

**MEMBRANE FLUIDITY AND FATTY ACIDS IN MULTIPLE
SCLEROSIS PATIENTS**

by

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DECLARATION

I, Gloudina Maria Hon, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

G Hon

Signed

28 August 2009

Date

ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), which leads to neuronal demyelination, and eventually to oligodendrocyte and axon loss, with subsequent lesion formation. The wide distribution of lesions in the CNS results in a variety of clinical features, such as cognitive impairment, vertigo, spasticity, ataxia tremors, progressive quadriparesis, pain and depression. Currently no cure exists for CNS disorders, resulting in a decline in quality of life, and an economic burden on society. Metabolic disturbances, especially lipid metabolic abnormalities, have been implicated in the development of MS. Although the disease cannot be cured, disease-modifiers, such as interferon beta, glatiramer acetate and mitoxantrone, as well as fatty acid supplementation have been used to delay the progression of the disease. Membrane fatty acids are precursors for mediators of inflammation, the eicosanoids, which are produced soon after stimulation and which regulate a number of inflammatory effects, such as the induction of fever, vasodilation and production of macrophage- and lymphocyte-derived cytokines. Eicosanoids, in contrast to their fatty acid precursors, have a short half-life and are therefore difficult to measure.

The objective in the present study was to determine the role of fatty acids from South African MS patients, by measuring the fatty acid composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SM) phospholipids in the plasma, red blood cell (RBC) and peripheral blood mononuclear cell (PBMC) membranes and correlate abnormalities with the neurological outcome as measured by the Kurtzke Expanded Disability Status Scale (EDSS) and inflammation assessed by C-reactive protein (CRP). A second objective was to establish whether possible changes in membrane lipids (phospholipids, fatty acids and cholesterol) would have an effect on membrane fluidity, and whether this would correlate with the EDSS and CRP.

The plasma, RBC and PBMC membrane lipid composition from 31 white female patients with MS and 30 age- and gender-matched control subjects were assessed. Fatty acids were quantified by gas chromatography (GC), phospholipids by colorimetric and cholesterol by enzymatic assays. Membrane fluidity, as measured by the membrane lipid composition, was calculated, using previously established formulae, and includes the following: the saturated nature of the membrane was measured by the phospholipid PC+PS/PE+PS ratio, fluidity and permeability were measured by the cholesterol concentration and the cholesterol to total phospholipid ratio and membrane deformability was measured by the phospholipid PE to PS

ratio. Membrane fluidity was also measured by the ordered-crystalline-phase to liquid-crystalline-phase lipid composition, which correlates with the phospholipid PE to PC ratio. The membrane saturated (SATS) to polyunsaturated fatty acid (PUFA) ratio was further used as an indication of the fluidity status of the membranes. CRP was measured in all participants using a Beckman nephelometer.

In MS, the n-6 fatty acids, particularly C18:2n-6, C20:4n-6 and C22:4n-6, were significantly decreased in plasma, RBC and/or PBMC membranes. In addition, the relationship between C20:3n-6 and C20:4n-6 showed a metabolic disturbance in both RBC and PBMC membranes from patients with MS, as compared to the control group. C20:4n-6 showed significant inverse correlations with the EDSS and CRP in MS patients, indicating that loss of these fatty acids from membranes correlated with higher disability as well as with increased inflammation. There were significant increases in free fatty acids C18:2n-6 and C20:4n-6 in plasma from MS patients. Saturated fatty acids, SM C14:0 and PI C22:0 were significantly increased in PBMC membranes from MS patients, and SM C14:0, C16:0 and C20:0 showed inverse correlations with the Functional System Scores. In contrast, the longer-chain SATS, C22:0 and C24:0 showed positive correlations with the Functional System Scores. Red blood cell membrane fluidity as measured by the SATS to PUFA ratio was significantly higher in patients than in controls. In patients with CRP ≥ 5.00 $\mu\text{g/ml}$ the ratio showed significant inverse correlation with disease outcome. The saturated nature correlated positively, whilst the ordered-crystalline-phase to liquid-crystalline-phase lipid ratio correlated inversely with the Functional System Scores.

In this study it was consistently shown that C20:4n-6, or its precursor and elongation products, C18:2n-6 and C22:4n-6 respectively, was lower in plasma, RBC and/or PBMC membranes from MS patients. Red blood cells lack the desaturase enzymes and depend on fatty acids sourced from the plasma. Therefore, lower C20:4n-6 in the RBC membranes from MS patients may be due to depleted plasma stores, or an indication of an increased demand of this fatty acid elsewhere. Furthermore, this study has demonstrated that lower RBC C20:4n-6, with an increase in plasma FFA C20:4n-6, resulted in worse disease outcome, perhaps due to the pro-inflammatory effect of eicosanoid production. This study also characterized the specific SATS, that is, longer-chain SATS that may increase the risk of developing MS, as higher shorter-chain SATS, C14:0 and C16:0 reflected better disease outcome, demonstrated by the inverse correlation with the EDSS and FSS. Lastly, this study has shown that in the presence of uncontrolled inflammation such as in MS, the altered lipid composition indirectly compromised cell membrane structure and fluidity, and thereby contributed to the disease progression in MS patients.

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DEDICATION

For Helene and Jolané, with love and in gratitude

PREFACE

This thesis has been written according to prescribed guidelines by the Cape Peninsula University of Technology (www.cput.ac.za/Research). The thesis is presented as an “article format thesis”.

Extracts from the Cape Peninsula University of Technology (CPUT) guidelines for the article-format thesis

- Rule 2.4.a “For a DTech thesis, the norm is that at least THREE journal articles, with the candidate as major contributor and of which at least two are already published, must be included. A third article “ready-for-publication” should also be included if not yet published”.
- Rule 4.1 Technical presentation of the thesis must still follow the guidelines as stipulated by the CPUT “Guide to postgraduate studies”.
- Rule 4.2 “The article format thesis requires that the text of the articles must be retyped for inclusion in the thesis according to CPUT standards. It is not acceptable to insert copies or off-prints of published articles directly into the thesis”.
- Rule 4.4 “References (included in articles) should follow the style of the journal to which the article was/will be submitted”. Therefore, references in this thesis may not necessarily be in one format.

This thesis is therefore presented as follows

- **Abstract**
- **Chapter One:** Literature review
- **Chapters Two to Six:** are the articles resulting from this thesis of which 3 have been accepted for publication and the other 2 have been submitted for publication

Articles

Hon, G.M., Hassan, M.S., Janse van Rensburg, S., Abel, S., Marais, De W., Van Jaarsveld, P., Smuts C.M., Henning, F. Erasmus, R.T. & Matsha, T. 2009. Erythrocyte membrane fatty acids in patients with multiple sclerosis. *Multiple Sclerosis*, 15:759-762.

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Hon. G.M., Hassan, M.S., Janse van Rensburg, S., Abel, S., Van Jaarsveld, P., Erasmus, R.T. & Matsha, T. 2009. Red blood cell membrane fluidity in the aetiology of multiple sclerosis. *Journal of Membrane Biology*, Submitted 18th February 2009.

- **Chapter Seven:** Discussion and Conclusion
- **Chapter Eight:** Materials and Methods not included in the articles
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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations and symbols	Definitions
$\Delta 5$	Delta-5
$\Delta 6$	Delta-6
$\Delta 9$	Delta-9
EDA	Eicosadienoic acid, C20:2n-6
AA	Arachidonic acid, C20:4n-6
ADA	Adrenic acid, C22:4n-6
AdrA	Adrenic acid
AGP	Alpha 1-acid glycoprotein
ALA	Alpha-linolenic acid, C18:3n-3
BBOT	2,5-bis-(5'-tert-butylbenzoxazolyl-[2'])thiophene
BCA	Bicinchoninic acid
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
C14:0	Myristic acid
C16:0	Palmitic acid
C16:1n-7	Palmitoleic acid
C18:0	Stearic acid
C18:1n-7	Vaccenic acid
C18:1n-9	Oleic acid
C18:2n-6	LA, linoleic acid
C18:3n-3	ALA, alpha-linolenic acid
C18:3n-6	GLA, gamma-linolenic acid
C18:4n-3	STA, stearidonic acid
C20:0	Arachidic acid
C20:1n-9	Gadoleic acid
C20:2n-6	EDA, Eicosadienoic acid

C20:3n-6	DGLA, dihomogamma-linolenic acid
C20:4n-3	ETA, eicosatetraenoic
C20:4n-6	AA, arachidonic acid
C20:5n-3	EPA, eicosapentaenoic acid
C22:0	Behenic acid
C22:1n-11	Cetoleic acid
C22:4n-6	ADA, adrenic acid
C22:5n-3	DPA, docosapentaenoic acid
C22:6n-3	DHA, docosahexaenoic acid
C24:0	Lignoceric acid
CM	Chloroform/methanol
CNS	Central nervous system
COX1 and 2	Cyclooxygenase 1 and 2
CRP	C-reactive protein
CS ₂	Carbon disulphide
CSF	Cerebrospinal fluid
DI	Decilitre
DGLA	Dihomogamma-linolenic acid, C20:3n-6
DHA	Docosahexaenoic acid, C22:6n-3
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid, C22:5n-3
EBV	Epstein-Barr virus
EDSS	Expanded Disability Status Scale
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid, C20:5n-3
ESR	Electron spin resonance
ETA	Eicosatetraenoic, C20:4n-3
FAME	Fatty acid methyl esters

FAs	Fatty acids
FFAs	Free fatty acids
FS	Functional Systems
FSS	Functional System Score
G	G-force
GC	Gas chromatography
GLA	Gamma-linolenic acid, C18:3n-6
HHV-6	Human herpesvirus-6
Hif-1 α ,	Hypoxia-inducible factor
HLA	Human leukocyte antigen
H ₂ O ₂	Hydrogen peroxide
H ₂ SO ₄	Sulfuric Acid
HASREC	Health and Applied Sciences Research Ethics Committee
HUFAs	Highly unsaturated fatty acids
I ₂	Iodide
IL-1 β	Interleukin-1beta
IL-10	Interleukin 10
IL-4R	Interleukin 4 receptor
IL-1 β	Interleukin 1 β
JAKI	Janus kinases
JCV	JC virus
KH ₂ PO ₄	Potassium dihydrogen orthophosphate
K ₂ HPO ₄ .3H ₂ O	Di-potassium hydrogen phosphate
KI	Potassium iodide
KOH	Potassium hydroxide
LA	Linoleic acid, C18:2n-6
LPS	Lipopolysaccharide
LT	Leukotriene

LTB ₄	Leukotriene B ₄
M	Mole
MCSF	Macrophage colony-stimulating factor
Mg	Milligram
MHC	Major histocompatibility complex
ml	Millilitre
mm	Millimetre
mM	Millimole
μl	Microlitre
MRI	Magnetic resonance image assessment
MS	Multiple sclerosis
MSRV/HERV-W	Multiple sclerosis-associated retrovirus/human endogenous retrovirus W
MUFAs	Monounsaturated fatty acids
N ₂	Nitrogen gas
N-3	Omega-3
N-6	Omega-6
NaCl	Sodium chloride
NaN ₃	Sodium azide
NEFA	Non-esterified fatty acids
NH ₂ -terminal	Amino-terminal
Nm	Nanometre
OD	Optical density
OND	Other neurological diseases
PAF	Platelet activating factor
PBLs	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine

PG	Prostaglandin
PGD2	Prostaglandin D2
PGE	Prostaglandin E
PGE1	Prostaglandin E1
PGE2	Prostaglandin E2
PGE3	Prostaglandin E3
PGF2 α	Prostaglandin F2- α
PGH2	Prostaglandin H2
PGI2	Prostaglandin I2
PGs	Prostaglandins
Pi	phosphate
PI	Phosphatidylinositol
PLA2	Phospholipase A2
PPMS	Primary progressive multiple sclerosis
PS	Phosphatidylserine
PUFAs	Polyunsaturated fatty acids
RBC	Red blood cells
RRMS	Relapsing remitting multiple sclerosis
SATS	Saturated fatty acids
SDS	Sodium dodecyl sulphate
sICAM-1	Soluble intercellular adhesion molecule-1
SM	Sphingomyelin
SPMS	Secondary progressive multiple sclerosis
STA	Stearidonic acid, C18:4n-3
STAT4	Transcription 4
STAT6	Transcription 6
sVCAM-1	Soluble vascular adhesion molecule-1
TGF- β	Transforming growth factor beta
TLC	Thin layer chromatography

TNF- α	Tumor necrosis factor alpha
TPL	Total phospholipid
TX	Thromboxane
TXA2	Thromboxane A2
VZV	Varicella zoster virus
WBCs	White blood cells

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), which leads to neuronal demyelination. As the disease progresses, oligodendrocytes and axons are destroyed, resulting in sclerotic plaque, or lesion formation (Brück, 2005; Adibhatla & Hatcher, 2007). The underlying cause of MS remains unknown, but both an autoimmune and an infectious aetiology are suspected. Genetic factors may also contribute to the disease etiology (Cooper, 1997; Stinissen et al. 1997; Hunter & Hafler, 2000; Brown, 2001; Chatzimanolis et al. 2004; Brück, 2005; Adibhatla & Hatcher, 2007). Metabolic disturbances, especially lipid metabolic abnormalities, have also been implicated in the development of MS (Baker et al. 1964; Cumings et al. 1965; Cunnane et al. 1989; Lassmann et al. 2001; Harbige & Sharief, 2007). Although treatment is available that can alleviate some of the symptoms, no cure is available. Therefore, as more lesions are formed in the brain, nerve impulse transmission becomes progressively impaired, and brain functions are increasingly compromised. As the disease develops, patients with MS experience a gradual decline in health and quality of life (Jelinek, 2000; Brown, 2001; Brück, 2005).

1.2 Epidemiology of multiple sclerosis

Multiple sclerosis is a relatively newly recognised disease. A few cases of MS were reported in the beginning of the 19th century, but the disease was recognised as a clinical disease only as late as 1860 (Compston & Coles, 2002). Currently, the disease is one of the more common neurological diseases. Worldwide, an estimated 2.5 million people have MS and the disease has an incidence and prevalence of 7 per 100,000 and 120 per 100,000 respectively, with a lifetime risk of 1 in 400 (Jelinek, 2000; Compston & Coles, 2002). There is a wide geographic variation in the prevalence of MS, with the highest in Northern Europe, Southern Australia, the middle part of North America and the United Kingdom (Jelinek, 2000; Noseworthy et al. 2000; Reipert, 2004). In the United Kingdom the incidence of MS is approximately 1 in 800 people and in northern Europe 1 in 1000. There is scarcity of literature on the prevalence and incidence of MS in the developing countries, including South Africa (Modi et al. 2008). However, a recent study on 430 patients with MS living in South Africa and based on patient-orientated questionnaires, presented similar findings to those reported in the literature (Modi et al. 2008).

According to recent studies, MS is the most common disease of the CNS of inflammatory and demyelinating origin, and also the most common neurological disorder in young adults

(Jelinek, 2000; Compston & Coles, 2002; Reipert, 2004; Leary et al. 2005; Okuda et al. 2005). Its onset is usually between 20 and 40 years of age, but about 5 % of patients are diagnosed before the age of 16 years. Females are more susceptible than males, with a ratio of about 2 to 1. Susceptibility to MS varies between different racial and ethnic groups. The disease is rare among North and South Amerindians, Chinese, Japanese, African blacks and New Zealand Maoris (Rosati, G. 2001). This difference in susceptibility to MS has resulted in an uneven geographic distribution of the disease. However, prevalence studies also show lower prevalence of MS among white people outside Europe than in many parts of northern Europe (Compston & Coles, 2002), and increased susceptibility in first-generation black people in the United Kingdom. One of the possible explanations for the differences in these surveys are said to be due to improved case recognition in the developed countries. This cannot explain the phenomenon for example that English-speaking people who migrate to South Africa as adults have a higher risk of developing MS than those who migrate as children (Compston & Coles, 2002). These findings suggested that an environmental factor could be contributing to the development of the disease.

1.3 Types of multiple sclerosis

There are a number of internationally recognized forms of MS, such as relapsing remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS) and primary progressive multiple sclerosis (PPMS). About 80% of people presenting with MS are diagnosed with RRMS. About 50% of these patients will progress to SPMS within 10 years of disease onset. This figure increases to about 90% over the next 25 to 30 years. Another 10 to 20 % of people presenting with MS are diagnosed with PPMS (Lublin & Reingold, 1996; Reipert, 2004).

Patients with RRMS experience a series of relapses, each followed by a complete or partial remission of symptoms. The duration and frequency of relapses vary, and recovery can be gradual or fast (Lublin & Reingold, 1996; Reipert, 2004).

Secondary progressive multiple sclerosis (SPMS) is characterized by a steady progression of neurological deterioration. Patients may experience relapses, minor remissions and plateaus, but these may also be absent (Lublin & Reingold, 1996; Reipert, 2004).

Primary progressive multiple sclerosis (PPMS) is characterized by a gradual progression of the disease that leads to a decline in the patients physical abilities. These patients experience only short periods of minor remissions of symptoms (Lublin & Reingold, 1996; Reipert, 2004).

1.4 Symptoms

Patients with MS can experience loss of any function that is controlled by the CNS. Clinical features include loss of sensation, muscle weakness, spasticity, visual loss, in-coordination, cognitive impairment, pain, as well as bladder and bowel disturbances (Noseworthy et al. 2000; Chwastiak et al. 2002; Compston & Coles, 2002; Reipert, 2004; Leary et al. 2005). Many patients also experience fatigue, which is accompanied by increases in body temperature. Patients with MS also take longer to recover from both physical and cognitive tasks. In time, symptoms such as cognitive impairment, vertigo, spasticity, ataxia tremors, progressive quadriparesis, pain and depression may become excessive. As patients become more disabled, their quality of life deteriorates and they may become an economic burden on society (Adibhatla & Hatcher, 2007). Life expectancy of MS patients is at least 25 years from disease onset, and many patients die from unrelated causes (Compston & Coles, 2002).

1.5 Diagnosis

Diagnostic criteria for MS (McDonald Diagnostic Criteria) were updated in 2001 and revised in 2005. These criteria include the integration of magnetic resonance image assessment (MRI) with clinical and para-clinical methods (Reipert, 2004; Polman et al. 2005). The 2005 revised diagnostic criteria for MS specify that at least two neurological events must occur before diagnosis may be established. These events, or relapses must be separated in time and also anatomically. The presence of multifocal lesions of various ages are evaluated together with additional tests such as cerebrospinal fluid (CSF) analysis and visual evoked potential (VEP) recordings, before a final diagnosis can be made (Noseworthy et al. 2000; Chwastiak et al. 2002; Reipert, 2004; Polman et al. 2005). Once diagnosis of MS is confirmed, the degree of disability can be monitored, using the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke, 1983; Reipert, 2004).

1.5.1 Kurtzke Expanded Disability Status Scale

The functional disability status (disease severity) of patients with MS can be measured using the Kurtzke Expanded Disability Status Scale (EDSS), which includes eight Functional Systems (FS). The Functional Systems are Pyramidal, Cerebellar, Brainstem, Sensory, Bowel and bladder, Visual, Cerebral and "other" (Kurtzke, 1983; Reipert, 2004). The EDSS, as well as the Functional Systems, are assigned a Functional System Score (FSS) by investigating neurologists. Higher values indicate greater disability. Scales for the EDSS are from 0 to 10, in which the 0 score indicates no disability at all and 10 indicates death due to MS.

1.6 Treatment

Neurodegenerative diseases, including MS, are of clinical importance because neurons regenerate slowly (Brown, 2001; Pino et al. 2005; Adibhatla & Hatcher, 2007). Neuronal remyelination in MS is limited, resulting in the multifocal sclerotic plaques from which the disease gets its name (Compston & Coles, 2002). Currently, there are treatments available to slow the course of the disease and alleviate the symptoms, but none that could cure the disease. Treatment of MS is mostly based on alleviation of inflammation, but the effects of these on the immune system have not been investigated fully (Compston & Coles, 2002; Reipert, 2004; Van Meeteren et al. 2005; Schippling et al. 2008).

Drugs that are able to modify the course of the disease (disease-modifiers) that have been approved for the treatment of MS are: interferon beta, glatiramer acetate, mitoxantrone and corticosteroids (Compston & Coles, 2002; Reipert, 2004). Interferon- β and glatiramer acetate are both immuno-modulatory drugs, while mitoxantrone and cyclophosphamide are immunosuppressive drugs (Schippling et al. 2008) Interferon- β 1b has been approved in the treatment of RRMS as it decreases the clinical relapse rate and disease activity and severity (Chatzimanolis et al. 2004). Corticosteroids are anti-inflammatory and have been used for acute treatment of MS relapses (Compston & Coles, 2002). However, the efficiency of these drugs in suppressing inflammation and clinical relapses in patients with MS is limited (Schippling et al. 2008).

Recent studies have shown promising results on the use of haemopoietic stem-cell transplantation in the treatment of severe forms of MS (Fassas et al. 2000; Fassas et al. 2002; Schippling et al. 2008), but the effectiveness of this treatment still needs to be evaluated in large randomized, prospective controlled trials. Although the mortality rate has dropped to about 1-2 % of treated cases, stem-cell transplantation therapy is still considered to be potentially a life-threatening procedure (Mancardi & Saccardi, 2008; Schippling et al. 2008).

Various membrane fatty acid metabolic abnormalities have been reported in patients with MS, especially fatty acids known to be involved in the inflammatory process, such as the n-6 (omega-6) and n-3 (omega-3) polyunsaturated fatty acids (PUFAs) (Cumings et al. 1965; Cherayil, 1984; Fisher et al. 1987; Navarro & Segura, 1989; Harbige & Sharief, 2007). Subsequently, a number of studies have evaluated the use of fatty acid supplementation in the treatment of MS. In general, improvement in neurological outcome in these patients was measured against a reduction in the EDSS, as well as against exacerbation rate and duration. Several studies reported an improvement in symptoms during n-6 and n-3 fatty acid supplementation (Bates et al. 1978; Nordvik et al. 2000; Weinstock-Guttman et al. 2005;

Harbige & Sharief, 2007). There were also reports with negative results (Paty et al. 1978) and a recent Cochrane review of all randomized trials of dietary regimens for MS, showed that PUFAs did not have a significant effect on disease progression as measured by the Disability Status Scale (Farinotti et al. 2007). The risk of developing MS is also associated with increased dietary intake of saturated fatty acids (Van Meeteren et al. 2005), but similar to findings for PUFA supplementation in MS, results from a prospective study showed that the amount and type of dietary fat, including saturated fatty acids, showed no effect on the risk of developing MS (Westlund & Kurland, 1953; Antonovsky et al. 1965; Cendrowski et al. 1969; Warren et al. 1982; Butcher, 1986; Zhang et al. 2000).

1.7 The aetiology of multiple sclerosis

The inflammatory response can be initiated by different factors, such as microbiological, immunological and toxic agents (Simopoulos, 2002). Findings on the mechanisms involved in MS development are inconclusive, but include the possibility of an autoimmune response, virus mediation, and metabolic disturbances. Some findings seem to suggest that metabolic disturbances could be responsible for primary oligodendrocyte damage, preceding autoimmune responses (Martino & Hartung, 1999; Lassmann et al. 2001; Hafler, 2004; Matute & Perez-Cerda, 2005; Harbige & Sharief, 2007). In MS, oligodendrocytes, the cells which synthesize and maintain the protective myelin sheath around neuronal axons, are the principal target of immune attack (Compston & Coles, 2002), whereas microglial cells, B and T lymphocytes, and macrophages are involved in the inflammatory response (Prineas & Wright, 1978; Bjartmar & Trapp, 2001; Brown, 2001; Brück, 2005; Adibhatla & Hatcher, 2007).

1.7.1 Inflammation

Inflammation is a defense mechanism of the body against various insults, which is usually a well-controlled process, although it may be accompanied by some damage to surrounding tissue (Brown, 2001; Calder, 2001; Kulmatycki & Jamali, 2006; Farooqui et al. 2007). The initial inflammatory response, innate immunity, is non-specific, and does not need previous exposure to pathogens to be activated (Roia-Pleszczynski & Stankova, 1992; Calder, 2001). Innate immunity functions by the elimination of pathogens by complement, toxic chemicals released by phagocytes, toxic proteins released by natural killer cells, or phagocytosis by macrophages (Calder, 2001). Acquired immunity, in contrast to innate immunity, involves the recognition of antigens on invading pathogens. Lymphocytes form a part of the acquired immune response; humoral immunity involves B-lymphocytes, which deal with extra-cellular pathogens through antibody production, while T-lymphocytes are part of a cell-mediated system, in which intra-cellular pathogens are dealt with. T-lymphocytes function by recognizing antigens, together with major histocompatibility complex (MHC) molecules,

displayed on cell surfaces of antigen presenting cells, such as macrophages, dendritic cells and B-lymphocytes (Calder, 2001). Re-infection by the same pathogen will result in a stronger and faster response (Calder, 2001).

Communication within the immune system is through direct cell-to-cell contact, involving adhesion molecules, and by the production of two classes of chemical messengers, cytokines and eicosanoids. Cytokines are synthesized by phagocytes and parenchymal cells. These messengers can alter the activity of target cells by binding to specific receptors on the cell surface of these cells. Eicosanoids are derived from cell membrane PUFAs, and modulate the more specific acquired immune response (Horrobin & Manku, 1990; Rola-Pleszezynski & Stankova, 1992; Calder, 2001; Bagga et al. 2003; Zamaria, 2004). To this group belong prostaglandins (PGs), leukotrienes (LTs), thromboxanes (TXs) and platelet activating factor (PAF). They are produced soon after stimulation and regulate many inflammatory effects, such as induction of fever, vasodilation, and production of macrophage- and lymphocyte-derived cytokines. They also modulate immune cell functions (De Pablo & De Cienfuegos, 2000; Tilley et al. 2001).

Glial cells are distributed throughout the CNS and are important cells for the survival of existing neurons. These cells include oligodendrocytes, microglia and astrocytes (Matute et al. 2001; Miller, 2005; Farooqui et al. 2007). Microglia are the main mediators of neuro-inflammation and are responsible for the recruitment of leukocytes from the blood stream into brain tissue. When activated, they migrate to injured sites where they remove dead cells through phagocytosis. Inflammatory responses usually result in self-limiting healing processes. In patients with MS, however, this leads to apoptosis of oligodendrocytes and lesion formation, causing the relapses experienced by patients. In this regard, glial involvement has been shown in CNS disorders, especially in MS. Barnett and Prineas (2004) reported that during the first few hours of the formation of a new lesion, microglia were the only immune cells present in the apoptotic zone, while T-lymphocytes, early activated macrophages and myelin phagocytes were rare or absent in the apoptotic zone, although they were present elsewhere in the lesion (Gay et al. 1997; Aboul-Enein, et al. 2003; Barnett & Prineas, 2004). Prineas and Wright (1978) have reported plasma cells (differentiated B-lymphocytes) to be present as well; both in MS normal white matter, but with increased numbers in chronic plaque tissue.

1.7.1.1 Markers of inflammation

There are several recognised markers of inflammation which include serum soluble vascular adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), C-reactive protein (CRP), neopterin, serum nitric oxide metabolites nitrate and nitrite, tumour

necrosis factor alpha (TNF- α) and alpha 1-acid glycoprotein (AGP) (Giovannoni et al. 2001; Adam et al. 2003; Henry et al. 2003; Weinstock-Guttman et al. 2005). Of these CRP is a marker of choice because its assay is commonly available in the routine biochemistry laboratories. CRP as an acute phase protein is widely used as a marker of both infection and inflammation. It has also been used as a pro-inflammatory marker in assessment of the degree of inflammation in patients with MS (Giovannoni et al. 2001; Sellner et al. 2008). A disadvantage of using serum inflammatory markers is that they don't always correlate with short-term disease progression (Giovannoni et al. 2001), but they have been shown to correlate with infectious episodes and clinical relapse in MS patients (Giovannoni et al. 1996).

1.7.2 Infectious agents in multiple sclerosis

More than 20 infectious agents have been reported to be associated with MS; but these associations have never been confirmed to be a direct cause of the disease. However, exposure to infectious agents in early childhood has been reported as a contributing factor which could initiate the onset of MS. Furthermore, relapses in MS frequently follow gastrointestinal or upper respiratory tract infections (Karpuj et al. 1997). There are different theories explaining virus action in the chronic inflammatory condition of patients with MS. One possibility is that certain microbes can mimic myelin fragments and can therefore trigger the immune response. Another theory involves superantigens, which are toxins produced by certain viruses and which can directly interact with T-lymphocyte receptors to trigger an inflammatory reaction (Karpuj et al. 1997; Willer & Ebers, 2000; Moses & Sriram, 2001).

Viruses that have been implicated in susceptibility to MS include both endogenous and exogenous viruses and range from retroviruses to herpes viruses and measles. Multiple sclerosis-associated retrovirus/human endogenous retrovirus W (MSRV/HERV-W) (endogenous retrovirus) and Human herpesvirus-6 (HHV-6) (exogenous virus) are the two most studied viruses as environmental co-factors in MS (Mameli et al. 2007). Other exogenous viruses such as Epstein-Barr virus (EBV), varicella zoster virus (VZV) and JC virus (JCV) have been implicated in the disease as well. Results are varied, but in general higher virus presence and expression in MS than in healthy subjects have been reported.

