THE ROLE OF N-6 AND N-3 PUFA RATIOS IN THE AETIOLOGY OF MULTIPLE SCLEROSIS

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.

Ghon
Signed

23 April 2007
Date

ABSTRACT

BACKGROUND

In multiple sclerosis (MS) the myelin sheaths surrounding the axons in the brain are mainly affected by the disease process. Myelin consists for the most part of lipids and proteins. An abnormality in essential fatty acid metabolism is known to be present in patients with MS (Horrobin, 1979), reflected in a high ratio of n-6 to n-3 fatty acids in cell membranes. It has also been established previously that the pathogenesis of inflammatory disorders is aggravated by excessive consumption of n-6 fatty acids relative to n-3 fatty acids (Guesnet *et al.*, 2005), and it has been shown that ingesting a larger proportion of n-3 fatty acids could be crucial in the regulation of cellular physiology and in the prevention of pathologies such as autoimmune and inflammatory diseases.

Modern Western medical treatment for autoimmune diseases, which includes MS, involves the administration of immunosuppressive drugs, such as beta interferon, cortisone (prednisone), methotrexate and cytoxan, which reduce the effectiveness of the entire immune system, and can have serious, sometimes life threatening, side effects (Perlmutter, 2006, http://www.msfacts.org). It would therefore be of interest to investigate other options for treatment.

Although there is an extensive literature on fatty acids in MS, the actual details of the mechanisms of fatty acid imbalances in MS have not been established. It would therefore be advisable to investigate the abnormality of the MS cell membrane fatty acid profile. Previous studies focused on individual fatty acids, but it would be more relevant to investigate the relationships within and between the n-6 and n-3 series, and their effect on outcome, and to establish any possible cumulative effects, because the metabolism of fatty acids within the two series does have an effect on one another.

PURPOSE OF STUDY

The purpose of this study was to investigate whether an imbalance in the ratio of n-6 to n-3 fatty acids in the membranes of erythrocytes and lymphocytes of MS patients is associated with their neurological outcome as measured by the Kurtzke EDSS.

Erythrocyte membranes were used in this study as a marker for the brain (Smuts *et al.*, 1999) and lymphocyte membranes because of their reported involvement in MS neuronal demyelination. Plasma fatty acid composition of total phospholipids was measured to investigate a possible dietary influence on disease status.

OBJECTIVES

1. To determine the n-6 and n-3 fatty acid ratios in total phospholipids, as well as in the PC and PE phospholipid sub fractions, in both the erythrocyte and lymphocyte membranes of MS patients, and
2. to establish whether there is an association between membrane fatty acid composition and severity of neurological outcome as measured by the Kurtzke EDSS.

METHODOLOGY

The research study design was quantitative in nature. All aspects of the study were designed before data was collected. Measurement of variables was numerical and statistical analysis was used to find an association between MS and fatty acid ratios.

The percentage composition of fatty acids in total phospholipids (in erythrocyte membranes, lymphocyte membranes and in plasma) as well as the percentage composition of the fatty acids of the PC and PE fractions in phospholipids (in erythrocyte membranes) of both the MS and control groups, were determined by gas chromatography (GC).

Disability was measured on a Kurtzke Expanded Disability Status Scale (numerical variables) and in the Functional System sub scores (numerical variables).

The fatty acid composition of blood was determined in MS patients and control persons. The MS patients were subdivided into two sub groups; A and B and the results were analysed separately. In one group, (group A) the fatty acids were determined on the total phospholipid fraction (in erythrocyte and lymphocyte membranes and in plasma) and in the second group (group B) the fatty acids of the PC and PE phospholipid sub fractions were determined in erythrocyte membranes.

In both groups (A and B) correlations between the disability scores, EDSS and sensory scores, and the fatty acid percentage composition, as well as with the ratios, were determined.

RESULTS

Significant correlations between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acid series were found systematically in all instances. In some instances the outcome was found to be statistically significant correlated with specific fatty acid ratios in both cell membranes and in both sub groups of patients. The most widely significant findings are listed below:

Ratios within the n-6 series:

The C20:4n-6 to C22:4n-6 ratio:

- in the erythrocyte membrane total phospholipids showed a statistically significant negative correlation with the EDSS ($r = -0.780$; $p = 0.013$)
- in lymphocyte membrane total phospholipids showed a statistically significant negative correlation with the EDSS and sensory scores (with the EDSS: $r = -0.875$; $p = 0.002$; with the sensory score: $r = -0.674$; $p = 0.047$)
- of the erythrocyte membrane PC fraction showed a statistically significant negative correlation with the EDSS and sensory scores (with the EDSS: $r = -0.786$; $p = 0.021$; with the sensory score: $r = -0.852$; $p = 0.007$)
- of the erythrocyte membrane PE fraction in phospholipids showed a trend ($p < 0.1$) toward a significant negative correlation with the EDSS ($r = -0.651$; $p = 0.081$)
- This relationship was not found between the plasma fatty acids and outcome (with the EDSS: $r = 0.113$; $p = 0.773$)

Ratios within the n-3 series:

Both the C20:5n-3 to C22:5n-3 ratio and the C22:5n-3 to C22:6n-3 ratio showed significant correlations with outcome:

- A significant negative correlation was found between the C20:5n-3 to C22:5n-3 ratio in the lymphocyte membrane total phospholipids, and the EDSS ($r = -0.685$; $p = 0.042$), and a positive correlation between the C22:5n-3 to C22:6n-3 ratio and the sensory score ($r = 0.727$; $p = 0.026$)
- The C22:5n-3 to C22:6n-3 ratio of the erythrocyte membrane PC fraction in phospholipids showed a trend towards significance with the sensory score ($r = 0.692$; $p = 0.057$)
- The C20:5n-3 to C22:5n-3 ratio of the erythrocyte membrane PE fraction in phospholipids showed a significant negative correlation with both the EDSS and the sensory scores

(EDSS: r = - 0.712; p = 0.048; and sensory: r = - 0.803; p = 0.016). The C22:5n-3 to C22:6n-3 ratio showed a significant positive correlation with both the EDSS and the sensory scores (EDSS: r = 0.761; p = 0.028; and sensory: r = 0.865; p = 0.006).

- However, in plasma total phospholipids, both these ratios also showed a trend toward significance with outcome. The C20:5n-3 to C22:5n-3 ratio showed a trend toward significance ($p < 0.1$) in a negative correlation with the EDSS (r = - 0.633; p = 0.068) and C22:5n-3 to C22:6n-3 ratio showed a trend toward significance in a positive correlation with the sensory score (r = 0.656; p = 0.055)

Ratios between the n-6 to n-3 series:

The C22:5n-6 to C22:6n-3 ratio:

- The C22:5n-6 to C22:6n-3 ratio in erythrocyte membrane total phospholipids showed statistically significant positive correlations with the EDSS (r = 0.910; p = 0.001)
- The C22:5n-6 to C22:6n-3 ratio in lymphocyte membrane total phospholipids showed a positive correlation with the sensory score (r = 0.709; p = 0.032)) and a trend towards significance ($p < 0.1$) with the EDSS (r = 0.650; p = 0.058)
- A significant positive correlation was found between the C22:5n-6 to C22:6n-3 ratio of the erythrocyte membrane PC fraction in phospholipids and the EDSS (r = 0.822; p = 0.012)
- The C22:5n-6 to C22:6n-3 ratio of the erythrocyte membrane PE fraction in phospholipids showed a significant positive correlation with the EDSS (r = 0.835; p = 0.010)
- A significant positive correlation was also found between the C22:5n-6 to C22:6n-3 ratio in plasma total phospholipids and the sensory score (r = 0.709; p = 0.032) and a trend toward significance ($p < 0.1$) with the EDSS (r = 0.650; p = 0.058)

CONCLUSION

Deficiencies of dietary origin as seen from plasma fatty acid determinations showed insufficient quantities of arachidonic acid (AA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). In erythrocyte and lymphocyte membranes, insufficient elongation and desaturation resulted in lower levels of both arachidonic acid (AA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3), the two structural fatty acids, as well as the possible up regulation of arachidonic acid (AA, C20:4n-6) specific phospholipase A_2. The inflammatory process could have been responsible for excessive arachidonic acid (AA, C20:4n-6) utilisation, which in turn could have been instrumental in further imbalances between the two fatty acids series at each elongation and desaturation step. Finally, this resulted in insufficient docosahexaenoic acid (DHA, C22:6n-3)

in all of its ratios with both the n-6 and n-3 fatty acid series, confirming results of earlier studies which showed that in MS, docosahexaenoic acid (DHA, C22:6n-3) concentrations were 50% lower in membranes from healthy subjects (Nightingale *et al.*, 1990). Therefore, both diet and dysregulation of fatty acid metabolism in MS may be major role players in the aetiology of the disease and warrants further investigation.

DEDICATION

To Helene and Jolané, with love and in gratitude.

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ABBREVIATIONS

Term	Definition
AA	Arachidonic acid
ALA	Alpha linolenic acid
CNS	Central nervous system
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EFAs	Essential fatty acids
EDSS (or Kurtzke EDSS)	Kurtzke Expanded Disability Status Scale
EM	Erythrocyte membrane
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAD	Flavin-adenine dinucleotide
FSS	Functional system score (e.g. sensory)
GC	Gas chromatography
GLA	Gamma-linolenic acid
HUFAs	Highly unsaturated fatty acids
IFNα (β) (γ)	Interferon-alpha (beta) (gamma)
IL-2, -4 and -6	Interleukin-2, -4 and -6
LA	Linoleic acid
LM	Lymphocyte membrane
LTB4	Leukotriene B4
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NADH	Nicotinamide adenine dinucleotide
NK	Natural killer cells
PBMCs	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGE2	Prostaglandin E2
PL	Phospholipids
PS	Phosphatidylserine
PUFAs	Polyunsaturated fatty acids

RRMS	Relapsing remitting multiple sclerosis
Th1 / Th2	T helper 1 (2) lymphocytes
TLC	Thin layer chromatography
Total Kurtzke EDSS (EDSS)	Kurtzke Expanded Disability Status Scale
TNF-α	*Tumor necrosis factor-alpha*
TPL	Total phospholipids

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INTRODUCTION

Modern Western medical treatment for autoimmune diseases, which includes MS, involves the administration of immunosuppressive drugs, such as beta interferon, cortisone (prednisone), methotrexate and cytoxan, which reduce the effectiveness of the entire immune system, and can have serious, sometimes life threatening, side effects (Perlmutter, 2006, http://www.msfacts.org).

An abnormality in essential fatty acid metabolism is also known to be present in MS patients (Horrobin, 1979). A high ratio of omega-6 to omega-3 fatty acids in cell membranes due to an excessive consumption of omega-6 fatty acids and a relative omega-3 fatty acid deficiency, may promote the pathogenesis of inflammatory disorders (Guesnet *et al.*, 2005) and ingesting these in equilibrated proportions could be crucial in the regulation of cellular physiology and in the prevention of pathologies such as autoimmune and inflammatory diseases.

Although there is an extensive literature on fatty acids in MS, the actual details of the mechanisms of fatty acid imbalances in MS have not been established. Further investigation on the abnormality of the MS cell membrane fatty acid profile is still needed. Previous studies had focused on individual fatty acids, but in this study it was deemed more relevant to investigate the relationships within and between these series, and their effect on outcome, and also to look for any possible cumulative effects, because the metabolism of fatty acids within the two series does have an effect on one another.

CHAPTER 1
LITERATURE REVIEW

To be diagnosed with MS can be stressful to patients and caregivers, because it predicts a progressive neurological disability for life. However, the outcome of MS is variable; some patients deteriorate fast, while others stay relatively symptom free for long periods of time. The gold standard to measure disease progression is the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). The EDSS is done by a neurologist or trained clinician, and registers signs of disability on a scale of 0 (no disability) to 10 (death due to MS).

Although there are indications that genetic factors play a role in MS, environmental factors are also implicated (Van Rensburg *et al.*, 2006). The aetiology of MS suggests that its occurrence depends on a combination of factors, such as possible viral infection, genotype and an initiating event within the central nervous system (Cooper, 1997). MS patients also have an abnormality in essential fatty acid metabolism (Horrobin, 1979).

The current consensus is that MS is an autoimmune disease (Chatzimanolis *et al.*, 2004), and follows a typical autoimmune pattern of demyelination, involving T lymphocytes and macrophages (Bruck, 2005), with an abnormality in lymphocyte function (Horrobin, 1979). However, Barnett and Prineas (2004) have found that lymphocytes may be absent from parts of early MS lesions, and when present, T cells are predominantly of the CD8 subtype and not of the CD4 subtype as had been reported previously (e.g. Brown, 2001). This is one of several immunopathological features of acute MS plaque pathology which casts doubt on the prevailing view that MS is a purely T cell-directed, macrophage-mediated process (Barnett *et al.*, 2006).

Work published recently by Barnett and Prineas (2004) suggests that oligodendrocyte injury may occur before macrophage activity is seen in newly forming MS lesions. In addition, apoptosis of oligodendrocytes, the cells that produce myelin, results in thinning of the myelin sheaths surrounding the axons. Apoptotic oligodendrocytes result in massive phosphatidylserine (PS) exposure, which acts as a scavenger signal to macrophages. Large numbers of activated microglia and macrophages, outnumbering lymphocytes by at least 10-20 times, are found in actively demyelinating lesions. Potential triggers of oligodendrocyte apoptosis in MS include locally generated proinflammatory cytokines, oxidative and nitrative stress and excitotoxicity (Barnett and Prineas, 2004). Markers for the production of the excitotoxin glutamate, have also been found in acute MS. In chronic MS, axonal demyelination (Van Meeteren *et al.*, 2005) eventually results in axon loss (Bruck, 2005).

Cumulative axonal loss leads to the progressive disability that MS patients experience (Bjartmar and Trapp, 2001).

Very clear evidence of the oxidative damage that takes place in MS has been demonstrated by Toshniwal and Zarling (1992). They measured hydrocarbons in the breath of MS patients, and found that patients with acute exacerbation of MS exhaled significantly higher concentrations of pentane compared to either controls or patients in remission. Pentane and ethane are degradation products of unsaturated fatty acids which are released during lipid peroxidation. They concluded that oxygen free radical activity is enhanced during exacerbation of MS.

Mammalian cells respond to any event which changes their cell membrane structure and induces the generation of lipidhydroperoxides from cell wall phospholipids (Spiteller, 2002). These are transformed to signalling compounds, some of which induce apoptosis. If the exerted impact exceeds a certain level, peroxylradicals are produced which cause severe damage by epoxidizing double bonds. Such reactions occur in all inflammatory diseases. Lipid peroxidation results in irreversible loss of fatty acids from membranes.

Phospholipids make up the basic structure of all cell membranes (Caret *et al.,* 1997) and their biological features are closely correlated with the type of fatty acids present in their structure (Manzoli *et al.*, 1970). Phospholipids and fatty acids determine cellular membrane fluidity and modulate membrane receptors (Zamaria, 2004). Polyunsaturated fatty acids (PUFAs) are precursors of active metabolites known collectively as eicosanoids which regulate cellular functions (Zamaria, 2004) such as nerve transmission and mediation of the inflammatory response (Caret *et al.,* 1997). Inflammation alters the composition of fatty acids incorporated into cells (Pond, 2005). The most abundant phospholipids in the body are phosphatidylcholine (lecithin) (PC) and phosphatidylethanolamine (PE) (Koay and Walmsley, 1999).

The fatty acid composition of the erythrocyte membrane is postulated to reflect the fatty acid composition of the central nervous system (Smuts *et al.,* 1995) and has been used in research studies on the aetiology of MS (Nightingale *et al.*, 1990). Erythrocyte fatty acid analysis can detect metabolic abnormalities and lipid peroxidation (Zamaria, 2004). Various erythrocyte abnormalities have been described in MS patients (Mayer, 1991). These membranes are believed to reflect defects associated with an altered unsaturated fatty acid content and metabolism.

The essential PUFAs, linoleic acid (LA, C18:2n-6) and alpha-linolenic acid (ALA, C18:3n-3), cannot be synthesised in the body and must be ingested from food (Zamaria, 2004). The parent dietary EFAs are converted to their metabolites by a series of alternating desaturations and elongations (6-desaturation, followed by elongation, then 5-desaturation, then elongation, and then 6-desaturation again) (Horrobin and Manku, 1990). The PUFAs are subdivided into n-3, n-6 and n-9 subtypes, depending on the position of the double bond closest to the methyl terminal of the fatty acid chain (Koay and Walmsley, 1999).

The process of desaturation, like that of chain elongation, occurs on the endoplasmic reticulum of, for example, the oligodendrocyte cell body (Broom, 2005). The desaturases are regulated at a transcriptional level and all three share a common feedback regulation mechanism to maintain homeostasis in membrane phospholipids (Nakamura and Nara, 2004). A desaturation enzyme has two iron atoms and requires molecular oxygen, NADH and cytochrome b_5. The reaction results in the oxidation of both fatty acids and NADH (Broom, 2005).

Unsaturated fatty acids are produced by terminal desaturases, which are membrane-bound, nonheme iron dependent enzymes containing two iron atoms in their active sites (Voet and Voet, 2004). Desaturases remove two hydrogen atoms, creating a carbon-carbon double bond (Sprecher, 2000). During this process a number of adverse reactions may occur. PUFAs have a characteristic methylene interrupted double bond structure which is particularly susceptible to the hydroxyl radical (•OH) mediated abstraction of H atoms (Lunec, 1990). Lipid peroxidation caused by free radical damage will cause damage to lipids and the cell membranes (myelin is just one big membrane). Hypoxia-like tissue damage had been described by Stadelmann *et al.* (2005) in the pathogenesis of Balo's concentric sclerosis, a form of acute MS. Iron deficiency may also influence the metabolism of specific fatty acids (Smuts *et al.*, 1994).

The degree of unsaturation of fatty acids affects the physical properties of membrane phospholipids (Nakamura and Nara, 2004). Most of the effects of linoleic acid (LA, C18:2n-6) and alpha-linolenic acid (ALA, C18:3n-3) are dependent not on the ingested EFAs themselves, but on their derivatives (Horrobin and Manku, 1990).

Phospholipids are particularly important in cell signalling and in the formation and remodelling of dendrites and synapses (Horrobin, 1999). The brain phospholipids are uniquely rich in highly unsaturated fatty acids with three to six double bonds. In neurones, in contrast to other tissues, there are only small amounts of the parent essential fatty acids, linoleic acid (LA, C18:2n-6) and alpha-linoleic acid (ALA, C18:3n-3). There are however large

amounts of arachidonic acid (AA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3), with smaller, but important amounts of dihomo-γ-linolenic acid (DGLA, C20:3n-6), adrenic acid (C22:4n-6), eicosapentaenoic acid (EPA, C20:5n-3) and docosapentaenoic acid (DPA, C22:5n-3). If conversion of linoleic acid (LA, C18:2n-6) to arachidonic acid (AA, C20:4n-6) and dihomo-γ-linolenic acid (DGLA, C20:3n-6), and alpha-linoleic acid (ALA, C18:3n-3) to eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) is impaired, the only way for the brain to obtain these EFAs is directly from the food. Arachidonic acid (AA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) constitute 80 – 90 % of the total weight of neuronal and retinal fatty acids. Docosahexaenoic acid (DHA, C22:6n-3) and arachidonic acid (AA, C20:4n-6) play the most important role in nerve function (Haag, 2003). Viral infections inhibit the formation of arachidonic acid (AA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) (Horrobin, 1999).

Docosahexaenoic acid (DHA, C22:6n-3) is the longest and most unsaturated fatty acid commonly found in membranes (Stillwell *et al.*, 2005). Long chain n-3 fatty acids such as eicosapentaenoic acid (EPA, C20:5n-3) or docosahexaenoic acid (DHA, C22:6n-3) are found naturally and mainly in marine lipids, which in most Western diets account for only a small proportion of ingested fatty acids (Nightingale *et al.*, 1990). Despite this, n-3 fatty acids such as docosahexaenoic acid (DHA, C22:6n-3) are present in substantial amounts in the phospholipids of the human central nervous system (CNS) (Nightingale *et al.*, 1990) and in retinal tissue (Stillwell *et al.*, 2005). docosahexaenoic acid (DHA, C22:6n-3) levels in the brains of different animals are constant despite a wide variation in diet, suggesting that docosahexaenoic acid (DHA, C22:6n-3) may have an important function in the CNS (Nightingale *et al.*, 1990). Docosahexaenoic acid (DHA, C22:6n-3) in the apparently normal white matter of the CNS of MS patients has been reported to be less than half that of normal values. MS may be associated with either a relative deficiency of dietary long chain n-3 fatty acids or with some metabolic impairment of elongation and desaturation of alpha-linoleic acid (ALA, C18:3n-3) to docosahexaenoic acid (DHA, C22:6n-3) in the CNS.

