Neurology 1994; 36 (supplement): 15-17.

Homes J, Madgwick T and Bates D. The cost of multiple sclerosis. British Journal of Medical Economics 1995; 18: 181-193.

Hulet SW, Powers S and Connor JR. Distribution of transferrin and ferritin binding in normal and multiple sclerotic human brains. Journal of Neurological Sciences 1999; 165: 48-55.

Jacobsen M, Schweer D, Ziegler A, Gaber R, Schock S, Schwinzer R, Wonigeit K, Lindert RB, Kantarci O, Schaefer-Klein J, Schipper HI, Oertel WH, Heidenreich F, Weinshenker BG, Sommer N and Hemmer B. A point mutation in PTPRC is associated with the development of multiple sclerosis. Nature Genetics 2000; 26: 495-499.

Jacobsen M, Hoffmann S, Cepok S, Stei S, Ziegler A, Sommer N and Hemmer B. A novel mutation in PTPRC interferes with splicing and alters the structure of the human CD45 molecule. Immunogenetics 2002; 54: 158-163.

Jersild C, Svejgaard A and Fog T. HL-A antigens and multiple sclerosis. Lancet 1972; 1: 1240-1241.

Kantarci OH, de Andrade M and Weinshenker BG. Identifying disease modifying genes in multiple sclerosis. Journal of Neuroimmunology 2002; 123: 144-159.

Kira J, Kanai T, Nishimura Y, Yamasaki K, Matsushita S, Kawano Y, Hasuo K, Tobimatsu S and Kobayashi T. Western versus Asian types of multiple sclerosis:

immunogenetically and clinically distinct disorders. Annals of Neurology 1996; 40: 569-574.

Kuokkanen S, Gschwend M, Rioux JD, Daly MJ, Terwilliger JD, Tienari PJ, Wikstrom J, Palo J, Stein LD, Hudson TJ, Lander ES and Peltonen L. Genomewide scan of multiple sclerosis in Finnish multiplex families. American Journal of Human Genetics 1997; 61: 1379-1387.

Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology 1983; 33: 1444-1452.

Kurtzke JF. Natural history and clinical outcome measures for multiple sclerosis studies. Why at the present time does EDSS scale remain a preferred outcome measure to evaluate disease evolution? Neurological Sciences 2000; 21: 339-341.

Larkin EC and Rao A. In Dobbing, J. (ed.), Brain, behaviour and iron in the infant diet. Importance of fetal and neonatal iron: adequacy for normal development of the central nervous system. New York, Springer-Verlag, 1990; 43-62.

Larsen F, Oturai A, Ryder LP, Madsen HO, Hillert J, Fredrikson S, Sandberg-Wollheim M, Laaksonen M, Harbo HF, Sawcer S, Fugger L, Sorensen PS and Svejgaard A. Linkage analysis of a candidate region in Scandinavian sib pairs with multiple sclerosis reveals linkage to chromosome 17q. Genes and Immunology 2000; 1: 456-459.

Lassmann H. Mechanisms of demyelination and tissue damage in multiple sclerosis. Acta Neurologica Belgica 1999; 99: 6-10.

Lassmann H. Mechanisms of demyelination and tissue destruction in multiple sclerosis. Clinical Neurology and Neurosurgery 2002; 104: 168-171.

Levin MC, Lee SM, Kalume F, Morcos Y, Dohan FC Jr, Hasty KA, Callaway JC, Zunt J, Desiderio D and Stuart JM. Autoimmunity due to molecular mimicry as a cause of neurological disease. Nature Medicine 2002; 8: 509-513.

LeVine SM, Lynch SG, Ou CN, Wulser MJ, Tam E and Boo N. Ferritin, transferrin and iron concentrations in the cerebrospinal fluid of multiple sclerosis patients. Brain Research 1999; 821: 511-515.

Ligers A, Teleshova N, Masterman T, Huang WX and Hillert J. CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms. Genes and Immunity 2001; 2: 145-152.

Lublin FD and Reingold SC. for the National Multiple Sclerosis Society (USA) Advisory Commitee on Clinical Trials of New Agents in Multiple Sclerosis. Defining the clinical course of multiple sclerosis: results of an international survey. Neurology 1996; 46: 907-911.

Masterman T, Ligers A, Olsson T, Andersson M, Olerup O and Hillert J. HLA-DR15 is associated with lower age at onset in multiple sclerosis. Annals of Neurology 2000; 48: 211-219.

McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, McFarland HF, Paty DW, Polman CH, Reingold SC, Sandberg-Wollheim M, Sibley W, Thompson A, van den Noort S, Weinshenker BY and Wolinsky JS. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Annals of Neurology 2001; 50: 121-127.

Miller A. Diagnosis of multiple sclerosis. Seminars in Neurology 1998; 18: 309-316.

Miterski B, Sindern E, Haupts M, Schimrigk S and Epplen JT. PTPRC (CD45) is not associated with multiple sclerosis in a large cohort of German patients. BMC Medical Genetics 2002; 3: 3.

Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M, Martin C, Fujimoto E, Hoff M, Kumlin E, et al. Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 1987; 235: 1616-1622.

Nelissen I, Dubois B, Goris A, Ronsse I, Carton H and Opdenakker G. Gelatinase B, PECAM-1 and MCP-3 gene polymorphisms in Belgian multiple sclerosis. Journal of Neurological Sciences 2002; 200: 43-48.

Noseworthy JH. Progress in determining the causes and treatment of multiple sclerosis. Nature 1999; 399: 40-47.

Noseworthy JH, Lucchinetti C, Rodriguez M and Weinshenker BG. Multiple sclerosis. New England Journal of Medicine 2000; 343: 938-952.

Olerup O and Hillert J. HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. Tissue Antigens 1991; 38: 1-15.

Oturai A, Larsen F, Ryder LP, Madsen HO, Hillert J, Fredrikson S, Sandberg-Wollheim M, Laaksonen M, Koch-Henriksen N, Sawcer S, Fugger L, Sorensen PS and Svejgaard A. Linkage and association analysis of susceptibility regions on chromosomes 5 and 6 in 106 Scandinavian sibling pair families with multiple sclerosis. Annals of Neurology 1999; 46: 612-616.

Perron H, Garson JA, Bedin F, Beseme F, Paranhos-Baccala G, Komurian-Pradel F, Mallet F, Tuke PW, Voisset C, Blond JL, Lalande B, Seigneurin JM and Mandrand B. Molecular identification of a novel retrovirus repeatedly isolated from patients with

multiple sclerosis. The Collaborative Research Group on Multiple Sclerosis. Proceedings of the National Academy of Sciences U S A 1997; 94: 7583-7588.

Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH and Tourtellotte WW. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Annals of Neurology 1983; 13: 227-231.

Pugliatti M, Sotgiu S and Rosati G. The worldwide prevalence of multiple sclerosis. Clinical Neurology and Neurosurgery 2002; 104: 182-191.

Rosati G. Descriptive epidemiology of multiple sclerosis in Europe in the 1980s: a critical overview. Annals of Neurology 1994; 36 (supplement 2): 164-174.

Rubio JP, Bahlo M, Butzkueven H, van Der Mei IA, Sale MM, Dickinson JL, Groom P, Johnson LJ, Simmons RD, Tait B, Varney M, Taylor B, Dwyer T, Williamson R, Gough NM, Kilpatrick TJ, Speed TP and Foote SJ. Genetic dissection of the human leukocyte antigen region by use of haplotypes of Tasmanians with multiple sclerosis. American Journal of Human Genetics 2002; 70: 1125-1137.

Sadovnick AD and Ebers GC. Epidemiology of multiple sclerosis: a critical overview. Canadian Journal of Neurological Sciences 1993; 20: 17-29.

Sadovnick AD, Yee IM and Ebers GC. The Canadian Collaborative Study Group. Factors influencing sib risks for multiple sclerosis. Clinical Genetics 2000; 58: 431-435.

Sadovnick AD. The Genetics of multiple sclerosis. Clinical Neurology and Neurosurgery 2002; 104: 199-202.

Sawcer S, Jones HB, Feakes R, Gray J, Smaldon N, Chataway J, Robertson N, Clayton D, Goodfellow PN and Compston A. A genome screen in multiple sclerosis reveals susceptibility loci on chromosome 6p21 and 17q22. Nature Genetics 1996; 13: 464-468.

Sawcer S, Maranian M, Setakis E, Curwen V, Akesson E, Hensiek A, Coraddu F, Roxburgh R, Sawcer D, Gray J, Deans J, Goodfellow PN, Walker N, Clayton D and Compston A. A whole genome screen for linkage disequilibrium in multiple sclerosis confirms disease associations with regions previously linked to susceptibility. Brain 2002; 125: 1337-1347.

Schiffer RB, Weitkamp LR, Ford C and Hall WJ. A genetic marker and family history study of the upstate New York multiple sclerosis cluster. Neurology 1994; 44: 329-333.

Simmons A. Herpesvirus and multiple sclerosis. Herpes 2001; 8: 60-63.

Sorensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, Qin S, Rottman J, Sellebjerg F, Strieter RM, Frederiksen JL and Ransohoff RM. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. Journal of Clinical Investigation 1999; 103: 807-815.

Stohlman SA and Hinton DR. Viral induced demyelination. Brain Pathology 2001; 11: 92-106.

Syburra C and Passi S. Oxidative stress in patients with multiple sclerosis. Ukrainskii Biokhimicheskii Zhurnal 1999; 71: 112-115.

Tchilian EZ, Dawes R, Ramaley PA, Whitworth JA, Yuldasheva N, Wells SR, Watera C, French N, Gilks CF, Kunachiwa W, Ruzibakiev R, Leetrakool N, Carrington CV, Ramdath DD, Gotch F, Stephens HA, Hill AV and Beverley PC. A CD45 polymorphism associated with abnormal splicing is absent in African populations. Immunogenetics 2002; 53: 980-983.

The Transatlantic Multiple Sclerosis Genetics Cooperative. A meta-analysis of genomic screens in multiple sclerosis. Multiple Sclerosis 2001; 7: 3-11.

Valberg LS, Flanagan PR, Kertesz A and Ebers GC. Abnormalities in iron metabolism in multiple sclerosis. Canadian Journal of Neurological Sciences 1989; 16: 184-186.

Vandenbroeck K, Fiten P, Heggarty S, Goris A, Cocco E, Hawkins SA, Graham CA, Marrosu MG and Opdenakker G. Chromosome 7q21-22 and multiple sclerosis: evidence for a genetic susceptibility effect in vicinity to the protachykinin-1 gene. Journal of Neuroimmunology 2002; 125: 141-148.

van Oosten BW, Barkhof F, Scholten PE, von Blomberg BM, Ader HJ and Polman CH. Increased production of tumor necrosis factor alpha, and not of interferon gamma, preceding disease activity in patients with multiple sclerosis. Archives of Neurology 1998; 55: 793-798.

Vitale E, Cook S, Sun R, Specchia C, Subramanian K, Rocchi M, Nathanson D, Schwalb M, Devoto M and Rohowsky-Kochan C. Linkage analysis conditional on HLA status in a large North American pedigree supports the presence of a multiple sclerosis susceptibility locus on chromosome 12p12. Human Molecular Genetics 2002; 11: 295-300.

Vorechovsky I, Kralovicova J, Tchilian E, Masterman T, Zhang Z, Ferry B, Misbah S, Chapel H, Webster D, Hellgren D, Anvret M, Hillert J, Hammarstrom L and Beverley PC. Does 77C-->G in *PTPRC* modify autoimmune disorders linked to the major histocompatibility locus? Nature Genetics 2001; 29: 22-23.

Walter T. Infancy: mental and motor development. American Journal of Clinical Nutrition 1989; 50: 655-661.

Weber JL and May PE. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. American Journal of Human Genetics 1989; 44: 388-396.

Weinshenker BG, Bass B, Rice GP, Noseworthy J, Carriere W, Baskerville J and Ebers GC. The natural history of multiple sclerosis: a geographically based study. I. Clinical course and disability. Brain 1989; 112: 133-146.

Weller M, List U, Schabet M, Melms A and Dichgans J. Elevated CSF lactoferrin in superficial siderosis of the central nervous system. Journal of Neurology 1999; 246: 943-945.

Wise LH, Lanchbury JS and Lewis CM. Meta-analysis of genome searches. Annals of Human Genetics 1999; 63: 263-272.

Xu C, Dai Y, Fredrikson S and Hillert J. Association and linkage analysis of candidate chromosomal regions in multiple sclerosis: indication of disease genes in 12q23 and 7ptr-15. European Journal of Human Genetics 1999; 7: 110-116.

Zeman D, Adam P, Kalistova H, Sobek O, Kelbich P, Andel J and Andel M. Transferrin in patients with multiple sclerosis: a comparison among various subgroups of multiple sclerosis patients. Acta Neurologica Scandinavica 2000; 101: 89-94.

Ziegle JS, Su Y, Corcoran KP, Nie L, Mayrand PE, Hoff LB, McBride LJ, Kronick MN and Diehl SR. Application of automated DNA sizing technology for genotyping microsatellite loci. Genomics 1992; 14: 1026-1031.

CHAPTER 2

METHODS, RESULTS AND DISCUSSION

CHAPTER 2.1

Analysis of the NRAMP1 gene implicated in iron transport: Association with multiple sclerosis and age effects.

Blood Cells, Molecules and Diseases 2001; 27: 44-53.

Blood Cells, Molecules, and Diseases (2001) 27(1) February: 44–53
doi:10.1006/bcmd.2000.0349, available online at http://www.idealibrary.com on IDEAL®

Analysis of the NRAMP1 Gene Implicated in Iron Transport: Association with Multiple Sclerosis and Age Effects

Submitted 11/20/00; revised 12/07/00
(Communicated by E. Beutler, M.D., 12/07/00)

Maritha J. Kotze,[1] J. Nico P. de Villiers,[1] Roberta N. Rooney,[1] Johanna J. Grobbelaar,[1] Erna P. G. Mansvelt,[2] Christianne S. H. Bouwens,[3] Jonathan Carr,[4] Ilse Stander,[5] and Lana du Plessis[1]

ABSTRACT: Multiple sclerosis (MS) is believed to be an autoimmune process occurring in genetically susceptible individuals after an appropriate environmental exposure. We have exploited the homogeneous Afrikaner population of European ancestry to investigate the likelihood that iron dysregulation, in association with infectious and/or autoimmune disease susceptibility, may underlie the MS phenotype in a subgroup of patients. The functional Z-DNA forming repeat polymorphism of the natural resistance-associated macrophage protein-1 (NRAMP1) gene was analyzed in 104 patients diagnosed with MS and 522 Caucasian controls. A family-based control group consisting of 32 parental alleles not transmitted to MS offspring was additionally studied to exclude the likelihood of population substructures. Statistically significant differences in allelic distribution were observed between the patient and control samples drawn from the same population ($P < 0.01$). Evidence is furthermore provided that alleles considered to be detrimental in relation to autoimmune disease susceptibility may be maintained in the population as a consequence of improved survival to reproductive age following infectious disease challenge. Although it remains to be determined whether the disease phenotype in MS patients with allele 5 of the NRAMP1 promoter polymorphism is directly related to dysregulation of iron or modified susceptibility to viral infection and/or autoimmunity, a combination of these processes most likely underlies the disease phenotype in these patients. In view of the emerging role of polymorphic variants in complex diseases and minimizing of possible confounding factors in this association study, we conclude that allelic variation in the NRAMP1 promoter may contribute significantly to MS susceptibility in the South African Caucasian population.

INTRODUCTION

The cause of multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS), remains unknown after more than a century of study. Current knowledge suggests that MS is associated with autoimmunity, with genetic susceptibility and infectious agents as important risk factors (1, 2). The overall lifetime risk for siblings of MS patients to manifest the disease is estimated at 4–5%, compared to a population prevalence of approximately 0.1%. Although data on family studies have excluded all but a polygenic mode of inheritance, and an autosomal dominant or recessive single locus with low penetrance, most evidence suggests that several genes control disease susceptibility (3). Autopsy studies in a cohort of MS patients indicated four fundamentally different patterns of demyelination, suggesting pathogenetic heterogeneity with important implications for the diagnosis and treatment of the disease (4).

Correspondence and reprint requests to: M. J. Kotze, Department of Human Genetics, Faculty of Medicine, P. O. Box 19063, Tygerberg 7505, South Africa. Fax: 27 21 9317810. E-mail: mjk@gerga.sun.ac.za.
[1] Department of Human Genetics, Faculty of Medicine, University of Stellenbosch, Tygerberg, South Africa.
[2] Department of Hematological Pathology, Faculty of Medicine, University of Stellenbosch, Tygerberg, South Africa.
[3] Department of Internal Medicine, Faculty of Medicine, University of Stellenbosch, Tygerberg, South Africa.
[4] Department of Neurology, Faculty of Medicine, University of Stellenbosch, Tygerberg, South Africa.
[5] Biostatistics Unit, South African Medical Research Council, Tygerberg, South Africa.

1079-9796/01 $35.00

Blood Cells, Molecules, and Diseases (2001) 27(1) February: 44–53
doi:10.1006/bcmd.2000.0349, available online at http://www.idealibrary.com on IDEAL®

Previous genome-wide gene searches and association studies suggest that the genetic susceptibility factors of MS may vary between different populations (3). The white Afrikaner population of South Africa represents an ideal group (5) for the identification of genes involved in this complex disease, since the prevalence of MS is relatively high in the European populations that contributed to the gene pool of this genetic isolate. Earlier studies indicated a relatively low incidence (~0.4/100,000) of the disease in the Caucasian population of South Africa (6), but more recently Dean (7) reported that the prevalence of MS is on the increase in the Afrikaner population. Although the Afrikaner population is mainly of Dutch, German and French origin, settlers from the United Kingdom with a relatively high prevalence (12/100,000) of MS amongst Scottish individuals (8), have also made a considerable contribution (~5%) to the gene pool (9). The unique genetic background of the Afrikaner population therefore offers the advantage to study the genetics of MS on the basis of an expected limited number of disease-associated genes/mutations introduced from Europe. Genetic analysis in the African context furthermore provides the opportunity to assess the relevance of potentially important sequence changes in indigenous population groups, where MS is considered to be extremely rare (10).

The importance of genetic factors underlying population/ethnic differences in disease risk was illustrated by the finding that the hereditary hemochromatosis (HH) mutation C282Y in the HFE gene (11), associated with this common iron overload disease in the majority (70–100%) of affected Caucasians worldwide, is virtually absent in the South African Black population (12). Of relevance to this study is the apparent absence of clinically manifested HH among 1700 patients with MS encountered by Valberg et al. (13), despite the higher serum ferritin levels detected in patients compared with controls and the fact that MS and HH affect the same ethnic group. Similar findings were reported in an Afrikaner family where two sisters with MS were found to be homozygous for the common HH mutation C282Y (14), which largely excluded the likelihood of a linkage disequilibrium effect with a putative MS-related gene on chromosome 6. These findings raised the possibility that MS and HH may be mutually exclusive due to gene-gene interaction in certain populations.

There are several lines of support for a role of iron dysregulation in the pathogenesis of MS (15–20). This pertains to the fact that iron overload may predispose an individual to virus infection, while on the contrary, inadequate supply of iron may impair immune function. To further investigate the potential role of iron metabolism in association with infectious and/or autoimmune disease susceptibility in MS, we genotyped 104 South African MS patients and 532 control individuals from the same population for the functional Z-DNA forming repeat polymorphism of the natural resistance-associated macrophage protein-1 (NRAMP1) gene. This gene that regulates iron, and is also regulated by cellular iron levels (21), has been linked to many infectious and autoimmune diseases (22–25). Although these findings may be related to any one of the multiple pleiotropic effects associated with macrophage activation, it seems highly likely that regulation of iron transport via NRAMP1 may contribute directly to disease susceptibility (23). Some of the diseases associated with the NRAMP1 gene may pathogenetically be related to MS (26–30) and apparently also to abnormalities in iron absorption (31, 32), that may be linked to defective iron supply for erythropoiesis (33). The anemia associated with autoimmune disease may be related to the proposed role of NRAMP1 in scavenging iron from senescent red cells via splenic macrophage phagosomes for transport back to the blood.

MATERIALS AND METHODS

Study population. Relevant information was obtained from 281 South African patients, following the distribution of a questionnaire that was published in the South African National Multiple Sclerosis Society newsletter. Blood samples were obtained, after informed consent, from 156 South African respondents who completed the questionnaire and 197 of their close relatives. The patients' neurologists were subsequently contacted

to verify the MS diagnoses, based on clinical features (34), lumbar puncture, magnetic resonance imaging, evoked potentials and/or exclusion of other diseases known to manifest similarly. Uncertain cases included in the study were re-examined by J.C. Non-Caucasian South African MS patients, recent immigrants, and respondents without a confirmed diagnosis of MS were excluded from the study, leaving 104 index cases diagnosed with definite MS for genetic analysis.

DNA samples of 329 South African individuals of European descent (mainly German, Dutch, French and British) below the age of 70 years were included as unrelated controls. The individuals consisted of 231 laboratory personnel and medical students (68 males and 163 females), and 98 spouses of patients with MS (16 males and 13 females) or familial hypercholesterolemia (34 males and 35 females). Additionally, 193 elderly individuals above the age of 70 years (67 males and 126 females) and a family-based control group consisting of 32 parental alleles not transmitted to MS offspring were included for comparison within and between subgroups. Inclusion and exclusion criteria were similar in the experimental and control groups. MS patients and controls found to be homozygous for the HH mutation C282Y were excluded from this study. Data obtained on laboratory personnel, medical students and spouses of index cases were pooled with the "affected-family-based control" (AFBAC) group (35) after verification of similar allele frequencies in the different groups. DNA samples from 448 non-Caucasians (278 Xhosas, 50 KhoiSan, and 120 subjects of Mixed Ancestry), 37 healthy Scottish individuals and 143 Germans were included as controls for population studies. Matched case-control groups are particularly important in diseases such as MS where environmental triggers may be involved. The study protocol has been approved by the Ethics Review Committee of the University of Stellenbosch.