1.7.2.1 Exogenous viruses

Mancuso et al. (2007) found that HHV-6 and JCV were present in the CSF of patients with MS and other neurological diseases (OND), whereas Mameli et al. (2007) reported no difference between patients with MS and healthy controls with regards to presence and replication of HHV-6 in the brain or peripheral blood mononuclear cells. Mancuso et al. (2007) reported the presence of VZV in the CSF of a subgroup of patients with MS, but

absent in the CSF of normal controls and that of other neurological disorders. The absence of VZV in some of the patients with MS showed the lack of a causative effect of VZV in the aetiology of MS however. Infectious mononucleosis (caused by EBV infection) has also been implicated as a risk factor in MS development, and furthermore this risk may persist for at least 30 years after the initial infection (Nielsen et al. 2007).

1.7.2.2 Endogenous viruses

Inherited retroviruses such as MSR/HERV-W are present in the human brain in healthy subjects, but in patients with MS they are actively expressed (Perron et al. 2005; Sotgiu et al. 2006; Mameli et al. 2007). Converging results from three independent groups, Perron et al. (2005) showed this difference in MSR/HERV-W GAG and ENV antigen expression (proteins) between normal and MS brains. These differences could be a reflection of differential regulation of inherited HERV-W copies, or expression of "infectious" MSR copies. The presence of MSR virions in the CSF from patients with early onset MS, was associated with a worse prognosis in follow-up studies (Sotgiu et al. 2006). De Villiers et al. (2006) reported expression of MSR pol gene in the serum of 69 % of patients with MS, but also in 70 % of unaffected close relatives of the patients.

Reports indicate that the endogenous virus HERV-W is active in patients with MS. Antony et al. (2004) reported that syncytin was expressed in microglia and astrocytes from patients with MS. Syncytin is a virus-expressed glycoprotein that can induce the release of redox reactants, which are cytotoxic to cells (Antony et al. 2004). Astrocytes, which recycle the neurotransmitter glutamate (Piani & Fontana, 1994; Miller, 2005; Van Meeteren et al. 2005), abandon this function when they are injured. Increased concentrations of glutamate cause over-activation of glutamate receptors on oligodendrocytes, which may result in cell death (Oka et al. 1993; Matute et al. 2001; Van Meeteren et al. 2005).

1.7.3 Genetic factors

Autoimmune diseases may be the result of an interaction between environmental factors and multiple, specific genes (Karpuj et al. 1997; Willer & Ebers, 2000). The development of MS may also be associated with genetic factors, as was shown by an increased relative risk of this disease in siblings when compared to the general population. Several regions of the human genome are associated with susceptibility to MS, but the impact of environmental conditions still needs clarification (Karpuj et al. 1997). The identification of genes that impart susceptibility to a disease is of great importance as this may result in finding preventive measures and improved therapy (Karpuj et al. 1997).

The genetic analysis of MS has been based mainly on studies in which the sharing of certain alleles of candidate genes in unrelated affected individuals is compared with that of a control population (Karpuj et al. 1997; Noseworthy et al. 2000; Willer & Ebers, 2000). Since MS is an inflammatory disease, the focus has therefore been on genes which are involved in the immune response. Zeis et al. (2007) reported that in brain tissue from patients with MS, consistent up-regulation of genes involved with anti-inflammatory and protective mechanisms, such as signal transducer and activator of transcription 6 (STAT6), Janus kinases (JAK1), interleukin 4 receptor (IL-4R), Interleukin 10 (IL-10), Chromogranin C and hypoxia-inducible factor (Hif- α), were expressed mainly by oligodendrocytes. In contrast, genes involved in pro-inflammatory mechanisms, such as STAT4, interleukin 1 β (IL-1 β) and macrophage colony-stimulating factor (MCSF) were predominantly up-regulated in microglia (Zeis et al. 2007). Up-regulation of genes involved in anti-inflammatory mechanisms may protect the CNS environment, whereas the activation of pro-inflammatory mechanisms in microglia may favour disease progression. Other immune responsive genes include the major histocompatibility complex (MHC) on chromosome 6, T-lymphocyte receptor genes on chromosomes 7 and 14, and immunoglobulin genes, all of which have been reported to have significant genetic effects on susceptibility to MS (Karpuj et al. 1997; Willer & Ebers, 2000). Further candidate genes include those involved with cytokines, proteolytic enzymes, nitric oxide, myelin precursors, interleukins, interferon, human leukocyte antigen and tumor necrosis factor (Willer & Ebers, 2000).

1.8 Metabolic disturbances in multiple sclerosis

1.8.1 Cell membrane lipids

Metabolic disturbances mostly involved in MS are lipid metabolic abnormalities. Lipids are important components of all mammalian cells and have a variety of biological functions, such as the formation of lipid bilayers that provide structural integrity necessary for protein functions, and as an energy reservoir, for example, triglycerides (Caret et al. 1997; Adibhatla & Hatcher, 2007). Membrane lipids consist of phospholipids, into which saturated and unsaturated fatty acids are incorporated, as well as cholesterol. The structures of membrane lipids are extremely diverse, and the functions of neuronal and other membranes depend on the type and composition of phospholipids and cholesterol present, as well as their association with proteins, while the types of fatty acids present in phospholipids are closely correlated with the biological features of the phospholipids (Manzoli et al. 1970; Williams, 1998; Horrobin, 1999).

1.8.1.1 Cell membrane phospholipids

All phospholipids (except sphingomyelin) have a 3-carbon glycerol backbone to which two fatty acid molecules and any one of a range of different head-groups are attached. The head-groups (polar groups) may be choline, ethanolamine, inositol or serine (Horrobin, 1999; Koay & Walmsley, 1999). Saturated fatty acids (SATS) and monounsaturated fatty acids (MUFAs) tend to be attached to the Sn1 position, polyunsaturated fatty acids (PUFAs) to the Sn2 position, and the headgroups to the Sn3 position (Horrobin, 1999). Cell membranes are formed by a double layer of phospholipids, with the head-groups of the outer layer facing the extra-cellular spaces, the fatty acids "tails" facing each other, and the head-groups of the inner layer facing the inter-cellular spaces. Choline containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM) are mainly on the outer leaflet of plasma membranes, while phosphatidylethanolamine (PE) and phosphatidylserine (PS) are located on the inner leaflet. Phosphatidylcholine is the most abundant phospholipid in animal cell membranes, and PE the second most (Williams, 1998; Barenholz & Thompson, 1999). In the brain however, normal myelin contains a higher percentage of PE than PC, with PE representing about 34 % of the total phospholipids (Manzoli et al. 1970).

The properties of each phospholipid molecule are dependent both upon the type of the head-group and on the nature of the other molecules (fatty acids) attached to the Sn1 and the Sn2 positions (Horrobin, 1999). The fatty acids contained within membrane phospholipids can be saturated (SATS) or mono- and polyunsaturated (MUFAs and PUFAs respectively). Polyunsaturated fatty acids are subdivided into n-9, n-6 and n-3 subtypes (Koay & Walmsley, 1999). The abbreviated chemical formulae and common names of major membrane fatty acids have been presented in table 1.1 (Nightingale et al. 1990; Horrobin, 1999; Sprecher, 2000; Pereira et al. 2003).

Essential fatty acids needed for PUFA synthesis come from the diet, but the body can synthesize both SATS and MUFAs from carbohydrates, if sufficient dietary fat is unavailable (Horrobin, 1999; German & Dillard, 2004). Fatty acids are transported in the blood, either bound to albumin, or in the form of triglycerides (fat) associated with lipoproteins (Horrobin, 1999; Koay & Walmsley, 1999). Fatty acids may be esterified in the form of neutral triglycerides and phospholipids, or non-esterified (free fatty acids) (Koay & Walmsley, 1999). Free fatty acids are the major plasma contributor to brain uptake (Chen et al. 2008).

Table 1.1: Abbreviated chemical formulae and common names of major membrane fatty acids

	Abbreviated chemical formulae	Common names	Abbreviated common names
Polyunsaturated fatty acids (PUFAs)			
N-6 fatty acids	C18:2n-6	Linoleic acid	LA
	C18:3n-6	Gamma-linolenic acid	GLA
	C20:2n-6	Eicosadienoic acid	EDA
	C20:3n-6	Dihomo-gamma- linolenic acid	DGLA
	C20:4n-6	Arachidonic acid	AA
	C22:4n-6	Adrenic acid	ADA
	C22:5n-6	Docosapentaenoic acid	DPA
N-3 fatty acids	C18:3n-3	Alpha-linolenic acid	ALA
	C18:4n-3	Stearidonic acid	STA
	C20:4n-3	Eicosatetraenoic	ETA
	C20:5n-3	Eicosapentaenoic acid	EPA
	C22:5n-3	Docosapentaenoic acid	DPA
	C22:6n-3	Docosahexaenoic acid	DHA
Monounsaturated fatty acids (MUFAs)			
	C16:1n-7	Palmitoleic acid	
	C18:1n-7	Vaccenic acid	
	C18:1n-9	Oleic acid	
	C20:1n-9	Gadoleic acid	
Saturated fatty acids (SATS)			
	C14:0	Myristic acid	
	C16:0	Palmitic acid	
	C18:0	Stearic acid	
	C20:0	Arachidic acid	
	C22:0	Behenic acid	
	C24:0	Lignoceric acid	

References: Nightingale et al. 1990; Horrobin, 1999; Sprecher, 2000; Pereira et al. 2003

1.8.1.2 Saturated fatty acids

Saturated fatty acids are made of a variable chain length with single carbon-carbon bonds and are straight and rigid (Horrobin, 1999). Both clinical and epidemiological evidence indicates that diets rich in SATS can be harmful to health (German & Dillard, 2004; Zamaria, 2004). However, myelin phospholipids contain a high percentage of SATS and MUFAs, including C16:0 (palmitic acid), C18:0 (stearic acid) and C18:1n-9 (oleic acid) (Manzoli et al. 1970). Furthermore, there is a lack of information about the metabolic aspects of individual dietary SATS (German & Dillard, 2004).

A variety of cellular and also viral membrane proteins are modified by fatty acid acylation (Grand, 1989; James & Olson 1990; Okubo et al. 1991). These modifications may play a role in protein targeting and/or signal transduction. C14:0 (myristic acid) and C16:0 (palmitic acid) are metabolites for myristoylation, and palmitoylation respectively. C14:0 is found only in small amounts, while C16:0 is a common metabolite in eukaryotic cells.

Saturated fatty acids have also been shown to display anti-viral and -bacterial properties (Sands, 1977; Cordo et al. 1999; Narasimhan et al. 2006). A series of esters and amides of C14:0 showed in vitro antibacterial activity against gram-positive and gram-negative bacteria which was comparable to that of the standard drug, antibiotic Ciprofloxacin (Narasimhan et al. 2006). Myristic acid analogs have been found to inhibit a late stage in the Junin virus replication cycle (Cordo et al. 1999). Sands (1977) showed that C14:0 and C16:0 did not inactivate enveloped bacteriophage $\phi 6$ virus, but did prevent plaque production (Sands, 1977), whereas both C18:1n-9 (oleic acid) and C16:1n-7 (palmitoleic acid) were found to be potent inactivators of the bacteriophage $\phi 6$ virus.

Reports on the saturated and monounsaturated fatty acid composition in patients with MS showed that in most studies SATS were increased, while MUFAs were decreased. An increase in SATS, especially C16:0 and C18:0 were found in brain tissue (Chia et al. 1984; Wilson & Tocher, 1991), in plasma (Cherayil, 1984; Cunnane et al. 1989; Holman et al. (1989) and RBCs (Cherayil, 1984; Navarro & Segura, 1989), while a decrease in C20:0 in brain tissue has been reported by Chia et al (1984). A decrease in MUFAs in brain tissue (Chia et al. 1984; Wilson & Tocher, 1991), but an increase in plasma (Holman et al. (1989) was also reported.

1.8.1.3 Unsaturated fatty acids

Monounsaturated fatty acids (MUFAs) get their name from the fact that they have one double carbon-carbon bond introduced to the single carbon-carbon bonds of saturated fatty acids. Polyunsaturated fatty acids (PUFAs) have two or more double carbon-carbon bonds (Horrobin, 1999). The double bonds of unsaturated fatty acids are angled and flexible and make the carbon chain more mobile. The more double bonds, the more fluid, flexible and apparently disordered the phospholipid molecule becomes. MUFAs can be synthesized from SATS (Nakamura & Nara, 2004), but PUFAs can only be synthesized from dietary precursors called essential fatty acids (EFAs). The essential fatty acids of the n-6 and n-3 fatty acid series, C18:2n-6 (linoleic acid, LA) and C18:3n-3 (alpha-linolenic acid, ALA) respectively, can not be synthesized in the body and must be ingested from the diet (Roche, 1999; De Pablo & De Cienfuegos, 2000; Zamaria, 2004). The parent dietary n-6 and n-3 essential fatty acids are converted to their fatty acid metabolites (the n-6 and n-3 fatty acid

series respectively), by a series of alternating desaturations (addition of a double bond) and elongations (addition of two carbon atoms) (Sprecher, 1982; Horrobin & Manku, 1990). The two series are not inter-convertible (Nightingale et al. 1990; De Pablo & De Cienfuegos 2000). The names of the n-6 and n-3 fatty acids refer to the position of the first double bond in the carbon chain, starting at the methyl end of the molecule (Horrobin, 1999; Koay & Walmsley, 1999). There are three known desaturases in humans: delta-5 ($\Delta 5$), delta-6 ($\Delta 6$) and delta-9 ($\Delta 9$). Delta-6 and delta-5 desaturases are required for the synthesis of PUFAs and highly unsaturated fatty acids (HUFAs) respectively, while delta-9 desaturase catalyze synthesis of MUFAs from SATS. Fatty acids are referred to by an abbreviation of their chemical formulae in the form "Cx:yn-z", where x is the number of carbon atoms (chain lengths), y is the number of double bonds and z is the position of the first double bond, in carbon atoms, from the methyl end of the fatty acid (Nightingale et al. 1990). Figure 1.1 describes the metabolic pathways for the n-6 and n-3 fatty acid series. Some of the metabolic products of C18:2n-6 include C20:3n-6 (dihomo-gamma-linolenic acid, DGLA) and C20:4n-6 (arachidonic acid, AA), while important fatty acids from C18:3n-3 metabolism include C20:5n-3 (eicosapentaenoic acid, EPA) and C22:6n-3 (docosahexaenoic acid, DHA) (Nightingale et al. 1990; Horrobin, 1999; Sprecher, 2000; Pereira et al. 2003).

In contrast to other tissues, there are only small amounts of C18:2n-6 and C18:3n-3 in neuronal phospholipids, but they have large amounts of C20:4n-6 and C22:6n-3, as well as smaller, but important amounts of C20:3n-6, C22:4n-6 (adrenic acid, AdrA), C20:5n-3 and C22:5n-3 (docosapentaenoic acid, DPA) (Horrobin, 1999). C20:4n-6, together with C22:6n-3 forms 80-90 % of the total PUFAs in neuronal and retinal tissue. The human brain has a high demand for C20:4n-6 and C22:4n-6 and requires 4 times the amount of C20:4n-6 than C22:6n-3 on a daily basis (Harbige & Sharief, 2007; Rapoport et al. 2007). The consumption of C20:4n-6 does not significantly change with age (Rapoport et al. 2007). Most neurons need all PUFAs to be preformed (Horrobin, 1999). Plasma C18:2n-6 and C18:3n-3 do not contribute significantly to the brain content of C20:4n-6 or C22:6n-3 respectively (Rapoport et al. 2007), therefore the incorporation of circulating C20:4n-6 and C22:6n-3 into brain tissue represents their rates of consumption by the brain. Glial cells may be involved in these reactions and may supply these fatty acids to neurons (Horrobin, 1999).

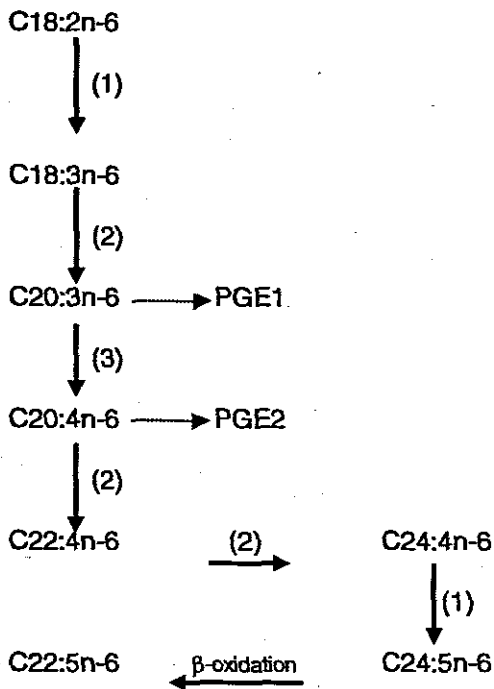
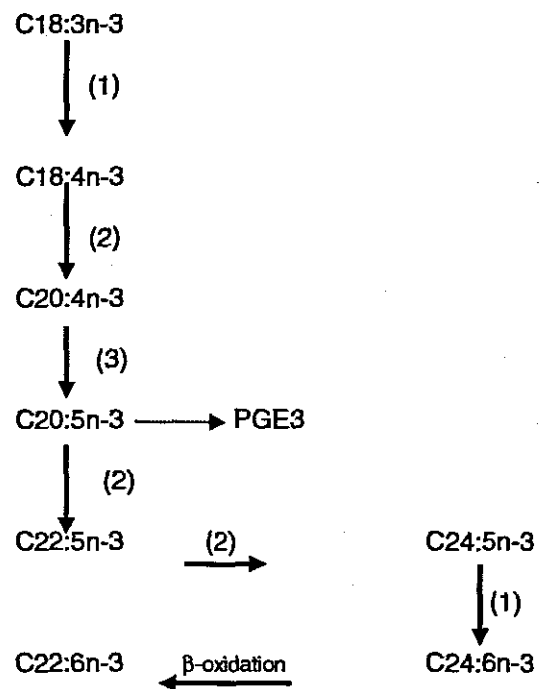
N-6 fatty acid series**N-3 fatty acid series**

Figure 1.1: Metabolic pathway of the n-6 (omega-6) and n-3 (omega-3) fatty acid series

References: Adapted from Nightingale et al. 1990; Horrobin, 1999; Sprecher, 2000; Pereira et al. 2003

Key

- C18:2n-6 (linoleic acid, LA)
- C18:3n-6 (γ -linolenic acid, GLA)
- C20:3n-6 (dihommo- γ -linolenic acid, DGLA)
- C20:4n-6 (arachidonic acid, AA)
- C18:3n-3 (α -linolenic acid, ALA)
- C20:5n-3 (eicosapentaenoic acid, EPA)
- C22:6n-3 (docosahexaenoic acid, DHA)
- Prostaglandin E1: PGE1
- Prostaglandin E2: PGE2
- Prostaglandin E3: PGE3
- (1) delta-6-desaturase
- (2) elongases
- (3) delta-5-desaturase

1.8.1.3.1 Function of unsaturated fatty acids

Polyunsaturated fatty acids are the basic constituents of phospholipid membranes. They are involved in enzyme and receptor expression, modulation of proteins and cell signaling (Horrobin & Manku, 1990; Horrobin, 1999; De Pablo & De Cienfuegos, 2000; Zamaria, 2004). They also influence membrane fluidity (Nakamura & Nara, 2004), neurotransmission and prostaglandin formation, processes that are vital in the maintenance of normal brain function (Horrobin, 1999; Haag, 2003). Altered lipid metabolism in the cell membrane is believed to be a key event which contributes to CNS injury (Adibhatla et al. 2006; Adibhatla & Hatcher, 2007). Likewise, the fatty acid composition in human immune cells influences their

function and can affect phagocytosis, T-lymphocyte signaling and antigen presentation (Calder, 2007). In this, changes in immune cell membrane C20:4n-6, C20:5n-3 and C22:6n-3, the precursors for eicosanoid production, may be important indicators of the pathogenesis of an inflammatory disease.

The PUFA status in blood can be assessed in plasma, platelets, red blood cells and white blood cells (Zamaria, 2004). The plasma fatty acid profile may vary considerably, whereas RBC membrane fatty acids reflect dietary fat intake in relation to the biological half life of these cells (Romon et al. 1995; Zamaria, 2004). RBC membranes, in contrast to other cells, lack the desaturase enzymes and must get their fatty acids from plasma (Allen et al. 2006). Various RBC membrane abnormalities in patients with MS are believed to reflect an altered unsaturated fatty acid content and metabolism (Gul et al. 1970; Homa et al. 1980; Nightingale et al. 1990; Mayer, 1991). Although plasma fatty acids vary considerably, plasma phospholipids are also said to reflect changes in PUFAs that occur in tissue phospholipids (Holman et al. 1989).

There have been several reports on abnormalities in fatty acid metabolism in cerebral white matter, white blood cells (WBCs), red blood cells (RBCs), platelets and plasma/serum from patients with MS (Baker et al. 1964; Cumings et al. 1965; Cherayil, 1984; Fisher et al. 1987; Holman et al. 1989; Navarro & Segura, 1989; Harbige & Sharief, 2007). The disease has been associated with n-3 fatty acid metabolic abnormalities in the CNS, or insufficient dietary intake of these fatty acids (Nightingale et al. 1990). N-6 fatty acids have also been shown to play a role in the pathogenesis and treatment of MS (Harbige & Sharief, 2007).

1.8.1.3.2 Unsaturated fatty acids in brain tissue

Abnormalities in fatty acid metabolism have been reported, in both apparently normal cerebral white matter and in plaques from MS brain tissue (Boggs & Moscarello, 1980; Chia et al. 1984; Wilson & Tocher, 1991). Myelin is formed from extensions of oligodendrocyte cell membranes. In healthy brains, these membranes have a balanced lipid and protein content (Toshniwal & Zarlring, 1992). An increased protein to lipid ratio in the myelin of affected patients has been reported, fully due to a decrease in lipids (Wilson & Tocher, 1991). The protein to lipid ratio showed a 2-fold increase in normal appearing white matter, a 3.3-fold increase in chronic plaques and a 4.3-fold increase in sub-acute plaques in comparison to normal brain tissue. The total myelin yield from normal appearing white matter from patients with MS has been reported to be reduced to two-thirds of that of age-matched control brains (Göpfert et al. 1980).

Increases in C20:4n-6, C22:6n-3 and C20:5n-3 have been reported in the brains from patients with MS (Chia et al. 1984; Wilson & Tocher, 1991), but a reduction in C22:4n-6 (Göpfert et al. 1980) and C22:6n-3 (Kishimoto et al. 1967; Nightingale et al. 1990) in the apparently normal white matter in the CNS of patients with MS have also been reported. The increased C20:4n-6 and C22:6n-3 concentrations reported by Wilson and Tocher (1991) were higher in chronic and sub-acute plaques than in normal appearing white matter. These increases could possibly be due to fatty acids of proliferating and invading astrocytes, which are known to be associated with plaques. However, it is not clear whether the reported alteration in PUFA balance plays a role in the progression of plaques, such as involvement of eicosanoïds in disease progression (Horrobin, 1979; Wilson & Tocher, 1991) or whether these changes were entirely secondary to the disease process (Wilson & Tocher, 1991).

Accumulation of free fatty acids (FFAs) in the brains from patients with MS was also reported. Chia et al. (1984) reported slightly higher levels of FFAs in diseased myelin as compared to normal myelin, including increases in FFA C20:4n-6, and Wilson and Tocher (1991) reported increases in total FFAs in plaques. In contrast, Craelius et al. (1981) reported lower FFA C20:4n-6 and FFA C24:4n-6 in diseased white matter. However, the ratio FFA C20:4n-6 to PC C20:4n-6 was higher in the brains from patients with MS than in normal brain tissue, indicating that in MS, FFA C20:4n-6 was released in higher quantities in relation to its phospholipid stock, than in control brains. Glutamate receptors require normal levels of C20:4n-6 in cell membrane phospholipids (Horrobin, 1999), while free fatty acid C20:4n-6 inhibit glutamate uptake into glial cells (Barbour et al. 1989).

1.8.1.3.3 Unsaturated fatty acids in biological fluids

Baker et al. (1964) reported a decrease in C18:2n-6 in the serum from patients with MS, while Holman et al. (1989) found plasma C18:2n-6 normal, but reported increased levels of C18:3n-6 and subnormal levels of subsequent n-6 fatty acids, including that of C20:4n-6. Cherayil (1984) found reduced C18:2n-6 and C20:4n-6 in plasma from patients with MS. Fisher et al. (1987) found no difference in serum C18:2n-6 between patients with MS and controls. Holman et al. (1989) found the concentration of all n-3 fatty acids to be subnormal in plasma of affected patients, and Cunnane et al. (1989) found the total n-3 fatty acid concentration to be decreased. Cumings et al. (1965) did not find any significant abnormalities in the blood fatty acid profile from patients with MS.

With regards to RBCs, Cherayil (1984) reported a decrease in C18:2n-6, Navarro and Segura (1989) decreases in both C18:2n-6 and C20:4n-6, Nightingale et al. (1990) an increase in C20:3n-6, while others reported no differences between patients with MS and controls (Evans & Dodd, 1989; Koch et al. 2006). Cunnane et al. (1989) reported metabolic

abnormalities between C18:2n-6 and C20:4n-6 in the RBCs from patients with MS. In the n-3 fatty acid series, Nightingale et al. (1990) reported reduced C20:5n-3 levels in patients with MS, which did not show any relationship with either the EDSS or duration of illness.

Significant decreases in the relative percentages of C18:2n-6 have been shown in peripheral blood lymphocyte (PBL) membranes from patients with MS, with accompanying lower plasma levels (Tsang et al. 1976; Cherayil, 1984), while Fisher et al. (1987) showed a similar decrease in C18:2n-6 in the white blood cells and platelets from patients with MS, but not in their serum. Harbige and Sharief (2007) reported C20:3n-6 and C20:4n-6 to be lower in peripheral blood mononuclear cell (PBMC) membranes in 20-30 percent of the patients studied. They also demonstrated a disturbed metabolic relationship between C18:2n-6 and C20:3n-6, as well as between C20:3n-6 and C20:4n-6, in the PBMC membranes from patients with MS, as compared to healthy controls. Fatty acid metabolic abnormalities in cell membranes could indicate a problem with desaturases, or greater requirement for C20:4n-6 in the studied group of patients. Abnormalities in the relationship between plasma and cell membrane fatty acids from patients with MS reported by Navarro and Segura (1989), suggested a relative deficiency of essential fatty acids (Navarro & Segura, 1989).

1.8.1.4 Cell membrane cholesterol

Cholesterol, a common steroid, is found in the membranes of most animal cells, and is involved in the regulation of membrane barrier properties, such as membrane fluidity and permeability (Voet & Voet, 1995; Caret et al. 1997; Barenholz, 2002). The fused ring system of cholesterol provides it with greater rigidity than other membrane lipids, reducing membrane fluidity and permeability (Mouritsen & Jorgensen, 1994; Voet & Voet, 1995; Mouritsen & Jorgensen, 1998; Mitchell & Litman, 2001; Barenholz, 2002). Wilson and Tocher (1991) reported the percentage cholesterol to be generally lower in MS plaques, but the differences were not significant. Chia et al. (1984) and Boggs and Moscarello (1980) did not find the cholesterol to phospholipid ratio in myelin different from that of normal myelin.

1.8.2 Cell membrane fluidity

Because phospholipids make up the structure of biological membranes, they can be used to evaluate membrane integrity. Phospholipids have different headgroups, PC, PE, PS, PI and SM, and because these differ in function and structure, their composition will determine how closely they can pack together in the cell membrane, and therefore how rigid or fluid the membrane structure will be. Cell membrane lipids may be more ordered (closely packed) or less ordered (less packed) (Williams, 1998). Phospholipids with smaller head-groups such as PE, will pack closer together than those with bigger head-groups, such as PC. In addition, because the phospholipids contain fatty acids with different chain lengths and different

degrees of unsaturation, these will have a modulating effect on the way membrane phospholipids can pack (Williams, 1998). Phospholipids with increased saturated (SATS) concentrations, which contain no double bonds, and monounsaturated (MUFAs), which contain one double bond, will therefore pack closer together than phospholipids with increased polyunsaturated (PUFA) concentrations, which contain two or more double bonds. The double bonds of MUFAs and PUFAs are angled and flexible and make the carbon chain more mobile, which prevent close packing. An increase in the number of double bonds will therefore result in a more fluid and apparently disordered membrane structure (Horrobin, 1999). The concentration of cholesterol present in the membrane will further determine membrane fluidity (Voet & Voet, 1995; Mouritsen & Jorgensen, 1998; Barenholz, 2002).

The composition of membrane phospholipids, their fatty acids and cholesterol specify the degree of membrane permeability and fluidity, which have an effect on imbedded protein interactions and membrane-bound enzyme and receptor expression (Mouritsen & Jorgensen, 1994; Voet & Voet, 1995; Mouritsen & Jorgensen, 1998; Williams, 1998; De Pablo & De Cienfuegos, 2000; Mitchell & Litman, 2001; Barenholz, 2002; Zamaria, 2004). In this, organisms have the ability to adjust the order or fluidity of their cellular membranes to optimize interaction with their physiochemical environment (Williams, 1998). However, there are changes in either the cell membrane composition itself, or induced from the environment, such as changes in temperature and pressure, that may impair membrane structure and function, and may even result in cell death.