Alpha-linoleic acid (ALA, C18:3n-3) is found in marine oils and some seeds, nuts and grains, and must presumably provide the dietary source of CNS docosahexaenoic acid (DHA, C22:6n-3) in those not ingesting marine oils (Nightingale *et al.*, 1990). However, it was found that increasing alpha-linoleic acid (ALA, C18:3n-3) intake in normal individuals will result in an increase in the proportion of eicosapentaenoic acid (EPA, C20:5n-3) in plasma lipids, as well as in erythrocytes, leukocytes and in platelets, but with no increase in docosahexaenoic acid (DHA, C22:6n-3), and docosahexaenoic acid (DHA, C22:6n-3) may even decline when alpha-linoleic acid (ALA, C18:3n-3) intake is very high (Burdge and Calder, 2005).

Both n-6 and n-3 fatty acids are required for the normal structure and functioning of the nervous system (Horrobin, 1999). N-6 fatty acids are essential for normal growth, development and health (Berry, 2001) and a deficiency results in disease (Nightingale et al, 1990). Theoretical and epidemiological evidence also suggests the involvement of n-6 PUFAs in both disease progression and prevention (Berry, 2001). N-6 function cannot be considered in isolation however, but needs to be seen as part of the complex of nutrient interactions with n-3 fatty acids (which compete for the same enzymatic pathways) and antioxidants. Dietary supplementations could affect the metabolic interactions both within and between the n-6 and n-3 fatty acid pathways, with a subsequent effect on the physiological balance of their derivatives such as eicosanoids. The administration of high doses of fish oil (n-3) to humans inhibits 6-desaturation and reduces the concentrations of metabolites of linoleic acid (LA, C18:2n-6) (n-6) (Horrobin and Manku, 1990). Therefore the concentrations of n-6 to n-3 fatty acids should be in balance.

The risk of developing MS is associated with increased intake of saturated fatty acids (Van Meeteren *et al.*, 2005). Additionally, both n-6 and n-3 fatty acids are known to have an effect on the immune system and a relative deficiency of n-3 in phospholipid components of the immune system or in the CNS itself may also be important in the pathogenesis of MS (Nightingale *et al.*, 1990). Many factors contribute to the complex course of inflammatory reactions (Simopoulos, 2002). Microbiological, immunological and toxic agents can initiate the inflammatory response by activating a variety of humoral and cellular mediators. N-6 polyunsaturated fatty acids, abundant in the Western diet, are precursors for a number of mediators of inflammation including prostaglandin E2 (PGE2) (Bagga *et al.*, 2003) from the metabolism of arachidonic acid (AA, C20:4n-6) (Simopoulos, 2002). PGE2 is a potent mediator of inflammation and cell proliferation (Bagga *et al.*, 2003) and are released from membrane phospholipids in the course of inflammatory activation (Simopoulos, 2002). N-3 PUFA, in particular eicosapentaenoic acid (EPA, C20:5n-3), have anti-inflammatory properties (Zamaria, 2004) and when released compete with arachidonic acid (AA, C20:4n-6) for enzymatic metabolism inducing the production of less inflammatory and chemotactic derivatives (Simopoulos, 2002). Dietary supplements rich in n-3 PUFA reduce the concentrations of PGE2 and increase the synthesis of PGE3, which are believed to be less inflammatory (Bagga *et al.*, 2003). Successful replacement of n-6 PUFA with n-3 PUFA in cell membranes can result in a decreased cellular response to mitogenic and inflammatory stimuli. eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) are more biologically potent than alpha-linoleic acid (ALA, C18:3n-3) (Simopoulos, 2002).

The n-6 and n-3 EFAs are competitive inhibitors of each other's metabolism (Horrobin and Manku, 1990). This competition is not readily apparent at the elongation steps, but at the

desaturation steps, where a large amount of one type of EFA will interfere with the metabolism of the other. All fatty acids are only effective if they are present in optimum amounts (De Villiers, 1992). In most cells of the body, quantities of n-6 EFAs exceed those of n-3 EFAs by 3-6 to 1, the main exceptions being the eye and the brain, where the amounts of the two series are not far from equal (Horrobin and Manku, 1990).

The ratio of membrane n-3 to n-6 PUFAs influences neurotransmission and prostaglandin formation, processes that are vital for the maintenance of normal brain function (Haag, 2003). The ratio of membrane n-3 to n-6 PUFAs can be modulated by dietary intake. A balanced intake of both n-6 and n-3 PUFA is essential for the well being of the human body (Zamaria, 2004).

Research studies in the early 1950s on fatty acids in MS were focused on the possible role of n-6 fatty acids, because n-3 fatty acids were not considered essential at that time (Nordvik *et al.*, 2000). Reduced levels of linoleic acid (LA, C18:2n-6) and arachidonic acid (AA, C20:4n-6) were found in the brain, serum, platelets and erythrocytes of MS patients. Conflicting results have been reported since then.

Cheravil (1984) found a significant decrease in the percentage composition of arachidonic acid (AA, C20:4n-6) in the plasma of MS patients. He also found a decrease in the percentage composition of linoleic acid (LA, C18:2n-6) in the erythrocyte and lymphocyte membranes and plasma of MS patients. Holman *et al.* (1989) found linoleic acid (LA, C18:2n-6) to have normal values in the plasma of MS patients, but all subsequent n-6 fatty acids to be subnormal. They also found all n-3 fatty acids in the plasma to be subnormal. Nightingale *et al.* (1990) found no reduction in MS patients in the percentage composition of erythrocyte membrane linoleic acid (LA, C18:2n-6) of the PC or PE fractions in phospholipids. They did find a significant reduction in eicosapentaenoic acid (EPA, C20:5n-3)and a significant increase in dihomo-gamma-linolenic acid dihomo-γ-linolenic acid (DGLA, C20:3n-6) of the PE fraction in the erythrocyte membranes of MS patients.

Treatment of MS with PUFAs is an approach that is firmly grounded in basic fundamental scientific concepts (Horrobin, 1979). However, careful consideration is necessary when supplementing with fatty acids. For example, recent studies in a variety of disciplines suggest the possible deleterious effects of high linoleic acid (LA, C18:2n-6) consumption on cardiovascular disease, cancer and insulin resistance (Berry, 2001).

Paty *et al.* (1978) could detect no effect of dietary supplementation with linoleic acid (LA, C18:2n-6) on the progression of neurological findings in MS, and the same results were

found in a study done by Bates *et al.* (1978). A significant improvement in symptoms was found in MS patients after an n-3 supplementation diet as measured by the EDSS (Nordvik *et al.*, 2000). Field and Joyce (1983) found improvement of MS symptoms on supplementation with n-6 fatty acids. In a study conducted by Zhang *et al.* (2000), no difference was found from any type of dietary fat intake. Van Rensburg *et al.* (2006) found significant neurological improvement in MS patients, as measured by the Kurtzke EDSS, after n-3 and n-6 supplementation in relatively small concentrations (500 mg/day of each).

The role of the differences in cell membrane fatty acids in MS has not been fully elucidated, and warrants further investigation. The effect of the ratios between the two essential fatty acid series on disease outcome should be a good indication of the activity of the enzymes in the two pathways in patients not on fatty acid supplementation.

CHAPTER 2
RESEARCH FOCUS

2.1 Problem statement

The focus of this study was to investigate whether an imbalance in the ratio of n-6 to n-3 fatty acids in the membranes of erythrocytes and lymphocytes of MS patients is associated with their neurological outcome as measured by the Kurtzke EDSS.

Erythrocyte membranes were used in this study as a marker for the brain (Smuts *et al.*, 1999) and lymphocyte membranes because of their reported involvement in MS neuronal demyelination. Plasma fatty acid composition of total phospholipids was measured to investigate a possible dietary influence on disease status.

2.2 Background to problem statement

2.2.1 The aetiology of multiple sclerosis

The aetiology of multiple sclerosis is unknown (Okuda *et al.*, 2005), however, both an autoimmune and infectious aetiology are suspected (Brown, 2001). Autoreactive T cells against CNS myelin antigens are thought to play a crucial role in the pathogenesis of MS (Okuda *et al.*, 2005). However Barnett and Prineas (2004) have found that lymphocytes may be absent from parts of nascent MS lesions and, when present, T cells are predominantly of the CD8 subtype.

The current consensus is that the mechanisms leading to the formation of lesions in MS brains include typical autoimmune patterns of demyelination involving T cells and macrophages as characteristic effector mechanisms (Bruck, 2005). Work done by Barnett and Prineas (2004) suggests however, that oligodendrocyte injury may occur before macrophage activity is seen in newly forming MS lesions, and that apoptotic oligodendrocytes could result in massive phosphatidylserine (PS) exposure, a dominant mammalian scavenger signal, and accompanying phagocyte activation.

The functional result of lesion formation in the MS brain is that nerve impulse transmission along the axon is impaired (Jelinek, 2000a) with subsequent motor and sensory disturbances and disabilities (Brown, 2001). As more of these lesions occur, more pathways are impaired, which result in a gradual decline in function (Jelinek, 2000a).

2.2.2 Pathology

The pathological hallmarks of MS lesions in the brain and spinal cord are inflammation, demyelination, and eventually axon loss and gliosis (Bruck, 2005). Additionally, oligodendrocyte dystrophy patterns of demyelination and oligodendrocyte apoptosis were observed in the MS brain (Bruck, 2005).

2.2.3 Epidemiology

MS has a much higher incidence among Caucasians than in any other race (Johnson, 2000). Females are also much more susceptible than males, in a ratio of about 3:2 (Jelinek, 2000a). In the United Kingdom the incidence of MS is approximately 1 in 800 people and in northern Europe 1 in 1000. An estimated 2.5 million people worldwide have MS.

MS is the most common neurological disorder in young adults (Jelinek, 2000a) and also the most common inflammatory and demyelinating disease of the CNS of possible autoimmune origin (Okuda et al, 2005).

2.2.4 Types and stages of MS

Although every individual may experience a different combination of MS symptoms, there are a number of distinct disease stages and/or types that have been identified as the naturally occurring course of MS (Leary *et al.*, 2005). They are benign MS, relapsing remitting MS, secondary progressive MS, primary progressive MS and progressive relapsing MS.

2.2.5 Treatment

Modern Western medical treatment for autoimmune diseases, which includes MS, involves the administration of immunosuppressive drugs, such as beta interferon,

cortisone (prednisone), methotrexate and cytoxan, which reduce the effectiveness of the entire immune system, and can have serious, sometimes life threatening, side effects (Perlmutter, 2006, http://www.msfacts).

The results of randomised controlled trials of recombinant interferon and glatiramer acetate in MS showed a 30% reduction of relapses in the first year, but no evidence of benefit beyond one year (De Jong *et al.*, 2005, http://neurology.thelancet.com). This finding differs from the Cochrane review which concluded that interferon slightly reduces the number of patients with exacerbations in the first year of treatment and that the clinical effect beyond 1 year is unknown (De Jong *et al.*, 2005).

The controversy surrounding treatment of MS patients (De Jong *et al.*, 2005) and problematic side effects of the treatment, indicate the need for a different route of investigation. No axon-protective therapy has been established and the mechanisms and effector molecules involved in axonal degeneration are still unknown (Bruck, 2005).

2.2.5.1 Corticosteroids

Corticosteroids are used as a therapy to suppress the immune system (Jelinek, 2000b). Steroids work by decreasing the levels of some of the eicosanoids. It has been found to have a beneficial effect in the recovery from acute relapses in MS patients. Side effects of steroids are however many, and include an increased risk of infection.

2.2.5.2 Interferon

Treatment of MS has advanced dramatically in recent years, with the introduction of drugs like beta-interferon (IFN-β) (Bruck, 2005). Interferon type I, alpha (IFN-α) and beta interferon, tend to suppress the immune system, while type II, gamma interferon (IFN-γ) promotes inflammation and deterioration (Jelinek, 2000c). Decreased IFN-β production has been demonstrated in the white cells of MS patients. The benefit of interferon treatment is a reduction in the relapse rate and a reduced development in new lesions in some of the types of MS.

Some patients however develop antibodies against the interferons, which neutralise the interferons, reduce effectiveness of treatment and accelerate disease progression (Namaka *et al.*, 2006). Some patients also develop side effects to the interferons. Adverse reactions listed for one of the commercial preparations of IFN-βIb are CNS disturbances such as depression and suicidal tendencies, leucopenia and severe hypersensitivity reactions.

2.2.6 Prognosis

Some patients deteriorate fast, while others stay relatively symptom free for long periods of time. However, no axon-protective therapy has been established and the mechanisms and effector molecules involved in axonal degeneration are still unknown (Bruck, 2005).

2.3 Research question

Does an imbalance in the ratio of n-6 to n-3 fatty acids in the erythrocyte and lymphocyte membranes of MS patients have an effect on their neurological outcome as measured by the Kurtzke EDSS?

2.4 Hypothesis

2.4.1 Null hypothesis

An imbalance in the ratio of n-6 to n-3 fatty acids in the erythrocyte and lymphocyte membranes of MS patients, will not correlate with their neurological outcome as measured by the Kurtzke EDSS.

2.4.2 Alternative hypothesis

An imbalance in the ratio of n-6 to n-3 fatty acids in the erythrocyte and lymphocyte membranes of MS patients, will correlate with their neurological outcome as measured by the Kurtzke EDSS.

2.5 Research objectives

1. To determine the n-6 and n-3 fatty acid ratios in total phospholipids, as well as in the PC and PE phospholipid subfractions, in both the erythrocyte and lymphocyte membranes of MS patients, and 2. to establish whether there is an association between membrane fatty acid composition and severity of neurological outcome as measured by the Kurtzke EDSS.

CHAPTER 3
METHODOLOGY

3.1 Research study design

The research study design was quantitative in nature. All aspects of the study were designed before data was collected. Structured procedures and formal instruments were used to collect data. Measurement of variables was numerical and statistical analysis was used to find an association between MS and fatty acid ratios.

The study design was observational and not experimental. Its focus was on outcome, which was known (disease), and exposure to risk factor, which was unknown.

3.2 Data collection

3.2.1 Recruitment of patients

MS patients were contacted and recruited through the MS Society, Western Cape Branch, South Africa.

3.2.1.1 Ethical considerations

3.2.1.1.1 Informed consent from the patients

Informed written consent was obtained from each of the participants.
(English and Afrikaans information and consent forms are included as appendices B and C respectively).

3.2.1.1.2 Ethical approval

Ethical approval has been obtained from the Faculty of Health Sciences and Wellness Ethics Committee, Cape Peninsula University of Technology.
Project leader: Dr Susan Janse van Rensburg.

3.2.1.1.3 Ethical statement

This study was conducted according to the ethical principles laid down in the World Medical Association Declaration of Helsinki (World Medical Association, 2005). It states that biomedical research that involves human beings must be to improve diagnostic, therapeutic and prophylactic procedures and to improve the understanding of the aetiology and pathogenesis of disease and that the research must at all times be conducted in such a way that the patient receives humane treatment.

Some of the important specifications of this Declaration are that:

- Both patients and healthy control persons should be volunteers.
- Patients should be adequately informed of the aims, methods, anticipated benefits and potential hazards of the study.
- They should give written informed consent.
- The researcher should withdraw any person from the study if it is in the best interest of that person.
- The integrity, personality and privacy of the person should be respected.
- A clear distinction is made between therapeutic and non-therapeutic research.

This was not an interventional study. The South African Medical Research Council (MRC, 2005) declared observational research, such as the anonymous examination of a specimen taken from a patient, as involving no risk and no interference with the mental or physical integrity of the person.

3.2.2 Inclusion / exclusion criteria

3.2.2.1 Inclusion criteria MS patients

Relapsing remitting MS patients
Patients in remission
Patients with a Kurtzke EDSS score of more than 1

3.2.2.2 Exclusion criteria MS patients

Patients on any steroid treatment for the preceding 3 months
Patients on any dietary supplementation likely to affect fatty acid levels
Patients diagnosed with an additional disease

3.2.2.3 Control subjects

The control group was recruited from the laboratory staff at the Department of Pathology, Tygerberg Hospital.

3.2.3 Justification

Even though MS has a relatively low incidence rate in South Africa there are a number of justifiable reasons why more research on some of the problems surrounding this disease is necessary.

3.2.3.1 Disability

MS is a chronic inflammatory disease of the CNS, which results in oligodendrocyte (Van Meeteren *et al.*, 2005) and axon loss (Bruck, 2005) and leads to the progressive disability that MS patients experience (Bjartmar and Trapp, 2001).

3.2.3.2 Treatment

Not all patients respond well to treatment with the prescribed drugs (Bruck, 2005).

3.2.3.3 International studies

In South Africa MS is a disease with low prevalence (Van Rensburg *et al.*, 2006). However, the disease has a high incidence rate in some countries, and worldwide an estimated 2.5 million people have MS (Jelinek, 2000a). Publications on this topic are abundant in the literature, and interest in the disease and its aetiology is widespread.

3.2.3.4 Infrastructure

This research project is part of an ongoing project on the aetiology of MS.

3.2.3.5 Availability of research subjects

The MS Society Western Cape Branch has been extremely helpful in establishing contact with the patients.

3.2.3.6 Relevance of findings to other diseases

MS is only one of the neurodegenerative pathologies that affect patients (Pino *et al.*, 2005). The others are Alzheimer's disease (AD), schizophrenia and Parkinson's disease (PD) (Pino *et al.*, 2005). Any knowledge gained in this study could be of benefit in these fields as well.

3.2.4 Sampling of study subjects

3.2.4.1 Health questionnaire

A health questionnaire was administered to each of the MS patients who consented to the study, and was completed by the respondents.
(English and Afrikaans health questionnaires are included as appendices D and E respectively).

3.2.4.2 Kurtzke Expanded Disability Status Scale (EDSS)

The functional disability status of each patient (not controls) was measured by a neurologist or a trained clinician and was registered on a Kurtzke Expanded Disability Status Scale (EDSS). The EDSS is a method of quantifying disability in MS patients (Kurtzke, 1983).

The EDSS quantifies disability in eight Functional Systems 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".

Functional disabilities are estimated in which higher numbers 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 a deceased patient, while the sub scores for the Functional Systems are from 0 to 6 (Kurtzke, 1983).

(A Kurtzke EDSS form is included as appendix A).

3.3 Sample size calculation

MS is not a disease of high prevalence in South Africa and no data was available to calculate sample size. The patients in this study were well-defined MS patients, therefore, even though the sample size was rather small, it was seen as sufficient for a pilot study. Recruitment of patients was a wide selection and inclusion and exclusion criteria were adhered to strictly. This research project is seen as observational and explorative work that needs to be further investigated.

3.4 Blood samples

Blood samples were collected from all participants into tubes with anti-coagulant ethylenediamine tetraacetic acid (EDTA). A laboratory number together with the initials of each subject were used to ensure confidentiality.

3.5 Data production methods

The percentage composition of fatty acids in total phospholipids (in erythrocyte membranes, lymphocyte membranes and in plasma) as well as the percentage composition of the fatty acids of the PC and PE fractions in phospholipids (in erythrocyte membranes) of both the MS and control groups, were determined by gas chromatography (GC).

3.6 Data handling

A database was set up on Microsoft Excel (computer software program) spreadsheets.

3.7 Data processing

3.7.1 Identification of data variables

3.7.1.1 Disability status

The disability status of the patients were seen as the dependent variable which measures the problem (outcome) under study: the Kurtzke EDSS and Functional System Scores (e.g. sensory score) (numerical variables).

3.7.1.2 Fatty acid percentage composition and ratios

The fatty acid percentage compositions as well as the ratios between the n-6 and n-3 fatty acid series in total phospholipids and of the PC and PE phospholipid sub-fractions were used as independent variables (numerical variables).

3.7.2 Units of analysis

3.7.2.1 Fatty acids

Fatty acids are reported as percentage composition of the total fatty acids identified. Both individual fatty acid values (numerical variables) and the ratios between and within the n-6 and n-3 fatty acid series (numerical variables) were used.

3.7.2.2 Disability status

Disability was measured on a Kurtzke Expanded Disability Status Scale (numerical variables) and in the Functional System subscores (numerical variables).

3.8 Statistical analysis

A software program, Statistica, was used to do statistical analysis (Statistica Stat Soft, Inc (2004) version 7, www.statsoft.com).

3.8.1 General ANOVA/MANOVA

One-way ANOVA was used to analyse designs with a single categorical independent variable. Statistical significant differences in the percentage fatty acid composition between the MS and the control groups were reported as mean ± standard error and p-value.