Hematological and biochemical analysis. Hemoglobin, serum iron, transferrin and ferritin concentrations were determined using standard methods. Serum iron was measured with a Beckman CX7 autoanalyzer, and transferrin with a Beckman array nephelometer. Transferrin saturation was subsequently calculated. None of the patients analyzed experienced episodes of MS at the time iron concentrations were determined.

DNA analysis. Genomic DNA was isolated from whole blood using a standard salting-out method (36). The PCR primers used to amplify a 483-bp fragment of the NRAMP1 promoter were 5′-GGGGTCTTGGAACTCCAGAT-3′ (forward) and 5′-GGGCAGCTCCTCAGCCTGCAC-3′ (reverse). The PCR conditions were as follows: 200 ng of genomic DNA, 0.2 μM of each dGTP, dCTP, dATP, and dTTP, 20 pmol of each primer, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, 50 mM KCl, and 1 U *Taq* DNA Polymerase. PCR cycle conditions included an initial denaturing step at 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 65°C for 45 s, and 72°C for 30 s. Screening of PCR-amplified DNA for potential disease-related mutations was performed using a combined HEX-SSCP (heteroduplex single-strand conformation polymorphism) method according to Kotze et al. (37). PCR products corresponding to aberrant bands were sequenced using an automated system (ABI 310, Perkin–Elmer Applied Biosystems). For better discrimination between different alleles of the (gt)n repeat, *Rsa*I restriction enzyme analysis was performed according to Graham et al. (25). Following the amplification of a shorter 116-bp fragment using a new reverse primer 5′-TACCCCATGACCACACCC-3′, PCR products were digested to completion with *Rsa*I, subjected to polyacrylamide (PAA) gel electrophoresis (12% PAA, 5% C), stained with ethidium bromide and visualized by ultraviolet light.

Statistical methods. NRAMP1 allele frequencies were analyzed by STATISTICA for Windows (38), which offers different statistical methods to determine significant associations. The Chi-square (χ^{2-}) test and/or Fisher-exact test were performed to assess significant associations with NRAMP1 in the study population. Due to the low frequency of certain variants in the study population, the Yates' correction was applied to improve the approximation of the χ^{2-} test,

Blood Cells, Molecules, and Diseases (2001) 27(1) February: 44–53
doi:10.1006/bcmd.2000.0349, available online at http://www.idealibrary.com on IDEAL®

TABLE 1

Biochemical Iron Status Parameters Measured in the Serum of 53 South African MS Patients Classified According to Relapsing–Remitting, Secondary Progressive, and Primary Progressive Disease Types

	Relapsing–remitting (n = 34)		Secondary progressive (n = 10)		Primary progressive (n = 9)	
Variable	Mean	SD	Mean	SD	Mean	SD
Age (years)	40.3	10.9	46.4	12.1	47.8	7.1
S-iron (μmol/liter)	17.0	6.8	17.8	6.8	16.6	6.4
Transferrin (g/liter)	2.8	0.4	2.9	0.4	2.6	0.4
Transf sat (%)	25.1	10.6	25.4	12.0	25.4	10.0
Ferritin (μg/liter)	77.9	63.5	117.0	83.1	171.8*	135.7

* Relapsing–remitting vs primary progressive MS: $P = 0.04$.

thereby providing a more conservative estimation. The Arlequin program (39) was used to test for various associations of polymorphic variants in the disease and control populations. This program assesses the likelihood that false associations may be obtained due to population substructures. For continuous variables groups were compared using unpaired t tests. The Wilcoxon (Kruskal–Wallis) and median tests were used for variables (ferritin) with skew distributions.

RESULTS

Serum Iron Parameters

Iron parameters were determined in 34 South African MS patients with relapsing-remitting (RR) MS, 10 with secondary progressive (SP) MS and 9 with primary progressive (PP) MS. Similar to that of a control group including 30 Caucasian individuals, the hemoglobin, serum iron, transferrin, transferrin saturation and ferritin values were within the normal range in the majority of cases. The serum ferritin concentration was significantly lower in the RR group than in the PP group ($P < 0.05$), but none of the other parameters tested differed between the three patient groups (Table 1). When males are excluded, the statistical significance disappears even though the trend remains the same (RR 71.6 μg/liter, SP 88.0 μg/liter, PP 109.6 μg/liter). The patients with the progressive illness showed greater variability in ferritin levels. Patients with a RR disease course were the youngest on average ($P < 0.08$). The age at onset was also the youngest in the RR MS group (29.5, SD 9.6), followed by the SP (34.3, SD 11.1) and PP (35.3, SD 6.8) groups.

Analysis of the Functional Z-DNA Forming NRAMP1 Repeat Polymorphism

Many variants have been described in the NRAMP1 gene, one of which is a functional Z-DNA forming repeat promoter polymorphism affecting NRAMP1 expression (22). The different alleles detected within the amplified DNA fragment spanning this polymorphic region are shown in Table 2, together with the allelic distribution observed in South African Caucasian MS patients and controls drawn from the same population. The allelic distribution of the Z-DNA forming

TABLE 2

Comparison of Allelic Distribution between South African MS Patients (22 Males, 82 Females) and Controls

		Controls			
Alleles	MS patients	General	AFBAC	Elderly	Total
2	41 (20%)	223 (34%)	11 (34%)	86 (22%)	320 (30%)
3	160 (77%)	434 (66%)	21 (66%)	297 (77%)	752 (70%)
5	7 (3%)	1 (0.1%)	0	3 (0.8%)*	4 (0.4%)**
Total	208	658	32	386	1076

Note. NRAMP1 alleles are numbered according to Ref. 25. The controls (1076 alleles) are grouped according to "affected family-based control" (AFBAC), general population-matched controls (118 males, 211 females), and elderly subjects (67 males, 126 females).

* General control group vs elderly subjects: $P < 0.01$, 2 df, $\chi^2 = 16.6$.

** MS vs total control group: $P < 0.01$, 2 df, $\chi^2 = 35.2$; MS vs general control group: $P < 0.01$, 2 df, $\chi^2 = 30.8$; MS (16 patients) vs AFBAC (32 parental alleles): $P < 0.01$, 2 df, $\chi^2 = 43.79$; MS vs elderly control group: $P = 0.05$, 2 df, $\chi^2 = 5.7$.

TABLE 3

Clinical Characteristics of South African MS Patients with NRAMP1 Allele 5

	Index patients (n = 7)							Relatives with definite MS (n = 2)	
Patient numbers	43	58	67	70	73	81	90	Sister of 43	Aunt of 81
Gender	F	F	F	F	M	F	F	F	F
Age (years)	48	38	27	35	45	27	29	39	50
Age of onset (years)	38	24	20	32	41	23	15	15	23
Age at diagnosis (years)	41	30	24	33	42	23	23	25	46
Disease course	RR	RR	RR	RR	SP	RR	RR	RR	SP
Iron parameters[a]									
Hemoglobin (11.5–16.5 g/dl)	13.5	15.0	14.4	12.6	14.0	12.0	13.7	ND	13.6
Serum iron (6–32 μmol/liter)	11.0	14.2	23.3	21.5	16.3	29.7	16.1	ND	17.1
Transferrin (1.8–3.8 g/liter)	3.0	2.4	3.0	3.1	2.3	2.5	3.0	ND	2.4
Transferrin saturation (15–50%)	15	24	31	30.3	28	52.7	21	ND	31
Ferritin (12–119 μg/liter)	33.5	88.8	70.2	157.9	329.5	216.6	14.5	51.2	77.3

Note. F, female; M, male; RR, relapsing–remitting; SP, secondary progressive; ND, not determined.

[a] Reference values for males: hemoglobin, 12.5–17.5 g/dl; serum iron, 7–35 μmol/liter; transferrin, 1.8–3.8 g/liter; saturation, 20–50%; ferritin, 29–396 μg/liter.

dinucleotide polymorphism differed significantly between the MS patients and population-matched controls ($P < 0.01$), including 329 individuals from the general population within the same age group, a family-based control (AFBAC) group including 32 parental alleles not transmitted to MS offspring, and 193 elderly Afrikaner individuals above the age of 70 years. Although the sample sizes of the different subgroups analyzed are relatively small, no population substructures were evident upon application of the Ewens–Watterson test of selective neutrality and the Chakraborty's test for population amalgamation (39). The genotype distribution and allele frequencies of the NRAMP1 promoter variant were in Hardy–Weinberg equilibrium in the populations tested.

Genotyping of the 193 elderly Afrikaner individuals from the general population also indicated a significant deviation from the younger control group ($P < 0.01$) (Table 2). This finding suggests that alleles considered to be detrimental in relation to autoimmune disease susceptibility may be maintained in the population because they improve survival to reproductive age following infectious disease challenge (22), a phenomenon that may be of particular relevance in the African context. This may imply that alleles 3 and 5 found at a higher frequency in the elderly group compared to younger controls might confer protection against infection, iron overload and/or oxidative processes implicated in aging. Iron deficiency anemia was evident in the two elderly males (ferritin, 12 and 39 μg/liter; transferrin saturation, 10.8 and 16.5%) with this genotype, while higher levels (ferritin, 273 μg/liter; transferrin saturation, 20%) were measured in the elderly female with alleles 3 and 5 of NRAMP1.

The clinical characteristics of the index MS patients with allele 5 are summarized in Table 3, together with that of two affected female relatives with this allele who were also diagnosed with definite MS. Extended clinical and family follow up are underway to clarify the potential role of NRAMP1 in the MS phenotype.

Population Studies

NRAMP1 allele 5 was not detected in Africans following DNA screening of 278 Xhosas, 50 KhoiSan, and 120 subjects of Mixed Ancestry. This finding is in accordance with the potential significance of allele 5 in MS, since this disease is extremely rare in Africans (10). Population and haplotype studies using intragenic NRAMP1 polymorphisms are in progress to trace the origin of NRAMP1 allele 5 in European populations that contributed to the Afrikaner gene pool. Prelimi-

Blood Cells, Molecules, and Diseases (2001) 27(1) February: 44–53
doi:10.1006/bcmd.2000.0349, available online at http://www.idealibrary.com on IDEAL®

nary data obtained in DNA samples of healthy Scottish individuals indicated a significantly higher frequency (2/37, 5.4%) of NRAMP1 allele 5 in this population compared with the general Afrikaner population (0.3%) ($P < 0.01$, 2 *df*, $\chi^2 = 14.33$), while an intermediate frequency was observed in the German population (3/143, 2.1%). These findings raise the possibility that NRAMP1 allele 5 may be one of the factors underlying the high prevalence (8) of MS in Scotland. Approximately 15% of the 104 South African MS patients (1/7 with allele 5) included in this study reported that at least one of their ancestors was of Scottish descent.

DISCUSSION

Iron is an essential nutritional element for normal cellular functioning of a large number of enzymes, including those involved in myelin formation, neurotransmitter synthesis and degradation, oxidative phosphorylation and heme biosynthesis (40–42). Hemeproteins are involved in electron transport, which could be critical to axonal as well as to myelin integrity. The human NRAMP1 gene was analyzed in this study as a first candidate to investigate the hypothesis that intermittent iron deficiency (19) may underlie the MS phenotype in a subgroup of patients. The potential value of iron concentration determination in the cerebrospinal fluid (CSF) in distinguishing between MS patients with different disease courses has been demonstrated (16, 17), and this may also apply to determination of serum iron parameters (20). Preliminary data furthermore indicated a significantly higher mean serum ferritin concentration in the South African patient group compared to controls (data not shown), which is in accordance with previous findings (13). This difference remained statistically significant when only females were compared, and in this group mean transferrin saturation was lower in patients compared with controls, although the values were within the normal range in both groups. The statistically significant differences in NRAMP1 allelic distribution observed between MS patients and control individuals drawn from the same population ($P < 0.01$) are in accordance with possible involvement of iron metabolism in the pathogenesis of MS. Notably, different cellular iron levels in Nramp1 wild type (low) versus mutant (high) macrophages in mice may affect mRNA stability for MHC class II molecules (23), which highlights the link between iron metabolism and cell-mediated immunity (43). The enhanced responsiveness of Nramp1 wildtype macrophages to a range of biological stimuli (44), including bacterial lipopolysaccharide (LPS), interferon-γ, glucocorticoids such as dexamethasone or cortisone (frequently applied in the treatment of MS), may relate to the enhancer activity of the Z-DNA forming dinucleotide repeat (22) analyzed in this study.

The functional significance of the NRAMP1 promoter polymorphism analyzed argues against the likelihood that the increased frequency of the potentially functional Z-DNA variant in South African MS patients is due to association with another mutation in the NRAMP1 gene or a nearby gene. Sequence changes which directly affect the gene product are more likely to demonstrate genetic and phenotypic differences between patient and control samples than indirect markers, particularly if it is present in only a small proportion of the study population as demonstrated in this study. This may explain why previous investigators failed to identify human chromosome 2q as a potentially important region in whole genome screens for MS and highlights the potential involvement of separate candidate loci in different populations (3). Association studies appear to be of greater power than linkage analysis in genetic studies of complex human diseases (45), since associated (disease-predisposing) polymorphisms are not necessarily linked to a disease. A mathematical explanation for this phenomenon has been provided by Greenberg (46), which emphasizes the fact that the detection of certain alleles (usually at a lower frequency) in a control population does not exclude the likelihood that the sequence change may cause or contribute to the disease phenotype. MS is considered to be a heterogeneous disease (4, 47) where many different genes may be defective. Consistent linkage to the HLA region on chromosome 6 (47) in different genomic data sets and different popula-

tions may be related to possible polygenic control of MS, where disease susceptibility in conjunction with environmental factors is determined by non-MHC genes and disease severity/course by modulation of T cell responses to particular antigens, much like the situation described in mice (48). Interestingly, Jacobsen et al. (49) have recently demonstrated an association between MS and the gene (PTPRC) encoding protein-tyrosine phosphatase, receptor-type C (CD45), known to be essential for the activation of T and B cells.

The many cellular functions dependent on iron and other metal ions as cofactors may explain the complex role of NRAMP1 in infectious and autoimmune disease. Future studies may reveal whether the frequent co-existence of MS and inflammatory bowel disease (26, 27), which share some common features (28), may be explained by the involvement of the NRAMP1 gene in both conditions (24). The increased frequency of allele 5 of the Z-DNA forming polymorphism detected in patients with primary biliary cirrhosis (PBC) studied by Graham et al. (25), further supports the view that PBC and MS may be related by a similar autoimmune process (29). The likelihood that the relatively high frequency of allele 5 detected in the South African MS cohort is caused by coexistence of PBC, was largely excluded by previous studies performed by Reich et al. (50), recently extended to our study population.

None of the South African MS patients with NRAMP1 allele 5 was anemic, which highlights the fact that standard parameters of iron status may not necessarily reflect abnormalities in iron-related genes (51, 52). Expression of iron-related genes is furthermore modulated by environmental (e.g., diet, the menstrual cycle, multiple pregnancies, regular blood donation) and genetic factors (53), as demonstrated by frequent detection of clinically unaffected subjects with the HH genotype, even in the elderly population (54). It is well known that a significant inflammatory process can cause interference of iron delivery for heme synthesis resulting in altered iron levels, such as raised ferritin levels and decreased transferrin saturation. Two index cases with a family history of MS and their two female relatives diagnosed with definite MS reported porphyria-like symptoms similar to those described by Rooney et al. (19), supporting the hypothesis that a disturbance of the heme biosynthesis pathway may be involved in the etiology of MS in a subgroup of patients. The likelihood that the allelic association described here may only be due to the atypical porphyria-like symptoms and is unrelated to MS per se, was largely excluded by the fact that only two of the five remaining allele 5-positive index cases without a family history of MS reported similar symptoms.

The data presented in this study are consistent with pathogenic roles for iron-related gene-environment interactions in MS and the previous speculation (22) that certain detrimental NRAMP1 alleles related to autoimmune disease susceptibility, may be associated with improve survival. Although it remains to be determined whether the disease phenotype in patients with NRAMP1 allele 5 is directly related to dysregulation of iron or modified susceptibility to viral infections and/or autoimmunity, a combination of these processes most likely underlie the disease phenotype in a subgroup of South African MS patients with the potential disease-predisposing promoter variant. In view of the emerging role of polymorphisms in complex diseases (55), the functional significance of the Z-DNA forming NRAMP1 dinucleotide repeat in autoimmune and infectious disease susceptibility linked to iron regulation, and minimizing of possible confounding factors in this association study, we conclude that allelic variation in the NRAMP1 promoter may contribute significantly to MS susceptibility in the South African population of European descent. A prominent role of iron in the etiology of many neurological diseases may be forthcoming via its role as a cofactor of hemeproteins which, when dysfunctional, may give rise to deficiencies in energy production, axonal degeneration and porphyric neuropathy (56–58).

ACKNOWLEDGMENTS

The University of Stellenbosch and the Harry and Doris Crossley Foundation supported the study financially. The South African National Multiple Sclerosis Society is acknowledged for distribution of patient ques-

Blood Cells, Molecules, and Diseases (2001) 27(1) February: 44–53
doi:10.1006/bcmd.2000.0349, available online at http://www.idealibrary.com on IDEAL®

tionnaires, collaborating neurologists for clinical data of MS patients, and the study participants for their cooperation. Dr. R. Hillermann, Professor L. Warnich, Dr. A. Olckers, Dr. L. van Niekerk, Dr. S. J. van Rensburg, Professor W. J. H. Vermaak, Dr. C. Lombard, and H. Cloete are thanked for support and helpful discussions. Professor H. Schuster (Germany) and Dr. Scott Baden (Scotland) are thanked for control DNA samples. P. Opperman and L. Breytenbach are thanked for sample collection. J. N. P. de Villiers received student bursaries from the Freda and David Becker Trust and the Poliomyelitis Research Foundation. This work forms part of his thesis to be submitted in fulfillment of the requirements for the degree of Doctor of Philosophy, University of Stellenbosch. The first two authors contributed equally to the study.

REFERENCES

1. Ebers, G. C., and Dyment, D. A. (1998) Genetics of multiple sclerosis. *Semin. Neurol.* **18,** 295–299.
2. Noseworthy, J. H. (1999) Progress in determining the causes and treatment of multiple sclerosis. *Nature* **399**(Suppl.), A40–A47.
3. Bell, J. I., and Lathrop, M. (1996) Multiple loci for multiple sclerosis. *Nat. Genet.* **13,** 377–378.
4. Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (2000) Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. *Ann. Neurol.* **47,** 707–717.
5. Groenewald, J. Z., Liebenberg, J., Groenewald, I., and Warnich, L. (1998) Linkage disequilibrium analysis in a recently founded population: Evaluation of the variegate porphyria founder in South African Afrikaners. *Am. J. Hum. Genet.* **62,** 1254–1258.
6. Dean, G. (1967) Annual incidence, prevalence, and mortality of multiple sclerosis in white South-African-born and in white immigrants to South Africa. *Br. Med. J.* **2,** 724–730.
7. Dean, G. (1985) A new multiple sclerosis epidemic? *S. Afr. Med. J.* **68,** 630.
8. Rothwell, P. M., and Charlton, D. (1998) High incidence and prevalence of multiple sclerosis in south east Scotland: Evidence of a genetic predisposition. *J. Neurol. Neurosurg. Psychiatry* **64,** 730–735.
9. Botha, M. C., and Beighton, P. (1983) Inherited disorders in the Afrikaner population of southern Africa. Part I. Historical and demographic background, cardiovascular, neurological, metabolic and intestinal conditions. *S. Afr. Med. J.* **64,** 609–612.
10. Dean, G., Bhigjee, A. I., Bill, P. L., Fritz, V., Chikanza, I. C., Thomas, J. E., Levy, L. F., and Saffer, D. (1994) Multiple sclerosis in black South Africans and Zimbabweans. *J. Neurol. Neurosurg. Psychiatry* **57,** 1064–1069.
11. Feder, J. N., Gnirke, A., Thomas, W., *et al.* (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat. Genet.* **13,** 399–408.
12. De Villiers, J. N. P., Hillermann, R., Loubser, L., and Kotze, M. J. (1999) Spectrum of mutations in the HFE gene implicated in haemochromatosis and porphyria. *Hum. Mol. Genet.* **8,** 1517–1522.
13. Valberg, L. S., Flanagan, P. R., Kertesz, A., and Ebers, G. C. (1989) Abnormalities in iron metabolism in multiple sclerosis. *Can. J. Neurol. Sci.* **16,** 184–186.
14. Kotze, M. J., de Villiers, J. N. P., Carr, J., Schmidt, S., Warnich, L., and Hillermann, R. (1999) Lack of clinical expression of mutation C282Y implicated in haemochromatosis and porphyria in two sisters diagnosed with multiple sclerosis. *Eur. J. Hum. Genet.* **7**(Suppl.), 59.
15. Schiffer, R. B., Wetkamp, L. R., Ford, C., and Hall, W. J. (1994) A genetic marker and family history study of the upstate New York multiple sclerosis cluster. *Neurology* **44,** 329–333.
16. LeVine, S. M., Lynch, S. G., Ou, C. N., Wulser, M. J., Tam, E., and Boo, N. (1999) Ferritin, transferrin and iron concentrations in the cerebrospinal fluid of multiple sclerosis patients. *Brain Res.* **821,** 511–515.
17. Weller, M., List, U., Schabet, M., Melms, A., and Dichgans, J. (1999) Elevated CSF lactoferrin in superficial siderosis of the central nervous system. *J. Neurol.* **246,** 943–945.
18. Hulet, S. W., Powers, S., and Connor, J. R. (1999) Distribution of transferrin and ferritin binding in normal and multiple sclerosis human brains. *J. Neurol. Sci.* **165,** 48–55.
19. Rooney, R. N., Kotze, M. J., de Villiers, J. N. P., Hillermann, R., and Cohen, J. A. (1999) Multiple sclerosis, porphyria-like symptoms and a history of iron deficiency anemia in a family of Scottish descent. *Am. J. Med. Genet.* **86,** 194–196.
20. Zeman, D., Adam, P., Kalistova, H, Sobek, O., Kelbich, P., Andel, J., and Andel, M. (2000) Transferrin in patients with multiple sclerosis: A comparison among various subgroups of multiple sclerosis patients. *Acta Neurol. Scand.* **101,** 89–94.
21. Atkinson, P. G. P., and Barton, C. H. (1998) Ectopic expression of Nramp1 in COS-1 cells modulates iron accumulation. *FEBS Lett.* **425,** 239–242.
22. Searle, S., and Blackwell, J. M. (1998) Evidence for a functional repeat polymorphism in the promoter of the human NRAMP1 gene that correlates with autoimmune versus infectious disease susceptibility. *J. Med. Genet.* **36,** 295–299.
23. Blackwell, J. M., and Searle, S. (1999) Genetic regulation of macrophage activation: Understanding the

function of Nramp1 (=*Ity/Lsh/Bcg*). *Immunol. Lett.* **65,** 73–80.