1.8.2.1 Measurement of cell membrane fluidity

Various methods, such as electron spin resonance (ESR) (Boggs & Moscarello, 1980; Kurantsin-Mills et al. 1982), fluorescence polarization spectroscopy (Boggs & Moscarello, 1980) and microfiltration (Pollock et al. 1982) have been used to investigate membrane fluidity in patients with MS. No difference was found between MS patients and controls in membrane fluidity (Boggs & Moscarello, 1980; Kurantsin-Mills et al. 1982) or RBC deformability (Pollock et al. 1982). Chia et al. (1984) used x-ray diffraction studies to investigate myelin from both healthy controls and MS patients. They found that at 25 °C, tissue from both normal and MS brains contained a mixture of liquid-crystalline-phase lipids and ordered-crystalline-phase lipids, as in normal subjects. Lipids in the liquid-crystalline-phase is in a fluid-phase, while lipids in the ordered-crystalline-phase is in a gel-phase and have close hexagonal packing of the hydrocarbon chain, that is the fatty acid tails are packed closely together. When these results were repeated at 50 °C, the authors found that myelin from MS patients contained liquid-crystalline-phase lipids only, while normal myelin still contained both liquid-crystalline-phase lipids and ordered-crystalline-phase lipids. These results suggested some abnormality in the lipid composition in myelin from MS patients.

The liquid-crystalline-phase lipids and ordered-crystalline-phase lipids reported by Chia et al. (1984), form lamellar and hexagonal structures respectively, of which the lamellar phase is dominant (Harlos & Eibl, 1981). Cell membrane fluidity is regulated by variation of the ratio of liquid-crystalline-phase lipids to ordered-crystalline-phase lipids in the membrane. A transition from the liquid-crystalline-phase (lamellar phase) to an ordered-crystalline-phase (hexagonal phase) only, will result in a non-functional membrane (Harlos & Eibl, 1981; Chia et al. 1984; Williams, 1998). At the other extreme, membrane order can also become steadily decreased and may undergo another phase transition, to the reversed-hexagonal phase, which is also non-lamellar, and in which the phospholipid molecules are in total disarray (Williams, 1998). Membrane function can however be impaired long before either of these transition points is reached (Williams, 1998). The ratio of ordered-crystalline-phase lipids to liquid-crystalline-phase lipids in the membrane corresponds to phospholipid PE to PC ratio (Williams, 1998).

Membrane lipids differ in their head-groups and hydrocarbon chains, and have therefore been used to investigate the degree of fluidity of membranes as well. Formulae that have been used to calculate membrane fluidity include the following: the membrane saturated nature as measured by the phospholipid PC + SM/PE + PS ratio (Allen et al. 2006), membrane fluidity and permeability as measured by the cholesterol to phospholipid ratio, the membrane ordered-crystalline-phase lipids to liquid-crystalline-phase lipids as measured by the phospholipid PE to PC ratio, as well as the rheologic properties (deformability) of RBCs, as measured by the phospholipid PE to PS ratio (Labrousche et al. 1996; Allen et al. 2006). RBCs can lose their flexibility due to changes in the cell membrane lipid composition and become rigid (Allen et al. 2006). Decreased deformability can make oxygen delivery through the capillaries difficult. Furthermore, RBCs with decreased deformability are known to respond to PGE₂, and the sensitivity of these cells to PGE₂ suggests that RBCs may be a primary target for prostaglandin action (Allen and Rasmussen, 1971; Harris et al. 2001). The membrane SATS to PUFA ratio has also been used as an indication of membrane fluidity (Candiloros et al. 1996; Allen et al. 2006).

No differences were reported in membrane cholesterol concentrations or in the relationship between membrane cholesterol and phospholipids between patients with MS and control subjects (Boggs & Moscarello, 1980; Chia et al. 1984). However, decreases in plasma and/or RBC membrane PUFAs were reported to be replaced by increases in SATS and/or MUFAs (Cherayil, 1984; Cunnane et al. 1989; Holman et

al. 1989; Navarro & Segura, 1989; Nightingale et al. 1990). If essential fatty acids (PUFAs) are unavailable, they will be replaced by the nonessential fatty acids, SATS and MUFAs (Horrobin, 1999). Reports on the fatty acid composition of the brain from patients with MS did not show this trend. A decrease in PUFAs was reported (Göpfert et al. 1980), but increases were also reported (Chia et al. 1984; Wilson & Tocher, 1991). Increases in individual SATS were reported (Chia et al. 1984; Wilson & Tocher, 1991), but decreases in others were also reported (Chia et al. 1984). In general, decreases were found in MUFAs (Göpfert et al. 1980; Chia et al. 1984; Wilson & Tocher, 1991).

1.8.3 Lipids as mediators of inflammation

Although the reports are inconsistent, particularly C18:2n-6 (linoleic acid, LA) and C20:4n-6 have been shown to be reduced in the plasma, platelets, RBCs, WBCs, and CSF from patients with MS (Harbige & Sharief, 2007). Harbige and Sharief (2007) reported that together with the loss of C18:2n-6 and C20:4n-6 from PBMC membranes, an increase was shown in the production of pro-inflammatory cytokines TNF- α and interleukin-1beta (IL-1 β), and a decrease in anti-inflammatory transforming growth factor beta (TGF- β). These results suggested that the loss of C18:2n-6 and C20:4n-6 in the PBMC membranes from patients with MS was due to the inflammatory process. These fatty acids are precursors for eicosanoid production, which are reported to be increased during the inflammatory response in neurodegenerative diseases (De Pablo & De Cienfuegos, 2000; Tilley et al. 2001; Farooqui et al. 2007; Khanapure et al. 2007). Because of the relatively high amount of C20:4n-6 in immune cell membrane phospholipids, it is typically the major precursor for eicosanoid synthesis e.g. prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) (Calder, 2007). Inflammatory activation of the enzyme phospholipase A2 (PLA2) leads to the release of C20:4n-6 from cell membrane phospholipids, which in turn is metabolized by cyclooxygenases (COX), yielding eicosanoids (Herschman, 1996; Calder, 2001; Simopoulos, 2002; Bagga et al. 2003; Gilroy et al. 2004; Farooqui et al. 2007). An increase in PLA2 activity is associated with neurological disorders such as MS (Farooqui et al. 2007).

Eicosanoids regulate cellular functions, but their effects also depend on each precursor fatty acid and the cell type concerned (Horrobin & Manku, 1990; De Pablo & De Cienfuegos, 2000; Zamaria, 2004). Prostaglandin H2 (PGH2) is produced by both COX isoforms (COX1 and COX2), and is the common substrate for a series of specific synthase enzymes that produce prostaglandin D2 (PGD2), PGE2, prostaglandin F2 α (PGF2 α), prostaglandin I2 (PGI2) and thromboxane A2 (TXA2) (Tilley et al. 2001). Mast cells and neutrophils generate predominantly PGD2, while monocytes and macrophages produce PGE2 and TXA2 (Tilley et

al. 2001; Calder, 2007). This profile will change according to the different stages of the immune response (Calder, 2007). In resting macrophages, TXA₂ is produced in excess of PGE₂, while this ratio changes in favor of PGE₂ production after an immune response is elicited (Tilley et al. 2001). In the brain, the proportion of C20:4n-6 lost to metabolic processes, such as eicosanoid production, is replaced by FFAs from the plasma (Chen et al. 2008).

PGE₂ is highly pro-inflammatory (Simopoulos, 2002; Bagga et al. 2003; Haag, 2003), while prostaglandin E₁ (PGE₁), derived from the metabolism of C20:3n-6 (dihomo-gamma-linolenic acid, DGLA), is considered to have intermediate properties (Horrobin & Manku, 1990; Haag, 2003). In contrast, prostaglandins derived from the metabolism of the n-3 PUFAs, show anti-inflammatory effects (Grimm et al. 2002; Culp et al. 1979; Simopoulos, 2002; Bagga et al. 2003; Calder, 2007). C20:5n-3 is metabolized to prostaglandin E₃ (PGE₃), which has anti-inflammatory properties (Hansen, 1983; Bagga et al. 2003; Haag, 2003; Zamaria, 2004). C22:6n-3 is metabolized to docosanoids (Farooqui et al. 2007; Chen et al. 2008), which include resolvins, docosatrienes and protectins (Farooqui et al. 2007), and which are anti-inflammatory in nature (Nakamura & Nara, 2004; Calder, 2007; Farooqui et al. 2007).

Altered prostaglandin production is associated with a variety of illnesses, including inflammation (Herschman, 1996; Bagga et al. 2003). The amplification of PGE₂ may be active in brain pathologies such as MS (Repovic et al. 2003). In this regard, Hofman et al. (1986) found immune cells in MS lesions that exhibited positive staining for prostaglandin E (PGE), which was not found in normal brain tissue. The presence of stimulated cells present in the brains of patients with MS suggested an active immune mechanism in the pathogenesis of MS. Prostaglandins are very short-lived substances, and measurements of the levels of eicosanoids in body fluids are difficult (Horrobin & Manku, 1990). Evaluating their fatty acid precursors could however give an indication of an abnormal metabolism.

1.9 The importance, aims and findings of the present study

1.9.1 The importance of this study

- The present study is important because it addresses inconclusive findings from previous reports on the fatty acid composition in patients with MS
- The study includes analysis of plasma, RBC and PBMC membrane fatty acids of the same group of patients, enabling comparisons between the different blood components

- The inclusion of plasma free fatty acid analysis provides an opportunity to compare phospholipid bound fatty acids with that of the released fatty acids, which is an important aspect of the inflammatory pathways mediated by fatty acids
- The study is comprehensive and includes the analysis of all membrane lipids, which allows for a detailed analysis of the relationships between these fractions

1.9.2 Aims of the present study

The aims of the present study therefore were

- to compare lipids in plasma, RBC and PBMC membranes from patients with MS and healthy controls
- to correlate the presence of these lipids with disease outcome as measured by the EDSS in patients with MS
- to correlate the presence of these lipids with the inflammatory condition, as measured by the CRP, in patients with MS and healthy control subjects
- to compare membrane fluidity as measured by the relationship between the different lipid fractions between patients with MS and healthy controls subjects
- to correlate membrane fluidity as measured by the relationship between the different lipid fractions with disease outcome and the inflammatory status as measured by the EDSS and CRP respectively, in patients with MS and healthy controls subjects

1.9.3 New information resulting from the study

- We have confirmed earlier reports on a decrease in C20:4n-6 in membranes from patients with MS, but have further shown that free fatty acid C20:4n-6 was increased in the plasma from the same group of patients
- We have also shown an inverse correlation between RBC C20:4n-6 and plasma free fatty acid C20:4n-6, thereby linking the decrease of the RBC cells to the increase of the released free fatty acids in plasma
- We have further shown that the decreased membrane C20:4n-6 correlated inversely with both the EDSS and CRP, linking this fatty acid with both the disease outcome and inflammatory status in MS
- We have identified the saturated fatty acids that are indicative of a worse diagnosis in MS, and also those that correlated with a better outcome, which corresponded to saturated fatty acids known to be involved in membrane metabolic processes
- We have confirmed earlier reports on an increased SATS to PUFA ratio in MS, but has shown that both fatty acids were lower in MS RBC membranes, and that this ratio should therefore be used with discretion
- We have also shown that the change in membrane lipid composition resulted in a change in RBC membrane fluidity in patients with MS

CHAPTER TWO

ERYTHROCYTE MEMBRANE FATTY ACIDS IN PATIENTS WITH MULTIPLE SCLEROSIS

2.1 Introduction

The article "Erythrocyte membrane fatty acids in patients with multiple sclerosis" has been accepted by the Multiple Sclerosis Journal and is available online via Pubmed: *12 May 2009, Multiple Sclerosis*. PMID: 19435752 [Epub ahead of print]

2.2 Acceptance letter for manuscript ID MSJ-08-0290.R2

From: onbehalf@scholarone.com on behalf of msjournal@ion.ucl.ac.uk

Sent: 22 January 2009 04:10 PM

To: matshat@cput.ac.za; Hon, GM, Mev <dhon@sun.ac.za>

Subject: Multiple Sclerosis - Decision on Manuscript ID MSJ-08-0290.R2

22-Jan-2009

Dear Dr. Matsha,

I am pleased to accept your manuscript entitled "Erythrocyte membrane fatty acids in patients with multiple sclerosis" in its current form for publication in the journal Multiple Sclerosis.

You will shortly receive a second email requesting you submit a contributor agreement online without which we cannot commence with publication.

If you would like your article to be freely available online immediately upon publication (as some funding bodies now require), you can opt for it to be published under the SAGE Open Scheme on payment of a publication fee. Please simply follow the link to the Contributor Agreement form in the next email and you will be able to access instructions and further information about this option within the online form.

Thank you for your contribution.

Yours sincerely,

Prof. Alan Thompson

Editor, Multiple Sclerosis

a.thompson@ion.ucl.ac.uk

Title page**Title of article**

Erythrocyte membrane fatty acids in patients with multiple sclerosis

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Abstract

Background

Reports on fatty acids levels in multiple sclerosis remain inconclusive.

Objective

To determine the erythrocyte membrane fatty acid levels in multiple sclerosis patients and correlate with Kurtzke Expanded Disability Status Scale.

Methods

Fatty acid composition of 31 multiple sclerosis and 30 control individuals were measured by gas chromatography.

Results

The membrane phosphatidylcholine C20:4n-6 concentration was lower in the multiple sclerosis patients compared to that of the control group, $P = 0.04$ and it correlated inversely with the EDSS and FSS.

Conclusion

Decrease of C20:4n-6 in the erythrocyte membrane could be an indication of depleted plasma stores, and a reflection of disease severity.

Introduction

In multiple sclerosis (MS) previous reports regarding the fatty acid (FA) composition in biological tissues have been inconclusive. Erythrocyte membrane FA composition reported by Koch et al, [1] was not significantly different, whilst a significant decrease in C18:2n-6 and/or C20:4n-6 in the erythrocyte membranes of MS patients compared to that of a healthy control group has been reported [2]. Cultural and ethnic differences, as well as dietary variability, especially in a diseased state have been implicated in the differences observed in these studies [3]. This study determined the erythrocyte membrane FA profile of MS patients and investigated a possible association between the erythrocyte membrane FA composition in MS patients and severity of neurological outcome as measured by the Kurtzke Expanded Disability Status Scale (EDSS) and its Functional System Scores (FSS) [4]. The exclusion criteria used in this study included the use of fatty acid supplements, Interferon and cortisone or presence of a second disease for both MS patients and control subjects.

Materials and methods

Ethical approval for the study was obtained from the Health and Applied Sciences Research Ethics Committee (HASREC) of the Cape Peninsula University of Technology (CPUT). Thirty-one Caucasian females of which 28 were relapsing remitting MS (RRMS), one with primary progressive MS (PPMS) and two with secondary progressive MS (SPMS), and 30 age- gender- and race matched control subjects were recruited through the Multiple Sclerosis Society, Western Cape Branch, South Africa. The patients recruited were diagnosed by a neurologist based on clinical, laboratory and magnetic resonance imaging (MRI) findings. Six of the patients were active disease cases, 11 had a relapse 5-12 months previously and 14 had not relapsed for more than a year. The median (interquartile range) for years since diagnoses was 7 (11) years. Ten patients were using non-steroidal anti-inflammatory drugs (NSAIDs) and 5 patients were using immunosuppressive medication. Therefore, the MS patients were subdivided into two groups: Group A consisted of the total number of patients (N = 31) and Group B (N = 15) consisted of patients not on anti-inflammatory or immunosuppressive drugs. The categorisation of cases was done to exclude the possible interference of medication on the eicosanoid pathway. The functional disability status (disease severity) of each patient was measured by a trained clinician using the Kurtzke Expanded Disability Status Scale (EDSS) and the median (interquartile range) for the EDSS was 5.5 (3.5).

Venous blood from both the patients and control subjects was collected into anti-coagulant ethylenediaminetetraacetic acid (EDTA) tubes (Beckman Coulter, South Africa) and immediately separated using histopaque-1077 separation medium as per manufacturer's instructions (Sigma-Aldrich, South Africa). Fatty acid composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM) in the erythrocyte membranes were measured by gas chromatography (GC) as previously described [5, 6] and results were quantified against an internal standard, C17:0. C-reactive protein (CRP) was determined on a Beckman nephelometer auto-analyser using reagents from Beckman, South Africa.

Statistical analysis

STATISTICA (STATISTICA 7, StatSoft Inc 1984 – 2004) was used to perform all statistical analyses. Descriptive data are presented as median (interquartile range). For asymmetrical data Mann Whitney U was used to compare distributions between the cases and control subjects. Correlations were calculated using Spearman's Rank correlation coefficient. Logistic regression was used to determine the adjusted odds ratio for fatty acids (FAs) by adjusting for duration of symptoms. In view of the small sample size, P-values were corrected for multiple testing by Bonferroni. For comparison of FAs between MS and

controls, P-value < 0.006; for correlations between FAs and EDSS and FSS, P < 0.003; for metabolic relationship between FAs P < 0.008 and for FAs and CRP, P < 0.006 were considered as statistically significant.

Results

There were no significant differences in FA composition between the cases and the controls, but PC C20:4n-6 was lower in cases, (quantified in $\mu\text{g/ml}$ packed erythrocytes) 21.75 (6.4) and 24.38 (6.3), $P = 0.04$, respectively. Also the PE C22:4n-6 was lower in cases than controls, respectively, 18.80 (4.9) and 21.06 (7.7), $P = 0.06$. PC C20:4n-6 demonstrated a significant inverse correlation with the EDSS ($R = -0.73$; $P = 0.002$) as well as with the Bowel and bladder FSS ($R = -0.73$; $P = 0.002$) (Figure 1). The effect of C20:4n-6 was studied after adjustments for the duration of symptoms and was shown to be significantly and independently associated with disease severity as measured by the EDSS (Beta = -0.72; $R^2 = 0.48$; $P = 0.002$). In MS, PC C20:3n-6 and C20:4n-6 demonstrated a more prominent disturbed relationship than that observed between C18:2n-6 and C20:3n-6 or C20:4n-6 (Table 1). No significant differences were observed in the CRP concentrations between the cases and the controls (MS Group B: 3.80 $\mu\text{g/ml}$ /ml (5.2); controls: 3.70 $\mu\text{g/ml}$ (3.8); $P = 0.86$). However, non significant inverse correlations were observed with PE C20:4n-6, C22:4n-6 and CRP ($R = -0.45$; $P = 0.01$; $R = -0.36$; $P = 0.04$ respectively).

Discussion and conclusion

In the present study we provided evidence that membrane PC C20:4n-6 levels in MS patients who were not on FA supplements, Interferon or cortisone treatment are lower, whilst C18:2n-6 levels are similar to that of control subjects. Furthermore, the decreased C20:4n-6 levels in MS correlated inversely with disease severity and inflammation as measured by the EDSS and CRP, respectively. Although a decrease in C20:4n-6 was only observed in the PC phospholipid fraction, PC is the most abundant phospholipid in animal cell membranes [7]. Erythrocyte membranes lack the desaturase enzymes and the membrane lipids are taken up from the plasma [5], but similar to previous reports [3, 8], we observed a disturbed relationship between the FAs of the n-6 FA series in MS patients. Likewise, Harbige and Sharief [3] reported a disturbed relationship between C20:3n-6 and C20:4n-6 as well as between C18:2n-6 and C20:3n-6 whilst Homa et al showed the relationship between C18:2n-6 and C20:4n-6 to be disturbed. We therefore postulate that in MS, a decrease in C20:4n-6 in the erythrocyte membranes could be a result of insufficient incorporation due to depleted plasma stores. In MS plasma, decreased C18:2n-6 and subnormal subsequent n-6 FAs including C20:4n-6 levels have been reported [9, 10]. C20:4n-6 and C22:6n-3 (DHA, docosahexaenoic acid), constitute 80 – 90 % of the essential FAs in the brain [11]. Erythrocyte membrane FA composition has previously been used as an indicator of neural FA composition [12]. Neurons, like erythrocyte cannot synthesize C20:4n-6 *ex novo*, but depend on the supply from plasma and other brain cells [13].

The limitation of this study was that only female patients were used. The main strength of this study is that neither the cases nor the controls were on any FA supplements, and the patients were not on interferon or corticosteroid treatment, however, that resulted in a small samples size as MS patients not on any of these medications/supplementations are not easily available. In addition, MS cases on anti-inflammatory or immunosuppressive drugs were further excluded in MS patients subgroup B. In conclusion our findings suggest that in MS patients, erythrocyte membrane FAs, particularly the decrease in C20:4n-6 in the erythrocyte membrane could be an indication of an increased demand of this FA elsewhere, as erythrocyte membranes lack the desaturase enzymes and the membrane lipids are taken up from the plasma but, a reflection of disease severity as demonstrated by the inverse correlation with the EDSS.

Acknowledgements

We would like to extend our sincere gratitude to the following: MS Society, Western Cape Branch, South Africa and Sister Treska Botha for the recruitment of patients, Zakariya Mohammed for statistical analysis, Johanna van Wyk for technical support in the analysis of FAs, and Dr Marius de Klerk for the measurement of the EDSS and FSS.

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Table 2.1: Correlations between the FAs of the n-6 FA series in MS and control erythrocyte membranes

		Controls; N = 30		MS Group A; N = 31		MS Group B; N = 15	
		R	P-value	R	P-value	R	P-value
PC C18:2n-6	PC C20:3n-6	0.53	0.002*	0.37	0.04	0.60	0.02
PC C18:2n-6	PC C20:4n-6	0.61	0.0004*	0.49	0.01	0.50	0.06
PC C18:2n-6	PC C22:4n-6	0.62	0.0002*	0.66	0.0001*	0.55	0.04
PC C20:3n-6	PC C20:4n-6	0.63	0.0002*	0.35	0.06	0.37	0.19
PC C20:3n-6	PC C22:4n-6	0.63	0.0002*	0.48	0.01	0.79	0.0004*
PC C20:4n-6	PC C22:4n-6	0.71	0.00001*	0.54	0.002*	0.69	0.004*
PE C18:2n-6	PE C20:3n-6	0.76	0.000001*	0.83	0.0000001*	0.86	0.00004*
PE C18:2n-6	PE C20:4n-6	0.67	0.0001*	0.75	0.000001*	0.85	0.0001*
PE C18:2n-6	PE C22:4n-6	0.62	0.0002*	0.72	0.00001*	0.73	0.002*
PE C20:3n-6	PE C20:4n-6	0.77	0.000001*	0.70	0.00001*	0.81	0.0003*
PE C20:3n-6	PE C22:4n-6	0.62	0.0002*	0.63	0.0001*	0.83	0.0002*
PE C20:4n-6	PE C22:4n-6	0.82	0.0000003*	0.83	0.00000001*	0.79	0.0005*

P*: P-values significant after corrected for multiple testing (Bonferroni correction method)

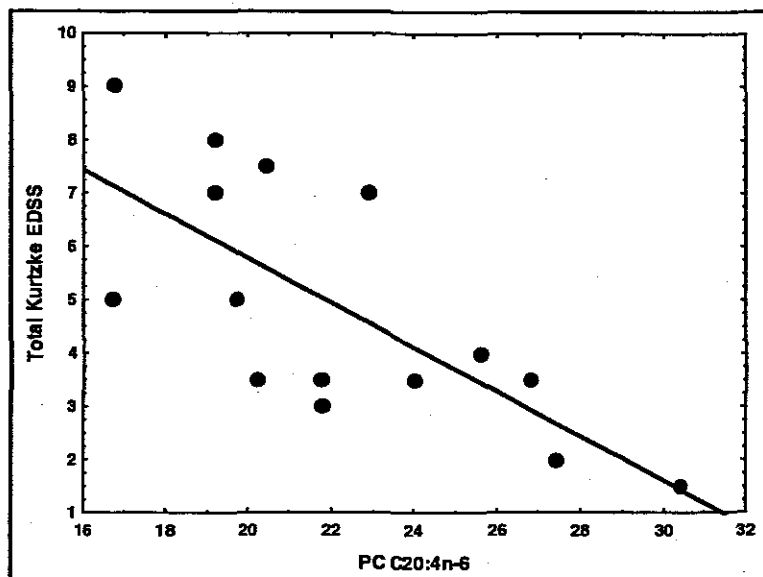


Figure 2.1: Correlation between MS Group B erythrocyte membrane PC C20:4n-6 and the Kurtzke EDSS (N = 15): $R = -0.727$; $P = 0.002$

CHAPTER THREE

IMMUNE CELL MEMBRANE FATTY ACIDS AND INFLAMMATORY MARKER, C-REACTIVE PROTEIN IN PATIENTS WITH MULTIPLE SCLEROSIS

3.1 Introduction

The article "Immune cell membrane fatty acids and inflammatory marker, C-Reactive protein in patients with multiple sclerosis" has been accepted by the British Journal of Nutrition.

3.2 Acceptance letter for manuscript ID BJN-2008-013464R

From: edoffice@nutsoc.org.uk

Sent: 22 April 2009 11:41 AM

To: matshat@cput.ac.za

Subject: BJN-2008-013464R Decision Letter

Dr. Tandi Matsha

Cape Peninsula University of Technology

Biomedical Sciences

Symphony Way

Bellville

Cape Town, Western Province 7530

South Africa

21st Apr 2009

Dear Dr. Matsha

Paper No. BJN-2008-013464R: "Immune cell membrane fatty acids and inflammatory marker, C-reactive protein in patients with multiple sclerosis".

We have now been able to assess the revised version of your paper, and I am very pleased to tell you that it has been accepted for publication in the British Journal of Nutrition.

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Yours sincerely

Philip C. Calder

Editor-in-Chief

Title page

Title: Immune cell membrane fatty acids and inflammatory marker, C-reactive protein in patients with multiple sclerosis

Running title: PBMC fatty acids in multiple sclerosis

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Abstract

Measurement of fatty acids in biological fluids and cell membranes including leukocytes from multiple sclerosis patients is inconsistent. The objective of this study was to investigate the fatty acid composition within the different membrane phospholipid fractions in peripheral blood mononuclear cells in multiple sclerosis patients, and correlate with severity of neurological outcome as measured by the Kurtzke Expanded Disability Status Scale and Functional System Scores. The fatty acid composition of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin and phosphatidylinositol phospholipids in the peripheral blood mononuclear cells of 26 multiple sclerosis and 25 control subjects, were measured by gas chromatography, and C-reactive protein was measured on all subjects. The elongation product of C20:4n-6, C22:4n-6 was significantly decreased in membrane phosphatidylethanolamine and phosphatidylserine in multiple sclerosis patients, $P = 0.01$ and 0.03 respectively, and correlated inversely with severity of disease and C-reactive protein. Also an inverse correlation was observed between the C-reactive protein and membrane phosphatidylcholine and phosphatidylserine C20:4n-6. Cultural and ethnic differences, as well as dietary variability, especially in a diseased state have been implicated in the differences observed in the fatty acid composition in peripheral blood mononuclear cell membranes of patients with multiple sclerosis. Our results suggest that the disease state may in part explain the reported inconsistencies in fatty acid levels in multiple sclerosis patients.

Introduction

The aetiology of multiple sclerosis (MS), a disease characterized by chronic inflammation of the central nervous system (CNS) is unknown, but an autoimmune and/or infectious component has been implicated ⁽¹⁻⁵⁾. A distinct feature of the MS brain is the appearance of lymphocytes around small blood vessels ^(4, 6). Fatty acids (FAs), particularly the n-6 FAs, have also been shown to have a role in the pathogenesis and treatment of the disease ⁽²⁾. Phospholipids make up the basic structure of all cell membranes and the type of FAs present in the phospholipids are closely related to the biological features of the phospholipids ^(7, 8). Membrane lipids differ in their headgroup and hydrocarbon chains ⁽⁹⁾. The polar groups can be choline, ethanolamine, serine, inositol, inositol phosphates or glycerol ⁽¹⁰⁾. The phospholipid sphingomyelin (SM), a sphingolipid, is a major component of myelin in the brain ⁽¹⁰⁾. Phosphatidylcholine (PC) is the most abundant phospholipid in animal cell membranes, and Phosphatidylethanolamine (PE) the second most ^(11, 12). The FAs contained within the different phospholipid fractions can be saturated or unsaturated (mono- or poly-), and the polyunsaturated fatty acids (PUFAs) are subdivided into different series, the n-9, n-6 and n-3 subtypes ⁽¹⁰⁾. Membrane FAs fulfill a variety of roles within immune cells and changes in the membrane phospholipid composition influence immune cell function in a variety of ways ⁽¹³⁾. These include alterations in the physical properties of the membrane, effects on cell signaling pathways, alterations in the pattern of lipid mediators produced, and an increase in eicosanoid production as part of the inflammatory process ^(13, 14).

Measurements of FAs in biological fluids and cell membranes including leukocytes from MS patients have been inconsistent. Significant decreases in the relative percentages of C18:2n-6 (LA, linoleic acid), the precursor for C20:4n-6, have been shown in peripheral blood lymphocyte (PBL) membranes in MS patients, with accompanying lower plasma levels ^(15, 16), while Fisher et al. ⁽¹⁷⁾ showed a similar decrease in the white blood cells of MS patients, but not in their plasma. Therefore in the present study our aim was to investigate the FA composition within the different phospholipid fractions in peripheral blood mononuclear cells (PBMC) membranes of MS patients, using a stringent exclusion criterion, and correlated it with severity of neurological outcome as measured by the Kurtzke Expanded Disability Status Scale (EDSS) and its Functional System Scores (FSS) ⁽¹⁸⁾. The exclusion criteria used in this study included the use of FA supplements, interferon and cortisone or presence of a second disease for both MS patients and control subjects. In addition we have used plasma C-Reactive protein (CRP) concentrations as an inflammatory marker. C-reactive protein has been used as an inflammatory marker in assessing inflammation in MS patients ^(19, 20).

Materials and methods

Ethical approval

Ethical approval for the study was obtained from the Health and Applied Sciences Research Ethics Committee (HASREC) of the Cape Peninsula University of Technology (CPUT). MS patients were contacted and recruited through the MS Society, Western Cape Branch, South Africa.