3.8.2 Spearman rank order correlation

Spearman rank order correlation analysis was used to establish statistical significant correlations between the ratios of the n-6 to n-3 fatty acids, and the Kurtzke EDSS and sensory sub scores (non-normally distributed data). Statistical significant differences were reported as the correlation value (r) and statistical significance was expressed as the p-value.

CHAPTER 4
MATERIALS AND METHODS

4 Introduction

The fatty acid (FA) percentage composition in total phospholipids (TPL) in the erythrocyte membranes (EM), lymphocyte membranes (LM) and plasma of sub group A, as well as the FA percentage composition of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in phospholipids (PL) in the EM of sub group B in MS patients and control persons, were determined by gas chromatography (GC).

4.1 Lymphocyte, erythrocyte and plasma separation from whole blood

4.1.1 Blood collection

Blood was collected in 2 x 9 ml vacuette tubes treated with anti-coagulant EDTA (Beckman Coulter, Howard Place, Cape Town, South Africa).

4.1.2 Separation method

Blood was layered over a separation medium, histopaque, in 50 ml centrifuge tubes (3 ml histopaque / 2 ml blood), without mixing of the two layers. The tubes were centrifuged at 400 g for 20 minutes at room temperature. The top layer of plasma was taken off, spun twice more to remove any possible platelet contamination and stored at –70 °C. The interface layer, containing lymphocytes, was taken off and washed twice with a balanced salt solution to remove possible platelet contaminants. It was then resuspended in 1 ml balanced salt solution and stored at –70 °C. Cells were taken from the erythrocyte pellet, washed twice with a balanced salt solution to get rid of possible white cell contaminants, frozen and stored at –70 °C.

The method followed for lymphocyte separation is from MP Biomedicals (MP Biomedicals, LLC, Technical Information http://search.cosmobio.co.jp/cosmo_search_p/search_gate2/docs/CPL_/50494.20010328).

4.2 Determination of the FA percentage composition in TPL and the FA composition of the PC and PE fractions in PL by TLC and GC.

4.2.1 Methods

The RBC, plasma and lymphocytes were thawed and extracted with chloroform/methanol (2:1; v/v) according to a modified method of Folch *et al.* (1957). All the extraction solvents contained 0.01% butylated hydroxytoluene (BHT) as an antioxidant. Phosphatidylcholine-diheptadecanoic acid (PC 17:0) was used as internal standard to quantify the individual fatty acids. Neutral lipids in plasma, RBC and lymphocytes 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 benzin (bp 40-60°C)/diethyl ether (peroxide free)/acetic acid (90:30:1, by vol) as previously described (Van Jaarsveld *et al.*, 2000). Individual phospholipid classes, analysed in some RBC samples, were separated by TLC on pre-coated silica gel 60 plates (10 x 10 cm) without a fluorescent indicator (Merck, Darmstadt, Germany) using chloroform/ethanol/ triethylamine/water (40:50:40:10, v/v) as solvent. The lipid bands containing phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from RBC extracts were visualized with longwave 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% H_2SO_4/methanol at 70°C for 2 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 removed and evaporated to dryness, redissolved in CS_2 and analyzed by GLC (Varian Model 3300 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: hydrogen, 25 mL/min; air, 250 mL/min; and hydrogen

(carrier gas), 2-4 mL/min. Temperature programming was linear at 4°C/min, initial temperature 160°C, final temperature 220°C, injector temperature 240°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).

4.2.2 Fatty acid identification

The individual fatty acids were calculated as a percentage composition of the total fatty acids identified. Table 21 lists the abbreviations of the chemical formulae and the common names of the fatty acids considered in this study.

CHAPTER 5
RESULTS

5 Introduction

The focus of this study was to investigate whether an imbalance in the ratio of n-6 to n-3 fatty acids in the membranes of erythrocytes and lymphocytes of MS patients is associated with their neurological outcome as measured by the Kurtzke EDSS.

The fatty acid composition of blood was determined in 2 sub groups of patients. The results were analysed separately: in one group (group A) the fatty acids were determined on the total phospholipid fraction and in the second group (group B) the fatty acids of the PC and PE phospholipid subfractions were determined.

The results were reported on in 5 different categories:
Firstly, on the fatty acid composition in total phospholipids in group A:

- in the erythrocyte membrane
- in the lymphocyte membrane
- and in plasma

Secondly, on the fatty acid composition of the PC and PE phospholipid subfractions in group B:

- of the PC subfraction in the erythrocyte membrane
- and of the PE subfraction in the erythrocyte membrane

In each of these categories differences between the patients and controls were reported on:

- the percentage composition of individual fatty acids
- as well as on their ratios with each other

In addition, in each of these categories correlation studies were also done between disease outcome (EDSS and sensory subscores) in the MS patients and:

- the fatty acid percentage composition
- as well as with the ratios of these fatty acids with each other

5.1 Demographic details

5.1.1 Gender and ethnicity

23 MS patients and 23 control persons consented to this study. The study population included 3 brown females, 17 white females, 1 brown male and 2 white males. No black patients were available for recruitment to the study. The control group contained 2 brown females, 19 white females and 2 white males.

Six of the patients were excluded from the study because they did not meet the inclusion or they met the exclusion criteria. Two patients had received steroid treatment, 1 patient had been diagnosed with haemochromatosis, 2 patients had an EDSS score of 1, indicating very low disease expression, and 1 patient was using fatty acid supplementation.

The remaining patients were subdivided into two groups; A and B. Group A included 9 white female patients and age-, ethnicity- and gender matched control persons (8 white female persons). MS sub group B included 2 brown females, 3 white females, 1 brown male and 2 white males group. The control group had 2 brown females, 11 white females and 2 white males.

5.1.2 Age

The mean ± SE age in sub group A was 38.444 ± 2.819 (range 28-50) for the MS patients, and 42.375 ± 3.590 (range 28-55) for the control persons. The mean ± SE age in sub group B was 48.375 ± 2.976 (range 31-58) for the MS patients and for the control persons it was 40.800 ± 2.407 (range 26-57).

5.1.3 Disability status

The mean ± SE for the total Kurtzke EDSS was 2.222 ± 0.265 for patient sub group A. The mean ± SE for the total Kurtzke EDSS was 4.438 ± 0.684 for patient sub group B.

5.2 Parameters reported on

5.2.1 *Fatty acids*

In sub group A the fatty acid percentage composition in erythrocyte and lymphocyte membrane and plasma total phospholipids of both the MS and control groups was determined. In sub group B the fatty acid percentage composition of the erythrocyte membrane PC and PE fractions in phospholipids of the MS and control groups were determined.

5.2.2 The Kurtzke EDSS and Functional Systems Scores

In sub group A only the total Kurtzke EDSS and the Functional system (FS) sensory sub score could be used in the correlation studies. The other FS sub scores were zero, indicating no disability. They were pyramidal, cerebellar, brainstem, bowel and bladder, visual and cerebral. The patients in sub group B had higher disability status and all FS scored higher than 0.

5.3 Statistical analysis

In both sub group A and B, differences in the fatty acid percentage composition, as well as in the ratios, were determined between the MS and control groups.

In both groups correlations between the disability scores, EDSS and sensory scores, and the fatty acid percentage composition, as well as with the ratios, were determined in MS patients.

5.4 Sub group A: Statistical analysis of fatty acids in erythrocyte membrane total phospholipids

5.4.1 Differences between the MS and control fatty acid percentage composition in erythrocyte membrane total phospholipids (Table1)

In this study no significant difference was found between the MS and control groups in the percentage composition of the n-3, n-6, total n-3 or total n-6 fatty acids. The only noteworthy differences were the trend towards significance ($p < 0.1$) of a higher C20:2n-6 and total n-6 in MS compared to controls.

Table 1

Differences between MS and control fatty acid percentage composition: in erythrocyte membrane total phospholipids

Fatty acids	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C18:3n-3	0.067 ± 0.010	0.066 ± 0.019	0.984
C20:3n-3	0.006 ± 0.006	< 0.001	0.362
C20:5n-3	0.418 ± 0.042	0.409 ± 0.037	0.881
C22:5n-3	1.650 ± 0.041	1.753 ± 0.107	0.358
C22:6n-3	4.883 ± 0.427	4.816 ± 0.348	0.906
C18:2n-6	10.739 ± 0.426	10.169 ± 0.809	0.529
C18:3n-6	0.055 ± 0.018	0.062 ± 0.018	0.788
C20:2n-6	0.447 ± 0.070	0.297 ± 0.030	0.095
C20:3n-6	1.843 ± 0.143	1.664 ± 0.065	0.293
C20:4n-6	14.887 ± 0.512	14.237 ± 0.373	0.331
C22:4n-6	3.334 ± 0.238	2.980 ± 0.121	0.222
C22:5n-6	0.718 ± 0.076	0.559 ± 0.093	0.203
Total n-3	7.024 ± 0.436	7.045 ± 0.448	0.974
Total n-6	32.021 ± 0.780	29.967 ± 0.837	0.093

5.4.2 Differences between MS and control fatty acid ratios in the n-3 and n-6 fatty acid series: in erythrocyte membrane total phospholipids

In this study no statistically significant difference was found between the MS and control groups in the ratios between and within the n-3 and n-6 fatty acids, or in the ratio of total n-6 to total n-3 fatty acids.

Table 2

Differences between MS and control fatty acid ratios in the n-3 and n-6 fatty acid series: in erythrocyte membranes total phospholipids

Fatty acid ratios	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C20:5n-3 to C22:5n-3	0.255 ± 0.027	0.236 ± 0.023	0.598
C22:5n-3 to C22:6n-3	0.360 ± 0.033	0.371 ± 0.023	0.786
C20:2n-6 to C20:3n-6	0.245 ± 0.038	0.180 ± 0.017	0.158
C20:3n-6 to C20:4n-6	0.125 ± 0.010	0.118 ± 0.006	0.563
C20:4n-6 to C22:4n-6	4.646 ± 0.358	4.815 ± 0.162	0.686
C22:4n-6 to C22:5n-6	4.878 ± 0.351	6.141 ± 0.774	0.143
C20:4n-6 to C20:5n-3	38.894 ± 4.318	37.937 ± 5.094	0.887
C22:4n-6 to C22:5n-3	2.026 ± 0.145	1.749 ± 0.140	0.191
C22:5n-6 to C22:6n-3	0.155 ± 0.019	0.121 ± 0.022	0.265
Total n-6 to total n-3	4.718 ± 0.345	4.395 ± 0.344	0.520

5.4.3 Correlation between the EDSS and sensory scores, and the n-3 and n-6 fatty acid percentage composition in erythrocyte membrane total phospholipids in MS patients (Table 3)

A statistically significant negative correlation was found between C22:6n-3 and the sensory score, and a highly significant negative correlation between the total n-3 fatty acids and the sensory score.

In the n-6 fatty acid series statistically significant positive correlations were found between C20:2n-6 and C22:4n-6 with the EDSS.

Table 3

Correlation between the EDSS and sensory scores, and the n-3 and n-6 fatty acids in erythrocyte membrane total phospholipids in MS patients. Significant correlations are in bold.

Fatty acids	Kurtzke	r	p-value
C18:3n-3	EDSS	-0.373	0.323
	Sensory	0.098	0.803
C20:3n-3	EDSS	< 0.001	1.000
	Sensory	0.219	0.572
C20:5n-3	EDSS	-0.433	0.244
	Sensory	-0.346	0.362
C22:5n-3	EDSS	0.087	0.825
	Sensory	-0.301	0.430
C22:6n-3	EDSS	-0.615	0.078
	Sensory	**-0.816**	**0.007**
C18:2n-6	EDSS	-0.035	0.929
	Sensory	-0.248	0.520
C18:3n-6	EDSS	-0.367	0.332
	Sensory	-0.019	0.962
C20:2n-6	EDSS	**0.676**	**0.046**
	Sensory	0.576	0.104
C20:3n-6	EDSS	0.347	0.361
	Sensory	0.124	0.750
C20:4n-6	EDSS	0.208	0.591
	Sensory	0.044	0.910
C22:4n-6	EDSS	**0.702**	**0.035**
	Sensory	0.248	0.520
C22:5n-6	EDSS	0.555	0.121
	Sensory	-0.071	0.856
Total n-3	EDSS	-0.607	0.083
	Sensory	**-0.940**	**0.000**
Total n-6	EDSS	0.477	0.195
	Sensory	0.071	0.856

5.4.4 Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acid series in erythrocyte membrane total phospholipids in MS patients (Table 4)

The C20:4n-6 to C22:4n-6 ratio showed a statistically significant negative correlation with the EDSS (Graph 1). The C22:4n-6 to C22:5n-3 ratio and the C22:5n-6 to C22:6n-3 ratio showed statistically significant positive correlations with the EDSS (Graphs 2 and 3). The ratio of total n-6 to total n-3 fatty acids had positive correlations with both the EDSS and sensory scores (Graphs 4 and 5).

Table 4

Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acid series in erythrocyte membranes in total phospholipids in MS patients

Fatty acid ratios	Kurtzke	r	p-value
C20:5n-3 to C22:5n-3	EDSS	-0.373	0.323
	Sensory	-0.337	0.375
C22:5n-3 to C22:6n-3	EDSS	0.572	0.108
	Sensory	0.612	0.080
C20:2n-6 to C20:3n-6	EDSS	0.451	0.224
	Sensory	0.257	0.504
C20:3n-6 to C20:4n-6	EDSS	0.130	0.739
	Sensory	0.115	0.768
C20:4n-6 to C22:4n-6	EDSS	**-0.780**	**0.013**
	Sensory	-0.452	0.222
C22:4n-6 to C22:5n-6	EDSS	-0.200	0.591
	Sensory	0.310	0.416
C20:4n-6 to C20:5n-3	EDSS	0.468	0.204
	Sensory	0.346	0.362
C22:4n-6 to C22:5n-3	EDSS	**0.685**	**0.042**
	Sensory	0.461	0.212
C22:5n-6 to C22:6n-3	EDSS	**0.910**	**0.001**
	Sensory	0.585	0.098
Total n-6 to total n-3	EDSS	**0.823**	**0.006**
	Sensory	**0.700**	**0.036**

5.5 Group A: Statistical analysis of fatty acids in lymphocyte membrane total phospholipids

5.5.1 Differences between the MS and control fatty acid percentage composition in lymphocyte membrane total phospholipids (Table 5)

In this study the total n-6 fatty acid percentage composition was found to be statistically significantly lower in the MS group. C20:4n-6 (largest n-6 fraction) was found to be nearly statistically significantly lower in MS.

Table 5

Differences between the MS and control fatty acid percentage composition in lymphocyte membrane total phospholipids

Fatty acids	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C18:3n-3	0.005 ± 0.005	< 0.001	0.362
C20:5n-3	0.211 ± 0.032	0.224 ± 0.039	0.805
C22:5n-3	1.196 ± 0.065	1.343 ± 0.108	0.248
C22:6n-3	1.819 ± 0.146	2.102 ± 0.223	0.294
C18:2n-6	5.391 ± 0.378	6.214 ± 0.346	0.132
C18:3n-6	0.044 ± 0.022	0.025 ± 0.025	0.593
C20:2n-6	0.446 ± 0.040	0.427 ± 0.050	0.759
C20:3n-6	1.562 ± 0.113	1.694 ± 0.091	0.386
C20:4n-6	23.126 ± 0.849	25.144 ± 0.463	0.062
C22:4n-6	2.444 ± 0.094	2.511 ± 0.149	0.704
C22:5n-6	0.348 ± 0.027	0.288 ± 0.021	0.107
Total n-3	3.230 ±0.198	3.669 ± 0.292	0.223
Total n-6	33.362 ± 1.136	36.303 ± 0.487	**0.038**

5.5.2 Differences between the MS and control fatty acid ratios in the n-3 and n-6 fatty acid series in lymphocyte membrane total phospholipids (Table 6)

No significant differences were found between the MS and control groups in the ratios between the two fatty acid series, but there was a trend toward significance ($p < 0.1$) of a higher C22:5n-6 to C22:6n-3 ratio in the MS compared to controls.

Table 6

Differences between MS and control fatty acid ratios in the n-3 and n-6 fatty acid series in lymphocyte membrane total phospholipids

Fatty acid ratios	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C22:5n-3 to C22:6n-3	0.696 ± 0.069	0.666 ± 0.054	0.740
C20:2n-6 to C20:3n-6	0.293 ± 0.025	0.254 ± 0.028	0.312
C20:3n-6 to C20:4n-6	0.068 ± 0.006	0.068 ± 0.004	0.935
C20:4n-6 to C22:4n-6	9.556 ± 0.480	10.309 ± 0.752	0.401
C22:4n-6 to C22:5n-6	7.337 ± 0.561	8.908 ± 0.553	0.062
C22:4n-6 to C22:5n-3	2.069 ± 0.077	1.938 ± 0.167	0.468
C22:5n-6 to C22:6n-3	0.204 ± 0.026	0.145 ± 0.016	0.074
Total n-6 to total n-3	10.515 ± 0.421	10.328 ± 0.811	0.835

5.5.3 Correlation between the EDSS and sensory scores, and the n-3 and n-6 fatty acids in lymphocyte membrane total phospholipids in MS patients (Table 7)

A statistically significant negative correlation was found between C18:2n-6 and the sensory score, as well as between the total n-6 fatty acids and the sensory score. There was also a trend toward significance ($p < 0.1$) in the negative correlation between C20:4n-6 and the sensory score, and a positive correlation between C22:4n-6 and the EDSS in the MS group.

Table 7

Correlation between the EDSS and sensory scores, and the n-3 and n-6 fatty acids in lymphocyte membrane total phospholipids in MS patients

Fatty acids	Kurtzke	r	p-value
C18:3n-3	EDSS	-0.427	0.252
	Sensory	-0.437	0.239
C20:5n-3	EDSS	-0.390	0.300
	Sensory	-0.470	0.202
C22:5n-3	EDSS	-0.035	0.929
	Sensory	-0.062	0.874
C22:6n-3	EDSS	-0.477	0.195
	Sensory	-0.656	0.055
C18:2n-6	EDSS	-0.364	0.336
	Sensory	**-0.789**	**0.011**
C18:3n-6	EDSS	-0.154	0.692
	Sensory	-0.095	0.808
C20:2n-6	EDSS	-0.035	0.929
	Sensory	-0.053	0.892
C20:3n-6	EDSS	0.069	0.859
	Sensory	0.115	0.768
C20:4n-6	EDSS	-0.503	0.168
	Sensory	-0.594	0.092
C22:4n-6	EDSS	0.633	0.068
	Sensory	0.293	0.445
C22:5n-6	EDSS	0.468	0.204
	Sensory	0.266	0.489
Total n-3	EDSS	-0.416	0.266
	Sensory	-0.426	0.253
Total n-6	EDSS	-0.338	0.374
	Sensory	**-0.754**	**0.019**

5.5.4 Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acid series in lymphocyte membrane total phospholipids in MS patients (Table 8)

A significant negative correlation was found between the C20:5n-3 to C22:5n-3 ratio and the EDSS (Graph 6), and a positive correlation between the C22:5n-3 to C22:6n-3 ratio and the sensory score (Graph 7).

In the n-6 series the C20:4n-6 to C22:4n-6 ratio showed statistically significant negative correlations with both the EDSS and sensory scores (Graphs 8 and 9).

The C22:5n-6 to C22:6n-3 ratio showed a positive correlation with the sensory score (Graph 10) and a trend towards significance ($p < 0.1$) with the EDSS..

Table 8

Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acid series in lymphocyte membrane total phospholipids in MS patients

Fatty acid ratios	Kurtzke	r	p-value
C20:5n-3 to C22:5n-3	EDSS	**-0.685**	**0.042**
	Sensory	-0.470	0.202
C22:5n-3 to C22:6n-3	EDSS	0.503	0.168
	Sensory	**0.727**	**0.026**
C20:2n-6 to C20:3n-6	EDSS	-0.364	0.336
	Sensory	0.035	0.928
C20:3n-6 to C20:4n-6	EDSS	0.234	0.545
	Sensory	0.248	0.520
C20:4n-6 to C22:4n-6	EDSS	**-0.875**	**0.002**
	Sensory	**-0.674**	**0.047**
C22:4n-6 to C22:5n-6	EDSS	-0.121	0.756
	Sensory	-0.133	0.733
C20:4n-6 to C20:5n-3	EDSS	0.520	0.151
	Sensory	0.390	0.299
C22:4n-6 to C22:5n-3	EDSS	0.416	0.266
	Sensory	0.355	0.349
C22:5n-6 to C22:6n-3	EDSS	0.650	0.058
	Sensory	**0.709**	**0.032**
Total n-6 to total n-3	EDSS	0.381	0.311
	Sensory	0.372	0.324

5.6 Group A: Statistical analysis of fatty acids in plasma total phospholipids

5.6.1 Differences between the MS and control fatty acid percentage composition in plasma total phospholipids (Table 9)

No significant difference was found between the MS and control groups. Total n-6 was found to be marginally significantly ($p < 0.1$) higher in MS versus controls.