24. Hofmeister, A., Neibergs, H. L., Pokorny, R. M., and Galandiuk, S. (1997) The natural resistance-associated macrophage protein gene is associated with Crohn's disease. *Surgery* **122,** 173–178.
25. Graham, A. M., Dollinger, M. M., Howie, S. E. M., and Harrison, D. J. (2000) Identification of novel alleles at a polymorphic microsatellite repeat region in the human NRAMP1 gene promoter: Analysis of allele frequencies in primary biliary cirrhosis. *J. Med. Genet.* **37,** 150–152.
26. Minuk, G. Y., and Lewkonia, R. M. (1986) Possible familial association of multiple sclerosis and inflammatory bowel disease. *N. Engl. J. Med.* **314,** 586.
27. Agranof, D., and Schon, F. (1995) Are focal white matter lesions in patients with inflammatory bowel disease linked to multiple sclerosis? *Lancet* **346,** 190–191.
28. Lossos, A., River, Y., Eliakim, A., and Steiner, I. (1995) Neurological aspects of inflammatory bowel disease. *Neurology* **45,** 416–421.
29. Pontecorvo, M. J., Levinson, J. D., and Roth, J. A. (1992) A patient with primary biliary cirrhosis and multiple sclerosis. *Am. J. Med.* **92,** 433–436.
30. Bergsteinsdottir, K., Yang, H. T., Pettersson, U., and Holmdahl, R. (2000) Evidence for common autoimmune disease genes controlling onset, severity and chronicity based on experimental models for multiple sclerosis and rheumatoid arthritis. *J. Immunol.* **164,** 1564–1568.
31. Weber, J., Werre, J. M., Julius, H. W., and Marx, J. J. (1988) Decreased iron absorption in patients with active rheumatoid arthritis, with and without iron deficiency. *Ann. Rheum. Dis.* **47,** 404–409.
32. Nielsen, O. J., Andersen, L. S., Hansen, N. E., and Hansen T. M. (1994) Serum transferrin receptor levels in anaemic patients with rheumatoid arthritis. *Scand. J. Clin. Lab. Invest.* **54,** 75–82.
33. Cazzola, M., Ponchio L., De Benedetti, F., *et al.* (1996) Defective iron supply for erythropoiesis and adequate endogenous erythropoietin production in the anemia associated with systemic-onset juvenile chronic arthritis. *Blood* **87,** 4824–4830.
34. Poser, C. M., Paty, D. W., Scheinberg, L., *et al.* (1983) New diagnostic criteria for multiple sclerosis: Guidelines for research protocols. *Ann. Neurol.* **13,** 227–231.
35. Thomson, G. (1995) Mapping disease genes: Family-based association studies. *Am. J. Hum. Genet.* **57,** 487–498.
36. Miller, S. A., Dykes, D. D., and Polesky, H. F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **16,** 1215.
37. Kotze, M. J., Theart, L., Callis, M., Peeters, A. V., Thiart, R., and Langenhoven, E. (1995) Nonradioactive multiplex PCR screening strategy for the simultaneous detection of multiple low-density lipoprotein receptor gene mutations. *PCR Methods Appl.* **4,** 352–356.
38. StatSoft, Inc. (1995) STATISTICA for Windows [Computer program manual]. StatSoft, Inc., Tulsa, OK (e-mail: info@statsoft.com; Website: http://www.statsoft.com).
39. Schneider, S., Roessli, D., and Excofier, L. (2000) Arlequin: A software for population genetics data analysis, Ver. 2.000. Genetics and Biometry Lab, Department of Anthropology, Univ. Geneva.
40. Aisen, P. (1994) Iron metabolism in health and disease. *In* Iron Metabolism: An Evolutionary Perspective (Brock, J. H., Halliday, J. W., Pippard, M. J., and Powell, L. W., Eds.), pp. 1–30. Saunders, London.
41. Roskams, A. J. I., and Connor, J. R. (1994) Iron, transferrin, and ferritin in the rat brain during development and aging. *J. Neurochem.* **63,** 709–716.
42. Ponka, P., and Lok, C. N. (1999) The transferrin receptor: Role in health and disease. *Int. J. Biochem. Cell. Biol.* **31,** 1111–1137.
43. Weiss, G., Wachter, H., and Fuchs, D. (1995) Linkage of cell-mediated immunity to iron metabolism. *Immunol. Today* **16,** 495–500.
44. Blackwell, J. M. (1996) Structure and function of the natural resistance-associated macrophage protein (NRAMP1), a candidate protein for infectious and autoimmune disease susceptibility. *Mol. Med. Today* **2,** 205–211.
45. Risch, N., and Merikangas, K. (1996) The future of genetic studies of complex human diseases. *Science* **273,** 1516–1517.
46. Greenberg, D. A. (1992) Linkage disequilibrium of "necessary" disease loci versus "susceptibility" loci. *Am. J. Hum. Genet.* **52,** 135–143.
47. Haines, J. L. Terwedow, H. A., Burgess, K., *et al.* (1998) Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. *Hum. Mol. Genet.* **7,** 1229–1234.
48. Blackwell, J., Freeman, J., and Bradley, D. (1980) Influence of H-2 complex on acquired resistance to *Leishmania donovani* infection in mice. *Nature* **283,** 72–74.
49. Jacobson, M., Schwee, D., Ziegler, A., *et al.* (2000) A point mutation in PTPRC is associated with the development of multiple sclerosis. *Nat. Genet.* **26,** 495–499.
50. Reich, B., Ehlers, I., Will, H., Heesen, C., de Villiers, J. N. P., Rooney, R. N., and Kotze, M. J. (1998) Autoantibodies against nucleolar structures correlate significantly with an unusual association between

Blood Cells, Molecules, and Diseases (2001) 27(1) February: 44–53
doi:10.1006/bcmd.2000.0349, available online at http://www.idealibrary.com on IDEAL®

multiple sclerosis and porphyria symptoms. *J. Neuroimmunol.* **90**(Suppl.), 77.

51. Sampietro, M., Piperno, A., Lupica, L., *et al.* (1998) High prevalence of the His63Asp HFE mutation in Italian patients with porphyria cutanea tarda. *Hepatology* **27,** 181–184.
52. Levy, J. E., Jin. O., Fujiwara, Y., Kuo, F., and Andrews, N. C. (1999) Transferrin receptor is necessary for development of erythrocytes and the nervous system. *Nat. Genet.* **21,** 396–399.
53. Whitfield, J. B., Cullen, L. M., Jazwinska, E. C., *et al.* (2000) Effects of HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. *Am. J. Hum. Genet.* **66,** 1246–1258.
54. Willis, G., Wimperis, J. Z., Smith, K. C., Fellows, I. W., and Jennings, B. A. (2000) Haemochromatosis gene C282Y homozygotes in an elderly male population. *Lancet* **354,** 221–222.
55. Bennet, S. T. (1995) Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. *Nat. Genet.* **9,** 284–292.
56. Meyer, U. A., Schuurmans, M. M., and Lindberg, R. L. P. (1998) Acute porphyrias: Pathogenesis of neurological manifestations. *Semin. Liver Dis.* **18,** 43–52.
57. Lindberg, R. L., Martini, R., Baumgartner, M., *et al.* (1999) Motor neuropathy in porphobilinogen deaminase-deficient mice imitates the peripheral neuropathy of human acute porphyria. *J. Clin. Invest.* **103,** 1127–1134.
58. Heales, S. J., Bolanos, J. P., Stewart, V. C., Brookes, P. S., Land, J. M., and Clark, J. B. (1999) Nitric oxide, mitochondria and neurological disease. *Biochim. Biophys. Acta* **1410,** 215–228.

CHAPTER 2.2

Association study of the functional GT-repeat polymorphism in the promoter region of the *SLC11A1* gene in German and French patients with multiple sclerosis

Association study of the functional GT-repeat polymorphism in the promoter region of the *SLC11A1* gene in German and French patients with multiple sclerosis

ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. Support for a role of iron metabolism in MS was obtained in a recent study of the gene encoding the solute carrier family 11 (proton-coupled divalent metal ion transporters) member 1 (*SLC11A1*), formerly known as the natural resistance-associated macrophage protein (*NRAMP1*), in the genetically homogeneous Afrikaner population of South Africa. In this study the proposed allelic association with *SLC11A1* was further investigated in MS patients from Germany and France, representing two of the parent populations that contributed significantly to the gene pool of the Afrikaner population. The German study population consisted of 267 German MS patients, subdivided into three groups according to disease course, and 176 population-matched controls. The French study group included 107 MS simplex families subjected to transmission disequilibrium testing (TDT) for the functional 5'-(GT)n repeat polymorphism in the promoter region of the *SLC11A1* gene. No allelic association between this polymorphism and MS could be detected in the case-control or family studies. However, the German patient group demonstrated statistically significant differences in genotype distribution between patients with primary- and secondary-progressive MS ($P<0.05$), and between patients with relapsing remitting- and primary progressive MS ($P<0.05$). These findings raise the possibility of a complex interplay between *SLC11A1* and environmental factors such as infectious agents and/or iron status, known to affect gene expression. *SLC11A1* may serve as a model for various diseases where gene-environment interaction results in a relatively small effect with regard to familial risk but a large effect on population risk.

INTRODUCTION

The aetiology of multiple sclerosis (MS) is unknown, although it is widely regarded as an autoimmune disease. It primarily affects young adults, particularly women. Three clearly distinguishable disease courses have been defined (Lublin and Reingold, 1996), namely relapsing-remitting (RR), secondary-progressive (SP) and primary-progressive (PP). In patients with RR disease, episodes occur with unpredictable frequency, variable severity, and often with residual neurological deficits. In 50-60% of patients with RR MS the disease course converts to gradual progression, again at different rates in different patients. In these patients with SP MS, superimposed relapses may occur, particularly early in the SP phase. PP disease is observed in a small minority of MS patients without any clear-cut relapses or sustained periods of improvement. Although PP MS has less inflammatory activity on magnetic resonance imaging (MRI) and histopathological examinations (Thompson et al., 1997), the histopathological picture varies greatly within disease types (Storch and Lassmann, 1997). It is not clear whether the differences in the clinical course and pattern of MRI abnormalities between RR and SP phases represent different immunopathologic processes or mechanisms, or are simply a result of repeated clinical and subclinical relapses and cumulative damage to the central nervous system (CNS). Since disease types cannot be subclassified by MRI, a combination of precise clinical descriptions of disease courses and MRI appearance, possibly in conjunction with immunological, virological and/or genetic analysis, may be the only way of subgrouping MS.

LeVine et al. (1999) have demonstrated the potential value of iron concentration determination in the cerebrospinal fluid (CSF) in distinguishing between chronic progressive and RR MS. This was substantiated in a recent study including analysis of serum transferrin concentrations in MS patients, demonstrating frequent detection of subnormal values in PP patients in comparison with the SP form and RR form in remission (Zeman et al., 2000). The transferrin index was furthermore significantly higher in patients with PP MS than in patients with RR or SP disease courses, while the transferrin quotient was significantly more frequently subnormal in patients with RR MS

in remission compared to those experiencing attacks. CSF transferrin and transferrin quotient were furthermore higher in male than in female patients. These findings suggest an important role of iron metabolism in the aetiology of MS, which appear to be in accordance with gender-related differences in iron status that may relate to the fact that MS is more common in females than males (2:1).

Further support for a role of iron homeostasis in the pathogenesis of MS was obtained from a recent study performed in the relatively genetically homogenous Afrikaner population of South Africa, demonstrating a significant association with the gene encoding the solute carrier family 11 (proton-coupled divalent metal ion transporters) member 1 (*SLC11A1*), formerly known as the natural resistance-associated macrophage protein (*NRAMP1*) (Kotze et al., 2001). *SLC11A1* has been linked to various autoimmune and infectious diseases, which led to speculation that regulation of iron by *SLC11A1* may be of major importance in this context (Blackwell et al., 2000). We have recently provided direct support for this hypothesis, when functional studies demonstrated increased expression upon iron loading for allelic variants 3 and 5 (Graham et al., 2000) of the *SLC11A1* promoter polymorphism shown to be associated with MS in South Africa (Kotze et al., in press). The unique genetic background of the Afrikaner population including Dutch (34.8%), German (33.7%), French (13.2%) and British (5.2%) origins (Botha and Beighton, 1983), provided the advantage to study the genetics of MS on the basis of an expected limited number of disease-associated genes/mutations introduced from Europe. This gene pool remained relatively isolated during the establishment of around 14 generations since the arrival of the first immigrants from Holland at the Cape more than 330 years ago. The aim of the present study was to further investigate the association detected between MS and the *SLC11A1* gene in other populations that contributed to the gene pool of the South African Afrikaner population.

SUBJECTS AND METODS

The diagnosis of MS in affected patients was based on clinical features (Poser et al., 1983), magnetic resonance imaging (MRI) and/or exclusion of other diseases known to manifest similarly.

German study group

Blood samples were obtained with informed consent from 267 German MS patients, attending an outpatient clinic at the University Hospital Eppendorf in Hamburg. These individuals included 129 patients with a RR disease course, 75 with SP and 63 with PP MS. DNA samples of 176 healthy German individuals kindly provided by Dr Herbert Schuster were included as controls.

French study group

A total of 109 case-parent MS families, all comprising one affected child with definite MS and two healthy parents, were recruited throughout France by the French Multiple Sclerosis Genetic Group. Written consent was obtained from each individual participating in the study in accordance with the Helsinki Convention and the French law relating to biomedical research. Geographic and ethnic origin, sex, age at onset, duration of the disease, disease course and grading in a MS disability scale were recorded for each patient.

DNA analysis

Genomic DNA was extracted using standard methods and subjected to polymerase chain reaction (PCR)-amplification using primers (15pmol), 5' – ggggtcttggaactccagat- 3' (forward) and 5' –taccccatgaccacaccc –3' (reverse) spanning the 5'-(GT) repeat polymorphism in the *SLC11A1* promoter region. The PCR was performed in a 25 µi reaction volume containing 50 ng of genomic DNA, 0.2 µM of each dGTP,dCTP,dATP and dTTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl and 0.5 U *Taq* DNA Polymerase (Roche - Diagnostics, Germany). PCR cycle conditions included an initial denaturing step at 95˚C for 2 min, followed by 40 cycles at 95˚C for 30 s, 60˚C for 45 s, and 72˚C

for 30 s and performed on a 9700 thermal Cycler (ABI, Foster City, CA, USA). The PCR products were digested to completion with 5 U *Rsa*I (Gibco, Gaithersburg, Md, USA) as described by Graham et al. (2002), and subjected to polyacrylamide (PAA) gel electrophoresis (12% PAA, 5% C), stained with ethidium bromide and visualised by ultraviolet light.

Statistical analysis

Statistical analysis was performed in the German study population by STATISTICA for Windows (StatSoft Inc. Tulsa OK, USA). Two-tailed Fisher exact test and the χ^2 calculation were applied as appropriate. Due to the low frequency of genotypes 22 and 35 it was excluded to obtain a statistically viable sample size (table 1). The transmission test for linkage disequilibrium (TDT) was performed in the French families as described by Spielman et al. (1993). *P*-values <0.05 were regarded as statistically significant.

RESULTS AND DISCUSSION

Analysis of the 5'-(GT)n repeat polymorphism in the promoter region of the *SLC11A1* gene in 267 German and 107 French patients diagnosed with definite MS, did not reveal statistically significant associations in either the German case-control study or in the French simplex families using TDT. However, when genoptype comparisons were made between German MS patients subgrouped according to disease course, a statistically significant difference ($P<0.05$) was detected between patients with SP and PP MS (table 1). A significant difference in genotypic distribution was also observed between the German patients with a RR and PP disease course ($P<0.05$). No significant differences were observed when the allele frequencies were compared between these MS patient groups (data not shown).

Table 1: Comparison of genotype distribution for the *SLC11A1* GT-repeat polymorphism among controls and German MS patients subdivided according to disease course.

Genotype 2 [t(gt)$_5$ac(gt)$_5$ac(gt)$_{10}$g] 3 [t(gt)$_5$ac(gt)$_5$ac(gt)$_9$g] 5 t(gt)$_4$ac(gt)$_5$ac(gt)$_{10}$ggcaga(g)]	Controls	German MS patients			
		Relapsing remitting (RR)	Secondary progressive (SP)	Primary progressive (PP)	Total MS
2/2	4 (2%)	4 (3%)	0	1 (2%)	5 (2%)
2/3	74 (42%)	40 (31%)	25 (33%)	31 (49%)	96 (36%)
3/3	96 (54%)	83 (64%)	50 (67%)	31 (49%)	164 (61%)
3/5		2 (2%)	0	0	2 (1%)
Total	176	129	75	63	267

Alleles are numbered according to Graham et al. (2000).
RR MS vs PP MS: $P<0.025$, 1 df, $\chi^2 = 5.33$
SP MS vs PP MS: $P<0.05$, 1 df, $\chi^2 = 3.9$

Failure to detect significant associations in the French families (table 2) may be due to the fact that TDT generally has a lower power than association studies based on case-control samples (Spielman and Ewens, 1996). *SLC11A1* allele 5 implicated as an important risk factor in South African MS patients (Kotze et al., 2001), was detected in only three of the 107 French families studied and in one of these cases this allele has not been transmitted from the mother to the MS affected individual. Allele 3, known to be associated with autoimmune disease susceptibility in general (Searle and Blackwell, 1998; Blackwell et al., 2000) and with MS in the South African population (Kotze et al., 2001), was more frequently transmitted to the affected offspring, but this was not statistically significant. Our previous finding indicating a significant association between the 5'-(GT)n repeat polymorphism of the *SLC11A1* gene and MS in the South African population (Kotze et al., 2001), is in accordance with the assumption that the Afrikaner population of European descent represents a valuable source of material for mapping studies to identify genes involved in complex diseases.

Table 2: Association analysis of the *SLC11A1* GT-repeat polymorphism in 107 French simplex MS families

Alleles	French simplex MS families	
	Transmitted	Non-transmitted
1 [t(gt)$_5$ac(gt)$_5$ac(gt)$_{11}$g]	0	1
2 [t(gt)$_5$ac(gt)$_5$ac(gt)$_{10}$g]	51	56
3 [t(gt)$_5$ac(gt)$_5$ac(gt)$_9$g]	161	156
5 t(gt)$_4$ac(gt)$_5$ac(gt)$_{10}$ggcaga(g)]	2	1
Total	214	214

Alleles are numbered according to Graham et al. (2000).

Detection of *SLC11A1* allele 5 in both the German and French populations confirmed the European origin of this variant (Graham et al., 2000). It is noteworthy that the highest frequency (5.4%) reported to date for *SLC11A1* allele 5 occurs in the Scottish population, reported to have a genetic predisposition for MS (Rothwell and Charlton, 1998, Graham et al., 2000). This allele appears to be absent in Africans, a finding which has previously been related to the virtual absence of MS in this ethnic group (Kotze et al., 2001). Failure to clearly demonstrate an association between *SLC11A1* and MS in the European populations studied, may be a consequence of selection criteria and differences in environmental exposures in the different populations.

The data provided in this study raise the possibility of a complex interplay between *SLC11A1* and environmental factors in the aetiology of MS, which may include infectious agents and iron status known to affect gene expression (Searle and Blackwell, 1998, Kotze et al., in press). It seems likely that the observed differences in allelic distribution of the 5'-(GT) promoter polymorphism among German MS subgroups reflect interaction of the gene with such potential modifiers of MS susceptibility or progression. Differing environmental exposures interacting with *SLC11A1* might furthermore contribute to the failure to demonstrate an association between the *SLC11A1* gene promoter polymorphism and MS in the French patients using TDT. *SLC11A1* may therefore serve as a model for various diseases where interaction of the gene with infectious agents and/or iron as modifying factors may have a relatively small effect on familial risk but a large effect on population risk.

ACKNOWLEDGEMENTS

The Harry and Doris Crossley Foundation, the University of Stellenbosch, South Africa, and the University of Hamburg, Germany, supported the study financially. The work of the French Group was supported by ARSEP, INSERM, INSERM and the French Ministry of Research (CRB) and FRM (Action 2000). The French Multiple Sclerosis Genetics Group, Genethon and the CIC Pitié-salpêtrière provided help with establishment of the DNA bank. Study participants and their families are acknowledged for their willingness and cooperation to participate in this study. Dr JH Louw and Stefan Maritz are thanked for helpful discussions on methods of statistical analysis. Dr C Heesen, Dr T Fock, Prof H Will and B Reich are thanked for clinical data and samples of the German study group. Prof Bertrand Fontaine is thanked for samples and data of French MS patients. JNP de Villliers received student bursaries from the Freda and David Becker Trust and the Poliomyelitis Research Foundation.

