The Effects of Omega-3, Omega-6 Polyunsaturated Fatty Acids (PUFAs) and Vitamin E on Patients with the Remitting-Relapsing (RR) Form of Multiple Sclerosis (MS) in Cyprus

A Thesis Submitted for the Degree of Doctor of Philosophy (Ph.D)

By

George N. Loucaides

Lipidomics and Nutrition Research Centre
Faculty of Life Sciences and Computing
London Metropolitan University

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Abstract

Multiple sclerosis is an inflammatory and demyelinating multifactorial disease that results from the interplay between environmental factors and a susceptible genetic background. Omega (ω)-3/ω-6 polyunsaturated fatty acids (PUFAs) and some vitamins have been shown to reduce the number and severity of relapses and the overall progression of disability in multiple sclerosis patients, however clinical trials remain inconclusive due to a plethora of reasons. In this randomized, double blinded, placebo controlled trial aiming to provide concrete conclusions for the role of PUFAs and vitamins A and E (both gamma (γ) and alpha (α)) in multiple sclerosis. By measuring the incorporation and changes of the lipid composition in red blood cell (RBC) membranes before and after the dietary intervention, and by correlating the efficacy of the different interventions with disease progression, it was shown that supplementation with these specific molecules (that can either act on their own or synergistically) could probably cause the decrease of arachidonic acid (AA) and linoleic acid (LA) from the RBC membranes and the subsequent substitution by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These events parallel the clinical observations where this can be correlated with the increased number of relapse activity occurring in the first six months of treatment and later with a prolonged period of remission. Supplementation with the mixture of PUFAs (both ω3 and ω6), vitamin E as gamma tocopherol significantly reduced the annualised relapse rate (ARR) and the risk of sustained disability progression without any reported serious adverse events.

In the memory of

my parents Nicos and Demetra and granny Joanna

Διαιτήμασί τε χρήσομαι ἐπ' ὠφελείῃ καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμὴν, ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἴρξειν. Απόσπασμα από τον Όρκο του Ιπποκράτη 460π.Χ.-377π.Χ. Translation: I will apply dietetic measures for the benefit of the sick according to my ability and judgment; I will keep them from harm and injustice. Quotation from The Hippocratic Oath 460 B.C-377B.C.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA: Arachidonic Acid

AI: Adequate Intake

ALA: Alpha Linolenic Acid

AMDR: Acceptable Macronutrient Distribution Range

AMPA: alpha (α)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ARE: Antioxidant Response Element

ARR: Annualised Relapse Rate

BBB: Blood-Brain-Barrier

BC: Breast cancer

beta-IFN 1b: Beta-interferon 1b

BHT: Butylated hydroxytoluene

CAT: Catalase

$CD4^+$: Cluster of Differentiation 4^+

CEHC: Carboxyethyl hydroxychromane

CHD: Coronary Heart Disease

CIS: Clinically Isolated Syndrome

CLO: Cod Liver Oil

CNS: Central Nervous System

COX: Cyclooxygenase

COMA: Committee on Medical Aspects of Food and Nutrition Policy

CRP: C-reactive protein

CSF: Cerebrospinal Fluid

DAG: Diacylglycerol

DGLA: Di-homo-gamma linolenic Acid

DHA: Docosahexaenoic Acid

DMTs: Disease-modifying Treatments

DRI: Dietary Reference Intake

DTA: Docosatetraenoic Acid

EAE: Experimental Autoimmune Encephalomyelitis

EDA: Eicosadienoic Acid

EDSS: Kurtzke Expanted Disability Status Scale

EFA: Essential Fatty Acids

EFSA: European Food Safety Authority

EPA: Eicosapentaenoic Acid

ER: Endoplasmic Reticulum

ERA: Eicosatrienoic Acid

ETA: Eicositetraenoic Acid

FA: Fatty Acid

FAME: Fatty acid methyl esters

FBO: Fish Body Oils

FFQ: Food frequency questionnaire

FS: Functional Systems

FSS: Functional System Score

GA: Glatiramer Acetate

GLA: Gamma Linolenic Acid

GluRs: Glutamate receptors

GPCR: G-protein coupled surface receptors

GSH-Px: Glutathione Peroxidase

$HO^{\bullet}$: Hydroxyl Radical

H_2O_2: Hydrogen peroxide

H6PD: Hexose-6-phosphate Dehydrogenase

15-HETrE: 15-(S)-hydroxy-8,11,13-eicosatrienoic acid

HLA: Human Leucocyte Antigen

HMC-1: Mastocystoma cell line

HR: Hazard Ratio

ICAM-1: Intracellular Adhesion Molecule 1

IFN-γ: (Interferon-gamma)

IL-12: Interleukin 12

IL-17: Interleukin 17

IL-22: Interleukin 22

IL-23: Interleukin 23

IL-6: Interleukin 6

ISFFAL: International Society for the Study of Fatty Acids and Lipids

ITT: Intention-to-treat

IU: International unit

cNOS: Constitutive Nitric Oxide Synthase

iNOS: Iducible Nitric Oxide Synthase

LA: Linoleic Acid

LCPUFA: Long Chain Polyunsaturated Fatty Acids

LDL: Low Density Lipoprotein

LMWA: Low-molecular-weight antioxidants

LOX: 5- Lipoxyganase

LPS: Lipopolysaccharide

LT: Leukotriene

MHC: Major Histocompatibility Complex

MMP: Matrix Metalloproteinases

MS: Multiple Sclerosis

MSC: Mesenchymal Stem Cell

MUFA: Mono-unsaturated fatty acid

NADPH: Nicotinamide adenine dinucleotide phosphate

5NγT: 5-nitro-γ-tocopherol

NCX: Na^{+}/Ca^{2+} exchanger

NMDA: N-methyl-D-aspartate

NO: Nitric Oxide

NOx: Nitrogen oxides

NF-kB: Nuclear Transcription Factor-kappa B

Nrf2: Nuclear Factor-E2-related factor

O_2^{-}: Superoxide radical

$ONOO^{-}$: Peroxynitrite

PBMC: Peripheral blood mononuclear cells

PC: Phosphatidylcholine

PDK: Phosphoinositide-depentant kinase

PE: Phosphatidylethanolamine

PG: Prostaglandins

PI: Phosphatidylinositol

PI3K: Phosphatidylinositol 3-kinase

PKB: Protein kinase B or Akt

PKC: Protein kinase C

PL: Phospholipases

PMCA2: plasma-membrane Ca2+-ATPase 2

PPAR: Peroxisome proliferator-activated receptors

PPMS: Primary Progressive Multiple Sclerosis

PPP: Pentose Phosphate Pathway

PRMS: Progressive–relapsing Multiple Sclerosis

PS: Phosphatidylserine

PUFAs: Polyunsaturated Fatty Acids

RA: Rheumatoid arthritis

RBC: Red Blood Cells

RIS: Radiologically Isolated Syndrome

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

PMCA2: Plasma-membrane Ca^{2+}-ATPase 2

RRMS: Relapsing-Remitting Multiple Sclerosis

rTG: Re-esterified Triglycerides

RXR-γ: Retinoid X receptor γ

SCD: Stearoyl CoA desaturases

SFAs: Saturated fatty acids

SM: Sphingomyelin

SOD: Superoxide Dismutase

SPMS: Secondary Progressive Multiple Sclerosis

STA: Stearodonic Acid

T: Tocopherol

TG: Triglycerides

TGFβ: Transforming Growth factor-beta

T_h1: T-helper cell 1

T_h17: T-helper cell 17

THA: Tetracosahexaenoic Acid

TLC: Thin Layer Chromatography

TLR: Toll-like Receptor

TNF-α: Tumor Necrosis Factor-α

TPA: Tetracosapentaenoic Acid

TRP: Transient Receptor Potential Channels

TX: Thromboxane

UFA: Unsaturated Fatty Acids

VCAM-1: Vascular-cell Adhesion Molecule 1

Abbreviated chemical formulae and common names of major membrane fatty acids

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

CHAPTER ONE

LITERATURE REVIEW

CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating multifactorial disease of the brain and spinal cord with the presence of large, multifocal, demyelinated plaques with reactive glial scar formation (Compston and Coles 2008). The inflammation consists of infiltration, mainly of T cells and macrophages, (Lassmann, Brück et al. 2001) leading to demyelination and remyelination, oligodendrocyte depletion and astrocytosis and eventually to neuronal and axonal degeneration (Compston and Coles 2008). Although demyelination can be repaired, at least in part, by remyelination, axonal destruction is irreversible (Lassmann, Brück et al. 2001); however the sequence of these events remains unknown. The mechanisms involved include: (a) immune-mediated inflammation, (b) oxidative stress and (c) excitotoxicity, and all of which contribute to disease progression via oligodendrocyte and neuronal damage and even cell death. (Evans 1993; Knight 1997; Smith, Kapoor et al. 1999; Matute, Alberdi et al. 2001; Owens 2003).

Although the cause and pathogenesis of MS still remains elusive (Chaudhuri and Behan 2004), two main theories have been put forward: the viral causation and the autoimmune (Chaudhuri and Behan 2004). According to Compston et al. 2008, MS involves environmental exposure and genetic susceptibility (Compston and Coles 2008); Chaudhuri et al. 2004 concluded that abnormal neuronal metabolism, and metabolic changes such as vitamin D and glucose metabolism as observed by Mathur et al. 2014 can influence glial and neuronal function, which may lead to the break-down of the blood-brain-barrier (BBB) which is observed in MS relapses (Chaudhuri and Behan 2004; Mathur, López-Rodas et al. 2014).

Functional impairment from relapsing–remitting phases is caused mainly by inflammation and demyelination, whereas the accumulation of an irreversible neurological deficit is caused mainly by axonal destruction and loss (Lassmann, Brück et al. 2001). In combination with the thus far ineffective therapies in MS, this leads to worsening of the patients' health and quality of life.

Other metabolic disturbances that have been observed in MS patients are those of the metabolism of polyunsaturated fatty acids (PUFAs) and antioxidants, along with decreased cellular antioxidant defence mechanisms (van Meeteren, Teunissen et al. 2005). So far clinical trials on the effects of PUFAs on the progression of the disease have been controversial due to different design and methodology (Mehta, Dworkin et al. 2009); furthermore no trials have ever used any combination of PUFAs or a combination of PUFAs with antioxidants in a single formulae (Mehta, Dworkin et al. 2009). In the present study the effects of PUFAs and tocopherols (alpha and gamma), alone or the mixture of the two were tested against placebo in a clinical design in order to provide evidence based results to the health care community for the prevention and eventually on the treatment of MS using the above named nutrients.

1.2 Nutrition and MS

Swank in 1950 attempted to correlate diet with MS and demonstrated that higher saturated fat consumption correlates with higher incidence of the disease (Swank 1950). In 1995 Esparza et al 1995 in a meta-analysis of epidemiological studies concluded that a relation between MS mortality and dietary fat does exist (Luisa Esparza, Sasaki et al. 1995) and that saturated fatty acids, animal fat and animal fat without fish fat, correlate positively with MS mortality (Ghadirian, Jain et al. 1998). Ghadirian et al 1998 observed an increased risk for MS associated with energy and animal fat intake (Ghadirian, Jain et al. 1998). In the same

study, it was observed that some nutrients, such as vitamin C, thiamine, riboflavin vegetable protein, dietary fibre, exhibit a protective effect, whereas in another study no association was observed (Zhang, Hernán et al. 2001).

1.2.1 Antioxidant deficiencies in MS

It is not clear yet if the antioxidant deficiency which exists in MS is the result of chronic inflammation that is accompanied by increased oxidative stress (van Meeteren, Teunissen et al. 2005). Besler et al 2002 found decreased levels of α-tocopherol, β-carotene, retinol, ascorbic acid in the serum of secondary progressive MS patients first experiencing exacerbations (Besler, Comoglu et al. 2002). Other studies showed lipid peroxidation in patients with MS (Toshniwal and Zarling 1992). Vitamin E levels from cerebrospinal fluid (CSF) samples of MS patients during exacerbations have no differences as compared to those of healthy individuals (Jiménez-Jiménez, de Bustos et al. 1998). In MS plaques, levels of glutathione (GSH) and vitamin E were significantly decreased as compared to adjacent and distant white matter (Langemann, Kabiersch et al. 1992). However antioxidant treatment with catalase in the experimental autoimmune encephalomyelitis (EAE) model (an experimental model of MS), disease severity was markedly suppressed (Sigrid, Jan et al. 1995). Guy et al 1998 in treatment of EAE with catalase suppressed optic neuritis (Guy, Qi et al. 1998). Ruuls et al 1995 showed no effect on treating EAE with superoxide dismutase (SOD) (Sigrid, Jan et al. 1995). Hooper et al 1998 treated EAE with uric acid, a scavenger of peroxynitrite and demonstrated inhibition of clinical signs of the disease (Hooper, Spitsin et al. 1998). In another study by Lehmann et al 1994 administration of N-acetylcysteine (NAC) (oxidant scavenger) inhibited EAE (Lehmann, Karussis et al. 1994). Desferrioxamine, an iron chelator, suppressed development of EAE (Pedchenko and LeVine 1998). Iron-deficient mice failed to develop EAE (Grant, Wiesinger et al. 2003). What Grand et al 2013 did not point out is that the EAE was not exacerbated in mice that were fed with

a high iron diet and that the iron deprivation was related to the impairment of CD4+ T cell development. Some investigators have shown iNOS inhibition, as well as NO scavenging, to suppress EAE whereas others have shown iNOS inhibition or administration of an NO donor aggravates or ameliorates EAE (van Meeteren, Teunissen et al. 2005).

1.2.1.1 Vitamin D

Although genetic background plays a significant role in MS development, it is however of lesser importance than environmental and lifestyle factors (Ascherio and Munger 2007; Ascherio, Munger et al. 2010; Ascherio 2013; Jelinek, Marck et al. 2015). This is supported by evidence from studies that show changes in MS risk among people of common ancestry upon migration (Munger, Zhang et al. 2004; Munger, Levin et al. 2006). Epidemiological studies on the development of MS have linked the incidence of the disease with increasing latitude, both north and south of the Equator (Compston and Coles 2008) and hence reduced exposure to ultraviolet B radiation (UVB) (Ascherio, Munger et al. 2010; Lucas, Ponsonby et al. 2013). However Ascherio et al. 2010 supported the idea that the latitude gradient is fading out within the USA and that the observed increase in MS incidence in low-risk regions (Middle-East, Southern Europe, Mexico) could be due to increased MS diagnosis (Ascherio, Munger et al. 2010).

Under conditions of low UVB exposure, vitamin D levels in the body are reduced (Ascherio, Munger et al. 2010). Reduced levels of vitamin D in the plasma may be associated with increased susceptibility to autoimmune disorders, as well as to the severity of diseases such as insulin dependent diabetes (IDD), rheumatoid arthritis (RA), and MS (Ponsonby, McMichael et al. 2002; Pozuelo-Moyano, Benito-Leon et al. 2013; Jelinek, Marck et al. 2015). Particularly in MS there is a correlation of lower MS prevalence, activity and mortality with high levels of vitamin D in the body (Ascherio, Munger et al. 2010; Simpson, Taylor et al. 2010; McDowell, Amr et al. 2011; Pozuelo-Moyano, Benito-Leon et al. 2013).

From the Nurses' Health Study there are evidence of a protective effect on MS for women that had high levels of daily vitamin D intake (Munger, Zhang et al. 2004). Simpson et al 2010 in a prospective population-based cohort study showed a reduced hazard of relapse (up to 12%) with increased levels of vitamin D for every 10 nmol/L increase in serum 25-hydroxyvitamin D (Simpson, Taylor et al. 2010). Furthermore seasonal effects on MS and RA incidence and clinical course have also observed (Ponsonby, McMichael et al. 2002).

Reduced serum levels of vitamin D have been observed in MS patients during relapses (Correale, Ysrraelit et al. 2009). Soilu-Hänninen et al.2005 observed lower serum levels of 25 $(OH)_2$ D in MS patients during the months of June to September as compared to controls (Soilu-Hanninen, Airas et al. 2005). Interestingly Holmoy et al (2009) found that 25 $(OH)_2$ D concentrations in the CSF correlate with serum concentrations and that there was no association with relapses or gadolinium-enhanced lesions (Holmøy, Moen et al. 2009). Furthermore higher serum levels of 25(OH)D3 and 1,25$(OH)_2$D3 were observed in healthy controls than RR MS patients (Correale, Ysrraelit et al. 2009).

Humans (see figure 1) can get Vitamin D from exposure to sunlight, their diet and dietary supplements (Holick 2007; Holick and Chen 2008; Ascherio, Munger et al. 2010), where the first source is the major one. Skin exposure to UVB in the 290-315 nm range photolyses 7-dehydrocholesterol located in the skin to form vitamin D_3 which isomerizes to cholecalciferol (Holick and Chen 2008; Ascherio, Munger et al. 2010; Newberry SJ, Chung M et al. 2014 Sep). The efficiency of the conversion of 7-dehydrocholesterol to vitamin D_3 is dependent on the time of day, season of the year, latitude, skin color, and age (Newberry SJ, Chung M et al. 2014 Sep). Both cholecalciferol and ergocalciferol when ingested are enzymatically converted in the liver to 25-hydroxyvitamin D (25 $(OH)_2$ D) (Ascherio 2013; Newberry SJ, Chung M et al. 2014 Sep). This molecule has a low biological activity but is the major form of vitamin D circulating in the blood stream and is thought to reflect the

nutritional status of a person's vitamin D levels (Ascherio 2013; Newberry SJ, Chung M et al. 2014 Sep). In the kidney 25 $(OH)_2$ D undergoes a second hydrolxylation to form 1,25-dihydroxyvitamin D (1,25 $(OH)_2$ D). This conversion also happens in the colon, prostate, mammary glands, macrophages, antigen-presenting cells, osteoblasts and keratinocytes which all possess the enzyme, 25-hydroxyvitamin D3-1-α-hydroxylase responsible for this conversion (Ascherio 2013; Newberry SJ, Chung M et al. 2014 Sep). 1,25 $(OH)_2$ D can bind and activate the vitamin D receptor (VDR) (Norman 2006; Norman 2008) that can regulate as many as 500 genes (Norman 2006; Norman 2008). Furthermore VDR is also present on cell membranes where it mediates some of the rapid responses to 1,25 $(OH)_2$ D (Norman 2006; Norman 2008; Newberry SJ, Chung M et al. 2014 Sep). Vitamin D's function in regulating calcium homeostasis is the biological function best studied thus far, but other effects of vitamin D on brain development and function, cell proliferation and apoptosis, regulation of blood pressure and insulin have been observed. (Holick and Chen 2008; Ascherio, Munger et al. 2010; Ascherio 2013; Newberry SJ, Chung M et al. 2014 Sep).

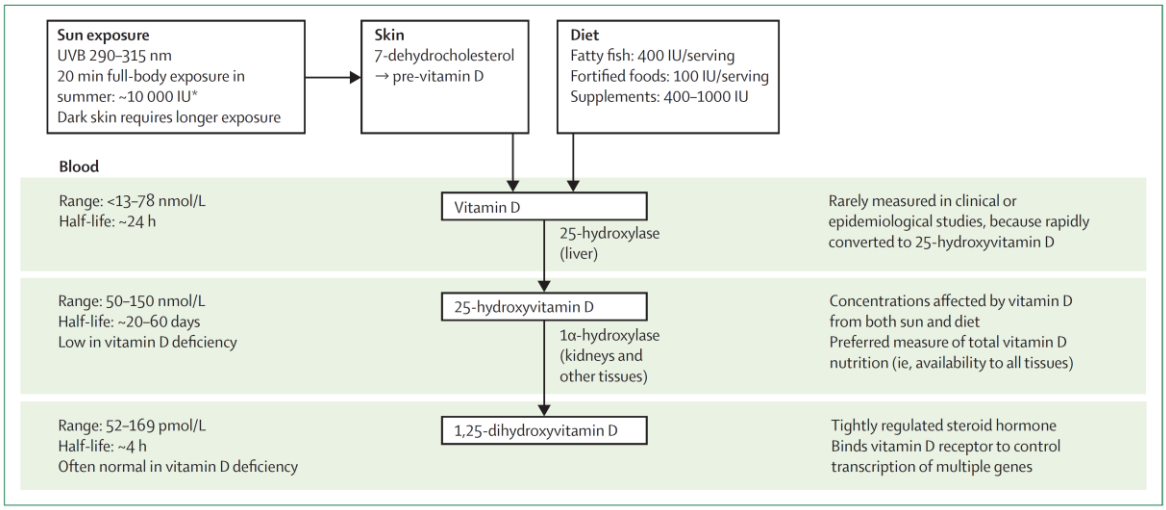

Figure 1. Sources and metabolism of vitamin D (from Ascherio et al. 2010).

The presence of VDR and D3-1-α-hydroxylase has been demonstrated in dendritic cells, macrophages (Overbergh, Decallonne et al. 2000; Veldman, Cantorna et al. 2000), B cells (Chen, Sims et al. 2007), and activated T cells (Veldman, Cantorna et al. 2000; Norman 2006). The differentiation of monocytes into dendritic cells can be inhibited *in vitro* by $1,25(OH)_2$; furthermore IL-12 secretion and surface expression of co-stimulatory molecules can be suppressed by $1,25(OH)_2$ (D'Ambrosio, Cippitelli et al. 1998; Penna and Adorini 2000; Adorini 2002) . $1,25(OH)_2$ can also facilitate a CD4+ T-cell shift to an anti-inflammatory status (Adorini 2002; Meehan and DeLuca 2002; Smolders, Damoiseaux et al. 2008). Inhibition of B cell proliferation, plasma differentiation and immunoglobulin production can also be mediated *in vitro* by $1,25\ (OH)_2$ (Chen, Sims et al. 2007).

The above mentioned immunomodulatory effects are consistent with the onset of EAE, in vitamin D-deficient mice (Cantorna, Hayes et al. 1996). $1,25\ (OH)_2$ D is also effective in the prevention and treatment of EAE (Spach, Nashold et al. 2006) mediated by a reduction in autoreactive T cells, peptide-specific proliferation and Th1 cell development (Lemire and Archer 1991); and probably through the nuclear VDR, the IL-10 receptor and IL-10 which inhibit APC function, inflammatory T cell activation, cytokine synthesis, and chemokine

synthesis (Spach, Nashold et al. 2006). Nashhold et al in 2001 supported the idea that 1,25 $(OH)_2$ D acts through Rag-1-dependent cells by promoting T regulatory cell function rather affecting Th1 or Th2 (Nashold, Hoag et al. 2001).

Studies on MS patients in relation to the levels of 25 $(OH)_2$ D or 1, 25 $(OH)_2$ D in the blood and their correlation with immunological markers (e.g. frequency or suppressive activity of regulatory T cells) have reported (Mahon, Gordon et al. 2003; Royal, Mia et al. 2009; Smolders, Thewissen et al. 2009; Jorde, Sneve et al. 2010). Further studies are needed to determine the protective or treatment effects of Vitamin D.

Furthermore more studies are also needed in order to predict vitamin D status and MS causation. For instance Black people have lower 25$(OH)_2$ D concentrations than white people because of the melanin pigment in skin which absorbs UVB and so they are often deficient in vitamin D. On the other hand the risk of MS in black people is lower than that in white people, probably due to genetic variations (Looker, Pfeiffer et al. 2008). However a more severe course of MS in black patients has been reported indicating adverse effects of vitamin D deficiency (Boster, Endress et al. 2009; Ascherio, Munger et al. 2010).

A study from Tremlett et al. 2008 observed an increase in MS relapses during the times where serum vitamin D concentrations are at the lower end, however this increase could also be explained from increase respiratory infections (Andersen, Lygner et al. 1993; Tremlett, van der Mei et al. 2008).

The reduction observed in the relapse rate during pregnancy is accompanied with an increase in serum vitamin D which falls after delivery. This could explain the increase of relapse after delivery; however other hormones change during pregnancy which can offer an explanation as well (Vukusic and Confavreux 2006).

The amount (dose) of vitamin D required for protection has to be investigated with large longitudinal studies that include MS patients at all stages of the disease (Ascherio, Munger et al. 2010). The Committee on Medical Aspects of Food and Nutritional Policy (COMA) has no reference nutrient intake (RNI) for vitamin D for those individuals that lead a healthy life style since it is produced internally by the body. However for individuals that are homebound, pregnant, and lactating women, an intake of 10 μg/day was recommended by COMA (COMA 1991). As far as the safety of vitamin D supplementation is concerned, in the UK it is set at 25 μg/day (1000 IU/day). In Canada and the USA the upper limit of intake is 50 μg/day (2000 IU/day). Ascherio et al. 2010 suggested that a supplementation of 100-250 μg/day (4000–10 000 IU/day) will maintain $25(OH)_2$ D to 100–150 nmol/L and at the same time will retain a low risk of toxic effects (Ascherio, Munger et al. 2010); therefore it is suggested that further studies on safety should be carried on since that the productive and treatment effects from vitamin D supplementation will require a much higher dose than the recommended one (Ascherio, Munger et al. 2010). Furthermore although the study by Mokry et al 2015 using Mendelian randomization demonstrated that a genetically lowered $25(OH)_2$D level is strongly associated with increased susceptibility to MS still further long-term randomized controlled trials are needs in order to investigate whether vitamin D sufficiency can delay, or prevent, MS onset (Mokry, Ross et al. 2015).

1.2.1.2 Vitamin C

Vitamin C or ascorbate is abundant in the central nervous system (CNS) (van Meeteren, Teunissen et al. 2005) and acts as an intracellular antioxidant against highly reactive radicals (van Meeteren, Teunissen et al. 2005). Ascorbate acts together with Vitamin E and GSH as well as with antioxidant enzymes SOD and GSH-peroxidase (van Meeteren, Teunissen et al. 2005). Ascorbate is continuously produced from dehydroascorbate and GSH in the CNS under physiological conditions (van Meeteren, Teunissen et al. 2005). Besler et al. in 2002

found that in 24 MS patients with the secondary progressive form of the disease have lower serum levels of antioxidant vitamins, alpha tocopherol, beta-carotene, retinol and ascorbic acid and higher serum levels of thiobarbituric acid reactive substances (TBARS) as compared with 24 healthy sex- and age matched person as controls (Besler, Comoglu et al. 2002); with a possible explanation that these antioxidants are depleted because of the continuous oxidative stress observed in MS (Besler, Comoglu et al. 2002; Carvalho, Lim et al. 2014). Zhang et al. in 2001 from 2 large cohorts of women found no evidence that higher intakes of dietary carotenoids, vitamin C, vitamin E and fruits and vegetables have any association with reduced risks of MS; not even supplementation with vitamin C, vitamin E or multivitamins has any association with reduce risks of MS (Zhang, Hernán et al. 2001). Ghadirian et al. 1998 in a case-control study among women with MS and matched controls, observed a significant protective effect of vitamin C and fruit juices (Ghadirian, Jain et al. 1998). Other case control studies showed no association (Antonovsky, Leibowitz et al. 1965; Berr, Puel et al. 1989; Gusev, Boiko et al. 1996). Vitamin C was found not be protective in EAE in a study by Spitsin et al. 2002 (Spitsin, Scott et al. 2002).

1.2.1.3 Vitamin E

Whereas vitamin C is found in the cytoplasm, vitamin E is localised into the cell membrane.

Vitamin E, which consists of tocopherols and tocotrienols, was originally studied for its ability to prevent fetal resorption in rats (tocos in Greek means child bearing) resulting from a vitamin E deficient diet. At the molecular and cellular level, vitamin E (all eight forms) acts as an antioxidant, scavenging reactive oxygen and nitrogen species (ROS) and (RNS) thus preventing damage to membranes, and interacts with and regulates specific enzymes and transcription factors, thus influencing cellular functions (Gohil, Oommen et al. 2007; Reiter, Jiang et al. 2007; Zingg 2007).

The four natural tocopherols and four tocotrienols (alpha (α), beta (β), gamma (γ) delta (δ)) (see figure 2) can affect a number of enzymes involved in signal transduction, with different potencies (Zingg 2007).

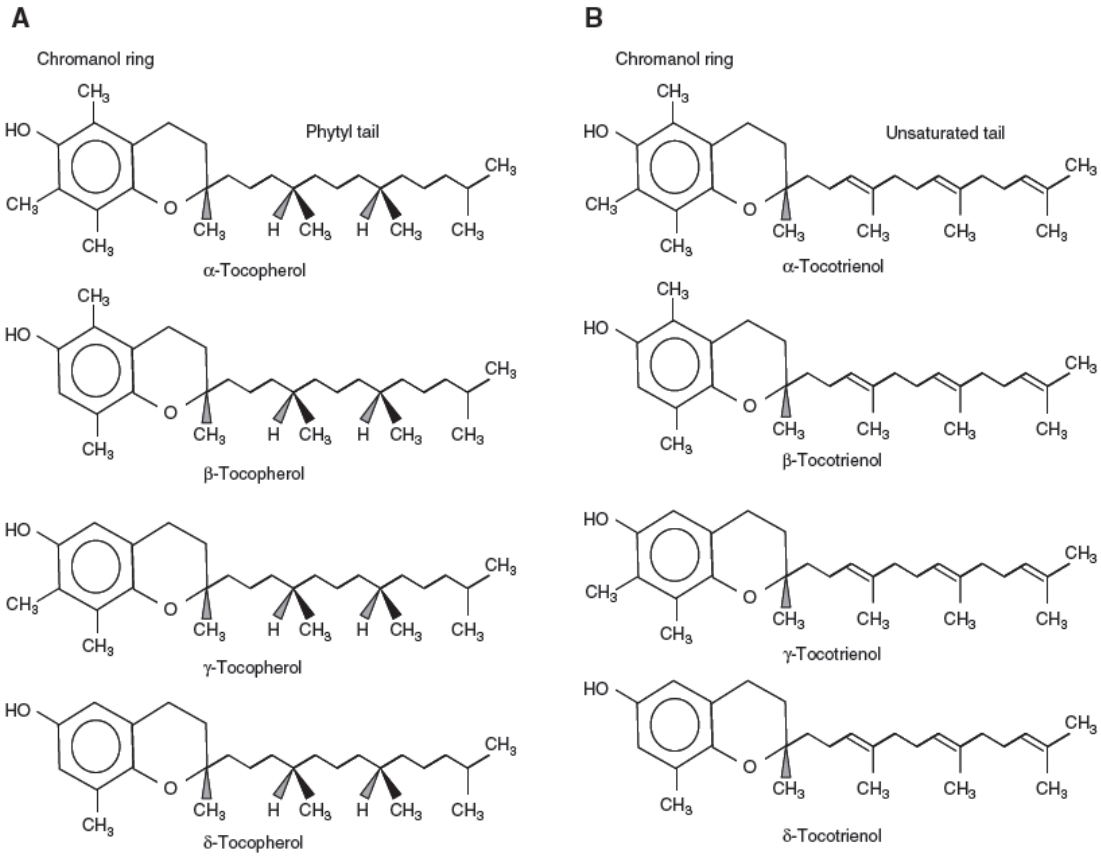

Figure 2. The constituents of vitamin E; (A) Tocopherols α,β,γ,δ and (B) Tocotrienols α,β,γ,δ (From Meydani et al 2005).