Table 9

Differences between the MS and control fatty acid percentage composition in plasma total phospholipids

Fatty acids	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C18:3n-3	0.096 ± 0.020	0.073 ± 0.031	0.549
C20:5n-3	0.512 ± 0.060	0.434 ± 0.054	0.357
C22:5n-3	0.671 ± 0.032	0.651 ± 0.043	0.700
C22:6n-3	3.878 ± 0.372	3.608 ± 0.431	0.640
C18:2n-6	21.404 ± 0.659	20.661 ± 1.325	0.611
C18:3n-6	0.052 ± 0.028	0.064 ± 0.020	0.741
C20:2n-6	0.348 ± 0.052	0.438 ± 0.077	0.333
C20:3n-6	3.774 ± 0.316	3.411 ± 0.250	0.390
C20:4n-6	11.105 ± 0.748	10.404 ± 0.970	0.571
C22:4n-6	0.459 ± 0.038	0.386 ± 0.034	0.183
C22:5n-6	0.362 ± 0.040	0.285 ± 0.039	0.195
Total n-3	5.157 ± 0.381	4.766 ± 0.476	0.527
Total n-6	37.503 ± 0.642	35.651 ± 0.783	0.085

5.6.2 Differences between MS and control fatty acid ratios in the n-3 and n-6 fatty acid series in plasma total phospholipids (Table 10)

No statistically significant difference was found between the MS and control groups.

Table 10

Differences between MS and control fatty acids ratios in the n-3 and n-6 FA series in plasma total phospholipids

Fatty acid ratios	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C20:5n-3 to C22:5n-3	0.769 ± 0.084	0.679 ± 0.079	0.451
C22:5n-3 to C22:6n-3	0.191 ± 0.026	0.193 ± 0.018	0.949
C20:2n-6 to C20:3n-6	0.094 ± 0.016	0.135 ± 0.026	0.197
C20:3n-6 to C20:4n-6	0.356 ± 0.041	0.350 ± 0.046	0.926
C20:4n-6 to C22:4n-6	25.177 ± 2.041	27.236 ± 2.060	0.490
C22:4n-6 to C22:5n-6	1.432 ± 0.228	1.461 ± 0.135	0.917
C20:4n-6 to C20:5n-3	24.247 ± 3.437	25.540 ± 3.115	0.786
C22:4n-6 to C22:5n-3	0.689 ± 0.058	0.600 ± 0.046	0.254
C22:5n-6 to C22:6n-3	0.100 ± 0.016	0.081 ± 0.008	0.323
Total n-6 to total n-3	7.583 ± 0.547	8.052 ± 0.833	0.638

5.6.3 Correlation between the EDSS and sensory scores, and n-3 and n-6 *fatty acids in plasma total phospholipids in MS patients (Table 11)*

No significant correlation was found between the disability scores and any of the fatty acids, but there was a trend toward significance ($p < 0.1$) in the negative correlation between C20:5n-3 and the EDSS.

Table 11

Correlation between the EDSS and sensory scores, and n-3 and n-6 *fatty acids in plasma total phospholipids in MS patients*

Fatty acids	Kurtzke	r	p-value
C18:3n-3	EDSS	-0.579	0.103
	Sensory	-0.165	0.672
C20:5n-3	EDSS	-0.659	0.054
	Sensory	-0.053	0.892
C22:5n-3	EDSS	0.477	0.195
	Sensory	0.576	0.104
C22:6n-3	EDSS	-0.347	0.361
	Sensory	-0.576	0.104
C18:2n-6	EDSS	-0.121	0.756
	Sensory	-0.497	0.174
C18:3n-6	EDSS	0.129	0.741
	Sensory	0.142	0.715
C20:2n-6	EDSS	0.303	0.428
	Sensory	0.133	0.733
C20:3n-6	EDSS	0.399	0.288
	Sensory	0.381	0.311
C20:4n-6	EDSS	0.269	0.485
	Sensory	0.009	0.982
C22:4n-6	EDSS	-0.035	0.929
	Sensory	0.381	0.311
C22:5n-6	EDSS	0.407	0.277
	Sensory	0.266	0.489
Total n-3	EDSS	-0.295	0.442
	Sensory	-0.532	0.140
Total n-6	EDSS	0.355	0.348
	Sensory	-0.275	0.474

5.6.4 *Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acid series in plasma total phospholipids in MS patients (Table 12)*

A significant positive correlation was found between the C22:5n-6 to C22:6n-3 ratio and the sensory score (Graph 11) and a trend toward significance ($p < 0.1$) with the EDSS. A trend toward significance ($p < 0.1$) was also found with the

5.6.3 Correlation between the EDSS and sensory scores, and n-3 and n-6 *fatty acids in plasma total phospholipids in MS patients (Table 11)*

No significant correlation was found between the disability scores and any of the fatty acids, but there was a trend toward significance ($p < 0.1$) in the negative correlation between C20:5n-3 and the EDSS.

Table 11

Correlation between the EDSS and sensory scores, and n-3 and n-6 *fatty acids in plasma total phospholipids in MS patients*

Fatty acids	Kurtzke	r	p-value
C18:3n-3	EDSS	-0.579	0.103
	Sensory	-0.165	0.672
C20:5n-3	EDSS	-0.659	0.054
	Sensory	-0.053	0.892
C22:5n-3	EDSS	0.477	0.195
	Sensory	0.576	0.104
C22:6n-3	EDSS	-0.347	0.361
	Sensory	-0.576	0.104
C18:2n-6	EDSS	-0.121	0.756
	Sensory	-0.497	0.174
C18:3n-6	EDSS	0.129	0.741
	Sensory	0.142	0.715
C20:2n-6	EDSS	0.303	0.428
	Sensory	0.133	0.733
C20:3n-6	EDSS	0.399	0.288
	Sensory	0.381	0.311
C20:4n-6	EDSS	0.269	0.485
	Sensory	0.009	0.982
C22:4n-6	EDSS	-0.035	0.929
	Sensory	0.381	0.311
C22:5n-6	EDSS	0.407	0.277
	Sensory	0.266	0.489
Total n-3	EDSS	-0.295	0.442
	Sensory	-0.532	0.140
Total n-6	EDSS	0.355	0.348
	Sensory	-0.275	0.474

5.6.4 *Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acid series in plasma total phospholipids in MS patients (Table 12)*

A significant positive correlation was found between the C22:5n-6 to C22:6n-3 ratio and the sensory score (Graph 11) and a trend toward significance ($p < 0.1$) with the EDSS. A trend toward significance ($p < 0.1$) was also found with the

negative correlation between the C20:5n-3 to C22:5n-3 ratio and the EDSS, a trend toward significance ($p < 0.1$) with the positive correlation between the C22:5n-3 to C22:6n-3 ratio and the sensory score and a trend toward significance ($p < 0.1$) with the positive correlation between the total n-6 to n-3 ratio and the EDSS.

Table 12

Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 FA series in plasma total phospholipids in MS patients

Fatty acid ratios	Kurtzke	r	p-value
C20:5n-3 to C22:5n-3	EDSS	-0.633	0.068
	Sensory	-0.018	0.964
C22:5n-3 to C22:6n-3	EDSS	0.286	0.456
	Sensory	0.656	0.055
C20:2n-6 to C20:3n-6	EDSS	0.061	0.877
	Sensory	-0.186	0.631
C20:3n-6 to C20:4n-6	EDSS	0.113	0.773
	Sensory	0.168	0.665
C20:4n-6 to C22:4n-6	EDSS	0.113	0.773
	Sensory	-0.541	0.133
C22:4n-6 to C22:5n-6	EDSS	-0.338	0.374
	Sensory	-0.106	0.785
C20:4n-6 to C20:5n-3	EDSS	0.503	0.168
	Sensory	-0.151	0.699
C22:4n-6 to C22:5n-3	EDSS	-0.312	0.414
	Sensory	-0.071	0.856
C22:5n-6 to C22:6n-3	EDSS	0.650	0.058
	Sensory	**0.709**	**0.032**
Total n-6 to total n-3	EDSS	0.633	0.068
	Sensory	0.497	0.174

5.7 Group B: Statistical analysis of the percentage composition of fatty acids of the erythrocyte membrane PC fraction in phospholipids

5.7.1 Differences between the MS and control percentage composition of fatty acids of the erythrocyte membrane PC fraction in phospholipids (Table 13)

C22:5n-6 was found to be significantly higher in MS than in the control group and C22:4n-6 was found to be marginally higher in MS than in controls ($p < 0.1$).

Table 13

Differences between the MS and control percentage composition of fatty acids of the erythrocyte membrane PC fraction in phospholipids

Fatty acids	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C18:3n-3	0.125 ± 0.044	0.117 ± 0.010	0.815
C20:5n-3	0.451 ± 0.091	0.508 ± 0.075	0.647
C22:5n-3	0.526 ± 0.045	0.449 ± 0.030	0.159
C22:6n-3	2.763 ± 0.376	2.292 ± 0.172	0.204
C18:2n-6	20.943 ± 0.574	21.799 ± 0.615	0.375
C18:3n-6	0.268 ± 0.117	0.225 ± 0.130	0.834
C20:2n-6	0.453 ± 0.045	0.386 ± 0.022	0.147
C20:3n-6	2.974 ± 0.328	2.417 ± 0.164	0.103
C20:4n-6	7.321 ± 0.649	6.833 ± 0.448	0.536
C22:4n-6	0.426 ± 0.051	0.349 ± 0.019	0.095
C22:5n-6	0.180 ± 0.034	0.062 ± 0.022	**0.006**
Total n-3	3.869 ± 0.438	3.365 ± 0.245	0.288
Total n-6	32.560 ± 0.843	32.071 ± 0.673	0.664

5.7.2 Differences between the MS and control ratios in the n-3 and n-6 fatty acids of the erythrocyte membrane PC fraction in phospholipids (Table 14)

No significant difference was found between the MS and control groups.

Table 14

Differences between the MS and control ratios in the n-3 and n-6 fatty acids of the erythrocyte membrane PC fraction in phospholipids

Fatty acid ratios	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C20:2n-6 to 20:3n-6	0.155 ± 0.010	0.172 ± 0.016	0.496
C20:3n-6 to 20:4n-6	0.433 ± 0.068	0.369 ± 0.033	0.355
C20:4n-6 to 22:4n-6	18.846 ± 2.352	19.700 ± 0.971	0.696
Total n-6 to total n-3	9.138 ± 1.014	10.208 ± 0.716	0.394

5.7.3 Correlation between the EDSS and sensory scores, and the n-3 and n-6 fatty acids of the erythrocyte membrane PC fraction in phospholipids in MS patients (Table 15)

A highly significant negative correlation was found between C20:5n-3 and the sensory score and a trend toward significance ($p < 0.1$) with the EDSS. The negative correlation between C22:6n-3 and the EDSS also showed a trend toward significance ($p < 0.1$). The total n-3 fatty acids showed a significant

negative correlation with both the EDSS and sensory scores.

C22:4n-6 showed a positive correlation with the sensory score and C18:2n-6 a trend toward significance ($p < 0.1$) in a positive correlation with the sensory score.

Table 15

Correlation between the EDSS and sensory scores, and the n-3 and n-6 fatty acids of the erythrocyte membrane PC fraction in phospholipids in MS patients

Fatty acids	Kurtzke	r	p-value
C18:3n-3	EDSS	-0.118	0.781
	Sensory	-0.369	0.369
C20:5n-3	EDSS	-0.679	0.064
	Sensory	**-0.969**	**0.000**
C22:5n-3	EDSS	-0.432	0.285
	Sensory	0.019	0.965
C22:6n-3	EDSS	-0.700	0.053
	Sensory	-0.420	0.300
C18:2n-6	EDSS	0.282	0.498
	Sensory	0.692	0.057
C18:3n-6	EDSS	-0.086	0.840
	Sensory	-0.012	0.977
C20:2n-6	EDSS	0.049	0.908
	Sensory	0.408	0.316
C20:3n-6	EDSS	-0.074	0.862
	Sensory	0.086	0.839
C20:4n-6	EDSS	-0.258	0.538
	Sensory	-0.086	0.839
C22:4n-6	EDSS	0.525	0.182
	Sensory	**0.708**	**0.049**
C22:5n-6	EDSS	0.381	0.352
	Sensory	0.284	0.495
Total n-3	EDSS	**-0.896**	**0.003**
	Sensory	**-0.815**	**0.014**
Total n-6	EDSS	0.160	0.706
	Sensory	0.581	0.131

5.7.4 Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acids of the erythrocyte membrane PC fraction in phospholipids in MS patients (Table 16)

Significant negative correlations were found between the C20:4n-6 to C22:4n-6 ratio and both the EDSS and sensory scores (Graphs 12 and 13).

A highly significant positive correlation was found between the C20:4n-6 to C20:5n-3 ratio and the sensory score (Graph 14). Significant positive correlations

were found between the C22:4n-6 to C22:5n-3 ratio and both the EDSS and sensory scores (Graphs 15 and 16). A significant positive correlation was also found between the C22:5n-6 to C22:6n-3 ratio and the EDSS (Graph 17). Significant positive correlations were also found between the ratio of total n-6 to total n-3 fatty acids and both the EDSS (Graph 18) and sensory scores.

Table 16

Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acids of the erythrocyte membrane PC fraction in phospholipids in MS patients

Fatty acid ratios	Kurtzke	r	p-value
C22:5n-3 to C22:6n-3	EDSS	0.602	0.115
	Sensory	0.692	0.057
C20:2n-6 to C20:3n-6	EDSS	0.098	0.810
	Sensory	0.618	0.103
C20:3n-6 to C20:4n-6	EDSS	0.233	0.578
	Sensory	0.259	0.535
C20:4n-6 to C22:4n-6	EDSS	**-0.786**	**0.021**
	Sensory	**-0.852**	**0.007**
C22:4n-6 to C22:5n-6	EDSS	0.037	0.931
	Sensory	0.222	0.597
C20:4n-6 to C20:5n-3	EDSS	0.577	0.134
	Sensory	**0.902**	**0.002**
C22:4n-6 to C22:5n-3	EDSS	**0.835**	**0.010**
	Sensory	**0.815**	**0.014**
C22:5n-6 to C22:6n-3	EDSS	**0.822**	**0.012**
	Sensory	0.568	0.142
Total n-6 to total n-3	EDSS	**0.896**	**0.003**
	Sensory	**0.815**	**0.014**

5.8 Group B: Statistical analysis of the percentage composition of fatty acids of the erythrocyte membrane PE fraction in phospholipids

5.8.1 Differences between the MS and control percentage composition of fatty acids of the erythrocyte membrane PE fraction in phospholipids (Table 17)

C18:2n-6, C18:3n-6 and C20:3n-6 had highly significantly higher percentage concentrations in the MS patients than in the controls, and C22:4n-6 was significantly lower in the MS group. There was also a trend toward significance ($p < 0.1$) in a higher C20:2n-6 in MS than in the controls.

Table 17

Differences between the MS and control percentage composition of fatty acids of the erythrocyte membrane PE fraction in phospholipids

Fatty acids	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C18:3n-3	0.119 ± 0.052	0.083 ± 0.016	0.411
C20:5n-3	0.741 ± 0.143	0.963 ± 0.075	0.141
C22:5n-3	3.166 ± 0.516	3.585 ± 0.142	0.328
C22:6n-3	8.391 ± 0.838	8.182 ± 0.360	0.791
C18:2n-6	8.466 ± 0.479	6.171 ± 0.309	**0.000**
C18:3n-6	0.115 ± 0.057	0.005 ± 0.005	**0.015**
C20:2n-6	0.465 ± 0.058	0.362 ± 0.022	0.062
C20:3n-6	1.715 ± 0.193	1.340 ± 0.071	**0.038**
C20:4n-6	20.718 ± 0.819	21.619 ± 0.644	0.407
C22:4n-6	5.084 ± 0.432	7.069 ± 0.355	**0.003**
C22:5n-6	1.063 ± 0.193	0.862 ± 0.043	0.196
Total n-3	12.419 ± 1.144	12.813 ± 0.472	0.710
Total n-6	37.624 ± 1.229	37.428 ± 0.994	0.906

5.8.2 Differences between the MS and control ratios in the n-3 and n-6 fatty acids of the erythrocyte membrane PE fraction in phospholipids (Table 18)

Both the C20:3n-6 to C20:4n-6 and C20:4n-6 to C22:4n-6 ratios were significantly higher in the MS group than in the control group and there was a trend toward significance ($p < 0.1$) in a lower C22:4n-6 to C22:5n-6 ratio and a higher C20:4n-6 to C20:5n-3 in the MS versus the control group.

Table 18

Differences between the MS and control ratios in the n-3 and n-6 fatty acids of the erythrocyte membrane PE fraction in phospholipids

Fatty acid ratios	MS	Controls	
	Mean ± SE	Mean ± SE	p-value
C20:5n-3 to C22:5n-3	0.259 ± 0.052	0.268 ± 0.018	0.846
C22:5n-3 to C22:6n-3	0.397 ± 0.069	0.448 ± 0.025	0.410
C20:2n-6 to C20:3n-6	0.294 ± 0.049	0.278 ± 0.020	0.722
C20:3n-6 to C20:4n-6	0.084 ± 0.011	0.063 ± 0.004	**0.035**
C20:4n-6 to C22:4n-6	4.268 ± 0.378	3.141 ± 0.147	**0.003**
C22:4n-6 to C22:5n-6	6.031 ± 1.362	8.318 ± 0.397	0.054
C20:4n-6 to C20:5n-3	35.651 ± 6.094	24.711 ± 2.194	0.052
C22:4n-6 to C22:5n-3	1.805 ± 0.248	2.019 ± 0.134	0.414
C22:5n-6 to C22:6n-3	0.140 ± 0.036	0.110 ± 0.009	0.294
Total n-6 to total n-3	3.256 ± 0.366	2.983 ± 0.145	0.417

5.8.3 Correlation between the EDSS and sensory scores, and the n-3 and n-6 fatty acids of the erythrocyte membrane PE fraction in phospholipids in MS patients (Table 19)

A significant negative correlation was found between C18:3n-3 and the sensory score and a trend toward significance ($p < 0.1$) with the EDSS. Significant negative correlations were found between C20:5n-3 and both the EDSS and the sensory scores. C22:6n-3 also had negative correlations with both the EDSS and the sensory scores. The total n-3 fatty acids showed a negative correlation with the sensory score and a trend toward significance ($p < 0.1$) with the EDSS.

C18:2n-6 showed a positive correlation with both the EDSS and the sensory scores. C20:2n-6 showed a positive correlation with the EDSS

Table 19

Correlation between the EDSS and sensory scores, and the n-3 and n-6 fatty acids of the erythrocyte membrane PE fraction in phospholipids in MS

Fatty acids	Kurtzke	r	p-value
C18:3n-3	EDSS	-0.706	0.050
	Sensory	**-0.895**	**0.003**
C20:5n-3	EDSS	**-0.741**	**0.035**
	Sensory	**-0.727**	**0.041**
C22:5n-3	EDSS	-0.061	0.885
	Sensory	0.062	0.884
C22:6n-3	EDSS	**-0.810**	**0.015**
	Sensory	**-0.840**	**0.009**
C18:2n-6	EDSS	**0.835**	**0.010**
	Sensory	**0.741**	**0.035**
C18:3n-6	EDSS	-0.524	0.182
	Sensory	-0.542	0.165
C20:2n-6	EDSS	0.540	0.167
	Sensory	**0.741**	**0.035**
C20:3n-6	EDSS	0.479	0.230
	Sensory	0.556	0.152
C20:4n-6	EDSS	-0.196	0.641
	Sensory	-0.185	0.660
C22:4n-6	EDSS	0.393	0.336
	Sensory	0.222	0.597
C22:5n-6	EDSS	0.491	0.217
	Sensory	0.198	0.639
Total n-3	EDSS	-0.675	0.066
	Sensory	**-0.778**	**0.023**
Total n-6	EDSS	0.503	0.204
	Sensory	0.593	0.121

5.8.4 Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acids of the erythrocyte membrane PE fraction in phospholipids in MS patients (Table 20)

The C20:5n-3 to C22:5n-3 ratio showed a significant negative correlation with both the EDSS and the sensory scores (Graphs 19 and 20), and the C22:5n-3 to C22:6n-3 ratio showed a significant positive correlation with both the EDSS and the sensory scores (Graphs 21 and 22).