REFERENCES

Blackwell JM, Searle S, Goswami T and Miller EN. Understanding the multiple functions of *NRAMP1*. Microbes and Infection 2000; 2: 317-321.

Botha MC and Beighton P. Inherited disorders in the Afrikaner population of southern Africa. Part I. Historical and demographic background, cardiovascular, neurological, metabolic and intestinal conditions. South African Medical Journal 1983; 64: 609-612.

Graham AM, Dollinger MM, Howie SE and Harrison DJ. Identification of novel alleles at a polymorphic microsatellite repeat region in the human *NRAMP1* gene promoter: analysis of allele frequencies in primary biliary cirrhosis. Journal of Medical Genetics 2000; 37: 150-152.

Kotze MJ, de Villiers JNP, Rooney RN, Grobbelaar JJ, Mansvelt EP, Bouwens CSH, Carr J, Stander I and du Plessis L. Analysis of the *NRAMP1* gene implicated in iron transport: association with multiple sclerosis and age effects. Blood Cells Molecules and Disease 2001; 27: 44-53.

Kotze MJ, de Villiers JNP, Zaahl MG and Robson KJH. The role of iron metabolism in multiple sclerosis. In: Metal Ions and Neurodegenerative Disorders. P. Zatta, Ed., World Sci. Singapore, London, 2003 (in press).

LeVine SM, Lynch SG, Ou CN, Wulser MJ, Tam E and Boo N. Ferritin, transferrin and iron concentrations in the cerebrospinal fluid of multiple sclerosis patients. Brain Research 1999; 821: 511-515.

Lublin F and Reingold S. Defining the clinical course of multiple sclerosis: results of an international survey. Neurology 1996; 46: 907-911.

Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH and Tourtellotte WW. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Annals of Neurology 1983; 13: 227-231.

Rothwell PM and Charlton D. High incidence and prevalence of multiple sclerosis in south east Scotland: Evidence of a genetic predisposition. Journal of Neurology, Neurosurgery and Psychiatry 1998; 64: 730-735.

Searle S and Blackwell JM. Evidence for a functional repeat polymorphism in the promoter of the human *NRAMP1* gene that correlates with autoimmune versus infectious disease susceptibility. Journal of Medical Genetics 1998; 36: 295-299.

Spielman RS, McGinnis RE and Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). American Journal of Human Genetics 1993; 52: 506-516.

Spielman RS and Ewens WJ. The TDT and other family-based tests for linkage disequilibrium and association. American Journal of Human Genetics 1996; 59: 983-989.

Storch M and Lassmann H. Pathology and pathogenesis of demyelinating diseases. Current Opinion in Neurology 1997; 10: 186-192.

Thompson AJ, Polman CH, Miller DH, McDonald WI, Brochet B, Filippi M Montalban X and De Sa J. Primary progressive multiple sclerosis. Brain 1997; 120: 1085-1096.

Zeman D, Adam P, Kalistova H, Sobek O, Kelbich P, Andel J and Andel M. Transferrin in patients with multiple sclerosis: a comparison among various subgroups of multiple sclerosis patients. Acta Neurologica Scandinavica 2000; 101: 89-94.

CHAPTER 2.3

Analysis of South African patients with multiple sclerosis: Population versus familial risk associated with the presence of viral sequences

Analysis of South African patients with multiple sclerosis: Population versus familial risk associated with the presence of viral sequences

ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). Although current knowledge suggests that MS is associated with autoimmunity, and that genetic susceptibility and infectious agents may be involved in the disease process, the cause of MS remains unknown. Recent studies performed in the South African population demonstrated a significant association between MS and the functional 5'-(GT)n polymorphism in the promoter region of the *SLC11A1* gene implicated in both autoimmune and infectious disease susceptibility. In this study serum and peripheral blood mononuclear cells (PBMCs) of MS patients, close relatives and unrelated controls were screened for the presence of MS-associated retrovirus (MSRV) and two herpes virus (HHV-6 and EBV) sequences, respectively, within the context of the *SLC11A1* genetic background. Viral sequences were not confined to a specific *SLC11A1* genotype, thereby excluding the possibility that *SLC11A1* allele 2 previously implicated in susceptibility to infectious diseases, correlates with viral infection in MS patients. Expression of the *pol* gene of MSRV was detected in the serum RNA of 34/49 (69%) MS patients and 23/33 (70%) of their unaffected close relatives, whilst absent in the serum of 39 unrelated healthy control individuals ($P<0.001$). No significant differences were observed with respect to presence or absence of EBV sequences. HHV-6 sequences were detected at a significantly lower frequency ($P<0.04$) in the PBMCs of unrelated controls (5%) compared with the MS patients (22.5%), most of whom also expressed MSRV RNA. The data provided in this study indicated that virus infections mostly affect the population risk and not the familial risk in MS.

INTRODUCTION

There is a considerable interest in the theory that exposure to a virus may lead to an immunopathologic condition resulting in multiple sclerosis (MS). Numerous infectious agents, both viral and bacterial, have been implicated in the aetiology of MS, but to date no infectious agent has been consistently found in affected patients (Cook et al., 1995). Some viruses produce superantigens capable of T-cell stimulation, which may result in an autoimmune process. A clear link has been shown between virus infection, autoimmunity and neurological disease in patients with human T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis, a disease that may be indistinguishable from MS (Levin et al., 2002). Molecular mimicry, a process whereby an immune response is mediated against an environmental agent that cross-reacts with a host antigen, has been proposed as another possible explanation for MS (Levin et al., 2002). In this case an antiviral response may be triggered against myelin due to molecular mimicry between viral proteins and proteins of the central nervous system (CNS). It has been shown that the myelin basic protein (MBP) shares extensive homologies at the amino acid level with a number of common pathogens, including measles, hepatitis B, influenza virus, adenovirus, Epstein-Barr virus (EBV) and papillomavirus.

Viruses such as the EBV that can establish persistent or latent infections in the CNS or the immune system are attractive candidates as aetiological agents in a chronic neurological disorder such as MS (Myhr et al., 1998). In a meta-analysis of published investigations, it was estimated that the odds of disease are more than 10 times higher in EBV-positive than EBV-negative individuals (Ascherio and Munch, 2000). Wandinger et al. (2000) have reported that active viral replication occurs more commonly in MS patients with exacerbations than in patients with stable disease. The known association between MS and the HLA class II protein, recently shown to act as a cofactor of EVB infection in B lymphocytes (Li et al., 1997), furthermore suggests a common genetic predisposition. However, in a study of long-term EBV-negative adult MS patients, the distribution of the DR2 alleles commonly associated with MS was similar to that of EBV-

seropositive adults (Jabs et al., 1999), which argues against the role of genes to explain the EBV association with MS. Since only a small proportion of individuals infected with EBV develop MS, it is clear that other cofactors are required, which may include age at primary infection or co-infection with other microbes. The finding that variant A of the human herpesvirus-6 (HHV-6) may infect EBV-positive B-cell lines and activate the latent EBV genome (Cuomo et al., 1995), suggest that interactions between herpesviruses may play a role in the pathogenesis of MS (Akhyani et al., 2000). Perron et al. (1997) have described a novel MS-associated retrovirus (MSRV) in patients with MS that was absent in control individuals, and has subsequently demonstrated (Perron et al., 2000) that endogenous retroviral elements are integrated into human chromosomal regions previously implicated in susceptibility to MS. A complex multi-step aetiology of MS is therefore proposed, involving triggering of specific genetic elements by EBV or other infectious agents.

The role of genetic influences in MS susceptibility has been demonstrated (Ebers et al., 1995), but there is controversy as to which genes are involved. The major histocompatibility (MHC) loci, particularly class II, as well as genes controlling T-cell receptors and cytokines, appear to be important. In a recent study performed in the genetically relative homogeneous Afrikaner population of South Africa, Kotze et al. (2001) reported an association between MS and the gene encoding the solute carrier family 11 (proton-coupled divalent metal iron transporters) member 1 (*SLC11A1*), formerly known as the natural resistance-associated macrophage protein 1 (*NRAMP1*) gene. Subsequent *in vitro* studies to determine the effect of iron loading on allelic expression of the functional Z-DNA forming polymorphism in the promoter region of the gene, provided a direct link between iron homeostasis and autoimmune versus infectious disease susceptibility (Kotze et al., in press). Since *SLC11A1* regulates iron and is also regulated by iron, this gene may serve as a model for various diseases where interaction with iron as a modifying (environmental) factor may have a relatively small effect on familial risk but a large effect on population risk.

The aim of the present study was to screen for the presence of MSRV and two herpes virus (HHV-6 and EBV) sequences in serum and/or peripheral blood (PBMC) of MS patients and population-matched controls, to investigate the possible role of these agents as environmental inducers of MS in genetically susceptible individuals. Comparison of frequencies of specific viral sequences among MS patients, close relatives and unrelated controls were performed within the context of the *SLC11A1* genetic background, in an attempt to provide a better definition of MS subgroups and disease expression within specific families.

SUBJECTS AND METHODS

Study population

Blood samples were obtained with written informed consent from 104 unrelated South African MS patients of European descent and their close family members (parents, spouses and siblings). Prior to inclusion in this study, all individuals have been genotyped for the '5-(GT)n repeat polymorphism in the promoter region of the *SLC11A1* gene (Kotze et al., 2001). The diagnoses of MS in affected patients were based on clinical features (Poser et al., 1983), magnetic resonance imaging (MRI) and/or exclusion of other diseases known to manifest similarly. The samples obtained form 49 MS patients and 33 of their close relatives were subjected to viral analysis in the present study. The unrelated control population consisted of 39 individuals, including laboratory personnel and healthy blood donors within the same age- and population group. The Ethics Review committee of the University of Stellenbosch approved the study protocol.

DNA and RNA extraction

Genomic DNA was isolated from EDTA-preserved whole blood using a standard salting-out method (Miller et al., 1988). DNA concentrations were quantified and diluted to a 20 ng/μl working solution. RNA was prepared from 140 μl serum using the QIAamp viral RNA purification kit according to the manufacturers' protocol (QIAGEN GmbH, Germany).

Amplification of virus sequences

A portion of the internal repeat sequence (Bam H1 W-fragment) of the EBV genome was amplified using the polymerase chain reaction (PCR) as described by van Heerden et al. (1995), with minor modifications. In the second round of amplification, 3 µi of the product generated during the first round was used as template. A cell lysate preparation of the EBV containing Raji cell line was used as positive control. The PCR results were confirmed by overnight spot blot hybridisation (van Rensburg et al., 1996) to the DIG-labelled Bam H1 W-fragment at 55°C.

For detection of HHV-6 sequences in peripheral blood lymphocytes, a 249-bp fragment was amplified in a first-round PCR using primers A and B as described (Aubin et al., 1991). The second round of amplification was performed by using 2 µi of the first round product. Primer B from the first round and primer 5U (5'-GCGAAGGGCTGATTAGGAT-3') (Kempf et al., 1995) was used as primer pair for the second round of amplification. The 50 µi PCR reaction mixtures contained 3 mM $MgCl_2$ and 1 U Taq polymerase enzyme. The annealing temperature for both the first and second round amplifications was 55°C. A DIG-labelled plasmid probe (HHV6M) containing an 830-bp fragment of the large tegument protein gene was used to confirm the PCR results by spot blot hybridisation.

RNA was treated with RNase-free DNase prior to reverse transcription-PCR (RT-PCR) with the MSRV specific primers for fragment B of the reverse transcriptase region of the *pol* gene as described by Perron et al. (1997). Only 5 µl of the DNase-treated RNA was used in the RT-PCR reaction using the Access RT-PCR system kit (Promega Corporation, Madison, Wisconsin, USA). A second round of amplification with the hemi-nested primer pair was performed and 2 µi RT-PCR product was carried over. The annealing temperature for PCR amplification during RT-PCR was 50°C. For the hemi-nested PCR 2.5 mM $MgCl_2$ and 1 U Taq polymerase enzyme was used in the reaction mixture, at an annealing temperature of 55°C. The nested PCR product was electrophoresed in a 2% agarose gel and stained with ethidium bromide for documentation by UV-illumination. The PCR results were confirmed by overnight spot

blot hybridisation at 50°C to the DIG-labelled plasmid containing the MSRV polymerase gene fragment (Perron et al., 1997).

Statistical analysis

Statistical analysis was performed by STATISTICA for Windows (StatSoft Inc. Tulsa OK, USA). Two-tailed Fisher exact test and the χ^2 calculation were applied as appropriate.

RESULTS

Table 1 shows the data obtained after screening for the presence of MS-associated retrovirus (MSRV) and two herpesviruses' (HHV-6 and EBV) sequences in serum or peripheral blood (PBMC) of 49 MS patients, 33 close relatives and 39 unrelated control individuals. A significantly higher frequency of MSRV (P<0.001) and HHV-6 sequences (P<0.04), and to a lesser extent of EBV (P=0.33) sequences, was observed in MS patients compared with unrelated controls. Overall, a statistically significant difference was observed between MS patients and unrelated controls (P<0.001, 3 df, χ^2= 45.95). Interestingly, the viral sequences were detected at a similarly high frequency in the unaffected close relatives of the MS patients.

Table 1: Comparison of the frequencies of viral sequences detected in MS patients, close family members and unrelated controls.

	MS patients n=49 (%)			Total (%)	Close Family members n=20 (%)	Control group n=39 (%)
	RR n=37	SP n=7	PP n=5			
MSRV	25 (68)	4 (57)	5 (100)	69	23 (70)*	0
EBV	17 (46)	6 (86)	2 (40)	51	8 (40)	15 (38.5)
HHV-6	9 (24)	1 (14)	1 (20)	22.5	2(10)	2 (5)
None	5 (13.5)	1 (14)	0	12	2(10)	23 (59)
Combinations						
All three	2 (5)	1 (14)	0	6	0	0
MSRV/EBV	8 (22)	3 (43)	2 (40)	27	6 (30)	0
MSRV/HHV-6	4 (11)	0	1 (20)	10	0	0
EBV/HHV-6	3 (8)	0	0	6	0	1 (3)

Total MS vs unrelated controls $P< 0.001$, df=3, $\chi^2= 45.95$

*33 close family members were analysed for the presence of MSRV sequences.

MSRV, multiple sclerosis associated retrovirus; EBV, Epstein Barr virus; HHV-6, human herpes 6 virus

RR, relapsing remitting; SP, secondary progressive; PP, primary progressive

Although the number of MS patients with a primary progressive disease course was small, it is noteworthy that all five individuals tested positive for MSRV sequences. Viral sequences were not confined to a specific genotype for the functional *SLC11A1* gene promoter polymorphism studied earlier (Kotze et al., 2001) in a subset of the study population, but it is noteworthy that all the MS patients with the primary progressive disease course were homozygous for allele 3 of the *SLC11A1* promoter polymorphism (table 2).

Table 2: Presence of viral sequences for MSRV, EBV and HHV-6 in MS patients with different disease courses subdivided according to *SLC11A1* genotype.

		MS patients n=49		
Virus	*SLC11A1* Genotypes	RR n=37	SP n=7	PP n=5
MSRV	2/2	1	1	
	2/3	11	1	
	3/3	8	1	5
	3/5	5	1	
EBV	2/2		1	
	2/3	9	2	
	3/3	7	2	2
	3/5	1	1	
HHV-6	2/2			
	2/3	4		
	3/3	5		1
	3/5		1	

Figure 1 illustrates the pedigree of one of the MS families included in this study. Co-existence of MSRV sequences and alleles 3 and 5 of the *SLC11A1* gene, which appears to predominate in South African MS patients (Kotze et al., 2001), was detected in the index patient. However, her aunt who was also diagnosed with definite MS, also has the *SLC11A1* 3/5 genotype but tested negative for all the virus sequences analysed. Furthermore, several of the family members without a diagnosis of MS tested positive for the virus sequences, *SLC11A1* genotype 3/5, or both.

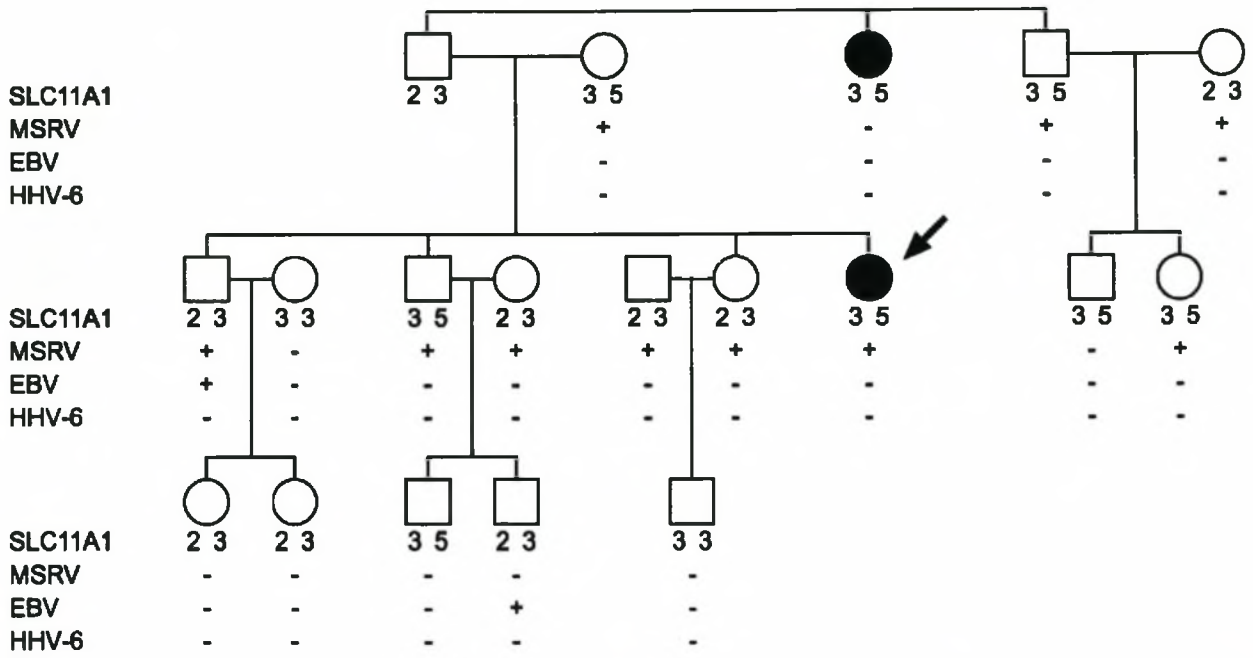


Figure 1: Detection of sequences for MSRV, HHV-6 and EBV in an MS family genotyped for the 5'-(GT)n repeat polymorphism in the promoter region of the *SLC11A1* gene. The index case is indicated by an arrow.

DISCUSSION

In the present study significant differences between MS patients and unrelated control individuals were demonstrated with respect to the presence of MSRV ($P<0.001$) and HHV-6 sequences ($P<0.04$), but not for EBV. However, no significant differences could be detected when the close family members of the MS patients were compared as unaffected controls. These findings suggest that the presence of viral sequences does not represent a primary causative agent for MS, but rather reflects the properties of viruses in the presence of relevant genetic and environmental factors involved in the pathogenesis of this complex disease. In one of the MS families studied (figure 1) the MSRV sequences were detected in only one of the two affected members, although both tested positive for *SLC11A1* alleles 3 and 5 shown to be associated with MS in the South African population (Kotze et al., 2001). Familial aggregation in this family therefore seems to be limited largely to genetic factors, some of which may be shared by the unaffected mother and father of the index case. These findings highlight the

complex nature of MS, where disease expression appears to involve more than one susceptibility gene (Vitale et al., 2002) in the presence of an appropriate environmental trigger(s). To our knowledge, this study represents the first analysis of MSRV sequences in unaffected family members of MS patients and therefore makes a valuable contribution to our current understanding of familial clustering in MS.

Evidence on potential mechanisms by which EBV can cause MS is limited. Failure to demonstrate EBV in MS plaques (Hilton et al., 1994; Morre et al., 2001) largely excludes the possibility that direct CNS infection is involved. It seems likely that the T-cell response to EBV infection could result in harmful cross-reaction with self-antigens. In a study performed by Knox et al. (2000) it was shown that blood samples of approximately 50% (22/41) of patients with definite MS contain active HHV-6 infections, compared with none (0/61) of the normal controls. Although there was no significant difference between HHV-6 viraemia-positive and –negative MS patients with respect to type of disease (relapsing-remitting or progressive), patients with active HHV-6 viraemia were significantly younger and experienced a shorter duration of disease than in HHV-6 viraemia-negative patients. In a recent study performed in Sardinia, Dolei et al. (2002) detected the MSRV in 12.8% of healthy blood donors versus 100% of patients with MS (P<0.000001). This finding remained significant when the study population was stratified in relation to other neurological conditions with respect to inflammatory disease, and it was concluded that the presence of MSRV in blood plasma may be related to the inflammatory nature of the disease, specifically those of both peripheral and CNS origin. Although the MSRV viral sequences were detected in other inflammatory CNS diseases like infectious encephalomyelitis and immune-mediated peripheral neuropathy, the occurrence was lower (Dolei et al., 2002). These findings were confirmed when no significance could be detected in comparison with other non-inflammatory neurological diseases. Differences in viral frequencies detected in MS cohorts in different studies may be related to population selection criteria applied or differences in experimental procedures.