α-Tocopherol (T) constitutes 80–90% of vitamin E in human blood plasma, and is the major form found in most tissues. This appears to be due to the preferential reincorporation of α-T into nascent, very low-density lipoproteins via the tocopherol-binding protein and the rapid catabolism of gamma γ –T in the liver (Jiang, Lykkesfeldt et al. 2002).

Vitamin E analogues have been recently discovered; palm oil contains small amounts of α-tocomonoenol, and some marine organisms contain marine-derived tocopherols (Ng, Choo

et al. 2004). The phosphorylated form of α-T, α-tocopheryl phosphate, occurs naturally in foods and in animal and human tissues (Zingg 2007). α-tocopheryl phosphate, is probably acting as a storage molecule, as a transport form or as 'lipid messenger' for the modulation of signal transduction and gene expression (Zingg 2007). Vitamin E is found in photosynthetic organisms including higher plants (Zingg 2007), in seeds and green leafy vegetables. The intake of each vitamin E analogue depends on dietary oil preferences. In olive oil and sunflower the major vitamin analogue is α-T, corn oil contains mainly γ-T while soy bean oils contain high amounts of δ-T. Palm oils contain mainly tocotrienols which are also found in significant amounts in barley, oats and rice bran (Sen, Khanna et al. 2007).

According to the expert group publication of 2003 "Safe upper levels for vitamins and minerals", vitamin E activity is expressed as RRR-α-T equivalents. Activity is given in international units (IU). One IU of vitamin E has been defined as the equivalent of 0.67 mg of RRR-α-T and 0.91 mg of racemic mixtures of α-T (Expert group on vitamins and minerals 2003). In 1991 COMA concluded that a daily intake of 4 mg and 3 mg of α-T equivalents is adequate for men and women respectively. γ-T is the predominant form of vitamin E in food sources (in vegetable oils, grains, nuts and seeds (Zhao 2014). In the USA, it was estimated that 70% of the vitamin E intake from food sources is in the form of γ-T (Jiang 2001). The EU Recommended Daily Allowances (RDA) 2008 and Institute of Medicine (IOM) Estimated Average Requirement (EAR) 2006 has been set to 12 mg/day (Jennifer J. Otten, Jennifer Pitzi Hellwig et al. 2006; Directive 2008) . Zhao et al. reported that the 12 mg/day is achieved with a combination of food and supplements however this is not the case for the US where 58% of adults have intakes below IOM EAR even when intake from supplements was taken into account (Zhao, Monahan et al. 2014).

The average plasma concentration of α-T in an un-supplemented adult human is about 23.2 μM and for γ-T and δ-tocopherol it is 10 and 100 times lower respectively (Zingg 2007).

The relative concentrations of different tocopherols in other tissues vary, suggesting tissue specific mechanisms of transport, enrichment and/or storage of tissue tocopherols (Rigotti 2007). In a study by K. Ghebremeskel et al. 1988 the vitamin E (α-T) levels in MS patients' plasma were lower than controls and that supplementation was effective in raising α-T in MS (Ghebremeskel, Williams et al. 1988); the same results were obtained by Harbige et al. 2011 (Harbige, Pinto et al. 2011). Immune cells are particularly enriched with vitamin E because of their high PUFA content which is required for the inhibition of their peroxidation. This puts these particular cells in a high risk of oxidative damage since free radical damage to immune cells can impair their ability to respond to pathogens (Meydani, Han et al. 2005).

Several observational and clinical studies have demonstrated the health benefits of vitamin E (tocopherols and tocotrienols) supplementation (Jiang 2014). such as in the case of cardiovascular disease (Gey, Puska et al. 1991; Brigelius-Flohé, Kelly et al. 2002; Munteanu and Zingg 2007; Jiang 2014), Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, ataxia, Huntington's disease, MS (Butterfield, Castegna et al. 2002; Fariss and Zhang 2003; Berman and Brodaty 2004; Mariotti, Gellera et al. 2004; Pantzaris, Loukaides et al. 2013) and in certain types of cancers (Coulter, Hardy et al. 2006).

Vitamin E can exhibit both antioxidant and non-antioxidant functions (Glauert 2007). Vitamin E deficiency impairs both humoral and cell-mediated immune functions (Moriguchi and Muraga 2000). Supplementation with vitamin E in doses above the recommended daily values results in enhancement of the immune response and therefore an increased resistance to pathogens (Moriguchi and Muraga 2000; Meydani, Han et al. 2005; Pekmezci 2011). Many studies as reported by Meydani et al. 2005 in a review paper have shown that doses of vitamin E above the recommended levels improve T cell-mediated function in the elderly (Meydani, Han et al. 2005).

Furthermore it was shown that γ-T and its metabolite 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC) can inhibit both COX-2, with the reduction of the pro-inflammatory PGE_2 (Jiang, Elson-Schwab et al. 2000) and 5-lipoxygenase, with the reduction of LTB_4, a potent chemotactic agent; in addition to the reduction of eicosanoids, γ-T was found in a study by Jiang et al. 2003 that can also decrease the release of TNFα and that γ-T is more efficient than α-T in trapping reactive nitrogen oxide, and hence protecting peroxynitrate- induced lipid peroxidation (JIANG and AMES 2003). The proinflammatory cytokines TNFα, IL1β, IL6 are produced following the activation of NFκB in MS (Gharagozloo, Mahvelati et al. 2015) (see figure 3).

Several animal and in vitro studies as reported in a review by Glauert et al. 2007 have shown that vitamin E analogues can alter the activation of NFκB by both its antioxidant and non-antioxidant function (blocking the formation of lipoxygenase metabolites inhibits oxidation of LDL, and this has been shown to activate NF-κB, inhibiting of protein kinase C (PKC))(Glauert 2007). *In-vitro* studies by Wiser et al., 2008 showed that NFκB and JNK production is inhibited by both γ-T and α-T (Wiser, Alexis et al. 2008; Jiang 2014) (see figure 3).

In a review by McGuire et al., 2013 the implication of the NF-κB signalling cascade in the regulation of immune and inflammatory responses and in the pathogenesis of MS and EAE were reported. NF-κB is important for both peripheral immune cell activation and in resident cells of the CNS and for the pathogenesis and the development of the disease (Mc Guire, Prinz et al. 2013). Furthermore NF-κB inhibition in immune cells of peripheral and CNS cells can be protective against the development of EAE and can have therapeutic effect in MS (Yan and Greer 2008). Drug therapies are targeting NFκB but NFκB is also involved in normal cellular physiology and systemic blockage of NFκB leads to undesirable side effects (Mc Guire, Prinz et al. 2013). Vitamin E from NFκB knock-out models does not seem

to result toa systemic decrease of NFκB activation; however other knock-out studies are necessary to examine the effects of vitamin E on a systemic blockage of NFκB, if any (Glauert 2007) (see figure 3).

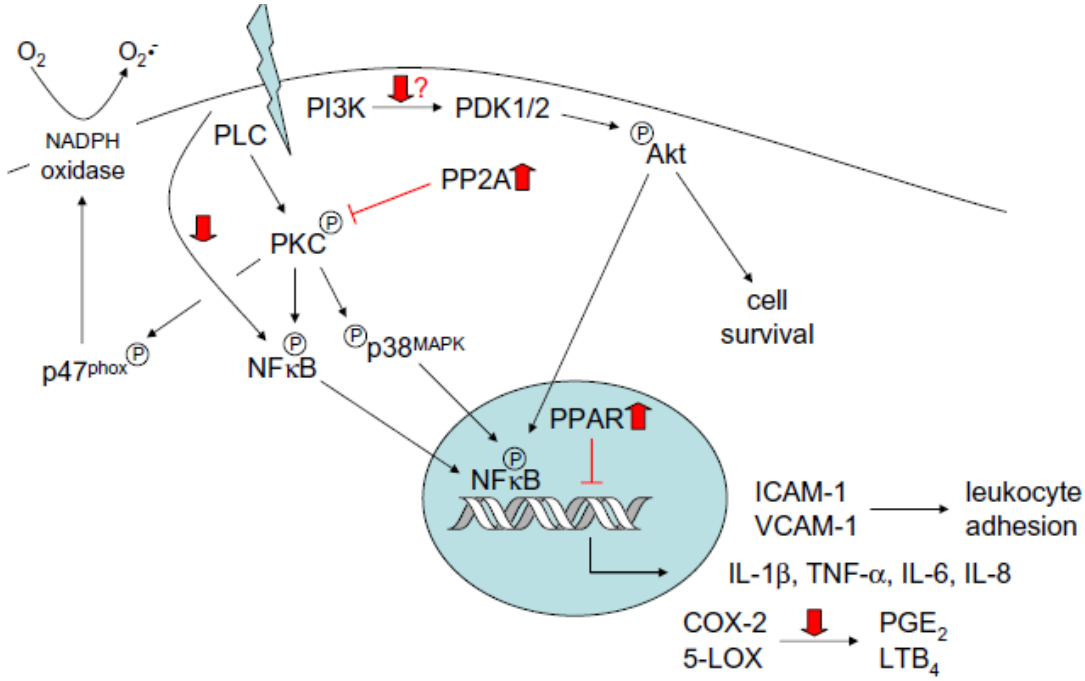

Figure 3. Schematic diagram showing the possible pro-inflammatory cytokines and other pro-inflammatory stimuli mediated by α-T and γ-T. (Figure from Reiter et al., 2007)

Mast cells are located perivascularly secreting proinflammatory and vasoactive molecules disrupting the BBB, which may precede clinical or pathological signs of MS (Theoharides, Kempuraj et al. 2008). Following acute stress brain mast cells in MS, are activated by molecules within the neural tissue such as substance P, MBP, and corticotropin-releasing hormone with the subsequent release of inflammatory madiators (Theoharides, Kempuraj et al. 2008). All tocopherol analogues can affect the proliferation and survival of HMC-1 mastocytoma cells through there action on signal transduction and even cause induction of apoptosis (Zingg 2007). These effects are mediated through inhibition of the c-kit/I3K/PKB pathway (Zingg 2007).

Taken the above into account, γ-T could be used for its involvement in suppressing NFκB signalling pathways in various types of cells in MS as it can affect the proliferation and survival of mast cells.

Moreover oligodentroglial excitotoxicity may be involved in the pathogenesis of demyelinating diseases (Matute, Alberdi et al. 2001) and γ-T can protect neuronal cells from glutamate excitotoxicity by scavenging ROS and suppressing lipid peroxidation (Saito, Nishio et al. 2010). γ-T can reduce the activity of MMP 9 and 2 (Sanches, Santos et al. 2013) hence protecting the integrity of BBB (Rosenberg, Estrada et al. 1998) . Any disruption of BBB will result in a migration of activated leukocytes seen in MS(Minagar and Alexander 2003).

Campbell et al. 2003 discovered that α & γ-T can upregulate the expression of PPARγ in colon cancer cell lines, with γ-T being a better modulator of PPARγ than α-T at the concentrations tested (Campbell, Stone et al. 2003). Moreover Drew et al. 2008 in a review article supported that PPARγ agonists can be used to treat MS (Drew, Xu et al. 2008) (see figure 4).

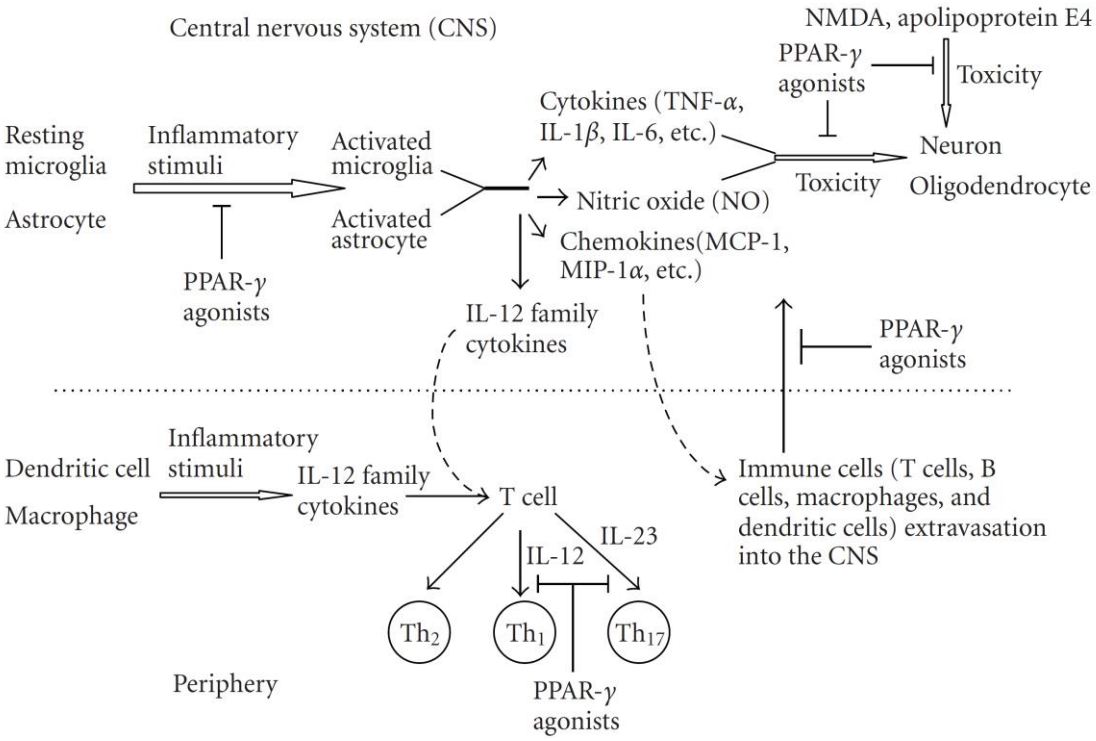

Figure 4. Effects of PPAR-γ on immune cell function (from Drew et al 2008).

Therefore, it can be concluded from the literature review above, that vitamin E is an important antioxidant that can interrupt the propagation of free radical chain reactions. In the α-T form, vitamin E can efficiently detoxify hydroxyl, perhydroxyl and superoxide free radicals and in the γ-T form it appears to be more efficient in trapping NO radicals. In addition, γ-T exerts non-antioxidant properties, including the modulation of cell signalling and immune functions, regulation of transcription and induction of apoptosis; processes that have been implicated in MS (Pantzaris, Loukaides et al. 2013).

1.2.2 Lipids

Lipid metabolic disturbances play an important role in MS (Corthals 2011). Lipids in mammalian cells have variety of biological functions and are important components of all cells. Lipids are used for both maintenance and energy needs of different tissues. E.g. Glial cells, particularly oligodentrocytes use lipids for myelin synthesis. Lipids such as linoleic

acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA), and prostaglandins can also act as endogenous ligands to the PPARs (Kota, Huang et al. 2005). The lipid bilayer forms the outer cell, mitochondrial, nuclear and lysosomal membranes, as well as membranes of the endoplasmic reticulum and vesicles. Lipids are of even greater importance in neurons due to the anatomical shape of axons and dendrites. The lipid bilayer provides the environment where many proteins are incorporated in or associated with, hence contributing in the protein's quaternary structure and subsequently their function both as individual proteins and as components of a larger cellular response system.

Three types of lipids can be found in biological membranes: Phospholipids, glycolipids and cholesterol, each having different properties and roles in the membrane.

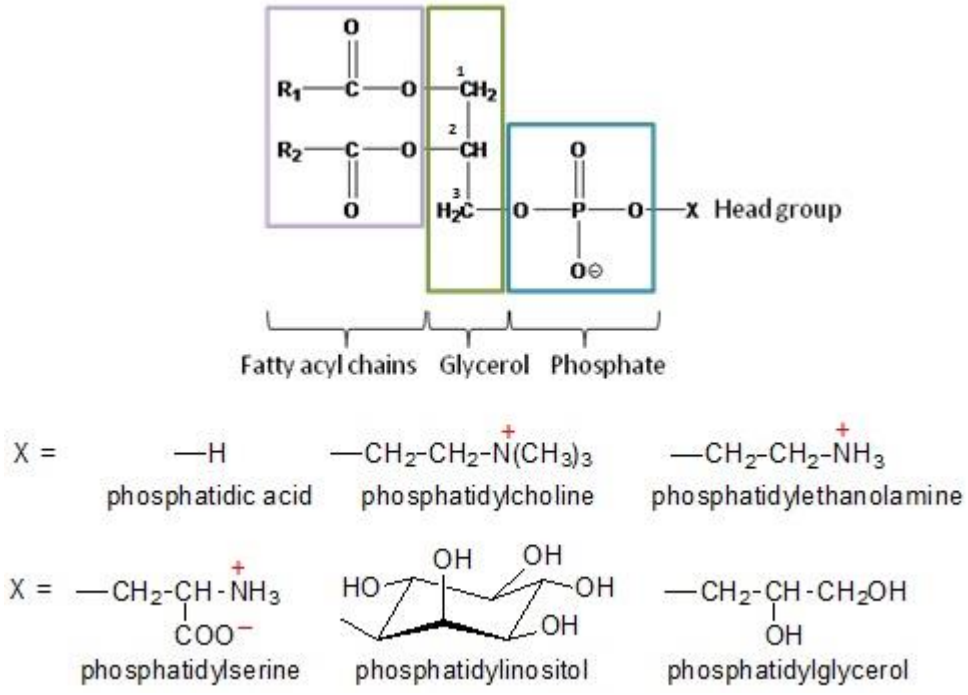

Figure 5. Phosphoplipids consist of glycerol, a 3-carbon alcohol, where the hydroxyl groups in positions C1, C2 of the glycerol are esterified to the carboxyl groups of the two fatty acids chains. The hydroxyl group at the C3 position of the glycerol molecule is esterified to phosphoric acid. Any further addition occurs by the formation of an ester bond between the phosphate group of the phosphatidate and the hydroxyl group of a hydrophilic compound such as choline or other molecules such as, the amino acid serine, ethanolamine, glycerol and the sugar derivative inositol.

Phospholipids (see figure 5) are abundant in all biological membranes and they consist of glycerol, a 3-carbon alcohol, where the hydroxyl groups in positions C1, C2 of the glycerol are esterified to the carboxyl groups of the two fatty acids chains. The fatty acid chains usually contain 14 to 24 carbon atoms. The one chain from the C2 position is typically unsaturated with 1 to 4 *cis* double bonds and saturated fatty acids or monounsaturated fatty acids are usually attached to the C1 position. The hydroxyl group at the C3 position of the glycerol molecule is esterified to phosphoric acid. Any further addition occurs by the formation of an ester bond between the phosphate group of the phosphatidate and the hydroxyl group of a hydrophilic compound such as choline or other molecules such as the amino acid serine, ethanolamine, glycerol and, the sugar derivative, inositol.

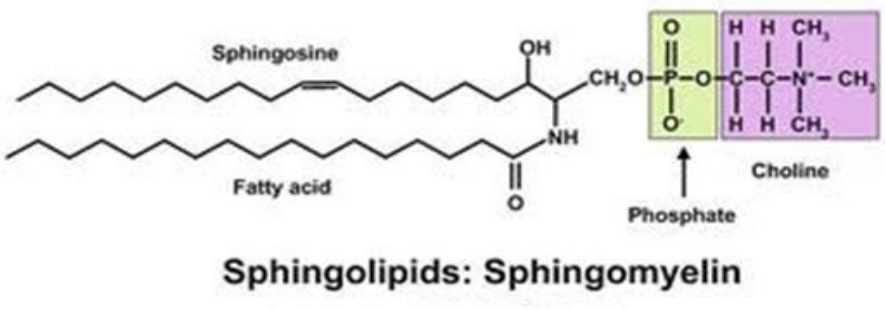

Figure 6. Sphingomyelin, a phospholipid with sphingosine instead of glycerol as backbone. Sphingosine is an amino alcohol with a long, unsaturated hydrocarbon chain. A fatty acid is linked by an amide bond to the amino group of the sphingosine and the primary hydroxyl group of sphingosine is esterified to phosphoryl choline (From Steve Blake 2009).

One other phospholipid found in biological membranes is sphingomyelin (SM) (see figure 6), a phospholipid with sphingosine instead of glycerol. Sphingosine is an amino alcohol with a long, unsaturated hydrocarbon chain. A fatty acid is linked by an amide bond to the amino group of the sphingosine and the primary hydroxyl group of sphingosine is esterified to phosphoryl choline.

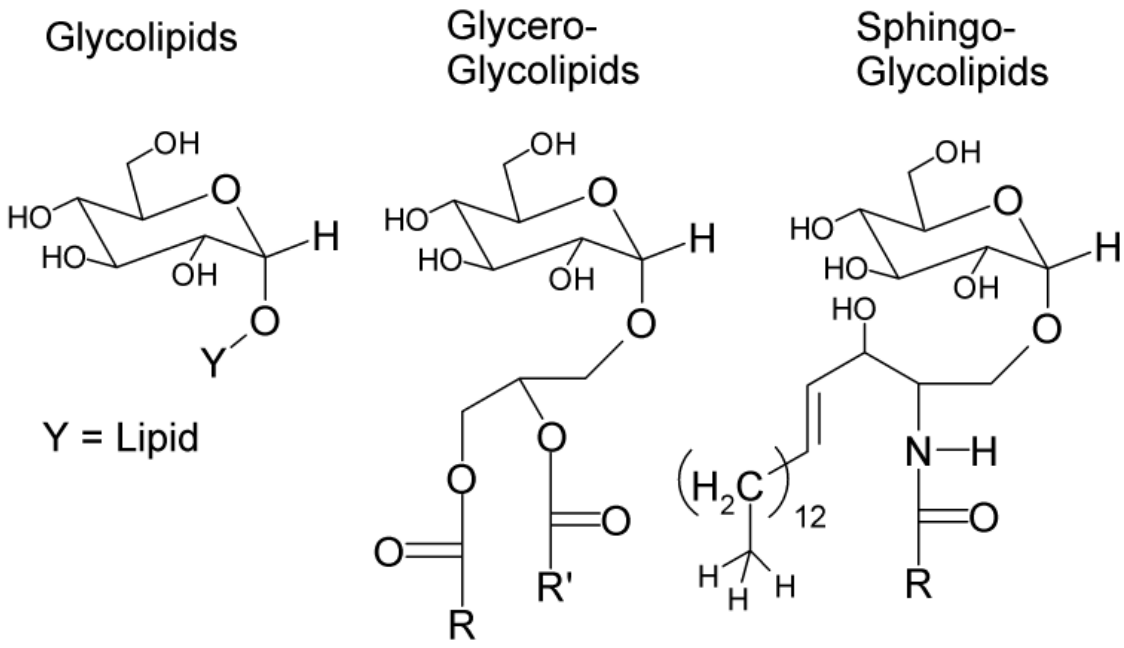

Figure 7. Glycolipids are sugar containing lipids.

Glycolipids (see figure 7) are sugar containing lipids with the sugar residue facing the extracellular side of the membrane. Structurally is a very heterogeneous group of compounds with monosaccharides attached to a fatty moiety. According to the primary structure of the interface region that connects the sugar with the hydrophobic moiety, glycolipids fall in to two different types; the glycolipids with glycerol backbone eg. glycoglycerolipids, usually as diacyl sugars and shingosine based eg. cerebrosides and gangliosides.

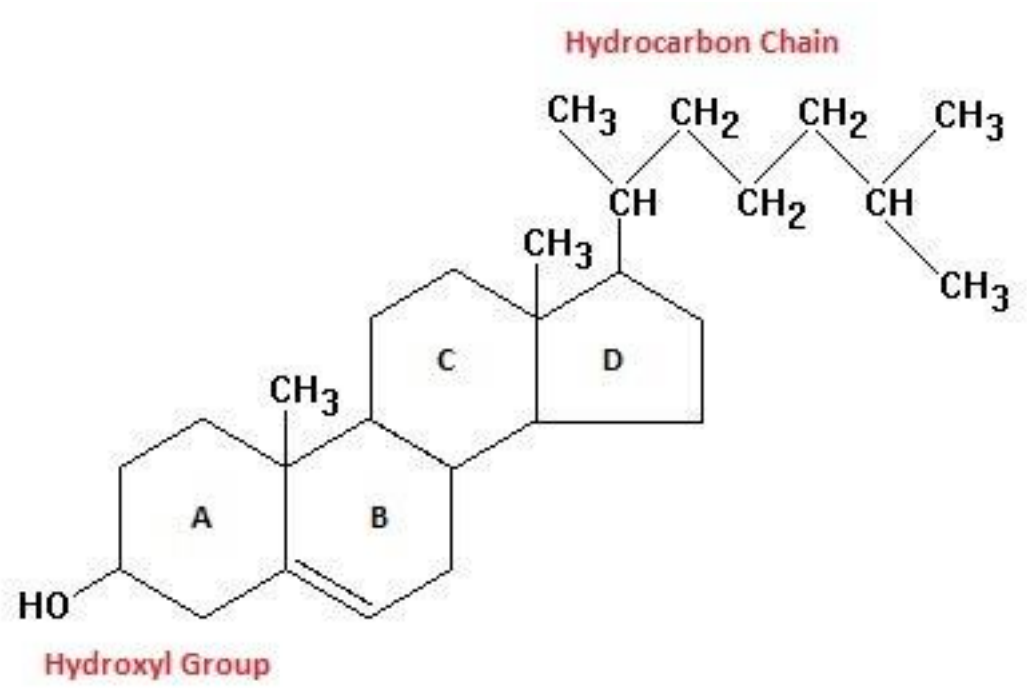

Figure 8. The structure of cholesterol with four hydrocarbon rings with a hydroxyl group attached to one end, and a hydrocarbon chain to the other end.

Cholesterol (see figure 8) in the membrane is oriented parallel to the fatty acids chains of phospholipids. Cholesterol is a steroid with four linked hydrocarbon rings, with a hydroxyl group attached to one end, which interacts with the phospholipid head groups and a hydrocarbon chain to the other end.

Phosphatidylcholine (PC) and SM, the choline containing phospholipids, are both positively charged and are mainly located on the outer leaflet of plasma membranes, while the negatively charged phosphatidylethanolamine (PE) and phosphatidylserine (PS) are located on the inner leaflet together with phosphatidylinositol (PI). PC is the most abundant phospholipid in animal cell membranes, followed by PE (Williams 1998). PE is however the most abundant phospholipid in the brain, accounting for 34% of the total brain phospholipids (Manzoli, Stefoni et al. 1970).

The properties of phospholipids depend on the type of head group and the nature of the fatty acid attached to the C1 and C2 positions of the glycerol molecule. Thus fatty acids in cell membranes can be saturated mono-unsaturated or polyunsaturated.

1.2.2.1 Saturated fatty acids

Saturated fatty acids (SFAs) exist as rigid chains of single carbon-carbon bonds with chain-length ranging from 16 up to 24 carbons, with a methyl group at one end of the molecule and a carboxyl group at the other end. From epidemiological and clinical studies, SFAs are associated with health risks, i.e. excess weight, insulin resistance, increased LDL cholesterol and are atherogenic (Zamaria 2004; Michas, Micha et al. 2014; Sieri, Chiodini et al. 2014). Higher proportions of SFAs such as, palmitic and stearic acids and monounsaturated fatty acids (MUFA), such as oleic and gadolitic acids are present in larger proportions in myelin sheaths as compared to white and grey matter of the brain (O'Brien and Sampson 1965).

Modification of membrane proteins in cellular and viral membranes by fatty acid acylation or farnesylation might play an important role in protein targeting and/or signal transduction. Myristoylation and palmitoylation are the two most common types of fatty acylation with metabolites such as myristic acid and palmitic acid respectively. Palmitoylation is more common in many membrane proteins (Okubo, Hamasaki et al. 1991; Bijlmakers 2009). Antiviral and antibacterial properties by myristic acid derivatives is described by various researches that show that myristoylation leads to anti-HIV activity and modification of G-protein-mediated signal transduction (Narasimhan, Mourya et al. 2006).

Hon et al in 2009 correlated MS outcome and SFA in the peripheral blood mononuclear cell membrane. They showed a positive correlation of behenic acid (C22:0) and lignoceric acid (C24:0) with the Functional System Scores (FSS) and inverse correlations myristic acid (C14:0), palmitic acid (C16:0) and arachidic acid (C20:0) with FSS and EDSS. Higher levels

of the C14:0 and C16:0 are also associated with a better disease outcome. One other finding in Hon's et al study was the increase of shorter long-chain SFA in MS patients (Hon, Hassan et al. 2009a; Hon, Hassan et al. 2009b).

1.2.2.2 Unsaturated fatty acids

Unsaturated fatty acids (see figure 9) are fatty acids with at least one double bond within the alkyl chain and can be monounsaturated (containing 1 double bond) or polyunsaturated (containing >2 double bonds), with the double bonds being separated by at least one methylene group. The configuration of double bonds (see figure 10) could be either *cis* configuration (with two hydrogen atoms on the same site of the molecule) or the *trans* configuration (the hydrogen atoms are on opposite sides).

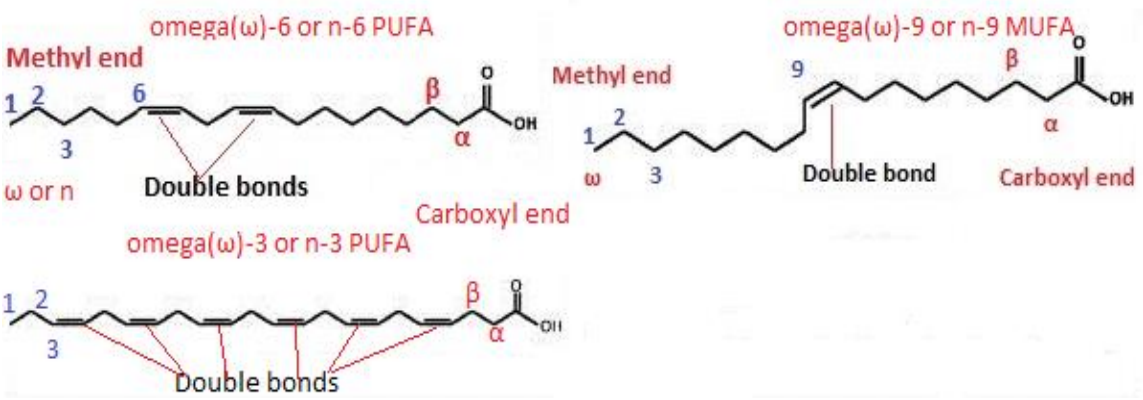

Figure 9. Polyunsaturated (left top and bottom) and Monounsaturated (right) fatty acids. The omega (ω or n) carbon is the carbon of the methyl group at the end of the chain on the left-hand site of the molecule. The alpha (α) carbon is the carbon next to the carboxyl group on the right-hand site of the molecule and the beta (β) carbon is the carbon next to the alpha. The counting of the carbon atoms starts from the omega (ω) carbon on the left of the molecule. Delta (Δ) desaturases introduce cis double bonds at positions counted from the carboxyl end of the FA, & omega (ω) desaturases on positions counted from the methyl end of the FA chain which are absent in vertebrates.