Study population

The study population consisted of 26 female Caucasians and 25 age- and gender-matched control subjects. The median (range) age in MS was 51 years (42-62) and in controls 52 years (40-60). The number of years since diagnosis was 9 years (6-17), and the median EDSS in MS was 5.25 (4.50-7.50). Five of the patients were active disease cases whilst 21 were in the remission phase. Of the patients in remission 11 had relapses 5-12 months previously, while 10 patients had no relapses during the previous year. The sample size was based on prevalence rate given for rare diseases which is 1% ⁽²¹⁾. The patients recruited were diagnosed by a neurologist based on clinical, laboratory and magnetic resonance imaging (MRI) findings. The exclusion criteria used in this study included the use of FA supplements, interferon and cortisone or presence of a second disease for both RRMS patients and control subjects.

Measurement of the disability status of patients

The functional disability status (disease severity) of each patient was measured by a trained clinician using the Kurtzke EDSS ⁽¹⁸⁾. The EDSS quantifies disability in eight Functional Systems (FS) and allows neurologists to assign a Functional System Score (FSS) in each of them. The Functional Systems are Pyramidal, Cerebellar, Brainstem, Sensory, Bowel and bladder, Visual, Cerebral and "other". Higher values indicate greater disability. Scales for the Total Kurtzke EDSS are from 0 to 10, in which the 0 score indicates no disability at all and 10 indicates death due to MS. The clinician was blinded to the results of the FA analysis (i.e. the clinical evaluation was not influenced by prior knowledge of the FA values).

Blood sample analysis

Fifteen ml venous blood from both the patients and control subjects were collected into anti-coagulant EDTA tubes (Beckman Coulter, South Africa) and immediately separated using histopaque-1077 separation medium as per manufacturer's instructions (Sigma-Aldrich, South Africa). The PBMCs were washed in a 0.85% saline solution and stored in 1 ml saline at -80°C. A 0.85% saline solution was used in this study instead of the prescribed balanced phosphate buffered saline solution as phosphate was inappropriate for additional tests done

in the study. For all participants an extra 5ml EDTA blood was collected for full blood count analysis. Full blood counts were determined on a Beckman Coulter and Lymphocytes were 25.9% $\{6.89 \times 10^9 \text{ (5.83-8.48)}\}$, Monocytes 5.8% $\{1.52 \times 10^9 \text{ (1.05-2.00)}\}$.

The PBMC membrane fatty acid composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SM) phospholipids of RRMS patients and control subjects were measured by gas chromatography (GC). PBMCs (800ul) were extracted with chloroform/methanol (18ml; 2:1; v/v) according to a modified method of Folch et al ⁽²²⁾. All the extraction solvents contained 0.01 % butylated hydroxytoluene (BHT) as an antioxidant. Free fatty acids were separated from the total phospholipid (TPL) fraction by TLC on pre-coated silica gel 60 plates (10 x 10 cm) without a fluorescent indicator (Art. 1.05721, Merck, Darmstadt, Germany) using the solvent system petroleum benzene (bp 40-60 °C)/diethyl ether (peroxide free)/acetic acid (90:30:1, by vol) as previously described ⁽²³⁾. Individual phospholipid classes were separated by TLC on pre-coated silica gel 60 plates (10 x 10 cm) without a fluorescent indicator (Merck, Darmstadt, Germany) using chloroform/petroleum benzene/methanol/acetic acid/boric acid (40:30:20:10:1.8 v/v/v/v/w) as solvent ⁽²⁴⁾. The lipid bands containing PC, PE, PS, PI and SM were visualized with long wave ultraviolet light after spraying the plates with chloroform/methanol (1:1, by vol) containing BBOT (2,5-bis-(5'-tert-butylbenzoxazolyl-[2])thiophene; 10 mg/100 mL; Sigma Chemical Co.). The lipids were transmethylated using 5% H₂SO₄/methanol at 70 °C for 2 hrs (SM 18 hrs). After cooling, the resulting fatty acid methyl esters (FAME) were extracted with 1 ml of water and 2 ml of *n*-hexane. The top hexane layer was evaporated to dryness, redissolved in CS₂ and analyzed by GLC (Finnigan Focus GC, Thermo Electron Corporation, USA, equipped with flame ionization detection) using 30 m BPX 70 capillary columns of 0.32 mm internal diameter (SGE International Pty Ltd, Australia). Gas flow rates were: Nitrogen (make up gas), 25 ml/min; air, 250 ml/min; and hydrogen (carrier gas), 25 ml/min and split ratio of 20:1. Temperature programming was linear at 5 °C/min, initial temperature 140 °C, final temperature 220 °C, injector temperature 220 °C, and detector temperature 250 °C. The FAME were identified by comparison of the retention times to those of a standard FAME mixture (Nu-Chek-Prep Inc., Elysian, Minnesota). The individual FAME were quantified against an internal standard (C17:0, Sigma-Aldrich).

CRP concentrations were determined in a routine Chemical Pathology laboratory on a Beckman nephelometer auto-analyser using reagents from Beckman, South Africa. The diagnostic values of this laboratory are considered positive for CRP values greater than or equal 5µg/ml.

Statistical analysis

A statistics programme, STATISTICA (STATISTICA 7, StatSoft Inc 1984 – 2004) was used to perform all statistical analyses. The PBMC membrane PC, PE, PS, SM and PI phospholipid FA profiles were reported in μg FAs/mg protein. Descriptive data are presented as median and range. For asymmetrical data Mann Whitney U was used to compare distributions between the cases and control subjects. Correlations between FAs, CRP and EDSS FSS were calculated using Spearman's Rank correlation coefficient. Results were considered significant if P-values were less than 0.05.

Results

Differences in the fatty acid profile between relapsing-remitting multiple sclerosis patients and controls

Significant differences were observed between the RRMS and control groups in their FA profile. PE, PS C22:4n-6, as well as total C22:4n-6, were lower in RRMS patients than in the control subjects, $P = 0.01$, 0.03 and 0.02 respectively (Table 1).

C-reactive protein concentrations

No significant differences were observed in the CRP concentrations between the RRMS patients and the control subjects, however, significant inverse correlations were observed between the RRMS membrane FA values and the CRP concentrations but not in the control group (Table 2). In RRMS patients with CRP values above $5\mu\text{g/ml}$, PC C20:4n-6 demonstrated a decreasing trend whilst the opposite was observed in controls (Figure 1). There was a significant positive correlation in the RRMS patients between the CRP concentrations and the EDSS; $R = 0.45$; $P = 0.03$ (Figure 2).

Metabolic relationship between the fatty acids in the n-6 fatty acid series in relapsing-remitting multiple sclerosis patients and control subjects

The metabolic relationships between the n-6 FAs are summarized in Table 3. 18:2n-6 demonstrated in general a positive correlation with C20:3n-6, C20:4n-6 and C22:4n-6 in both RRMS and control subjects. A positive correlation was also observed between C20:3n-6 and C20:4n-6, as well as between C20:4n-6 and C22:4n-6. However, total C18:2n-6 and C20:3n-6 was positively correlated in the RRMS patients only.

Correlation between the n-6 and n-3 fatty acids and the Kurtzke Expanded Disability Status Scale and Functional System Scores in relapsing-remitting multiple sclerosis patients

Table 4 summarizes the significant correlations between PBMC membrane FAs and the Kurtzke EDSS and FSS. PC, PS and PI C18:2n-6 showed positive correlations with the Pyramidal, Brainstem and Bowel and Bladder FSS respectively. PC and PS C20:3n-6 showed positive correlations with the EDSS and Cerebral FSS respectively. Total C20:3n-6 also showed a positive correlation with the EDSS. Both PE and PI C20:4n-6 showed inverse correlations with the Sensory FSS and PC C22:4n-6 showed an inverse correlation with the Visual FSS. Total C20:4n-6 also showed an inverse correlation with the Sensory FSS. In the n-3 FA series, both PC C20:5n-3 (EPA, eicosapentaenoic acid) and PC C22:6n-3 (DHA, docosahexaenoic acid), showed positive correlations with the Brainstem FSS.

Discussion

Fatty acids, particularly C18:2n-6 and C20:4n-6 have been shown to be reduced in the plasma, platelets, red cells, leucocytes, and cerebrospinal fluid of RRMS patients, but the reports are inconsistent ⁽²⁾. Cultural and ethnic differences, as well as dietary variability, especially in a diseased state, have been implicated in the differences observed in these studies. In the present study, the elongation product of C20:4n-6, C22:4n-6 in PBMC membrane PE and PS phospholipids was significantly decreased in RRMS patients studied. In addition, C18:2n-6 and C20:3n-6 showed positive correlations with the EDSS. It is unlikely that the reduced C22:4n-6 levels were due to dietary requirements as C18:2n-6, the parent dietary FA was similar in both RRMS and control subjects. The n-6 PUFAs are precursors for a number of mediators of inflammation that are released from membrane phospholipids in the course of inflammatory activation ^(25, 26). It is possible that lower n-6 PUFA's in MS could be due to an increased eicosanoid production that regulate a great number of inflammatory effects, which depends on each precursor ⁽²⁷⁾. This in turn could affect the total fatty acids pool, hence neural requirements since the human brain requires 4 times the amount of C20:4n-6 than C22:6n-3 on a daily basis ^(2, 28, 29). Similar to Harbige and Sharief ⁽²⁾, we have shown a disturbed relationship between C20:3n-6 and C20:4n-6 in the PC fraction in MS. Furthermore, we have found a reversal of the C20:3n-6 and C20:4n-6 relationship in the PC and PE fractions of MS PBMC membranes compared with controls. Since PE is the second most abundant phospholipid in animal cell membrane the positive correlation between C20:3n-6 and C20:4n-6 in the PE fraction may be compensating for the negative correlation between C20:3n-6 and C20:4n-6 in the PC fraction.

C-reactive protein has been used as an inflammatory marker in assessing inflammation in RRMS patients ^(19, 20). Several studies have shown inverse correlation with CRP and PUFAs, but inconsistencies exist in specific fatty acids ^(30, 31, 32, 33). Petersson et al. ⁽³³⁾ demonstrated inverse (C18:2n-6) and positive (C20:3n-6) association with CRP. Similarly, significant inverse correlations were observed between the CRP and membrane PC and PS C20:4n-6, as well as with PS and PI C22:4n-6 in RRMS cases. In contrast, control subjects with CRP levels above 5 µg/ml, PC C20:4n-6 demonstrated an increasing trend whilst in RRMS patients the opposite was observed. These results suggest that the degree of inflammation is an important determinant of resultant FA concentrations. In addition, we observed a positive correlation between CRP and disease severity as measured by the EDSS. It has been reported that serum levels of many inflammatory markers do not correlate with short-term disease progression ⁽¹⁹⁾. Giovannoni et al. ⁽¹⁹⁾ found no correlation between inflammatory markers, including CRP, and the EDSS, or changes in any of the MRI measured in a group of RRMS patients they have studied over an 18-month period. However in an earlier study the same authors ⁽³⁴⁾ demonstrated that raised CRP concentrations correlated with infectious

episodes and clinical relapse. We think that our results could possibly be explained by the fact that we have excluded patients on disease-modifying agents, interferon and corticosteroids which are potent anti-inflammatory agents.

The limitation of this study was that only female patients were used and the small sample size. As we also did not consider dietary fatty acid intake, this could have affected the fatty acid levels observed in this study. The main strength of this study is that neither the cases nor the controls were on any fatty acid supplements, and the patients were not on interferon or corticosteroid treatment. Since it is difficult to recruit patients with RRMS not on any of these medications/supplementations this resulted in a small sample size. In conclusion, our results concur with Harbige and Sharief ⁽²⁾ that the relationship between n-6 fatty acids C20:3n-6 and C20:4n-6 is disturbed in RRMS patients and affects the physiological integrity of immune cells. Furthermore we have shown the phospholip fractions involved. It would however be of interest to investigate the role of desaturases and elongases in FA metabolism in multiple sclerosis and other inflammatory conditions. The essential parent fatty acids (EFAs), C18:2n-6 and C18:3n-3 (ALA, alpha-linolenic acid), cannot be synthesised in the body and must be obtained from the diet ⁽³⁵⁾. Most of the effects of C18:2n-6 and C18:3n-3 are dependent not only on the ingested EFAs themselves, but on their derivatives ⁽³⁶⁾. The parent dietary EFAs are converted to their metabolites (the n-6 and n-3 series respectively) by a series of alternating desaturations and elongations ^(36, 37). The inverse correlation between fatty acids, CRP and disease severity and fatty acids suggest that the disease state may in part explain the reported inconsistencies in fatty acid levels in multiple sclerosis patients. Based on our results, we suggest that further studies should take into account CRP levels in the interpretation of results.

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Conflict of Interest Statement

None declared

Author Contribution

Gloudina Hon: Designed the study and wrote the text

Mogamat Hassan: Collaborator, interpretation and proofreading of the manuscript

Susan Janse van Rensburg: Co-supervisor and involved in the recruitment of volunteers for this study, proofreading of the manuscript

Stefan Abel: Co-supervisor, fatty acid analysis, interpretation, proofreading of the manuscript

De Wet Marais: Fatty acid analysis and interpretation, proofreading of the manuscript

Paul van Jaarsveld: Fatty acid analysis and interpretation, proofreading of the manuscript

Cornelius Smuts: Collaborator and proofreading of the manuscript

Franco Henning: Measurement of neurological outcome, proofreading of the manuscript

Rajiv Erasmus: Collaborator and proofreading of the manuscript

Tandi Matsha: Supervisor and coordinator of this group and was involved in the recruitment of volunteers for this study, proofreading of the manuscript.

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Table 3.1: Differences in the peripheral blood mononuclear cell membrane fatty acids between relapsing-remitting multiple sclerosis (RRMS) patients and control subjects

	Controls		RRMS patients		P-value	Controls		RRMS patients		P-value
	µg FAs/mg protein		Percentage FAs							
	Median	Range	Median	Range		Median	Range	Median	Range	
PC C18:2n-6	4.13	3.77-4.50	4.12	3.53-4.68	0.84	9.21	8.49-10.12	8.94	8.10-9.95	0.66
PE C18:2n-6	0.80	0.72-0.89	0.75	0.67-0.81	0.13	3.04	2.65-3.38	3.01	2.72-3.43	0.76
PS C18:2n-6	0.11	0.10-0.13	0.13	0.10-0.14	0.16	1.25	1.11-1.50	1.37	1.23-1.82	0.10
PI C18:2n-6	0.11	0.09-0.12	0.09	0.08-0.11	0.25	1.48	1.23-1.98	1.46	1.23-1.82	0.92
SM C18:2n-6	0.11	0.08-0.18	0.11	0.07-0.16	0.98	1.00	0.82-1.37	0.89	0.65-1.60	0.78
Total C18:2n-6	5.31	4.96-5.72	5.30	4.46-5.79	0.98	15.76	15.03-17.51	16.16	14.60-18.60	0.72
PC C20:3n-6	0.92	0.79-1.03	0.89	0.72-1.02	0.49	2.07	1.84-2.26	2.05	1.74-2.62	0.94
PE C20:3n-6	0.25	0.22-0.29	0.23	0.19-0.27	0.19	0.99	0.91-1.12	0.97	0.86-1.29	0.59
PS C20:3n-6	0.20	0.15-0.22	0.18	0.14-0.22	0.65	2.12	1.94-2.46	2.17	1.79-2.64	0.78
PI C20:3n-6	0.09	0.07-0.10	0.08	0.07-0.10	0.86	1.17	1.03-1.31	1.15	0.96-1.32	0.95
Total C20:3n-6	1.46	1.30-1.70	1.40	1.17-1.58	0.35	6.27	5.88-7.04	5.94	5.17-7.55	0.59
PC C20:4n-6	7.09	6.34-8.11	7.00	6.37-7.81	0.63	16.11	15.03-17.13	15.62	14.73-16.93	0.34
PE C20:4n-6	12.00	11.18-13.10	11.70	11.15-12.86	0.73	47.10	46.09-48.16	47.53	45.63-49.35	0.56
PS C20:4n-6	2.43	2.11-2.51	2.26	2.01-2.84	0.99	27.52	24.99-29.67	26.94	24.99-28.38	0.67
PI C20:4n-6	3.34	3.01-3.48	3.34	2.93-3.59	0.92	44.83	43.23-45.57	44.21	41.20-45.19	0.16
SM C20:4n-6	0.10	0.08-0.14	0.10	0.07-0.12	0.92	0.82	0.60-1.11	0.84	0.60-1.33	0.77
Total C20:4n-6	25.07	23.5-26.8	25.17	22.4-26.5	0.64	137.19	132.0-140.3	138.67	132.3-141.2	0.57
PC C22:4n-6	0.49	0.41-0.56	0.43	0.37-0.55	0.19	1.10	0.98-1.30	1.09	0.93-1.27	0.42
PE C22:4n-6	1.72	1.55-1.99	1.52	1.15-1.73	0.01	6.72	5.92-7.25	5.81	4.89-7.09	0.02
PS C22:4n-6	0.15	0.13-0.16	0.13	0.11-0.16	0.03	1.84	1.50-2.04	1.63	1.28-1.94	0.10
PI C22:4n-6	0.06	0.04-0.07	0.06	0.04-0.07	0.38	0.84	0.70-0.98	0.76	0.63-0.90	0.24
Total C22:4n-6	2.47	2.08-2.83	2.12	1.66-2.50	0.02	10.91	9.05-11.43	9.27	7.96-10.72	0.01
PC C22:6n-3	0.56	0.52-0.67	0.59	0.44-0.73	0.79	1.33	1.11-1.60	1.36	1.18-1.68	0.79
PE C22:6n-3	1.15	1.00-1.30	1.09	0.90-1.31	0.53	4.44	3.69-5.26	4.74	3.95-5.11	0.94
PS C22:6n-3	0.20	0.16-0.22	0.17	0.15-0.22	0.31	2.14	1.95-2.61	2.08	1.77-2.49	0.40
Total C22:6n-3	1.91	1.73-2.25	1.92	1.63-2.22	0.52	7.91	7.10-10.28	8.51	7.28-9.74	0.99

Table 3.2: Correlations between C-reactive protein values and the peripheral blood mononuclear cell membrane fatty acids in controls and relapsing-remitting multiple sclerosis (RRMS) patients

	Controls		RRMS patients	
	R	P-value	R	P-value
PC C20:4n-6	0.01	0.95	-0.45	0.03
PS C20:4n-6	0.17	0.45	-0.57	<0.01
PS C22:4n-6	0.37	0.07	-0.60	<0.01
PI C22:4n-6	0.16	0.48	-0.50	0.01
Total C18:2n-6	0.13	0.57	-0.16	0.48
Total C20:3n-6	0.22	0.32	0.19	0.37
Total C20:4n-6	0.09	0.67	-0.33	0.13
Total C22:4n-6	0.15	0.50	-0.17	0.42

Table 3.3: Correlations between the fatty acids within the n-6 fatty acid series in controls and relapsing-remitting multiple sclerosis (RRMS) patients

		Controls		RRMS patients	
		R	P-value	R	P-value
PC C18:2n-6	PC C20:3n-6	0.36	0.08	0.71	<0.01
PE C18:2n-6	PE C20:3n-6	-0.05	0.80	0.57	<0.01
PS C18:2n-6	PS C20:3n-6	0.48	0.01	0.41	0.04
PI C18:2n-6	PI C20:3n-6	0.51	0.01	0.68	<0.01
PC C18:2n-6	PC C20:4n-6	0.45	0.03	0.54	0.01
PE C18:2n-6	PE C20:4n-6	0.40	0.05	0.45	0.02
PS C18:2n-6	PS C20:4n-6	-0.12	0.57	0.42	0.03
SM C18:2n-6	SM C20:4n-6	0.76	<0.01	0.48	0.01
PI C18:2n-6	PI C20:4n-6	0.47	0.02	0.39	0.04
PC C20:3n-6	PC C20:4n-6	0.46	0.02	0.25	0.21
PE C20:3n-6	PE C20:4n-6	0.18	0.40	0.42	0.03
PS C20:3n-6	PS C20:4n-6	0.41	0.04	0.72	<0.01
PI C20:3n-6	PI C20:4n-6	0.62	<0.01	0.60	<0.01
PC C20:4n-6	PC C22:4n-6	0.86	<0.01	0.60	<0.01
PE C20:4n-6	PE C22:4n-6	0.54	0.01	0.42	0.03
PS C20:4n-6	PS C22:4n-6	0.28	0.18	0.52	0.01
PI C20:4n-6	PI C22:4n-6	0.82	<0.01	0.81	<0.01
Total C18:2n-6	Total C20:3n-6	0.32	0.12	0.65	<0.01
Total C18:2n-6	Total C20:4n-6	0.59	<0.01	0.57	<0.01
Total C20:3n-6	Total C20:4n-6	0.52	0.01	0.41	0.04
Total C20:4n-6	Total C22:4n-6	0.70	<0.01	0.55	<0.01

Table 3.4: Correlations between membrane fatty acids and the Expanded Disability Status Scale and Functional System Scores in relapsing-remitting multiple sclerosis patients

FAs	EDSS and FSS	RRMS patients	
		R	P-value
PC C18:2n-6	Pyramidal	0.42	0.04
PS C18:2n-6	Brainstem	0.40	0.04
PI C18:2n-6	Bowel/Bladder	0.49	0.02
PC C20:3n-6	EDSS	0.46	0.02
PS C20:3n-6	Cerebral	0.46	0.02
PE C20:4n-6	Sensory	-0.45	0.02
PI C20:4n-6	Sensory	-0.45	0.02
PC C22:4n-6	Visual	-0.48	0.02
PC C20:5n-3	Brainstem	0.41	0.04
PC C22:6n-3	Brainstem	0.43	0.03
Total C20:3n-6	EDSS	0.47	0.02
Total C20:4n-6	Sensory	-0.46	0.02

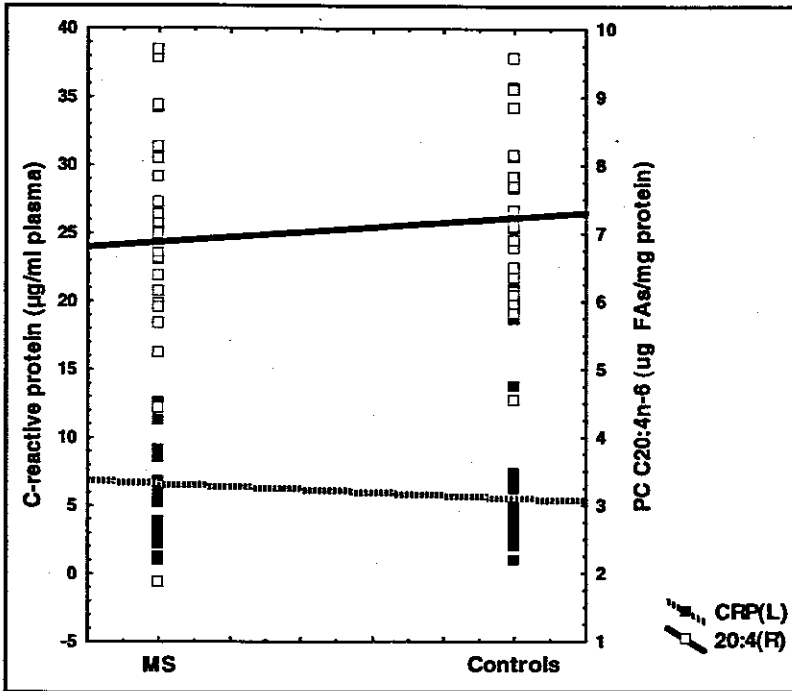


Figure 3.1: C-reactive protein (CRP) and phosphatidylcholine (PC) C20:4n-6 and CRP levels. In relapsing-remitting multiple sclerosis (RRMS) patients with CRP values above 5 µg/ml PC C20:4n-6 demonstrated a non-significant decreasing trend whilst the opposite was observed in controls.

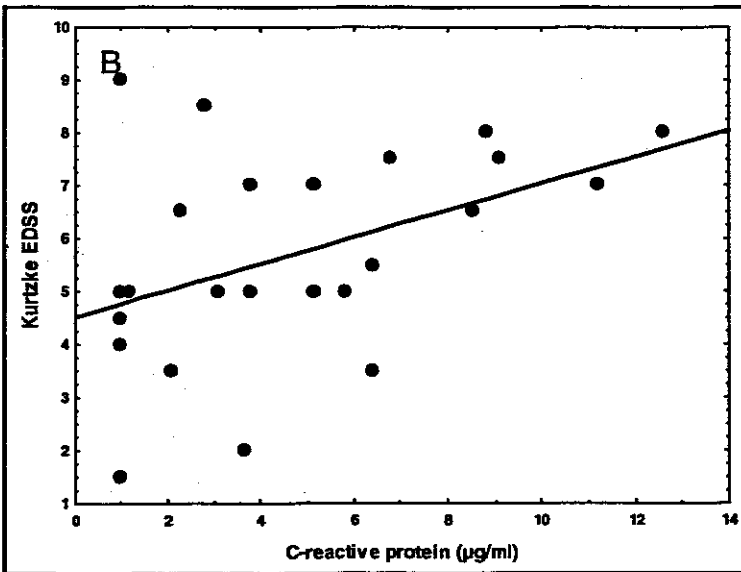
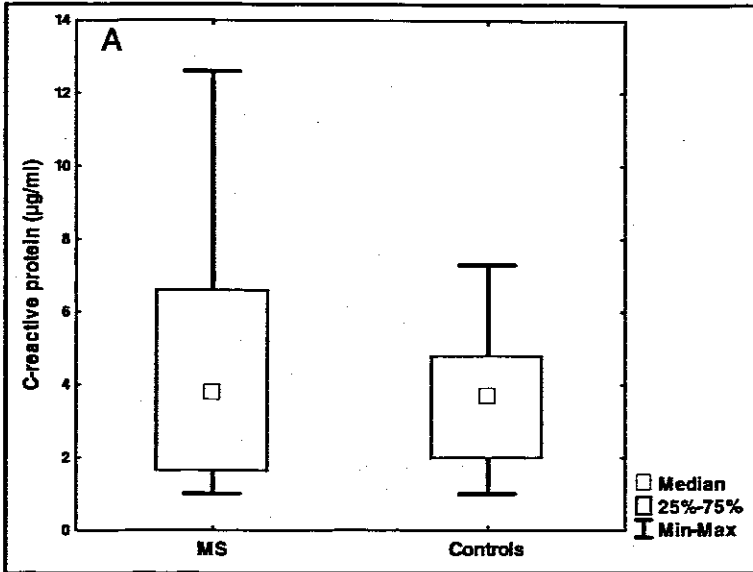


Figure 3.2: C-reactive protein (CRP) levels and the Expanded Disability Status Scale (EDSS) (a) CRP levels in relapsing-remitting multiple sclerosis (RRMS) patients and control subjects: RRMS 3.80 (1.65-6.60) $\mu\text{g/ml}$; controls: 3.70 (2.00-4.80) $\mu\text{g/ml}$; $P = 0.35$; (b) Correlation between CRP levels and the EDSS in RRMS patients. The correlation was significantly positive ($R = 0.45$; $P = 0.03$).

CHAPTER FOUR

PLASMA FATTY ACIDS AND FREE FATTY ACIDS IN MULTIPLE SCLEROSIS

4.1 Introduction

The article "Plasma fatty acids and free fatty acids in Multiple Sclerosis" has been submitted to the Journal of Metabolic Brain Disease. It is currently under review.

4.2 Letter of submission received from the Journal of Metabolic Brain Disease

>>> "Vivienne Russell" <Vivienne.Russell@uct.ac.za> 2009/03/25 04:08 PM

>>>

Dear Tandi

Thank you very much for submitting your manuscript to Metabolic Brain Disease for consideration for publication in the special issue.

I wish to confirm that I received a manuscript entitled "Plasma fatty acids and free fatty acids in Multiple Sclerosis" by G.M. Hon, M.S. Hassan, S.J. van Rensburg, S. Abel, De W Marais, C.M. Smuts, R.T. Erasmus, and T. Matsha. The manuscript will be sent to two reviewers for peer-review.

With best wishes

Viv

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Title page**Title:** Plasma fatty acids and free fatty acids in Multiple Sclerosis**Authors:** G.M. Hon¹, M.S. Hassan¹, S.J. van Rensburg², S. Abel³, De W. Marais⁴, C.M. Smuts^{4,5}, R.T. Erasmus⁶, and T. Matsha^{1*}**Affiliations:** ¹Department of Bio-Medical Sciences, Faculty of Health and Wellness Science, Cape Peninsula University of Technology, P O Box 1906, Bellville, 7530, Cape Town, South Africa. ²Division of Chemical Pathology, National Health Laboratory Services, Tygerberg, South Africa. ³PROMEC Unit and NIRU, South African Research Council, Tygerberg, South Africa. ⁴South African Medical Research Council, South Africa. ⁵North-West University (Potchefstroom Campus), South Africa. ⁶Division of Chemical Pathology, University of Stellenbosch, South Africa.**Running head:** Plasma fatty acids in Multiple Sclerosis***Corresponding author**

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Abstract

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system in which inconsistent findings on plasma fatty acids have been reported. Plasma free fatty acids (FFAs) are the major source of brain fatty acids.

Objective

To investigate whether there was a difference in the fatty acid and FFA profiles in the plasma from MS patients and a healthy control group, and to investigate a possible relationship between previously reported lower n-6 fatty acids in the red blood cell (RBC) membranes from MS patients and plasma fatty acids from the same group of patients.

Methods

The plasma phosphatidylcholine (PC) and sphingomyelin (SM) fatty acid composition and plasma FFAs from patients and control subjects were measured by gas chromatography.