Although the number of patients with primary progressive MS included in this study is small, it is noteworthy that all the patients with this type of disease course tested positive for MSRV sequences. In addition, all of these individuals were homozygous for allele 3 of the *SLC11A1* promoter polymorphism, shown to be associated with an increased susceptibility to autoimmune diseases (Blackwell et al., 2000). These findings raise the possibility that viral gene expression remains active in patients with a progressive disease course while its activation from time to time in patients with relapsing-remitting MS may trigger the attacks. Further studies are warranted to determine whether this proposed effect in primary progressive MS is related to absence of allele 2 of the *SLC11A1* 5'-(GT)n repeat, which is protective against autoimmunity (Searle and Blackwell, 1999). Activation of a latent virus due to co-infection with another infectious agent remains a possibility in MS patients. Our data on co-existence of different virus sequences in MS subgroups does not provide sufficient evidence to explain such a role of MSRV in MS, although 61.8% of MS patients with MSRV expression harbored herpes viruses compared to 42.9% of all MS patients.

Although the exact role of infectious agents in the pathogenesis of MS has not been determined, it has been suggested that the presence of viral sequences may represent useful markers for diagnosis, prognosis and/or therapeutic monitoring of MS patients. From the results obtained in this study it seems justified to include detection of relevant viral sequences by PCR as a diagnostic tool, to complement the standard methods that are currently applied to make a diagnosis of MS. Although the presence of relevant viral sequences may be of prognostic value due to the association with disease activity (Wandinger et al., 2000), available antiviral drugs have little effect on MS (Cohen, 2000). It is therefore unlikely that a virus is the primary cause of MS and proof that this may be the case should come from the demonstration that a suitable vaccine prevents MS. Nevertheless, beta interferon, the neuroprotective drug currently used in the management of MS, has antiviral activity against HHV-6. Acquisition of one or more of the pathogens investigated in this study, presumably between early childhood and adolescence, may be responsible for triggering the autoimmune process underlying MS in genetically susceptible individuals, thereby contributing to the complex multi-step

aetiology of MS. When the immune system is affected adversely by external exposures, commonly circulating virusses may be activated and under appropriate conditions may develop their full pathological potential. The likelihood that MSRV is produced endogenously and contribute to MS reactivation in the presence of predisposing conditions, can therefore not be excluded. Among the fundamental questions yet to be answered in understanding the pathophysiology of MS and other autoimmune diseases are (1) what initiates the inflammation and (2) what drives the inflammation in the target organ. Elucidation of the mechanisms responsible for the initiation of MS has obvious implications for future development of preventative therapy.

The data presented in this study support the hypothesis that virus infections may represent co-factors in the pathogenesis of some cases of MS and are associated with a more disabling disease course (Sotgiu et al., 2002). The detection of MSRV sequences in the majority of South African MS patients and their unaffected family members, whilst absent in unrelated control individuals, indicates that virus infections mostly affect the population risk and not familial risk. Other environmental factors such as iron, which interact with *SLC11A1* known to regulate macrophage activation in infectious and autoimmune diseases (Blackwell et al., 2000), may contribute to environmental or familial risk, or both. Since the potential importance of this process has been clearly demonstrated in MS (Kotze et al., 2001; in press), further studies are warranted to elucidate the role of iron in the pathogenesis of MS. It is known that the continuous battle for iron between the host and invading pathogens may result in cytokine-mediated responses, which are involved in various infectious and autoimmune diseases.

ACKNOWLEDGEMENTS

Study participants and their families are acknowledged for their willingness and cooperation to participate in this study. The Department of Medical Virology, University of Stellenbosch and Florette Treurnicht are thanked for guidance and technical assistance. The University of Stellenbosch and the Harry and Doris Crossley Foundation supported the study financially. JNP de Villiers received student bursaries from the Freda and David Becker Trust and the Poliomyelitis Research Foundation.

REFERENCES

Akhyani N, Berti R, Brennan MB, Soldan SS, Eaton JM, McFarland HF and Jacobson S. Tissue distribution and variant characterization of human herpesvirus (HHV)-6: increased prevalence of HHV-6A in patients with multiple sclerosis. Journal of Infectious Disease 2000; 182: 1321-1325.

Ascherio A and Munch M. Epstein-Barr virus and multiple sclerosis. Epidemiology 2000; 11: 1-5.

Aubin JT, Collandre H, Candotti D, Ingrand D, Rouzioux C, Burgard M, Richard S, Huraux JM and Agut H. Several groups among human herpesvirus 6 strains can be distinguished by Southern blotting and polymerase chain reaction. Journal of Clinical Microbiology 1991; 29: 367-372.

Blackwell JM, Searle S, Goswami T and Miller N. Understanding the multiple functions of *NRAMP1*. Microbes and Infection 2000; 2: 317-321.

Cohen JI. Epstein-Barr virus infection. New England Journal of Medicine 2000; 343: 481-492.

Cook SD, Rohowsky-Kochan S, Bansil S and Dowling PC. Evidence for multiple sclerosis as an infectious disease. Acta Neurologica Scandinavica 1995; 161 (supplement): 34-42.

Cuomo L, Angeloni A, Zompetta C, Cirone M, Calogero A, Frati L, Ragona G and Faggioni A. Human herpesvirus 6 variant A, but not variant B, infects EBV-positive B lymphoid cells, activating the latent EBV genome through a BZLF-1 dependent mechanism. AIDS research and human Retrovirus 1995; 11: 1241-1245.

Dolei A, Serra C, Mameli G, Pugliatti M, Sechi G, Cirotto MC, Rosati G and Sotgiu S. Multiple sclerosis-associated retrovirus (MSRV) in Sardinian MS patients. Neurology 2002; 58: 471-473.

Ebers GC, Sadovnick AD and Risch NJ. A genetic basis for familial aggregation in multiple sclerosis. Nature 1995; 377: 150-151.

Hilton DA, Love S, Fletcher A and Pringle JH. Absence of Epstein-Barr virus in multiple sclerosis as assessed by in situ hybridisation. Journal of Neurology, Neurosurgery and Psychiatry 1994; 57: 975-976.

Jabs WJ, Paulsen M, Wagner HJ, Kirchner H and Kluter H. Analysis of Epstein-Barr virus (EBV) receptor CD21 on peripheral B lymphocytes of long-term EBV- adults. Clinical Experimental Immunology 1999; 116: 468-473.

Kempf W, Adams V, Pfaltz M, Briner J, Schmid M, Moos R and Hassam S. Human herpesvirus type 6 and cytomegalovirus in AIDS-associated Kaposi's sarcoma: no evidence for an etiological association. Human Pathology 1995; 26: 914-919.

Knox KK, Brewer JH, Henry JM, Harrington DJ and Carrigan DR. Human herpesvirus 6 and multiple sclerosis: systemic active infections in patients with early disease. Clinical Infectious Disease 2000; 31: 894-903.

Kotze MJ, de Villiers JNP, Rooney RN, Grobbelaar JJ, Mansvelt EP, Bouwens CSH, Carr J, Stander I and du Plessis L. Analysis of the *NRAMP1* gene implicated in iron transport: association with multiple sclerosis and age effects. Blood Cells Molecules and Disease 2001; 27: 44-53.

Kotze MJ, de Villiers JNP, Zaahl MG and Robson KJH. The role of iron metabolism in multiple sclerosis. In: Metal Ions and Neurodegenerative Disorders. P. Zatta, Ed., World Sci. Singapore, London, 2003 (in press).

Levin MC, Lee SM, Kalume F, Morcos Y, Dohan FC Jr, Hasty KA, Callaway JC, Zunt J, Desiderio D and Stuart JM. Autoimmunity due to molecular mimicry as a cause of neurological disease. Nature Medicine 2002; 8: 509-513.

Li Q, Spriggs MK, Kovats S, Turk SM, Comeau MR, Nepom B and Hutt-Fletcher LM. Epstein Barr virus uses HLA class II as a cofactor for infection of B lymphocytes. Journal of Virology 1997; 71: 4657-4662.

Miller SA, Dykes DD and Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 1988; 16: 1215.

Morre SA, van Beek J, De Groot CJ, Killestein J, Meijer CJ, Polman CH, van der Valk P, Middeldorp JM and van Den Brule AJ. Is Epstein-Barr virus present in the CNS of patients with MS? Neurology 2001; 56: 692.

Myhr K-M, Riise T, Barrett-Connor E, Myrmel H, Vedeler C, Gronning M, Kalvenes MB and Nyland H. Altered antibody pattern to Epstein-Barr virus but not to other herpesviruses in multiple sclerosis: a population based case-control study from western Norway. Journal of Neurology, Neurosurgery and Psychiatry 1998; 64: 539-542.

Perron H, Garson JA, Bedin F, Beseme F, Paranhos-Baccala G, Komurian-Pradel F, Mallet F, Tuke PW, Voisset C, Blond JL, Lalande B, Seigneurin JM and Mandrand B. Molecular identification of a novel retrovirus repeatedly isolated from patients with multiple sclerosis. Proceedings of the National Academy of Sciences of the United States of America. 1997; 94: 7583-7588.

Perron H, Perin JP, Zrieger F and Alliel PM. Particle-associated retroviral RNA and tandem RGH/HERV-W copies on human chromosome 7q: possible components of a "chain-reaction" triggered by infectious agents in multiple sclerosis. Journal of Neurovirology 2000; 6 (supplement 2): 67-75.

Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH and Tourtellotte WW. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Annals of Neurology 1983; 13: 227-231.

Searle S and Blackwell JM. Evidence for a functional repeat polymorphism in the promoter of the human *NRAMP1* gene that correlates with autoimmune versus infectious disease susceptibility. Journal of Medical Genetics 1999; 36: 295-299.

Sotgiu S, Serra C, Mameli G, Pugliatti M, Rosati G, Arru G and Dolei A. Multiple slerosis-associated reyrovirus and MS prognosis: an observational study. Neurology 2002; 59: 1071-1073.

Van Heerden WE, van Rensburg EJ, Engelbrecht S and Raubenheimer EJ. Prevalence of EBV in oral squamous cell carcinomas in young patients. Anticancer Research 1995; 15: 2335-2339.

Van Rensburg EJ, Engelbrecht S, Van Heerden WF, Raubennheimer EJ and Schoub BD. Human papillomavirus DNA in oral squamous cell carcinomas from an African population sample. Anticancer Research 1996; 16: 969-973.

Vitale E, Cook S, Sun R, Specchia C, Subramanian K, Rocchi M, Nathanson D, Schwalb M, Devoto M and Rohowsky-Kochan C. Linkage analysis conditional on HLA status in a large North American pedigree supports the presence of a multiple sclerosis susceptibility locus on chromosome 12p12. Human Molecular Genetics 2002; 11: 295-300.

Wandinger K, Jabs W, Siekhaus A, Bubel S, Trillenberg P, Wagner H, Wessel K, Kirchner H and Hennig H. Association between clinical disease activity and Epstein-Barr virus reactivation in MS. Neurology 2000; 55: 178-184.

CHAPTER 2.4

Analysis of the 77C-G mutation in exon 4 of the *PTPRC* gene in multiple sclerosis

Analysis of the 77C-G mutation in exon 4 of the *PTPRC* gene in multiple sclerosis

ABSTRACT

An analysis between multiple sclerosis (MS) and the gene encoding protein-tyrosine phosphatase, receptor-type C (*CD45*, *PTPRC*) known to be essential for activation of T and B cells, has recently been demonstrated in German and Italian MS patients, but not in patients from North America, Sweden or in a German follow-up study. In an attempt to clarify the role of the C to G transition at nucleotide position 77 in exon 4 of the *PTPRC* gene in MS, 124 unrelated South African and 353 German MS patients were subjected to mutation detection using polymerase chain reaction (PCR)-based methods. The exon 4 mutation causing altered expression of CD45 isoforms on immune cells, was detected in one of the South African patients with MS, but was absent in her less severely affected sister who was also diagnosed with definite MS. This finding indicates that the mutation does not increase the risk of MS in South African patients, or that it is associated with earlier disability in this family. In the population-matched South African control group, none of the 85 subjects within the same age group tested positive for the mutation, while 5 of 52 elderly individuals above the age of 70 years (9.6%) carried the mutation (Fisher exact test, P=0.0085). This finding is similar to that described for the *SLC11A1* gene in an earlier study and raises the possibility that the deleterious *PTPRC* mutation has been maintained in the population as a consequence of improved survival following infectious disease challenge. Although 13 of the 353 German MS patients (4%) were heterozygous for the *PTPRC* mutation compared with two of 140 (1.4%) control individuals from the same population, this difference was not statistically significant. The mutation was not confined to German patients with a specific disease course and no difference in mutation frequency could be detected between MS patients with different disease courses. The study included 134 patients with relapsing-remitting MS (38%), 74 patients with secondary progressive MS (21%), 59 patients with primary progressive MS (17%) and 86 uncharacterised MS patients (24%). Our findings support the notion that genetic susceptibility factors of MS may vary between different population groups,

although it seems more likely that the failure to confirm the association between MS and the *PTPRC* mutation in different study populations is due to the simplistic single-gene approach being used for data comparison in a complex disease.

INTRODUCTION

Multiple sclerosis (MS) is the most common demyelinating disorder of the central nervous system (CNS). Current knowledge suggests that MS is a multifactorial disease with a supposed autoimmune aetiology on the ground of genetic susceptibility and probably also disease-triggering infectious factors. No predictable pattern of inheritance is observed and clinical features of MS cannot yet be explained through an effect of a definable gene product or functional abnormalities (Chataway et al., 1998). The estimated familial occurrence in Caucasian MS populations is approximately 15%. The age-adjusted risk is higher for siblings (3%), parents (2%), and children (2%) than for second-and third-degree relatives (Compston and Coles, 2002).

Familial association in German MS patients has recently been reported by Jacobsen et al. (2000), as a consequence of a C-to-G transition at nucleotide position 77 in exon 4 of the protein-tyrosine phosphatase, receptor type C *(PTPRC)* gene. This alternatively spliced gene with 33 exons gives rise to 5 isoforms and encodes for a transmembrane protein tyrosine phosphatase that is expressed on all nucleated hematopoetic cells. In T-cells, its function is to prime the T-cell receptor that allows for its activation when interacting with an antigen presenting cell (Weiss and Littman, 1994). In humans a deficiency of this protein causes severe combined immunodeficiency disease (Kung et al., 2000). Interestingly, mice that contain an active form of this protein are hyper-reactive to antigen and are prone to develop autoimmune disease (Majeti et al., 2000). The 77C-G mutation does not change the encoded amino acid, but prevents splicing of exon 4 pre-mRNA, an effect that could explain the reported association between MS and the *PTPRC* gene.

In a follow-up study of the 77C-G *PTPRC* mutation performed in a relatively large number of German MS patients and control individuals, no statistically significant

difference in mutation frequency could be detected (Miterski et al., 2002). Likewise, no evidence of genetic association between MS and this mutation could be detected in American and Swedish MS patients (Barcellos et al., 2001; Vorechovsky et al., 2001). Ballerini et al. (2002) detected the 77C-G mutation in a small number of Italian MS patients but not in a matched group of healthy controls (Fisher exact test, $P = 0.02$). This finding suggests a role, in at least certain patient groups, for the *PTPRC* mutation in genetic susceptibility to MS.

Other mutations in the *PTPRC* gene may also be of relevance in MS, since Jacobsen et al. (2002) have also detected a novel C to A mutation in exon 4 at position 59 in one of 22 MS families with aberrant CD45 expression, but without the 77C-G mutation. This mutation interferes with alternative splicing and with antibody binding to the CD45RA domain, similar to the effect previously described for the 77C-G mutation (Jacobsen et al., 2000).

In the present study DNA samples of 124 South African MS patients and 353 German MS patients were screened for the 77C-G mutation in exon 4 of the *PTPRC* gene, in order to determine possible allelic associations when compared with population-matched controls. The South African control group included 85 age-matched control individuals, as well as 52 elderly individuals above the age of 70 years, all from the Afrikaner population of European descent. The aged population provides a valuable source of material to determine whether certain deleterious sequence changes are retained in the population, possibly as a consequence of a selective advantage which may be related to the finding that certain alleles associated with autoimmunity protects against infectious diseases, while alleles associated with increased susceptibility to infection may conversely protect against autoimmune diseases. This phenomenon has been demonstrated earlier for the *SLC11A1* gene shown to be associated with MS in the South African population (Kotze et al., 2001).

SUBJECTS AND METHODS

Blood samples were obtained with written informed consent from 124 unrelated South African patients and 353 unrelated German patients diagnosed with definite MS, as well as relevant close family members. The patients were subdivided according to disease course: 134 relapsing remitting (RR), 74 secondary progressive (SP), 59 primary progressive (PP) and 86 uncharacterised MS patients. The South African patients were recruited mainly through the South African Multiple Sclerosis Society (Kotze et al., 2001), while the German patients attended an outpatient clinic at the University Hospital Eppendorf in Hamburg, Germany. The diagnoses of MS in affected patients were based on clinical features (Poser et al., 1983), magnetic resonance imaging (MRI) and/or exclusion of other diseases known to manifest similarly. DNA samples of 85 healthy South African and 140 German individuals within the same age group as the MS patients, were included as controls. The 85 South African subjects included laboratory personnel, medical students and healthy blood donors within the same age- and population group. In addition, 52 elderly South Africans above the age of 70 years were recruited as population matched controls to study possible age effects. The Ethics Review committees of the Universities of Stellenbosch and Hamburg approved the study.

DNA analysis

Genomic DNA was extracted from peripheral blood, using a standard salting-out method (Miller et al., 1988) or commercial DNA extraction solution DNAzol (Invitrogen, UK), with minor modifications. *PTPRC* exon 4 specific primers (15pmol), 5' – atttattttgtccttctccca-3' (forward) and 5' –gttaacaacttttgtgtgcc –3' (reverse) resulting in a 260-bp fragment following polymerase chain reaction (PCR)-amplification, were used in a 25 µi PCR reaction containing 50 ng of genomic DNA, 0.2 µM of each dGTP, dCTP, dATP and dTTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl and 0.5 U *Taq* DNA Polymerase (Roche - Diagnostics, Germany). PCR cycle conditions included an initial denaturing step at 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 60°C for 45 s, and 72°C for 30 s and performed in a 9700 Thermal Cycler (ABI, Foster City, CA, USA). PCR

products were digested to completion with 5 U *Msp*I (Gibco, Gaithersburg, MD, USA) and subjected to polyacrylamide (PAA) gel electrophoresis (12% PAA, 3,4% C), stained with ethidium bromide and visualised by ultraviolet light.

Statistical analysis

Statistical analysis was performed by STATISTICA for Windows (StatSoft Inc. Tulsa, OK, USA). Two-tailed Fisher exact test was applied as appropriate. *P*-values <0.05 were regarded as statistically significant.

RESULTS AND DISCUSSION

Detection of mutation 77C-G in exon 4 of the *PTPRC* gene is shown in figure 1, following restriction enzyme analysis of PCR-amplified genomic DNA. In the mutation-negative control sample, fragments of 199-bp and 61-bp (lanes 1 and 3) are observed, while in the heterozygote the 199-bp fragment is cut into two additional fragments of 115-bp and 84-bp in the mutant allele (lane 2). Only one of the 124 South African MS patients tested positive for the exon 4 mutation, compared to none of the 85 age-matched control individuals. The frequency of the 77G-C mutation was significantly increased in the elderly population compared with the younger controls (Fisher exact test, *P*=0.0085), since 5 of the 52 (9.6%) aged individuals were heterozygous for this mutation. The significant difference in mutation frequency observed between the two age groups in the South African control group raises the possibility that the deleterious *PTPRC* mutation has been maintained in the population as a consequence of improved survival following infectious disease challenge. A similar age effect has previously been demonstrated for the *SLC11A1* gene shown to be associated with MS in the South African population (Kotze et al., 2001). Enrichment of the aged group for sequence changes associated with autoimmune disease suggests that these alleles may have some selective advantage. This may be of particular relevance in the South African context where epidemics of infectious diseases occurred in the past.

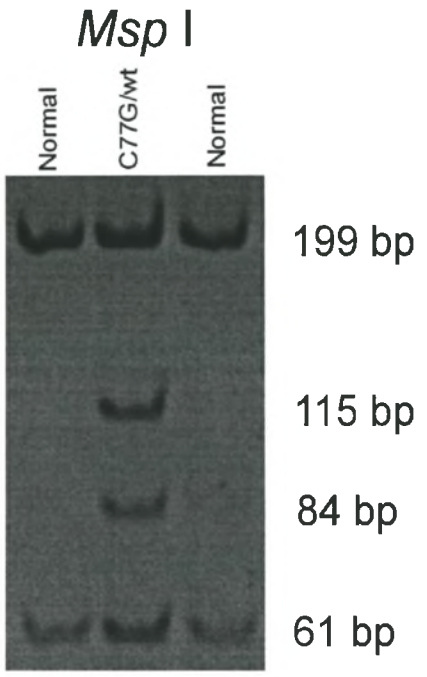


Figure 1: Detection of mutation 77C-G using restriction enzyme analysis. DNA samples of mutation-negative control individuals (lanes 1 and 3) and a heterozygote for the 77C-G mutation (lane 2) is shown after digestion with *Msp*I and electrophoresis on a 12% polyacrylamide gel.

Table 1 compares the mutation frequency for mutation 77C-G between different population groups analysed in this study and elsewhere. In the German study population, the G-allele at nucleotide position 77 of the *PTPRC* gene was present in 14 out of 353 (4%) German and in 2 out of 140 (2%) control individuals. Contrary to the report of Jacobsen et al. (2000), who reported an association between this mutation and the development of MS in two independent German study cohorts, the role of the 77C-G mutation could not be demonstrated or confirmed in our German study cohort. In addition, no association could be detected when the mutation frequency in this patient group was compared to that reported for the German control samples studied by Miterski et al. (2002), which were recruited from the same geographical area.

Table 1: Study comparisons of the 77C-G transition in the *PTPRC* gene.