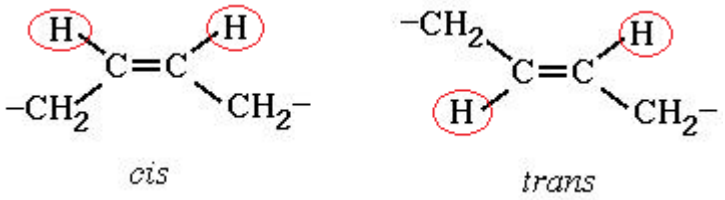

Figure 10. The configuration of double bonds could be either cis configuration (with two hydrogen atoms on the same site of the molecule) or the trans configuration (the hydrogen atoms are on opposite sides).

This configuration reflects the shape and the physical properties of the molecule. For example *cis* fatty acids have a kink in the chain and trans have a straight configuration similar to saturated fatty acids (Roche 1999). The chain length and the degree of saturation will also contribute to the properties of the fatty acids and the lipids that they originate from. The double bonds in the fatty acid molecules are non-linear and flexible making the chain more mobile. The phospholipid molecule becomes more fluid, flexible and disordered with an increasing number of double bonds (Stillwell and Wassall 2003).

MUFAs can be synthesised from saturated fatty acids (Nakamura and Nara 2004) whereas PUFAs can only be synthesised from PUFAs provided from food and are therefore termed essential fatty acids (EFA). Linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) are the classical dietary essential fatty acids. MUFAs, and specially oleic acid, may have beneficial effects on cholesterol metabolism and protective roles on cardiovascular diseases (Zamaria 2004). The Mediterranean diet, which includes olive oil and olives that are rich in oleic acid and a series of phenols, have both an inhibitory effect on platelet function and on synthesis of thromboxane (Karantonis, Antonopoulou et al. 2006).

EFA (for more on EFA see paragraph below) deficiency was first described by Burr in 1929 (Le, Meisel et al. 2009). EFA deficiency can occur when less than 1-2% of total calories are provided from EFA (Ichi, Kono et al. 2014). In EFA deficient animals, 5,8,11-eicosatrienoic acid (Mead acid, C20:3ω-9) is endogenously synthesized from oleic acid and is detected in

plasma and tissues and is thought to be used in biological membranes as a substitute for other PUFAs (see figure 11). Furthermore products of 5-lypoxygenase from mead acid can act as proinflammatory mediator as reported by Patel et al 2008. In order for the mead acid to be synthesized two desaturases and one elongase are needed (Ichi, Kono et al. 2014). EFA deficiency in animals can lead to several symptoms including delayed development, decreased fertility as well as dry scaly skin, dry hair, dandruff, polydipsia, polyuria, follicular keratoses (Kirby, Woodward et al. 2010). Although severe EFA deficiency in humans is rare and it was reported by Bjerve et al 1987 with symptoms such as scaly and dry, leathery skin which were improved after supplementation with EFA (Bjerve, Mostad et al. 1987). These symptoms are only observed when diet is deficient in AA or LA. In ALA, EPA, DHA deficiency no similar symptoms have been produced hence more difficult to diagnose (Le, Meisel et al. 2009); supplementation with EFA alleviate symptoms (Kirby, Woodward et al. 2010).

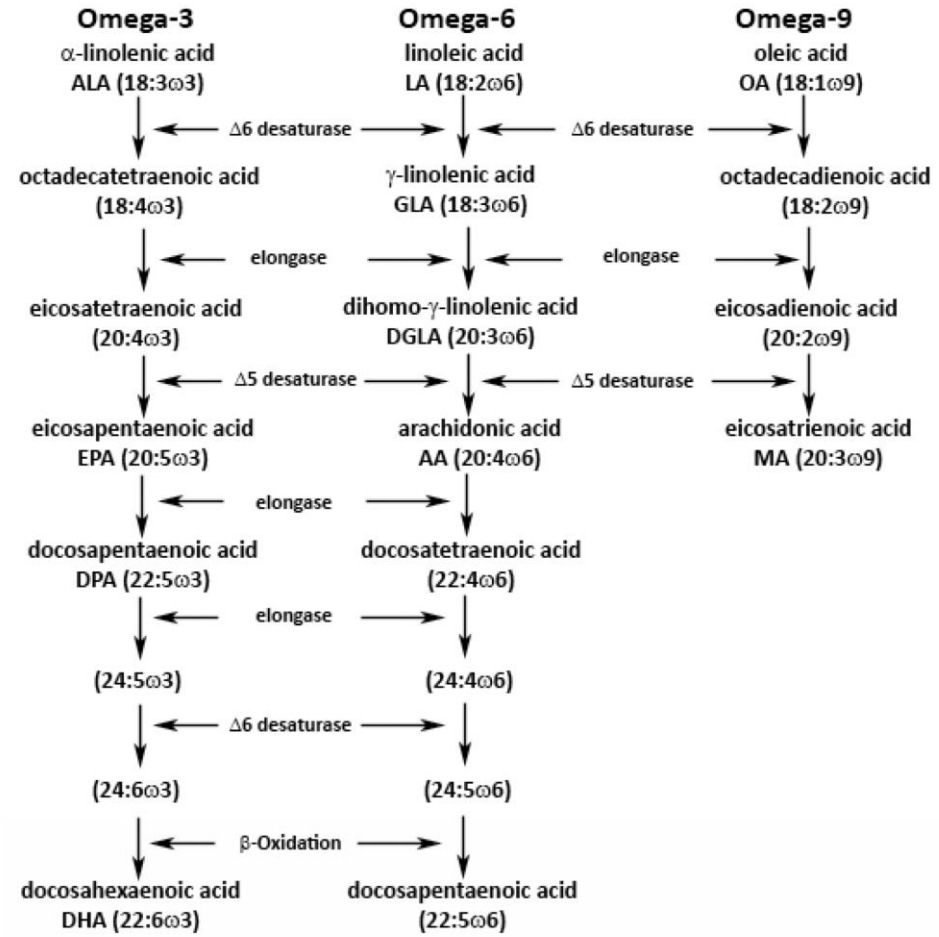

Figure 11. Pathway of metabolism and synthesis of ω-3, ω-6, and ω-9 PUFAs (from Lee et. al. 2012).

The EFAs are divided into two families, the omega (ω)-6 or n-6 series and omega (ω)-3 or n-3 series (see figure 12) depending on the position of the first double bond, counting from the methyl end of the fatty acid (Venegas-Caleron, Sayanova et al. 2010). Linoleic acid (LA), a C:18-2ω-6 and alpha linolenic acid (ALA), a C:18-3ω-3 (Zamaria 2004) are tEFAs and converted to the to their metabolites of the omega-6 family and omega-3 family respectively. The conversion occurs through a series of desaturation (addition of double bonds) and elongation (addition of two carbon atoms) reactions (Venegas-Caleron, Sayanova et al. 2010). Desaturases introduce a double bond between carbons of the FA chain, delta (Δ) desaturases introduce *cis* double bonds (Park, Kothapalli et al. 2009) at

positions counted from the carboxyl end of the FA, and ω desaturases on positions counted from the methyl end of the FA chain, the latter are absent in vertebrates (Williams and Burdge 2006). Stearoyl CoA desaturases (SCDs) catalyse synthesis of MUFAs, Δ-6 desaturase and Δ-5 desaturase which are required for the synthesis of PUFAs, are three types of desaturases known in humans and they are located in endoplasmic reticula and peroxisomes (Sprecher 2002). Humans are not able to produce LA and ALA from oleic acid, as the genetic absence of specific desaturases (Δ12 & Δ15) have resulted in the inability to synthesize long chain PUFAs (Venegas-Caleron, Sayanova et al. 2010). Although long chain PUFAs (LCPUFA) can be synthesized by LA and ALA only a small percentage of LA and ALA is converted to LCPUFAs. The conversion efficiency of ALA to eicosapentaenoic acid (EPA) is 5-10%, whereas less than 1% of ALA is converted to docosahexaenoic acid (DHA) (Venegas-Caleron, Sayanova et al. 2010). Most dietary ALA and LA undergo β-oxidation (Venegas-Caleron, Sayanova et al. 2010).

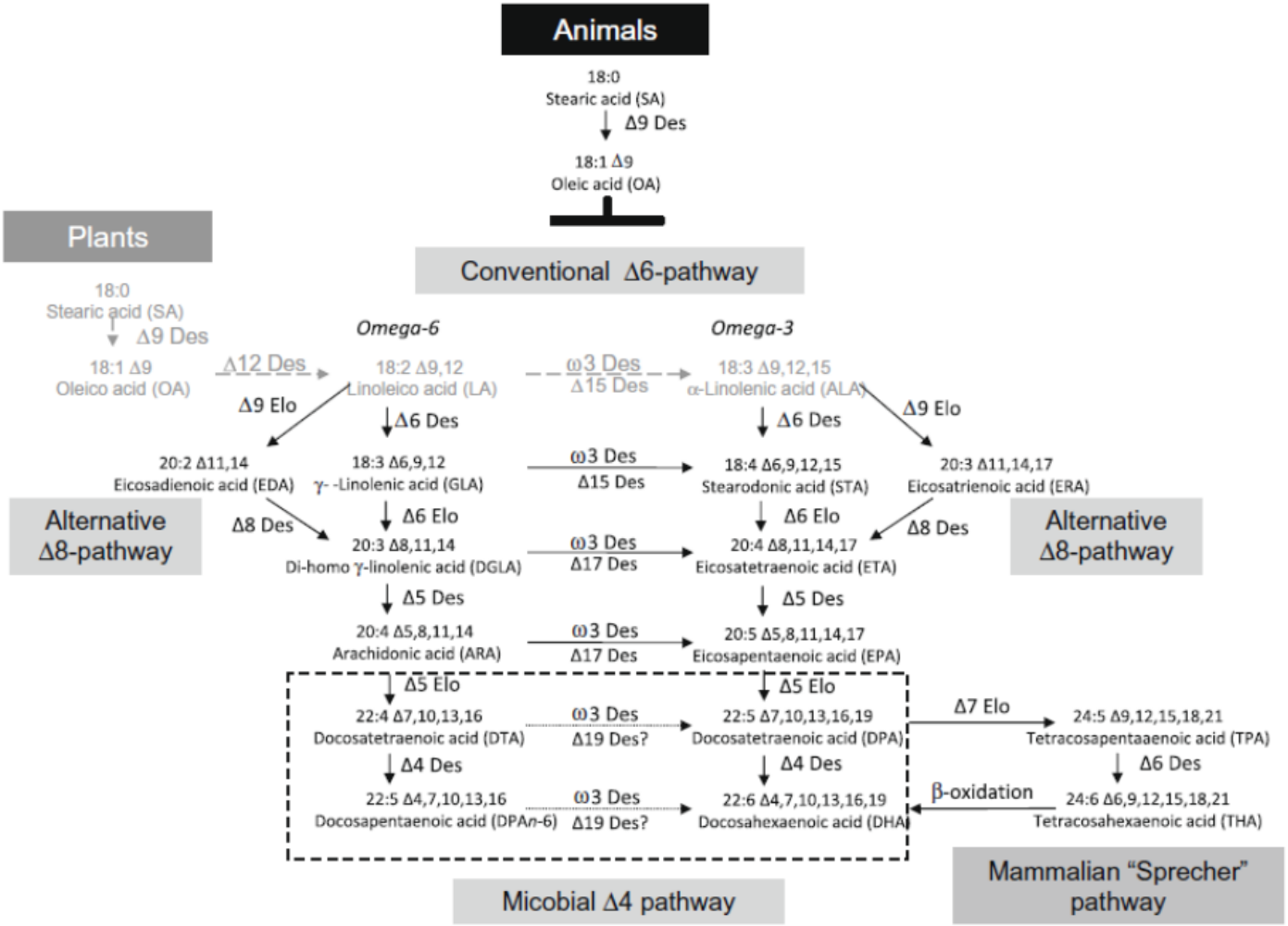

Figure 12. A schematic diagram illustrating the aerobic LCPUFA biosynthetic pathways. The various routes for synthesis of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are shown, as mediated by the consecutive action of desaturases and elongases. The predominant Δ6-pathway is shown, as is the alternative Δ8-pathway. Two routes for DHA synthesis are shown, microbial Δ4-pathway and mammalian ‘‘Sprecher” pathway. Des = desaturase, Elo = elongase (From Venegas-Caleron, M. et al.2010).

The conversion of ALA to LCPUFA (figure 12) involves firstly the introduction of a double bond by the Δ-6 desaturase, thus converting ALA to stearodonic acid (STA), an 18:4n-3 FA, and this step is the rate limiting one of the pathway (Williams and Burdge 2006; Venegas-Caleron, Sayanova et al. 2010). The Δ-6 desaturase is also part of the omega-6 pathway for the conversion of LA to LCPUFA. The Δ-6 desaturase has a higher affinity for ALA than for LA, however LA concentrations in cells are higher than those of ALA and this results in to a greater conversion of LA to LCPUFA. One other limiting factor is the higher dietary intake of omega-6 PUFAs which divert towards the formation of LCPUFA from LA (Simopoulos 2002; Williams and Burdge 2006). The next step is an elongation step which involves the addition of two carbons by elongases, forming eicositetraenoic acid (ETA) and then this is followed by the synthesis of EPA via desaturation by Δ-5 desaturase. DHA is then synthesised by the addition of two carbons. The synthesis of DHA from EPA (figure 12) is as follows: First EPA undergoes a 2 carbon elongation forming docosatetraenoic acid (DTA) followed by a second elongation process to form tetracosapentaenoic acid (TPA) 24:5n-3, followed by desaturation to form tetracosahexaenoic acid (THA) a 24:6n-3 FA by the action of Δ-6 desaturase. The latter is translocated from the ER to the peroxisome where the production of DHA occurs by β-oxidation, with a loss of two carbons per round of β-oxidation. (Williams and Burdge 2006; Venegas-Caleron, Sayanova et al. 2010).

LA conversion to gamma linolenic acid (GLA) is also catalysed by the Δ-6 desaturase. GLA is then elongated to di-homo-gamma linolenic acid (DGLA) and a Δ-5 desaturase converts it to arachidonic acid (AA), a C20:4ω-6 FA. Then follows the addition of 4 carbons in a two-step elongation process with the production of tetracosatetraenoic acid a C24:4ω-6. This product then undergoes desaturation acting as a substrate for the Δ-6 desaturase resulting in the formation of TPA, a C24:5ω-6 which undergoes β-oxidation forming a docosapentaenoic acid a C22:5ω-6 (Sprecher 2002). As with the case of ω-3 LC-PUFAs, the

24-carbon desaturation occurs in the ER, and then moves to peroxisomes where β-oxidation takes place. The resulting DPAis then moved back to the ER (Sprecher 2002).

The existence of an alternative pathway using a Δ8 desaturase was reported in unicellular organisms as well as in the humans testes, (Park, Kothapalli et al. 2009), although its presence in humans has not been verified by molecular cloning. In this pathway EPA and AA are formed from ALA and LA respectively through intermediates of elongation by the action of the Δ9 elongase. These intermediates respectively are: Eicosatrienoic acid (ERA) C20:3ω-3 and eicosadienoic acid (EDA) C20:2ω-3. The action of a Δ8 desaturase completes the formation of EPA and DGLA. Intermediates of the alternative pathway were reported to be found in human plasma, red blood cells and in other tissues (Schenck, Rakoff et al. 1996; Park, Kothapalli et al. 2009).

LA and AA are the fatty acids that accumulate in relatively large quantities in the liver and most other tissues of animals while the GLA and DGLA intermediates are present in much lower quantities in tissues. The most abundant n-3 FAs in animal tissues such as the cerebral cortex, retina, testes and muscle are the EPA and DHA with 40-60% of the FA content in retinal rod outer segments being DHA (Cook 1996). The fatty acid composition of nervous tissue is characterised by the high content of AA, DHA and adrenic acid, a 22:4n-6 and in neuronal synapses the highest concentration is that of AA and DHA (Wilson and Bell 1993; Martínez and Mougan 1998). AA and DHA are incorporated during the development of brain tissue and changes in concentration of these specific PUFAs are minimal after the age of 2 years (Wilson and Bell 1993). Brain AA and DHA cannot be synthesised *de novo* and the plasma ALA and LA do not contribute towards the formation of brain DHA and AA. In the adult human brain the consumption of AA and DHA is at rates of 17.8 and 4.6 mg/day respectively (Rapoport, Rao et al. 2007). Normal brain DHA levels can be maintained in the case of a lack in dietary uptake, by the conversion of liver ALA, as long as there is a sufficient

dietary supply of this EFA. Rapport et al. in 2007 observed a reduced expression of DHA-metabolising enzymes, and a slow DHA loss from the brain in case of dietary DHA deprivation. This also causes upregulation of n-6 PUFA metabolism in the brain (Rapoport, Rao et al. 2007).

AA in plasma can be formed by the action of the Δ6 elongase and the Δ5 desaturase on LA to form GLA and DGLA. Because of the absence of the Δ5 desaturase in human neutrophils, AA from GLA does not form. However the increased production of AA in the plasma after supplementation with GLA is sometimes undesired because AA enhances the formation of platelet-aggregating endoperoxides and thromboxanes, which are undesired effects (Barham, Edens et al. 2000). Barham et al. 2000 tested and proved that the addition of EPA supplements attenuate the conversion of DGLA to AA in the plasma by blocking the Δ5 desaturase (Barham, Edens et al. 2000).

Sources of unsaturated fatty acids

Dietary, ω-3 PUFAs are derived from both animal and plant sources; more precisely EPA and DHA are mainly derived from fish, which along with other seafood are much better sources for long chain PUFAs (Calder 2012). Fish can store lipids in their liver and are, in this case, described as lean, for e.g. cods sardines, mackerel, herring, salmon, and tuna can store lipids in their flesh and these are described as fatty or oily, (Calder 2012). It is worth noting that different types of fish contain different ratios of EPA: DHA depending on their diet and metabolic characteristics, the temperature of the water the fish live in and the season. ALA sources are of plant origin for e.g. rapeseed and soya bean oils, green leafy vegetables and nuts such as walnuts (Williams and Burdge 2006). However in humans, the conversion of consumed ALA to longer-chain PUFAs particularly DHA appears to be limited (Burdge and Calder 2005). There is in addition a difference in the capacity of synthesis between men and women, probably affected by pregnancy or lactation, when for example the demand for

DHA is especially high (Burdge and Calder 2005). During normal functions where EPA and DHA are used for membrane turnover and renewal, there might be a limited capacity for synthesis of EPA and DHA from ALA in adult humans consuming a balanced diet (Burdge and Calder 2005). Since observations that PUFAs can have a beneficial effect on health, and because the western diet provides small amounts of the PUFAs in respect to the amount required, it was sought to deliver the required higher dosages of these PUFAs by means of fish oils (Dyerberg, Madsen et al. 2010).

Fish oils can be obtained from cod liver oil (CLO) and fish body oils (FBO) and the ω-3 PUFAs are esterified as triglycerides (TG). This form of fish oils however requires that relative large amounts have to be ingested in order to deliver their therapeutic effect (Dyerberg, Madsen et al. 2010). In order to overcome this issue, a more concentrated form of fish oils has been prepared containing 30-90% of EPA and DHA in the form of free fatty acids (FFA), ethyl esters (EE) and re-esterified TG (Rtg) (Dyerberg, Madsen et al. 2010). Re-esterified is the term used to show that the product is obtained from FBO in which approximately 30% of the TG content is first converted to ethyl esters and are then molecularly distilled in order to remove the short chains and SFs thus increasing the EPA and DHA contents to around 60%. The ethyl esters are enzymatically reconverted to glycerides (Dyerberg, Madsen et al. 2010). The bioavailability of these forms of fish oil is under study by different researchers showing inconsistent results as far as their absorption is concerned (Dyerberg, Madsen et al. 2010); FFA formulations are showing a more efficient absorption through the small intestine as compared to the EE formulations. This is due to the fact that EE need to be hydrolysed first to FFA by pancreatic enzymes and then absorbed. Therefore FFA are not dependant on pancreatic enzymes (Pirillo and Catapano 2015). However all of the studies are emphasizing the effect of a rich lipid meal on ω-3 PUFA absorption (Dyerberg, Madsen et al. 2010).

Functions of polyunsaturated fatty acids

Lipids constitute about 50-60% of the dry weight of the mature human brain, 35% of which is the long chain PUFAs, AA and DHA. Frontal cortex analysis of the fatty acid profile showed that the ω-6 PUFA content is 17% (mainly AA) and ω-3 PUFA content is 14% (mainly DHA) and other ω-3 PUFAs represent less than 1% (McNamara and Carlson 2006). AA is found in all tissues, whereas DHA in grey matter, retina and testes. The AA and DHA content increases three months before delivery and for short time in the postnatal life (Zeman, Jirak et al. 2012). Furthermore Carver et al., found that there is a statistically significant correlation between the AA and DHA content in RBCs and that of the cortex after post mortem analysis of the fatty acid profile of 58 individuals (Carver, Benford et al. 2001)

As it was said earlier one of the main constituents of the cell membranes are polyunsaturated fatty acids PUFAs. The PUFAs precursors (DGLA, AA and EPA) can be released from the C2 position of PCs by the action of phospholipase A_2, (Calder 1996). The effects produced by these PUFAs are different (see figure 13). For instance eicosanoids such as leukotrienes, prostaglandins and thromboxanes of classes 1, 2 and 3 are produced by DGLA, AA and EPA respectively. Class 2 has a strong pro-inflammatory effect, class 3 has an anti-inflammatory effect and class 1 has an intermediate effect (Haag 2003).

The production of these eicosanoids prostaglandins and thromboxanes by the action of enzymes such COX-1/COX-2, and leukotrienes by LOX and the action of cytochrome P450 mono-oxygenases DGLA, AA and EPA are the three substrates of those enzymes. DGLA produced from GLA is only partially converted to AA and this is because of a limited activity of the Δ5 desaturase necessary for this conversion. As a result there is an attenuation of eicosanoid production from AA by COX-1/2, shifting the production of PGE1 which possess anti-inflammatory properties (Wang, Lin et al. 2012). Furthermore DGLA can also be

metabolised by the 15-LOX into 15-(S)-hydroxy-8,11,13-eicosatrienoic acid (15-HETrE) (Simopoulos 2008). Both 15-HETrE and PGE1 can exert clinical efficacy in a variety of diseases, including suppression of chronic inflammation (Wang, Lin et al. 2012). On the other hand, EPA inhibits the release of AA from membrane phospholipids by phospholipase A2, and can also competitively inhibit the metabolism of AA by COX-1/2. Furthermore EPA can also act as a substrate for both COX-1/2 and 5-LOX giving rise to the increase in production of anti-inflammatory eicosanoids and at the same time decreasing those derived from AA that exert pro-inflammatory action (Calder 2002) (see figure 13).

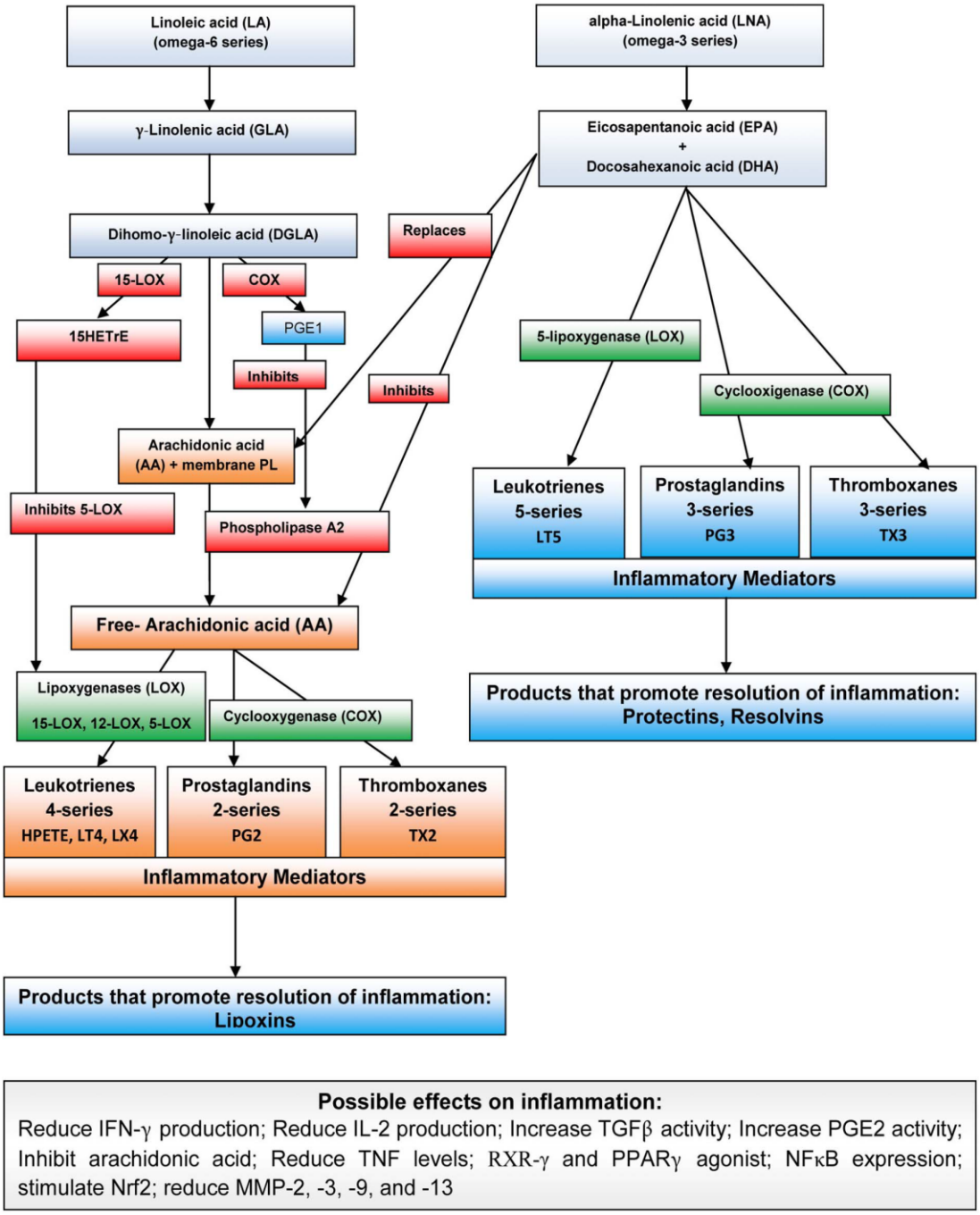

Figure 13. ω-6 and ω-3 PUFAs, their respective metabolic derivatives and their possible effects on inflammation. PUFAs can be metabolised via several pathways (not shown) to active compounds that mediate inflammation and to products that promote the resolution of inflammation. COX, cyclooxygenase; HETrE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; IFN-γ, interferon γ; IL-2, interleukin 2; LOX, lipoxygenase; LT, leukotriene; MMP, metalloproteinase; NFκB, nuclear factor kappa B; Nrf2, nuclear respiratory factor; PG, prostaglandin; PGE2, prostaglandin E2; PL, phospholipid; PPARγ, peroxisome proliferator-activated receptor γ; PUFAs, polyunsaturated fatty acids; RXR-γ, retinoid X receptor/γ; TGFβ, transforming growth factor β; TNF, tumour necrosis factor; TX, thromboxane (From Patzaris et al., 2013).

PUFAs participate in modulation of signal transduction mechanisms especially in neuronal membranes (Calder 2002). PUFAs are also involved in a variety of functions in the body such as regulation of blood pressure, platelet function, blood coagulation, plasma TG concentrations, vascular function, cardiac rhythm, heart rate, inflammation, immune function, fatty acid and TG metabolism, regulation of bone turnover, insulin sensitivity, tumour cell growth and visual signalling, as well as being a structural component of brain and central nervous system (Calder 2012).

Four mechanisms have been characterized by which ω-3 PUFA can demonstrate their action in cells, providing intra- and extra-cellular links between various processes. (Shaikh, Rockett et al. 2009; Calder 2012). These four mechanisms are:

(1) Influencing the concentrations of metabolites and/or hormones that in turn can influence the cell or tissue;

(2) Influencing oxidation of LDL, oxidative stress or other factors that in turn can again influence the cell or tissue; As discussed previously on page 14, all these factors are critical in MS.

(3) Affecting cells or tissues by directly influencing surface or intracellular fatty acid 'receptors' or 'sensors. An example is the way that ω-3 PUFA and eicosanoid mediators activate PPARγ hence promoting regulation of inflammation and other cell and tissue responses. In particular DHA in dendritic cells induced PPARγ with the effects of decrease production of inflammatory cytokines; furthermore ω-3 PUFAs exhibit inhibitory effects on NFκB activation by decreasing phosphorylation of the inhibitory subunit IκB with the resulting inhibition of the production of inflammatory proteins including COX-2, NO synthase, TNFα, IL-1, IL-6, IL-8. By activating PPAR, ω-3 PUFAs can inhibit upregulation of NFκB target genes and this is done by the PPAR physically interacting with NFκB

preventing its translocation to the nucleus. NFκB is also inhibited by ω-3 PUFAs through the inhibitory effects of ω-3 PUFAs on G-protein coupled surface receptors (GPCR). One other mechanism by which unsaturated and saturated fatty acids can alter gene expression is by modulating the activation of the toll-like receptor (TLR) 4 and its down-stream signalling pathways. Unsaturated FAs supress the activation of NFκB activation and COX2 from TLR-2 agonists and saturated ones potentiate TLR-2 activation and induction of NFκB and COX-2 (Mills, Windsor et al. 2005);

(4) Affecting cell or tissue physiology by changes in cell phospholipid membrane composition. The correct membrane composition is necessary to maintain membrane order, lipid rafts and to provide the correct environment for protein function (Calder 2012). Phospholipids can be hydrolysed to produce second messengers, or PUFAs that are released from the membrane can act as signalling molecules, ligands, or precursors for the biosynthesis of lipid mediators like eicosanoids, resolvins, and protectins. The production of eicosanoids from AA in the presence of ω-3 PUFA is decreased by making AA unavailable as a substrate and at the same time the metabolism of AA is inhibited in the presence of ω-3 PUFAs (Calder 2012). Resolvins and protectins have an anti-inflammatory and immunomodulatory effect as shown by different studies (Serhan, Clish et al. 2000; Serhan, Hong et al. 2002; Serhan, Chiang et al. 2008). For example protectin D1 is synthesised from DHA and can protect different tissues including neuronal tissues form excessive damage, such as oxidative stress in the brain and can also inactivate proapoptotic and proinflammatory signals as concluded by Bazan et al 2009 (Bazan 2009). Although dietary PUFAs cannot be incorporated into the rafts due to their low affinity to cholesterol, PUFAs can influence rafts by displacing proteins as shown by Fan et al 2003 in immune cells (Fan, McMurray et al. 2003) and others (Shaikh, Rockett et al. 2009).