The C20:3n-6 to C20:4n-6 ratio showed a trend ($p < 0.1$) towards a significant positive correlation with the sensory score and the C20:4n-6 to C22:4n-6 ratio a trend ($p < 0.1$) toward a significant negative correlation with the EDSS.

The C22:5n-6 to C22:6n-3 ratio showed a significant positive correlation with the EDSS (Graph 23). The ratio of total n-6 to total n-3 fatty acids showed a positive correlation with both the EDSS and the sensory scores (Graphs 24 and 25).

Table 20

Correlation between the EDSS and sensory scores, and the ratios in the n-3 and n-6 fatty acids of the erythrocyte membrane PE fraction in phospholipids in MS patients

Fatty acid ratios	Kurtzke	r	p-value
C20:5n-3 to C22:5n-3	EDSS	**-0.712**	**0.048**
	Sensory	**-0.803**	**0.016**
C22:5n-3 to C22:6n-3	EDSS	**0.761**	**0.028**
	Sensory	**0.865**	**0.006**
C20:2n-6 to C20:3n-6	EDSS	0.098	0.817
	Sensory	0.111	0.793
C20:3n-6 to C20:4n-6	EDSS	0.552	0.156
	Sensory	0.655	0.078
C20:4n-6 to C22:4n-6	EDSS	-0.651	0.081
	Sensory	-0.507	0.200
C22:4n-6 to C22:5n-6	EDSS	-0.135	0.750
	Sensory	-0.074	0.862
C20:4n-6 to C20:5n-3	EDSS	0.565	0.145
	Sensory	0.618	0.103
C22:4n-6 to C22:5n-3	EDSS	0.258	0.538
	Sensory	0.086	0.839
C22:5n-6 to C22:6n-3	EDSS	**0.835**	**0.010**
	Sensory	0.531	0.175
Total n-6 to total n-3	EDSS	**0.810**	**0.015**
	Sensory	**0.840**	**0.009**

CHAPTER 6
DISCUSSION

Introduction

Similar to Smuts *et al.* (1999), erythrocyte membranes were used in this study as a marker for the brain, and lymphocyte membranes because of their reported involvement in MS neuronal demyelination. Plasma fatty acid composition of total phospholipids was measured to investigate a possible dietary influence on disease status.

The fatty acid percentage composition in total phospholipids was measured in the study population sub group A and the fatty acid percentage composition of the PC and PE fractions in phospholipids was measured in the study population sub group B.

The mean value of the disability score, EDSS, was 2.222 ± 0.265 in sub group A and 4.438 ± 0.684 in sub group B, i.e. the patients in sub group B were more disabled than the patients in sub group A. Both sub groups were diagnosed with relapsing remitting MS and all patients were in a remittent stage of the disease. Only the EDSS and sensory scores were measurable in sub group A because of lower disability status, and these results were used in the statistical analysis. (The sensory score of the EDSS measures early signs of MS, such as "pins and needles" and touch sensation).

The fatty acid composition in total phospholipids (sub group A) and of the PC and PE fractions in phospholipids (sub group B) was used for correlation and variance studies. No publications could be found in the literature in which the ratios of n-6 to n-3 fatty acids were investigated in the aetiology of MS patients. Reference to the ratio between these fatty acids referred to dietary supplementation only (e.g. Haag, 2003). In the current study the ratios between the fatty acids were correlated with disease outcome to examine the roles that individual concentrations could have played in the aetiology of MS.

Discussion of the results

Linoleic acid (LA, C18:2n-6) and alpha linolenic acid (ALA, C18:3n-3)

Research studies in the early 1950s on fatty acids in MS were focused on the possible role of n-6 fatty acids, because n-3 fatty acids were not considered essential at that time (Nordvik *et al.*, 2000). Reduced levels of linoleic acid (LA, C18:2n-6) were found in the brain, serum,

platelets and erythrocytes of MS patients (Nordvik *et al.*, 2000). Conflicting results have been reported since then. Cheravil (1984) found a decrease in the percentage composition of linoleic acid (LA, C18:2n-6) in the erythrocyte and lymphocyte membranes and plasma of MS patients. Holman *et al.* (1989) found linoleic acid (LA, C18:2n-6) to have normal values in the plasma of MS patients, but all subsequent n-6 fatty acids to be subnormal. Nightingale *et al.* (1990) found no reduction in MS patients in the percentage composition of erythrocyte membrane linoleic acid (LA, C18:2n-6) of the PC or PE fraction in phospholipids.

Since some of the metabolic products of linoleic acid (LA, C18:2n-6) and alpha linolenic acid (ALA, C18:3n-3) had very low levels, the ratios between and within the n-6 and n-3 fatty acid series could only effectively be analysed from fatty acid eicosapentaenoic acid (EPA, C20:5n-3) in the n-3 series and its counterpart in the n-6 series, arachidonic acid (AA, C20:4n-6), onwards (See diagram 1).

Arachidonic acid (AA, C20:4n-6)

In this study it was found that MS patients with higher disability had lower concentrations of C20:4n-6 within the respective ratios within the n-6 and n-3 fatty acid series.

Ratio arachidonic acid (AA, C20:4n-6) to adrenic acid (C22:4n-6) (elongation)

Significant correlations were found between the ratio of fatty acids C20:4n-6 to C22:4n-6 and disease outcome. In erythrocyte membrane total phospholipids there was a negative correlation with the EDSS ($r = -0.786$; $p = 0.013$) (Graph 1) and in lymphocyte membrane total phospholipids a strong negative correlation with the EDSS ($r = -0.876$; $p = 0.002$) (Graph 8), i.e. when the C20:4n-6 concentration was too low relative to the concentration of the elongation product C22:4n-6, the disease outcome was worse. The same was true for the sensory score ($r = -0.674$; $p = 0.047$) (Graph 9). In the erythrocyte PC fraction in phospholipids the ratio had a negative correlation with the EDSS ($r = -0.786$; $p = 0.021$) (Graph 12) and with the sensory score ($r = -0.852$; $p = 0.007$) (Graph 13). In the PE fraction it had a negative relationship with the EDSS but not significantly ($r = -0.651$; $p = 0.081$). This means that when the C20:4n-6 concentration was too low, relative to the concentration of the elongation product C22:4n-6, the disease outcome was worse.

However, in plasma total phospholipids, the ratio of C20:4n-6 to C22:4n-6 showed no correlation with either the EDSS or sensory scores (Correlation with the EDSS: $r = 0.113$; $p = 0.773$), which suggested that this problem was not only directly related to diet.

The effect of this fatty acid ratio on disease status was found in both sub group A and B patients, despite a substantial difference in the mean EDSS. The patients in sub group A had a low mean EDSS value, implicating an imbalance in this ratio early in the progression of the disease. The effect of this ratio imbalance was also seen in both cell types; erythrocyte membranes are traditionally seen as a marker for the brain tissue, but lymphocytes are viewed as one of the key problems in the neuronal demyelination process.

The relatively lower concentrations of arachidonic acid (AA, C20:4n-6) could possibly be due to the production of eicosanoids, leading to inflammatory conditions in the MS patients, which would deplete arachidonic acid (AA, C20:4n-6) levels in the membranes. Phospholipase A_2 is a key enzyme responsible for turnover of membrane phospholipids and is enriched in the neuronal membranes (Yao, 1999). Increased activity of the enzyme could result in reduced levels of the phospholipids/fatty acid substrate (Ross, 1999). In response to many inflammatory stimuli, phospholipase A_2 is generated and cleaves the arachidonic acid (AA, C20:4n-6) out of the cell membrane phospholipid and the fatty acids are released as free fatty acids, which are then oxygenated and further modified, yielding eicosanoids (Gilroy *et al.*, 2004). An upregulation of arachidonic acid (AA, C20:4n-6) selective phospholipase A_2 and its coupled COX-2, by low docosahexaenoic acid (DHA, C22:6n-3) intake may further explain the decreased levels of arachidonic acid (AA, C20:4n-6) in the membranes investigated in MS patients. Therefore, brain changes due to insufficient n-3 PUFA intakes would be expected to contribute to and exacerbate certain human diseases (Rapoport and Bosetti, 2002).

In this study MS patients had lower C20:4n-6 concentrations than controls. A reduction was found in the percentage composition of C20:4n-6 in lymphocyte membrane total phospholipids, though not significantly (MS: 23.126 ± 0.849; Controls: 25.144 ± 0.463; p = 0.062). Cheravil (1984) found a small, but significant decrease in C20:4n-6 in the plasma fatty acid composition of MS patients. C20:4n-6 is needed in high concentrations in the brain and according to the results of this study also of the fatty acid composition of the erythrocyte and lymphocyte membranes, since reduction of the relative C20:4n-6 concentration in its ratio with C22:4n-6, correlated with a worse disease status.

Ratio arachidonic acid (AA, C20:4n-6) to eicosapentaenoic acid (EPA, C20:5n-3)

In this study the ratio of fatty acids C20:4n-6 to C20:5n-3 in the erythrocyte membrane PC fraction in phospholipids showed a very strong positive correlation with the sensory score (r = 0.902; p 0.002) (Graph 14), which indicated that a lower C20:5n-3 resulted in a worse

disease outcome. C20:5n-3 inhibits phospholipase A_2's release of C20:4n-6 from cell membranes (Rao *et al.*, 1996), and lower C20:5n-3 concentrations in relation to C20:4n-6 would be insufficient to inhibit C20:4n-6 's release from the cell membrane.

Eicosapentaenoic acid (EPA, C20:5n-3)

Ratio eicosapentaenoic acid (EPA, C20:5n-3) to docosapentaenoic acid (DPA, C22:5n-3) (elongation)

The ratio of C20:5n-3 to C22:5n-3 showed significant correlations with disease status that supports our previous suggestion on the C20:5n-3 / C20:4n-6 ratio. The ratio of C20:5n-3 to C22:5n-3 in lymphocyte membrane total phospholipids had a negative correlation with the EDSS, i.e. the less C20:5n-3 relative to C22:5n-3, the worse the disease outcome ($r = -0.685$; $p = 0.042$: Graph 6). The ratio of C20:5n-3 to C22:5n-3 in the erythrocyte PE fraction in phospholipids also had a negative correlation with the EDSS ($r = -0.712$; $p = 0.048$: Graph 19) and sensory score ($r = -0.803$; $p = 0.016$: Graph 20).

The percentage concentration of C20:5n-3 in the erythrocyte PE fraction in phospholipids in this study was found to be lower in the MS patients compared to controls, but not significantly so: (MS: 0.741 ± 0.143; controls: 0.963 ± 0.075; $p = 0.141$). Nightingale *et al.* (1990) found a significant reduction in the percentage concentration of C20:5n-3 in erythrocyte membranes.

Eicosapentaenoic acid (EPA, C20:5n-3) suppresses the production of inflammatory cytokines (Shahidi and Miraliakbari, 2005). In this study eicosapentaenoic acid (EPA, C20:5n-3) was found to be insufficient in its ratios with both docosapentaenoic acid (DPA, C22:5n-3) and with the n-6 fatty acid arachidonic acid, as measured by disease status.

Ratio adrenic acid (C22:4n-6) to docosapentaenoic acid (DPA, C22:5n-3)

The ratio of fatty acids C22:4n-6 to C22:5n-3 in erythrocyte membrane total phospholipids had a positive correlation with the EDSS score ($r = 0.685$; $p = 0.042$) (Graph 2). The ratio of fatty acids C22:4n-6 to C22:5n-3 of erythrocyte membrane PC fraction in phospholipids had a positive correlation with both the EDSS and the sensory scores (EDSS: $r = 0.835$; $p = 0.010$ (Graph 15) and sensory: $r = 0.815$; $p = 0.014$) (Graph 16). In this ratio fatty acid C22:4n-6 seemed to inhibit the metabolism of C22:5n-3, as C20:4n-6 did with that of C20:5n-3.

Docosahexaenoic acid (DHA, C22:6n-3)

Ratio docosapentaenoic acid (DPA, C22:5n-3) to docosahexaenoic acid (DHA, C22:6n-3)(desaturation)

The ratio of fatty acids C22:5n-3 to **C22:6n-3** in lymphocyte membrane total phospholipids showed a positive correlation with the sensory score ($r = 0.727$; $p = 0.026$: Graph 7). The ratio of C22:5n-3 to **C22:6n-3** in the erythrocyte membrane PE fraction in phospholipids showed a positive correlation with the EDSS ($r = 0.761$; $p = 0.028$: Graph 21), and sensory scores ($r = 0.865$; $p = 0.006$: Graph 22). This means that the higher the **C22:6n-3** concentration relative to C22:5n-3, the better the disease outcome. This further emphasizes the importance and involvement of this brain fatty acid in the severity of the disease.

Ratio C22:5n-6 to docosahexaenoic acid (DHA, C22:6n-3)(desaturation)

The ratio of fatty acids C22:5n-6 to C22:6n-3 had a positive correlation with disease outcome in all the parameters tested, i.e. once again a higher C22:6n-3 concentration predicted a better outcome. C22:5n-6 will only increase when there is an insufficient supply of C22:6n-3 via the diet or from its n-3 precursors. The ratio of fatty acids C22:5n-6 to C22:6n-3 had a positive correlation with disease outcome in:

- erythrocyte membrane total phospholipids: with the EDSS score ($r = 0.910$; $p = 0.001$) (Graph 3)
- lymphocyte membrane total phospholipids: with the sensory score ($r = 0.709$; $p = 0.032$) (Graph 10)
- erythrocyte membrane PC and PE fractions in phospholipids: with the EDSS (PC fraction: $r = 0.822$; $p = 0.012$; Graph 17: PE fraction: $r = 0.835$; $p = 0.010$; Graph 23).
- plasma total phospholipids: with the sensory score ($r = 0.709$; $p = 0.032$) (Graph 11) and also a high but not significant correlation with the EDSS ($r = 0.650$; $p = 0.059$). This implicated a dietary imbalance (insufficient n-3 intake) in the MS group.

Arachidonic acid (AA, C20:4n-6), together with docosahexaenoic acid (DHA, C22:6n-3), constitutes 80-90% of the total essential fatty acids in neuronal and retinal tissue (Horrobin, 1999). Docosahexaenoic acid (DHA, C22:6n-3) in the white matter of the CNS of MS patients has been reported to be less than half that of normal values (Nightingale *et al.*, 1990). In this study, both these fatty acids were found to be insufficient within their respective ratios to enable a healthy outcome in MS patients.

According to the literature MS may be associated with either a relative deficiency of dietary long chain fatty acids or with some metabolic impairment of elongation (and/or desaturation) of alpha linolenic acid (ALA, C18:3n-3) to docosahexaenoic acid (DHA, C22:6n-3) in the CNS (Nightingale *et al.,* 1990). In this study no significant decrease compared to controls was found in the percentage composition of any of the n-3 fatty acids in lymphocyte or erythrocyte membranes or plasma total phospholipids or in the fatty acids of the erythrocyte membrane PC or PE fractions in phospholipids. However, significant positive correlations were found between all the ratios of the n-6 to n-3 fatty acids that were measurable with disease status, as well as with the ratios within the two fatty acid series. Lower arachidonic acid (AA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) in their respective ratios were largely implicated in higher disability in the MS patients. Both these fatty acids have structural functions in the cell membrane and insufficiency may result in or reflect MS neuronal demyelination. The effect of lower concentrations of arachidonic acid (AA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) would have been missed if the ratios had not been correlated statistically with disease outcome.

Correlation between disease status in MS patients and the ratio of total n-6 to total n-3 fatty acids in total phospholipids and of that of the PC and PE fractions in phospholipids

Correlation between the ratio of total n-6 to total n-3 fatty acids:

- in erythrocyte membrane total phospholipids with the EDSS ($r = 0.823$; $p = 0.006$) (Graph 4) and with the sensory score ($r = 0.700$; $p = 0.036$) (Graph 5)
- in erythrocyte membrane PC fraction in phospholipids: with both the EDSS and the sensory score (EDSS: $r = 0.896$: $p = 0.003$) (Graph 18); (sensory: $r = 0.815$; $p = 0.014$)
- in erythrocyte membrane PE fraction in phospholipids with both the EDSS and the sensory score (EDSS: $r = 0.810$; $p = 0.015$) (Graph 24); (sensory: $r = 0.840$; $p = 0.009$) (Graph 25).

From these results it was evident that an increased concentration of n-6 relative to n-3 contributed to disease severity.

In plasma total phospholipids the ratio of total n-6 to total n-3 fatty acids showed a positive correlation, though not statistically significant, with the EDSS ($r = 0.633$; $p = 0.068$). Even though plasma fatty acids seemed unsatisfactory as a marker for disease outcome, it did indicate a possible dietary influence on outcome, in the ratio of total n-6 to total n-3 fatty acids, and more specifically in the ratio of fatty acids C22:5n-6 to C22:6n-3.

Lymphocyte membranes

Traditionally lymphocytes, as part of the inflammatory process, are implicated in the demyelination process in MS brains. The results of this study showed however an imbalance between the two fatty acid series in both the erythrocyte and lymphocyte membranes. It would seem reasonable to extrapolate this problem to most cells in the body, which would include neuronal myelin. Imbalances in the fatty acid composition of lymphocyte membranes may affect cytokine production adversely. However, the ratio of total n-6 to total n-3 fatty acids in lymphocyte membrane total phospholipids had no correlation with either the EDSS or the sensory scores.

Erythrocyte membranes

In conclusion, the erythrocyte membrane fatty acid analysis in this study has been informative. The membrane fatty acids in total phospholipids and of the PC and PE fractions in phospholipids seemed to have a number of imbalances throughout the ratios between and within the n-6 and n-3 series which correlated with disease severity.

CHAPTER 7
CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The results of this study indicate that fatty acids play a major role in the aetiology of MS. This is easily understood if one takes into consideration the large area of membranes that is produced by oligodendrocytes. Most of the information gained in this study was from the ratios between the different fatty acids, and which would have been lost otherwise.

Deficiencies of dietary origin as seen from plasma fatty acid determinations showed insufficient quantities of eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). In erythrocyte and lymphocyte membranes, insufficient elongation and desaturation resulted in lower levels of both arachidonic acid (AA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3), the two structural fatty acids, as well as the possible upregulation of arachidonic acid (AA, C20:4n-6) specific phospholipase A_2. The inflammatory process could have been responsible for excessive arachidonic acid (AA, C20:4n-6) utilisation, which in turn could have been instrumental in further imbalances between the two fatty acids series at each elongation and desaturation step. Finally, this resulted in insufficient docosahexaenoic acid (DHA, C22:6n-3) in all of its ratios with both the n-6 and n-3 fatty acid series, confirming results of earlier studies which showed that in MS, docosahexaenoic acid (DHA, C22:6n-3) concentrations were 50% lower than in membranes from healthy subjects (Nightingale *et al.*, 1990). Therefore, both diet and dysregulation of fatty acid metabolism in MS may be major role players in the aetiology of the disease and warrants further investigation.

The membrane fatty acids in total phospholipids and of the PC and PE fractions in phospholipids had a number of imbalances throughout the ratios between and within the n-6 and n-3 series, which correlated with disease severity. Therefore, the null hypothesis which stated that an imbalance in the ratio of n-6 to n-3 fatty acids in the erythrocyte and lymphocyte membranes of MS patients, will not correlate with their neurological outcome as measured by the Kurtzke EDSS, must be rejected and the alternative hypothesis which stated that an imbalance in the ratio of n-6 to n-3 fatty acids in the erythrocyte and lymphocyte membranes of MS patients, will correlate with their neurological outcome as measured by the Kurtzke EDSS, can be accepted.

Recommendations

From the results of this study it was clear that both dietary and metabolic imbalances in the n-6 and n-3 ratios were contributing towards the disability status of the MS patients. Intervention to optimize fatty acid intake could be a safe and inexpensive way of restoring membrane equilibrium. Fatty acid supplementation should include both eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). Patients should be monitored to prevent any possible over dosage.

The decrease in membrane arachidonic acid (AA, C20:4n-6) is an important aspect in disease progression and both environmental factors (e.g. viral infection) and metabolism (e.g. increased phospholipase function) should be investigated.

The present study has also identified several putative blocks in the production of lipids needed for myelin synthesis. It would therefore be appropriate to investigate the enzymes catalyzing the final stages of PUFA biosynthesis: the elongases, desaturases and beta oxidases as well.

The antioxidant status of the patients should also be monitored and dietary supplementation with antioxidants should address a possible high free radical formation appropriately (e.g. vitamin supplementation).