Study area	Study group	Total	77C-G variant	*P*-value MS vs Controls
Marburg I (German) (Jacobsen et al., 2000)	Multiple Sclerosis	219	14 (6.4%)	0.00015
	Healthy donors	189	0	
Marburg II (German) (Jacobsen et al., 2000)	Multiple Sclerosis	108	7 (6.4%)	0.0058
	Healthy donors	114	0	
American study (Jacobsen et al., 2000)	Multiple Sclerosis	122	4 (3.2%)	0.55
	Healthy donors	244	9 (3.6%)	
Hanover study (German) (Jacobsen et al., 2000)	Multiple Sclerosis	76	5 (6.6%)	0.034
	Healthy donors	119	1 (0.8%)	
Swedish study (Vorechovsky et al., 2001)	Multiple Sclerosis	630	19 (3%)	0.62
	Healthy donors	358	9 (3.2%)	
American study (Barcellos et al., 2001)	Multiple Sclerosis	450	15 (3.4%)	0.32
	Healthy donors	253	6 (2.4%)	
Bochum and Göttingen (German) (Miterski et al., 2002)	Multiple Sclerosis	454	7 (1.6%)	0.15
Hamburg (German)	Healthy donors	347	10 (2.8%)	
Italian (Ballerini et al., 2002)	Multiple Sclerosis	194	5 (5.2%)	0.02
	Healthy controls	222	0	
Hamburg (German)*	Multiple Sclerosis	353	13 (4%)	0.15
	Healthy controls	140	2 (1.4%)	
South Africa*	Multiple Sclerosis	124	1 (0.8%)	0.59
	Healthy controls	85	0	

* this study; *P*-values <0.05 were regarded as statistically significant.

Figure 2 shows the pedigree of the South African MS patient who tested positive for mutation 77C-G. This mutation was present in several unaffected family members, whilst absent in her sister who was also diagnosed with definite MS. The 54-year old South African MS patient with the *PTPRC* mutation has been diagnosed with RR MS at the age of 25 and has been wheelchair bound for the past 12 years. Her mutation-negative sister (aged 51 years) has been diagnosed with RR MS at the age of 32 years, and is still able to walk. This finding raises the possibility that the presence of the PTPRC mutation is associated with clinical severity of disease expression in this family.

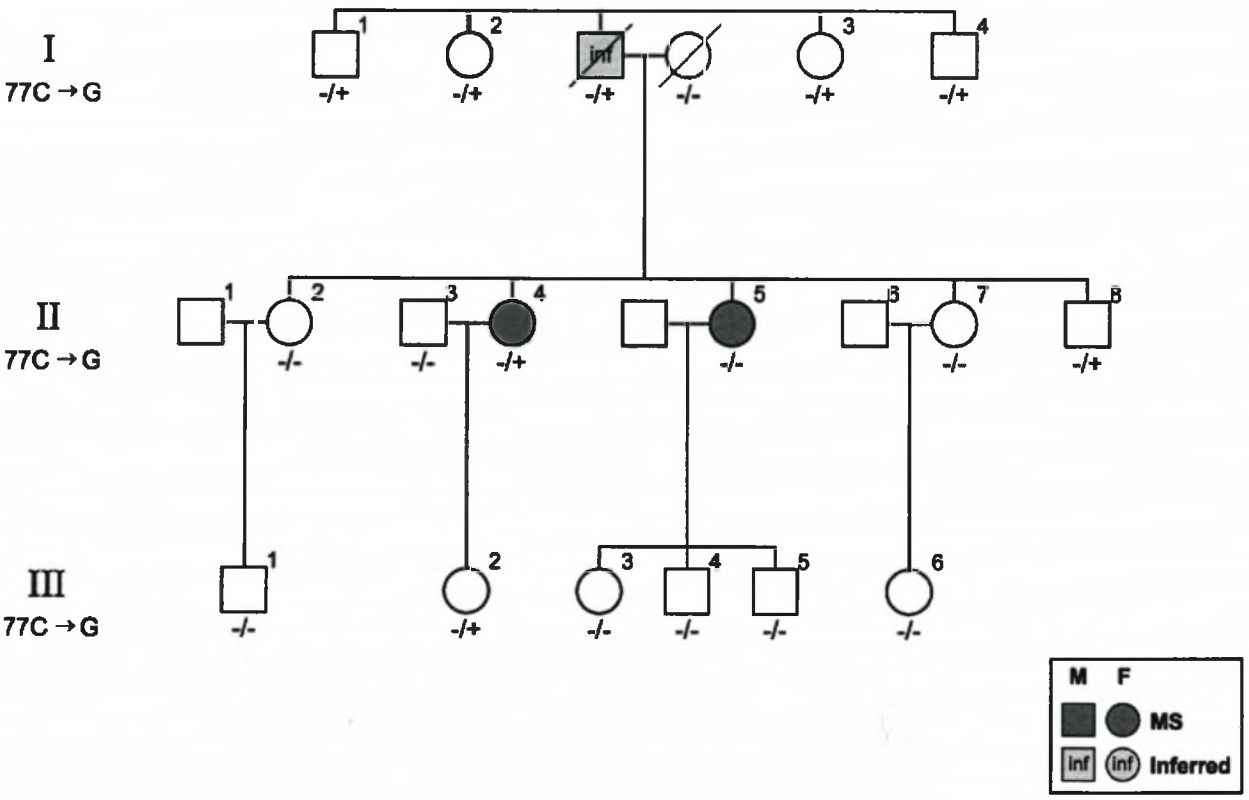


Figure 2: Pedigree of the South African family with two MS-affected sisters. Several family members tested positive for mutation 77C-G, including only one of the two sisters diagnosed with definite MS.

This study does not provide direct support for previous findings indicating an association between mutation 77C-G and MS susceptibility and points out that differences may occur between different population groups with regard to genetic susceptibility to MS. Meta analysis studies are warranted to clarify the role of the *PTPRC* mutation in MS.

The selection of control populations are of mayor importance in case-control studies since population substructures may lead to false associations (Little et al., 2002). Although the role of mutation 77C-G in the South African and German populations studied is unclear, the mutation could possibly play a role in disease expression in combination with other environmental and genetic influences in certain families.

ACKNOWLEDGEMENTS

The University of Stellenbosch and the Harry and Doris Crossley Foundation supported the study financially. Study participants and their families are acknowledged for their willingness and cooperation to participate in this study. Dr JH Louw and Stefan Maritz are thanked for helpful discussions on methods of statistical analysis. Dr C Heesen, Dr T Fock, Prof H Will and B Reich are thanked for clinical data and samples of the German study group. JNP de Villliers received student bursaries from the Freda and David Becker Trust and the Poliomyelitis Research Foundation.

REFERENCES

Ballerini C, Rosati E, Salvetti M, Ristori G, Cannoni S, Biagioli T, Massacesi L, Sorbi S, Vergelli M. Protein tyrosine phosphatase receptor-type C exon 4 gene mutation distribution in an Italian multiple sclerosis population. Neuroscience Letters 2002; 328: 325-327.

Barcellos LF, Caillier S, Dragone L, Elder M, Vittinghoff E, Bucher P, Lincoln RR, Pericak-Vance M, Haines JL, Weiss A, Hauser SL and Oksenberg JR. *PTPRC* (CD45) is not associated with the development of multiple sclerosis in U.S. patients. Nature Genetics 2001; 29: 23-24.

Chataway J, Feakes R, Coraddu F, Gray J, Deans J, Fraser M, Robertson N, Broadley S, Jones H, Clayton D, Goodfellow P, Sawcer S and Compston A.. The genetics of multiple sclerosis: principles, background and updated results of the United Kingdom systematic genome screen. Brain 1998; 121: 1869-1887.

Compston A and Coles A. Multiple sclerosis. Lancet 2002; 359: 1221-1231.

Jacobsen M, Schweer D, Ziegler A, Gaber R, Schock S, Schwinzer R, Wonigeit K, Lindert RB, Kantarci O, Schaefer-Klein J, Schipper HI, Oertel WH, Heidenreich F, Weinshenker BG, Sommer N and Hemmer B. A point mutation in *PTPRC* is associated with the development of multiple sclerosis. Nature Genetics 2000; 26: 495-499.

Jacobsen M, Hoffmann S, Cepok S, Stei S, Ziegler A, Sommer N and Hemmer B. A novel mutation in *PTPRC* interferes with splicing and alters the structure of the human CD45 molecule. Immunogenetics 2002; 54: 158-163.

Kotze MJ, de Villiers JNP, Rooney RN, Grobbelaar JJ, Mansvelt EP, Bouwens CSH, Carr J, Stander I and du Plessis L. Analysis of the *NRAMP1* gene implicated in iron transport: association with multiple sclerosis and age effects. Blood Cells Molecules and Disease 2001; 27: 44-53.

Kung C, Pingel JT, Heikinheimo M, Klemola T, Varkila K, Yoo LI, Vuopala K, Poyhonen M, Uhari M, Rogers M, Speck SH, Chatila T and Thomas ML. Mutations in the tyrosine phosphatase CD45 gene in a child with severe combined immunodeficiency disease. Nature Medicine 2000; 6: 343-345.

Little J, Bradley L, Bray MS, Clyne M, Dorman J, Ellsworth DL, Hanson J, Khoury M, Lau J, O'Brien TR, Rothman N, Stroup D, Taioli E, Thomas D, Vainio H, Wacholder S and Weinberg C. Reporting, appraising, and integrating data on genotype prevalence and gene-disease associations. American Journal of Epidemiology 2002; 156: 300-310.

Majeti R, Xu Z, Parslow TG, Olson JL, Daikh DI, Killeen N and Weiss A. An inactivating point mutation in the inhibitory wedge of CD45 causes lymphoproliferation and autoimmunity. Cell; 103: 1059-1070.

Miller SA, Dykes DD and Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 1988; 16: 1215.

Miterski B, Sindern E, Haupts M, Schimrigk S and Epplen JT. *PTPRC* (CD45) is not associated with multiple sclerosis in a large cohort of German patients. BMC Medical Genetics [electronic resource] 2002; 3: 3.

Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH and Tourtellotte WW. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Annals of Neurology 1983; 13: 227-231.

Vorechovsky I, Kralovicova J, Tchilian E, Masterman T, Zhang Z, Ferry B, Misbah S, Chapel H, Webster D, Hellgren D, Anvret M, Hillert J, Hammarstrom L and Beverley PC. Does 77C-->G in *PTPRC* modify autoimmune disorders linked to the major histocompatibility locus? Nature Genetics 2001; 29: 22-23.

Weiss A and Littman DR. Signal transduction by lymphocyte antigen receptors. Cell 1994; 76: 263-274.

CHAPTER 2.5

Co-existence of multiple sclerosis, hereditary haemochromatosis and variegate porphyria in a South African family: C282Y homozygosity in two sisters with multiple sclerosis

Co-existence of multiple sclerosis, hereditary haemochromatosis and variegate porphyria in a South African family: C282Y homozygosity in two sisters with multiple sclerosis

ABSTRACT

Hereditary haemochromatosis (HH) is considered to be the most common (~1/250) autosomal recessive disorder in Caucasians of European descent, an ethnic group also known to have a genetic predisposition for multiple sclerosis (MS). Clinically manifested HH was, however, not recognised among 1700 MS patients previously studied in Canada. In order to determine whether this finding may be due to a linkage disequilibrium effect or gene-gene interaction, DNA samples of South African MS patients were screened for mutations H63D and C282Y in the HH gene. Identification of two copies of mutation C282Y in one of the index patients and her MS-affected sister, both without clinical manifestations of HH, provided evidence for possible gene-gene interaction. Haplotype studies performed in the MS family using microsatellite markers flanking the HH locus, demonstrated that the two MS patients were homozygous for specific alleles at marker loci D6S273 (A5, 135-bp), D6S105 (A5, 121-bp), D6S2239 (A1, 105-bp) and heterozygous for marker D6S461 (A1, 253-bp; A9, 273-bp). This genotypic combination has not been observed in two healthy sisters, or their brother who tested positive for the R59W mutation causing variegate porphyria (VP). Evaluation of the study population for the specific alleles of each of the four markers identified in the C282Y homozygous MS patients, revealed a significantly increased frequency of the 135-bp fragment of marker locus D6S273 in MS patients (51%) versus the control group (41%) ($P<0.05$). This finding is in accordance with previous studies, which demonstrated significant linkage between MS and marker locus D6S273.

INTRODUCTION

Iron is an essential nutritional element for normal functioning of a large number of enzymes involved in haem biosynthesis, neurotransmitter synthesis and degradation, myelin formation and oxidative phosphorylation (Aisen, 1994; Roskams and Connor; 1994, Ponka, 1997). Changes in the normal distribution pattern of intracerebral iron, which may result in neurological dysfunction, have been described in both iron overload and iron deficiency (Larkin and Rao; 1990, Dexter et al., 1992; Jellinger and Kienzl, 1993). The high demand of iron in the brain and the central nervous system (CNS) and their sensitivity to iron-induced peroxidative damage, suggests the need for stringent regulation of iron availability in these organs. Studies performed on brain tissue of patients with multiple sclerosis (MS) indicated disruption of the normal pattern of transferrin and ferritin binding (Hulet et al.,1999). These findings suggest that loss of ferritin binding may be involved in or is a consequence of demyelination associated with this chronic inflammatory disease of the CNS.

The functional linkage between immune function and iron absorption became evident with the identification of the major histocompatibility complex (MHC)-encoded HFE protein, underlying hereditary haemochromatosis (HH) (Feder et al., 1996, 1998). This finding provided evidence that MHC-encoded class I molecules might play a role in iron metabolism (Salter-Cid et al., 2000). Studies on iron accumulation in blood-brain barrier endothelial cells cultured under conditions mimicking iron status were in agreement with clinical studies in HH patients, suggesting that alterations in the peripheral iron status may affect the intracerebral iron concentration (van Gelder et al., 1998).

DNA screening of the South African population for two common haemochromatosis mutations, H63D and C282Y, has demonstrated that compound heterozygosity for these mutations or homozygosity for mutation C282Y is responsible for HH in more than 80% of affected Caucasian individuals (de Villiers et al., 1999a). These iron overload genotypes could also explain the porphyria phenotype in a relatively large proportion of patient referrals displaying symptoms suggestive of this group of haem biosynthesis

disorders (de Villiers et al., 1999b), particularly patients who carry a definite diagnosis of sporadic porphyria cutanea tarda (PCT) (Hift et al., 1997). The carrier frequency of mutation C282Y was found to be 1 in 6 in the general Caucasian population of South Africa, with an estimated 1/115 homozygotes. Interestingly, a significantly lower carrier frequency (1 in 25) was detected in clinically affected index patients with the founder-related mutation R59W causing variegate porphyria (VP), implying that the HH gene may be a modifier locus for VP (de Villiers et al., 1999b). Although this autosomal dominant disease is extremely common in South Africa due to a founder effect (Dean, 1972; Meissner et al., 1996; Warnich et al., 1996), approximately 80% of the estimated 20 000 South African individuals with a defective VP gene remain clinically undetected (Day, 1986). The photosensitivity associated with VP may occur alone or in combination with acute symptoms such as abdominal pain, the passage of dark urine, and neuropsychiatric crises including bulbar paralysis, quadriplegia, motor neuropathy and weakness of the limbs. Some of the neurological manifestations of an acute porphyria are similar to that observed in MS (Rooney et al., 1999; Macy et al., 1991; Downey, 1992; Pierach 1993). This is probably due to CNS dysfunction in both conditions, although the acute porphyrias also affect the peripheral nervous system (PNS). The overlap in clinical presentation of MS and VP is of particular relevance in South Africa where MS is considered to be rare (Dean, 1967), and many patients have been misdiagnosed with VP prior to the availability of a DNA diagnostic test (Kotze et al., 1998).

HH is considered to be the most common autosomal recessive disease in Caucasians of Northern European decent, occurring in 1/200-300 individuals. It is therefore noteworthy that there were no subjects with clinically manifested HH recognised among 1700 patients with multiple sclerosis (MS) encountered by Valberg et al. (1989), particularly since MS affects the same ethnic group. In this study DNA samples of 123 apparently unrelated South African patients diagnosed with MS were subjected to mutation screening of the HH gene, in order to determine whether this finding may be due to a linkage disequilibrium effect or gene-gene interaction.

SUBJECTS AND METHODS

Subjects

Relevant personal information was obtained from 281 South African patients diagnosed with MS, following distribution of a specifically designed questionnaire (published in the National Multiple Sclerosis Society newsletter) to support group meetings and physicians. Blood samples were obtained with informed consent from 142 apparently unrelated patients. The completed questionnaires were subsequently forwarded to the patients' neurologists for verification of the MS diagnoses. Non-Caucasian South African MS patients, recent immigrants and patients without a definite diagnosis of MS were excluded from this study, leaving 123 index cases for DNA analysis. The diagnosis of MS in these patients was based on standard criteria, including selected clinical features (Poser et al., 1983), lumbar puncture, MRI and/or exclusion of other diseases known to manifest similarly. The study protocol was approved by the Ethics Review committee of the University of Stellenbosch.

DNA screening for the common HH mutation C282Y revealed homozygosity in one of the index patients, a 51-year old female. She developed gait difficulty with leg weakness in 1982 following pregnancy, numbness of the trunk and a burning sensation in her legs in 1984, when the MS diagnosis was made at the age of 32 years. In 1985 she had an episode of probable optic neuritis. Her hands have become progressively weaker and her face intermittently numb. Nerve conduction studies were normal. The disease course was relapsing-remitting initially, subsequently becoming chronic progressive. She has a history of iron deficiency anaemia evidenced by low transferrin saturation concentration during child-bearing years and received treatment (dietary iron supplementation) for this condition in the past. The family history revealed that four subjects in the first generation of her family (figure 1) were previously diagnosed with variegate porphyria (VP) based on routine biochemical analysis of blood, stool and urine specimens. Follow-up clinical investigations performed for this study at Tygerberg Hospital include MRI of the brain (Dr J Carr), liver function tests and radioisotope studies of the liver (Dr S Schmit), using standard techniques.

Haematological, Biochemical and Immunological analysis

Full blood counts, performed on a Technicon H2 blood cell counter and serum iron, transferrin and ferritin concentrations, were determined in a routine diagnostic laboratory. Serum iron was measured with a Beckman CX7 autoanalyser, and transferrin with a Beckman Array Nephelometer. Transferrin saturation was subsequently calculated. Transferrin receptor concentration was measured using an enzyme immunoassay (Human Transferrin Receptors Kit, Ramco Laboratories). Liver biopsies could not be performed, but since unusually high CD4:CD8 ratios were shown to precede the development of severe iron overload (Porto et al., 1997), T-cell phenotyping was carried out in four subjects to serve as a marker for iron loading. This procedure was performed after red cell lysis of whole blood analysed by flow cytometry (Becton Dickinson).

Mutation screening

Genomic DNA was isolated from whole blood using a standard salting-out method (Miller et al., 1988). Screening for the HH gene mutations H63D and C282Y was performed by restriction enzyme analysis of polymerase chain reaction (PCR)-amplified DNA using *Mbo*I and *Rsa*I, respectively (Feder et al., 1996; Roberts et al., 1997). Possible mistyping of C282Y homozygosity was excluded by *Mse*I restriction enzyme analysis as previously described (de Villiers and Kotze, 1999). R59W mutation screening of the protoporphyrinogen oxidase (PPOX) gene underlying VP was performed according to Warnich et al. (1996).

Haplotype analysis

Microsatellite genotyping was performed on ABI310 and ABI3100 automated sequencers using fluorosceinated oligonucleotide primers corresponding to loci D6S273, D6S105, D6S2239 and D6S461 (table 1). Primer sequences are available at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=UniSts. The map order is cen - D6S273 - D6S105 - (HFE gene) - D6S2239 – D6S461. D6S2239 is the closest marker (<20 kb) to the HFE gene (Feder et al., 1996). Previous genome screens have

indicated that D6S273 (Haines et al., 1996) and D6S461 (Ebers et al., 1996) may be closely linked to putative MS loci. Between markers D6S273 and D6S461 there are 295 genes.

Table 1: Numbering of alleles for marker loci D6S237, D6S105, D6S2239 and D6S461 according to increasing repeat length.

	Marker loci and PCR fragment sizes in base pairs			
	D6S273	D6S105	D6S2239	D6S461
A1	127	113	105	253
A2	129	115	107	255
A3	131	117	109	261
A4	133	119	111	263
A5	135	121	113	265
A6	137	123		267
A7	139	125		269
A8		127		271
A9		129		273
A10		131		275
A11		133		
A12		135		
A13		139		

Statistical analysis

Statistical analysis was performed by STATISTICA for Windows (StatSoft Inc. Tulsa, OK, USA). Two-tailed Fisher exact test and the χ^2 calculation were applied as appropriate. *P*-values <0.05 were regarded as statistically significant.

RESULTS

DNA screening for HH mutations H63D and C282Y in 118 (of the available 123) apparently unrelated South African MS patients revealed homozygosity for mutation C282Y in one individual. None of the MS patients were compound heterozygotes for mutations C282Y and H63D, whilst the carrier frequencies of both mutations were approximately 15% in the MS patient group (table 2). Although a statistically significant

difference was not observed between MS patients and controls, the frequency of the generally more common H63D mutation was lower in the patient group.

Table 2: Comparison of genotype distribution and allele frequencies of HH mutations C282Y and H63D in MS patients and controls drawn from the same population.