The effects of PUFAs in the Central Nervous System (CNS)

PUFAs can exhibit their effects on the CNS through 5 different mechanisms (Zeman, Jirak et al. 2012):

1. Physical-chemical characteristics of neuronal membranes
2. Intra- and intercellular signalization, gene expression and transcription
3. Modulation of inflammation and oxidative stress
4. Neurotransmission
5. Neurogenesis and neuronal survival

Physical-chemical characteristics of neuronal membranes

The physical-chemical characteristics of neuronal membranes can be influenced by PUFAs and subsequently all the structures connected with the membranes e.g. ion channels, receptors, cellular transporters (Zeman, Jirak et al. 2012). For instance Kurnellas et al. in a review on the role of the plasma-membrane Ca^{2+}-ATPase 2 (PMCA2) calcium pump and the Na^{+}/Ca^{2+} exchanger (NCX) in neuronal damage in MS and its animal models, concluded that restoration of normal PMCA2 and NCX activity may prevent or slow down disease progression by preventing neurodegeneration (Kurnellas, Donahue et al. 2007). Gerbi et al. showed that supplementation of diabetic rat models with fish oils restored Na,K-ATPase activity (Gerbi, Maixent et al. 1998). Kearns et al. showed that both DHA and EPA inhibit the Na^{+}, K^{+}-ATPase, which drives the NCX (Kearns and Haag 2002). Transient receptor potential channels (TRP) in the CNS are involved in the development of neuronal tissue, neuronal sprouting, cell communication, microglial activation, hydroelectrolytic balance, control of body temperature, and sensory signalling; TRP also permits the influx of Ca^{2+} and might be involved in excitotoxicity and oxidative stress which can damage neuronal tissue, as in the case of MS. TRP function can be modulated directly or indirectly by PUFAs (both EPA and DHA) hence protecting the neurons (Leonelli, Graciano et al. 2011).

Intra- and intercellular signalization, gene expression and transcription

As was mentioned earlier in pages 34-38 eicosanoids and docosanoids can be produced from C20 and C22 PUFAs. These can defuse through the neuronal membranes acting as autocrine or paracrine signalling molecules with effects that have already been discussed (see page 34). PUFAs can also act as intracellular secondary messengers in signalling can influence the function of transcriptional factors and gene expression as discussed earlier. For instance DHA can enhance the activation of protein kinase C as part of diacylglycerol (DAG) (Zeman, Jirak et al. 2012). Furthermore DHA through its docosanoid products can protect neurons from oxidative damage (Bazan 2009). In the case of gene expression, PUFAs (see figure 14) can influence their expression in the liver and adipose tissue as well as in the brain (Deckelbaum, Worgall et al. 2006; Zeman, Jirak et al. 2012) by controlling synaptic plasticity, signal transduction, interactions of cytoskeleton with cellular membrane, the formation of ion channels, regulatory proteins etc. (Zeman, Jirak et al. 2012).

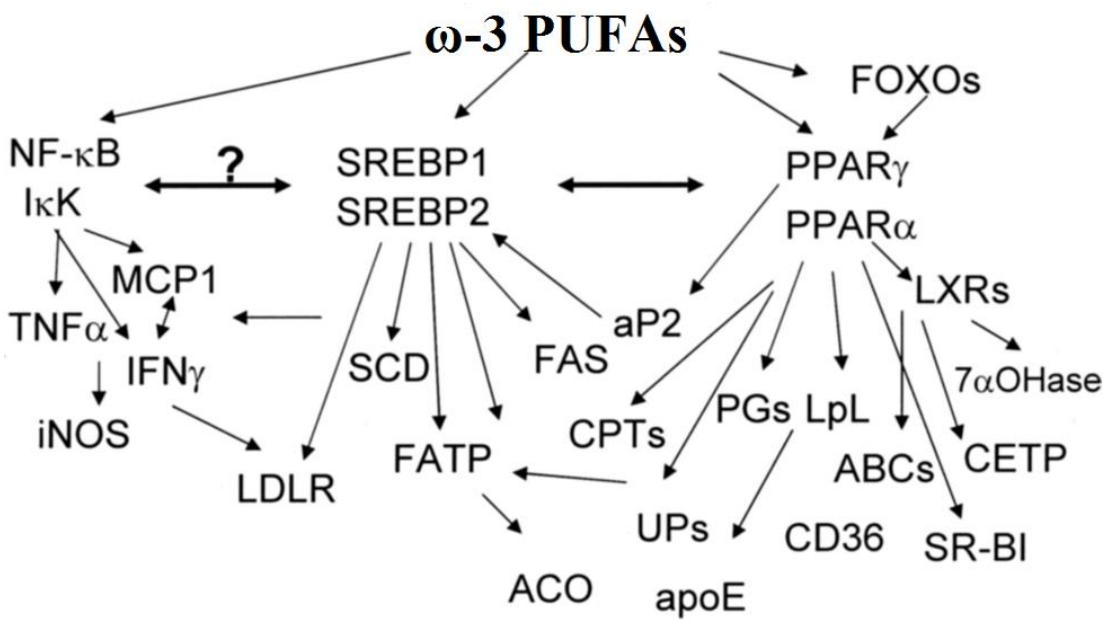

Figure 14. Examples of the different gene families regulated by ω-3 PUFAs and their interaction. ABCs, ATP binding cassette transporters; ACO, acyl CoA oxidase; Ap2, adipocyte fatty acid binding protein; apoE, apolipoprotein E; CETP, cholesteryl ester transfer protein; CPTs, carnitine palmitoyl transferases; FATP, fatty acid transport protein; FAS, fatty acid synthase; FOXOs, fork head transcription factors; IFNγ, interferon γ; IκK, inhibitory κ B kinase; Inos, inducible nitric oxide synthase; LDLR, LDL receptor; LpL, lipoprotein lipase; LXRs, liver X receptors; MCP1, monocyte chemo attractant protein 1; NF-Kb, nuclear factor Kb; 7 αOHase, 7α-hydroxylase; PGs, prostaglandins; PPAR, peroxisome proliferator activated receptor; SCD, stearoyl CoA desaturase; SR-BI, scavenger receptor B1; SREBP, sterol-responsive-element binding protein; TNFα, tumor necrosis factor α; Ups, uncoupling proteins.(From Deekelbaun et al., 2006)

Modulation of inflammation and oxidative stress

The eicosanoids and dcosanoids derivatives of AA, EPA, DGLA and DHA can be proinflammatory or anti-inflammatory (Calder 2012). AA is preferentially bound to the membrane phospholipids PE, PC and PI (Zeman, Jirak et al. 2012). EnhancedPLA_2 and the subsequent release of AA from the C2 position of the membrane phospholipids can generate highly reactive PGs, free fatty acids, lysophospolipids, eicosanoids, platelet-activating factors, and ROS all of which participate in cellular injury and neurodegeneration (Bazan, Colangelo et al. 2002; Bosetti 2007). In particular PLA_2 activation and PG production can lead to long-term neurological deficits; both activation and production are considered to be the earliest events that can initiate triggering of brain-damage pathways (Bazan, Colangelo et al. 2002; Bosetti 2007). Acute and chronic brain injury, that includes cerebral trauma,

ischemic damage, induced seizures in the brain and epilepsy, schizophrenia, and Alzheimer's disease are all associated with PLA_2 altered membrane-associated activities (Bazan, Colangelo et al. 2002). AA and its metabolites are involved in synaptic signalization, neurotransmitter release, cerebral blood flow and as was repeatedly said gene expression (Bosetti 2007). Altered AA metabolism has been associated with various neurological, neurodegenerative and psychiatric disorders (Zeman, Jirak et al. 2012). AA also stimulates the biosynthesis of glutamate, which as discussed earlier in section X which when in excess can cause neuronal destruction through the overproduction of oxygen radicals. Moreover EPA and DGLA compete with AA for the production of eicosanoids. On the other hand DHA can act as a radical scavenger of H_2O_2 $O_2^{-\bullet}$ and $HO^{\bullet}$ hence exerting its neuroprotective function (Shimazawa, Nakajima et al. 2009; McNamara 2010).

Neurotrasmission

Neurotrasmitters elicit excitatory and inhibitory postsynaptic potential. Their concentration, storage in vesicles and the function of post- and pre-synaptic receptors can influence inter- and intra-cellular signal transduction. Chalon et al. 2001 reported that PUFAs might have an effect in the monoaminergic and neurotransmission processes resulting in potential preventive and therapeutic effects of ω-3 PUFAs in a number of neurological and psychiatric diseases (Chalon, Vancassel et al. 2001). Moreover a number of animal studies examining the effects of ω-3 PUFAs deficiencies investigate the relation to impaired behavioural responses which usually involve learning ability and sensory, motor or motivational processes (Chalon 2006). Chalon et al. in 2006 also concluded that ω-3 PUFAs deficiency is able to alter dopanergic and serotonergic neurotransmission systems; Aid et al. in 2003 reported the same for the cholinergic neurotransmitter system (Aid, Vancassel et al. 2003; Chalon 2006). Studies on humans are not yet conclusive, however evidence seems to provide support for beneficial effects (Appleton, Rogers et al. 2010). As a result of ω-3

PUFAs deficiencies, there is an increase of Δ-6 desaturase-mediated ω-6 PUFAs and membrane AA; PGE_2 is derived from AA from the immune cell membrane and PGE_2 stimulates the synthesis of IL-6 via NFκB and the enzymes that regulate synthesis of PGE_2 from AA are up-regulated in ω-3 PUFAs deficiency (McNamara, Jandacek et al. 2010). Furthermore ω-3 PUFAs deficiencies and peripheral administration of IL6 in mice will result in elevated extracellular serotonin (5-HT) concentrations and 5-hydroxyindoleacetic acid (5-HIAA) levels and the 5-HIAA:5-HT ratio in brain (Zhang, Terreni et al. 2001). McNamara et al. 2010 suggested that up-regulation of AA to PGE_2 and IL6 production could be responsible for the elevated central 5-HT turnover in response to an ω-3 fatty acid deficiency; elevation of IL-6 and elevated central 5-HT can all be prevented by regulation of the ω-3 PUFAs status (McNamara, Jandacek et al. 2010).

Neurogenesis and neuronal survival

The lipid content in brain is 36-60%, DHA and AA are the main brain PUFAs; Docosatetreanoic acid together with DHA and AA are the three main PUFAs in grey matter, and DHA it is found in higher concentrations than AA whereas the opposite is true in the white matter (Tassoni, Kaur et al. 2008). In humans DHA is accumulated in the brain during the perinatal period (beginning of third trimester of gestation) and continues until 2 years after birth (Su 2010). Whereas DHA can be synthesized in the humans' developing brain, DHA synthesis in adults is low or none; therefore DHA must be supplied with food in order to maintain brain DHA levels (Su 2010). Kawakita et al. 2006 suggested that DHA can promote neurogenesis by generating new hippocampal neurons in adult and aged rat brain (Kawakita, Hashimoto et al. 2006). Furthermore Eriksson et al. 1998 demonstrated that new neurons are generated in the dentate gyrus and that the human hippocampus can generate neurons throughout life (Eriksson, Perfilieva et al. 1998).

Health benefits of ω-3 PUFAs

It was discussed earlier that ω-3 PUFA both EPA and DHA, possess different physiological functions for the body with some potential health benefits that target different disease states.

The beneficial effects of both EPA and DHA ω-3 PUFAs were observed in Greenland Eskimos where low incidence of autoimmune and inflammatory disorders such as psoriasis, asthma, type-1 diabetes and multiple sclerosis as compare with Danish population (Kromann and Green 1980; Lavie, Milani et al. 2009; Yessoufou, Nekoua et al. 2015) . Beneficial effects of both EPA and DHA ω-3 PUFAs on the prevention of coronary heart disease (CHD) were also observed in many prospective epidemiological studies and secondary prevention trials for CHD in Greenland Eskimos (Whelan and Rust 2006; Williams and Burdge 2006; Lavie, Milani et al. 2009; Yessoufou, Nekoua et al. 2015). The beneficial effects of both EPA and DHA ω-3 PUFAs in preventing CHD (Whelan and Rust 2006; Williams and Burdge 2006) can be attributed to the effects that they have on individual risk factors such as blood pressure a mild hypotensive effect in both normotensive and hypertensive individuals (Calder 2004), platelet reactivity and thrombosis, plasma triglyceride concentration, vascular function, cardiac arrhythmias, heart rate variability, lowering cholesterol plasma levels and inflammation (Leaf and Weber 1988; Burr, Fehily et al. 1989; Pauwels and Kostkiewicz 2008; Calder 2012). EPA and DHA, ω-3 PUFAs, due to their beneficial effects can reduce mortality and morbidity from cardiovascular disease, and can protect from fatal myocardial infarction (Calder 2004). EPA and DHA, ω-3 PUFAs, can also exert beneficial effects by reducing the risks for a number of other non-cardiovascular conditions, either by increasing the intake of such PUFAs as a preventative measure or even in some cases as adjuvants to drug therapy (Simopoulos 2002).

The beneficial effects observed in inflammatory or autoimmune disorders can be assigned to EPA and DHA ω-3 PUFAs(Simopoulos 2002). These PUFAs when supplemented with

the diet can replace the ω-6 PUFAs mainly AA that in the western diets accounts for the majority in food. ω-3 PUFAs compete with ω-6 PUFAs at the cyclooxygenase and lipoxygenase pathways for the synthesis prostaglandin (PG) and leukotriene (LT). In particular EPA and DHA competes with AA and increase ingestion of EPA and DHA will result in: decrease production of PGE_2 ; decrease production of thromboxane (TX) A_2 (a potent aggregator and vasoconstrictor); decrease production in LTB_4 (inflammation inducer and potent inducer of leukocyte chemotaxis and adherence); increase production of TXA_3 which is a weak vasoconstrictor; increase of total prostacyclin PGI_3 which cause an increase in total prostacyclin but at the same time the levels of PGI_2 are not affected (both PGI_3 PGI_2 are active vasodilators and inhibitors of platelet aggregation); LTB_5 a weak inducer of inflammation and a weak chemotactic agent is increased (Simopoulos 2002).

The increase intake by supplementation of EPA and DHA in healthy human volunteers showed a decrease production of PGE_2 and LT4 series by the immune cells, same decrease was observed in patients with rheumatoid arthritis (RA) (Sperling, Weinblatt et al. 1987; Miles and Calder 2012).

One other effect of EPA and DHA on phagocytic cells is the modulation of the production of superoxide anion radical and nitric oxide which will give rise to reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both ROS and RNS are physiologically produced as microbicidal but in excess can damage tissues causing injury and contributing to diseases such RA (Stamp, James et al. 2005).

Other ways that supplementation of EPA and DHA can contribute to the anti-inflammatory action in RA is by decreasing antigen presentation through MHCII, reduced T cell reactivity, reduced production of Th1 type cytokines and reduced production of inflammatory cytokines. (Miles and Calder 2012).

Some benefits from EPA and DHA supplementation in inflammatory bowel diseases have been observed in some studies in relation to improved gut histology, decreased disease activity, decreased use of corticosteroids, and decreased relapse; these effects of EPA and DHA are exerted either by acting via transcription factors (NFκB, PPAR-γ) or as lipid mediators for the production of specific eicosanoids(Calder 2008). The decrease of the incidence of malignancies (mammary, pancreatic, prostatic and gastrointestinal neoplasms) in experimental animal after increasing into the diet ω-3 PUFAs, was observed in many studies (Mills, Windsor et al. 2005).

Benefits of ω-3 PUFAs in adults have been observed in psychiatric illnesses such as major depressive disorder, bipolar disorder, schizophrenia, dementia including Alzheimer's disease, borderline personality disorder and impulsivity, attention-deficit/hyperactivity disorder and learning disabilities (Freeman, Hibbeln et al. 2006). The mechanisms by which the effects of ω-3 PUFAs on the psychiatric illnesses can be explained with their involvement in the increase of serotonergic neurotransmission, in alteration of dopaminergic function, in the regulation of corticotropin-releasing factor, in the inhibition of protein kinase C, in suppression of phosphatidylinositol-associated second messenger activity, in modulating heart rate variability via vagal mechanisms, in increased dendritic arborization and synapse formation, in the prevention of neuronal apoptosis, improved cerebral blood flow, in the regulation of gene expression, competition of EPA with AA for enzymatic action (Freeman, Hibbeln et al. 2006).

It is also known than ω-3 PUFAs are essential for growth and development; especially DHA accumulates in utero, with relatively increased quantities, to be deposited in the second half of gestation; after birth DHA is continued to be accumulated reaching 4g between 2 and 4 years of age. DHA is also important for retina in rods and cone(Koletzko, Lien et al. 2008). Therefore a good supply of DHA during development would ensure optimal visual and

neurological development (SanGiovanni, Berkey et al. 2000). Is worth mentioning that no other ω-3 PUFAs is accumulated with the same way as DHA, AA an n-6 PUFA is the only other LCPUFA that is accumulated in the brain (Koletzko, Lien et al. 2008). Moreover an increase of maternal consumption of the ω-3 PUFAs, DHA and EPA, is responsible for beneficial effects on longer gestation rates and higher birth weights (Williams and Burdge 2006).

It can therefore be concluded from all the above that from in vitro and in vivo studies, EPA, DHA, LA, GLA can modulate, with their involvement, almost all known networks of events and pathways in MS pathophysiology. For instance, PUFAs composition of membrane phospholipids plays an important role in inflammation (immune-related and non-immune-related inflammation). Increase levels of EPA and DHA in the membranes of inflammatory cells can reduce levels of AA with resulting modulation of an increased production of anti-inflammatory eicosanoids (Calder 2002; Calder 2006). Furthermore there is also increase production of resolvins and protectins from EPA and DHA via COX-2/LOX pathways hence promoting control inflammation in neuronal tissues (Serhan, Arita et al. 2004; Ariel and Serhan 2007; Serhan, Chiang et al. 2008). T-cell proliferation as seen in acute and chronic inflammation can also be reduced by GLA, EPA, DHA (Rossetti, Seiler et al. 1997). Ω-3 PUFAs electrophilic derivatives formed by COX-2 in activated macrophages can stimulate the Nrf, which induces the transcription of neuroprotective and antioxidant-related genes and can activate the peroxisome proliferator-activated PPARγ for an anti-inflammatory response (Calder 2008; Edwards and O'Flaherty 2008; Wymann and Schneiter 2008; Groeger, Cipollina et al. 2010). In animal studies, EPA and DHA have proved to be endogenous ligands of the retinoid RXR, with positive effects on neurogenesis (Dyall, Michael et al. 2010).

Additionally, in 2008, Salvati et al reported evidence of accelerated myelination in DHA-treated and EPA-treated animals (Salvati, Natali et al. 2008).

Moreover, DHA and EPA have been reported to significantly decrease the levels of metalloproteinases MMP-2, MMP-3, MMP-9 and MMP-13, which have a significant role in the migration of lymphocytes into the central nervous system by inducing the disruption of the blood brain barrier, an important step in the formation of MS lesions (Abraham, Shapiro et al. 2005).

Recommended intakes of ω-3 PUFAs

Currently there is no consensus for the recommended intakes of ω-3 PUFAs. In Tables 1 and 2 different recommendations are shown and can also be found in the Global Organization for EPA and DHA omega 3s (GOED) as revised in 2014 (GOED 2014). The purpose of these recommendations is the reduction of risks for coronary disease and lowering blood triglyceride levels; furthermore recommendations for pregnant women, infants, vegetarians and vegans have been put forward (Kris-Etherton, Grieger et al. 2009). The Institute of Medicine (IOM) of The National Academies set a Dietary Reference Intake (DRI), more specifically, an Adequate Intake (AI) for the ω-3 PUFA, ALA and recommended that 10% of the Acceptable Macronutrient Distribution Range (AMDR) for ALA can be consumed as EPA and/or DHA. DHA and EPA recommendations were made based on an intake that supports normal growth and neural development and without deficiency, and this intake is the mean intake in the USA which is approximately 100mg/day (Kris-Etherton, Grieger et al. 2009). Moreover as stated in Kris-Etherton et al. 2009 and Flock et al. 2013 there is a need for individual recommendations for EPA and DHA separately and not the total amount of EPA and DHA as are the current recommendations (Kris-Etherton, Grieger et al. 2009; Flock, Harris et al. 2013). Despite the numerous studies that have shown the protective effects that DHA and EPA have on health via known mechanisms, there has been no DRI ;

A number of organizations such as the European Food Safety Authority (EFSA) and expert groups such as the International Society for the Study of Fatty Acids and Lipids (ISFFAL) have issued evidence-based recommendations for EPA and DHA (see table 1) (Kris-Etherton, Harris et al. 2003; Kris-Etherton, Innis et al. 2007; Harris, Kris-Etherton et al. 2008; Food and Agriculture Organization 2009; EFSA 2010; U.S. Department of Agriculture and and U.S. Department of Health and Human Services 2010).

Table 1. Recommended intakes of EPA and DHA (From Flock, M. R., et al. 2013).

Organization	Population	EPA and DHA recommendation
Academy of Nutrition and Dietetics	Adults	≥500 mg/day
American Heart Association	Adults without CHD	Fatty fish ≥2 times/week (~500 mg/day)[a]
	Patients with CHD	~1 g/day
	Patients with high TG	2–4 g/day
US Department of Agriculture	Adults	≥250 mg/day
International Society for the Study of Fatty Acids and Lipids	Adults	≥500 mg/day
	Pregnant/lactating women	≥500 mg/day (≥300 mg/day of DHA)
European Food Safety Agency	Adults	≥250 mg/day
	Pregnant/lactating women	≥250 mg/day (and100–200 mg/day DHA)
World Health Organization	Adults	≥250 mg/day

[a] Calcuated to be approximately 500 mg/day.
Abbreviations: CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TG, triglycerides.

Table 2. Recommended dietary intakes for ω-3 PUFAs from national and international bodies (adults)[1] (From EFSA Journal 2012;10(7):2815).

National/International Body	ω-3 PUFA		ALA		EPA+DHA[2]	
	% of energy	g/day	% of energy	g/day	% of energy	mg/day
(WHO/FAO, 2003)	1-2	-	-	-	-	200-1000/wk
United Kingdom, (DoH, 1991, 1994)	-	-	>0.2	-	-	**200**
(SACN, 2004)	-	-	-	-	-	**450**
(Eurodiet, 2000)	-	-	-	2	-	200
Belgium, Superior Health Council (CSS, 2009; SHC, 2004)	1.3-2.0	-	>1	-	≥0.3	
Australia, (Ministry of Health-Department of Health and Ageing - National Health and Medical Research Council, 2006)						
Adult men	-	-	-	1.3	-	**610**[3]
Adult women	-	-	-	0.8	-	**430**[3]
Pregnancy	-	-	-	1.0	-	**115**[4]
Lactation	-	-	-	1.2	-	**145**[4]
The Netherlands, (Health Council, 2001, 2006)	-	-	1	-	-	**450**
Nordic Countries, (NNR, 2004)	≥1	-	-	-	-	-
France, (ANSES, 2010)			1			500 (250 DHA)
USA, (IoM, 2005)						
Adult men	-	-	-	1.6	-	-
Adult women	-	-	-	1.1	-	-
Pregnancy	-	-	-	1.4	-	-
Lactation	-	-	-	1.3	-	-
Germany, Austria, Switzerland, (D-A-CH, 2012)	-	-	0.5	-	-	-
Pregnancy	-	-	-	-	-	200 (DHA)
Lactation	-	-	-	-	-	200 (DHA)
(EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), 2010)			0.5			250
Pregnancy and lactation						+100-200 (DHA)

[1] Values for pregnancy and lactation are only indicated if different from those for adult women. [2]Values in bold refer to n-3 LCPUFA (EPA, DHA and DPA); [3]Suggested Dietary Target; [4] Adequate Intakes.

Metabolic disturbances in Multiple Sclerosis

Metabolic disturbances that have been observed in MS mostly involve those of lipid metabolism. For instance, it has been proposed by Horrobin et al. in 1981 that in MS patients there is a dysregulation of phospholipid-based signal transduction process which affects both the immune system and neurons (Horrobin and Lieb 1981). For example PGE1 blocks mobilization of AA, therefore lack of PGE1 will cause an increase in the mobilization of AA and an increase production of PG2. Stores of DGLA are limited and in case of excess

production of PGE1 this will cause depletion of DGLA with a subsequent depletion of PGE1 (Horrobin and Bennett 1999). On the other hand Rossetti et al, suggested that administration of GLA which can then be converted to DGLA which then competes with AA for the COX and LOX enzymes, favours the production of PGE1 and inhibits the production of PGE2 and LTB4 (Rossetti, Seiler et al. 1997). Harbige et al reported a 2-fold higher concentration of EDA in MS during remission and a 4-fold higher concentration during relapse, as determined from peripheral blood mononuclear cells (PBMC) (Harbige and Sharief 2007). They postulated that in MS there is an active elongation of LA (18:2-n6) to EDA in PBMC which increases during relapse, probably due to the higher requirement for DGLA and AA and/or a disturbance in the normal metabolism in PBMC (Harbige and Sharief 2007). They also observed a disturbance in the relationship between LA and DGLA and DGLA and AA indicating a probable disturbance in Δ6, Δ5 desaturation and/or an increase in demand of these n-6 fatty acids in MS (Harbige and Sharief 2007). Baker et al, have demonstrated a reduction of LA in MS patients as compared to controls (Baker, Thompson et al. 1964). Furthermore, as discussed by Harbige et al 2007, there are a number of reports from studies showing that the levels of LA and AA are reduced in MS patients; whereas other studies showed that the reduction is not specific for MS (Harbige and Sharief 2007).Taking into account the involvement of PUFAs in inflammation and the observed lipid metabolic disturbances in MS, few studies have been conducted in order to correct the dysregulation of lipids observed by supplying the PUFAs and observing the effects that might have (Horrobin and Lieb 1981; Horrobin and Bennett 1999; Horrobin and Bennett 1999)).

1.3 Multiple Sclerosis

1.3.1 Epidemiology of MS

As mentioned earlier both environmental factors and genetic susceptibility have both been shown to contribute to the manifestation of MS. Whether the environment prevails over genetics or vice-versa is a matter of discussion. Compston et al. in 2008 also noted the following "Each one is implicated together with cultural condition of age at which the interaction between environment and genetics took place" (Compston and Coles 2008).

MS is one of the most common neurological diseases causing disability in young adults and was first described in 1868 by Jean-Martin Charcot (Milo and Miller 2014). Its onset is usually between 20 and 40 years of age, and only ~5 % of patients are diagnosed before the age of 16 years. Worldwide, there are approximately 2.3 million MS patients of whom 400,000 reside in Europe, 1,200,000 in the Americas, 342,700 in Central America, 760 in South-East Asia and 60,000 in Africa (Thompson 2013). Globally, the median estimated prevalence of MS is 33 per 100,000 (with a range of 2–140) (Thompson 2013), (prevalence refers to the number affected within the community at one point in time expressed per unit of population, that is total cases present at a year per 100,000 population (Koutsouraki, Costa et al. 2010)). The highest prevalence in age is between 35 and 64 (Koutsouraki, Costa et al. 2010).

According to Kurtzke 2001 and the Multiple Sclerosis International Federation report (Thompson 2013), there is a geographical distribution that includes areas of high, medium and low frequency. High frequency refers to rates of >30/100,000 and includes most countries of Europe, (including Cyprus), Israel, Canada, Northern USA south-eastern Australia, New Zealand, and easternmost Russia. Medium frequency areas include southern USA, most of Australia, South Africa, the southern Mediterranean basin, Russia into Siberia, the Ukraine and parts of Latin America. Prevalence rates under 5 per 100,000 are found in

the rest of Asia, Africa and northern South America. The native Cypriot population has a high MS prevalence, 44.5/100,000 (Middleton L. 1991; Dean, Aksoy et al. 1997).

In the geographical distribution of MS one can observe a gradient from north to south in the northern hemisphere and a south to north gradient in the southern hemisphere, with almost no incidence along the equator. There are some exceptions to the north-south gradient such as the incidence and prevalence of MS in the Canary Islands which questioned the belief that such a gradient exists (Alonso and Hernán 2008) and that this gradient does not play a key role in determining the incidence of MS (Zivadinov, Iona et al. 2003). This indicates that other factors exist to explain the origins of MS and these factors could be genetic, environmental, climate, behaviour, infections, availability of sunlight and intake of vitamin D.

It has been suggested that environmental factors during childhood may play a role in developing MS later in life. This was observed from migration studies where if migration occurs before the age of fifteen, the migrant acquires the new region's susceptibility. If however the migration occurs after the age of fifteen the migrant retains the susceptibility of his country of origin. MS occurred in epidemic form in Northern Atlantic islands (Iceland, Shetland-Orkneys, and in the Faroe Islands). In the Faroes the first appearance was in 1943, followed by four successive epidemic outbreaks at 13-year intervals. The disease was presumably introduced by the occupying British troops during World War II (Kurtzke 2000).

Interestingly, MS is 3 times more common in women than in men. The overall incidence rate of MS is 3.6/100,000 in women and 2.0 in men. (The incidence rate is defined as the number of newly diagnosed cases in a unit of time within the specific population (Koutsouraki, Costa et al. 2010)). This difference in the susceptibility to MS is probably due

to sex hormones as suggested by animal studies in which testosterones were protective against the disease (Reipert 2004).

Another aspect in the development of MS involves genetic factors. Evidence that genetic factors have a substantial effect on susceptibility to multiple sclerosis is unequivocal (Noseworthy, Lucchinetti et al. 2000). Strong evidence stems for twin studies. A concordance rate of 31 percent among monozygotic twins is approximately six times the rate among dizygotic twins (5 percent) (Sadovnick, Armstrong et al. 1993). Furthermore MS is known that co-segregate in families (Nielsen, Westergaard et al. 2005); with an absolute recurrence risk for the disease (i.e. the risk of developing the disease over a time period, in a first-degree relative of a patient with multiple sclerosis) being less than 5%. However, the relative risk (i.e. the risk of developing MS as compared between the group of relatives and the general population) is 20 to 40 times higher in the relatives when compared to general population risk (Sadovnick, Baird et al. 1988; Nielsen, Westergaard et al. 2005). Studies have shown a higher prevalence of maternal transmission of MS with the most likely explanation a gene–environment effect that takes place *in utero* (Hoppenbrouwers I. A, Liu F et al. 2008). Hypotheses have been proposed that these genes are affecting both disease susceptibility and progression (Chataway, Mander et al. 2001). This has been observed in all populations, with the exception of individuals from Sardinia (Marrosu, Murru et al. 1998). Until very recently and despite many linkage and association studies, only the HLA class II region of the HLA-DR2 haplotype of chromosome 6p21 showed significant association with MS (Jersild, Fog et al. 1973). More powerful studies have however implicated *HLA-DRB1*1501* as the main susceptibility allele in MS (Sospedra and Martin 2005). In the Japanese, *DRB1*0405* and *DPB1*0301* are susceptibility alleles for MS (Yoshimura, Isobe et al. 2012). Other loci have also been identified that show convincing evidence for an association with MS, such as the IL2RA, IL7RA, KIF1B, IRF5, EVI5, CD226 and

CLEC16A genes (Alcina, Ramagopalan et al. 2010). Alcina et al 2010 identified in a south of Spain population a hexose-6-phosphate dehydrogenase (H6PD) gene as a risk gene for MS and validated this finding in an independent cohort of Canadian MS patients; H6PD encodes an enzyme that catalyzes the initial steps of the pentose phosphate pathway (PPP) within the lumen of the endoplasmic reticulum providing the body with $NADPH(H^+)$. $NADPH(H^+)$ in neurons derived from PPP it is used for the regeneration of antioxidant system glutathione peroxidase. The decrease of H6PD activity could probably lead to neurodegeneration (Alcina, Ramagopalan et al. 2010).