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APPENDIX A
KURTZKE EXPANDED DISABILITY STATUS SCALE (EDSS)

(Kurtzke, 1983)

Disability score	Neurological status
0.0	Normal neurological examination.
1.0	No disability, minimal signs in one functional score (FS).
1.5	No disability, minimal signs in more than one FS.
2.0	Minimal disability in one FS.
2.5	Mild disability in one FS or minimal disability in two FS.
3.0	Moderate disability in one FS, or mild disability in three or four FS. Fully ambulatory.
3.5	Fully ambulatory but with moderate disability in one FS and more than minimal disability in several others.
4.0	Fully ambulatory without aid, self-sufficient, up and about some 12 hours a day despite relatively severe disability; 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; 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 (work a full day without special provisions).
5.5	Ambulatory without aid or rest for about 100 meters; disability severe enough to preclude full daily activities.
6.0	Intermittent or unilateral constant assistance (cane, crutch, brace) required to walk about 100 meters with or without resting.
6.5	Constant bilateral assistance (canes, crutches, braces) required to walk about 20 meters without resting.
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.
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.

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.
8.5	Essentially restricted to bed much of day; has some effective use of arms, retains some self care functions.
9.0	Confined to bed; can still communicate and eat.
9.5	Totally helpless bed patient; unable to communicate effectively or eat/swallow.
10.0	Death due to MS.

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.

APPENDIX B
SUBJECT INFORMATION AND CONSENT FORM

TITLE OF PROJECT: THE ROLE OF THE FATTY ACID COMPOSITION OF BLOOD CELL MEMBRANES IN THE ETIOLOGY OF MULTIPLE SCLEROSIS.

PROJECT NUMBER: 96 / 099

DECLARATION:

I,.....the undersigned,

[ID.....]

of.....(address)

.....

and contact phone number.....(W) .....(H)

.....(Cell)

A Declare that:

1 I was invited to participate in the above mentioned research project, which is being undertaken by the Department of Chemical Pathology of the University of Stellenbosch.

2 It has been explained to me that:

2.1 The project is being undertaken to investigate the possible significance of the relationship between dietary fatty acid consumption and the progression and severity of the disease symptoms in multiple sclerosis (MS) patients. MS affects the central nervous system and can cause paralysis and premature death.

2.2 If I participate in the project, 20-50 ml of blood will be collected from my arm. The serum and white blood cells extracted from the blood will be used in laboratory tests.

2.3 The research may provide important insights into the pathogenic mechanisms of MS and may also have implications for the development of future therapeutic approaches to the disease. The project should not take longer than one year.

3 I have been warned that the drawing of blood may result in slight discomfort, which can be coupled with bleeding where the needle pierces the skin.

4 It has been explained to me that participation in this project will result in the broadening of medical knowledge.

5 I have been informed that all information collected will be treated confidentially. The results will be used for a MTech thesis and for publication in scientific journals, without revealing the identity of any individual.

6 I may, during or on completion of the project, request the results of the tests without any conditions attached thereto, since the results could be advantageous to my family and me.

7 I have been told that participation is voluntary and that I may refuse to participate in this project and that I may also at any time withdraw my participation from the project. Refusal or withdrawal from the project will in no way affect my present or future treatment at this institution. I also understand that the researcher may withdraw me from the project if he/she considers it in my best interest.

8 The information above has been explained to me by.....in English/Afrikaans, and that I am fully conversant in this language. I was given the opportunity to ask questions which were answered to my satisfaction.

9 I was not pressurized to participate in this project and I know that I may at any time withdraw from this project without penalization.

10 Participation in this project will not result in unnecessary expenses for me. However, I will not be paid for participation.

B. I voluntary agree to participate in the above-mentioned project.

Signed/Affirmed at.....On.....200...

....

Signature of patient Name / Signature of witness.

DECLARATION BY OR ON BEHALF OF THE RESEARCHER

I, ...declare that I

1. Have explained the information in this document to.....
2. Requested him/her to ask questions where anything was unclear,
3. This conversation was conducted in English/Afrikaans.

Signed at.....On.....200...

....

Researcher/Research representative Name / signature of witness.

IMPORTANT INFORMATION

Dear patient

Thank you for participating in this project.

If at any time during the course of the project:

1. An emergency situation arises resulting from the research, or
2. You require further information regarding the project, or
3. If your condition suddenly deteriorates, or
4. If you change your address and/or telephone number,

Please contact me, Dinie Hon; tel 021 938 4107 or cell 0828384558, or
Dr Susan van Rensburg, Project Leader, tel 021 938 4611 or cell 0835647654.

APPENDIX C
INFORMASIE EN TOESTEMMINGS VORM

TITEL VAN PROJEK: DIE ROL VAN DIE VETSUUR SAMESTELLING VAN BLOEDSEL MEMBRANE IN DIE AETIOLOGIE VAN VEELVULDIGE SKLEROSE

PROJEK NOMMER: 96 / 099

VERKLARING:

Ek,.....die ondergetekende

[ID.....]

van.....(adres)

.....

en kontak foon nommers .....(W) .....(H)

.....(Sel)

A. Verklaar dat

1 Ek genader was om deel te neem in bogenoemde navorsings projek wat deur die Departement van Chemiese Patologie van die Universiteit van Stellenbosch onderneem is.

2 Daar is aan my verduidelik dat:

2.1 Die projek onderneem is om navorsing te doen om vas te stel of daar n moontlike betekenisvolle verhouding is tussen vetsuur inname in die dieet en die verloop en hewigheid van die siekte simptome in veelvuldige sklerose pasiente. Veelvuldige sklerose affekteer die sentrale senuwee stelsel en kan verlamming en premature dood veroorsaak.

2.2 As ek sou deelneem in die projek, sal 20-50 ml bloed getrek word uit my arm. Die serum en witbloedsel ekstraksies sal in die laboratorium vir toetsing gebruik word.

2.3 Die studie mag belangrike inligting oplewer aangaande die patogeniese meganismes van veelvuldige sklerose en kan ook moontlik gebruik word in die ontwikkeling van toekomstige terapeutiese behandeling. Die projek behoort binne een jaar afgehandel te wees.

3 Ek was ingelig dat die trek van bloed n bietjie ongemaklik kan wees, en dat bloeding n moontlikheid is waar die naald die vel binne gaan.

4 Daar is aan my verduidelik dat my deelname aan die projek kan lei tot n uitbreiding van mediese kennis.

5 Ek was meegedeel dat alle informasie van my verkry vertroulik behandel sal word. Die resultate van die studie sal gebruik word vir n MTech verhandeling en vir publikasie in wetenskaplike tydskrifte, sonder om die identiteit van enige individu te openbaar.

6 Ek mag, gedurende of met die afhandeling van die projek, die resultate van toetse gedoen aanvra sonder dat enige voorwaardes daaraan geheg word, omdat die resultate voordelig vir my en my familie kan wees.

7 Ek was meegedeel dat deelname in die projek vrywillig is en dat ek kan weier om deel te neem aan die projek en dat ek ter eniger tyd my kan onttrek van die projek. Weiering of onttrekking van die projek sal op geen wyse my huidige of toekomstige behandeling by die institusie affekteer nie. Ek verstaan ook dat die navorser my mag onttrek van die projek as sy/hy dit in my belang sou ag.

8 Die bostaande inligting is aan my verduidelik deur .....in Afrikaans/Engels. Ek is die taal volkome magtig. Ek was die geleentheid gegee om vrae te stel wat na my bevrediging beantwoord was.

9 Ek was onder geen druk geplaas om deel te neem aan die projek nie en ek verstaan dat ek my kan onttrek van die projek sonder enige strafbaarheid.

10 Deelname in die projek sal nie enige onnodige uitgawes vir my meebring nie. Ek sal ook geen betaling ontvang vir deelname nie.

B. Ek stem vrywillig in om aan bostaande projek deel te neem.

Geteken/Bevestig te .....op.....200...

....

Handtekening van pasient Naam / Handtekening van getuie.

VERKLARING DEUR DIE NAVORSER/VERTEENWOORDIGER

Ek, ...verklaar dat ek

1. Die informasie in die dokument verduidelik het aan .....
2. Haar/hom gevra het om vrae te stel waar enige onduidelikheid was.
3. Die gesprek was gevoer in Afrikaans/Engels.

Geteken te.....op.....200...

....

Navorser/verteenwoordiger Naam / handtekening van getuie.

BELANGRIKE INLIGTING

Liewe pasient

Baie dankie dat u deelgeneem het aan die projek.

As daar ter enige tyd gedurende u deelname aan die projek:

1. n Nood situasie opduik as gevolg van die projek, of
2. As u meer inligting nodig het aangaande die projek, of
3. As u toestand onverwags verswak, of
4. As u adres en/of telefoon nommer verander,

Kontak my asb, Dinie Hon; tel 021 938 4107 of sel 0828384558, of

Dr Susan van Rensburg, Projek leier, tel 021 938 4611 of sel 0835647654.

APPENDIX D
HEALTH QUESTIONNAIRE

Name.....

DOB.....

Age.....

Sex..... Race.....

Tel no: (W)..... (H)..... (Cell).....

	Yes	Time / date	No
Diagnosed with MS?			
Diagnosis confirmed by an MRI scan?			
Any relapses during the preceding 12 months?			
On any Interferon beta treatment?			
On any unsaturated fatty acid supplementation?			
On any iron supplementation?			
On any vitamin supplementation?			
On any mineral supplementation?			
On any generic medication?			
On any prescription medication?			

Type of MS.....

Confirmed by Dr..... Tel no.....

Date.....

....

Signature of patient Name / Signature of witness

(Information gained from studies done by Dr S. J. van Rensburg, Department of Chemical Pathology, Tygerberg Hospital).

APPENDIX E
WELSTAND OPNAME

Naam.....

Geboortedatum.....

Ouderdom.....

Geslag..... Ras.....

Tel no: (W)..... (H)..... (Cell).....

	Ja	Tyd / datum	Nee
Gediagnoseer met MS?			
Is diagnose met n MRI skandering bevestig?			
Enige terugslae gedurende die afgelope 12 maande?			
Op enige Interferon-beta behandeling?			
Op enige onversadigde vetsuur aanvullings?			
Op enige yster aanvullings?			
Op enige vitamine aanvullings?			
Op enige minerale aanvullings?			
Op enige generiese medikasie?			
Op enige voorgeskrewe medikasie?			

Tipe MS.....

Bevestig deur Dr..... Tel no.....

Datum.....

....

Handtekening van pasient Naam / Handtekening van getuie

(Informasie van Dr S. J. van Rensburg, Department van Chemiese Patologie, Tygerberg Hospitaal).

TABLES

Table 21

A list of the abbreviations of the chemical formulae and the common names of the fatty acids considered in this study
(Nightingale *et al.*, 1990)

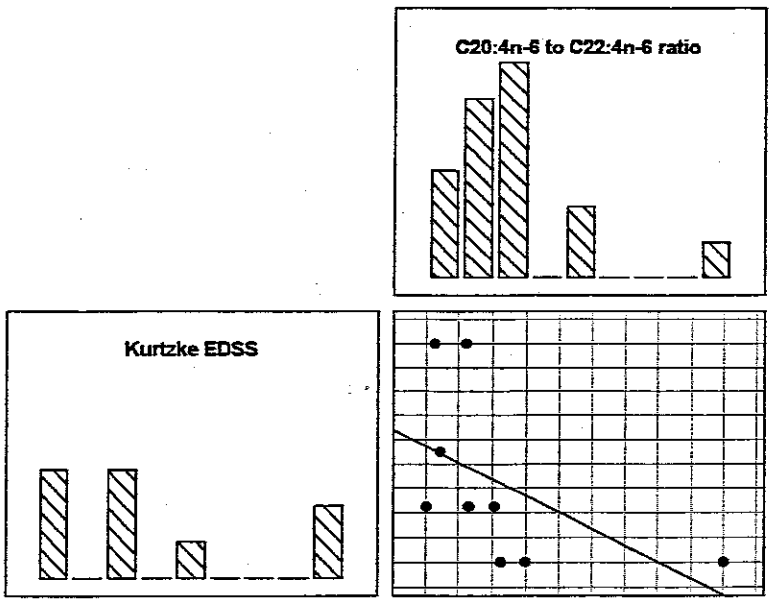
Fatty acids in the n-6 series

Abbreviation of chemical formulae	Common name
C18:2n-6	Linoleic acid (LA)
C18:3n-6	Gamma-linolenic acid (GLA)
C20:2n-6	
C20:3n-6	Dihommo-gamma-linolenic acid (DGLA)
C20:4n-6	Arachidonic acid (AA)
C22:4n-6	Adrenic acid
C22:5n-6	

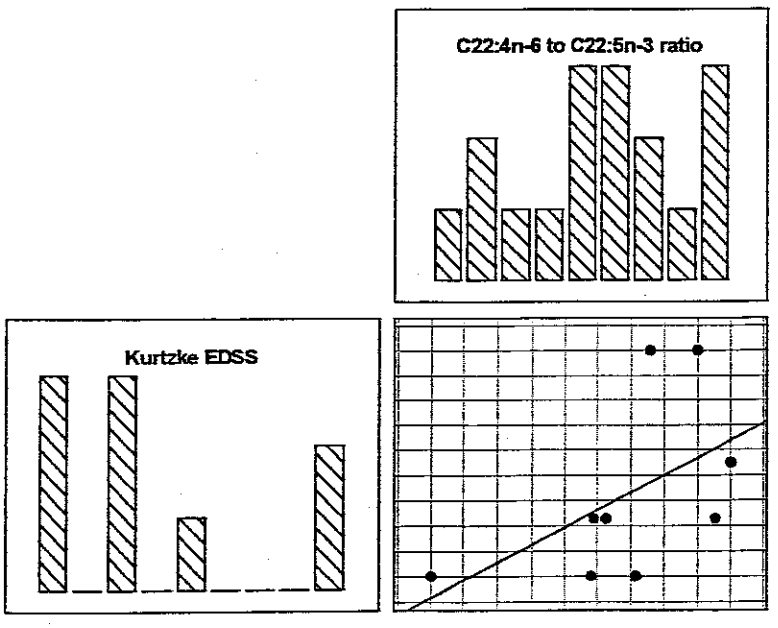
Fatty acids in the n-3 series

Abbreviation of chemical formulae	Common name
C18:3n-3	Alpha-linolenic acid (ALA)
C20:3n-3	
C20:5n-3	Eicosapentaenoic acid (EPA)
C22:5n-3	Docosapentaenoic acid (DPA)
C22:6n-3	Docosahexaenoic acid (DHA)

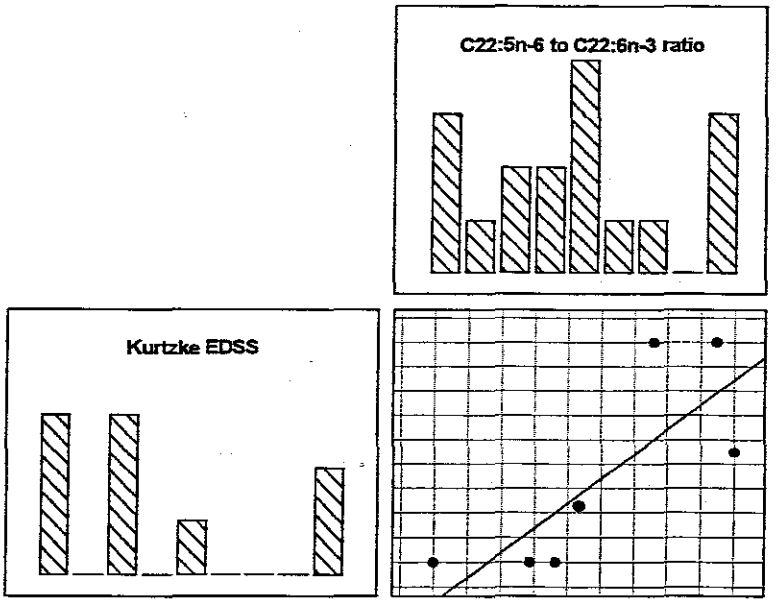
GRAPHS



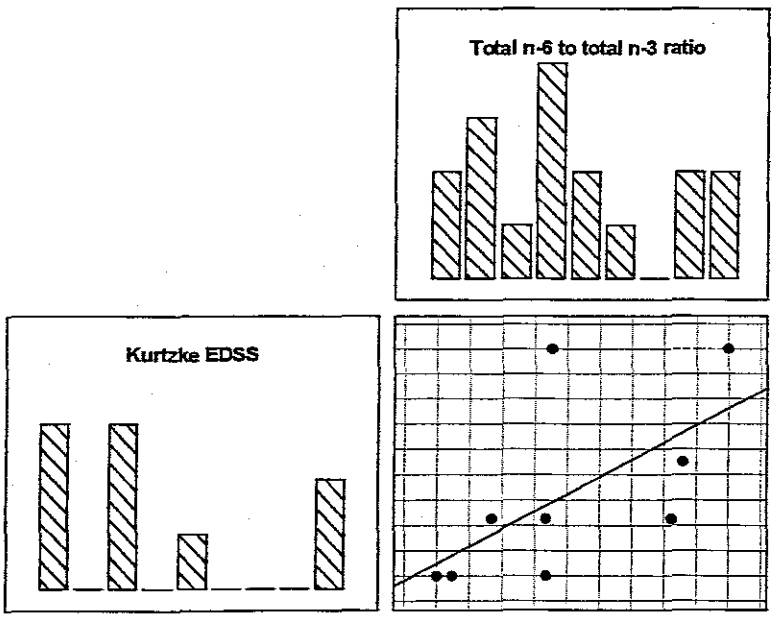
Graph 1: Correlation between the Kurtzke EDSS and the C20:4n-6 to C22:4n-6 ratio in erythrocyte membrane total phospholipids in MS patients
r = - 0.780; p = 0.013



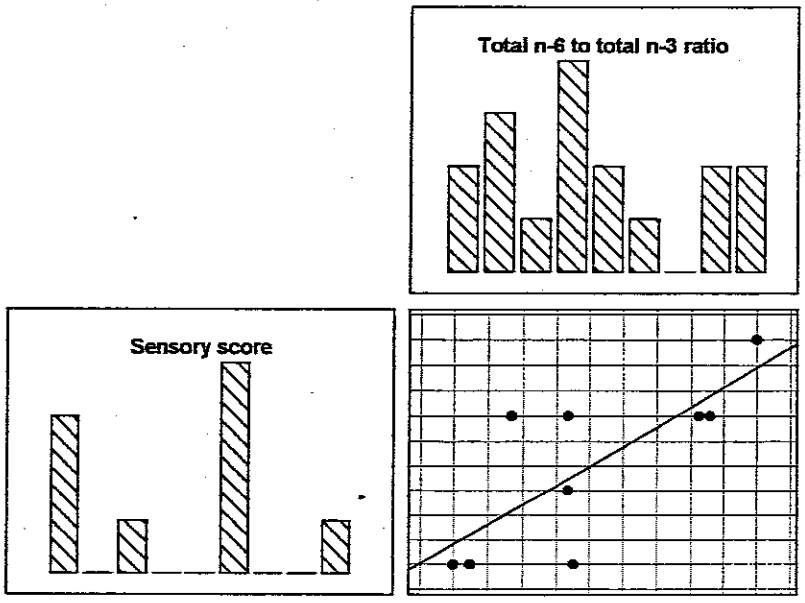
Graph 2: Correlation between the EDSS and the C22:4n-6 to C22:5n-3 ratio in erythrocyte membrane total phospholipids in MS patients
r = 0.685; p = 0.042



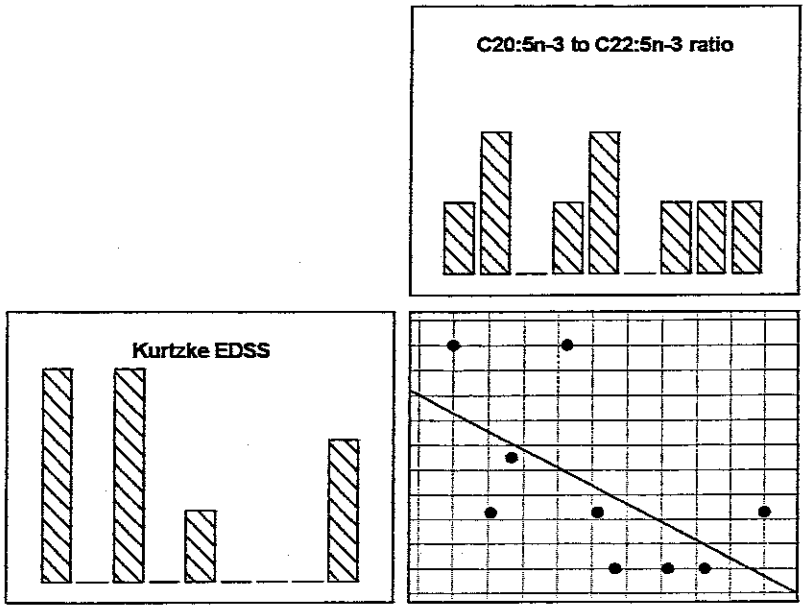
Graph 3: Correlation between the EDSS and the C22:5n-6 to C22:6n-3 ratio in erythrocyte membrane total phospholipids in MS patients
r = 0.910; p = 0.001