	Controls* (n=102)	Patients (n=118)
Genotype		
C282Y++/H63D--	2 (2%)	1 (0.9%)
C282Y+-/H63D+-	1(1%)	1 (0.9%)
C282Y+-/H63D--	14 (13.7%)	21 (18%)
C282Y--/H63D++	0	1 (0.9%)
C282Y--/H63D+-	24 (23.5%)	16 (14%)
C282Y--/H63D--	61 (59.8%)	78 (66%)
Allele		
282-G (normal)	0.91	0.90
282-A (mutant)	0.09	0.10
Carrier frequency	16.9%	18%
63-C (normal)	0.88	0.92
63-G (mutant)	0.12	0.08
Carrier frequency	21.1%	14.7%

*de Villiers et al., 1999a

The pedigree of the MS patient found to be homozygous for mutation C282Y is shown in figure 1. Mutation analysis in 17 additional family members of the index case (II-5) revealed the same genotype in her sister (II-4), who also has a diagnosis of MS. None of the other first- or second-generation relatives of these sibs were homozygous for the C282Y mutation, while this genotype was detected in all three children (aged 16-23 years) of the index patient. Since patient II-5 reported a family history of VP, mutation screening of the PPOX gene was also performed. As shown in figure 1, the founder-related mutation R59W was detected in five family members, of whom four are sibs in the first generation.

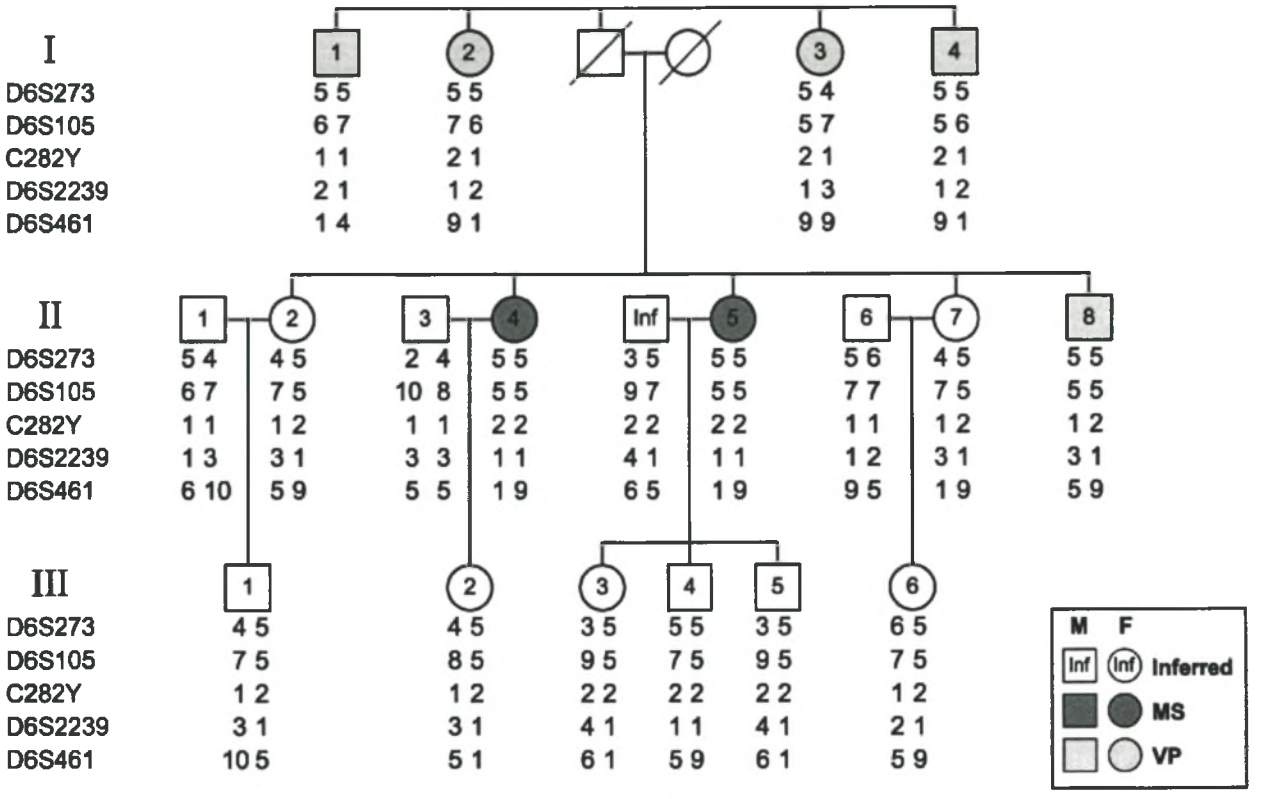


Figure 1: Pedigree of the index MS patient (II-5) found to be homozygous for mutation C282Y. Subjects diagnosed with multiple sclerosis (MS) are indicated in dark-shaded symbols, while those with mutation R59W causing variegate porphyria (VP) are indicated in light-shaded symbols. The genotypes for mutation C282Y in the HH gene, and microsatellite marker loci D6S273, D6S105, D6S2239 and D6S461 (in alleles, table 1), are indicated below the pedigree symbols. Only those family members of whom DNA samples were available are numbered.

Haplotype studies performed in the MS family using microsatellite markers flanking the HH locus, demonstrated that the two MS patients were homozygous for specific alleles at marker loci D6S273 (A5, 135-bp), D6S105 (A5, 121-bp), D6S2239 (A1, 105-bp) and heterozygous for marker D6S461 (A1, 253-bp; A9, 273-bp). This genotypic combination has not been observed in their unaffected brother and two sisters (figure 1).

Evaluation of the study population for the specific alleles of each of the four markers identified in the C282Y homozygous MS patients (highlighted in table 3), indicated that the 135-bp fragment of marker locus D6S273 previously shown to be linked to MS (Haines et al., 1996), predominates in the South African MS patients (51%) versus the

control group (41%) (P<0.05, 1 df, χ^2=3.9). No significant difference was however detected when the allelic distribution was considered for all the alleles combined (P>0.05, 6 df, χ^2=12.58). Detection of the 135-bp allele in 31/50 chromosomes of C282Y homozygotes previously diagnosed with HH (data not shown), suggests that a gene involved in MS susceptibility may occur on the chromosome carrying the C282Y mutation in some cases.

Table 3: Genotype distribution and allele frequencies of microsatellite marker loci D6S105, D6S273, D6S2239 and D6S461 on chromosome 6. The alleles detected in the two sisters with MS are highlighted.

Marker	Fragment size (base pairs)	Allele	MS	Control
D6S273	127	A1	5 (2.6%)	12 (7%)
	129	A2	7 (3.6%	16 (9.4)%
	131	A3	29 (15%)	24 (14%)
	133	A4	27 (14%)	29 (17%)
	135	**A5**	**101 (52%)**	**70 (41%)**
	137	A6	14 (7%)	8 (4.7%)
	139	A7	13 (6.6%)	11 (6.5%)
Total			196	170
D6S105	113	A1	2 (0.8%)	0
	115	A2	4 (1.7%)	7 (4.9%)
	117	A3	1 (0.8%)	1 (0.7%)
	119	A4	4 (1.6%)	5 (3.5%)
	121	**A5**	**66 (27%)**	**30 (21%)**
	123	A6	27 (11%)	25 (17.6%)
	125	A7	62 (25.6%)	31 (21.8%)
	127	A8	35 (14%)	18 (12.7%)
	129	A9	24 (10%)	14 (9.9%)
	131	A10	6 (2.5%)	4 (2.8%)
	133	A11	9 (3.7%)	7 (4.9%)
	135	A12	1 (0.4%)	0
	139	A13	1 (0.4%)	0
Total			242	142

Table 3 continue

D6S2239	**105**	**A1**	**81 (33%)**	**50 (33.3%)**
	107	A2	54 (22%)	36 (24%)
	109	A3	107 (43.5)	61 (40.7%)
	111	A4	4 (1.6%)	3 (2%)
Total			246	150
D6S461	**253**	**A1**	**5 (2.4%)**	**5 (4.7%)**
	261	A3	7 (3.3%)	2 (1.9%)
	263	A4	29 (14%)	6 (5.7%)
	265	A5	64 (30.7%)	28 (26.4%)
	267	A6	57 (27.4%	28 (26.4%)
	269	A7	9 (4.3%)	4 (3.8%)
	271	A8	20 (9.6%)	18 (17%)
	273	**A9**	**16 (7.6%)**	**15 (14.2%)**
	275	A10	1 (0.5%)	0
Total			208	106

Determination of iron parameters (after an overnight fasting period) revealed abnormal profiles in several family members of index patient II-5 (table 4). Elevated transferrin saturation and ferritin levels, correlating with homozygosity for mutation C282Y, were evident in the two sisters (II-4, II-5) diagnosed with MS, as well as the oldest son (III-4) of the index case. Although the raised ferritin levels detected in family members I-3, I-4 (and elevated transferrin saturation), II-2, II-7 and II-8 may partly be due to heterozygosity for mutation C282Y, the reason for the high serum levels of this acute phase protein in two mutation-negative male spouses (II-1, II-6) is unknown. The youngest son (III-5) of the index patient, who inherited two copies of mutation C282Y from his parents, presented with a normal iron profile, except for slightly raised transferrin saturation (53.5%). The daughter (III-3) demonstrated an increased serum transferrin receptor concentration suggestive of mild iron deficiency, which appears to contradict the detected homozygosity for mutation C282Y.

Table 4: Iron profiles determined in members of the index family following an overnight fasting period

No	Gender	Age (yrs)	Haemoglobin F: (11.5-16.5 g/dl) M: (12.5-17.5 g/dl)	Serum Iron* (6-32 µmol/l) (7-35 µmol/l)	Transferrin (1.8-3.8 g/l) (1.8-3.8 g/l)	Tr Saturation (15-50%) (20-50%)	Ferritin (20-150 µg/l) (20-228 µg/l)	Tr Receptor (42-69 ng/ml) (42-69 ng/ml)
I-1	M	88	n.d.	12.6	2.7	20.2	38	**92.9**
I-2	F	81	13.3	14	3.1	20.2	69	**89.6**
I-3	F	73	14.3	18.2	2.31	34.7	**248**	64
I-4	M	71	14.6	25.3	1.89	**58.9**	**285**	**41.1**
II-1	M	53	**17.9**	30.1	2.9	45.2	**559**	57.9
II-2	F	52	14.1	17.2	2.4	31	**253**	49.8
II-3	M	52	n.d.	19.6	2.8	31	228	**70.4**
II-4	F	50	n.d.	27.8	**1.7**	**65, 96**	**280**	**34.1**
II-5*	F	48	13.3	32.8	**1.7**	**77**	**347, 238**	**18.1, 25.8**
II-6	M	49	15.8	16.8	2.53	29.2	**312**	60.19
II-7	F	45	15.4	18.4	2.5	32.9	**251**	**71.1**
II-8	M	40	17.2	23.2	2.3	43.6	**416**	47.1
III-1	M	21	16.9	20.7	2.7	34.4	210	53.6
III-2	F	28	n.d.	13	2.6	22.4	125	**78.7**
III-3	F	23	15.5	20.7	2.4	38	76	**77.1**
III-4	M	22	15.8	**39.8**	**1.5**	**92, 100**	**480**	**37.5**
III-5	M	16	15.9	26.5	2.2	**53.5**	43	49
III-6	F	19	14.0	13.3	2.71	21.6	**9**	n.d.

*Index case. Her most recent ferritin level was 175 µg/l without treatment for iron overload.
Numbering refers to the Pedigree in figure 1
Reference ranges for females and males are given in parenthesis for each parameter. Levels outside these values are highlighted.
F, female; M, male; n.d., not determined; Tr, transferrin

Since evidence has previously been provided that heterozygosity for mutation C282Y may protect against iron deficiency (Datz et al., 1998), we compared the iron profiles of 17 of the female MS patients of European descent found to be heterozygous for this mutation against reference values (table 5). In five of these patients (29.4%) low serum ferritin levels, a reliable parameter of iron depletion, were measured. Two patients (11.8%) presented with raised serum ferritin concentrations.

Table 5: Non-fasting iron profiles of 17 female MS patients heterozygous for mutation C282Y in the HH gene.

No.	Age (yrs)	Haemoglobin (11.5-16.5 g/dl)	Serum Iron (6-32 µmol/l)	Transferrin (1.8-3.8 g/l)	Tr Saturation (15-50%)	Ferritin (20-150 µg/l)
13	41	12.5	14.2	2.8	20	**19.4**
28	39	n.d.	6.7	**4.0**	**7**	**6.2**
41	26	14.5	29.3	2.8	42	54.4
45	22	13.5	20.9	3.7	23	105.8
52	61	13.8	21.2	3.5	24	**10.8**
54	46	n.d.	9.0	2.4	16.5	25.1
58	36	15.0	14.2	2.4	24	88.8
60	61	12.5	15.8	2.5	25	**17.6**
66	24	13.3	28.7	3.0	38	90.2
77	69	n.d.	17.0	2.4	28	114.9
84	35	12.5	**4.2**	2.7	**6.9**	**7.0**
99	52	n.d.	**3.1**	1.8	**7.4**	99
108	53	14.7	19.4	1.9	41	210.7#
111	64	14.9	24.7	3.2	33.9	70.7
112	41	n.d.	20.9	3.2	29.3	40.3
117	43	16.0	n.d.	n.d.	n.d.	39
120	43	12.8	14.9	2.0	32.9	203.4#

#Elevated serum ferritin concentration; Levels suggestive of iron deficiency are highlighted

Since the two sisters previously diagnosed with MS were both shown to be homozygous for the C282Y mutation, follow-up neurological examinations were requested. The results were consistent with the diagnosis of MS in both cases. The index patient (II-5) had mild bilateral optic atrophy, saccadic smooth pursuit, spastic legs, generalised hyperreflexia, diminished light touch over the legs and trunk with a level corresponding to the T4 dermatome, slowed fine finger movements and slowed rapid alternating movements. Magnetic resonance imaging (MRI) results were consistent with a diagnosis

of definite MS in this patient and no iron deposition could be detected in the brain (figure 2).

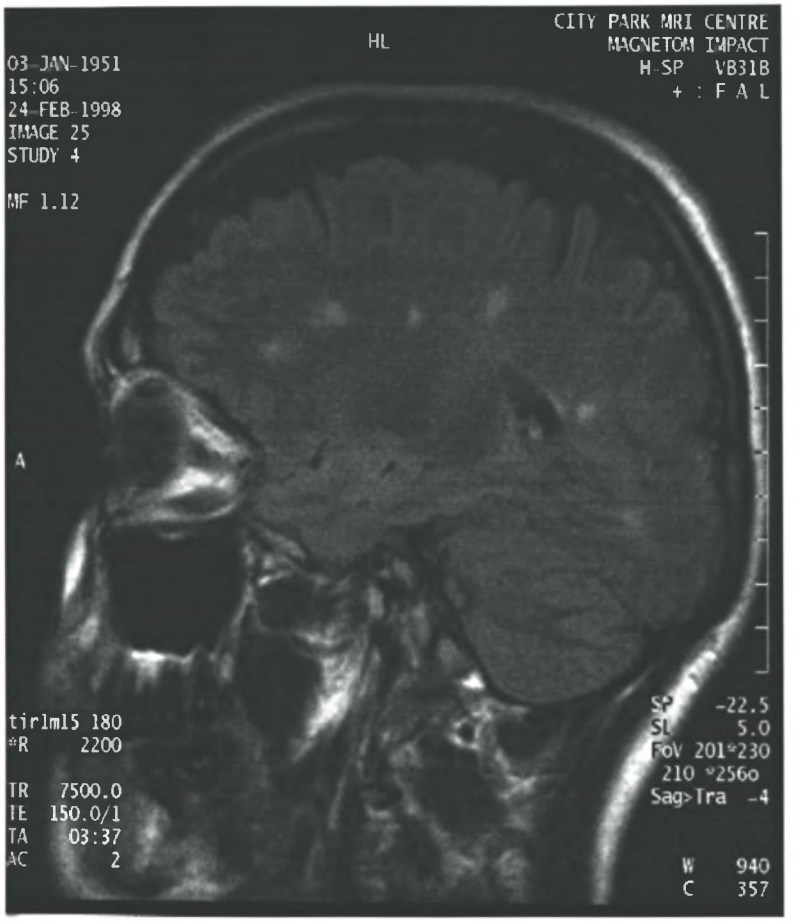


Figure 2: T-2 weighted magnetic resonance imaging lesions in the brain of patient II-5. (Image reproduced with permission of Dr R Hewlett, Christiaan Barnard Memorial Hospital, Cape Town).

The sister of the index patient (II-4) was also diagnosed with definite MS with a relapsing remitting course. She is a 54-year old female whose neurological symptoms (onset age 25) include gait difficulty and numbness of both legs. She has been wheelchair bound for the past 12 years. On examination she had presented with bilateral optic atrophy, impaired smooth pursuit, saccadic dysmetria, mild dysarthria, bilateral internuclear ophthalmoplegia, spastic quadriparesis (with lower limbs markedly more affected than the upper), extensor plantars bilaterally, diminished light touch (more severe over her

left side and including the left side of her face), mildly impaired joint position sense in the toes and markedly impaired co-ordination in all limbs. Nerve conduction studies were normal. During recent years her condition remained relatively static without active exacerbations.

The abnormal iron profiles of subjects II-4 and II-5 did not result in organ damage. None of the patients are known to have any dietary idiosyncrasies, they never donated blood and both underwent hysterectomies approximately 5 years ago. In the index case this procedure was performed due to the presence of myomas, and in her (wheelchair-bound) sister mostly for practical purposes. Liver-function tests and radio-isotope studies were normal in both cases (data not shown). Liver biopsies were not performed to further assess the likelihood of iron accumulation caused by elevated serum iron levels, since this could not be justified ethically, but T-cell phenotyping largely excluded the possibility of iron overload due to the presence of mutation C282Y. The CD4:CD8 ratios (Porto et al., 1997) were very low (<3) in the index patient (1.26). Low CD4:CD8 ratios were also detected in her oldest son (III-4, 1.33) and youngest sister (II-7, 1.67). Comprehensive phenotyping of the immune system in mice revealed no detectable anomalies within various T cell compartments, suggesting that high CD4:CD8 ratios observed in HH patients may, at least in part, be secondary manifestations of severe iron overload.

DISCUSSION

Multiple lines of evidence, ranging from population to family studies, support the role of genetic factors in susceptibility to MS (Ebers et al., 1995; Haines 1996; Haines et al., 1998; Rothwell and Charlton, 1998). The importance of human leukocyte antigen (HLA) status in MS susceptibility and the complex nature of the disease have recently been demonstrated in a large North American pedigree with an apparent autosomal dominant inheritance pattern, since co-inheritance of two loci were shown to be involved in disease susceptibility (Vitale et al., 2002). Risch (1987) reported that the effect of a HLA-

linked locus accounts for only a 2.5-fold increased risk of MS to sibs over the population prevalence, compared to the observed value of 20.

In this study we investigated the possible significance of the HH genotype (C282Y, 22), co-existing with the MS phenotype, in a family with VP. The aetiology of MS is poorly understood, and therefore co-existence of genetic disorders in large families may contribute to the elucidation of the disease mechanism(s). In contrast to the high prevalence of VP in South Africa, MS is considered to be uncommon among white Afrikaners (Dean, 1967, 1972; Kotze et al., 1998). Some striking similarities between MS and the acute porphyrias (Macy et al., 1991; Downey, 1992) raised the possibility that several South African patients with partial expression of MS, or a proposed new subtype of the disease (Rooney et al., 1999), have been misdiagnosed with VP prior to the availability of a DNA test for this condition. Since the opposite can probably also occur, we first excluded VP in the index case and her sister by screening for mutation R59W segregating in this family. The absence of peripheral nervous system involvement, an important feature distinguishing MS from the acute porphyrias (both affect the central nervous system) and many other diseases that may masquerade as MS (Natowicz and Bejjani, 1994), supported a diagnosis of MS in the family. Mutation R59W causing VP was detected in five family members of index patient II-5, facilitating accurate diagnosis of this condition which is dependent on interaction with other genetic and/or environmental factors (Kaupinnen and Mustajoki, 1992) for clinical expression.

A strong correlation has been demonstrated between signal intensity and iron concentration in the liver (Thomsen et al., 1992), and these data suggest that increased iron accumulation may occur in the brain of patients with HH. Neurological features associated with HH are rare, but psychomotor retardation, lethargy, fatigue, confusion, hearing loss and polyneuropathy have previously been described (Milder et al., 1980). Nielsen et al. (1995) furthermore reported a patient with HH who presented with dementia, dysarthria, a slowly progressive gait disturbance, muscle weakness, rigidity, tremor and ataxia, which were most likely caused by excessive iron accumulation in the

basal ganglia and cerebellum. The likelihood that (some of) the neurological features of subject II-5 are caused by deposition of iron in the brain was excluded by MRI.

Measurement of serum iron levels in the 17 female MS patients found to be heterozygous for mutation C282Y revealed diverse iron profiles that may represent a non-specific response due to inflammation associated with the disease process. Detection of values suggestive of iron deficiency in 6 cases (35%) nevertheless appears to contradict the notion that a single copy of this gene may protect women against iron deficiency (Datz et al., 1998). This may, however, apply to the general population but not to subjects with an inherited defect to absorb or transport iron normally. Earlier, Valberg et al. (1989) reported partial expression of the HH gene in heterozygotes from a control group, although a similar trend was not apparent in MS patients analysed by the authors. These findings provide additional support for the notion that intermittent iron deficiency may be related to the disease phenotype in a subgroup of MS patients (Rooney et al., 1998).