Variable environmental factors implicated in the pathogenesis of MS include vitamin D, human cytomegalovirus infection (CMV) and circadian disruption. Smoking and Epstein-Barr virus (EBV) infection are the best confirmed contributors. The connection between gut microbiota and incidence and severity of CNS inflammation was demonstrated in studies from EAE. However studies on humans connecting demonstrated that changes in gut microbiota studies have yet to be demonstrated. Gut microbiota is affected by environment and hence diet(Riccio and Rossano 2015).

Other environmental factors that recently have been investigated are the effects of salt or sodium chloride on the aetiology of MS (Kleinewietfeld, Manzel et al. 2013; Wu, Yosef et al. 2013). For instance the pivotal role of Th17 (type of T cell) in the promotion of autoimmune diseases is only recently identified (Korn, Bettelli et al. 2009). Recently both *in vitro* and *in vivo* studies on dietary salt showed that changes in concentrations can induce Th17 differentiation through SGK1 expression which in turn promotes interleukin (IL) 23R expression enhancing Th17 differentiation and hence promotion of MS (Kleinewietfeld, Manzel et al. 2013; Wu, Yosef et al. 2013).

Therefore susceptibility to MS is a combination of genetic and environmental factors.

1.3.2 Types of multiple sclerosis

There are four main types of MS as standardised in 1996 (1) Relapsing-Remitting (RRMS), (2) secondary progressive (SPMS) and (3) primary progressive (PPMS), (4) Progressive–relapsing MS (PRMS) (Lublin, Reingold et al. 1996; Milo and Miller 2014). The majority of MS patients (80%) initially present an acute episode affecting one or several (occasionally) sites known as the clinically isolated syndrome; the chances of a second attack of demyelination occurring, so fulfilling the diagnostic criteria for RRMS, increases from 50% at 2 years to 82% at 20 years. The rate of new episodes will seldom exceed 1,5 per year (Compston and Coles 2008).

Relapsing-Remitting (RRMS)

Eighty percent (80%) of people presenting MS are diagnosed with the relapsing-remitting form. Patients with this type of MS experience a series of attacks (relapses) followed by complete or partial disappearance of the symptoms (remissions). Remissions can last for days to years. Relapses can last for days to weeks. With each remission, recovery from deficits acquired during the relapse phase can be partial or full, with partial recovery gradually leading to disability over the years. About 50% of these patients will develop secondary progressive MS within 10 years. It has been estimated that this number will rise to 90% in 25-30 years. (Lublin, Reingold et al. 1996; Reipert 2004).

Primary progressive (PPMS)

Primary progressive MS is seen from the start of the disease. Ten to twenty percent (10–20%) of people with MS are diagnosed with primary progressive MS (PPMS). Neurological deterioration is present from the onset prior to the presence of relapses. This form is characterized by a gradual progression of the disease involving a decline in the patient's physical abilities with only short periods where the decline seems to stop with some minor relief. (Lublin, Reingold et al. 1996; Noseworthy, Lucchinetti et al. 2000; Reipert 2004).

Secondary progressive multiple sclerosis (SPMS)

SPMS is characterized by a steady progression of the patient's disability with or without superimposed relapses. The SPMS is associated with less inflammatory responses than the RRMS form but with a higher level of degeneration and axonal loss with accumulation of physical disability (Lublin, Reingold et al. 1996; Reipert 2004).

Progressive–relapsing MS (PRMS)

The disease is progressive from onset, with clear acute relapses, with or without full recovery; characterised by progression of the disease between relapses (Milo and Miller 2014).

With additional MS subtypes including (Milo and Miller 2014):

1. Clinically isolated syndrome (CIS): a first clinical episode indicatory of MS.
2. Radiologically isolated syndrome (RIS) or asymptomatic or pre-clinical MS: the incidental finding of typical MS lesions on MRI without signs of clinical disease.
3. Benign MS: the patients' 15 neurologic systems remain fully functional 15 years after onset.
4. Malignant (fulminant) MS: rapid progressive course, leading to disability or death in a relatively short time after onset.
5. Single-attack progressive MS: a rare condition is considered to be a subtype of SPMS with a single initial attack followed by the progressive phase.
6. Transitional MS: the transition phase between RRMS and SPMS.

1.3.3 Symptoms

MS patients can experience loss of any of the functions that are under the control of the central nervous system (CNS) and therefore clinical manifestations involve motor, sensory, visual and autonomic systems. Of course many other symptoms and signs can occur

(Cognitive impairment, hemisensory and motor, affective (mainly depression), epilepsy (rare), focal cortical deficits (rare), unilateral painful loss of vision, tremor, clumsiness and poor balance, diplopia, oscillopsia, vertigo, impaired swallowing, impaired speech and emotional lability, paroxysmal symptoms, weakness, stiffness and painful spasms, bladder dysfunction, erectile impotence, constipation, fatigue, temperature sensitivity and exercise intolerance) (Compston and Coles 2008). Other clinical features are Lhermitte's symptoms (an electrical sensation running down the spine or limbs on neck flexion) and Uhthoff phenomenon (transient worsening of symptoms and signs when core body temperature increases, such as after exercise or a hot bath) (Compston and Coles 2008). As the disease progresses, patients become disabled, their quality of life deteriorates and they may become an economic burden on society. Life expectancy of MS patients is at least 25 years from disease onset, and many patients die from unrelated causes (Compston and Coles 2002).

1.3.4 Diagnostic criteria

New diagnostic criteria for MS (The McDonald Diagnostic Criteria) (McDonald, Compston et al. 2001; Polman, Reingold et al. 2005), allow for safe and early diagnosis, avoiding incorrect diagnosis and allowing for early management before tissue injury compromises the daily activities of patients (Compston and Coles 2008). In some cases clinical evidence is sufficient, in some others when diagnosis is ambiguous, MRI visual evoked potentials and cerebrospinal fluid (CSF) analysis are necessary in order to make the diagnosis for MS (Compston and Coles 2008). Once diagnosis is confirmed, the disability can be monitored using Kurtzke Expanted Disability Status Scale (EDSS).

Kurtzke Expanted Disability Status Scale

The functional disability status (disease severity) of patients with MS can be measured using the EDSS, which includes eight Functional Systems (FS), which they are pyramidal, cerebellar, brainstem, sensory, bowel and bladder, visual, cerebral and 'other" (Kurtzke

1983; Reipert 2004). The EDSS, as well as the FS, are assigned a Functional System Score (FSS) by neurologists. Higher values indicate greater disability. Scales for the EDSS are from 0 to 10, in which the 0 score indicates no disability at all and 10 indicates death due to MS.

1.3.5 Pathogenesis of the disease

The course of pathogenesis, as suggested by Compston 2008, of MS can be summarised as follows:

The first event is lymphocyte-driven inflammation. This inflammation might impede the saltatory propagation of the action potential in three ways: soluble inflammatory mediators might cause a conduction block in structurally intact axons, demyelination or dysfunctional axonal transaction.

Microglia are activated, and contribute both to inflammation and to the repair process by the removal of myelin debris and promotion of remyelination (Barnett and Prineas 2004). If remyelination fails, demyelinated axons adapt by redistributing ion channels which might prove to be maladaptive and promote chronic neurodegeneration, and degenerating unmyelinated axons. Microglia can become chronically activated, in the absence of lymphocytic inflammation, in areas of normal appearing white matter and this may also lead to neuronal loss in later stages of the disease. In response to chronic tissue injury, astrocytes cause gliosis, which can then act as a mechanical barrier for repair. At the same time, peripheral macrophages are recruited to the brain as scavengers, amplifying the inflammatory response (Barnett, Henderson et al. 2006) while oligodendrocyte precursors cells are observed in the vicinity of the apoptotic process (Barnett and Prineas 2004). These cells mature into new oligodendrocytes and remyelinate the axons (van Rensburg, Kotze et al. 2012).

It is now known that the disease progression depends on the accumulated axon degeneration (Compston and Coles 2008). Whether pathogenesis of the disease involves demyelination preceding inflammation or is the other way round remains an unanswered question. Compston 2008 proposed four possible formulations in order to explain the relative contributions of acute and chronic axonal loss and their dependence on inflammation. A) Inflammation is the exclusive pathogenic event from which all else follows. B) Neurodegeneration occurs first and inflammation is purely a secondary response. C) Inflammation and neurodegeneration both contribute to the clinical course but are fully independent processes. D) Inflammation exposes an intrinsic neurodegenerative susceptibility that renders axons vulnerable to cumulative injury. The conclusion is that progression in MS is due to cumulative loss of axons, initiated and maintained by complex inflammatory responses acting on individuals who are inherently susceptible to neurodegeneration (Compston and Coles 2008).

Corthals 2011 proposes a new understanding of MS, by stating that MS is a dysfunction of lipid metabolism, involving mechanisms similar to those observed in atherosclerosis. In this way the pathophysiology, genetic susceptibility, and environmental triggers could be explained in MS (Corthals 2011).

1.3.6 Disease Mechanisms

As mentioned earlier the mechanisms involved in MS are: (a) immune-mediated inflammation (Owens 2003), (b) oxidative stress (Evans 1993; Knight 1997; Smith, Kapoor et al. 1999)and (c) excitotoxicity (Matute, Alberdi et al. 2001). These mechanisms may lead to processes such as inflammation, demyelination and remyelination, oligodendrocyte depletion and astrocytosis (Evans 1993; Knight 1997; Smith, Kapoor et al. 1999; Matute, Alberdi et al. 2001; Owens 2003). These processes will lead to the formation of the sclerotic plaque. The order and relation of these separate events have not yet been understood. Worth

noting is that the inflammatory activity in the CNS might precede clinical symptoms (Compston and Coles 2008).

1.3.7 Immunopathogenesis of Multiple Sclerosis

Whereas MS is typically considered a T-cell mediated autoimmune disease, with T cells appearing early in lesion formation, recent studies show that the pathogenesis of MS involves both the innate and adaptive immune systems, with slow progressive neurodegeneration and acute inflammation (Milo and Miller 2014; Dendrou, Fugger et al. 2015).

Oxidative stress

The cellular antioxidant defence mechanisms can be classified into two major groups: enzymatic (e.g., catalase, superoxide dismutase, hydrogen peroxidase) and nonenzymatic or low-molecular-weight antioxidants (LMWA). The LMWAs can further be classified into directly acting antioxidants (e.g., scavengers, and chain breaking antioxidants, (vitamin C and E, thiol-based antioxidants, flavonoids, polyphenols, curcumin, carotenoids)) and indirectly acting antioxidants (e.g., chelating agents) (van Meeteren, Teunissen et al. 2005; Mirshafiey and Mohsenzadegan 2009).

An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed 'oxidative stress' (Sies 1997). The main function of the reactive oxygen species (ROS), together with other defence mechanisms, is the defence against microbial agents and pathogens. ROS is involved in antigen processing and oxidation of target cells. However over-production of ROS, such as in the case of ischemia, inflammation, and the presence of catalytic iron ions, may become harmful by causing DNA mutations, lipid peroxidation, and protein oxidation (Mirshafiey and Mohsenzadegan 2009). Oxidative stress activates the stress response mechanism which is aims to protect cells against the over-production of ROS and includes transcriptional activation of various genes encoding antioxidant and detoxification enzymes via the nuclear factor-E2-related factor (Nrf2) and the antioxidant response element (ARE) pathways (Mirshafiey and Mohsenzadegan 2009).

The nervous system is for a number of reasons (biochemical, physiological, anatomical) vulnerable to reactive oxygen species (ROS) and reactive nitrogen species (RNS) and in order to counteract their effects in MS antioxidant treatment could be beneficial to patients. ROS and RNS are capable in causing injury to nervous system for many reasons as discussed in Evans et al. 1993, and Mirshafiey et al. 2009 (Evans 1993; Mirshafiey and Mohsenzadegan 2009) and summarised below:

A. High rate of oxidative metabolic activity.
B. High concentration of readily oxidisable substrates, in particular, membrane lipid PUFAs.
C. Low level of protective antioxidant enzymes, namely catalase and glutathione (GSH) peroxidase.
D. Endogenous generation of ROS by specific neurochemical reactions, for example, dopamine oxidation.
E. Specialised neuronal conduction and synaptic transmission activity dependent on efficient membrane function.
F. High ratio of membrane surface area to cytoplasmic volume.
G. Extended axonal morphology prone to peripheral injury.
H. Neural anatomical network vulnerable to disruption.
I. Non-replicating Neuronal cells.

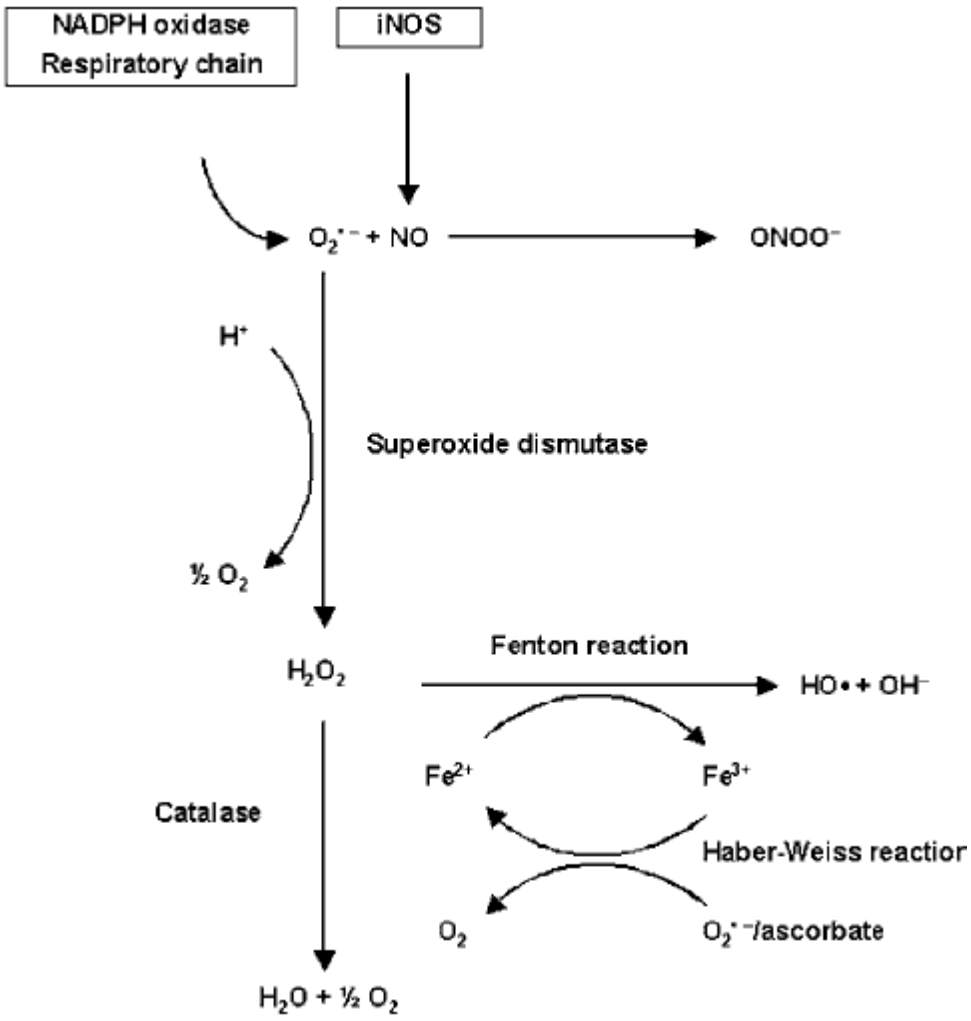

Figure 15. Interactions and converting enzymes in ROS and RNS produced during normal cell metabolism and oxidative stress (From Van Meeteren et al 2005).

ROS are produced primarily by mitochondria as side-products of cellular respiration. The side-products produced include superoxide (O_2^-) and hydroxyl ($HO^\bullet$) radicals and hydrogen peroxide (H_2O_2). Over production of O_2^- and H_2O_2 can result in tissue damage, which often involves generation of highly reactive $HO^\bullet$ and other oxidants in the presence of "catalytic" iron or copper ions (Halliwell 1992).

Production of O_2^- results from the transfer of an electron to oxygen by cytochrome *c* oxidase and flavin enzymes of the oxidative phosphorylation phase of cellular respiration (Lewén, Matz et al. 2000).

ROS and RNS are produced as shown in figure 15.

Superoxide dismutase (SOD) converts O_2^- into H_2O_2 and O_2. Activated macrophages during oxidative burst will produce increase amounts of ROS and RNS by upregulation of NADPH oxidase producing oxygen radical and inducible nitric oxide synthase (iNOS) (high levels of mRNA specific for iNOS can be observed in brain lesions from MS patients (Bo, Dawson et al. 1994; Bagasra, Michaels et al. 1995), producing NO radical.

Oxygen and nitric oxide radicals together will form peroxynitrite ($ONOO^-$), a very potent oxidant.

All these highly reactive species induce lipid peroxidation, affect DNA structure and react with cellular proteins via tyrosine nitration (van Meeteren, Teunissen et al. 2005).

When H_2O_2 is formed from the detoxification of O_2^- by SOD most of it is converted by catalase to H_2O and O_2, but some may escape into the cell when the H_2O_2 metabolising capacity of catalase is insufficient. Through Fenton chemistry, H_2O_2 will then be converted to $HO^\bullet$, the most reactive oxygen radical. Under certain conditions, formation of $HO^\bullet$, is through the nonenzymatic Haber–Weiss reaction, requiring free intracellular iron and O_2 or ascorbate (Halliwell 1992; Nappi and Vass 1997).

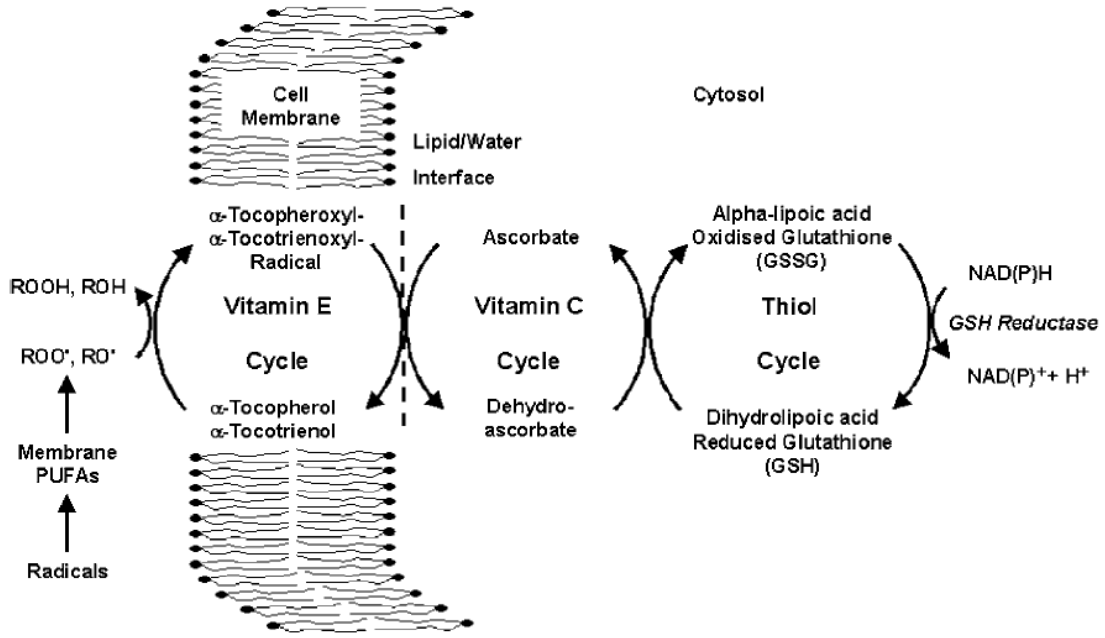

Figure 16. The mechanisms of cell protection from ROS. (From Van Meeteren et al 2005).

Mechanisms for protecting cells (see figure 16) from the toxic activity of ROS include the SOD-mediated conversion of O_2^- to H_2O_2, the enzymes catalase (CAT) and glutathione peroxidase (GSH-Px) that rapidly degrade H_2O_2 to H_2O, and various endogenous radical scavengers and reductants such as ascorbic acid, α-tocopherol (vitamin E), and the tripeptide thiol glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) (Nappi and Vass 1997).

ROS are potentially cytotoxic, but their increased production during infection is essential in order to destroy intracellular parasites or pathogens. Activated phagocytes manifest a respiratory burst which involves increased uptake of oxygen and its reduction to superoxide O_2^-, a reaction mediated by the plasma membrane-bound enzyme NADPH-oxidase which transfers electrons from NADPH to O_2. In addition to their role in defence reactions, ROS regulate signal transduction pathways, activate transcription factors, and control the expression of several genes concerned with growth, differentiation, and immune function (Nappi and Vass 1997).

Antioxidants and enzymes such as CAT and GSH-Px can convert H_2O_2 to H_2O, inhibiting the production of HO•. GSH provides reducing elements for GSH-Px activity, and NADPH for glutathione reductase GSH-Rx function. NADPH is regenerated from oxidized pyridine nucleotide NADP through various intracellular reactions including the oxidation of glucose 6-phosphate via glucose 6-phosphate dehydrogenase (Nappi and Vass 1997).

Damages by ROS/RNS on neural function have been described. These include: increased permeability of the blood-brain-barrier (BBB), inhibition of mitochondrial respiration and others. Also changes in the brain with age increase ROS production, and together with the compromised antioxidant defence mechanisms of the CNS and neuronal loss, will contribute to the increased vulnerability of the aged brain (Evans 1993).

Several reports show the involvement of ROS/RNS in the pathogenesis of MS (van Horssen, Drexhage et al. 2010; van Horssen, Witte et al. 2011); this is possible through the induction of lipid peroxidation, peroxidative damage to myelin, damage to the BBB with resulting migration of macrophages into the CNS. Macrophages also produce a variety of proinflammatory cytokines, inflammatory ROS, nitric oxide (NO); ROS enhance both monocyte adhesion and migration across the endothelial cells (Mirshafiey and Mohsenzadegan 2009) leading to demyelination, axonal damage, and disease progression (Evans 1993; Mirshafiey and Mohsenzadegan 2009). ROS also activates certain transcription factors, such as the nuclear transcription factor-kappa B (NF-kB) which upregulates the expression of many genes involved in MS, such as the tumor necrosis factor-α (TNF-α), the inducible nitric oxide synthase (iNOS), the intracellular adhesion molecule 1 (ICAM-1), and the vascular-cell adhesion molecule 1 (VCAM-1) (Lucas, Rodriguez et al. 2003). Redox reactions are involved in the activity of matrix metalloproteinases (MMP), which are important to T cell trafficking into the CNS (LeVine 1992; Lu, Selak et al. 2000), while the redox status regulates editing of the MHC class repertoire (Park, Lee et al. 2006),

which as mentioned before (see page 6) are important in the immune response in MS and established genetic factors.

Excitotoxicity

Excitotoxicity is the pathological process that can damage and kill nerve cells by excessive stimulation caused by neurotransmitters such as glutamate (Matute, Alberdi et al. 2001) (reference). Excitotoxicity occurs when receptors, such as the N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are overactivated by glutamate (Li and Stys 2000; Matute, Alberdi et al. 2001). This will allow high levels of calcium to enter the cell, which in turn activate enzymes such as phospholipases, endonucleases and proteases. These enzymes may eventually damage cell structures such as membranes and DNA (Matute, Alberdi et al. 2001).

Oligodendrocytes and other nerve cells are vulnerable to glutamate excitotoxicity which is the result of overactivation of glutamate receptors (GluRs) of the AMPA and kainate classes (Lipton, Epstein et al. 1994). This phenomenon has been implicated in acute injury to the CNS and in chronic neurodegenerative disorders (Lipton, Epstein et al. 1994). Oligodendrocytes' vulnerability to overactivation of GluRs may suggest that oligodendroglial excitotoxicity might also be involved in the pathogenesis of demyelinating diseases, oligodendrocyte cell death and inflammation (Lipton, Epstein et al. 1994). During inflammation large amounts of glutamate are released from activated immune cells which can lead to excitotoxicity (Matute, Alberdi et al. 2001). Cellular Ca^{2+} overload is one other important factor of neuronal death and it results from high extracellular glutamate generated after traumatic or ischemic injury of the CNS leading to the overstimulation of GluRs (Matute, Alberdi et al. 2001). It appears that CNS axons have repair mechanisms that are effective after an acute excitotoxic attack but are compromised in chronic attack (Matute, Alberdi et al. 2001). In MS patients, glutamate levels observed in the CSF are higher in acute

MS as compared to silent MS and controls. Extracellular glutamate levels increase during oxidative stress because ROS reduces the efficacy of glutamate transporters (Lipton, Epstein et al. 1994; Matute, Alberdi et al. 2001; van Meeteren, Teunissen et al. 2005).

1.3.8 Treatment

Currently there are a number of treatments for MS that are the products of reductionism and are partially effective with severe side effects (Pantzaris, Loukaides et al. 2013; Martinez-Altarriba, Ramos-Campoy et al. 2014). Since 1993 when beta-interferon (beta-IFN) 1b was approved by the USA Food and Drug Administration (FDA) and 1995 by the European Medicines Agency (EMA), nine other disease-modifying treatments (DMTs) have been approved by the FDA. The available DMTs are different in respect to the route and frequency of administration, adverse effects, tolerability, toxicity and treatment adherence. For the newer oral DMTs, long-term safety and efficacy are yet to be examined (Cross and Naismith 2014; Melzer and Meuth 2014).

The nine DMTs (see figure 17) approved by USA are beta-IFN 1a (two formulations), beta-IFN 1b, glatiramer acetate (GA), immune-modulatory drugs, the immunosuppressant mitoxantrone, the monoclonal antibody natalizumab, fingolimod, teriflunomide and dimethyl fumarate (Cross and Naismith 2014; Melzer and Meuth 2014).

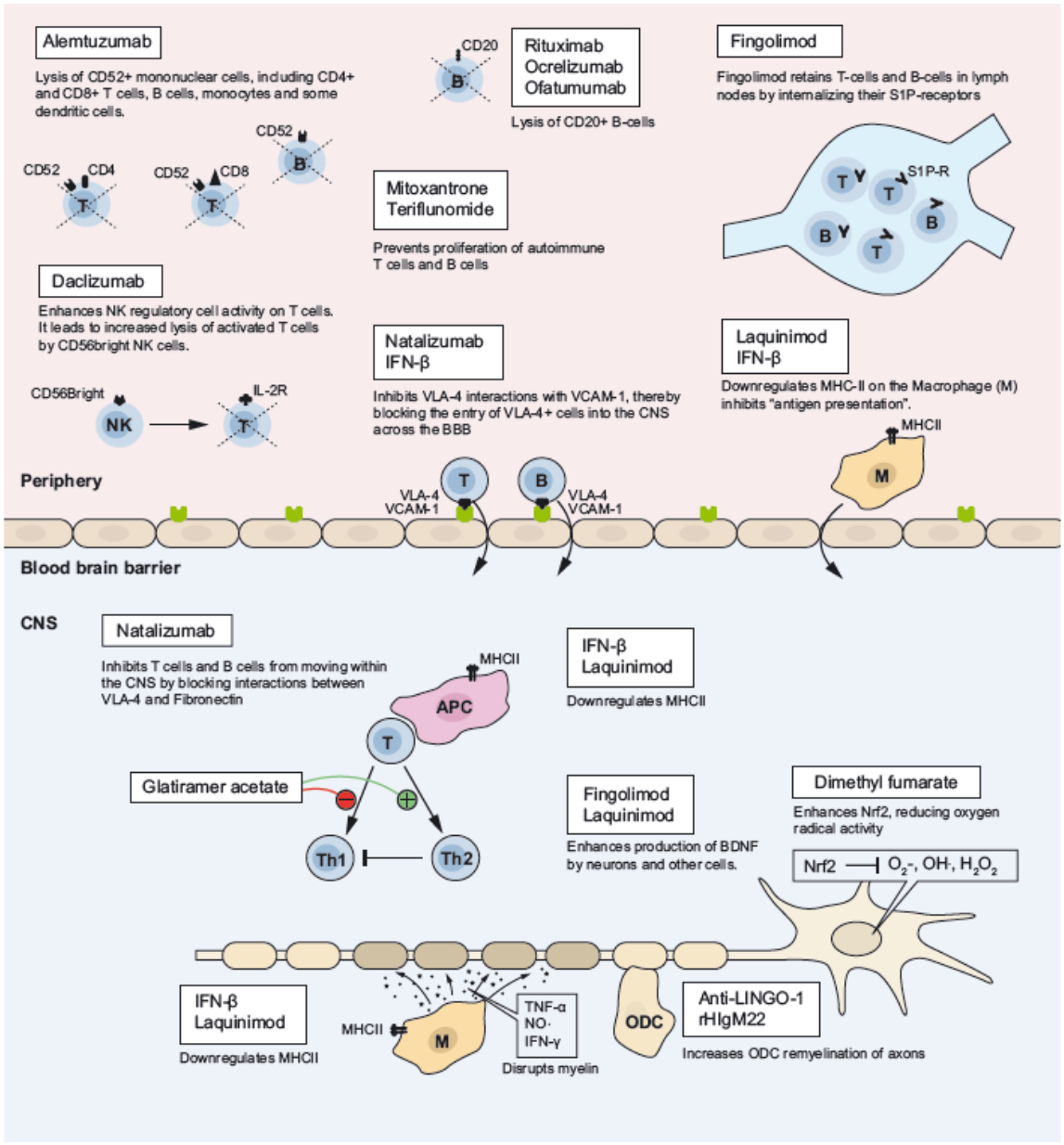

Figure 17. Established and novel disease-modifying treatments in MS and their site of action. (From Cross et al. 2014.)

For the acute treatment of RRMS (during MS relapses), corticosteroids are used which are anti-inflammatory (Compston and Coles 2008), however, there is not enough evidence for the long term efficiency of these drugs in delaying progression of long term disability in MS (Ciccone, Beretta et al. 2008).

Other medications in the pipeline are alemtuzumab, laquinimod, ocrelizumab and daclizumab. Antibody therapy drugs anti-LINGO-1 and anti-oligodenrocyte

immunoglobulin that may improve remyelination are being investigated for future therapies (Cross and Naismith 2014).

A number of animal studies have demonstrated the beneficial effect of mesenchymal stem cell (MSC) therapies in experimental autoimmune encephalomyelitis (EAE), an induced animal model of MS. Furthermore MSC therapies can alleviate symptoms and pathology of MS as was shown by clinical research (Bowles, Scruggs et al. 2014).

The experimental autoimmune encephalomyelitis (EAE) model is an experimental mouse model, considered to be similar to human MS (Harbige, Layward et al. 2000; Constantinescu, Farooqi et al. 2011). Using the EAE model the immune-pathological cascade of events of MS have been identified (immune regulation of CD4+ Th1/Th17 mediated tissue damage) (Miller, Karpus et al. 2007; Constantinescu, Farooqi et al. 2011).