Results

There were significant increases in FFAs C18:2n-6 and C20:4n-6 in plasma from patients compared to controls ($P = 0.003$ and $P = 0.007$ respectively). The plasma FFA C20:4n-6 to plasma PC C20:4n-6 ratio was significantly higher in patients {median and interquartile range for patients: 0.011 (0.006); controls: 0.007 (0.003); $P = 0.00008$ }, as was plasma FFA C20:4n-6 to RBC PC C20:4n-6 ratio { patients: 0.039 (0.026); controls: 0.031 (0.014); $P = 0.00051$ } There was a significant inverse correlation between plasma FFA C20:4n-6 and RBC PC C20:4n-6 from patients ($R = -0.36$; $P = 0.04$).

Discussion

The inflammatory condition experienced by MS patients, is in part mediated by the metabolic products of the n-6 and n-3 fatty acids. The changes in plasma and red blood cell membrane C20:4n-6, suggested that this fatty acid could be involved in the pathogenesis of the disease. Reports of an increase in prostaglandin in brain tissue from MS patients confirms the possibility that excessive release of FFA C20:4n-6 could be indicative of an increased demand for prostaglandin production.

Key words

Multiple sclerosis, inflammation, fatty acids, free fatty acids

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) (Adibhatla and Hatcher, 2007; Brück, 2005). During CNS injury microglial cells are activated to initiate a rapid response that involves cell migration, proliferation, release of cytokines/chemokines and trophic and/or toxic effects. Cytokines/chemokines stimulate phospholipase A2 (PLA2) and cyclooxygenase (COX) production (Farooqui, 2007). Phospholipase A2 releases fatty acids C20:3n-6, C20:4n-6 and C20:5n-3 from membrane phospholipids for prostaglandin 1, 2 and 3 (PGE1, 2, and 3) synthesis respectively (Haag, 2003). Collective evidence from many recent studies suggests that increased PLA2-activity and PLA2-generated mediators play a central role not only in acute inflammatory responses, but also in oxidative stress associated with neurological disorders such as MS (Farooqui et al. 2007). In MS, fatty acid metabolic abnormalities, particularly lower levels of C18:2n-6 and C20:4n-6 have been reported in cerebral white matter (Cumings et al. 1965), white blood cells (Harbige and Sharief, 2007; Fisher et al. 1987), red blood cells (RBC) (Navarro and Segura, 1989; Cherayil, 1984) and platelets (Fisher et al. 1987). Impaired polyunsaturated fatty acid (PUFA) status has been observed in numerous physiological and chronic diseases (Zamaria, 2004). There is scarcity of literature on free fatty acids (FFAs) in plasma from multiple sclerosis patients. However, increased FFAs in plasma has been implicated in a number of diseases, such as sudden cardiac death, insulin resistance, atherosclerosis and hypertension (Pilz et al. 2007; Blaak, 2003; Oram and Bornfeldt, 2004; Sarafidis and Bakris, 2007).

Fatty acids are sub-divided into saturated (SATs), mono-unsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs), and the PUFAs are further sub-divided into different series such as the n-6 and n-3 fatty acid series. The essential parent fatty acids (EFAs), C18:2n-6 and C18:3n-3 cannot be synthesised in the body and must be ingested from food (Zamaria, 2004). The parent dietary essential fatty acids are converted to their metabolites (the n-6 and n-3 fatty acid series respectively) by a series of alternating desaturation and elongation reactions. Most of the effects of C18:2n-6 and C18:3n-3 are dependent not only on the ingested essential fatty acids themselves, but on their derivatives (Horrobin and Manku, 1990). A balanced intake of both n-6 and n-3 PUFA is essential for good health (Zamaria, 2004). This may in part explain the interest in PUFA supplementation by investigators and the use of dietary supplements by MS patients.

The aim of this study was to investigate whether the plasma fatty acids and FFA profile in MS patients differ from that of a healthy control group, and to investigate whether there was any relationship between the lower PC C20:4n-6 previously reported in RBC membranes from MS patients (Hon et al. 2009a) and plasma fatty acids from the same group of MS patients.

Both the patients and control subjects were specifically screened to exclude the use of interferon, cortisone and/or fatty acid supplements, and blood sampling was done after an overnight fast.

Materials and methods

Ethical approval

Ethical approval for the study was obtained from the Health Sciences Research Ethics Committee (HSREC) of the Cape Peninsula University of Technology (CPUT). MS patients were contacted and recruited through the MS Society, Western Cape Branch, South Africa.

Study population

The study population consisted of 31 Caucasian female patients and 30 age- and gender-matched control subjects. Twenty eight patients presented with relapsing remitting MS (RRMS), 1 with primary progressive MS (PPMS) and 2 with secondary progressive MS (SPMS). The patients recruited were diagnosed by a neurologist based on clinical, laboratory and magnetic resonance imaging (MRI) findings. The exclusion criteria used in this study included the use of fatty acid supplements, interferon and cortisone or presence of a second disease for both MS patients and control subjects. Six of the patients reported increased disease activity during the 3 month period prior to the study, but the moderate extent of the exacerbations did not warrant cortisone treatment. Eleven patients had a relapse 5-12 months prior to the study, and 14 had not relapsed for more than a year. The median (quartile range) for years since diagnosis was 7 (11) years.

Blood sample processing and analysis

Venous blood from both the patients and control subjects was collected into anti-coagulant EDTA tubes (Beckman Coulter, South Africa) and immediately separated and stored at -80°C. The plasma was used for fatty acid analysis.

Fatty acid composition

The fatty acid composition of the PC and SM phospholipid fractions, as well as FFAs in plasma from MS patients and control subjects were measured by gas chromatography (GC) as previously described (Folch et al. 1957; Van Jaarsveld et al. 2000) and results were quantified against an internal standard, C17:0, in $\mu\text{g/ml}$ plasma analyzed.

Statistical analysis

A statistics programme, STATISTICA (STATISTICA 7, StatSoft Inc 1984 – 2004) was used to perform all statistical analyses. Descriptive data are presented as median (quartile range). For asymmetrical data Mann Whitney U was used to compare distributions between the cases and control subjects. Correlations were calculated using Spearman's Rank correlation coefficient. A P-value of less than 0.05 was taken as statistically significant.

Results

Plasma fatty acids and FFAs were quantified and results were reported as μg FAs per ml plasma. Phospholipids PC and SM were the only two phospholipids detected in plasma from both the patients and control subjects. Phospholipid SM contains mostly saturated fatty acids and only a few of the polyunsaturated fatty acids. In this study, C20:4n-6 was too low to measure in SM phospholipids, therefore the focus of the study was on PC fatty acids, which contained the complete polyunsaturated fatty acid range. The plasma n-6 FFAs were measurable, but in the n-3 series, only FFA C22:6n-3 was measurable.

Differences between MS patients and control subjects are summarized in Table 1. In the PC fraction, there were no significant differences between MS patients and control subjects in the plasma n-6 or n-3 fatty acids. There were however highly significant increases in FFAs C18:2n-6 and C20:4n-6 in MS patients compared to the control group ($P = 0.003$ and $P = 0.007$ respectively). Both total n-6 FFAs and total n-3 FFAs were increased in the plasma from MS patients ($P = 0.003$ and $P = 0.046$ respectively).

The plasma FFA C20:4n-6 to plasma PC C20:4n-6 ratio was significantly higher in MS patients {patients: 0.011 (0.006); controls: 0.007 (0.003); $P = 0.00008$ }, as was plasma FFA C20:4n-6 to RBC PC C20:4n-6 ratio {patients: 0.039 (0.026); controls: 0.031 (0.014); $P = 0.00051$ } (Figures 1 and 2).

There was an insignificant positive correlation between the plasma FFA C20:4n-6 and plasma PC C20:4n-6 from MS patients ($R = 0.23$; $P = 0.22$) (Figure 3), while a significant inverse correlation was found between the plasma FFA C20:4n-6 and RBC membrane PC C20:4n-6 from MS patients ($R = -0.36$; $P = 0.04$) (Figure 4).

Discussion

Reports on the n-6 fatty acid concentration of phospholipids in the plasma and cell membranes from MS patients are inconsistent, but C18:2n-6 and/or C20:4n-6 are repeatedly reported to be lower in MS than in control subjects. Baker et al. (1964) reported a decrease in C18:2n-6 in the serum of MS patients, while Holman et al. (1989) found plasma C18:2n-6 to be normal, with C18:3n-6 increased, while subsequent n-6 fatty acids (inclusive of C20:4n-6) were subnormal. Reduced C18:2n-6 and C20:4n-6 in plasma from MS patients was also reported by Cheravil (1984), while Fisher et al. (1987) found no difference in C18:2n-6 between MS and control serum.

Similar to these reports, we have reported lower PC C20:4n-6 in the RBC membranes from patients with MS (Hon et al. 2009a). C20:4n-6 was not lower in any of the other phospholipid fractions within the RBC membranes from these patients and neither was it decreased in peripheral blood mononuclear cell (PBMC) membranes (Hon et al. 2009b). In the present study, there was no difference between plasma from MS patients and control subjects in any of the n-6 or n-3 fatty acids contained within the PC phospholipid fraction, which indicated sufficient dietary intake of the essential parent fatty acids. However, there was a highly significant increase in FFA C18:2n-6 and FFA C20:4n-6 (Table 1) in the plasma from MS patients, which, according to Leaf (2001) is not affected by recent dietary intake of fat. FFA C20:4n-6 was too low to measure in both the RBC and PBMC membranes from both MS patients and control subjects. Therefore, plasma seems to be the only easily available source to investigate the FFA profile in an inflammatory disease such as MS.

The relationship (Figures 1-4) between plasma FFAs C20:4n-6 and plasma and RBC C20:4n-6 contained in the PC phospholipid fractions showed that the increase in FFA C20:4n-6 in plasma could have been the result of increased release from RBC membrane PC C20:4n-6, even possibly more so than from plasma PC C20:4n-6. Similarly, Craelius et al. (1981) reported phospholipid PC C20:4n-6 lower in relation to FFA C20:4n-6 in the white matter of MS patients as compared to that of control brains. C20:4n-6 is released from membrane phospholipids by the enzyme, phospholipase A2 (PLA2) and an increase in PLA2 activity is associated with neurological disorders such as MS (Farooqui et al. 2007). Recent studies (Harbige and Sharief, 2007) have shown an increase in pro-inflammatory cytokines TNF- α and IL-1 β and a decrease in anti-inflammatory cytokine TGF- β in PBMCs of MS patients, together with a decrease in membrane C20:4n-6, indicating a decrease in membrane C20:4n-6 to be part of the immune response. These findings could possibly explain the lower C20:4n-6 in RBC membranes from MS patients previously reported by this group (Hon et al. 2009).

Fatty acids in cell membranes are incorporated into phospholipids, while in plasma they are in the form of neutral triglycerides (fat) or phospholipids contained in lipoproteins (Horrobin, 1999; Koay and Walmsley, 1999). Fatty acids that are released from these lipid stores are called FFAs or non-esterified fatty acids. FFAs are toxic to surrounding cells (Leaf, 2001), and in healthy persons, the release of FFAs from the lipid stores is tightly regulated. In addition, because FFAs in plasma are from lipid stores, the concentration of plasma FFAs is little affected by the immediate dietary consumption of fat (Leaf, 2001). The concentration of FFAs in plasma can vary, depending on hormonal, metabolic and nutritional status (Leaf, 2001). In this study, dietary fat intake was not considered; however, participants were on an overnight fast before blood was collected, in order to minimize possible interference with fatty acid measurements as much as possible.

It is not known whether the increased FFAs in the plasma from MS patients are involved in the aetiology of MS, or whether they are a consequence of the disease process. Possible explanations for an increase in FFA C20:4n-6 from MS plasma includes dysregulation with re-uptake of the FFA into phospholipids, or, in view of the inflammatory nature of MS, could be due to an active enzymatic release of C20:4n-6 from the phospholipids, produced to facilitate an increased production of eicosanoids. In this regard, the n-6 fatty acids, C20:3n-6 and C20:4n-6, and the n-3 fatty acids, C20:5n-3 and C20:6n-3, are precursors for cell-signaling eicosanoids (which include the prostaglandins) and which, together with cytokines mediate the immune response (Horrobin and Manku, 1990; Calder, 2001; Bagga et al. 2003). Prostaglandin E2 (PGE2) is metabolized from FFA C20:4n-6, and is associated with inflammation. The amplification of PGE2 may be active in brain pathologies such as MS (Repovic et al. 2003). In this regard, Hofman et al. (1986) reported immune cells from MS brain lesions which stained positive for prostaglandin E (PGE), which was not found in normal brain tissue.

The production of eicosanoids is known to be markedly increased during inflammation, and because membrane phospholipids contain high concentrations of C20:4n-6, this fatty acid is used as a major precursor for eicosanoid production, including that of PGE2 (Calder, 2001; Calder, 2007). RBCs with diminished fluidity are specifically vulnerable to hydrolysis by sPLA2 (Harris et al. 2001). RBCs are also known to respond to PGE2, with a decrease in deformability (Allen and Rasmussen, 1971; Harris et al. 2001). The sensitivity of these cells to prostaglandins suggests that RBCs may be a primary target for prostaglandin action (Allen and Rasmussen, 1971). However, prostaglandins are short-lived substances and therefore measurements of the levels of eicosanoids in body fluids are difficult (Horrobin and Manku, 1990). Measuring the fatty acid precursors for these substances can therefore be used as a substitute in an initial investigation to establish possible changes in fatty acid composition.

Whether increased FFA C20:4n-6 in the plasma from MS patients is part of an altered inflammatory response, or the result of impaired metabolism of fatty acids, an increase in FFA C20:4n-6 in the plasma can cause damage to surrounding cells. An excess of circulating FFAs has been implicated in sudden cardiac death, insulin resistance, atherosclerosis and hypertension (Pilz et al. 2007; Blaak, 2003; Oram and Bornfeldt, 2004; Sarafidis and Bakris, 2007). There is evidence that C20:4n-6 acid acts via generation of free radicals in the course of its metabolism by cyclooxygenase and lipoxygenase pathways (Abbott, 2000). In particular, a product of C20:4n-6 metabolism, reactive oxygen species (ROS), react with cellular lipids to generate lipid peroxides and reactive aldehydes, which induce cellular damage by binding to proteins and alter their function (Adibhatla and Hatcher, 2008). C20:4n-6 is also elevated in some neural pathologies and causes opening of the blood-brain barrier to large molecules including proteins (Abbott, 2000).

The PC n-3 fatty acids and n-3 FFAs in the plasma from MS patients did not differ from that of the control subjects and neither did the n-3 fatty acids in RBC membranes from MS patients (Hon et al. 2009a). However, in the present study total n-3 FFAs were increased ($P = 0.04$), possibly indicating an increased turnover of the n-3 fatty acids to less inflammatory eicosanoids as well. In this regard, prostaglandins derived from the metabolism of the n-3 fatty acids are anti-inflammatory, in contrast to the pro-inflammatory nature of those derived from C20:4n-6 metabolism (Simopoulos, 2002; Calder, 2007).

Only female patients were used in this study, as the hormonal status may have an effect on FFA concentrations in plasma (Leaf, 2001). Selecting female patients only may have resulted in a more homogenous study population. The limitation of this study was that dietary fatty acid intake was not considered, however, the effect of dietary factors on the concentration of FFAs in plasma is possibly not as substantial as their release from membrane phospholipids,

Conclusion

The cause of MS is unknown, but the disease is characterized by increased immune cell activity and inflammation of the CNS. The increased ratios of plasma FFA C20:4n-6 to both plasma and RBC PC C20:4n-6 from patients with MS indicated excess release of this fatty acid from phospholipids. RBC membranes are known to be a primary target for PLA2 activity, the enzyme that cuts C20:4n-6 from membrane phospholipids during prostaglandin production, and C20:4n-6 is an important precursor for PGE2 synthesis. The inverse correlation between RBC C20:4n-6 and plasma FFA C20:4n-6 may therefore be interpreted as an increased release of C20:4n-6 from RBCs as a result of inflammation. In this regard, prostaglandin production is increased during inflammation, and the abnormal presence of prostaglandin in brain tissue from MS patients has been reported. Taken together, these

results suggest that the decrease in membrane C20:4n-6 and increase in plasma FFA C20:4n-6, as measured by the inverse correlation between these fatty acids, could be related to the pathogenesis of the disease.

Acknowledgements

We would like to extend our sincere gratitude to the following: MS Society, Western Cape Branch, South Africa and sister Treska Botha for the recruitment of patients and Johanna van Wyk for technical support in the analysis of fatty acids. This study was funded by a grant from the University Research Fund of the Cape Peninsula University of Technology, South Africa.

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Table 4.I: Differences between multiple sclerosis patients and control subject in plasma fatty acids and free fatty acids

	Controls; N = 30	MS patients; N = 31	P-value
	Median (quartile range)		
Plasma n-6 fatty acids			
PC C18:2n-6	218.0 (59.8)	202.2 (58.7)	0.30
PC C20:3n-6	38.3 (13.6)	33.3 (20.3)	0.47
PC C20:4n-6	103.5 (42.3)	99.2 (30.0)	0.06
PC C22:4n-6	3.47 (1.54)	3.43 (1.63)	0.48
Total PC n-6 fatty acids	374.3 (118.5)	354.2 (107.6)	0.17
Plasma n-6 free fatty acids			
C18:2n-6	12.95 (5.75)	19.61 (12.16)	0.00288
C20:4n-6	0.82 (0.30)	0.92 (0.50)	0.00682
C22:4n-6	0.70 (0.35)	0.64 (0.33)	0.97
Total n-6 free fatty acids	15.06 (5.76)	21.04 (11.80)	0.00318
Plasma n-3 fatty acids			
PC C20:5n-3	6.68 (5.06)	5.64 (4.08)	0.62
PC C22:6n-3	35.69 (17.09)	38.89 (26.16)	0.64
Total PC n-3 fatty acids	52.24 (20.80)	53.06 (31.27)	0.98
Plasma n-3 free fatty acids			
C22:6n-3	0.60 (0.20)	0.72 (0.41)	0.10
Total n-3 free fatty acids	1.05 (0.60)	1.39 (0.87)	0.04677

FFAs: free fatty acids

FAs: fatty acid

Plasma FAs and FFAs were quantified in µg/ml plasma

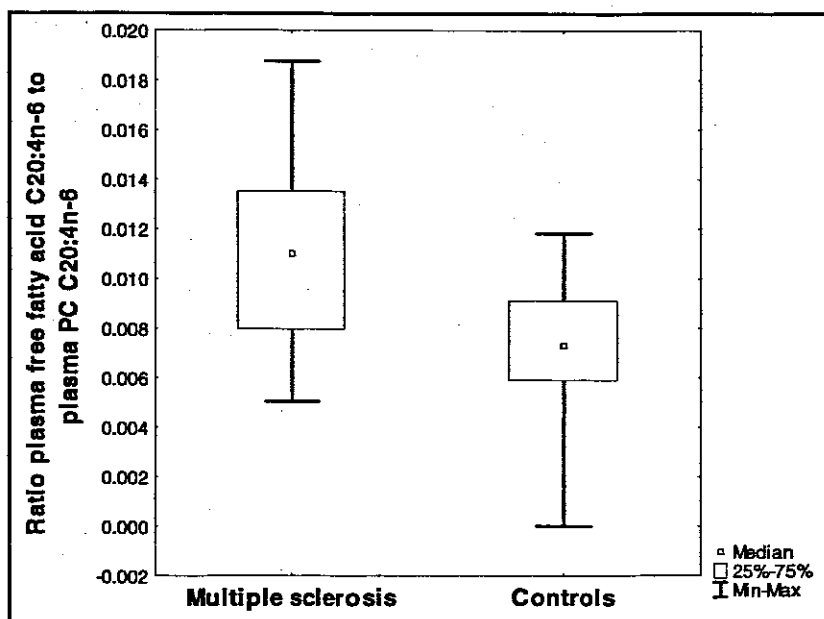


Figure 4.1: Difference between multiple sclerosis patients and control subjects in the ratio plasma free fatty acid C20:4n-6 to plasma PC C20:4n-6 (MS patients: 0.011 (0.006); controls: 0.007 (0.003); P = 0.00008)

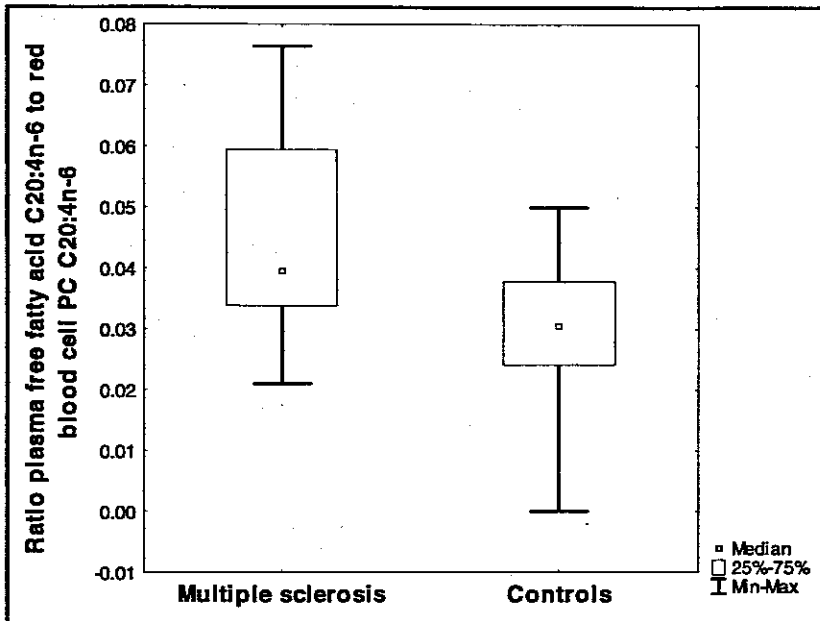


Figure 4.2: Difference between multiple sclerosis patients and control subjects in the ratio plasma free fatty acid C20:4n-6 to red blood cell PC C20:4n-6 {MS patients: 0.039 (0.026); controls: 0.031 (0.014); P = 0.00051}

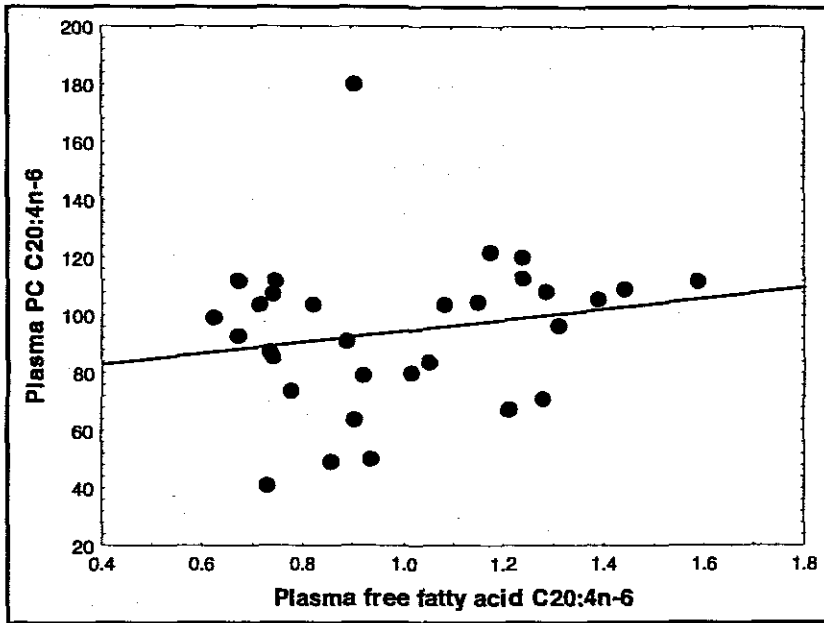


Figure 4.3: Insignificant positive correlation between the plasma PC C20:4n-6 and plasma free fatty acid C20:4n-6 from multiple sclerosis patients ($R = 0.23$; $P = 0.22$)

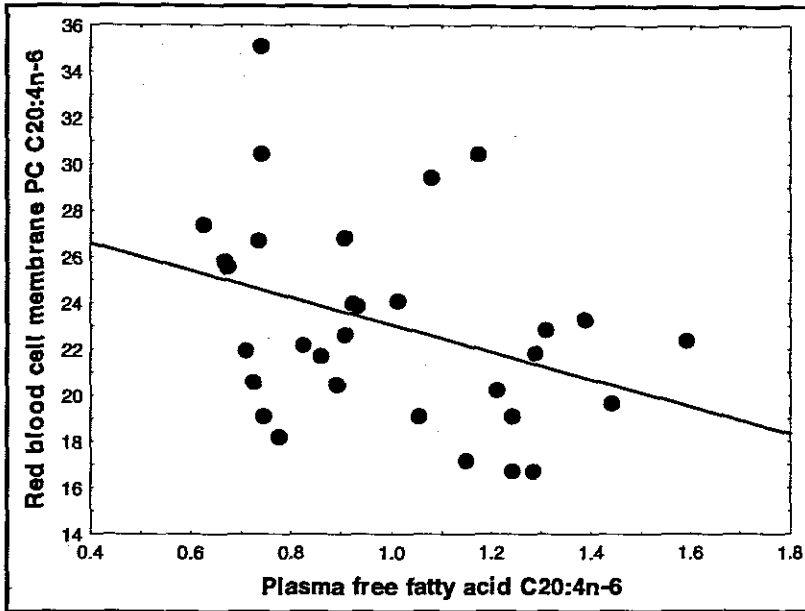


Figure 4.4: Significant inverse correlation between the red blood cell membrane PC C20:4n-6 and plasma free fatty acid C20:4n-6 from multiple sclerosis patients ($R = -0.36$; $P = 0.04$)

CHAPTER FIVE

MEMBRANE SATURATED FATTY ACIDS AND DISEASE PROGRESSION IN MULTIPLE SCLEROSIS PATIENTS

5.1 Introduction

The article "Membrane saturated fatty acids and disease progression in Multiple Sclerosis patients" has been submitted to the Journal of Metabolic Brain Disease. It is currently under review.

5.2 Letter of submission received from the Journal of Metabolic Brain Disease

>>> "Vivienne Russell" <Vivienne.Russell@uct.ac.za>

2009/03/06 06:22 PM

>>>

Dear Tandi Matsha

Thank you for submitting your manuscript to Metabolic Brain Disease for consideration for publication in the special issue.

I wish to confirm that I received a manuscript entitled "Membrane saturated fatty acids and disease progression in Multiple Sclerosis patients" by G.M. Hon, M.S. Hassan, S.J. van Rensburg, S. Abel, R.T. Erasmus, and T. Matsha. The manuscript will be sent to two reviewers for peer-review.

With best wishes

Viv

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Title page

Title: Membrane saturated fatty acids and disease progression in Multiple Sclerosis patients

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Running head: Saturated fatty acids in Multiple Sclerosis.

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Abstract

The risk of developing multiple sclerosis is associated with increased dietary intake of saturated fatty acids. We determined the fatty acid composition within the different phospholipid fractions of red blood and peripheral blood mononuclear cell membranes of 31 patients diagnosed with multiple sclerosis and 30 healthy control subjects using gas chromatography. Individual saturated fatty acids were correlated with the severity of neurological outcome as measured by the Kurtzke Expanded Disability Status Scale. Significant increases were found in multiple sclerosis peripheral blood mononuclear cell membrane sphingomyelin C14:0 and phosphatidylinositol C22:0. In the peripheral blood mononuclear cell membranes, C22:0 and C24:0 showed positive correlations, while C14:0, C16:0 and C20:0 showed inverse correlations with the Functional System Scores. In conclusion, this study is in accordance with previous studies that have shown an increase in shorter long-chain SATS short-chain SATS in MS patients. In addition this study also showed that higher C14:0 and C16:0 reflected better disease outcome as demonstrated by the inverse correlation with the EDSS and FSS. We have also characterized the specific SATS, that is, long-chain SATS that may increase the risk of developing MS.

Key words

Multiple sclerosis, saturated fatty acids, Expanded Disability Status Scale, C-reactive protein

Introduction

Saturated fatty acids (SATS) are non essential and harmful if ingested excessively (Zamaria, 2004) and the risk of developing multiple sclerosis (MS) is associated with increased dietary intake of these fatty acids (FAs) (Van Meeteren et al. 2005). However, in a prospective study done by Zhang et al. (2000), no evidence was found that the amount and type of dietary fat affect the risk of developing MS (Zhang et al. 2000; Westlund and Kurland, 1953; Butcher, 1986; Antonovsky et al. 1965; Cendrowski et al. 1969; Warren et al. 1982). Saturated fatty acids play a variety of roles within cell membranes, such as membrane protein binding (myristoylation and palmitoylation) (Okubo et al. 1991; Grand, 1989), and have been shown to display anti-viral and -bacterial properties (Narasimhan et al. 2006; Cordo et al. 1999; Sands, 1977). In this regard, MS is a disease characterized by chronic inflammation of the central nervous system (CNS) (Brück), with a possible autoimmune (Stinissen et al. 1997) and/or infectious component (Brown, 2001; Hunter and Hafler, 2000).

Studies on the red blood cell (RBC) and peripheral blood mononuclear cell (PBMCs) membranes have shown that membrane FAs, especially that of the n-6 series are altered in MS patients (Hon et al. 2009a; Hon et al. 2009b; Harbige and Sharief, 2007). We have also shown previously that both SATS and polyunsaturated fatty acids (PUFAs) in membrane total phospholipids are related to cell membrane changes in MS patients (data unpublished). Studies on the red blood cell (RBC) and peripheral blood mononuclear cell (PBMCs) membranes have shown that membrane FAs, especially that of the n-6 series, are altered in MS patients (Hon et al. 2009; Harbige and Sharief, 2007). We have also shown previously that both SATS and polyunsaturated fatty acids (PUFAs) in membrane total phospholipids are related to cell membrane changes in MS patients (data unpublished). In the present study we investigated the RBC and PBMC membrane SATS composition in the different phospholipid fractions in MS patients and control subjects for possible differences between the two groups, and correlated that of the MS patients against the disability status of the patients as measured by the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). In addition the C-reactive protein (CRP) concentrations were used as a marker of inflammation.