Homozygosity for mutation C282Y in index patient II-5 was unexpected, since she received treatment for iron deficiency (low transferrin saturation) in the past. Liver function tests and radio-isotope studies did not reveal any abnormalities in this patient, or her C282Y homozygous sister who also has MS, despite the fact that biochemical testing demonstrated elevated transferrin saturation and ferritin levels indicative of HH in both subjects. It is noteworthy that the MS phenotype of the sister, who did not supplement her diet with iron on a regular basis in the past, is more severe than that of the index case. Symptoms of HH presenting in 40-60% of HH homozygotes usually become apparent between the ages of 40 and 50 years, unless certain protective measures are taken, such as regular donation of blood, or if other gene(s) modulate the expression of disease-related mutations. In a report on a family displaying non-expressing C282Y homozygosity (Rhodes et al., 1997), variability in disease expression induced by gender did not account for differences in expression, as evidenced by the development of overt disease in two C282Y homozygous sisters (aged 50 and 53 years) who had both borne children. It is also interesting to note that the HLA-identical sister of

the non-expressing C282Y homozygote studied by Rhodes et al. (1997) had a history of iron deficiency, probably as a consequence of coeliac disease, despite the fact that her transferrin saturation (57/76%) and ferritin levels (157/258 µg/l) were elevated. This finding suggests that serum concentrations of certain iron parameters are not always a true reflection of iron status in tissue and that iron deficiency is possible in C282Y homozygotes, as previously reported in patient II-5. Consequently, we speculate that such a situation is unlikely to be ascribed solely to environmental factors, and that unknown gene mutations predisposing individuals to an unusual form of (intermittent) iron deficiency may be involved.

This study demonstrated that homozygosity for mutation C282Y (genetic HH), in association with the 135-bp allelic fragment of marker locus D6S273 shown to be associated with a putative MS susceptibility gene (Haines et al., 1996), has not caused organ damage in the two sisters with MS. This finding, together with the data reported by Valberg et al. (1989), implies that MS and clinically manifested HH may be mutually exclusive as a consequence of gene-gene interaction, and provides further support for a role of iron metabolism in the aetiology of MS.

ACKNOWLEDGEMENTS

The University of Stellenbosch and the Harry and Doris Crossley Foundation supported the study financially. The National Multiple Sclerosis Society of South Africa is acknowledged for distribution of questionnaires to MS patients and data collection. Study participants and their families are acknowledged for their willingness and cooperation to participate in this study. Dr. G. Porto from the St. Antonio General Hospital, Portugal, is thanked for advice on T-cell phenotyping, and Prof Patric Bouic and Dr Justin Devine for performing this analysis. C.J. van Heerden of the Central DNA sequencing Facility at the University of Stellenbosch is acknowledged for genotyping of the four microsatellite markers. Prof Erna Mansvelt, Elba Fourie and Dr Susan van Rensburg of Tygerberg Hospital are thanked for determination of iron parameters. Dr JH Louw and Stefan Maritz is acknowledged for advice with the statistical analyses. Prof J

Carr and Dr S Schmidt of Tygerberg Hospital, and Drs F Badenhorst (Panorama Medi-clinic) and Dr GA Prinsloo (Wellington) are acknowledged for clinical assessment and information of the index patient and her sister. JNP de Villliers received student bursaries from the Freda and David Becker Trust and the Poliomyelitis Research Foundation.

REFERENCES

Aisen P. In Brock JH, Halliday JW, Pippard MJ and Powell LW (eds.). Iron Metabolism in Health and Disease. *Iron metabolism: an evolutionary perspective*. Saunders Co. Ltd. London, 1994; pp. 1-30.

Connor JR and Menzies SL. Relationship of iron to oligodendrocytes and myelination. GLIA, 1996; 17: 83-93.

Datz C, Haas T, Rinner H, Sandhofer F, Patch W and Paulweber B. Heterozygosity for the C282Y mutation in the hemochromatosis gene is associated with increased serum iron, transferrin saturation, and hemoglobin in young women: a protective role against iron deficiency? Clinical Chemistry 1998; 44: 2429-2432.

Day RS. Variegate porphyria. Seminars in Dermatology 1986; 5: 138-154.

de Villiers JNP and Kotze MJ. Significance of linkage disequilibrium between mutation C282Y and a MseI polymorphism in population screening and DNA diagnosis of hemochromatosis. Blood Cells Molecules and Disease 1999; 15: 250-252.

de Villiers JNP, Hillermann R, de Jong G, Langenhoven E, Rossouw H, Marx MP and Kotze MJ. High prevalence of the Cys282Tyr HFE mutation facilitates an improved diagnostic service for hereditary haemochromatosis in South Africa. South African Medical Journal, 1999a; 89: 279-282.

de Villiers JNP, Hillermann R, Loubser L and Kotze MJ. Spectrum of mutations in the HFE gene implicated in hemochromatosis and porphyria. Human Molecular Genetics 1999b; 8: 1517-1522.

Dean G. Annual incidence, prevalence, and mortality of multiple sclerosis in white South-African-born and in white immigrants to South Africa. British Medical Journal 1967; 2: 724-730.

Dean G. The porphyrias. A story of inheritance and environment. Philadelphia: J B Lippincott (2nd ed) 1972.

Dexter DT, Jenner P, Schapira AVH and Marsden CD. Alterations in levels of iron, ferritin, and other trace metals in neurodegenerative diseases affecting the basal ganglia. Annals of Neurology 1992; 32 (supplement): 94-100.

Downey DC. Fatigue syndromes: New thoughts and reinterpretation of previous data. Medical Hypotheses, 1992; 39: 185-190.

Ebers GC, Sadovnick AD and Risch NJ. A genetic basis for familial aggregation in multiple sclerosis. Canadian Collaborative Study. Nature 1995; 377: 150-151.

Ebers GC, Kukay K, Bulman DE, Sadovnick AD, Rice G, Anderson C, Armstrong H, Cousin K, Bell RB, Hader W, Paty DW, Hashimoto S, Oger J, Duquette P, Warren S, Gray T, O'Connor P, Nath A, Auty A, Metz L, Francis G, Paulseth JE, Murray TJ, Pryse-Phillips W, Risch N, et al. A full genome search in multiple sclerosis. Nature Genetics 1996; 13: 472-476.

Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R Jr, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna

DT, Risch NJ, Bacon BR and Wolff RK. A novel MHC class-I like gene is mutated in patients with hereditary haemochromatosis. Nature Genetics 1996; 13: 399-408.

Feder JN, Penny DM, Irrinki A, Lee VK, Lebron JA, Watson N, Tsuchihashi Z, Sigal E, Bjorkman P and Schatzman RC. The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. Proceedings of the National Academy of Sciences (USA) 1998; 95: 1472-1477.

Haines JL and The Multiple Sclerosis Genetics Group. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. Nature Genetics 1996; 13: 469-471.

Haines JL, Terwedow HA, Burgess K, Pericak-Vance MA, Rimler JB, Martin ER, Oksenberg JR, Lincoln R, Zhang DY, Banatao DR, Gatto N, Goodkin DE and Hauser SL. Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. Human Molecular Genetics 1998; 7: 1229-1234.

Hift R J, Corrigall A V, Meissner PN and Kirsch RE. Significance of HLA mutations in South African patients with porphyria cutanea tarda. Haematology 1997; 98(suppl): 112.

Hulet SW, Powers S and Connor JR. Distribution of transferrin and ferritin binding in normal and multiple sclerotic human brains. Journal of Neurological Sciences 1999; 165: 48-55.

Jellinger K and Kienzl E. In Riederer, P. and Youdim, M.B.H. (eds.), Iron in Central Nervous System Disorders. *Iron deposits in brain disorders.* Springer-Verlag, New York, 1993; 19-36.

Kaupinnen R and Mustajoki P. Prognosis of acute porphyria: occurrence of acute attacks, precipitating factors and associated diseases. Medicine, 1992; 71: 1-13.

Kotze MJ, de Villiers JNP, Groenewald JZ, Rooney RN, Loubser O, Thiart R, Oosthuizen CJJ, van Niekerk MM, Groenewald IM, Retief AE and Warnich L. Molecular analysis reveals a high mutation frequency in the first untranslated exon of the PPOX gene and largely excluses variegate porphyria in a subset of clinically affected Afrikaner families. Molecular and Cellular Probes 1998; 12: 293-300.

Larkin EC and Rao A. In Dobbing. J. (ed), Brain, behaviour and iron in the infant diet. Importance of fetal and neonatal iron: adequacy for normal development of the central nervous system. New York, Springer-Verlag, 1990; 43-62.

Macy JA, Gilroy J and Perrin JC. Hereditary coproporphyria: an imitator of multiple sclerosis. Archives of Physical Medicine and Rehabilitation 1991; 72: 703-704.

Meissner PN, Dailey TA, Hift RJ, Ziman M, Corrigall AV, Roberts AG, Meissner D M, Kirsch RE and Dailey HA. A R59W mutation in human protoporphyrinogen oxidase results in decreased enzyme activity and is prevalent in South Africans with variegate porphyria. Nature Genetics 1996; 13: 95-97.

Milder MS, Cook JD, Stray S and Finch CA. Idiopathic hemochromatosis, an interim report. Medicine 1980; 59: 34-49.

Miller SA, Dykes DD and Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 1988; 16: 1215.

Natowicz MR and Bejjani B. Genetic disorders that masquerade as multiple sclerosis. American Journal of Medical Genetics 1994; 49: 149-169.

Nielsen JE, Neerup L, Jensen K and Krabbe K. Hereditary haemochromatosis: a case of iron accumulation in the basal ganglia associated with a parkinsonian syndrome. Journal of Neurology, Neurosurgery and Psychiatry 1995; 59: 318-321.

Pierach CA. Coproporphyria versus multiple sclerosis. Archives of Physical Medicine and Rehabilitation 1993; 74: 677-678.

Ponka P. Tissue-specific regulation of iron metabolism and heme synthesis: Distinct control mechanisms in Erythroid cells. Blood 1997; 89: 1-25.

Porto G, Vicente C, Teixeira MA, Martins O, Cabeda JM, Lacerda R, Goncalves C, Fraga J, Macedo G, Silva BM, Alves H, Justica B and de Sousa M. Relative impact of HLA phenotype and CD4-CD8 ratios on the clinical expression of hemochromatosis. Hepatology 1997; 25: 397-402.

Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH and Tourtellotte WW. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Annals of Neurology 1983; 13: 227-231.

Rhodes DA, Raha-Chowdhury R, Cox TM and Trowsdale J. Homozygosity for the predominant Cys282Tyr mutation and absence of disease expression in hereditary haemochromatosis. Journal of Medical Genetics 1997; 34: 761-764.

Roberts AG, Whatley SD, Morgan RR, Worwood M, Elder GH, Roth MP and Giraldo P. Increased frequency of the haemochromatosis Cys 282 Tyr mutation in sporadic porphyria cutaea tarda. Lancet 1997; 349: 321-323.

Rooney RN, Kotze MJ, de Villiers JNP, Hillermann R and Cohen JA. Multiple sclerosis, porphyria-like symptoms and a history of iron deficiency anemia in a family of Scottish descent. American Journal of Medical Genetics 1999; 86: 194-196.

Roskams AJI and Connor JR. Iron, transferrin, and ferritin in the rat brain during development and aging. Journal of Neurochemistry 1994; 63: 709-716.

Rothwell PM and Charlton D. High incidence and prevalence of multiple sclerosis in south east Scotland: evidence of a genetic predisposition. Journal of Neurology, Neurosurgery and Psychiatry 1998; 64: 730-735.

Risch N. Assessing the role of HLA-linked and unlinked determinants of disease. American Journal of Human Genetics 1987; 40: 1-14.

Salter-Cid L, Brunmark A, Peterson PA and Yang Y. The major histocompatibility complex-encoded class I-like HFE abrogates endocytosis of transferrin receptor by inducing receptor phosphorylation. Genes and Immunity 2000; 1: 409-417.

Thomsen C, Wiggers P, Ring-Larsen H, Christiansen E, Dalhoj J, Henriksen O and Christoffersen P. Identification of patients with hereditary haemochromatosis by magnetic resonance imaging and spectroscopic relaxation time measurements. Magnetic Resonance Imaging 1992; 10: 867-879.

Valberg LS, Flanagan PR, Kertesz A and Ebers GC. Abnormalities in iron metabolism in multiple sclerosis. Canadian Journal of Neurological Sciences 1989; 16: 184-186.

van Gelder W, Huijskes-Heins MIE, Cleton-Soeteman MI, van Dijk JP and van Eijk HG. Iron uptake in blood-brain barrier endothelial cells cultured in iron-depleted and iron-enriched media. Journal of Neurochemistry 1998; 71: 1134-1140.

Vitale E, Cook S, Sun R, Specchia C, Subramanian K, Rocchi M, Nathanson D, Schwalb M, Devoto M and Rohowsky-Kochan C. Linkage analysis conditional on HLA status in a large North American pedigree supports the presence of a multiple sclerosis susceptibility locus on chromosome 12p12. Human Molecular Genetics 2002; 11: 295-300.

Warnich L, Kotze MJ, Groenewald IM, Groenewald JZ, van Brakel MG, van Heerden CJ, de Villiers JNP, van de Ven WJ, Schoenmakers EF, Taketani S and Retief AE.

Identification of three mutations and associated haplotypes in the protoporphyrinogen oxidase gene in South African families with variegate porphyria. Human Molecular Genetics 1996; 5: 981-984.

CHAPTER 3

CONCLUSIONS

CONCLUSIONS

Defining the cause of MS represents a major challenge because little is certain about the pathogenesis of this neurological disease. The varied clinical picture of MS raises the possibility that it is not a well-defined aetiology, but consists of genetically different subtypes in addition to subtypes with no genetic contribution (phenocopies) (Rasmussen and Clausen, 2000). It seems highly likely that different genes and environmental factors are involved in the induction and progression of MS and that external factors may affect the population risk and genetic susceptibility the familial risk. The proposed role of iron in the aetiology of MS may explain this complexity.

Analysis of the iron transporter gene encoding the natural resistance-associated macrophage protein (*NRAMP1*), recently renamed the solute carrier family 11 (proton-coupled divalent ion transporters) member 1 (*SLC11A1*), represented the first attempt to investigate the likelihood that iron dysregulation in association with infectious and/or autoimmune disease susceptibility, may underlie the MS phenotype in a subgroup of patients. The Z-DNA forming repeat polymorphism in the promoter region of the gene was significantly associated with MS ($P<0.01$) in the genetically homogeneous Afrikaner population of South Africa. The potential significance of the Afrikaner population as a valuable source of material for identification of genes involved in complex conditions was highlighted by failure to confirm this association in the German and French populations using a case-control study and transmission linkage disequilibrium approach, respectively. Although this could mean that the *SLC11A1* gene is not involved in MS, it seems more likely that the unique genetic background of the Afrikaner population enabled the detection of a true association due to the fact that an expected limited number of disease-associated genes/mutations were probably introduced from Europe. Such a phenomenon would exclude some of the possible confounding factors complicating the identification of genes involved in complex diseases. Support for this conclusion was obtained by the detection of significant differences in genotype distribution among German MS patients with different disease courses. Due to the

important role of SLC11A1 in iron regulation, it would appear that genotyping of the 5'-(GT) repeat polymorphism, together with determination of iron concentration in CNS and serum (LeVine et al., 1999; Zeman et al., 2000) may represent important determinants of disease course in MS patients.

Based on the above findings, the Afrikaner population was furthermore exploited to confirm or exclude an association reported between MS and a deleterious point mutation (77C→G) in the gene encoding protein-tyrosine phosphatase, receptor-type C (*PTPRC*). This gene plays an essential role in the activation of T and B cells and was found to be associated with MS in the German population, although most of the follow-up studies performed to date failed to confirm this association. Analysis of the Afrikaner population did not indicate a causative role for the *PTPRC* gene in MS, a finding substantiated by the fact that the German study population included in our study also failed to demonstrate an association between MS and mutation 77C→G in the *PTPRC* gene. However, it seems likely that this mutation may contribute to disease expression, since in one of the South African families with two MS affected sibs, the most severely affected sister was heterozygous for the 77C→G mutation. The *PTPRC* mutation may therefore be of significance in disease prognosis, mainly within the family context.

To investigate a viral influence in the apparent multi-step aetiology of MS, serum and peripheral blood mononuclear cells (PBMCs) of MS patients, close relatives and unrelated controls were screened for the presence of MS-associated retrovirus (MSRV) and two herpes virus (HHV-6 and EBV) sequences. Comparison of frequencies of viral sequences among these groups was performed within the context of the genetic background, in an attempt to provide a better definition of MS subgroups and disease expression within specific families. Detection of virus sequences at similar frequencies in South African MS patients and their unaffected close relatives (MSRV), whilst absent or present at a significant lower frequency (HHV-6) in the serum of unrelated healthy control individuals, indicated that virus infections affect the population risk but not the familial risk in MS.

Viral sequences were not confined to a specific genotype for the functional *SLC11A1* gene promoter polymorphism, which excluded the possibility that the presence of allele 2 is related to the detection of viral sequences in MS patients. This allele increases the risk of infection but conversely protects against autoimmune disease (Searle and Blackwell, 1998). Although the number of South African MS patients with a primary progressive disease course was relatively small, all of them tested positive for MSRV sequences and were homozygous for allele 3 of the *SLC11A1* promoter polymorphism, which is associated with increased susceptibility to autoimmune diseases. These findings raise the possibility that the viral sequences remain active in patients with a progressive disease course, particularly in the absence of an autoimmune-protective allele 2, while activation of viral sequences in patients with relapsing-remitting MS may trigger the attacks from time to time.

Finally, some evidence for gene-gene interaction suggesting genetic influences involving iron metabolism, was provided in MS patients with a genetic predisposition for iron overload. In two MS-affected sisters with hereditary haemochromatosis (HH) due to the presence of two copies of mutation C282Y, no organ damage could be detected despite elevated serum iron parameters and the fact that both patients underwent hysterectomies several years ago. Since co-existence of genetic disorders in large families may contribute to the elucidation of disease mechanism(s) in complex conditions such as MS where the aetiology remains poorly understood, further investigation is warranted to define the genetic factors underlying the clinical phenotype in this family. It seems likely that a MS susceptibility locus could in some cases occur on the same chromosome carrying the C282Y mutation, thereby explaining the relatively high incidence of MS in Northern European populations where HH is prevalent.

The multidisciplinary approach used in this study towards elucidation of the aetiology of MS, has led to a stepwise accumulation of scientific information, which resulted in a new perspective on the disease process. It became clear that a simplistic single-gene search for MS susceptibility genes is not likely to provide the information required for development of a comprehensive diagnostic assay for this complex condition, due to

inconsistent findings in different population groups and even in subgroups of the same population. Based on the data provided in this study, it seems possible that a combined screening strategy including analysis of viral sequences and certain population-specific genetic factors might, together with current methods of diagnosing MS, provide a more conclusive means of early disease diagnosis or to distinguish between MS subtypes that may be of prognostic value. The ultimate approach would, however, be to utilise new sophisticated technology allowing simultaneous analysis of expression patterns of thousands of genes in a single experiment for individual profiling aimed at disease diagnosis and treatment.

Future Prospects

The human genome project has provided a tool for large-scale high-throughput analysis of differential gene expression, allowing progress in functional interpretation of genomic information. Of these methods, DNA microarrays and quantitative real-time PCR have been utilised most extensively. Microarrays are sophisticated detection systems that can read and quantify minute amounts of cDNA to simultaneously detect over- and under-expression of multiple genes. The results obtained could then be validated by gene expression studies utilising the sensitive real-time PCR technology. Only recently these methods of mutation detection and gene expression became available to the research community and will dramatically affect the direction and way research will be performed in future. Developing countries still lack the financial ability to develop facilities where such high-throughput genotyping and transcriptional profiling can be performed, but collaboration with international centres of excellence could alleviate this problem.

In a collaborative study with researchers at the University of Oxford, the monocyte chemotactic protein-3 (*MCP-3*) gene was shown to be significantly over-expressed in the samples of South African MS patients, but not in the control samples (M.G. Zaahl et al., unpublished data – abstract submitted for congress presentation). This finding prompted us to analyse a CA/GT repeat in the promoter-enhancer region of *MCP-3* to further investigate the role of this gene in MS. A significant difference was observed in

allelic distribution between 117 South African MS patients and 73 population-matched controls ($P<0.05$, 3 df, $\chi^2=9.14$). Sequencing of the coding region of three MS patients showing over-expression of MCP-3 did not reveal any sequence changes, which is in accordance with involvement of the promoter region in MCP-3 over-expression. This does not necessarily point towards a genetic susceptible gene for MS, but possibly more towards the mechanism related to the formation of lesions in the brains of MS patients.

Since the data obtained with microarray analysis confirm previous findings of a significant association between MS and the *MCP-3* gene (Nelissen et al., 2002), a comprehensive genetic test based on these and related findings will be developed for diagnostic purposes. This represents a classical example of how new technology can fast forward genetic research by defining disease-related mechanisms in a single experiment.

REFERENCES

Compston A and Sawcer S. Genetic analysis of multiple sclerosis. Current Neurology and Neuroscience reports 2002; 2: 259-266.

Kotze MJ, de Villiers JNP, Zaahl MG and Robson KJH. The role of iron metabolism in multiple sclerosis. In: Metal Ions and Neurodegenerative Disorders. P. Zatta, Ed., World Sci. Singapore, London, 2003 (in press).

LeVine SM, Lynch SG, Ou CN, Wulser MJ, Tam E and Boo N. Ferritin, transferrin and iron concentrations in the cerebrospinal fluid of multiple sclerosis patients. Brain Research 1999; 821: 511-515.

Nelissen I, Dubois B, Goris A, Ronsse I, Carton H, Opdenakker G. Gelatinase B, PECAM-1 and MCP-3 gene polymorphisms in Belgian multiple sclerosis. Journal of the Neurological Sciences 2002; 200: 43-48.

Rasmussen HB and Clausen J. Genetic risk factors in multiple sclerosis and approaches to their identification. Journal of Neurovirology 2000; 6 (supplement 2): 23-27.

Searle S and Blackwell JM. Evidence for a functional repeat polymorphism in the promoter of the human *NRAMP1* gene that correlates with autoimmune versus infectious disease susceptibility. Journal of Medical Genetics 1998; 36: 295-299.

Zeman D, Adam P, Kalistova H, Sobek O, Kelbich P, Andel J and Andel M. Transferrin in patients with multiple sclerosis: a comparison among various subgroups of multiple sclerosis patients. Acta Neurologica Scandinavica 2000; 101: 89-94.