EAE is a CD4+ T cell–mediated autoimmune disease model with characteristic perivascular CD4+ T cell and mononuclear cell inflammation and demyelination of axonal tracts in the CNS (Miller, Karpus et al. 2007); leading to progressive hind-limb paralysis (Miller, Karpus et al. 2007). Different strains are used that have different characteristics of the disease. For instance the SJL (H-2s) mouse strain is used to represent the disease in the relapsing remitting course of paralysis. C57BL/6 (H-2b) mice represent the chronic-progressive type, PL/J (H-2u) and B10.PL (H-2u) represent the acute form of the disease without clinical relapses (Miller, Karpus et al. 2007).

The activation of EAE consists of two phases: the induction and the effector phase. In the induction phase, priming of myelin epitope-specific CD4+ T cells with myelin proteins (PLP, MOG, MBP) in complete Freund's adjuvant. The effector phase is a phase of numerous stages, beginning with the initial migration of activated myelin-specific T cells to the CNS, followed by chemokine and cytokine production, activation of monocyte/macrophages and

microglia, which leads to demyelination of CNS axonal tracts from the cytotoxic effects of cytokines. The effector phase of the disease is represented by the adoptive-transfer model EAE model (Miller, Karpus et al. 2007).

Autologous haematopoietic stem-cell transplantation has been evaluated over the past few years as a possible new therapy in severe forms of multiple sclerosis. Since 1995 when it was first applied, more than 400 patients worldwide have been treated until today. Transplant-related mortality rates are now down to 1-2% from 5-6% five years ago, with relapses decreased and inflammatory activity almost completely suppressed as shown by MRI (Mancardi and Saccardi 2008). However its clinical efficacy has still to be proven as a larger number of patients and prospective controlled trials are needed (Mancardi and Saccardi 2008).

Due to the partial effectiveness of the available treatments and their side effects, MS patients make use of dietary supplements with the aim being to improve the disease outcome (Farinotti, Simi et al. 2007). Amongst the most common dietary interventions are gluten and milk free diets and supplementation with vitamins, micronutrients and antioxidants such as selenium, Gingko biloba extracts, coenzyme Q10 and PUFAs (Farinotti, Simi et al. 2007). Dietary interventions appear to favour the disease prognosis (Farinotti, Simi et al. 2007).

Moreover PUFA abnormalities have also been reported in patients with MS, especially of PUFAs involved in inflammatory processes. As a result, a substantial number of studies aimed to investigate the effects of supplementing MS patients with different PUFAs to determine their effects on the pathology of MS (Mehta, Dworkin et al. 2009; Pantzaris, Loukaides et al. 2013). However the evidence for the effectiveness of PUFAs is under investigation and as noted by Mehta et al 2009 several issues such as the duration of trials,

types of PUFAs, quantities and ratios need to be addressed further in order to prove their effectiveness on the disease prognosis (Mehta, Dworkin et al. 2009).

1.4 Trials of PUFAs in MS

A number of animal studies (Meade, Mertin et al. 1978; Hughes, Keith et al. 1980; Harbige, Yeatman et al. 1995; Harbige, Layward et al. 2000) have shown that LA and GLA could partially suppress or fully protect respectively from EAE induced animals. Furthermore several clinical trials, controlled (Millar, Zilkha et al. 1973; Bates, Fawcett et al. 1977; Bates, Fawcett et al. 1978; Paty, Cousin et al. 1978; Dworkin, Bates et al. 1984; Bates, Cartlidge et al. 1989; Gallai, Sarchielli et al. 1995; Weinstock-Guttman, Baier et al. 2005; Harbige, Pinto et al. 2008; Torkildsen Ø and et al. 2012) and non-controlled (Cendrowski 1986; Fitzgerald, Harbige et al. 1987; Swank and Dugan 1990; Nordvik, Myhr et al. 2000; Swank and Goodwin 2003) have investigated the effects of different PUFAs (EPA, DHA, GLA, LA, AA) on the disease progression, duration and severity of relapses. However their effects on the disease are inconclusive (Mehta, Dworkin et al. 2009). Therefore the need of conclusive results is of major importance. For instance Mehta et al 2009, placed first in the list "the question of appropriate clinical trial design". Either there were problems of selection bias, or disease activity differences among enrolled patients, or a miscellaneous number of patients that were in a different stage of the disease (relapsing remitting, primary or secondary progressive) or as a result of a small number of patients in the study. Duration of the trial was another issue that needed to be taken in consideration as reported by Mehta et al, 2009. Moreover, the different trials used different kind of PUFAs or used different concentrations as well as different ratios between the different kinds of lipids (Omega-3 vs. Omega-6). Therefore the need of an accepted clinical design/protocol according to the

international standards for outcome measures that can detect differences in the disease progression are pivotal for conclusive results (Mehta, Dworkin et al. 2009).

1.4.1 Aims of the study and hypothesis

For more than 70 years scientists are trying to correlate diet habits and multiple sclerosis (MS), mostly focusing on the omega-3/omega-6 polyunsaturated fatty acids and on specific vitamins. All nutrients have been tested for efficacy on disease evolution by decreasing the number and the severity of relapses and progression of disability (Farinotti, Simi et al. 2007). Several studies in USA, Canada, the Netherlands, Germany and Australia demonstrated that more than half of MS patients have been using complementary and alternative medicine as treatments, and mostly in the form of special diets /dietary supplements (Farinotti, Simi et al. 2007).

The mechanisms involved in the pathogenesis of MS include immune-mediated inflammation, formation of free radicals and oxidative stress. The products of oxidative stress, such as peroxynitrite and superoxide (reactive oxygen species (ROS)) have been reported as toxic to neurons (Farinotti, Simi et al. 2007). Free radical formation as well as cellular injury from free radical formation may be decreased by the antioxidant activity of vitamins A, C and E. Hence these vitamins have been used in many trials in order to be tested for their effectiveness in the course of MS (Gilgun-Sherki, Melamed et al. 2004; Ferretti, Bacchetti et al. 2005; Koch, Ramsaransing et al. 2006).

Several clinical trials using PUFAs that have been conducted in order to investigate their effect on the disease and the results have been inconclusive (Mehta, Dworkin et al. 2009) which further strengthens the need of conclusive results. Mehta et al. 2009 listed "the question of appropriate clinical trial design" as the first thing that needs to be addressed. Selection bias, disease activity differences among enrolled patients, patients that are in a

different stage of the disease (relapsing remitting, primary or secondary progressive) and a small number of patients enrolled in the study are potentially flaws in the experimental design. Duration of the trial is another issue that needed to be taken in consideration as reported by Mehta et al, 2009. Moreover, the different trials used different kind of PUFAs or used different concentrations as well as different ratios between the different kind of lipids (Omega-3 vs. Omega-6). Therefore the need for an accepted clinical design/protocol according to the international standards for outcome measures that can detect differences in the disease progression is pivotal for conclusive results.

The hypothesis of the present investigation is that supplementing MS patients with a combination of both omega-3 and omega-6 in a specific ratio 1:1 and in a total amount and ratio between EPA: DHA 1:3 omega-3 and with a ratio of GLA: LA 1:2 omega-6, and accompanied by the presence of gamma tocopherol (Vitamin E), relapse episodes and accumulation of disability in those patients will be significantly decreased.

The specific objective of the study is the quantitative and qualitative analysis of the incorporated PUFA phospholipids on RBC membranes as a result of the consumption of the specific intervention formulas. Blood was collected at time zero and at every six-month intervals for a total of 24 months. The analyzed data will be correlated to the MS disease progression by monitoring the mean annual relapse rate and the EDSS score.

CHAPTER TWO

ADHERENCE TO THE MEDITERRANEAN DIETARY PATTERN OF THE MS PATIENTS

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2.1 Introduction

For many years now dietary factors have been implicated as a possible cause for MS (Schwarz and Leweling 2005). Fat consumption in relation to its influence on MS has been the subject of research for more than 50 years (Payne 2001).

An association between incidence of MS and saturated fat intake is suggested in population-based epidemiological studies (Tola, Granieri et al. 1994). Other dietary products that were also found to have an association with MS are sweets, alcohol, smoked meat products, coffee and tea (Antonovsky, Leibowitz et al. 1965; Berr, Puel et al. 1989; Sepcic, Mesaros et al. 1993; Tola, Granieri et al. 1994). However Habek et al 2010 reported that these finding have not been confirmed by subsequent studies (Habek, Hojsak et al. 2010). The effects of the duration of breast feeding and MS were also investigated by two retrospective studies, however the results were inconclusive (Spencely and Dick 1982; Pisacane, Impagliazzo et al. 1994). Furthermore Isaacs et al in 2010 supported the hypothesis that constituents in breast milk have an effect on the development of white matter (Isaacs, Fischl et al. 2010). On the other hand it is of a great importance to note that the CNS and myelin sheath are made up of 70% lipids, one third of which are PUFAs with an ω-6: ω-3 ratio of 1:1 (Crawford, Budowski et al. 1979; Schwarz and Leweling 2005).

MS can be elicited by environmental factors in combination with a complex genetic risk profile (Compston and Coles 2008). The observed latitude gradient of MS incidence with increasing number from the equator to the north and south is now disappearing, however birth place remains a risk factor (Alonso and Hernán 2008). Environmental factors include infectious agents, with the Epstein-Barr virus being the most prominent (Ascherio and

Munger 2007). A low maternal exposure to ultraviolet radiation in the first trimester was inversely associated with increased risk of multiple sclerosis in offspring (Willer, Dyment et al. 2005; Staples, Ponsonby et al. 2010). Cigarette smoke, stressful life events, malnutrition and obesity at the age of 18-20, all seem to have a positive association and subsequent exacerbation in MS (Riise, Nortvedt et al. 2003; Mohr, Hart et al. 2004; Munger, Chitnis et al. 2009; von Geldern and Mowry 2012).

It is of paramount importance when investigating the effects of diet on MS, to take into account that individuals do not consume single foods but a combination of several foods that contain nutrient and non-nutrient substances. Furthermore due to the complexity of the human diet, the nutrient-to-nutrient interactions, the correlation of intake of some nutrients with the intake of others, and the effects that nutrient could have in the human organism, makes it difficult to come to a conclusion regarding the effects of consuming a single nutrient or a food group or a food supplement on a specific health outcome and may perhaps be even eventually misleading. It is therefore useful to examine patterns of nutrient intake, in this way several related aspects of dietary intake can be looked at simultaneously (Kant 1996; Velie, Schairer et al. 2005).

The "Mediterranean diet" is a dietary pattern that has been the subject of many studies and it is well accepted to be a well-balanced diet (Panagiotakos, Pitsavos et al. 2007). The Mediterranean diet became widely known for the first time in the 1980s from the "Seven Countries Study" (Keys, Menotti et al. 1986). Since then, the Mediterranean diet has been at the centre of numerous studies that indicate that implementation of such a diet is associated with decreased all-caused mortality and improvements in cardiovascular risk factors levels (Panagiotakos, Pitsavos et al. 2007).

Despite the variation (different religions, economies and cultures) between the Mediterranean countries and within individual countries themselves, the Mediterranean diet has common characteristics such as high consumption of fruits, vegetables, minimally processed cereals, potatoes, legumes, nuts and seeds; low to moderate consumption of dairy products, eggs, and poultry; infrequent consumption of red meat; moderately high intake of fish; high intake of monounsaturated lipids, with oleic acid being the major fat from olive oil. All these lead to a favourable balance of fatty acids i.e. decreased consumption of saturated fat, increased consumption of MUFAs and higher consumption of ω-3 PUFAs in relation to ω-6 PUFAs. Wine is consumed in low to moderate amounts (Binukumar and Mathew 2005; Couto, Boffetta et al. 2011; Demetriou, Hadjisavvas et al. 2012; Buckland, Travier et al. 2013).

The Mediterranean diet pattern includes foods that provide anti-oxidants such as flavonoids, carotenoids, and antioxidant vitamins (D, C, E), phytochemicals that include phytoestrogens and polyphenols, sufficient quantities of fiber, adequate folate, and a favourable fatty acid profile (Couto, Boffetta et al. 2011). All these nutrients could have a potential capability of influencing the course of MS (von Geldern and Mowry 2012).

Furthermore conclusions from several epidemiological studies reveal that Greece and especially on the Island of Crete, have the lowest deaths rate amongst the other countries of the basin. Furthermore incidence of cancer and heart disease led to almost three times as many deaths proportionally in the United States as in Crete (Simopoulos 2001). The Cretan diet is rich in the following nutrients: (1) Balanced intake of essential fatty acids from vegetable, animal and marine sources; a ratio ω-6: ω-3 of 2:1 instead of a 15:1 in Western and Northern Europe and 16.74:1 in the USA. Animals in Crete grazed rather than feeding on grains; in this way meat, milk and cheese contain ω-3 PUFAs. In Crete, people also consume wild plants which are rich in both ω-3 PUFAs and vitamin E for e.g. purslane.

Furthermore chicken are also fed with purslane and other kinds of grass and as a result eggs contain a ratio of ω-6: ω-3 of 1.2 as compare to the USA with a ratio of 19.4. (2) Rich in antioxidants such as: vitamin C, vitamin E, vitamin b-carotene, glutathione, resveratrol, selenium, phytoestrogens, folate, and other phytochemicals from green leafy vegetables; phenolic compounds from wine and olive oil; in cooking vegetables. meat and fish, high consumption of tomatoes, onions, garlic and herbs especially oregano, mint, rosemary, parsley and dill, which contain lycopene, allyl thiosulfinates, salicylates, carotenoids, indoles, monoterpenes, polyphenols, flavonoids and other phytochemicals (Simopoulos 2001).

There are 16 countries within the Mediterranean basin with diets that differ from one another, as well as different dietary patterns within different regions of one country. A number of studies revealed a geographic pattern of MS in the Mediterranean and Near East; high to medium range prevalence rates (> 20 per 100 000) in southern Europe, Israel, and Cyprus, low rates (< 10 per 100 000) in Libya, Tunisia, Saudi Arabia, and native Kuwaitis (Lauer 1991). In a study by Lauer in 1991, it was attempted to relate MS with the Mediterranean diet by comparing countries with high and low prevalence rates but no conclusive result was reached, with the exception of smoked and cured meat, something that was also observed in relation to the high risk of MS in the Orkney, Shetland and Faroe Islands (Lauer 1991).

The use of dietary scores is commonly used in epidemiological studies in order to assess how closely a subject's diet adheres to the Mediterranean diet pattern. A food frequency questionnaire (FFQ) was designed to evaluate whether the degree of adherence to the Mediterranean diet pattern is associated with decreased breast cancer (BC) risk amongst Greek-Cypriot women in the MASTOS (the Greek word for breast) study (Demetriou, Hadjisavvas et al. 2012).

2.2 Method

Twenty healthy individuals, 13 female and 7 male, which in their knowledge don't suffer from any known disease, were recruited. These individuals were between the ages of 22-57 and were given a food frequency questionnaire (FFQ) which was designed and used previously with another study comparing dietary patterns in Cypriot women with and without breast cancer (MASTOS ((Demetriou, Hadjisavvas et al. 2012)). Blood was also collected for fatty acid analysis of RBC membranes. The collection of blood and the fatty analysis is described in the method on chapters 3 and 4.

Data were collected using a standardized interview and with the use of a specially designed FFQ. The FFQ was then scored with two different scoring systems as discussed below. The FFQ was designed in order to assess consumption of food items that their consumption is of a higher contribution amongst the Greek Cypriot population for energy and other nutrients. The FFQ was also designed to provide information for an individual's habitual food intake over the previous year. Frequency of consumption for each food item was reported as number of portions daily, weekly or monthly, rarely consumed, or never consumed. The questionnaire also included questions on the kind of oil used while cooking, whether olive oil was used in salads, and what kinds of spreads were used on bread. Portion sizes for the foods consumed were assessed by showing subjects, pictures of standard medium portions. These specific portions shown were chosen form the "A photographic atlas of food portion sizes" (Nelson, Atkinson et al. 1997). Thirty-two food items were included in the questionnaire under eight distinct categories i.e. fruit and vegetables, fish, meat, meat products and substitutes, cereals, dairy, agricultural products and sweets.

Panagiotakos et al 2007 were the first to assess adherence to the Mediterranean diet pattern using a diet score (Panagiotakos, Pitsavos et al. 2007). This score incorporates the essential

characteristics of the Mediterranean diet pattern as well as its relationship with cardiovascular disease risk and biological markers (Panagiotakos, Pitsavos et al. 2007).

The score includes the weekly consumption of 9 food groups: non-refined cereals, potatoes, fruit, vegetables, legumes, fish, red meat and products, poultry, full fat dairy products as well as olive oil in cooking, and alcohol intake. Table 3 describes the scoring system for the level of consumption of each group with a score that ranges from 0-55. Higher values of this diet score indicate greater adherence to the Mediterranean diet, in particular, ratings from 0 to 5 or reverse in each of the food groups according to their position in the Mediterranean pyramid. Zero was assigned when no consumption was reported for food groups that their suggested consumption is either on a daily basis or more than 3 times a week. 1-5 for rare to daily consumption (this includes: cereals, fruits, vegetables, legumes, olive oil, fish and potatoes). On the other hand food groups that their consumption is suggested to be rare or monthly (this includes: meat and meat products, poultry and full fat dairy products) was assigned scores on a reverse scale. A score of five was assigned when no consumption was reported and 0 when daily consumption was reported. In the case of alcohol consumption, Panagiotakos et al. 2007, uses a monotonic function, a score of 5 was used for a 300 ml or less of alcohol consumption per day, a score 0 for no consumption or for consumption above 700 ml per day and score 4 to 1 for consumption of 600-700, 500-600, 400-500 and 300-400 ml per day (12g ethanol per 100ml). In order for the results from the 20 healthy individuals to be comparable with the MASTOS FFQ results alcohol consumption score were assume to be given 5 for zero consumption and 0 for highest consumption; despite the fact that some alcohol consumption is beneficial to cardiovascular disease (as in the Panagiotakos study).

Table 3. The Mediterranean Diet Score by Panayiotakos et al. as modified for the present study.

How often	**Frequency of consumption (servings/week or otherwise stated)**					
All cereals (bread, pasta, rice etc.)	Never	1-6	7-12	13-18	19-31	>32
	0	1	2	3	4	5
Potatoes	Never	1-4	5-8	9-12	13-18	>18
	0	1	2	3	4	5
Fruits	Never	1-4	5-8	9-15	16-21	>22
	0	1	2	3	4	5
Vegetables	Never	1-6	7-12	13-20	21-32	>33
	0	1	2	3	4	5
Legumes	Never	<1	1-2	3-4	5-6	>6
	0	1	2	3	4	5
Fish	Never	<1	1-2	3-4	5-6	>6
	0	1	2	3	4	5
Red Meat Products	≤1	2-3	4-5	6-7	9-10	>10
	5	4	3	2	1	0
Poultry	≤3	4-5	5-6	7-8	9-10	>10
	5	4	3	2	1	0
Full fat dairy products (cheese, yogurt, milk)	≤10	11-15	16-20	21-28	29-30	>30
	5	4	3	2	1	0
Use of olive oil	Never	Rare	<1	1-3	3-5	Daily
	0	1	2	3	4	5
Alcohol (ml/day)	0	<300	400	500	600	>700
	5	4	3	2	1	0

It was not possible to calculate the energy intake due to the short nature of the FFQ and the grouping of several different foods in the same food group category. Nutrient intake could not be estimated with accuracy for the same reason. Diet scores were based on food group intakes and did not involve any nutrient component. For the purpose of this study the original diet score categories were modified for the analysis. For instance in the cereals category, both refined and unrefined cereals were included in the same category as nutritional information on types of cereals was not available. Unrefined and refined cereals differ mostly in their fibre content but since nutrient measures were not estimated they were grouped together for the purpose of this study. Olive oil in cooking includes olive oil usage as a salad dressing since olive oil is the most common dressing used for salads amongst Greek Cypriots.

A diet score has also been developed by Martinez-Gonzalez (Martinez-Gonzalez, Fernandez-Jarne et al. 2004). This score is a quicker and simpler nutritional assessment tool developed to estimate quantitatively adherence to the Mediterranean diet in correlation to its cardio-protective elements. In table 4, the nine items that were in the diet score are shown.

The resulting combined score ranged from 0 to 9 points. The original score component was modified for the purpose of this study substituting wine consumption with any alcohol consumed and in component 9 pasta, rice and potatoes were included since the Mediterranean diet is characterized with high consumption of cereals which includes pasta and potatoes. Consumption of one to three portions of any cereal daily was scored with +1 and lower or higher amounts were scored with zero.

Table 4. Dietary items included in the Martinez-Gonzalez Score as modified for the present study.

	Yes
1. Olive Oil (≥ 1 spoon/day)	+1
2. Fruit (≥ 1 serving/day)	+1
3. Vegetables or Salad (≥ 1 serving/day)	+1
4. Fruit (≥ 1 serving/day) and Vegetables (≥ 1 serving/day)[a]	+1
5. Legumes (≥ 2 servings/week)	+1
6. Fish (≥ 3 servings/week)	+1
7. Any alcohol (≥ 1 serving/day)	+1
8. Meat (<1 serving/day)	+1
9. All cereals (Bread, rice etc.) (1-3 servings/day)	+1

[a] One point is added when ≥ serving/day of both fruits and vegetables is consumed

The FFQ were then analysed based according to Panagiotakos et al. and Martinez et al. (Martinez-Gonzalez, Fernandez-Jarne et al. 2004; Panagiotakos, Pitsavos et al. 2007).

2.3 Statistical Analysis

A two-tailed t-test statistical analysis was performed with a Bonferonni correction for comparison of PUFA concentration between the control and the MS groups. SPSS Statistics V. 20 (IBM) for statistical analysis was used.

2.4 Results

The results for the FFQ were collected and presented in table 5, 6 and 7.

Table 5. The Mediterranean Diet score by Panagiotakos and Martinez-Gonzales among the study population.

Mediterranean Diet Score – Panagiotakos	**n=20 (%)**
≤30	5 (25)
31-35	5 (25)
36-40	6 (30)
>40	4 (20)
Mediterranean Diet Score – Martinez-Gonzales	
0-4	5 (25)
5-6	5 (25)
6-7	7 (35)
8-9	3 (15)

50% of the subjects have a score of above 35 using the Panagiotakos Mediterranean diet score, and more than 50% of the subjects scored above 6 using the Martinez-Gonzalez score (Table 5).

Table 6. The Mediterranean Diet score by Panagiotakos and Martinez-Gonzales among the study population by gender.

Mediterranean Diet Score – Panagiotakos	n=20 (%)	Female	Male
≤30	5 (25)	2	3
31-35	5 (25)	3	2
36-40	6 (30)	5	1
>40	4 (20)	3	1
Mediterranean Diet Score – Martinez-Gonzales			
0-4	5 (25)	3	2
5-6	5 (25)	2	3
6-7	7 (35)	5	2
8-9	3 (15)	3	0

Table 7. The mean intake and SD of the 11 food indices used to construct the Mediterranean Diet score by Panagiotakos.

	Food Indexes	Mean Intakes and SD, n=20
1.	All cereals	17.35±6.48
2.	Potatoes	1.75±0.70
3.	Fruits	9.45±4.60
4.	Vegetables	5.70±1.90
5.	Legumes	2.75±1.76
6.	Fish	1.55±0.92
7.	Red meat and products	3.10±2.14
8.	Poultry	1.90±0.62
9.	Full fat dairy products	8.20±5.29
10.	Use of olive oil (times/week)	6.90±1.73
11.	Alcohol	24.80±26.60
	Mediterranean Diet Score	34.85±5.51

Table 8 shows the fatty acid concentrations of the phosphatidylcholine (PC) fraction of RBCs, for both healthy controls (n=20) and MS patients' (Intervention group) at recruitment (n=40). Significance was found in the following fatty acids: C14:0, C16:0, C20:0, C24:0, C18:1n-9, C20:3n-9, C20:2n-6, C20:5n-3, C16:0 DMA, C18:0 DMA (higher percentage concentration in the controls than in patients), C18:1n-7, C20:1n-9, C18:2n-6, C18:3n-6, C22:4n-6, C18:3n-3 (higher percentage concentration in the patients than in controls).

Table 8. Fatty acid concentration for PC fraction of RBCs.

RBC PC				
	Intervention Groups (n=40)Time Zero		Healthy Controls (n=20)	
	Mean	SD	Mean	SD
[a]C14:0	0.32	±0.08	0.22	±0.09
[a]C16:0	33.17	±1.78	35.77	±2.24
C18:0	14.97	±1.33	14.07	±1.35
[b]C20:0	0.06	±0.03	0.14	±0.09
C22:0	0.06	±0.05	0.12	±0.10
[c]C24:0	0.05	±0.04	0.15	±0.10
Σ Saturates	48.59	±3.27	50.47	±3.98
C16:1n-7	0.21	±0.07	0.22	±0.09
[a]C18:1n-7	1.48	±0.22	1.14	±0.24
[a]C18:1n-9	14.14	±1.02	15.86	±1.45
[d]C20:1n-9	0.24	±0.03	0.20	±0.05
C22:1n-9	0.03	±0.02		
C24:1n-9	0.05	±0.04	0.09	±0.03
Σ Monoenes	16.15	±1.39	17.53	±1.86
[a]C20:3n-9	0.03	±0.02	0.11	±0.06
[e]C18:2n-6	21.70	±1.96	19.70	±1.42
[a]C18:3n-6	0.16	±0.03	0.07	±0.04
[a]C20:2n-6	0.21	±0.06	0.29	±0.08
C20:3n-6	1.96	±0.57	2.03	±0.57
C20:4n-6	7.39	±0.88	6.74	±0.84
[a]C22:4n-6	0.43	±0.20	0.31	±0.09
C22:5n-6	0.21	±0.08	0.17	±0.10
N-6 Metabolites	10.37	±1.82	9.60	±1.72
Σ N-6	32.07	±3.78	29.30	±3.13
[a]C18:3n3	0.33	±0.05	0.09	±0.04
C20:3n3			0.06	±0.03
[b]C20:5n3	0.23	±0.10	0.35	±0.17
C22:5n3	0.36	±0.13		
C22:6n3	1.46	±0.50	1.20	±0.37
N-3 Index	1.69	0.60	1.56	0.53
N-3 Metabolites	2.05	±0.72	1.62	±0.57
Σ N-3	2.38	±0.78	1.71	±0.60
[a]16:0 DMA	0.18	±0.10	0.43	±0.14
[a]18:0 DMA	0.10	±0.07	0.20	±0.07
18:1 DMA	0.06	±0.02		

[a] C14:0, C:16:0, C18:1n-7, C18:1n-9, C20:3n-9, C18:3n-6, C20:2n-6, C22:4n-6, C18:3n-3, C16:0 DMA, C18:0 DMA p=≤0.001; [b] C20:0, C20:5n-3, p=0.004; [c] C24:0, p=0.033; [d] C20:1n-9, p=0.017, [e] C18:2n-6, p=0.011

Table 9 shows the fatty acids concentrations of the phosphatidylethanolamine (PE) fraction of RBCs for both healthy controls (n=20) and MS patients' (Intervention groups) at recruitment (n=40). Significance was found in the following fatty acids: C18:0, C20:0, C20:3n-9, C20:4n-6, C16:0 DMA, C18:0 DMA, C18:2 DMA (higher percentage concentration in the controls than in patients) C18:1n-7, C18:2n-6, C20:3n-6, C20:5n-3, C22:6n-3, (higher percentage concentration in the patients than in controls).

Table 9. Fatty acid concentration for PE fraction of RBCs.

RBC PE				
	Intervention Groups (n=40)Time Zero		**Healthy Control (n=20)**	
	Mean	**SD**	**Mean**	**SD**
C14:0	0.16	±0.13	0.09	±0.05
C16:0	14.10	±0.77	15.23	±2.45
[a]C18:0	8.17	±0.76	10.28	±1.08
[a]C20:0	0.08	±0.04	0.17	±0.10
C22:0	0.14	±0.03	0.13	±0.07
C24:0	0.15	±0.03	0.17	±0.11
Σ Saturates	22.80	±1.76	26.09	±3.85
C16:1n-7	0.24	±0.20	0.16	±0.10
[a]C18:1n-7	0.97	±0.31	0.39	±0.11
C18:1n-9	15.87	±1.24	15.60	±1.38
C20:1n-9	0.51	±0.36	0.33	±0.09
C22:1n-9	0.04	±0.03		
C24:1n-9	0.12	±0.12	0.17	±0.14
Σ Monoenes	17.74	±2.25	16.66	±1.81
[b]C20:3n-9	0.08	±0.09	0.11	±0.08
[a]C18:2n-6	6.48	±1.13	5.04	±0.78
C18:3n-6	0.13	±0.12	0.15	±0.09
C20:2n-6	0.25	±0.08	0.32	±0.08
[a]C20:3n-6	1.11	±0.25	0.91	±0.18
[a]C20:4n-6	19.04	±0.88	23.33	±1.47
C22:4n-6	6.54	±0.93	6.30	±0.94
C22:5n-6	0.75	±0.31	0.64	±0.18
N-6 Metabolites	27.82	±2.57	31.66	±2.94
Σ N-6	34.31	±3.70	36.70	±3.72

C18:3n3	0.35	±0.43	0.12	±0.07
C20:3n3			0.08	±0.04
[a]C20:5n3	1.98	±0.20	0.64	±0.31
C22:5n3	3.14	±0.60		
[c]C22:6n3	6.38	±0.64	5.13	±1.28
N-3 Index	8.35	0.84	5.77	1.59
N-3 Metabolites	11.50	±1.44	5.84	±1.63
Σ N-3	11.85	±1.88	5.96	±1.71
[a]16:0 DMA	3.03	±0.66	4.63	±0.93
[d]18:0 DMA	6.79	±0.75	7.94	±1.45
18:1 DMA	1.34	±0.26		
[e]18:2 DMA	0.11	±0.05	0.26	±0.07

[a] C18:0, C20:0, C18:1n-7, C18:2n-6, C20:3n-6, C20:4n-6, C20:5n-3, C16:0 DMA p=≤0.001
[b] C20:3n-9, p= 0.047; [c] C22:6n-3, p=0.002; [d] C18:0 DMA, p=0.033; [e] C18:2 DMA, p=0.002

In table 10 are presented the fatty acids' concentrations of the sphingomyelin (SM) fraction of RBC for both healthy controls (n=20) and Multiple Sclerosis patients' (Intervention group) at recruitment (n=40). Significance was found in the following fatty acids: C18:0, C20:0, C22:0, C22:4n-6, C20:5n-3, C22:6n-3 (higher percentage concentration in the controls than in patients). C24:0, C18:1n-9, C24:1n-9, C18:2n-6, C20:2n-6, (higher percentage concentration in the patients than in controls).

Table 10. Fatty acid concentration for SM fraction of RBCs.