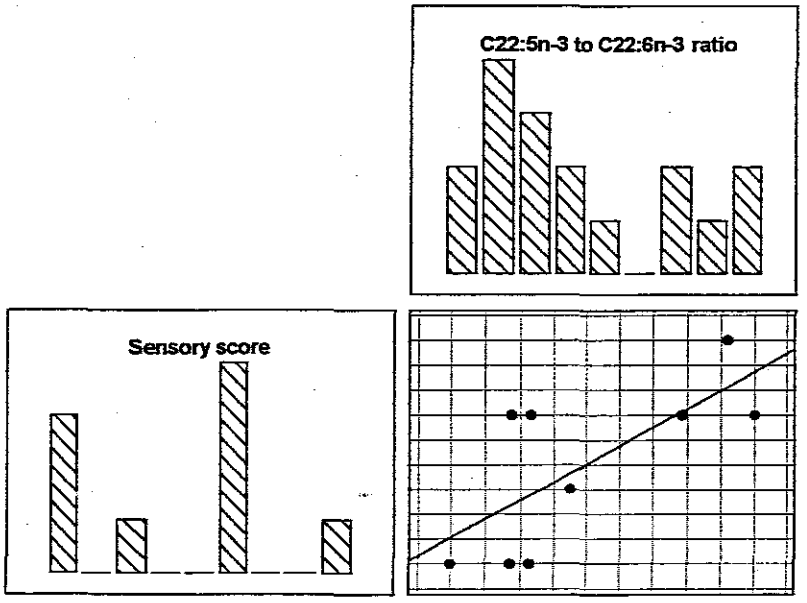
Graph 4: Correlation between the EDSS and the total n-6 to total n-3 ratio in erythrocyte membrane total phospholipids in MS patients
r = 0.823; p = 0.006



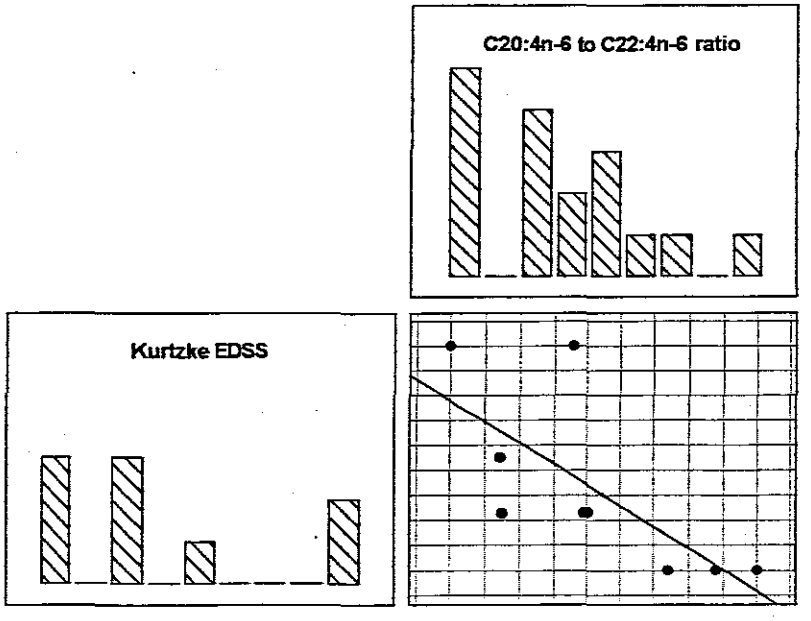
Graph 5: Correlation between the sensory score and the total n-6 to total n-3 ratio in erythrocyte membrane total phospholipids in MS patients
r = 0.700; p = 0.036



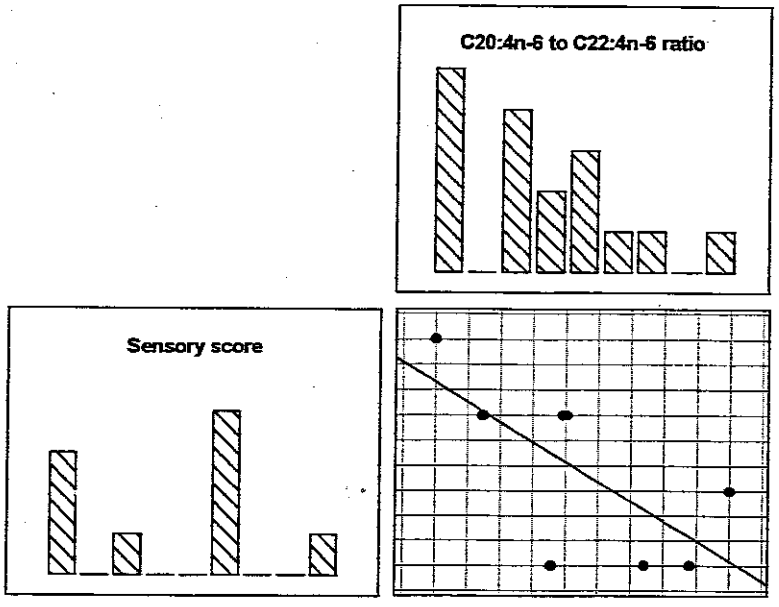
Graph 6: Correlation between the EDSS and the C20:5n-3 to C22:5n-3 ratio in lymphocyte membrane total phospholipids in MS patients
r = - 0.685; p = 0.042



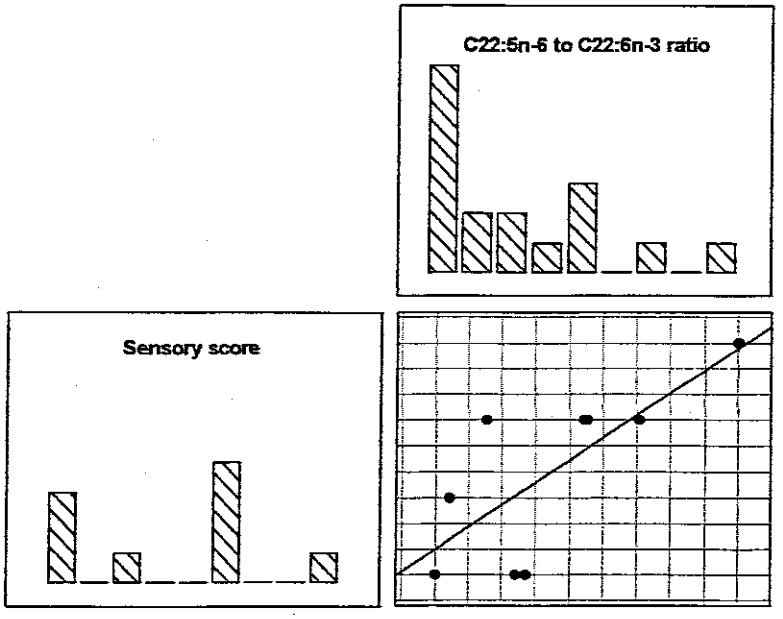
Graph 7: Correlation between the sensory score and the C22:5n-3 to C22:6n-3 ratio in lymphocyte membrane total phospholipids in MS patients r = 0.727; p = 0.026



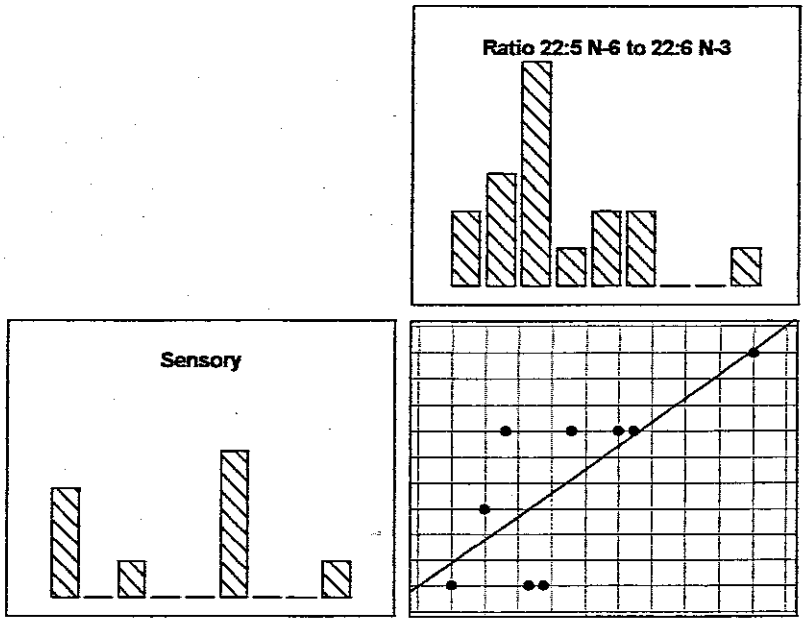
Graph 8: Correlation between the EDSS and the C20:4n-6 to C22:4n-6 ratio in lymphocyte membrane total phospholipids in MS patients r = -0.876; p = 0.002



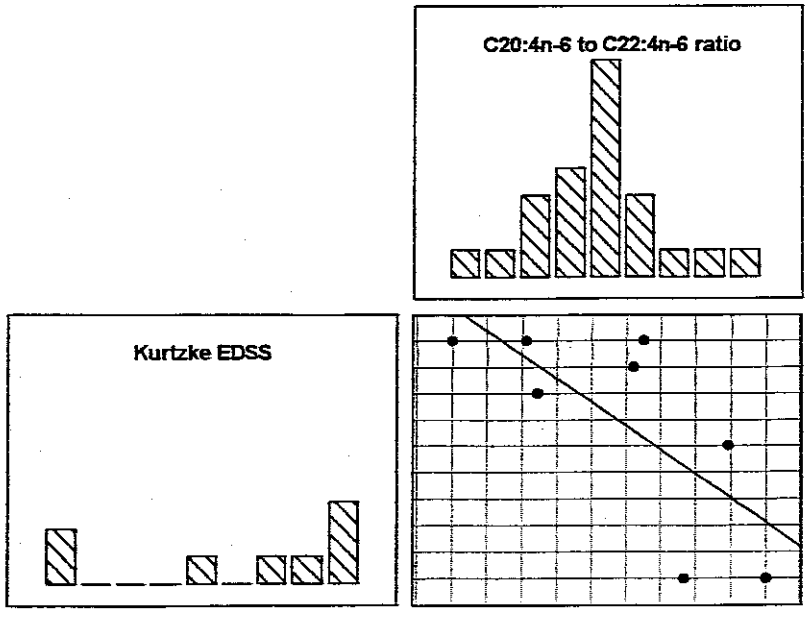
Graph 9: Correlation between the sensory score and the C20:4n-6 to C22:4n-6 ratio in lymphocyte membrane total phospholipids in MS patients r = - 0.674; p = 0.047



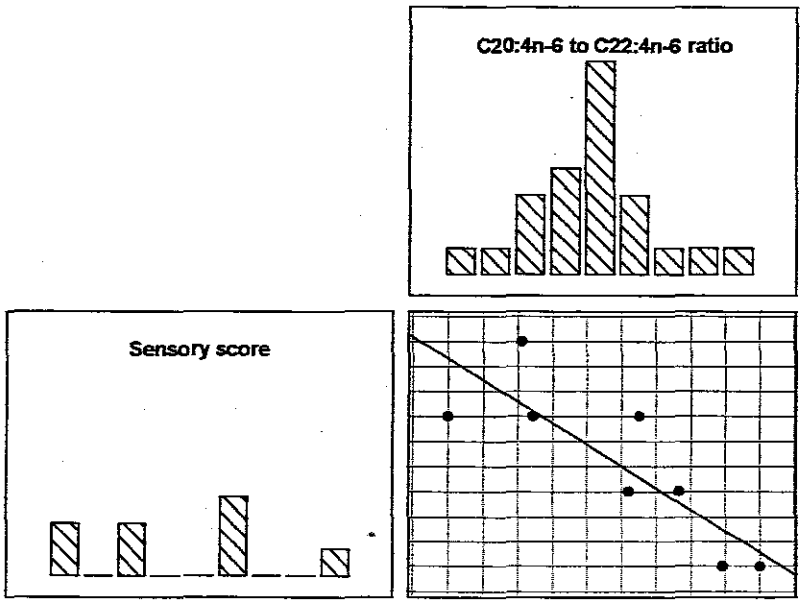
Graph 10: Correlation between the sensory score and the C22:5n-6 to C22:6n-3 ratio in lymphocyte membrane total phospholipids in MS patients r = 0.709; p = 0.032



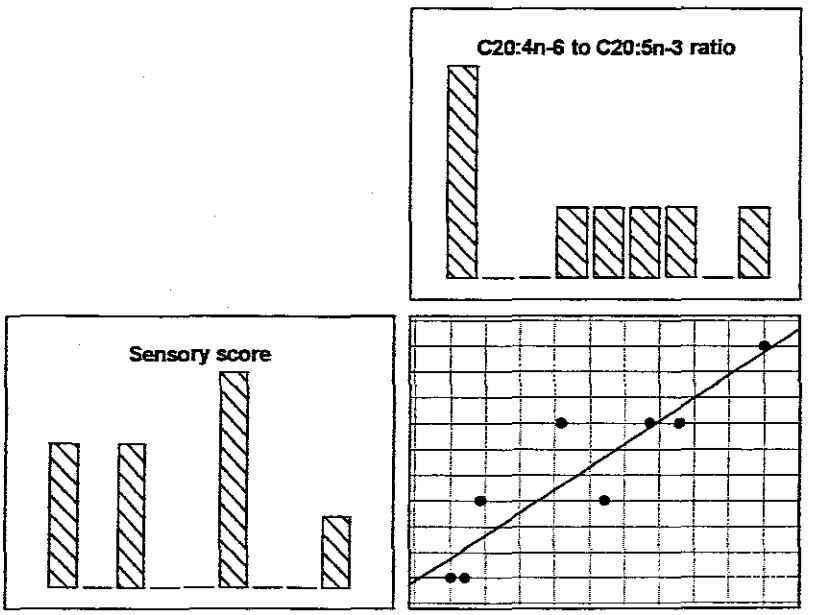
Graph 11: Correlation between the sensory score and the C22:5n-6 to C22:6n-3 ratio in plasma total phospholipids in MS patients
r = 0.709; p = 0.032



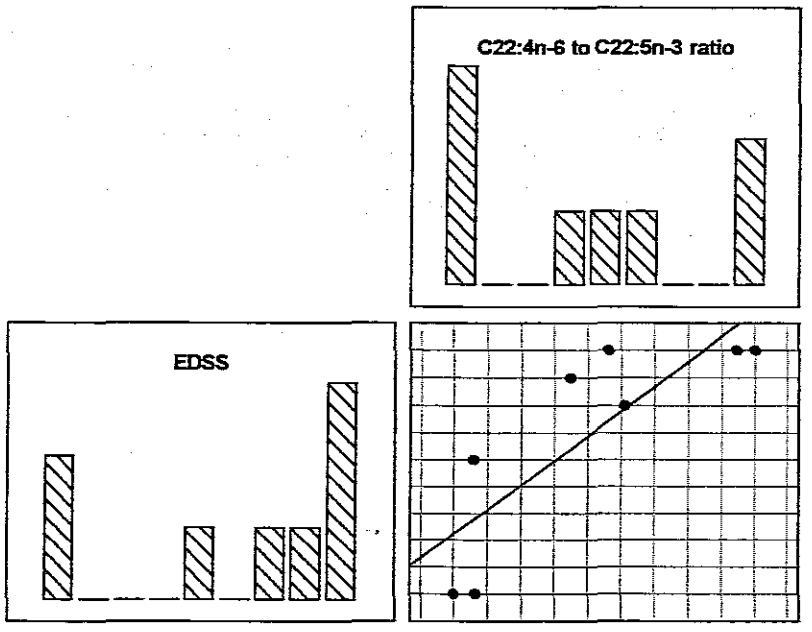
Graph 12: Correlation between the EDSS and the erythrocyte membrane PC C20:4n-6 to C22:4n-6 ratio in MS patients
r = - 0.786; p = 0.021



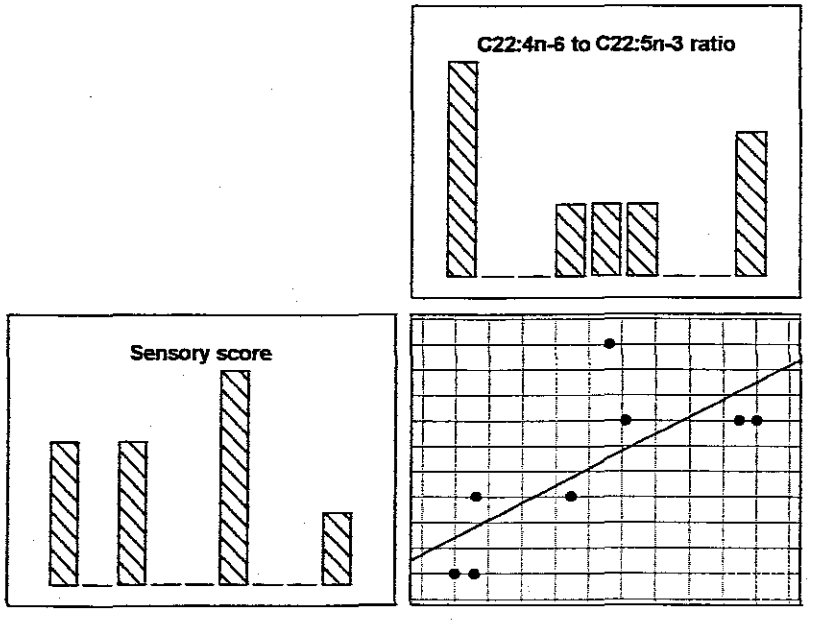
Graph 13: Correlation between the sensory score and the erythrocyte membrane PC C20:4n-6 to C22:4n-6 ratio in MS patients
r = - 0.852; p = 0.007



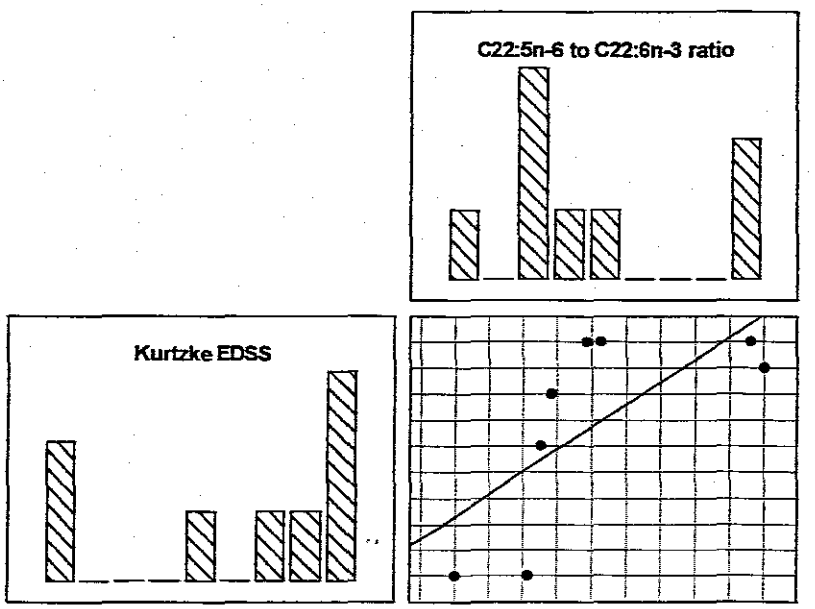
Graph 14: Correlation between the sensory score and the erythrocyte membrane PC C20:4n-6 to C20:5n-3 ratio in MS patients
r = 0.902; p = 0.002