Materials and methods

Ethical approval

Ethical approval for the study was obtained from the Health and Applied Sciences Research Ethics Committee (HASREC) of the Cape Peninsula University of Technology (CPUT). MS patients were contacted and recruited through the MS Society, Western Cape Branch, South Africa.

Study population

The study population consisted of 31 Caucasian female patients, 2 male patients and 30 age- and gender-matched female control subjects. Male patients were excluded from the study as the numbers were insufficient for meaningful statistical analysis leaving 31 patients for the study of which 28 patients presented with relapsing remitting MS (RRMS), 1 with primary progressive MS (PPMS) and 2 with secondary progressive MS (SPMS). The patients recruited were diagnosed by a neurologist based on clinical, laboratory and magnetic resonance imaging (MRI) findings. The exclusion criteria used in this study included the use of FA supplements, interferon and cortisone or presence of a second disease for both MS patients and control subjects. Six of the patients were active disease cases, 11 had a relapse 5-12 months previously and 14 had not relapsed for more than a year. The median (quartile range) for years since diagnoses was 7 (11) years and the median (quartile range) for the EDSS was 5.5 (3.5).

Measurement of the disability status of patients

The functional disability status (disease severity) of each patient was measured by a trained clinician using the Kurtzke EDSS (Kurtzke, 1983). The EDSS quantifies disability in eight Functional Systems (FS) and allows neurologists to assign a Functional System Score (FSS) in each of them. The Functional Systems are Pyramidal, Cerebellar, Brainstem, Sensory, Bowel and bladder, Visual, Cerebral and "other". Higher values indicate greater disability. Scales for the Total Kurtzke EDSS are from 0 to 10, in which the 0 score indicates no disability at all and 10 indicates death due to MS.

Blood sample processing and analysis

Venous blood from participants was collected into anti-coagulant ethylenediaminetetraacetic acid (EDTA) tubes (Beckman Coulter, South Africa) and immediately separated using histopaque-1077 separation medium as per manufacturer's instructions (Sigma-Aldrich, South Africa). The RBCs and PBMCs were washed in a 0.85% saline solution and stored at -80°C. The plasma was used for CRP determination, using a Beckman nephelometer auto-analyser, reagents from Beckman, South Africa. The PBMC membrane PC, PE, PS, PI and

SM FAs and RBC membrane PC, PE, PS and SM FAs of MS patients and control subjects were measured by gas chromatography as previously described (Van Jaarsveld et al. 2000; Folch et al. 1957) and percentages of fatty acids were measured against an internal standard, C17:0. PBMC FAs were quantified against membrane proteins present in μg FA/mg protein, and RBC membrane FAs as μg FA/ml packed RBC analyzed. A bicinchoninic acid protein determination assay was used to determine the protein content in the PBMC membranes (Kaushal and Barnes, 1986).

Statistical analysis

A statistics programme, STATISTICA (STATISTICA 7, StatSoft Inc 1984 – 2004) was used to perform all statistical analyses. Descriptive data are presented as median (inter-quartile range). For asymmetrical data Mann Whitney U was used to compare distributions between the cases and control subjects. Correlations were calculated using Spearman's Rank correlation coefficient. A P-value of less than 0.05 was taken as statistically significant.

Results

Results are reported on phospholipid PC, PE, PS, PI and SM SATS in MS and control RBC and PBMC membranes, the individual SATS in each phospholipid fraction (e.g. SM C16:0), as well as on the individual FA fractions totaled (e.g. total C16:0). The clinical profile of patients and controls as well as differences in membrane FAs between these groups are summarized in Table 1. In the PBMC membranes, SM total SATS, SM C14:0, total C14:0 and PI C22:0 were significantly higher in MS than in the control group, while no differences were observed in PBMC PC, PE, PS, PI SATS, or in any of the RBC membrane SATS.

Correlation studies

Correlations between membrane SATS and the EDSS and FSS in MS are summarized in Table 2. PBMC PI and SM total SATS correlated positively with the FSS. PBMC membrane PC C14:0 correlated inversely with the EDSS and PC C14:0, PC C16:0, PE C20:0, PI C20:0, total C14:0 and total C16:0 correlated inversely with the FSS, while PI C22:0 and PC C24:0 correlated positively. RBC PC total SATS correlated inversely with the Pyramidal FSS and positively with the Visual FSS. RBC PC C16:0, PS C16:0, SM C16:0, total C16:0 correlated inversely with the FSS, while RBC PE C16:0, PC C18:0, SM C18:0, PC C22:0 and total C18:0 correlated positively with the FSS.

Discussion

In the present study we found PBMC SM total SATS, SM C14:0 and PI C22:0 higher in MS than in the controls. Previously, we have shown in the same group of patients reduced PUFAs in RBC and PBMC membranes (Hon et al. 2009a; Hon et al. 2009b). Similar to our study, other investigators have also shown increased SATS in MS patients, particularly shorter long-chain SATS (Holman et al. 1989; Boggs and Moscarello, 1980). Furthermore, increases in SATS and/or MUFAs have been reported to replace plasma and/or RBC membrane PUFA deficiencies (Cherayil, 1984; Cunnane et al. 1989; Holman et al. 1989; Navarro & Segura, 1989). Increased dietary SATS intake is associated with an increased risk of developing MS ((van Meeteren et al. 2005), however SATS play a vital role in metabolism. For example, cellular membrane proteins need to be modified by (saturated) fatty acid acylation to be functional (Okubo et al. 1991). C14:0 (myristic acid) and C16:0 (palmitic acid) are respectively used in fatty acylation; myristoylation and palmitoylation (Okubo et al. 1991; Grand, 1989). C14:0 is found only in small amounts, while C16:0 is a common metabolite in eukaryotic cells, including the human RBC membrane proteins (Okubo et al. 1991). In addition, C14:0 has also been shown *in vitro* to have antibacterial activity (Narasimhan et al. 2006), to inhibit virus replication (Cordo et al. 1999) and both C14:0 and C16:0 were found to prevent bacteriophage plaque production (Sands, 1977).

Multiple sclerosis is a chronic inflammatory disease of the central nervous system (CNS) which leads to neuronal demyelination, and eventually to oligodendrocyte and axon loss (Brück, 2005). The mechanisms and effector molecules involved in axonal degeneration are still unknown (Brück, 2005), but an infectious aetiology is suspected (Brown, 2001; Cooper, 1997). Therefore it is possible that the increase in shorter long-chain SATS observed in this study could be a mechanism against viral replication as viral infections have been implicated in the development of MS (Cooper, 1997). In MS patients C14:0 and C16:0 insignificantly correlated inversely with CRP, the measure of inflammation (data not shown). In addition, these two SATS correlated negatively with EDSS and FSS. The relationship between individual SATS and EDSS demonstrated that not all SATS have the same association with disease outcome. Individual shorter long-chain SATS PC, SM and PS C14:0, C16:0, total C14:0 and total C16:0 showed inverse correlations with the EDSS and FSS. In contrast, longer chain PBMC SATS PI C22:0, PC C24:0, RBC PC C18:0, SM C18:0 and PC C22:0 showed positive correlations with the FSS. These correlations with the EDSS and/or FSS were not limited to any specific phospholipid type, but rather appeared to be a characteristic of the specific SATS themselves. Currently available evidence suggested that an increase in SATS is associated with an increased risk of developing MS, while increased PUFAs are thought to improve disease outcome (Van Meeteren et al. 2005). However, case-control studies have failed to demonstrate an association between intake of animal fat or saturated

fat and the risk of MS (Zhang et al. 2000; Westlund and Kurland, 1953; Butcher, 1986; Antonovsky et al. 1965; Cendrowski et al. 1969; Warren et al. 1982). Furthermore, there is paucity of understanding of the role of specific SATS and different types of SATS have been shown to have different health effects (German and Dillard, 2004).

The limitation of this study was that only female patients were used and dietary fatty acid intake was not considered. However, neither the cases nor the controls were on any fatty acid supplements, and the patients were not on interferon or corticosteroid treatment. In conclusion, this study is in accordance with previous studies that have shown an increase in shorter long-chain SATS in MS patients. In addition, this study also showed that higher C14:0 and C16:0 were associated with better disease outcome as demonstrated by the inverse correlation with the EDSS and FSS. We have also characterised the specific SATS, that is, long-chain C20:0, C22:0 and C24:0 that may increase the risk of developing MS. However, more research is needed before any conclusions as to dietary intake can be made, as MS is a complex disease. In this regard, irregularities in MS plasma and membrane n-6 fatty acids have also been reported by a number of investigators, showing major metabolic changes in the fatty acid profile in MS membranes in general.

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Table 5.J: Differences between MS and controls in PBMC and RBC membrane SATS

	Control subjects; N = 30	MS patients; N = 31	P-value
Age in years	50.0 (23.0)	51.0 (23.0)	0.77
Years since diagnoses	Not applicable	7 (11)	
EDSS	Not applicable	5.50 (3.50)	
CRP	3.40 (3.80)	3.80 (4.30)	0.28
PBMC membranes			
SM C14:0	0.03 (0.05)	0.06 (0.05)	0.01
SM C16:0	3.12 (1.06)	3.52 (0.82)	0.06
PI C20:0	0.05 (0.02)	0.05 (0.02)	0.10
PI C22:0	0.05 (0.04)	0.06 (0.05)	0.04
Total C14:0	0.12 (0.04)	0.18 (0.07)	0.01
Total C16:0	16.85 (2.30)	17.53 (2.86)	0.34
Total C18:0	18.88 (1.53)	18.52 (2.69)	0.69
Total C20:0	1.49 (0.30)	1.62 (0.54)	0.11
Total C22:0	3.44 (1.18)	3.55 (0.83)	0.36
Total C24:0	1.82 (0.37)	1.71 (0.56)	0.95
Total PC SATS	21.46(3.76)	21.97 (2.99)	0.98
Total PE SATS	7.91 (0.80)	7.88 (0.85)	0.65
Total PS SATS	5.07 (0.68)	5.22 (1.05)	0.53
Total PI SATS	4.17 (0.71)	4.42 (0.57)	0.33
Total SM SATS	10.54 (1.74)	11.38 (1.93)	0.03
RBC membranes			
Total C14:0	2.22 (1.10)	1.99 (1.03)	0.84
Total C16:0	185.59 (43.33)	183.84 (34.49)	0.48
Total C18:0	120.77 (41.40)	115.76 (41.45)	0.59
Total C20:0	4.57 (1.34)	4.17 (1.57)	0.43
Total C22:0	19.37 (3.54)	17.73 (4.10)	0.17
Total C24:0	54.71 (15.42)	49.47 (15.81)	0.25
Total PC SATS	144.37 (36.71)	140.80(29.29)	0.87
Total PE SATS	60.48 (10.73)	59.82(21.48)	0.88
Total PS SATS	48.02(22.01)	42.97(21.46)	0.19
Total SM SATS	125.76(27.83)	118.40(28.88)	0.33

PBMC FAs quantified in μg FA/mg protein

RBC FAs quantified in μg FA/ml packed RBC

Table 5.2: Correlation between MS SATS and the EDSS and FSS

		R	P-value
PBMC membranes			
PC C14:0	EDSS	-0.41	0.04
PC C14:0	Cerebral	-0.55	0.01
PC C16:0	Cerebral	-0.44	0.03
PE C20:0	Visual	-0.47	0.02
PI C20:0	Sensory	-0.41	0.04
PI C22:0	Bowel and bladder	0.41	0.04
PC C24:0	Pyramidal	0.42	0.03
Total C14:0	EDSS	-0.52	0.01
Total C14:0	Pyramidal	-0.43	0.03
Total C16:0	Cerebral	-0.45	0.02
Total PI SATS	Bowel/bladder	0.47	0.02
Total SM SATS	Brainstem	0.46	0.02
Total SM SATS	Visual	0.49	0.01
RBC membranes			
PC C16:0	Pyramidal	-0.52	0.01
PS C16:0	Pyramidal	-0.52	0.01
SM C16:0	Sensory	-0.38	0.04
PE C16:0	Cerebellar	0.36	0.04
PC C18:0	Visual	0.41	0.02
SM C18:0	Visual	0.37	0.04
PC C22:0	Visual	0.37	0.04
Total C16:0	Pyramidal	-0.36	0.04
Total C16:0	Sensory	-0.36	0.04
Total C18:0	Visual	0.43	0.01
Total PC SATS	Pyramidal	-0.47	0.01
Total PC SATS	Visual	0.36	0.04

CHAPTER SIX

RED BLOOD CELL MEMBRANE FLUIDITY IN THE AETIOLOGY OF MULTIPLE SCLEROSIS

6.1 Introduction

The article "Red blood cell membrane fluidity in the aetiology of multiple sclerosis" has been submitted to the Journal of Membrane Biology. It is currently under review.

6.2 Letter of submission received from the Journal of Membrane Biology

Subject: Fwd: Journal of Membrane Biology - Manuscript ID JMB-H-09-0015

>>> <twoolf@jhu.edu> 2009/02/18 01:08 PM >>>18-Feb-2009

Dear Dr. Matsha

Your manuscript entitled "Red blood cell membrane fluidity in the aetiology of Multiple Sclerosis" has been successfully submitted online and is presently being given full consideration for publication in the Journal of Membrane Biology.

Your manuscript ID is JMB-H-09-0015.

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Title page

Title of article: Red blood cell membrane fluidity in the aetiology of Multiple Sclerosis

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Abstract

Background: Organisms adjust the order, or fluidity, of their cellular membranes, in response to changes in their physiochemical environment, by adjusting the lipid composition of their membranes.

Objective: To investigate membrane fluidity using the phospholipid, fatty acid and cholesterol content of red blood cells from multiple sclerosis patients and to correlate this with C-reactive protein, as well as with the severity of neurological outcome as measured by the Kurtzke Expanded Disability Status Scale and its Functional System Scores.

Methods: The study group consisted of 31 patients with multiple sclerosis and 30 healthy control subjects. Phospholipids were determined using a colorimetric assay, fatty acids by gas chromatography, cholesterol by an enzymatic assay and C-reactive protein by a Beckman nephelometer. Cell membrane fluidity was calculated according to previously established formulae.

Results: Red blood cell membrane fluidity as measured by the saturated to polyunsaturated fatty acid ratio was higher in patients than in controls ($P = 0.04$). The phosphatidylethanolamine saturated to polyunsaturated fatty acid ratio showed highly significant positive correlations with the EDSS and $CRP < 5 \mu\text{g/ml}$. C-reactive protein showed significant inverse correlations with the saturated nature, but positive correlations with the ordered-crystalline-phase to liquid-crystalline-phase lipid ratio.

Conclusion: In this study we have shown that membrane fluidity as measured by the relationship between membrane fatty acids, phospholipids and cholesterol are closely interrelated with inflammation and disease outcome in patients with multiple sclerosis. In conclusion, our findings suggest that the membrane lipid composition of patients with multiple sclerosis is altered, and consequently membrane fluidity, which seems to be influenced by the inflammatory status.

Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (Ohler et al. 2004; Brück, 2005), in which a decreased lipid to protein ratio has been reported (Göpfert et al. 1980; Wilson & Tocher, 1991). Loss of polyunsaturated fatty acids (PUFAs) from plasma and blood cell membranes has also been reported, especially that of the n-6 fatty acids, C18:2n-6 and C20:4n-6 (Harbige & Sharief, 2007). These fatty acids are precursors for inflammatory messengers, eicosanoids, and because MS is an inflammatory disease, the loss of membrane PUFAs could be part of the pathogenesis of the disease. Although the membrane fatty acid composition has been studied in the brain and blood cells from patients with MS, there is scarcity of literature on the fluidity status of blood cell membranes, including that of red blood cells (RBCs). Decreases in PUFAs are replaced by saturated fatty acids (SATS) (Horrobin, 1999), and could therefore be expected to contribute to changes in membrane fluidity in patients with MS. However, studies have shown little differences in membrane fluidity in patients with MS. Chia et al. (1984) found no significant differences in the SATS to PUFA ratios in myelin from patients with MS and normal myelin, although the authors reported some changes in the fatty acid composition. Furthermore, using different techniques such as electron spin resonance (ESR) and fluorescence polarization spectroscopy, no significant differences were observed between the fluidity or deformability of RBC membranes from patients with MS or that of control subjects (Boggs & Moscarello, 1980; Kurantsin-Mills et al. 1982; Pollock et al. 1982). Using wide-angle x-ray diffraction Chia et al. (1984) was able to show significant differences in the physical organization of the myelin lipid bilayer. They could detect liquid-crystalline-phase lipids as well as ordered-crystalline-phase lipids in membranes, which were not discernible by ESR, and which showed a changed transition temperature in patients with MS (Chia et al. 1984).

Biological cell membranes are composed of lipids and protein (Caret et al. 1997), and are involved in a variety of cellular functions (Williams, 1998). Membrane phospholipids differ in their head-groups and hydrocarbon chains (Hazel & Williams, 1990; Williams, 1998; Barenholz, 2002). Their polar head-groups can be choline, ethanolamine, serine, inositol, inositol phosphates or glycerol (Koay & Walmsley, 1999). Choline containing phospholipids, phosphatidylcholine (PC), is the most abundant phospholipid in animal cell membranes, and phosphatidylethanolamine (PE) the second most (Williams, 1998), with sphingomyelin (SM) also contributing significantly to the membrane phospholipid composition (Barenholz & Thompson, 1999). Phospholipids PC and SM are contained on the outer leaflet of the membrane, while PE and phosphatidylserine (PS) are on the inside (Williams, 1998; Koay & Walmsley, 1999). PE phospholipids are ordered-crystalline-phase lipids and can pack closely in membranes, while PC phospholipids are liquid-crystalline-phase lipids, and do not pack

close in the membrane (Harlos & Eibl, 1981; Williams, 1998; Hamai et al. 2006). A combination of ordered-crystalline-phase lipids and liquid-crystalline-phase lipids are needed in cell membranes to regulate membrane fluidity.

The type of fatty acids contained within the different phospholipid fractions are closely related to the biological features of the phospholipids (Manzoli, 1970). The fatty acids can be saturated or unsaturated (mono- or poly-), and the polyunsaturated fatty acids (PUFAs) are subdivided into different series, the n-9, n-6 and n-3 subtypes (Koay & Walmsley, 1999). Fatty acid desaturation has an important function in changing membrane fluidity in all cells (Allen et al. 2006). The double carbon-carbon bonds introduced into the single carbon-carbon bonds of SATS, makes unsaturated fatty acids more angled and flexible and make the carbon chain more mobile. The more double bonds there are, the more fluid, flexible and apparently disordered does the phospholipid molecule become (Horrobin, 1999). PUFAs have two or more double carbon-carbon bonds, and both monounsaturated fatty acids (MUFAs) and PUFAs have a range of chain lengths. In addition, sterols such as cholesterol also contribute to the regulation of membrane fluidity and permeability, mainly because its steroid ring system minimise free volume in the membrane (Voet & Voet, 1995; Caret et al. 1997; Barenholz, 2002).

Organisms adjust the order, or fluidity, of their cellular membranes in response to changes in their physiochemical environment (Hazel & Williams, 1990; Williams, 1998; Barenholz, 2002). They do this by changing the membrane lipid composition, and therefore changes in this composition may be an indication of disease. Cells can change their membrane lipid composition to make it more ordered or more fluid (Labrousche et al. 1996; Williams, 1998; Allen et al. 2006). This includes balancing the ratio of ordered-crystalline-phase lipids (PE) and liquid-crystalline-phase lipids (PC) (Harlos & Eibl, 1981; Chia et al. 1984; Williams, 1998), as measured by the phospholipid PE to PC ratio (Williams, 1998). Environmental changes may also result in changes in the membrane saturated nature, as measured by the phospholipid PC + SM/PE+ PS ratio, as well as in the cholesterol to phospholipid ratio (Allen et al. 2006). The phospholipid chains (fatty acids) may also become more ordered or more fluid, by varying the SATS to PUFA ratio.

Membrane lipid changes can also result in changes in RBC membrane fluidity and deformability, which is important to these cells when passing through small capillaries (Allen et al. 2006; Labrousche et al. 1996). Changes in RBC membrane deformability could compromise oxygen delivery, and could therefore contribute to disease outcome. RBC deformability correlates with the phospholipid PE to phospholipid phosphatidylserine (PS) ratio (Allen et al. 2006). Maintaining a balanced degree of membrane fluidity is important to

RBCs, because these cells are vulnerable to hydrolysis by serum phospholipase A2 (sPLA2) and a target for prostaglandin action with diminished fluidity and deformability (Allen & Rasmussen, 1971; Harris et al. 2001). These changes in membrane lipid composition may all therefore contribute to changes in membrane fluidity (Allen et al. 2006).

Therefore, the aim of the present study was to investigate whether there would be differences in RBC membrane fluidity and permeability, as measured by the relationship between membrane phospholipids, fatty acids, and cholesterol, between patients with MS and healthy control subjects. Furthermore, to correlate these parameters against the disability status of the patients as measured by the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). C-reactive protein (CRP) concentrations were used as a marker for inflammation and studied for correlation with both possible membrane lipid changes in MS and disease progression.

Materials and methods

Ethical approval

Ethical approval for the study was obtained from the Health and Applied Sciences Research Ethics Committee (HASREC) of the Cape Peninsula University of Technology (CPUT). MS patients were contacted and recruited through the MS Society, Western Cape Branch, South Africa.

Study population

The study population consisted of 31 female patients and 30 age- and gender-matched control subjects. The study population consisted of 31 Caucasian female patients and 30 age- and gender-matched control subjects. Twenty eight patients presented with relapsing remitting MS (RRMS), 1 with primary progressive MS (PPMS) and 2 with secondary progressive MS (SPMS). The patients recruited were diagnosed by a neurologist based on clinical, laboratory and magnetic resonance imaging (MRI) findings. The exclusion criteria used in this study included the use of fatty acid supplements, interferon and cortisone or presence of a second disease for both MS patients and control subjects. Six of the patients reported increased disease activity during the 3 month period prior to the study, but the moderate extent of the exacerbations did not warrant cortisone treatment. Eleven patients had a relapse 5-12 months prior to the study, and 14 had not relapsed for more than a year. The median (quartile range) for years since diagnosis was 7 (11) years.

Measurement of the disability status of patients

The functional disability status (disease severity) of each patient was measured by a trained clinician using the Kurtzke EDSS (Kurtzke, 1983). The EDSS quantifies disability in eight Functional Systems (FS) and allows neurologists to assign a Functional System Score (FSS) in each of them. The Functional Systems are Pyramidal, Cerebellar, Brainstem, Sensory, Bowel and bladder, Visual, Cerebral and "other". Higher values indicate greater disability. Scales for the Total Kurtzke EDSS are from 0 to 10, in which the 0 score indicates no disability at all and 10 indicates death due to MS.

Blood sample processing and analysis

Venous blood from consenting participants was collected into anti-coagulant EDTA tubes (Beckman Coulter, South Africa), and separated using histopaque-1077 separation medium as per manufacturer's instructions (Sigma-Aldrich, South Africa). The RBCs were washed in 0.85% saline solution and stored at -80°C. Plasma CRP was determined using a Beckman nephelometer auto-analyser, using reagents from Beckman, South Africa. Membrane phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine

(PS) and sphingomyelin (SM) were determined using a colorimetric assay as previously described (Smuts et al. 1994; Itaya and Ui, 1966). Phospholipids were quantified according to their phosphate (Pi) content, and were reported as membrane phospholipids in $\mu\text{g Pi/ml}$ packed RBCs.

The fatty acid composition of RBC membrane PC, PE, PS and SM phospholipids of MS patients and control subjects were measured by gas chromatography (GC) as previously described (Van Jaarsveld et al. 2000; Folch et al. 1957) and results were quantified against an internal standard, C17:0. RBC membrane fatty acids were quantified in $\mu\text{g/ml}$ packed RBC analyzed. Membrane cholesterol was determined according to an enzymatic assay and quantified in $\mu\text{g cholesterol/ml}$ packed RBC analyzed (Richmond, 1973).

Factors which were used as possible contributors to changes in membrane fluidity

I. The membrane SATS to PUFA ratio: the quantified SATS and PUFAs in membrane phospholipid fractions were totaled and the SATS to PUFA ratios were calculated and used as an indication of membrane fluidity (Allen et al. 2006).

II. The membrane saturated nature: the membrane saturated nature was calculated according to the phospholipid ratio PC+PS/PE+PS and used as an indication of membrane fluidity (Allen et al. 2006).

III. Cholesterol and cholesterol to total phospholipid ratio: the membrane cholesterol, and cholesterol to total phospholipid ratio was calculated and used as an indication of membrane fluidity and permeability (Voet and Voet, 1995; Barenholz, 2002; Allen et al. 2006).

IV. Membrane ordered-crystalline-phase lipid to liquid-crystalline-phase lipid ratio: the composition of membrane ordered-crystalline-phase lipids to liquid-crystalline-phase lipids was measured by the phospholipid PE (ordered-crystalline-phase lipids) to phospholipid PC (liquid-crystalline-phase lipids) ratio (Chia et al. 1984; Williams, 1998).

V. Rheologic property (deformability): Membrane phospholipid PE to PS ratio was calculated and used as an indication of the deformability of RBCs (Allen et al. 2006).

Statistical analysis: A statistics programme, STATISTICA (STATISTICA 7, StatSoft Inc 1984 – 2004) was used to perform all statistical analyses. Descriptive data are presented as median (quartile range). For asymmetrical data Mann Whitney U was used to compare

distributions between the cases and control subjects. Correlations were calculated using Spearman's Rank correlation coefficient. All statistical analysis was done on all patients as a group as well as a subgroup that comprised of patients with CRP < 5 µg/ml. This was done to correct for the differences in the clinical presentations of patients since relapses in MS are associated with a more generalised inflammation. In view of the sample size a P-value of less than 0.01 was taken as statistically significant in order to correct for multiple testing.

Results

Differences in membrane lipids between patients with multiple sclerosis and control subjects

Red blood cell membrane fluidity as measured by the SATS to PUFA ratio was higher in patients with MS than in controls: MS, median and quartile range: 1.21 (0.15); controls: 1.17 (0.09); $P = 0.038$, but with both SATS and PUFAs marginally lower in patients with MS (Table 1). No significant differences were observed between the cases and the controls in RBC membrane fluidity as measured by its saturated nature (phospholipid PC + SM/PE + PS ratio), cholesterol values or in the membrane ordered-crystalline-phase and liquid-crystalline-phase lipid composition (phospholipid PE to PC ratio) (Table1). The phospholipid SM was higher in patients than controls in the sub-group with CRP $< 5 \mu\text{g/ml}$.

Correlation between C-reactive protein and membrane lipids in patients with multiple sclerosis and control subjects

The CRP showed inverse correlations with the RBC SM SATS to PUFA ratio in patients with CRP $< 5 \mu\text{g/ml}$ (Table 2). The CRP also showed inverse correlations with the saturated nature (phospholipid PC + SM/PE + PS ratio) and phospholipid PI, but positive correlations with the ordered-crystalline-phase to liquid-crystalline-phase lipid ratio (phospholipid PE to PC ratio). The CRP showed a near-significant inverse correlation with cholesterol in both MS patients and controls (Table 2).

Correlation between membrane lipids the Kurtzke Expanded Disability Status Scale in patients with multiple sclerosis

Significant correlations between membrane lipids and the EDSS in patients with MS are summarized in Table 3. The RBC PE SATS to PUFA ratio showed highly significant positive correlations with the EDSS, Pyramidal and Cerebellar FSS in patients with CRP values $< 5 \mu\text{g/ml}$. Membrane deformability as measured by the phospholipid PE to PS ratio, showed a positive correlation with the Sensory FSS, while the membrane ordered-crystalline-phase to liquid-crystalline-phase lipid ratio (phospholipid PE to PC ratio) showed an inverse correlation with the Bowel and bladder FSS in patients with CRP $< 5 \mu\text{g/ml}$. Phospholipids PC, PE as well as total phospholipids, showed inverse correlations with the EDSS.

Figure 1 shows the decrease in red blood cell membrane total fatty acids in patients with MS when CRP $\geq 5.00 \mu\text{g/ml}$; Median (quartile range) when CRP $< 5.00 \mu\text{g/ml}$: 881.8 (231.0); and when CRP $\geq 5.00 \mu\text{g/ml}$: 740.1 (157.6) ; $P = 0.036$. Both membrane total saturated and polyunsaturated fatty acids also showed a decrease in patients with CRP $\geq 5.00 \mu\text{g/ml}$. Saturated fatty acids: when CRP $< 5.00 \mu\text{g/ml}$: 378.4 (93.5) and when CRP $\geq 5.00 \mu\text{g/ml}$:

326.1 (84.8); $P = 0.053$. Polyunsaturated fatty acids: when CRP < 5.00 $\mu\text{g/ml}$: 337.0 (110.7) and when CRP ≥ 5.00 $\mu\text{g/ml}$: 286.1 (29.9) ; $P = 0.032$ (data not shown). The membrane total phospholipids and cholesterol did not show a difference in patients with CRP < 5.00 $\mu\text{g/ml}$ or CRP ≥ 5.00 $\mu\text{g/ml}$. Phospholipids: when CRP < 5.00 $\mu\text{g/ml}$: 1393.4 (588.6) and when CRP ≥ 5.00 $\mu\text{g/ml}$: 1356.0 (154.7) ; $P = 0.49$ and cholesterol: when CRP < 5.00 $\mu\text{g/ml}$: 422.4 (54.7) and when CRP ≥ 5.00 $\mu\text{g/ml}$: 411.0 (39.1); $P = 0.20$.