RBC SM				
	Intervention Groups (n=40)Time Zero		Healthy Control (n-20)	
	Mean	SD	Mean	SD
C14:0	0.26	±0.22	0.21	±0.12
C16:0	25.58	±2.38	25.75	±4.15
[a]C18:0	12.92	±1.53	17.90	±2.52
[b]C20:0	1.51	±0.25	1.81	±0.39
[a]C22:0	4.32	±0.59	6.02	±1.23
[c]C24:0	17.03	±0.97	15.13	±3.38
Σ Saturates	61.62	±5.94	66.81	±11.79
C16:1n-7	0.17	±0.03	0.15	±0.11
C18:1n-7	0.27	±0.10	0.31	±0.15
[a]C18:1n-9	6.44	±1.25	3.36	±1.16
C20:1n-9	0.20	±0.04	0.20	±0.07
C22:1n-9	0.44	±0.08		
[a]C24:1n-9	17.20	±1.60	13.56	±2.22
Σ Monoenes	24.72	±3.11	17.59	±3.71
C20:3n-9	0.11	±0.10	0.14	±0.12
[a]C18:2n-6	3.58	±0.71	2.10	±0.67
C18:3n-6	0.14	±0.15	0.09	±0.06
[d]C20:2n-6	0.48	±0.10	0.39	±0.18
C20:3n-6	0.41	±0.19	0.47	±0.18
C20:4n-6	4.92	±0.50	5.84	±2.32
[a]C22:4n-6	0.53	±0.18	1.51	±0.82
C22:5n-6			0.60	±0.19
N-6 Metabolites	6.48	±1.12	8.90	±3.74
Σ N-6	10.06	±1.83	10.99	±4.42
C18:3n3	0.04	±0.02		
C20:3n3				
[e]C20:5n3	0.21	±0.08	0.34	±0.12
C22:5n3	1.05	±0.12		
[a]C22:6n3	1.41	±0.32	3.01	±0.94
N-3 Index	1.62	0.41	3.35	1.06
N-3 Metabolites	2.67	±0.53	3.35	±1.06
Σ N-3	2.71	±0.55	3.35	±1.06
16:0 DMA	0.14	±0.10	0.18	±0.06
18:0 DMA	0.37	±0.41	0.30	±0.08

[a] C18:0, C22:0, C18:1n-9, C24:1n-9, C18:2n-6, C22:4n-6, C22:6n-3, p=<0.001; [b] C20:0, p=0.016; [c] C24:0, p=0.009; [d] C20:2n-6, p=0.002; [e] C20:5n-3, p=0.024

2.5 Discussion

Analysis of the results of the FFQ showed that 40% adhere to the Mediterranean diet scoring above 35 of the Panagiotakos diet score and above 6 of the Martinez-Gonzales diet score. This supports the findings of Klaus Lauer in 1991 who tried to correlate MS with Mediterranean diet however with no conclusive results (Lauer 1991).

It would be expected that because of the constituents in the Mediterranean diet the MS incidence in Cyprus would be similar to that of Japan or another Mediterranean country, for example Malta. It turns out that this is not the case. On the other hand somebody might argue that because of MS, patients changed their dietary pattern to a healthier Mediterranean diet. One could also argue that following Mediterranean diet would have a better outcome in the development of the disease. This probably will require a better controlled trial-design since an individual's diet is not an easy parameter to control.

The dietary pattern of the twenty healthy individuals is a pattern that resembles patterns observed in the Mediterranean diet and is the same pattern observed in the MASTOS study where the investigators reached the same conclusion. This can lead to a tentative result that Mediterranean diet pattern is not protective to MS.

The results in combination with those from the MASTOS study could be used to discuss whether dietary patterns have any effect on the incidence of MS amongst the Greek-Cypriot population. The results from both studies showed that the majority of the participants (over two thirds) adhered to the Mediterranean dietary pattern. For this reason we can safely assume that there should be no significant dietary differences between the participants of the MS study. Although in the MASTOS study only women were recruited, this should not be a limitation for two main reasons: Firstly, there is a gender bias in MS where the incidence ratio of females to males is 2-3:1. Secondly, in Cyprus, one can assume that the majority of

food purchasing and preparation in the average household is being done by females (anecdotal observation). Therefore, the MASTOS results are transferable to the study presented here and can provide enough information on the dietary patterns of MS patients. Overall, these twenty healthy volunteers have a ratio female to male 2:1 close to MS incidence in the island and worldwide and the dietary pattern doesn't differ from that of MASTOS.

The results from the analysis of the percentage concentration of the fatty acids of the RBC membranes between the control and the MS group showed statistical significance difference for some FA in fractions PC, PE, and SM (see tables 8-10); For instance there was significant lower percent composition of the AA of the PE fraction of the MS population compared to healthy control. This goes along with the observations of other researchers that reported decreased concentrations of this FA in the plasma, platelets, erythrocytes, leucocytes and cerebrospinal fluid in MS patients and other neurological conditions (Homa, Belin et al. 1980; Cherayil 1984; Fisher, Johnson et al. 1987; Holman, Johnson et al. 1989; Navarro and Segura 1989). As far as the LA is concerned there were no statistical differences found. This however is in contrast with the above observations. This different finding could be explained to the different dietary habits of Cypriots or it could be that the number of both patients and controls is not high enough for statistical significand results. Furthermore, methodological differences could also account for the non-significant differences. Worth noting that the insufficiency in LA and AA which was described by different researchers in plasma, platelets, RBC, leucocytes and cerebrospinal fluid is also accompanied with changes in the unsaturated fatty acid composition of brain white matter in MS patients when compared to healthy individuals (Navarro and Segura 1988; Harbige and Sharief 2007). In addition, Thompson 1973 proposed an inborn error in the metabolism of the essential fatty acids as a contributing factor of MS (Thompson 1973). Love et al in 1974 consider that the decrease

concentration of LA it is not to be specific only for MS since this was also observed in other neurological diseases Furthermore other authors observed no differences in the concentration of the said FA (Yoshida, Takase et al. 1983; Nightingale, Woo et al. 1990)). Moreover Harbige et al 2007 reported that in the total phospholipid concentrations of peripheral blood mononuclear cells (PBMC) in MS patients in remission and healthy controls, the relationships between LA and AA, LA and DGLA and DGLA and AA in MS seemed to be different to those of healthy controls. This may be an indication of a Δ6, and Δ5 desaturase problem and/or a greater requirement for these FA (Harbige and Sharief 2007). Harbige at al 2007 results were in agreement with that of Homa et al 1980 (Homa, Belin et al. 1980). Furthermore a comparison of 20:2ω-6 in PBMC total phospholipids between MS in remission and healthy controls revealed a significant 2 fold increase of 20:2ω-6 while a 4-fold increase was observed in patients in the relapse phase (Harbige and Sharief 2007). This was interpreted by the authors to be due to the active elongation of 18:2ω-6 (LA) to 20:2ω-6 (GLA), a process which is very active in the relapse phase of MS, indicating either a disturbance in the metabolism of DGLA and AA or higher requirements of the two (Harbige and Sharief 2007).

The concentration of myristic acid (C14:0) was found to be statistically significantly higher in the PC fraction of the MS patients as compared to healthy controls (table 8), whereas palmitic acid (C16:0) and arachidic acid (C20:0) were found to be significantly lower. The concentration of stearic acid (C18:0) and arachidic acid (C20:0) were found to be significantly lower in the PE fraction (table 9) and in the SM fraction (table 10) of the MS patients as compared to healthy controls. The concentration of behemic acid (C22:0) was found to be statistically significantly lower in the SM fraction of the MS patients as compared to controls. All the fatty acids mentioned above always occupy the sn-1 position on the membrane phospholipid, a position that otherwise may be occupied by either

saturated or unsaturated fatty acids.(Hulbert, Turner et al. 2005). As demonstrated by Hulbert et al 2005, although there may be very large differences in the relative content of saturated fatty acids in diets the relative saturation of membrane lipids of the tissues examined (liver plasmalemma, cardiac sarcolemma, cerebral synaptosomes and cerebral myelin) remains constant at ~50%. The same finding is true for monounsaturated fatty acids (Hulbert, Turner et al. 2005). However, it is worth noting that the association between the incidence of MS and the intake of saturated fat of animal origin has been suggested by population-based epidemiological studies (Schwarz and Leweling 2005).

The concentration of lignoceric acid (C24:0) was statistically lower in the PC fraction of the MS patients as compared to healthy controls (table 8). Interestingly, the concentration of lignoceric acid in the SM fraction of the MS patients was statistically higher as compared to healthy controls (table 10). A similar observation was described by Homa et al 1981 (Homa, Conroy et al. 1981), according to whom this difference in concentration of this long chain FA may affect the physical properties of the membrane lipids and that in turn may be related to the abnormalities in size and osmotic fragility in RBC's that have also been observed by several researchers (Homa, Conroy et al. 1981).

The concentration of nervonic acid (C24:1-ω9)in the SM fraction of the MS patients was statistically higher as compared to healthy controls (table 10). This is in contrast to what others have observed and proposed. Harbige and colleagues have suggested that nervonic acid may be a marker of CNS myelin damage in MS (Harbige and Sharief 2007). Sargent et al 1994 found that sphingolipids from post mortem MS brain samples have decreased concentrations of nervonic acid and that this FA is depressed in erythrocytes in MS patients (Sargent, Coupland et al. 1994).

One other monoene, oleic acid (C18:-ω9) was found to in statistically higher in concentration in the SM fraction (table 10) and lower in the PC fraction (table 8) of the MS patients as compared to healthy controls. Although oleic acid has known anti-inflammatory effects (Yaqoob 2002) it has been shown that there are no effects in prevention or of any beneficit in the progression of MS (Yaqoob 2002; Mehta, Dworkin et al. 2009). Cunnane et al 1989 attempted to correlate the increased levels of Zn^{2+} observed in MS to the abnormal oleic acid concentrations, however no correlation was found (Cunnane, Ho et al. 1989). It was also shown that oleic acid can increase Zn^{2+} uptake by RBC membranes. (Cunnane, Ho et al. 1989).

2.6 Conclusion

One may conclude that the Mediterranean diet has no protective effect for the development of MS. The Mediterranean diet may probably have an effect in correcting any disturbances, of the ω-6 PUFAs in MS and other neurological patients were observed by different researchers. In this study differences were observed for AA but not for LA. Furthermore, more investigations are needed for nervonic acid so as to be used as a marker or 'early predictor' for CNS myelin damage in MS, as well as other neurodegenerative diseases as proposed by Jones R & Harbige L 1987. In conclusion more, well-designed studies are needed in order to verify the effects of the Mediterranean diet on MS or in other neurological diseases. Larger sample size and extended FFQ are required in order to correlate dietary patterns and disease development Furthermore more studies are needed in order to assess the optimal levels of different fatty acids in healthy individuals, as well as to make comparisons between different populations.

CHAPTER THREE

INTERVENTION WITH FATTY ACIDS AND GAMMA TOCOPHEROL AND CLINICAL OUTCOMES

CHAPTER THREE: INTERVENTION WITH FATTY ACIDS AND GAMMA TOCOPHEROL AND CLINICAL OUTCOMES

3.1 Introduction

MS is a complex multifactorial disease and is the result of interactions between environmental factors and genetic susceptibility (Baranzini, Oksenberg et al. 2002; Lobo 2008). The result of the interplay of these factors trigger a cascade of events that involve the engagement of the immune system, inflammatory injury of myelin, axons and glia, functional recovery and structural repair, gliosis and neurodegeneration (Compston and Coles 2008). Immune-mediated inflammation, oxidative stress and excitotoxicity are the mechanisms involved and can all contribute to oligodendrocyte and neuronal damage and even cell death, hence promoting disease progression (Evans 1993; Knight 1997; Smith, Kapoor et al. 1999; Matute, Alberdi et al. 2001; Owens 2003). The prevalence of MS is increasing (Thompson 2013) and in combination with the partial efficacy and side effects of existing treatments, other, new, innovative, safe and preventive treatments have been tested in animals and humans for a probable efficacy on number, duration and severity of relapses as well as in the progression of the disease. Amongst these treatments, a correlation between dietary habits and MS has been always been postulated for more than 70 years; mostly focusing on the omega-3/omega-6 polyunsaturated fatty acids but also on specific vitamins namely vitamin D. Farinotti et al 2007 in a cochrane review "Dietary interventions for multiple sclerosis" reported that GLA, LA, EPA, DHA have been investigated for their clinical efficacy on the progression of MS (Farinotti, Simi et al. 2007).

For this study a 1:1 of omega-3:omega 6 PUFAs was used in order to overpass any differences amongst populations (regional traditions and habits) irrespective of the quantities consumed as far as the consumption of these PUFAs is concerned and at the same time to

correct to the physiological ratio (1:1-4)(Simopoulos 2002). However The American Heart Association recommends that omega-6 in order to reduce the risks for coronary heart disease should be 5%-10% of total energy despite large bodies of controversies (Harris, Mozaffarian et al. 2009; Ramsden, Hibbeln et al. 2010; Mathias, Sergeant et al. 2011). Moreover the current consumption of these PUFAs is 1:16 as reported by Simopoulos 2002 (Simopoulos 2002). The specific dosage also aimed to correct existing insufficiencies (no insufficiencies were observed when in a later stage (see chapter 2) 20 healthy individuals were recruited and they were compared with MS patients' at time zero for the fatty acid composition of RBC membranes), cell membrane abnormalities, specifically of the immune system and blood mononuclear peripheral cells (Navarro and Segura 1988; Navarro and Segura 1989), and at the same time be available in order to modulate all the pathogenic mechanisms and network of events in MS. This PUFA dosage can also aid cellular incorporation, especially in the central nervous system of humans with a process that is age dependent and more importantly it is possibly quantity-dependent of the dietary/supplemented PUFAs (Youdim, Martin et al. 2000). Furthermore, the digestion of fatty acids (FAs) constitutes another barrier since FAs must first cross the intestinal epithelium before reaching the different tissues therefore bioavailability of FAs is of paramount importance. The omega-3 PUFAs used are in the re-esterified form for two main reasons: 1) to increase bioavailability of FAs; it was shown that the triglyceride form of PUFAs has higher bioavailability than free fatty acids (Dyerberg, Madsen et al. 2010) and 2) to eliminate unwanted disturbances at the sides of action by other FAs and other molecules present in body fish oils (Carlier, Bernard et al. 1991; Dyerberg, Madsen et al. 2010). The omega-6 PUFA, LA, is an essential structure molecules and important for any physiological (re)generation of cell membranes. GLA in not a structural molecule; GLA can be converted to DGLA which can compete with AA for the production of anti-inflammatory eicosanoids (Calder 2012). GLA to LA is in a 1:2 ratio

in order to ensure production of dihomo-gamma-linolenic acid (DGLA), from GLA when LA cannot be metabolized, due to desaturase deficiency or malfunction (Thompson 1966; Navarro and Segura 1988; Navarro and Segura 1989; Harbige and Sharief 2007). Such a reduced capacity to convert LA to GLA has been associated with aging, diabetes, alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and cardiovascular diseases (Horrobin 1990; Leventhal, Boyce et al. 1993; Bolton-Smith, Woodward et al. 1997).

3.2 Description of the study

3.2.1 Subjects and Methods

The study presented here is a randomized, double blind, placebo controlled study aiming to investigate the rate and number of relapses in relation to the incorporation of PUFAs on RBC membranes after supplementation.

3.2.2 Subjects and recruitment

Eighty patients (all Greek Cypriots of Greek Cypriot ancestry) that represent ~20% of the total MS population in Cyprus with RR MS and were eligible for treatment (see next paragraph for criteria) were enrolled in this study at the Cyprus Institute of Neurology and Genetics in July 2007. All patients gave written informed consent. The study protocol was developed by the investigators (Dr Marios Pantziaris, Neurologist, Dr Ioannis Patrikios, Biochemist, and George Loucaides Clinical Dietitian) and it was approved by Cyprus National Bioethics committees according to European Union (EU) guidelines. Data were collected and blindly analyzed by the investigators.

Enrolment was limited to men and women who were between the ages of 18 and 65 years and had a diagnosis of RR MS, (according to McDonald criteria) (Polman, Reingold et al. 2005) had a score of 0.0 to 5.5 on the Expanded Disability Status Scale (EDSS) (the scale

starts with 0.0 (normal) up to 10.0 (death)), had undergone magnetic resonance imaging (MRI) showing lesions consistent with multiple sclerosis, had at least one medically documented relapse within 24 months prior to the beginning of the study and had been receiving approximately the same medical treatment during the two years before enrolment. Exclusion criteria were the use of immunosuppressants or monoclonal antibody therapy, pregnancy or nursing, the presence of progressive multiple sclerosis or any other severe disease compromising organ function or had a history of recent alcohol or drug abuse. Exclusion criteria were a recent (<30 days) relapse, prior of immunosuppressants or monoclonal antibody therapy, pregnancy or nursing, the presence of progressive multiple sclerosis or any other severe disease compromising organ function or had a history of recent alcohol or drug abuse, use of any additional food supplement formula, vitamin of any type or any form of PUFA (ω-3 or ω-6) for the last four months prior enrolment and a history of severe allergic or anaphylactic reactions or known specific nutritional hypersensitivity.

3.2.3 Randomisation and design

Patients were randomly assigned to four intervention groups in a 1:1:1:1 ratio stratified by gender (women to men, 3:1). Randomisation was facilitated by a lottery-type pool of numbered balls. Patients were randomly assigned to the treatments in blocks of four by flipping a coin as follows: for the first two drawn balls, heads stratified them to the groups A/B and tails stratified them to the groups C/D. The other two balls were stratified accordingly. A second toss of the coin assigned the two patients to group A (head)/B (tail) or to group C (head)/D (tail). The randomisation scheme was generated, performed and securely stored by the Helix Incubator Organization of Nicosia University (HIONU).

As summarised in table 11 the first intervention group A, was administered a mixture of PUFAs comprising ω-3 and ω-6 in a ratio 1:1. The ω-3 used are Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) in a ratio 3:1, and the ω-6 are Linoleic acid (LA) and

gamma (γ)-linolenic acid (GLA) in a ratio 2:1. Also present in smaller amounts were vitamin A in the form of beta carotene and vitamin E in the form of α-tocopherol. The second intervention group, B/PLP10, was administered the same mixture as in group A but with the addition of vitamin E in the form of γ-tocopherol; the third intervention group, C, was administered vitamin E in the form of γ-tocopherol; and Group D was administered the placebo containing olive oil which doesn't have any known immunomodulatory effects (positive or negative) on the disease (Millar, Zilkha et al. 1973; Mehta, Dworkin et al. 2009). All four intervention mixtures were liquids, citrus flavoured, and sealed in brown coloured glass bottles under nitrogen, which were labelled by a pharmacist. Labels included medication code numbers that were unidentifiable to patients as well as investigators. All different formulas and placebo were liquids and had identical appearance and smell. The interventions were administered once a day in a dose of 15 ml, preferably at night, 30 minutes before dinner.

Table 11. Showing all treatment arms and the administered mixtures.

Treatment arms			
A*	**B (PLP10)***	**C***	**Placebo***
Intervention: EPA (1650 mg)/DHA (4650 mg)/ GLA (2000 mg)/LA (3850 mg)/total other Ω-3 (600 mg)†/total MUFA‡ (1714 mg)+total SFA (18:0 160 mg, 16:0 650 mg)/vitamin A (0.6 mg)/ vitamin E (22 mg) plus citrus aroma	*Intervention*: EPA (1650 mg)/DHA (4650 mg)/ GLA (2000 mg)/LA (3850 mg)/total other Ω-3 (600 mg) †/total MUFA‡ (1714 mg)+total SFA (18:0 160 mg, 16:0 650 mg)/vitamin A (0.6 mg)/vitamin E (22 mg)+pure γ-tocopherol (760 mg) plus citrus aroma	*Intervention*: Pure natural γ-tocopherol (760 mg) dispersed in pure virgin olive oil (16137 mg) as delivery vehicle plus citrus aroma	*Intervention*: Olive oil (pure virgin) plus citrus aroma

*Total daily dose: 19.5 ml.
†Other ω-3: C18:3n−3 37 mg, C18:4n−3 73 mg, C20:4n−3 98 mg, C22:5n−3 392 mg.
‡MUFA: 18:1 1300 mg, 20:1 250 mg, 22:1 82 mg, 24:1 82 mg.
EPAX 1050, EPAX AS, Aalesund, Norway, was used as the source for the ω-3 PUFAs, as re-esterified glycerides from fish body oils; borage seed oil (organic, cold pressed) 'Borago officinalis' Goerlich Pharma International GmbH, Edling, Germany, was used as the source for the ω-6 PUFAs, MUFAs and SFAs, as triglycerides. The pure natural γ-tocopherol was purchased from Tama Biochemical Co Ltd, Shinjuku-ku Tokyo, Japan; vitamin A, as β-carotene, from HealthAid Ltd, Middlesex, UK and the citrus aroma from Givaudan Schwaiz AG, Dubendorf,
Switzerland.DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid (From Pantzaris et al. 2013).

Group A consisted of 20 patients (15 female and 5 male) with a mean age of 37.95 years, a mean disease duration of 9.00 years, an annual relapse rate (range) of 1.17 (1 to 6), a mean (range) baseline expanded disability status scale (EDSS) score of 2.52 (1.0 to 5.5) and 55% were on conventional treatment (Disease Immunomodulators-DMT) and 45 % were on no DMT. Group B/PLP10 consisted of 20 patients (15 female and 5 male), with a mean age of 36.90 years, a mean disease duration of 8.55 years, an annual relapse rate (range) of 1.21 (1 to 7), a mean (range) baseline EDSS score of 2.15 (1.0 to 4.0) and 45% were on conventional treatment (Disease Immunomodulators-DMT) and 55 % were on no DMT. Group C consisted of 20 patients (15 female and 5 male), with a mean age of 37.65 years, a mean disease duration of 8.55 years, an annual relapse rate (range) of 1.16 (1 to 6), a mean (range) baseline EDSS score of 2.42 (0.0 to 5.0) and 60% were on conventional treatment and 40 % were on no DMT. Group D consisted of 20 patients (15 female and 5 male) with a mean age of 38.10 years, a mean disease duration of 7.65 years and an annual relapse rate (range) of 1.05 (1 to 4), a mean (range) baseline EDSS score of 2.39 (1.0 to 4.0) and 50% were on

conventional treatment and 50 % were on no DMT (Table 12). They were no statistical significant differences between the four Groups in regard to the demographic data.

Table 12. Demographic and Pre-Study Baseline Characteristics for Total Study Population by Treatment Arm.

Characteristics	Group A	Group B/PLP10	Group C	Placebo	P value
	(n=20)	(n=20)	(n=20)	(n=20)	
Sex					
Male	5 (25%)	5 (25%)	5 (25%)	5 (25%)	
Female	15 (75%)	15 (75%)	15 (75%)	15(75%)	1.000
Age (yr)					
Mean	37.95	36.90	37.65	38.10	0.982
Range	22 - 65	25 – 61	24 – 54	21 - 58	
Pre-study disease duration (yr)					
Mean	9.00	8.55	8.55	7.65	0.908
Range	2 - 37	2 – 20	3 – 24	2 - 25	
Pre-study Relapses rate					
Mean	2.33	2.41	2.31	2.10	0.946
Range	1 – 6	1 – 7	1 – 6	1 – 4	
Annual relapse rate	1.17	1.21	1.16	1.05	
Study Base line EDSS score					
Mean	2.52	2.15	2.42	2.39	0.775
Range	1.0 – 5.5	1.0 – 4.0	0.0 – 5.0	1.0 – 4.0	

3.2.4 Clinical assessment

All subjects were examined by the assigned Neurologist at time zero (recruitment time), at every 6 months intervals and at every relapse for a period of 24 months. Clinical examination included completion of the EDSS score for every subject. In addition, all relevant demographic, clinical and nutritional information was rigorously and carefully documented. The study supplement was not expected to have any clinical or laboratory adverse effects different from those of the placebo that could disturb the double-blind nature

of the trial. Therefore, the study neurologist functioned as both the treating and evaluating physician.

The 10 point Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke 1983) was used by the neurologist in examining the patients.

Procedures and end points

The raw materials, ω-3 and ω-6, were purchased considering the molecular structure, quantity/ ratio and quality of these PUFAs and with the vitamin E as alpha-tocopherol used as antioxidant stabilizer by the supplier. Vitamin A, vitamin E as gamma-tocopherol, and aroma were purchased separately. The mixing of the different fractions of the required intervention composition was performed by the same three scientists of the project under appropriate conditions every six months and then stored under nitrogen in a dark cold environment. See table 11 for detailed descriptions of interventions.

The selection of the ingredients was based on their biophysical and biochemical properties that could have on the effects of the disease. For instance, there is contradicting data in literature regarding the effects of the ω-6 and the ω-3 PUFAs on different aspects of the MS, in particular on the frequency and severity of relapses and platelet function. This can be ascribed to the different criteria of patient selection in relation to the form and severity of disease at the beginning of the various studies, as well as on the type of PUFAs for e.g. the use of only ω-3, or different dosages of ω-3 or different ratios of EPA:DHA ((6g daily with EPA 51%: DHA 31%, Gallai et al. 1995) (Gallai, Sarchielli et al. 1995), (10g daily with EPA 18%: DHA 12% Bates et al. 1989) (Bates, Cartlidge et al. 1989)) or only ω-6 LA:GLA 8:1, Bates et al. 1978) (Bates, Fawcett et al. 1978)), (only 8,6g LA Millar et al. 1973) (Millar, Zilkha et al. 1973))). Several human studies with PUFAs, ω-3 and ω-6 or γ tocopherol showed a relatively positive correlation between progression of the disease and PUFA

supplementation in the diet (Danilov, Andersson et al. ; Gallai, Sarchielli et al. 1995; van der Veen, Hinton et al. 1997; Stewart and Bowling 2005). Supplementing with ω-3 and ω-6 PUFAs showed a decrease of different types of pro-inflammatory and immunoenhancers cytokines secretion (Bates, Cartlidge et al. 1989). Furthermore, PUFAs have been shown to ameliorate the disease by regulate the production of eicosanoids and also able to be incorporated to the damaged myelin sheath as a building blocks.

Supplements with PUFAs showed some decrease in the number and severity of relapses on MS patients (MSP) (Nordvik, Myhr et al. 2000). Two controlled clinical trials have tested EPA and DHA in the form of fish oil. The largest study, involving 312 patients, used 10g fish oil supplementation daily as the intervention and olive oil as a placebo. The results showed fewer people progressing 1 point of the disability status scale (DSS), (P=0.07), the DSS is an earlier version of the Expanded Disability Status Scale (EDSS), that is used to measure disability changes in modern clinical trial (Bates, Cartlidge et al. 1989). Another small randomized study, involving 31 patients, investigated fish oil and other dietary fat changes as a treatment among users of approved MS therapies (Weinstock-Guttman, Baier et al. 2005). The authors reported on the physical component scale (PCS) favouring the fish oil group that was not statistically significant at the end of 12 months study. EDSS scores improved in the fish oil group and worsened in the olive oil (placebo) group.

Swank and co/worker 1990 (Swank and Dugan 1990) followed the effect of strict low fat diet in 144 MSP for 43 years the diet was supplemented with 5g of fish oil. In the final evaluation a significant reduction of deterioration and mortality was found.

A controlled double blind study focus on the ω-6 PUFAs was performed by Millar at al 1973 (Millar, Zilkha et al. 1973). After 2 years of supplementation, the lower frequency and severity of relapse was observed in the LA group. Similar results were reported by Bates et

al. 1978 (Bates, Fawcett et al. 1978) in the RR form of the disease. Other authors didn't find any benefit of LA supplementation in MS (Paty, Cousin et al. 1978). Meta-analysis of the clinical studies of ω-6 PUFAs treatment in MS was published by Bates 1990 who concluded that the LA supplementation had a beneficial effect in mild and RR forms of the disease(Bates 1990).

Martinez 2003 observed restoration of myelin and clinical improvement after supplementing patients suffering from generalized peroxisomal disorders which results in a severe DHA deficiency with a high dose of DHA in order to "normalize DHA concentrations"; a dose of 50-500mg of pure form of DHA ethyl esters was used for all infants (Martínez, Vázquez et al. 2000; Martinez 2003). Moreover, 2.3-cyclic nucleotide 3- phsphodyesterase (CNP) protein expression was shown to be significantly increase by GLA supplementation (van Meeteren, Baron et al. 2006). Other scientific studies involving PUFAs as food supplements for MSP suggested that the supplement concentration used was quantitatively insufficient (Dworkin (Dworkin, Bates et al. 1984).

Furthermore EPA and DHA was chosen firstly because DHA is the main PUFA in myelin sheath and in the CNS (Martinez 2003; Stillwell, Shaikh et al. 2005); secondly DHA together with EPA will substitute excess AA and SFA within the cell membranes (Calder 2002; Simopoulos 2002; Calder 2006); EPA will contribute to the inhibition (competitive to AA) of PLA2, together with the GLA and LA (Simopoulos 1991; Harbige and Sharief 2007). GLA can be converted to DGLA. GLA will ensure production of DGLA which can then compete with AA promoting production of prostaglandin (PG)E1 but also inhibiting PLA2 (Rossetti, Seiler et al. 1997). EPA will also participate in the production of anti-inflammatory leukotrienes, prostaglandins of the 3-series (PG3) and thromboxane (TX3) along with DHA for the production of protectins and resolvins (Serhan, Arita et al. 2004). DHA is used in 3:1 ratio to EPA to cover for the inability of EPA to be metabolized further to DHA, and high

enough to promote high production of the aforementioned anti-inflammatory eicosanoids and cytokines including protectins and resolvins and to be incorporated into CNS cell including myelin. Furthermore it was observed that MS patients are insufficient in LA and that supplementing patients with LA can normalize concentration levels in serum, RBC, leucocytes, cerebrospinal fluid (Harbige and Sharief 2007); Considering the above was decided to use the specific PUFAs ω-3 and ω-6 in the specific 1:1 ratio and in the specific high amounts.

Furthermore, the high dosage of the PUFAs in the interventions was selected in order to optimizing the body composition of omega-3 and omega-6 to a 1:1 wt/wt ratio irrespective of the dietary habits of the MS patients participating in the study. Olive oil has been chosen as placebo since as mentioned above doesn't have any known immunomodulatory effects (positive or negative) on the disease. Mehta et al 2009 in a review on the potential therapeutic effects of PUFAs in MS attributed the lack of efficacy of PUFAs in the studies using olive oil, as a placebo, to the effects of olive oil on inhibiting the activation of NF-κB, and that olive oil can also induce the release of prostacyclin which can in turn activate PPARγ (Mehta, Dworkin et al. 2009). On the other hand oleic acid and no any other trace elements in olive oil is responsible for the observed effects on the modulation of some functions of the immune system in animal studies (Yaqoob 2002). Several studies on the consumption of MUFAs-rich diet by healthy humans as reported by Yaqoob 2002 doesn't have any general suppression effect on the immune system functions (Yaqoob 2002) which according to the same author this can be attributed to the much higher experimental consumption of olive oil in animal studies difficult to be achieved in human studies. Furthermore, Cypriots consume diets rich in olive oil (unpublished data) but still MS in Cyprus it is presenting in a rate seen only in countries with poor consumption of olive oil.