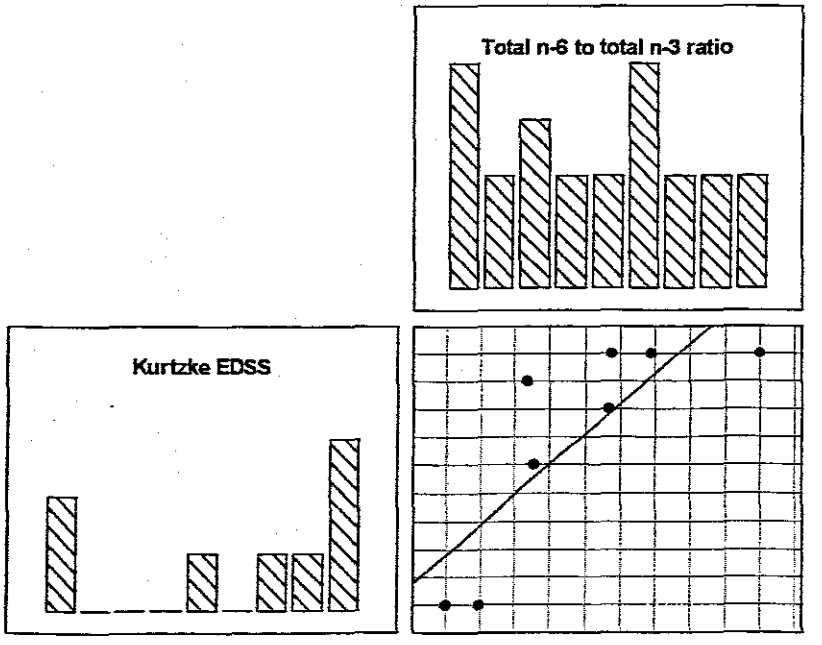
Graph 15: Correlation between the EDSS and the erythrocyte membrane PC C22:4n-6 to C22:5n-3 ratio in MS patients
r = 0.835; p = 0.010



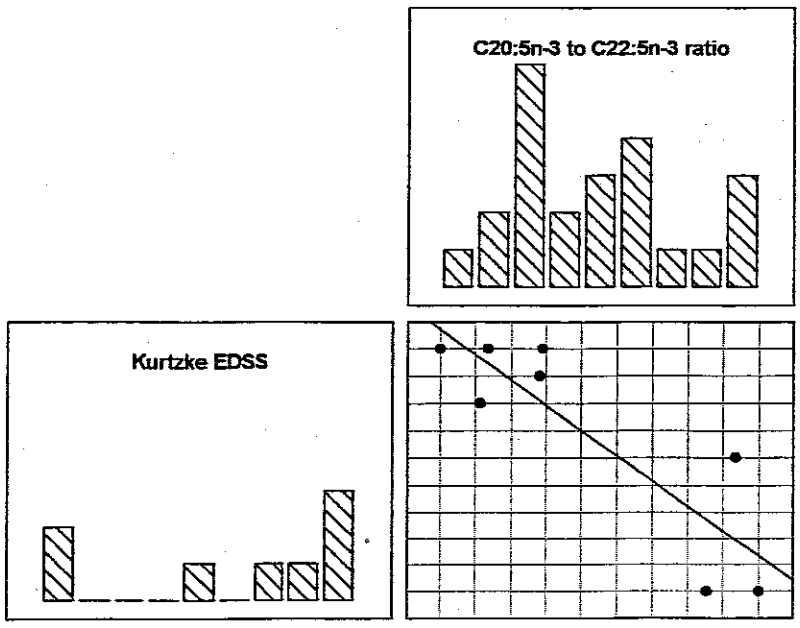
Graph 16: Correlation between the sensory score and the erythrocyte membrane PC C22:4n-6 to C22:5n-3 ratio in MS patients
r = 0.815; p = 0.014



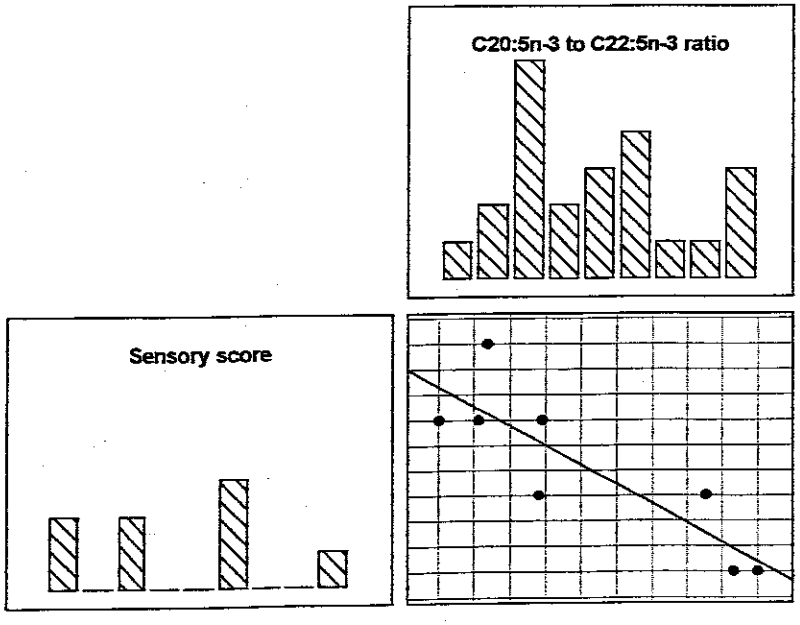
Graph 17: Correlation between the EDSS and the erythrocyte membrane PC C22:5n-6 to C22:6n-3 ratio in MS patients
r = 0.822; p = 0.012



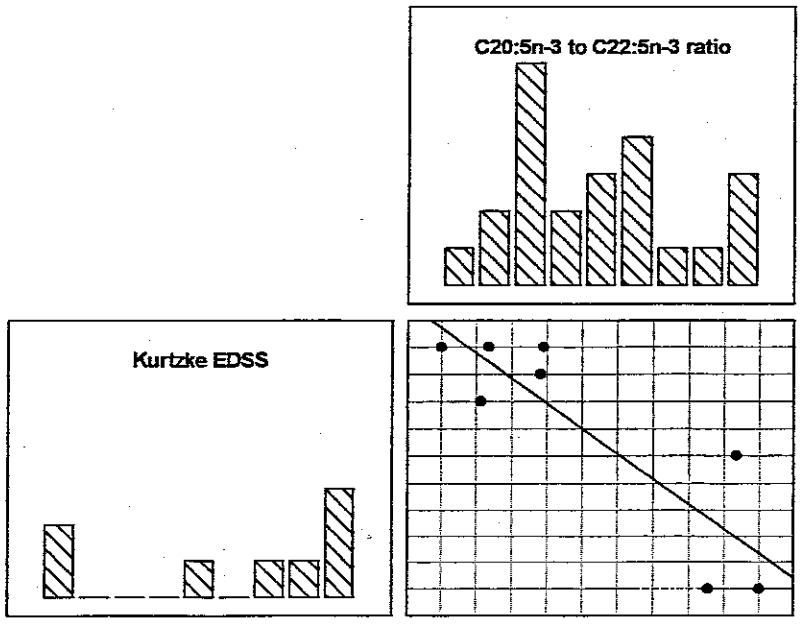
Graph 18: Correlation between the EDSS and the erythrocyte membrane PC total n-6 to total n-3 ratio in MS patients
r = 0.896; p = 0.003



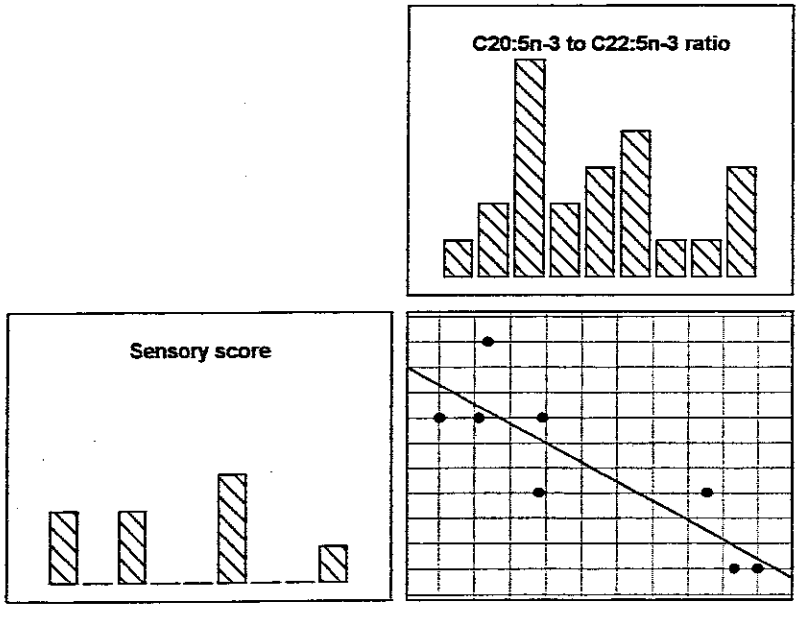
Graph 19: Correlation between the EDSS and the erythrocyte membrane PE C20:5n-3 to C22:5n-3 ratio in MS patients
r = - 0.712; p = 0.048



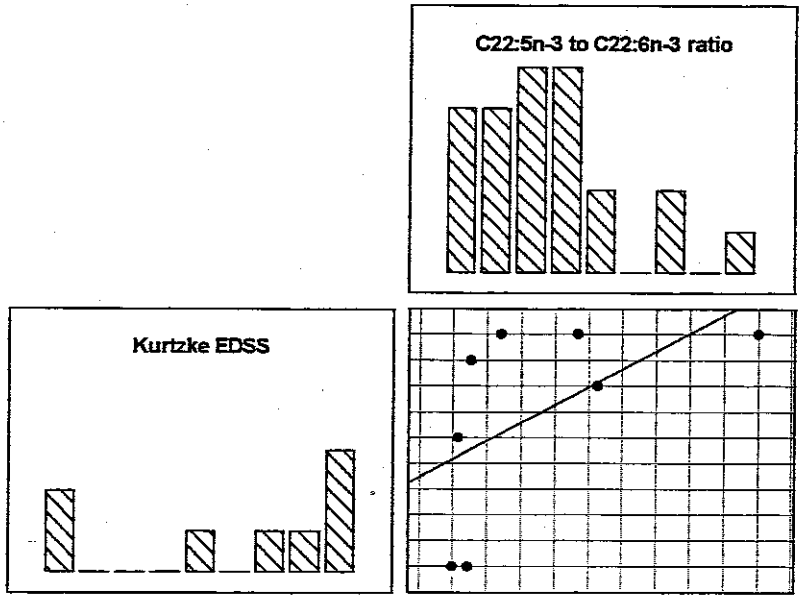
Graph 20: Correlation between the sensory score and the erythrocyte membrane PE C20:5n-3 to C22:5n-3 ratio in MS patients
r = - 0.803; p = 0.016



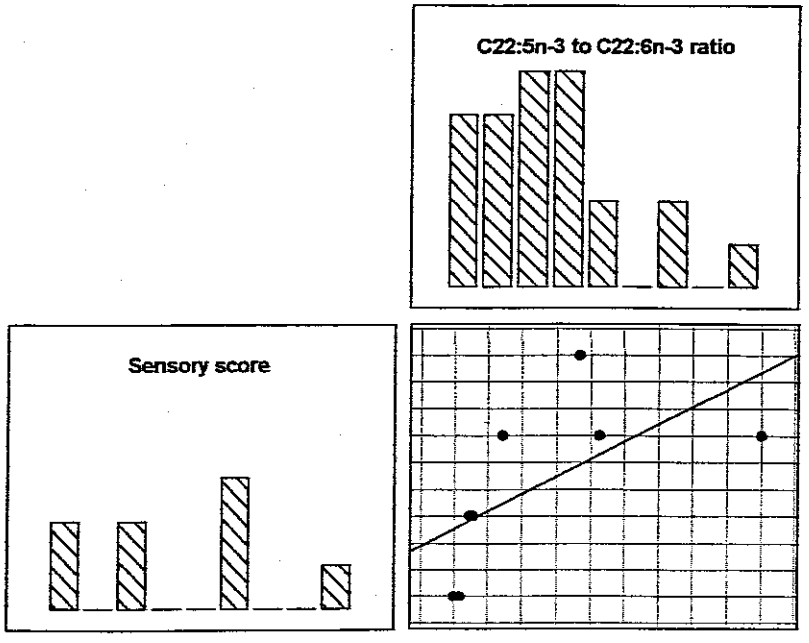
Graph 19: Correlation between the EDSS and the erythrocyte membrane PE C20:5n-3 to C22:5n-3 ratio in MS patients
r = - 0.712; p = 0.048



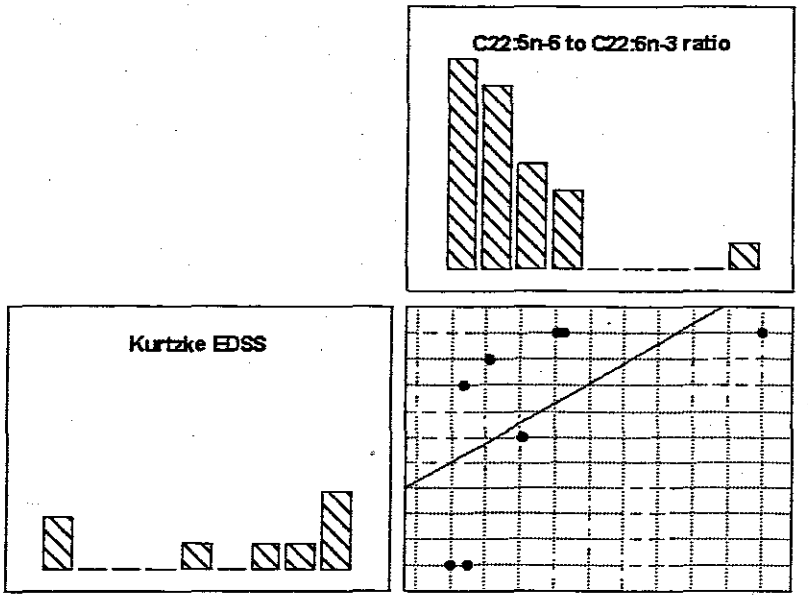
Graph 20: Correlation between the sensory score and the erythrocyte membrane PE C20:5n-3 to C22:5n-3 ratio in MS patients
r = - 0.803; p = 0.016



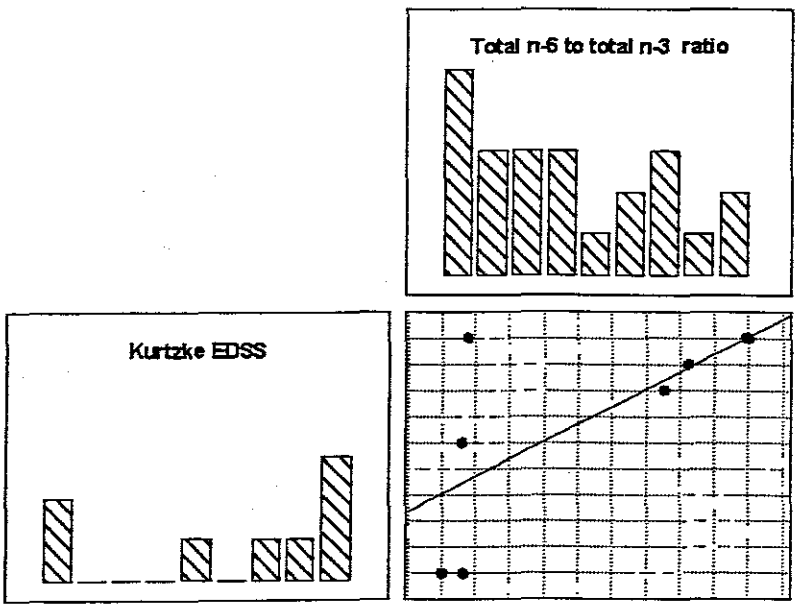
Graph 21: Correlation between the EDSS and the erythrocyte membrane PE C22:5n-3 to C22:6n-3 ratio in MS patients
r = 0.761; p = 0.028



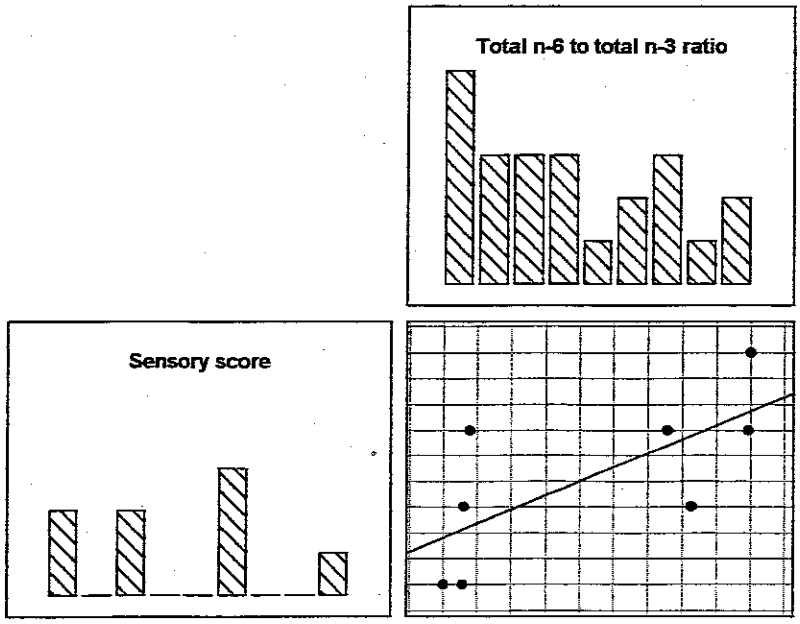
Graph 22: Correlation between the sensory score and the erythrocyte membrane PE C22:5n-3 to C22:6n-3 ratio in MS patients
r = 0.865; p = 0.006



Graph 23: Correlation between the EDSS and the erythrocyte membrane PE C22:5n-6 to C22:6n-3 ratio in MS patients
r = 0.835; p = 0.010



Graph 24: Correlation between the EDSS and the erythrocyte membrane PE total n-6 to total n-3 ratio in MS patients
r = 0.810; p = 0.015



Graph 25: Correlation between the sensory score and the erythrocyte membrane PE total n-6 to total n-3 ratio in MS patients
r = 0.840; p = 0.009

DIAGRAMS

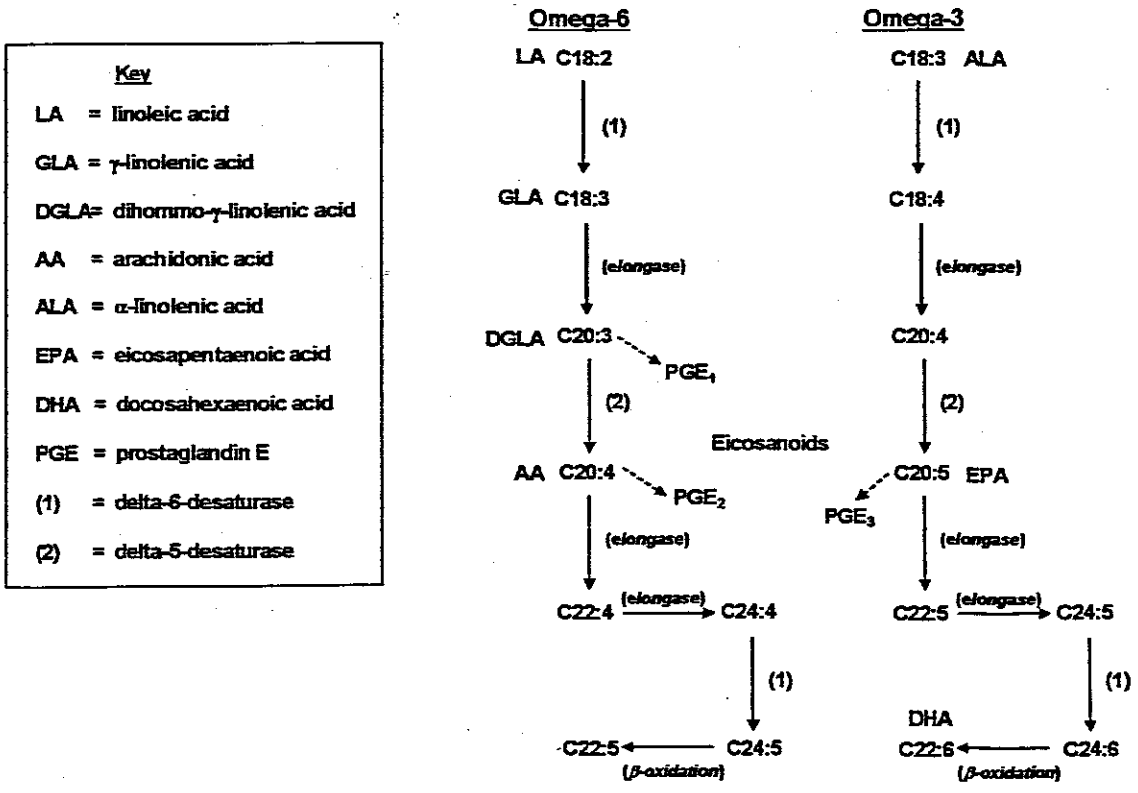


Diagram 1: Metabolic path of essential fatty acids (Sprecher, 2000) (Pereira *et al.*, 2003)