Discussion and conclusion

In response to changes in their physiochemical environment, organisms adjust the order, or fluidity, of their cellular membranes (Williams, 1998). The most commonly observed alterations are changes in the membrane ordered-crystalline-phase to liquid-crystalline-phase lipid ratio (phospholipid PE to PC ratio) (Williams, 1998), the membrane saturated nature, as measured by the phospholipid PC + SM/PE + PS ratio, the cholesterol to total phospholipid ratio, and the SATS to PUFA ratio; factors which may all contribute to changes in membrane fluidity (Zamaria, 2004; Allen et al. 2006). Various investigators have assessed the levels of fatty acids in a range of biological tissues, such as RBC and peripheral blood mononuclear (PBMC) membranes. Generally, lower levels of PUFAs and increased SATS are reported (Cherayil, 1984; Holman et al. 1989; Navarro & Segura, 1989; Nightingale et al. 1990; Hon et al. 2009a; Hon et al. 2009b; Hon et al. 2009c). In this study, both total SATS and PUFAs were marginally lower in RBC membrane from patients with MS than in controls, however, a decrease in RBC membrane fluidity (higher rigidity) as measured by the increase in the SATS to PUFA ratio, was observed. Previous studies have not shown significant differences between MS and control RBC membrane fluidity and deformability, using different methods, such as electron spin resonance (ESR) (Kurantsin-Mills et al. 1982), and microfiltration (Pollock et al. 1982).

The increase in the SATS to PUFA ratio in the RBC membranes from patients with MS is unlikely to be the result of adjustment by these cells to changes in their environment; but more likely the result of decreases in the PUFAs, C18:2n-6 and C20:4n-6, which have been shown to be reduced in the plasma, platelets, red cells, leucocytes, and cerebrospinal fluid of patients with MS (Harbige & Sharief, 2007). The loss of C20:4n-6 from RBC and PBMC membranes has been shown to correlate with an increase in inflammation as measured by the CRP (Hon et al. 2009a) as well as an increase in pro-inflammatory cytokines, tumor necrosis factor alpha (TNF- α) and interleukin-1beta (IL-1 β) (Harbige & Sharief, 2007) respectively, indicating a possible association with the pathogenesis of the disease. Furthermore, these results indicated strongly that the inflammatory processes are not confined to the brain alone. This is especially important, as RBCs with compromised membrane fluidity and deformability are targeted by PLA2 and PGE2 action, which are released and synthesized respectively during the inflammatory process (Allen & Rasmussen, 1971; Harris et al. 2001).

Multiple sclerosis (MS) is a chronic inflammatory disease characterized by the destruction of myelin (demyelination) with resultant loss of sensory and motor functions and disability (Ohler et al. 2004; Brück, 2005). Previously, we and other investigators have used C-reactive

protein as an inflammatory marker in assessing inflammation in MS patients (Giovannoni et al. 2001; Sellner et al. 2008, Hon et al. 2009b). Hon et al. (2009b) showed that the degree of inflammation may be one of the determinants of the resultant fatty acid concentrations. Similarly, in the present study the total red blood cell membrane fatty acids from patients with MS were decreased in patients with CRP ≥ 5.00 $\mu\text{g/ml}$. Evidence from the literature suggests that lipids, particularly fatty acids, play a role in the pathogenesis of MS (Harbige & Sharief, 2007). Increased dietary SATS intake is associated with an increased risk of developing MS, whilst supplementation with PUFAs is thought to improve disease outcome (van Meeteren et al. 2005). In this regard, RBCs with changed membrane fluidity could be compromised and have diminished oxygen carrying capacities, and thereby affect disease outcome. Similarly, we also found that the PE SATS to PUFA ratio correlated positively with the EDSS and FSS, especially in the subgroup with CRP < 5 $\mu\text{g/ml}$. These results highlight the importance of considering the fatty acids contained in the different phospholipids fractions as well as the inclusion of CRP measurements in the analysis of fatty acid levels in patients with MS.

Phospholipid fractions are used to calculate the saturated nature and ordered- and liquid-crystalline-phase lipid ratios as indications of membrane fluidity (Allen et al. 2006; Chia et al. 1984; Williams, 1998). In this study, no changes were found in membrane fluidity as assessed by its saturated nature (phospholipid PC + SM/PE + PS ratio), its composition of ordered- and liquid-crystalline-phase lipids (phospholipid PE to PC ratio) or membrane cholesterol to phospholipid ratio, between patients and control subjects. Although the membrane saturated nature did not differ between patients and controls, the CRP concentrations in patients with MS correlated inversely with the saturated nature. The saturated nature is measured by the phospholipid PC + SM/PE + PS ratio, therefore these findings may indicate that during increased inflammation the phospholipids on the outer membrane layer becomes progressively displaced from the outer membrane leaflet in relation to that of the inner layer, suggesting damage to the outer layer by the inflammatory processes. Phospholipids PC and SM are situated on the outer leaflet, while PE and PS are on the inner leaflet. PC is present in large amounts with PE the second largest. Furthermore, the CRP demonstrated positive correlation with the phospholipid PE to PC ratio, showing that during higher inflammation there is a trend towards the formation of ordered-crystalline-phase lipids, which would result in a decrease in membrane fluidity.

Although keeping the RBC membrane integrity is of importance, its rheologic properties (deformability) is an important aspect when cells are passing through capillaries, and membrane lipid changes can also result in changes in deformability (Allen et al., 2006; Labrousche et al., 1996). Similar to Pollock et al. (1982) we did not find differences in the

RBC/deformability between MS patients and control subjects. However, the RBC membrane PL ratio PE/PS (deformability) correlated positively with the Sensory FSS in MS patients, indicating that higher deformability (less rigidity), could have resulted in less protection to the cells.

The damaging effect of inflammation on RBCs from patients with MS, is highlighted by the significant decrease in membrane saturated, polyunsaturated and total fatty acids in patients with CRP ≥ 5.00 $\mu\text{g/ml}$ as compared to the concentrations when CRP < 5.00 $\mu\text{g/ml}$ (figure 1). The membrane total phospholipids and cholesterol did not show a difference in patients with CRP < 5.00 $\mu\text{g/ml}$ or CRP ≥ 5.00 $\mu\text{g/ml}$. These results strongly suggest that membrane fatty acids are targeted by inflammatory processes more so than the other membrane lipids, and that this can result in cell death in a disease such as MS in which relapses may occur frequently. These results indicate that decreased lipid weight as shown by a lower lipid to protein ratio in myelin from patients with MS (Göpfert et al. 1980; Wilson & Tocher, 1991), could be reflected in RBC membranes as well. On the other hand, it appears that membrane phospholipids and cholesterol does not differ between patients with CRP < 5.00 $\mu\text{g/ml}$ or CRP ≥ 5.00 $\mu\text{g/ml}$. Furthermore, membrane phospholipids showed inverse correlations with the EDSS and FSS, suggesting a protective role of phospholipids against these conditions.

The limitation of this study was that only female patients were used. The sample size was too small to allow corrections for all the drugs that could influence membrane composition of erythrocytes. However, subjects were grouped according to patients using either non-steroidal anti-inflammatory drugs (NSAIDs) or immunosuppressive medication and no differences were observed in the membrane fluidity between the two groups. As we also did not consider dietary fatty acid intake, which could have affected the lipid levels observed in this study. The main strength of this study is that neither the cases nor the controls were on any fatty acid supplements, and the patients were not on interferon or corticosteroid treatment. However, this resulted in a small samples size, as MS patients not on any of these medications/supplementations, are not easily available. Collectively, we have shown that membrane fluidity as measured by the relationship between membrane fatty acids, phospholipids and cholesterol, are closely interrelated with inflammation and disease outcome in MS patients. In conclusion, our findings suggest that the membrane lipid composition of MS patients is altered, and consequently membrane fluidity, which seems to be influenced by the inflammatory status.

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Table 6.1: Differences in clinical characteristics and in red blood cell membrane fluidity as measured by membrane lipids, between patients with multiple sclerosis and control subjects

	All CRP values			CRP < 5.00 µg/ml		
	Controls; 30	MS; 31	P-value	Controls; 21	MS; 17	P-value
	Median (quartile range)			Median (quartile range)		
Age in years	50.0 (23.0)	51.0 (23.0)	0.77	52.0 (24.0)	55.0 (23.0)	0.75
Years diseased	Not applicable	15 (16)	NA	Not applicable	14.0 (13.0)	NA
EDSS	Not applicable	5.50 (3.50)	NA	Not applicable	5.00 (3.00)	NA
CRP	3.40 (3.80)	3.80 (4.30)	0.28	2.80 (2.80)	2.30 (2.70)	0.40
FATTY ACIDS						
SATS	384.4 (95.9)	354.9 (96.6)	0.46	392.7 (103.7)	378.4 (93.5)	0.75
PUFA	341.5 (100.3)	293.5 (91.8)	0.39	343.1 (106.9)	337.0 (110.7)	1.00
Total fatty acids (+ MUFAs)	881.1 (220.7)	808.1 (239.8)	0.36	913.0 (269.7)	881.8 (231.0)	0.95
FATTY ACID RATIOS						
PC SATS to PUFA	1.25 (0.13)	1.26 (0.13)	0.14	1.23 (0.13)	1.26 (0.13)	0.17
PE SATS to PUFA	0.40 (0.07)	0.43 (0.10)	0.07	0.38 (0.07)	0.42 (0.09)	0.27
PS SATS to PUFA	0.86 (0.08)	0.88 (0.07)	0.17	0.86 (0.06)	0.88 (0.07)	0.14
SM SATS to PUFA	50.4 (24.2)	52.0 (24.8)	0.35	44.3 (24.3)	49.9 (18.9)	0.24
Total SATS to PUFA	1.17(0.09)	1.21 (0.15)	0.038	1.16 (0.07)	1.21 (0.14)	0.15
PHOSPHOLIPIDS						
PC	538.1 (108.6)	528.9 (81.6)	0.96	511.3 (83.9)	526.6 (193.2)	0.43
PE	475.1 (71.9)	472.2 (97.2)	0.43	465.9 (73.6)	471.8 (137.0)	0.58
PS	147.3 (23.3)	146.4 (32.5)	0.86	147.3 (32.0)	145.8 (43.6)	0.68
PI	43.8 (24.8)	44.4 (14.8)	0.72	43.9 (23.8)	48.7 (21.8)	0.12
SM	159.8 (51.2)	176.1 (83.5)	0.12	162.8 (47.8)	194.1 (75.7)	0.026
Total phospholipids	1367.5 (346.3)	1360.5 (365.7)	0.92	1367.5 (326.6)	1393.4 (588.6)	0.42
PHOSPHOLIPID RATIOS						
<i>Saturated nature</i> PC + SM/PE + PS	1.11 (0.21)	1.13 (0.24)	0.25	1.11 (0.21)	1.26 (0.19)	0.05
<i>Deformability</i> PE to PS	3.17 (0.91)	3.32 (0.76)	0.67	3.17 (1.08)	3.37 (0.41)	1.00
<i>Ordered- to liquid-</i> <i>crystalline-phase lipids</i> PE to PC	0.90 (0.21)	0.89 (0.08)	0.98	0.90 (0.23)	0.87 (0.16)	0.27
CHOLESTEROL						
Cholesterol	423.7 (37.8)	420.8 (52.4)	0.84	423.7 (29.0)	422.4 (54.7)	0.50
Cholesterol to phospholipid	0.30 (0.07)	0.30 (0.07)	0.93	0.31 (0.08)	0.32 (0.11)	1.00

Fatty acids are quantified in µg/ml packed cells

Membrane phospholipids are quantified in µg Pi/ml packed RBC

Membrane cholesterol is quantified in µg cholesterol/ml packed RBC

C-reactive protein (CRP) is quantified in µg/ml plasma

Table 6.2: Correlation between C-reactive protein and red blood cell membrane fluidity as measured by membrane lipids, in patients with multiple sclerosis and control subjects

	All CRP values				CRP < 5.00 µg/ml			
	Controls; 30		MS; 31		Controls; 21		MS; 17	
	R	P-value	R	P-value	R	P-value	R	P-value
FATTY ACIDS								
Total SATS	-0.11	0.58	-0.22	0.26	-0.21	0.37	0.12	0.64
Total PUFA	-0.01	0.94	-0.27	0.15	-0.10	0.66	0.08	0.75
Total fatty acids (+ MUFAs)	-0.13	0.50	-0.26	0.17	-0.19	0.40	0.08	0.76
FATTY ACID RATIOS								
PC SATS to PUFA	-0.03	0.88	0.11	0.57	-0.14	0.55	0.02	0.93
PE SATS to PUFA	0.08	0.71	0.30	0.12	-0.13	0.58	0.31	0.23
PS SATS to PUFA	0.02	0.92	-0.00	0.99	-0.01	0.96	-0.26	0.31
SM SATS to PUFA	0.22	0.28	-0.17	0.37	-0.03	0.90	-0.63	0.007
Total SATS to PUFA	-0.27	0.17	0.12	0.54	-0.36	0.11	-0.02	0.94
PHOSPHOLIPIDS								
PC	0.12	0.58	-0.14	0.48	0.08	0.74	-0.26	0.31
PE	-0.02	0.91	-0.03	0.87	0.20	0.38	-0.19	0.46
PS	-0.20	0.33	-0.28	0.15	-0.46	0.04	-0.23	0.37
PI	-0.05	0.81	-0.38	0.048	-0.07	0.78	0.04	0.87
SM	-0.30	0.13	-0.27	0.16	-0.45	0.04	-0.33	0.20
Total phospholipids	0.04	0.85	-0.12	0.53	0.11	0.62	-0.18	0.49
PHOSPHOLIPID RATIOS								
<i>Saturated nature</i> PC + SM/PE + PS	-0.05	0.81	-0.41	0.029	-0.04	0.86	-0.38	0.14
<i>Deformability</i> PE to PS	0.20	0.33	0.07	0.71	0.39	0.09	0.12	0.64
<i>Ordered- to liquid- crystalline-phase lipids</i> PE to PC	-0.08	0.71	0.47	0.011	-0.02	0.95	0.51	0.038
CHOLESTEROL								
Cholesterol	-0.34	0.09	-0.33	0.09	-0.49	0.023	-0.29	0.25
Cholesterol to phospholipid	-0.39	0.05	-0.11	0.59	-0.46	0.036	-0.02	0.94

Table 6.3: Correlation between red blood cell membrane fluidity as measured by membrane lipids, and the Kurtzke Expanded Disability Status Scale and Functional System Scores in multiple sclerosis patients

		All CRP values MS; 31		CRP < 5.00 µg/ml MS; 17	
		R	P-value	R	P-value
FATTY ACIDS					
Total SATS	Visual	0.38	0.03	0.43	0.08
Total PUFA	Pyramidal	-0.31	0.09	-0.37	0.15
Total fatty acids (+ MUFAs)	Pyramidal	-0.31	0.09	-0.32	0.21
Total fatty acids (+ MUFAs)	Sensory	-0.30	0.10	-0.19	0.46
Total fatty acids (+ MUFAs)	Visual	0.30	0.10	0.40	0.11
FATTY ACID RATIOS					
PC SATS to PUFA	Cerebellar	0.31	0.09	0.32	0.21
PE SATS to PUFA	EDSS	0.22	0.25	0.67	0.003
PE SATS to PUFA	Pyramidal	0.27	0.15	0.66	0.004
PE SATS to PUFA	Cerebellar	0.45	0.012	0.85	0.00001
PS SATS to PUFA	Sensory	0.31	0.08	0.18	0.50
PS SATS to PUFA	Bowel/bladder	0.11	0.55	0.54	0.03
Total SATS to PUFA	EDSS	0.10	0.60	0.47	0.06
Total SATS to PUFA	Pyramidal	0.05	0.81	0.41	0.10
Total SATS to PUFA	Cerebellar	0.20	0.29	0.46	0.06
Total SATS to PUFA	Brainstem	0.28	0.13	0.46	0.07
PHOSPHOLIPIDS					
PC	EDSS	-0.38	0.037	-0.61	0.009
PC	Pyramidal	-0.35	0.06	-0.45	0.07
PE	EDSS	-0.45	0.011	-0.61	0.009
SM	EDSS	-0.28	0.13	-0.57	0.018
SM	Pyramidal	-0.26	0.15	-0.45	0.07
SM	Bowel/bladder	-0.29	0.12	-0.46	0.06
Total phospholipids	EDSS	-0.44	0.015	-0.66	0.004
Total phospholipids	Pyramidal	-0.38	0.037	-0.45	0.07
PHOSPHOLIPID RATIOS					
<i>Saturated nature</i>					
PC + SM/PE + PS	Sensory	0.30	0.11	0.30	0.24
<i>Deformability</i>					
PE to PS	Sensory	0.41	0.021	0.27	0.30
<i>Ordered- to liquid-crystalline-phase lipids</i>					
PE to PC	Bowel/bladder	-0.21	0.26	-0.51	0.037
CHOLESTEROL					
Cholesterol	EDSS	-0.25	0.18	-0.45	0.07
Cholesterol to phospholipids	EDSS	0.24	0.20	0.40	0.11

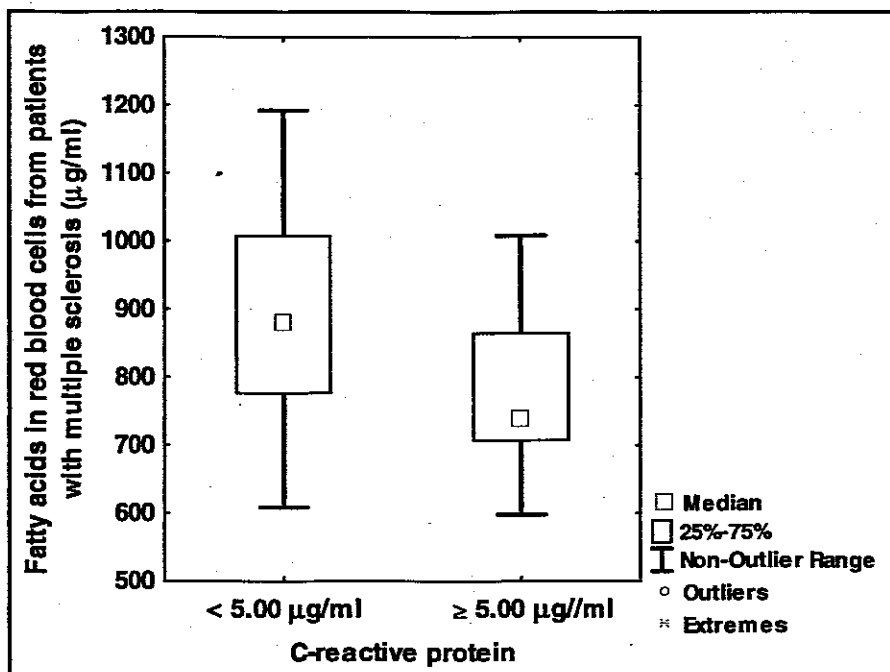


Figure 6.1: Total Fatty acids and CRP. Patients with MS were sub-grouped according to their CRP levels. The red blood cell membrane total fatty acids in patients with MS when CRP ≥ 5.00 $\mu\text{g/ml}$ was lower; Median (quartile range) when CRP < 5.00 $\mu\text{g/ml}$: 881.8 (231.0); and when CRP ≥ 5.00 $\mu\text{g/ml}$: 740.1 (157.6); $P = 0.036$

CHAPTER SEVEN

DISCUSSION AND CONCLUSION

Although the underlying cause of multiple sclerosis (MS) is unknown, it is a disease that is characterized by inflammation of the central nervous system (CNS), which has been contributed to possible auto-immune conditions and/or the presence of foreign agents, such as viruses. Increased or unregulated expression of the modulators of the inflammatory process may therefore contribute to the pathogenesis of the disease, which presents with destruction of myelin, the protective, insulating membrane surrounding neurons. Inflammatory substances are produced by activated immune cells, consisting of plasma cells, microglial cells, lymphocytes and macrophages. Cytokines and eicosanoids, which are both mediators of inflammation, are produced by these cells during the course of inflammatory activation. In this regard, membrane fatty acids are the precursors for eicosanoid production. It is therefore possible that an increased turn-over of membrane fatty acids to eicosanoid production, could contribute to the pathogenesis of the disease, both through depletion of membrane fatty acid concentrations, and damage caused by the released eicosanoids.

Membrane fatty acid metabolic abnormalities have been reported in MS and have been linked to the pathogenesis of the disease. Reports are inconsistent, but a reduction in both n-6 and n-3 polyunsaturated fatty acids (PUFAs), with an increase in saturated fatty acids (SATS) and monounsaturated fatty acids (MUFAs), has been reported. In particular, a reduction in the n-6 fatty acids, C18:2n-6 and its elongation product, C20:4n-6 have consistently been reported in the plasma and cell membranes from MS patients. In the present study, C20:4n-6 was lower in red blood cell (RBC) membranes, with metabolic disturbances between C20:3n-6 and C20:4n-6 in both RBC and peripheral blood mononuclear cell (PBMC) membranes from patients with MS. Although these results may indicate depleted plasma stores, they may also represent the increased release of these fatty acids for eicosanoid (prostaglandin) production. Both phospholipase A2 (PLA2) activity and prostaglandin E2 (PGE2) production is known to be increased during inflammatory activation. PLA2 releases C20:4n-6 from membrane phospholipids, making the free fatty acids (FFAs) available for PGE2 synthesis. In this regard, the released FFA C20:4n-6 itself is known to be toxic to surrounding cells, and would therefore add to the effects of a possibly increased pro-inflammatory PGE2 concentration. Because of the relatively high amount of C20:4n-6 in immune cell membrane phospholipids, eicosanoids synthesized from C20:4n-6, such as PGE2, are known to be produced in increased amounts upon stimulation.

In this study, plasma FFAs, C18:2n-6 and C20:4n-6 were increased in the plasma from the same study group of patients with MS. Furthermore, the RBC C20:4n-6 correlated inversely

with plasma FFA C20:4n-6, showing a relationship between the decrease in membrane C20:4n-6 and the increase in plasma FFA C20:4n-6. It seems unlikely that lower RBC C20:4n-6 was due to insufficient uptake from plasma, because there was an increased concentration of FFA C20:4n-6 available in the plasma, but rather that plasma FFA C20:4n-6 was increased because of increased release of C20:4n-6 from RBC membranes, suggesting inflammatory activation. In this study FFA C20:4n-6 was too low to measure in the RBC membranes themselves, therefore the relationship between RBC membrane C20:4n-6 and plasma FFA C20:4n-6 is important, as it allows insight into the possible mechanisms involved in the pathogenesis of the disease.

The inverse correlation between RBC membrane C20:4n-6 and plasma FFA C20:4n-6 may indicate either plasma and/or dietary irregularities, or even compromised PLA2 activity. However, it is known that there is an increased demand for PGE2 synthesis during inflammation, therefore this finding strongly suggests that the inverse relationship between RBC membrane C20:4n-6 and plasma FFA C20:4n-6 may be due to an increased demand for the production of inflammatory mediator, PGE2. Results reported by Harbige and Sharief (2007), who showed an increase in pro-inflammatory cytokines with a decrease in PBMC membrane C20:4n-6, suggested that a decrease in membrane C20:4n-6 could be indicative of pro-inflammatory effects. In this regard, synthesis of both pro-inflammatory cytokines and pro-inflammatory prostaglandins are known to be upregulated during inflammation. Results from this study also showed that an increase in inflammation, as measured by the CRP, correlated inversely with RBC membrane C20:4n-6; suggesting that increased CRP could be indicative of increased PGE2 production from RBC C20:4n-6, further supporting a possible role for C20:4n-6 in inflammation.

Similar to previous reports, we found the metabolic relationship between C20:3n-6 and C20:4n-6 disturbed in the PBMC membranes from patients with MS, but C20:4n-6 was not decreased in these membranes. These results indicated that in blood, the release of C20:4n-6 was primarily from RBC membranes. In this regard, it is known that RBC membranes with diminished fluidity are vulnerable to hydrolysis by sPLA2 (serum PLA2) (Harris et al. 2001), and in this study RBC membrane fluidity was decreased; thereby confirming the vulnerability of these cells from patients with MS in this study to sPLA2 activity.

Inflammatory responses usually result in self-limiting healing processes, but increased immune cell activity has been reported in the CNS from patients with MS. Microglia are the main mediators of neuro-inflammation and also initiate recruitment of leukocytes from the blood stream into brain tissue. Glial cells have been shown to be involved in CNS disorders, especially in MS. They precede other immune cells such as T-lymphocytes, differentiated B-

lymphocytes and early activated macrophages in apoptotic zones. Leukocytes are not present in healthy brains, and therefore increased immune cell activity in the brain could change the fatty acid requirements of brain tissue from patients with MS, and could possibly deplete sources elsewhere, such as from compromised RBC membranes. Results from this study showed that PBMC membranes maintained normal concentrations of C20:4n-6, possibly suggesting preservation of immune cell function a high priority in patients with MS. It is not clear whether C20:4n-6 will be sourced from neuronal tissue as well as from RBC membranes. However, the amplification of PGE₂ may be active in brain pathologies such as MS (Repovic et al. 2003) and Hofman et al. (1986) reported immune cells from MS brain lesions which stained positive for prostaglandin E (PGE), which was not found in normal brain tissue.

In this study membrane fatty acid concentrations were correlated with disease outcome as measured by the Kurtzke Expanded Disability Status Scale and its Functional System Scores (EDSS and FSS respectively). RBC membrane C20:4n-6 showed inverse correlations with the EDSS and FSS, and PBMC membrane C20:4n-6 with the FSS, linking decreased membrane C20:4n-6 directly to the pathogenesis of the disease. Whether this relationship is due to impairment of the RBC membrane and therefore diminished ability to deliver oxygen to the brain and other tissue, and/or because of an indication of increased eicosanoid production, is not clear, and needs further investigation.

There was no difference in the n-3 fatty acid composition between patients with MS and control subjects; neither did membrane n-3 fatty acids correlate with either the disease outcome, as measured by the EDSS, or the inflammatory status, as measured by the CRP. These findings indicated that the n-3 fatty acids did not contribute to disease outcome directly. When inflammation becomes resolved, the effects of C20:4n-6 are compensated for by the anti-inflammatory properties of the n-3 fatty acids, C20:5n-3 and C22:6n-3. Therefore, normal n-3 fatty acid concentrations in patients with MS, may suggest that the inflammatory process was allowed to proceed. These findings suggested that the anti-inflammatory effects of the n-3 fatty acids may not independently be sufficient to halt the inflammatory process in MS and perhaps partially provide an explanation to the controversial outcomes on PUFA supplementation in MS patients.

In MS, decreases in plasma and/or membrane PUFAs were reported to be replaced by increases in SATS and/or MUFAs. Diets rich in SATS can be harmful to health and is generally associated with the development of many diseases, including MS (Van Meeteren et al. 2005). However, there is lack of information about the individual dietary SATS (German & Dillard, 2004). Therefore, in the present study we identified the specific SATS that in MS

patients at least, were found to be associated with a worse outcome, which were the longer-chain saturated fatty acids, while the shorter-chain saturated fatty acids correlated with a better disease outcome as measured by the EDSS and FSS. These included C14:0 and C16:0, metabolites for myristoylation, and palmitoylation respectively.

Changes in membrane lipid composition can be expected to have an effect on membrane structure and function. The composition of membrane phospholipids, their fatty acids and cholesterol specify the degree of membrane rigidity, permeability and fluidity. Likewise, in this study, the altered fatty acid profile resulted in changes in membrane fluidity as measured by the relationship between membrane lipids, and this was closely interrelated with inflammation and disease outcome in patients with MS. Lower RBC membrane fluidity (higher rigidity) as measured by the SATS to PUFA ratio was observed in patients with MS. The membrane fluidity as assessed by its saturated nature (phospholipid PC + SM/PE + PS ratio), and membrane cholesterol was not different between patients with MS and control subjects, but in patients with CRP ≥ 5.00 $\mu\text{g/ml}$, it correlated positively with the FSS.

In conclusion therefore, this study has enhanced our understanding on the role of fatty acids, and the probable role they play in the inflammatory aspect of MS. It has clearly demonstrated that changes in C20:4n-6 concentration is an important factor in the pathogenesis of MS. Lastly, the present study has shown that in the presence of uncontrolled inflammation such as in MS, altered fatty acid composition indirectly compromises the cell membrane structure and function, and thereby contributes to the disease progression. It would however be of importance to confirm these findings by the quantification of relevant phospholipases, eicosanoids, desaturases and elongases, as well as a controlled comparative study between the brain matter and biological fluids from patients with multiple sclerosis.

CHAPTER EIGHT

MATERIALS AND METHODS

8.1 Ethics statement

Ethical approval for the study was obtained from the Health and Applied Sciences Research Ethics Committee (HASREC) of the Cape Peninsula University of Technology (CPUT) (Appendix A). MS patients were contacted and recruited through the MS Society, Western Cape Branch, South Africa. Informed written consent was obtained from all study participants (Appendices B and C). All results were treated confidentially.

8.2 Results made available to participants

Where possible, results, such as that of C-reactive protein and the haematology results were reported to the patients and control subjects, with consent from the Health and Applied Sciences Research Ethics Committee (HASREC) (Appendices D, E and F).

8.3 Study population

The study population was recruited using a comprehensive approach, including several local radio and newspaper advertisements, as well as active participation of a nursing sister affiliated to the MS Society, Western Cape, South Africa. Recruitment and sampling were done during October to December 2007. No black patients responded to the recruitment campaign, and only two male patients replied. Finally the study population consisted of 31 white female patients with MS and 30 age- race- and gender-matched control subjects.

The diagnosis of the recruited patients was verified by a neurologist based on clinical, laboratory and magnetic resonance imaging findings. Six of the patients had active disease, 11 had a relapse 5-12 months previously and 14 did not relapse for more than a year. The number of years since the patients were diagnosed was 7 (11) years (median and interquartile range). Ten patients were using non-steroidal anti-inflammatory drugs (NSAIDs) and five patients were using immunosuppressive medication. Therefore, the patients with MS were subdivided into two groups: Group A consisted of the total number of patients (N = 31) and Group B (N = 15) consisted of patients not on any anti-inflammatory or immunosuppressive drugs. This was done to exclude the possible interference from the medication on the eicosanoid pathway. The functional disability status (disease severity) of each patient was measured by a trained clinician using the Kurtzke EDSS (Appendix G).

8.3.1 Inclusion criteria

- Multiple sclerosis diagnosis verified by the study neurologist based on clinical, laboratory and magnetic resonance imaging (MRI) findings
- Overnight fasting by study participants before blood was taken

8.3.2 Exclusion criteria

- Use of fatty acid supplementation
- Treatment with interferon and steroids
- Co-existence of any other disease

8.4 Data collection and production methods

8.4.1 Blood sampling and processing

Fifteen millilitre (ml) venous blood was collected from each participant into anti-coagulant ethylenediaminetetraacetic acid (EDTA) tubes (Beckman Coulter, South Africa). For all participants an extra 5 ml blood was collected for a full blood and differential white cell count.

Blood was separated using histopaque-1077 separation medium as per manufacturer's instructions (Sigma-Aldrich, South Africa). Blood was layered onto histopaque in a ratio of 15 ml blood/12 ml histopaque, and centrifuged at 400 g for 20 minutes at room temperature. The plasma layer was kept, spun again at 1500 g for 5 minutes to get rid of platelet contaminants, and frozen in 1 ml aliquots. The peripheral blood mononuclear cell (PBMC) interface was recovered, washed twice with 0.85 % saline solution, resuspended in 1 ml of a 0.85 % saline solution and frozen. Three mls red blood cells (RBCs) were washed twice with 0.85 % saline solution and frozen as packed cells without added saline. Samples were frozen at -80 °C immediately after separation of blood fractions. A 0.85 % saline solution was used in this study instead of the prescribed balanced phosphate buffered saline solution as phosphate was inappropriate for additional tests done in the study, such as membrane phosphate evaluation.

8.4.2 Lipid analysis

Phospholipids and cholesterol were quantified in RBCs and PBMCs, while fatty acids were quantified in RBCs, PBMCs and plasma. The red blood cell (RBC) and peripheral blood mononuclear cell (PBMC) membrane phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SM) of patients and control subjects were measured. Of these the RBC membrane fatty acid composition of PC, PE, PS and SM and PBMC membrane PC, PE, PS,

PI and SM were measured. The plasma fatty acid composition of PC and SM were measured. Plasma, RBC and PBMC FFAs were measured as well.

8.4.2.1 Thin layer chromatography

Thin layer chromatography was used in the extraction and separation of phospholipids, fatty acids and cholesterol. Fatty acids were further converted to methyl esters and quantified by gas chromatography (GC) (Folch et al. 1957; Gilfillan et al. 1983; Van Jaarsveld et al. 2000). Phospholipids were quantified using a colorimetric assay with malachite green dye (Itaya & Ui, 1966; Smuts et al. 1994). Cholesterol was determined according to an enzymatic assay adapted from Richmond (1973).

The samples were extracted with 18 ml chloroform/methanol (CM) (ratio 2:1 v/v) according to a modified method of Folch et al. (1957). All the extraction solvents contained 0.01 % butylated hydroxytoluene (BHT, Sigma-Aldrich, South Africa) as an antioxidant. The RBC samples were extracted and resuspended in 80 ul chloroform/methanol (CM) (ratio 2:1 v/v) for TLC. Of this 20 ul was used for fatty acid and 40 ul for free fatty acid (FFA) separation on TLC. A separate extraction was done for phospholipid and cholesterol analysis: RBC samples were extracted and resuspended in 150 ul CM, of which 40 ul was used for phospholipid and 20 ul for cholesterol determination. The PBMC samples were resuspended in 70 ul volumes for TLC; 20 ul was used for phospholipid analysis, 20 ul for fatty acids and 30 µl for FFAs. Cholesterol esters and free cholesterol spots (separated lipids on TLC plates) were harvested from plates used for separation of FFAs. Plasma was extracted, resuspended in 80 ul CM for TLC of which 20 ul was used for fatty acid analysis and 30 ul for that of FFAs.

Free fatty acids were separated from the total phospholipid (TPL) fraction by TLC on pre-coated silica gel 60 plates (10 x 10 cm) without a fluorescent indicator (Merck, Darmstadt, Germany) using the solvent system petroleum benzene (boiling point 40-60 °C)/diethyl ether (peroxide free)/acetic acid (ratio 90:30:1; v/v/v; Sigma-Aldrich, South Africa) as previously described (Van Jaarsveld et al. 2000). Individual phospholipid classes were separated by TLC on pre-coated silica gel 60 plates (10 x 10 cm) without a fluorescent indicator (Merck, Darmstadt, Germany) using chloroform/petroleum benzene/methanol/acetic acid/boric acid (ratio 40:30:20:10:1.8; v/v/v/v/w) as solvent (Giffillan et al. 1983). The lipid bands containing PC, PE, PS, PI and SM were visualized with long wave ultraviolet light after spraying the plates with chloroform/methanol (ratio 1:1; v/v) containing 10 mg/100 ml BBOT (2,5-bis-(5'-tert-butylbenzoxazolyl-[2'])thiophene; Sigma Chemical Company, South Africa). These fractions were scraped off the TLC plates and used for phospholipid and fatty acid analysis (separate plates).

8.4.2.2 Gas chromatography

For fatty acid analysis, the phospholipid fraction and FFAs were transmethylated using 5 % sulfuric acid (H_2SO_4)/methanol at 70 °C for 2 hours (SM; 18 hours). After cooling, the resulting fatty acid methyl esters (FAME) were extracted with 1 ml of distilled water and 2 ml of n-hexane. The top hexane layer was removed and evaporated to dryness, re-dissolved in carbon disulphide (CS_2) and analyzed by GC (Finnigan Focus GC, Thermo Electron Corporation, USA, equipped with flame ionization detection), using 30 meter BPX 70 capillary columns of 0.32 millimetre (mm) internal diameter (SGE International Pty Ltd, Australia). Gas flow rates were as follows: Nitrogen (make up gas), 25 ml/minute; air, 250 ml/minute; and hydrogen (carrier gas), 25 ml/minute and split ratio of 20:1. Temperature programming was linear at 5 °C/minute, initial temperature 140 °C, final temperature 220 °C, injector temperature 220 °C, and detector temperature 250 °C. The FAME were identified by comparison of the retention times to those of a standard FAME mixture (Nu-Chek-Prep Inc., Elysian, Minnesota). The individual FAME were quantified against an internal standard (C17:0, Sigma-Aldrich, South Africa) and as percentage composition of total fatty acids present. Red blood cell membrane fatty acids were quantified in absolute values in μg fatty acid/ml packed RBC analyzed. PBMC membrane protein was measured and fatty acids were quantified against membrane proteins present in μg fatty acid/mg protein (see protein assay below).

8.4.3 Phospholipid determination

Red blood cell and PBMC membrane phospholipids were determined using a colorimetric assay with malachite green dye, as previously described (Itaya & Ui, 1966; Smuts et al. 1994). After phospholipid fractions were separated by TLC, they were reduced to inorganic phosphates and quantified according to their phosphate (Pi) content. The samples were incubated in 400 μl , 200 μl , 100 μl , 100 μl , 100 μl perchloric acid for PC, PE, SM, PI and PS phospholipids respectively, at 170 °C for 2 hours. Tubes were allowed to cool down to room temperature and then distilled water was added at a ratio of 5:1. Samples were centrifuged at 10 g-force (g) for 15 minutes. The phospholipid reagent (malachite green:ammonium molybdate:tween (ratio 15:5:1; v/v/v; Sigma-Aldrich, South Africa) was prepared and allowed to stand for about 15-20 minutes until clear brown. Serial dilutions of standards {1, 2, 3 and 4 μg Pi; potassium dihydrogen orthophosphate (KH_2PO_4); Merck, South Africa} were evaporated, to which 400 μl perchloric acid and 2 ml distilled water were added, while 400 μl perchloric acid and 2 ml distilled water were used as blanks. A 250 μl aliquot from the standard/blank/sample was added to 1 ml reagent and incubated for 20 minutes. The optical density (OD) was read on a spectrophotometer at 660 nanometer (nm).

8.4.3.1 Calculation of phospholipids

Membrane phospholipids were quantified according to their phosphate content, which was then converted to phospholipids. Quantification was done using the following formulae:

- $f \times \text{total spot volume/volume used} \times \text{specific phospholipid factor} \times 1/\text{protein concentration} \times \text{absorption of sample} \times \text{phospholipid ratio} = \mu\text{g Pi/mg protein}$

[where f = concentration of standard/absorption reading]

- The different factors used for conversion of phosphorous to phospholipids were:
for PC: 25.4; PE: 23.22, PS: 25.4 PI: 27.64 and SM: 24.21
- RBC membrane phospholipids were quantified in $\mu\text{g Pi/ml}$ packed RBCs and PBMC membrane phospholipids in $\mu\text{g Pi/mg protein}$ (see protein assay below)

8.4.4 Protein analysis

A bicinchoninic acid protein determination assay was used to determine the protein content of PBMC membranes (Kaushal & Barnes, 1986) for quantification of PBMC membrane phospholipids, fatty acids and cholesterol. PBMC membrane lipids were quantified against membrane protein present because of the high variation normally found in white blood cell counts. 200 μl starting material was diluted with 2 % sodium dodecyl sulphate solution (SDS; Fluka, Sigma-Aldrich, South Africa) to denature protein prior to protein determination. Of each of the samples 40, 60 and 80 μl aliquots were used for the assay. Doubling dilutions (10, 20, 40, 60, 80 and 100 μl) of a 0.1 mg/ml stock bovine serum albumin (BSA; Sigma-Aldrich, South Africa) were used as standards. An extra sample without BSA added to it was used as a blank. Bicinchoninic acid (BCA) protein assay reagents A and B (Pierce, Separations, South Africa) were mixed in a ratio of 50:1, and diluted 400 $\mu\text{l}/1$ ml distilled water. The each sample, standard and blank 400 μl reagent (A+B mix) and distilled water were added to a final volume of 1 ml, and then incubated at 60 °C for 60 minutes. The ODs were read at 562 nm. Sample values were obtained from the BSA linear standard curve. PBMC membrane phospholipids, fatty acids and cholesterol were quantified against membrane proteins and expressed in $\mu\text{g Pi/mg protein}$, $\mu\text{g fatty acids/mg protein}$ and $\mu\text{g cholesterol/mg protein}$ respectively.

8.4.5 Cholesterol determination

Red blood cell and PBMC membrane cholesterol was determined using an enzymatic assay (iodide (I_2)/cholesterol method) adapted from Richmond (1973). This is 2-stepped method based on the use of 2 enzymes: cholesterol esterase and cholesterol oxidase. In the first step cholesterol esterase releases cholesterol and fatty acids from cholesterol esters. In the second step, cholesterol oxidase is used to catalyze catabolism of free cholesterol, producing cholest-4-en-3-one plus hydrogen peroxide (H_2O_2). The free cholesterol reacts with iodide (I_2) to generate a yellow colour.

PBMC cholesterol esters and free cholesterol (equals total cholesterol) were harvested from TLC plates, extracted from silica gel and further analyzed for total cholesterol concentration. For RBC, 20 μ l RBC lipid extract was used for total cholesterol determination. To these samples, 50:1 chloroform/methanol (CM) (ratio 2:1 v/v) was added. 10x dilutions of 100, 150, 200, 300, 400 milligrams/decilitre (mg/dl) cholesterol biochemical standard (Preciset Cholesterol kit; Boehringer-Mannheim, Germany) were used. A corresponding blank was prepared for each standard. To both samples and standards 300:1 of a 1 % polyethylene glycol monoether (Triton x100, peroxide free; Roche, South Africa) was added. The emulsion was then evaporated under nitrogen gas (N_2) in a 37 °C water-bath until clear, after which 1.7 ml cholesterol reagent (see below) was added. Both samples and standards were divided into two separate test tubes, containing 1 ml solution each. To one set of tubes 20:1 enzyme mix was added (ratio, 83.3:1 cholesterol esterase and 125:1 oxidase enzymes; Roche, South Africa). To the second set of tubes, 20:1 3 M sodium chloride (NaCl) was added, mixed and used as blanks for samples and standards. Samples, standards and blanks were allowed to stand for 30 minutes at room temperature, and then read at 365 nm in a spectrophotometer, calibrating together with its corresponding blank.

8.4.5.1 Cholesterol reagent

The cholesterol reagent used in the above assay contained the following chemicals: 163 mM potassium dihydrogen orthophosphate (KH_2PO_4) (Sigma-Aldrich, South Africa), 37 mM dipotassium hydrogen phosphate ($K_2HPO_4 \cdot 3H_2O$) (Sigma-Aldrich, South Africa), 120 mM potassium iodide (KI) (Sigma-Aldrich, South Africa), 0.15mM sodium azide (NaN_3) (Sigma-Aldrich, South Africa), 0.274 mM alkylbenzyltrimethylammonium chloride (Merck, South Africa), 0.01mM ammonium molybdate (Riedel de Haen, South Africa), 3.09 mM Triton X100 (Roche, South Africa). The buffer was made up and the pH was adjusted to 6.2 with a 33 % potassium hydroxide (KOH) solution (Sigma-Aldrich, South Africa).

8.4.5.2 Cholesterol calculation

Quantification of cholesterol was done using the following formulae:

- $f \times (\text{sample absorption}/1) \times (\text{total spot volume}/\text{volume used}) \times (1/\text{protein concentration}) = \mu\text{g cholesterol}/\text{mg protein}$

[where f = concentration of standard/absorption reading]

- RBC membrane cholesterol was quantified in $\mu\text{g cholesterol}/\text{ml}$ packed RBCs and PBMC membrane cholesterol was quantified in $\mu\text{g cholesterol}/\text{mg protein}$

8.4.6 C-reactive protein determination

Plasma C-reactive protein (CRP) concentrations were determined in a routine Chemical Pathology laboratory on a Beckman nephelometer auto-analyser using reagents from

Beckman, South Africa. The diagnostic values of this laboratory are considered positive for CRP values equal to or greater than 5 µg/ml.

8.4.7 Statistical analysis

A statistics programme, STATISTICA (STATISTICA 7, StatSoft Inc 1984 – 2004) was used to perform all statistical analyses. The RBC and PBMC membrane phospholipids, fatty acids, cholesterol and membrane fluidity parameters were evaluated, against that of control subjects, as well as against the EDSS (patients only) and the CRP. Descriptive data are presented as median and quartile range. The Mann Whitney U test was used to compare data between the cases and control subjects. Correlations between phospholipids, fatty acids, cholesterol and membrane fluidity versus the EDSS and CRP were calculated using Spearman's Rank correlation coefficient. Results were considered significant if P-values were less than 0.05.

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APPENDIX A

ETHICS APPROVAL

1. An ethics approval certificate from the Cape Peninsula University of Technology Health and Applied Sciences Research Ethics Committee is included here.



20 July 2007

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**OFFICE OF THE CHAIRPERSON:
HEALTH AND APPLIED SCIENCES RESEARCH ETHICS COMMITTEE (HASREC)**

At a meeting of the HASREC on the 15 June 2007, ethics approval was granted to Gloudine Marie HON for research activities related to the D Tech Biomedical Technology of the Cape Peninsula University of Technology. This approval was pending amendments and further information which has been satisfactorily completed.

TITLE: The role of viral factors and membrane fatty acids in the loss of cell membrane integrity in multiple sclerosis patients.

Comment:

Research activities are restricted to those detailed in the research proposal and ethics application submitted on 25 July 2007. This CPUT ethics approval is valid up to December 2008.

**Dr PENELOPE ENGEL-HILLS
CHAIRPERSON: HEALTH AND APPLIED SCIENCES RESEARCH ETHICS COMMITTEE**

e-mail: engel@asp@cpu.ac.za

APPENDIX B

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT

The role of viral factors and membrane fatty acids in the loss of membrane integrity in multiple sclerosis patients

PRINCIPAL INVESTIGATOR: Gloudina Maria Hon

ADDRESS: Division of Chemical Pathology, P O Box 19113, Tygerberg Hospital, 7505.

CONTACT TELEPHONE NUMBER: 021 938 4107

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the researcher any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you will be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health and Applied Sciences Research Ethics Committee (HASREC)** at the Cape Peninsula University of Technology.

What is this research study all about?**Research sites and participants**

The research study will be conducted at the Division of Chemical Pathology, Tygerberg Hospital in collaboration with scientists from the Cape Peninsula University of Technology and the Medical Research Council and will be funded by the Cape Peninsula University of Technology. Some participants will have relapsing remitting multiple sclerosis and others will be persons without the disease as a control group.

Project aims

The project is being undertaken to investigate whether cell membranes in multiple sclerosis patients, especially the fatty acids that make up these membranes, are affected or can affect

the process by which the body fight infections and whether this has an effect on how the disease progresses. The research may provide important insights into MS and may have implications for how people with MS are treated. The project should not take longer than three years.

Procedures

20-50 ml of blood will be collected from each participant. The drawing of blood may result in a slight discomfort, which can be coupled with bleeding, where the needle pierces the skin. The blood will be used in laboratory tests including the extraction of DNA to detect possible viral presence. This is a single event and no repeat blood samples will be needed. In addition we will ask you to complete a health questionnaire so that we can obtain information relevant to the research study from each participant. All samples collected will be used in our study. We will adhere to the following inclusion and exclusion criteria:

Inclusion criteria

Patients diagnosed with relapsing remitting MS

Patients who have been diagnosed by a neurologist and have had an MRI scan

Exclusion criteria

Patients on any fatty acid supplementation

Patients diagnosed with an additional disease

Patients with a Kurtzke Expanded Disability Status Scale of less than 2. This evaluation will be done in the Division of Chemical Pathology, Tygerberg Hospital by a neurologist or a trained clinician

Medication

No medication or supplements of any kind whatsoever will be prescribed for participants.

Why have you been invited to participate?

You have been invited to participate in this research project so that scientific knowledge about MS can be broadened.

What will your responsibilities be?

All that will be asked of you is the donation of 50 ml of blood, as well as to fill in a short health questionnaire to establish some medical facts relevant to the study.

Will you benefit from taking part in this research?

You may, during or on completion of the project, request the results of the tests without any conditions attached thereto. However, the primary aim of this project will be to enhance knowledge about the disease.

Are there any risks involved in your taking part in this research?

There are no risks involved other than that of the drawing of blood from the arm.

If you do not agree to take part, what alternatives do you have?

This project does not aim at addressing any medical treatment whatsoever and participants should adhere to their routine medical treatment. If in doubt about future treatment, a general practitioner should be contacted for referral to a neurologist.

Who will have access to your medical records?

All information will be treated confidentially. A code system will be used and the names of participants will not be used. Only the researchers involved in this project will have access to any of the data. The results will be used for a DTech thesis and for publication in scientific journals, without revealing the identity of any individual.

Will you be paid to take part in this study and are there any costs involved?

Participation in this project will not result in unnecessary expenses for you and you will not be paid for participation.

Is there any thing else that you should know or do?

You can contact Ms D. Hon at telephone: 021 938 4107 or cell: 082838 4558 or Dr Susan van Rensburg at telephone: 083 564 7654 if you have any further queries or encounter any problems. You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I agree to take part in a genetic research study entitled: "The role of viral factors and membrane fatty acids in the loss of membrane integrity in multiple sclerosis patients".

I declare that:

I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

I have had a chance to ask questions and all my questions have been adequately answered.

I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.

I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

Signed at (*place*) on (*date*)2007

Signature of participant.....Signature of witness.....

Declaration by investigator

I (*name*) declare that:

I explained the information in this document to

I encouraged him/her to ask questions and took adequate time to answer them.

I am satisfied that he/she adequately understands all aspects of the research, as discussed above

I did/did not use a translator. (*If a translator is used then the translator must sign the declaration below.*)

Signed at (*place*)on (*date*)2007

Signature of investigator.....Signature of witness.....

Declaration by translator

I (*name*) declare that:

I assisted the investigator (*name*) to explain the information in this document to (*name of participant*) using the language medium of Afrikaans/Xhosa.

We encouraged him/her to ask questions and took adequate time to answer them.

I conveyed a factually correct version of what was related to me.

I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) on (*date*)2007

Signature of translator.....Signature of witness.....

APPENDIX C

HEALTH QUESTIONNAIRE

Name.....
 DOB.....
 Age.....
 Sex..... Race.....
 Tel no: (W)..... (H)..... (Cell).....

	Yes	Time/date	No
Have you been diagnosed with MS?			
Was diagnosis confirmed by an MRI scan?			
When did symptoms started?	NA		NA
Have you had any relapses during the preceding 12 months?			
Are you on any treatment specific for MS?			
Are you on interferon-beta treatment?			
Are you on any prescription medication?			
Are you on any generic medication?			
Do you suffer from acute infection?			
Do you suffer from chronic infections?			
Are you on any unsaturated fatty acid supplementation?			
Are you on any iron supplementation?			
Are you on any vitamin supplementation?			
Are you on any mineral supplementation?			
Have you been diagnosed with an additional disease?			
Have you fasted overnight?		NA	

Type of MS.....

Diagnoses confirmed by Dr..... Tel no.....

Date.....

.....
 Signature of patient

.....
 Name/Signature of witness

APPENDIX D**COVERING LETTER TO MEDICAL PRACTITIONER
WITH RESULTS SEND TO PATIENTS**

Date

To whom it may concern

Dear Doctorhas participated in the study titled **"The role of viral factors and membrane fatty acids in the loss of membrane integrity in multiple sclerosis patients"**. This study is a research project undertaken by the Cape Peninsula University of Technology and the Medical Research Council. The attached results were obtained: A Full Blood and Differential White Cell Count as well as a C-Reactive Protein.

This is for your attention and management of the patient.

Thanking you.

Dr T. Matsha
Biomedical Sciences
Cape Peninsula University of Technology
Bellville
Tel: 021 460 3209
Cell: 072 679 0885
Email: matshat@cput.ac.za

APPENDIX E**COVERING LETTER TO MEDICAL PRACTITIONER
WITH RESULTS SEND TO CONTROL PARTICIPANTS**

Date.....

To whom it may concern

Dear Doctor:has participated in the study titled **"The role of viral factors and membrane fatty acids in the loss of membrane integrity in multiple sclerosis patients"**. This study is a research project undertaken by the Cape Peninsula University of Technology and the Medical Research Council. The attached results were obtained: A Full Blood and Differential White Cell Count as well as a C-Reactive Protein.

This is for your attention and management of the control subject.

Thanking you.

Dr T. Matsha
Biomedical Sciences
Cape Peninsula University of Technology
Bellville
Tel: 021 460 3209
Cell: 072 679 0885
Email: matshat@cput.ac.za

APPENDIX F**COVERING LETTER TO PARTICIPANTS**

Dear

Enclosed please find the available results, as well as the consent form, accompanied by a letter to your General Practitioner explaining the study you have been a part of. In case of any uncertainty regarding these results, please contact your GP for interpretation thereof.

At this stage, please allow us to thank you again sincerely for your participation in this study of ours. We are grateful for your sacrifice and willingness to help towards the efforts of unraveling the complexities of Multiple Sclerosis (MS).

We remember you fondly and wish you well.

Kindest regards

Sr Treska Botha

B.Cur. (RGN, RMW, RPN, Dip. Comm Health)

Clinician: MS Study, Cape Peninsula University of Technology

APPENDIX G

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

Patient.....

Date.....

Examiner.....

Final score.....

A. The Kurtzke Expanded Disability Status Scale (EDSS)

Disability score	Neurological status
0.0	Normal neurological examination (all grade 0 in all Functional System (FS) scores) (Excludes cerebral function grade 1)
1.0	No disability, minimal signs in one functional score (FS*) (i.e. grade 1)
1.5	No disability, minimal signs in more than one FS* (more than 1 FS grade 1)
2.0	Minimal disability in one FS (one FS grade 2, others 0 or 1)
2.5	Minimal disability in two FS (two FS grade 2, others 0 or 1)
3.0	Moderate disability in one FS (one FS grade 3, others 1 or 0), or mild disability in three or four FS (three or four FS grade 2, others 0 or 1) though fully ambulatory
3.5	Fully ambulatory but with moderate disability in one FS (one grade 3) and one or two FS grade 2, or two FS grade 3 (others 0 or 1) or five grade 2 (others 0 or 1)
4.0	Fully ambulatory without aid, self-sufficient, up and about some 12 hours a day despite relatively severe disability consisting of one FS grade 4 (others 0 or 1), or combination of lesser grades exceeding limits of previous steps; able to walk without aid or rest some 500 meters
4.5	Fully ambulatory without aid, up and about much of the day, able to work a full day, may otherwise have some limitation of full activity or require minimal assistance; characterized by relatively severe disability usually consisting of one FS grade 4 (others or 1) or combinations of lesser grades exceeding limits of previous steps; able to walk without aid or rest some 300 meters
5.0	Ambulatory without aid or rest for about 200 meters; disability severe enough to impair full daily activities (e.g. to work a full day without special provisions): (Usual FS equivalents are one grade 5 alone, others 0 or 1; or combinations of lesser grades usually exceeding specifications for step 4.0)
5.5	Ambulatory without aid for about 100 meters; disability severe enough to preclude

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

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

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

B. The Functional Systems Scores (FSS)

Pyramidal Function

- 0 - Normal
- 1 - Abnormal signs without disability
- 2 - Minimal disability
- 3 - Mild to moderate paraparesis or hemiparesis (detectable weakness but most function sustained for short periods, fatigue a problem); severe monoparesis almost no function)
- 4 - Marked paraparesis or hemiparesis (function is difficult); moderate quadriparesis (function is decreased but can be sustained for short periods) or monoplegia
- 5 - Paraplegia, hemiplegia, or marked quadriparesis
- 6 - Quadriplegia
- 9 - Unknown

Cerebellar Function

- 0 - Normal
- 1 - Abnormal signs without disability
- 2 - Mild ataxia (tremor or clumsy movements easily seen, minor interference with function)
- 3 - Moderate truncal or limb ataxia (tremor or clumsy movements interfere with function in all spheres)
- 4 - Severe ataxia in all limbs (most function is very difficult)
- 5 - Unable to perform coordinated movements due to ataxia
- 9 - Unknown

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

Brainstem Function

- 0 - Normal
- 1 - Signs only
- 2 - Moderate Nystagmus or other mild disability
- 3 - Severe Nystagmus, marked extraocular weakness, or moderate disability of other cranial nerves
- 4 - Marked dysarthria or other marked disability
- 5 - Inability to speak or swallow
- 9 - Unknown

Sensory Function

- 0 - Normal
- 1 - Vibration or figure - writing decrease only in 1 or 2 limbs
- 2 - Mild decrease in touch or pain or position sense, and/or moderate decrease in vibration in 1 or 2 limbs, or vibratory (c/s figure writing) decrease alone in 3 or 4 limbs
- 3 - Moderate decrease in touch or pain or position sense, and/or essentially lost vibration in 1 or 2 limbs; or mild decrease in touch or pain and/or moderate decrease in all proprioceptive tests in 3 or 4 limbs
- 4 - Marked decrease in touch or pain or loss of proprioception, alone or combined in 1 or 2 limbs; or moderate decrease in touch or pain and/or severe proprioceptive decrease in more than two limbs
- 5 - Loss (essentially) of sensation in 1 or 2 limbs; or moderate decrease in touch or pain and/or loss of proprioception for most of the body below the head
- 6 - Sensation essentially lost below the head
- 9 - Unknown

Bowel and Bladder Function

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

- 0 - Normal
- 1 - Mild urinary hesitancy, urgency, or retention
- 2 - Moderate hesitancy, urgency, or retention of bowel or bladder, or rare urinary incontinence (intermittent self-catheterization, manual compression to evacuate bladder, or finger evacuation of stool)
- 3 - Frequent urinary incontinence
- 4 - In need of almost constant catheterization (and constant use of measures to evacuate stool)
- 5 - Loss of bladder function
- 6 - Loss of bowel and bladder function
- 9 - Unknown

Visual Function

- 0 - Normal
- 1 - Scotoma with visual acuity (corrected) better than 20/30
- 2 - Worse eye with scotoma with maximal visual acuity (corrected) 20/30 to 20/59
- 3 - Worse eye with large scotoma, or moderate decrease in fields, but with maximal visual acuity (corrected) 20/60 to 20/99
- 4 - Worse eye with marked decrease of fields and maximal visual acuity (corrected) of 20/100 to 20/200; grade 3 plus maximal acuity of better eye of 20/60 or less

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

Cerebral (or mental) Functions

- 0 - Normal
- 1 - Mood alteration only (does not affect EDSS score)
- 2 - Mild decrease in mentation
- 3 - Moderate decrease in mentation
- 4 - Marked decrease in mentation (chronic brain syndrome – moderate)
- 5 - Dementia or chronic brain syndrome – severe or incompetent
- 9 - Unknown

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

Reference

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