The period of the study was started 1 July 2007 (enrolment) to 31 December 2009 and the recording of relapses continued until 31 December 2010. Depending on their clinical status and in accordance with common practice, the participants continued to receive their indicated regular treatment, with persistent evaluation for any side effects and adverse events. For the clinical evaluation the study included 'normalization period' from July 2007 to December 2007, 'on treatment' period from January 2008 (baseline) to December 2009 and a 12-month 'post-study monitoring period' January 2010 to December 2010. The 'normalisation period'(Martínez, Vázquez et al. 2000; Martinez 2003) would allow the interventions to exert their beneficial effects as oral PUFAs need 4-6 months to incorporate to cells of the immune and neural cells, achieve optimal normalization of the EPA and DHA ratios, correction of antioxidant deficiencies and body PUFA redistribution (Marangoni, Angeli et al. 1993; Martínez, Vázquez et al. 2000; Huang, Tang et al. 2007). Clinical assessment visits were schedule at enrolment, baseline, and 3, 9, 15, 21 and 24 month on-treatment. The patients were also clinically examined by the treating neurologist within 48 h after the onset of new or recurrent neurological symptoms.

The primary end point was the annualised relapse rate (ARR) at 2 years. A relapse was defined as new or recurrent neurological symptoms not associated with fever or infection that lasted for at least 24 h and was accompanied by new neurological signs. Relapses were treated with methyl-prednisolone at a dose of 1 g intravenous per day for 3 days, followed by prednisone orally at a dose of 1 mg/kg of weight per day on a tapering scheme for 3 weeks. The secondary end point at 2 years was the time to disability progression, defined as an increase of 1.0 or more on the EDSS and confirmed after 6 months (Kappos, Radue et al. 2010). Progression could not be confirmed during a relapse and the final EDSS score was confirmed 6 months after the end of the study (Kappos, Radue et al. 2010). A post hoc analysis was performed to assess the proportion of patients free from new or enlarging T2

lesions on brain MRI scans at the end of the study for the per-protocol participants of the group receiving the most effective intervention versus placebo. This comparison was made versus the available archival MRI scans up to 3 months before the enrolment date. The MRI scans were performed and blindly analysed at an MRI evaluation centre. The patients were monitored for an additional 12 months after completion of the trial, and relapses were recorded. The patients were strongly encouraged to remain in the study for follow-up assessments even if they had discontinued the study drug. Safety measures were assessed from the time of enrolment until 12 months following the study completion. Haematological and biochemical tests were performed at enrolment and every 12 months, including a full blood count, renal and liver function tests, and protein, cholesterol, triglyceride, glucose and electrolyte levels. The patients were able to contact the involved neurologist at any time if there was any adverse event, side effect or allergic reaction. The study drug was not expected to have any clinical or laboratory adverse effects different from those of the placebo that could disturb the double-blind nature of the trial. Therefore, the study neurologist functioned as both the treating and evaluating physician. The whole procedure followed the clinical trial guidelines as required by the USA Food and Drug Administration, European Medicines Agency and the Committee for Medicinal Products for Human Use (Directive 2001/20/EC; Committee for medicinal products for human use 2006).

3.2.5 Blood collection and processing

Blood sample (25ml) was drawn in heparinised tube (anticoagulant solution) from all subjects at recruitment, subsequently at every six months and at every relapse for a period of 24 months. Blood samples collected were used in order to assess the fatty acid composition of the RBC membrane. The analysis of red blood cells' fatty acids was performed after study termination and thus did not influence the blinding. An aliquot of the blood was used for haematological and biochemical analysis. The remaining whole blood

was centrifuge at 1500g for 15min to separate RBC and plasma. The plasma was carefully removed and stored at -70ºC. The RBC pellet was re-suspended in an equal volume of physiological saline (0.85% Sodium Chloride) and centrifuged at 1500g for 15 min and the supernatant discarded. This process was repeated to ensure a complete removal of plasma. The resulting washed RBC pellet was then stored at -70ºC until analysis. RBC lipids were extracted by the method of Folch et al., (Folch, Lees et al. 1957).

3.3 Statistical analysis

Since there were no data from previous studies on the potential effect sizes, power calculations could not be performed before the study. The prevalence of MS in Cyprus in 2005 was 120/100000, and the total population was 600,000. Based on these numbers and information from The Cyprus Institute of Neurology and Genetics, the reference centre, 20% of the total RRMS patients were eligible for treatment and enrolled in the study.

Baseline analysis of variance or the Kruskal-Wallis rank test for continuous variables and Fisher's exact test for categorical variable were performed in order to compare baseline characteristics across all intervention groups. For the primary outcome, the annual relapse rate (ARR), a negative binomial regression model was used in order to compare the interventions with the placebo in a pair-wise fashion and adjusted for the number of relapses within 2 years, the EDSS score at baseline and DMT. The relapse rate was calculated as the total number of relapses divided by the total number of patient-years followed for each treatment group. ARR differences were also calculated among all comparable parameters and reported as the per cent difference. For the secondary end point, the time to disability progression and the Kaplan-Meier curves were constructed. The progression of disability and time thereof were compared in a pair-wise fashion for the active interventions versus placebo by the log-rank test in the main analysis and by the Cox proportional-hazards models

with adjustments for the baseline EDSS score, age and DMT in the supportive analysis. Multivariate models considered all variables with $p<0.1$ in the univariate models. There was no overt violation of the proportionality assumption. Both per-protocol and intention-to-treat (ITT) analyses were performed for different sets of research questions to be answered, and both are reported. Missing data of the five patients lost to follow-up were imputed by the last-observation-carried-forward approach. Owing to the proof-of-concept design of the study, the considerable non-adherence rate (49%) and the resulting interpretation issues regarding the ITT analysis, the per-protocol analysis was considered to be the more informative and appropriate method to answer the research questions addressing the efficacy of the interventions when the participants continuously followed the protocol. All statistical analyses were well-defined a priori. All analyses for the clinical results were performed with STATA SE V.10.0 (College Station, Texas, USA).

3.4 Results

3.4.1 Study population

20 patients were randomly assigned to each of the three intervention groups and 20 patients to receive the placebo totalling 80 patients (figure 18). The base line characteristics were similar between the groups for both the intention to treat (ITT) and per-protocol populations (table 13A B). Drop-out patients, completed the follow-up for the duration of the study and were included in the ITT analyses (table 14). Five patients were lost to follow-up after their first schedule visit. Two other patients progressed to secondary progressive MS and dropped-out before their first schedule visit. Fifteen patients dropped out within the first six months of the study including five women due to pregnancy. Seventeen other patients dropped out after six months within the study, because of palatability of the supplements. From the drop-out patients seven were given monoclonal antibody treatment (natalizumab). Forty-one (51%) patients completed the study. One patient from group A and two from the placebo

group transferred to natalizumab. Thirty-nine (49%) patients either withdrew or lost to follow-up (figure 18).

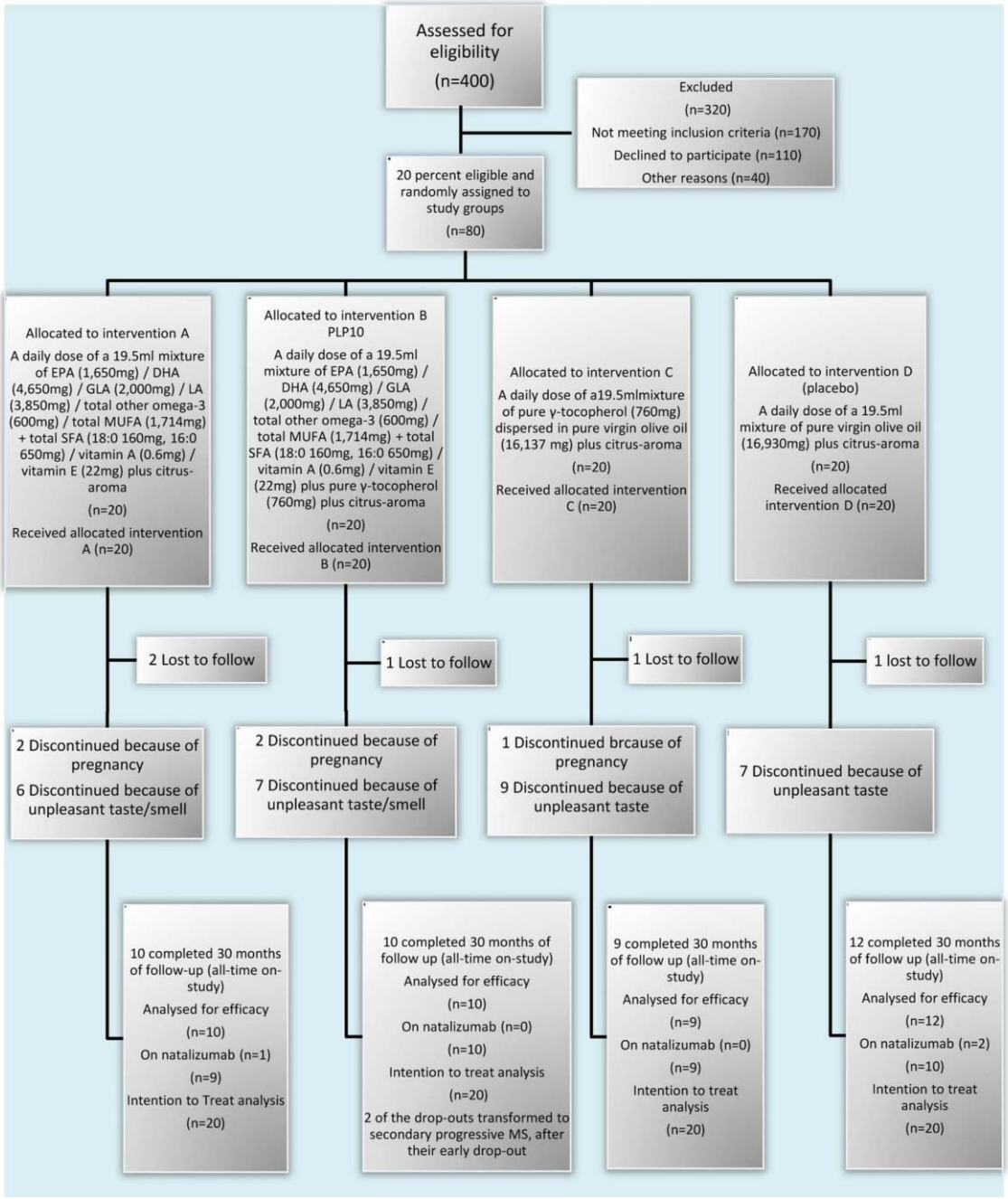

Figure 18. Study flowchart (From Pantzaris et al. 2013).

Table 13. Section A reports the demographics and baseline disease characteristics for the total randomised population by treatment arm and section B reports the demographics and baseline disease characteristics of the all-time on-study population by treatment arm (From Pantzaris et al 2013).

Section A Characteristics	Group A (n=20)	Group B*(n=20)	Group C (n=20)	Placebo (n=20)	p Value
Sex					
Female—no. (%)	15 (75)	15 (75)	15 (75)	15 (75)	1.000
Age (years)					
Mean±SD	38.0±11.9	36.9±8.4	37.7±8.7	38.1±10.9	0.982
Median (range)	38.0 (22 –65)	37.0 (25 –61)	36.5 (24–54)	36.0 (21–58)	
Treatment history					
Patients on DMT—no. (%)	11 (55)	9 (45)	12 (60)	10 (50)	0.875
Pretreatment disease duration (years)					
Mean±SD	9.0±7.6	8.6±4.8	8.6±5.3	7.7±5.7	0.909
Median (range)	7.5 (2–37)	8.0 (2–20)	8.0 (3–24)	6.5 (2–25)	
Pretreatment relapses†					
Mean±SD	2.33±1.68	2.41±1.73	2.31±1.66	2.10±1.32	0.946
Median (range)	2.0 (1–6)	2.0 (1–7)	2.0 (1–6)	2.0 (1–4)	
ARR	1.17	1.21	1.16	1.05	0.946
Patients % with ≤1 relapse	40	45	40	35	
Baseline EDSS score†					
Mean±SD	2.52±1.23	2.15±1.05	2.42±1.21	2.39±0.93	0.775
Median (range)	2.5 (1.0–5.5)	2.0 (1.0–4.0)	2.5 (0.0–5.0)	2.5 (1.0–4.0)	
Section B Characteristics	**Group A (n=10)**	**Group B*(n=10)**	**Group C (n=9)**	**Placebo (n=12)**	**p Value**
Sex					
Female—no. (%)	5 (50)	7 (70)	6 (66.6)	10 (83.3)	0.419
Age (years)					
Mean±SD	36.6±13.5	34.8±5.4	40.9±8.1	39.8±13.2	0.572
Median (range)	34.5 (22–65)	34.5 (26–43)	40.0 (29–54)	37.5 (21–58)	
Treatment history					
Patients on DMT—no. (%)	6 (60)	4 (40)	6 (67)	6 (50)	0.949
Pretreatment disease duration (years)					
Mean±SD	9.7±10.0	8.3±5.3	11.3±6.1	8.7±7.1	0.807
Median (range)	7.5 (2–37)	8.0 (2–20)	8.0 (4–24)	5.5 (2–25)	
Pretreatment relapses					
Mean±SD	2.20±1.47	2.70±1.25	1.78±0.66	1.67±1.37	0.241
Median (range)	2.0 (1–6)	2.5 (1–4)	2.0 (1–3)	1.5 (1–4)	
ARR	1.10	1.35	0.89	0.83	
Patients % with ≤1 relapse	30	20	33	50	
Baseline EDSS score					
Mean±SD	2.65±1.37	2.40±1.12	2.11±1.02	2.16±0.96	0.698
Median (range)	3.0 (1.0–5.5)	2.5 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–3.5)	

There were no significant between study-group differences at baseline for any characteristic.
†Available data at entry baseline (n=18 for group A, n=17 for group B, n=19 for group C, n=19 for group D).
ARR, annual relapse rate; DMT, disease-modifying treatment.

Table 14. Section A reports the 2-year primary end point of relapses based on the study design as reported by the dropoutpatients by treatment arm and section B reports the comparison of the 24-month pretreatment ARR (baseline) with the 24-month on-treatment ARR for the total randomised population by treatment arm (From Pantzaris et al 2013).

Section A Characteristics	**Group A (N=8)**		**Group B*(N=7)**		**Group C (N=10)**		**Placebo (N=7)**	
End point	X	Y	X	Y	X	Y	X	Y
Number of relapses	20	14	14	14	27	26	20	13
Annual relapse rate	1.25	0.88	1.00	1.00	1.35	1.30	1.42	0.92

Section B Characteristics	**Group A (N=20)**		**Group B*(N=20)**		**Group C (N=20)**		**Placebo (N=20)**	
End point	X	Y	X	Y	X	Y	X	Y
Number of relapses	45	34	49	30	46	41	43	41
Annual relapse rate	1.13	0.85	1.23	0.75	1.15	1.03	1.08	1.03
ARR reduction % (Y to X)†		–25		–39		–10		–5
p Value against baseline		0.120		0.005		0.475		0.652
Percentage of reduction of the ARR compared with placebo (Ys)†		–18		–27		0.0		N/A
p Value against placebo		0.447		0.121		0.996		

Section A: The most dropout patients who transferred to disease-modified therapy (DMT) were from group A and the placebo group, with three and two patients, respectively, on natalizumab. These parameters justify the decreased number of relapses recorded within the group A and placebo dropouts and could affect the ITT analysis in favour of the placebo when the total 2-year recorded data are used. For the group B, 14 relapses were reported at baseline, which remained the same during the 2-year study period. For the placebo group, 20 relapses were reported at baseline and decreased to 13 during the 2-year study period. These results are expected because for group B, 43%
of the dropouts were under DMT at entry baseline and remained the same until the end of the study, with no patient on natalizumab, but the 57% of the placebo group dropouts who were under DMT at entry baseline increased to 86% at the end of the study, including two patients on natalizumab.
Section B: The ARR of group B was 1.23 at baseline and 0.75 at the end of the study (39% reduction, p=0.005), and that for the placebo group was 1.08 at baseline and 1.03 at the end of the study (5% reduction, p=0.652). No significant difference was calculated for the other two treatment arms. During the 24-month on-treatment, group B presented a 27% reduction in the ARR versus the placebo group (p=0.121), with all groups lacking statistically significant results
X: total number of relapses for the 24-month pretreatment (baseline).
Y: total number of relapses for the 24-month on-treatment.
†Unadjusted estimate.

3.4.2 Efficacy

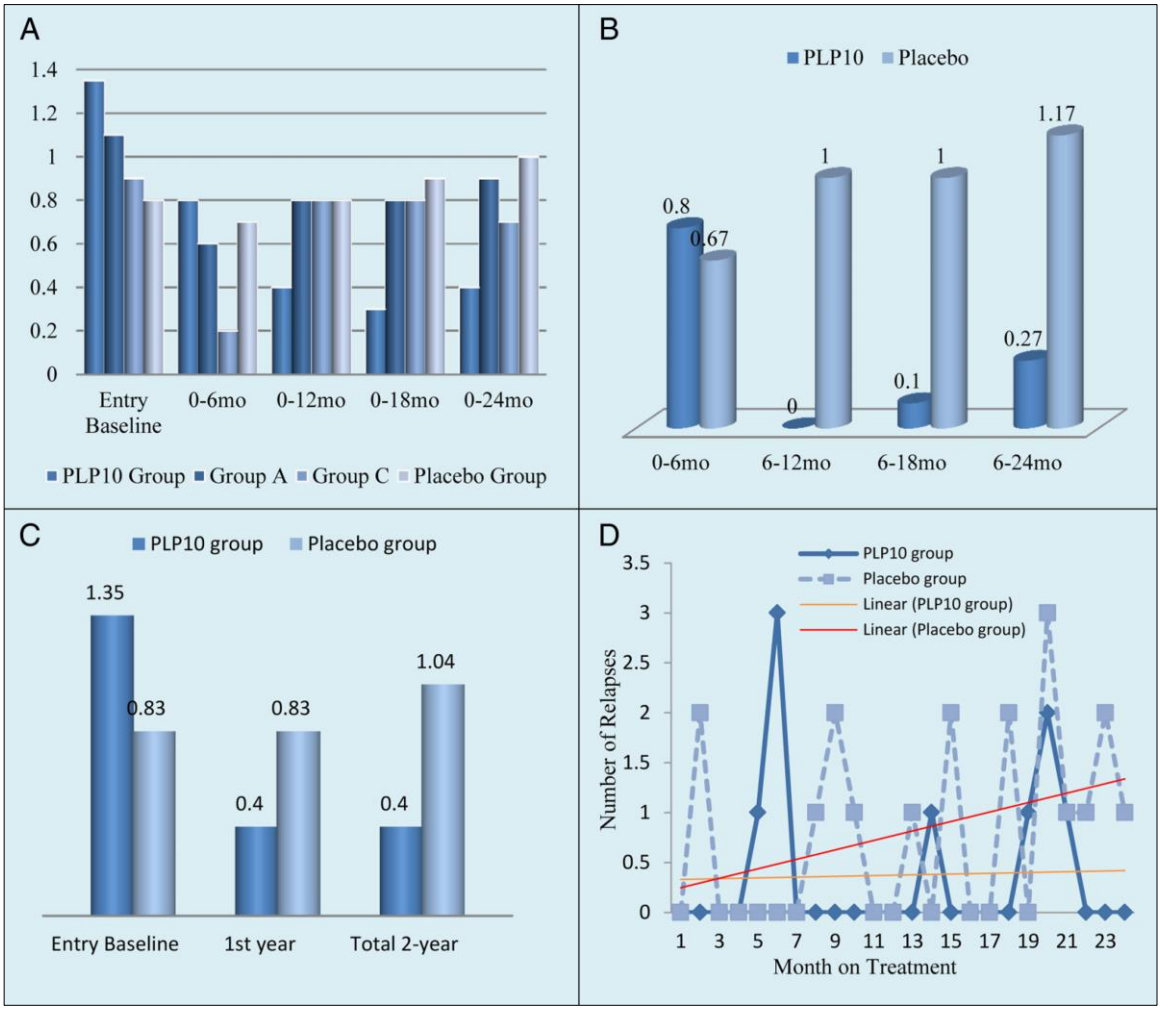

Figure 19. (A) Demonstrates the ARR of the all-time on-study patients during the 24-month pretreatment (baseline ARR) and at different on-study intervals (6, 12, 18 and 24 months) per-treatment arm.* (B) Demonstrates the ARR of the all-time on-study population between the 0–6, 6–12, 6–18 and 6–24-month period intervals for group B versus placebo group.*(C) Demonstrates the ARR of the all-time on-study population for group B versus placebo group at baseline, during the first year, and during the second year on-treatment.* (D) Demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) for group B (n=10) versus placebo (n=10). The placebo group showed an irregular dispersion of relapses compared with group B, with a linear increasing trend, whereas the group B showed a stabilised linear trend. Using the per-protocol model in which the patients on natalizumab were excluded, the number of relapses could be compared on the same number of patients.* Including the patients on natalizumab (From Pantzaris et al 2013).

3.4.3 Relapses

The per-protocol analysis was performed in order to assess the effectiveness of the intervention formulas on the patients adhering in the study. However an ITT analysis was also performed for methodological comprehensiveness and to be able to answer what happened to all 80 patients in the trial (the effect of assignment) (Committee for medicinal products for human use 2006). In the per-protocol analysis, the ARR for the first year of treatment was 0.80, 0.40, 0.78, and 0.83 for the four groups respectively. During the second year, the ARR was 0.90, 0.40, 0.67, and 1.25 for the four groups respectively. For the primary end point in the two years of study, 8 relapses were recorded for the 10 patients in group B (0.40 ARR) versus 25 relapses for the 12 patients in the placebo (1.04 ARR), a 64% adjusted relative rate reduction (RRR) for group B (RRR 0.36, 95% CI 0.15 to 0.87, p=0.024; tables 15 A and 16 and figure 19 A and C).

Table 15. Section A reports the 2-year primary end points of the ARR for the all-time on-study population by treatment arm and per cent difference from the placebo and section B reports the comparison of the 24-month pretreatment ARR with the 24-month on-treatment ARR of the all-time on-study population excluding patients on natalizumab and the comparison of the ARR during the 24-month period on-treatment (primary end point) for each treatment group compared with the placebo.

Section B: During the 24-month period on-treatment, the ARR of group A was 0.85, with an 18% decrease compared with placebo (p=0.468); that of group B was 0.40, with a 62% decrease (p=0.024); and that of group C was 0.72, with a 30% decrease (p=0.578). This section also reports the comparison of the 24-month pretreatment ARR (baseline ARR) with the 24-month on-treatment ARR of the all-time on-study population, including patients on natalizumab.

Section A **Characteristics**	**Group A** **(N=10)**		**Group B*** **(N=10)**		**Group C** **(N=9)**		**Placebo** **(N=12)**	
End point	X	Y	X	Y	X	Y	X	Y
Total number of relapses	22	17	27	8	16	13	20	25
Annual relapse rate (ARR)	1.10	0.85	1.35	0.40	0.88	0.72	0.83	1.04
Percentage of reduction compared with placebo (primary end point)†		−18		−62		−30		N/A
p Value against placebo		0.468		0.024		0.578		
ARR change % (Y to X)†		−23		−70		−18		+25
p Value against baseline		0.425		0.003		0.578		0.500

Section B **Excluding patients on natalizumab**	**Group A** **(N=9)**		**Group B*** **(N=10)**		**Group C** **(N=9)**		**Placebo** **(N=10)**	
End point	X	Y	X	Y	X	Y	X	Y
Total number of relapses	16	15	27	8	16	13	13	19
ARR	0.88	0.83	1.35	0.40	0.88	0.72	0.65	0.95
Percentage reduction compared with placebo (primary end point)†		−13		−58		−24		N/A
p Value against placebo		0.493		0.016		0.412		
ARR change % (Y to X)†		−6		−70		−18		+46
p Value against baseline		0.857		0.003		0.578		0.354

X: total number of relapses for the 24 months pretreatment (baseline).
Y: total number of relapses for the 24 months on-treatment.
†Unadjusted estimate.
(From Pantzaris et al 2013)

Table 16. Clinical end points according to study group for the all-time on-study population (From Pantzaris et al 2013).

Characteristics*	Group A (n=10)	Group B (n=10)	Group C (n=9)	Placebo (n=12)	p Value of Group B versus placebo
Annual relapse rate over 1 year†	0.80	0.40	0.78	0.83	
Total number of relapses†	8	4	7	10	
Primary end points					
Annual relapse rate over 2 years (95% CI)†	0.85	0.40 (0.15–0.87)	0.72	1.04	0.024
Total number of relapses†	17	8	13	25	
Excluding patients on natalizumab	**(n=9)**	**(n=10)**	**(n=9)**	**(n=10)**	
Annual relapse rate over 2 years (95% CI)	0.83	0.40 (0.10–0.79)	0.72	0.95	0.016
Total number of relapses	15	8	13	19	
Secondary end points					
Cumulative probability of sustained progression increase by 1 point on EDSS, confirmed after 6 months, over 2 years %†	43	10 (1/10)	24	58 (7/12)	0.019
Excluding patients on natalizumab					
Cumulative probability of sustained progression increase by 1 point on EDSS, confirmed after 6 months, over 2 years %	33	10 (1/10)	24	70 (7/10)	0.006
Exploratory results					
Patient proportion with ≤1 relapse over 2 years %†	50 (5/10)	90 (9/10)	56 (5/9)	42 (5/12)	0.030
MRI					
Patient proportion with new or enlarging T2 lesions %†	–	29 (2/7)	–	67 (4/6)	
Excluding patients on natalizumab					
Patient proportion with no new or enlarging T2 lesions %	–	29 (2/7)	–	80 (4/5)	
DMT (interferons, glatiramer acetate) and natalizumab					
Patient proportion on DMT and natalizumab at the end of 2 years %†	80 (8/10)‡	60 (6/10)	67 (6/9)	75 (9/12)§	0.747

*CI denotes confidence interval.
†Including patients on natalizumab.
‡1 of 10 on natalizumab.
§2 of 12 on natalizumab.
DMT, disease-modifying treatment.

After excluding patients on monoclonal antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over the 2 years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016, Table 15B and 16). Pair-wise comparisons for the other two groups against the placebo did not yield significant results (Table 15A and B). The proportion of patients with ≤1 relapse for the 2 years on-study was higher in group B than in the placebo group (90% vs 42%, p=0.030, Table 16). The relapse rate was also compared during the 24 months before the entry into the study to the 24 months on-treatment for each intervention group. It was observed a significant relative reduction in the ARR (70%) only in group B (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 15A); within-group comparisons for the ARR reduction of the three other groups were not significant and remained not significant when the natalizumab-treated patients were further excluded from the analysis. The effect of

intervention group B through time at different time-windows versus placebo for all-time on-study patients were recorded (figure 19 A–D) although this analysis wasn't an assigned end point. It could however help to evaluate the efficacy profile through time. Intervention B reached maximum effect within 1 year on-treatment and remained stable thereafter at an ARR of 0.4 with some free-relapse time-windows. In Figure 19 D it is shown the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) for intervention B (n=10) versus placebo (n=10). The placebo group showed an expected trend of increased relapse incidence and this in accordance with the existing knowledge of how relapse history works in relation to future relapses in MS patients (contagion phenomenon) (Wang, Meyerson et al. 2009). This it is also true for all groups. Moreover the on-study patients in group B showed, during 12 month post study extended period benefit in the ARR compared with the placebo group (six relapses for the 10 participants within group B, 0.6 ARR vs. 19 for the 12 participants within the placebo group, 1.58 ARR) with a statistically significant 62% adjusted RRR in the ARR for group B (RRR 0.38, 95% CI 0.12 to 0.99, p=0.046).

For the ITT analysis the number of relapses for the drop-out patients is reported in table 8A. No statistically significant differences in ARR were found in any intervention group compare to placebo for the 24 months on-treatment (table 14B) and figure 20 for ITT on DMT. Despite high drop-out rate there was found a statistically significant difference for the comparison of the ARR in the 24 months before entry with 24 month on-treatment for group B (RRR 0.45, 95% CI 0.26 to 0.78, p=0.005).

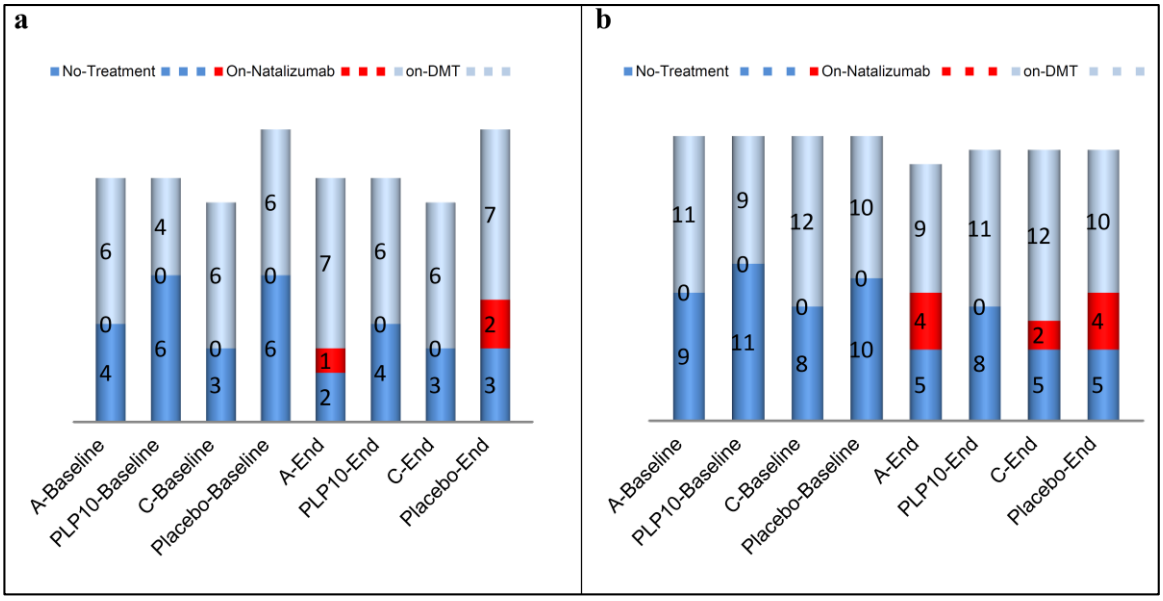

Figure 20. Population on DMT and/or natalizumab (From Pantzaris et al 2013).

(a) Demonstrates the all-time on-study population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial (including patients on natalizumab). No statistical significant differences were calculated. The all-time on-study patients per treatment-arm that were receiving DMT at entry baseline were six patients out of ten (60%) within group A, four out of ten (40%) within group B, six out of nine (66%) within group C and six out of 12 (50%) within placebo. When the study completed, 80% of the patients in group A, 60% in group B, 66% in group C and 75% of the patients in placebo ended up on treatment. Within group A one out of eight and within placebo two out of nine patients on DMT transferred on natalizumab. (b) Demonstrates the total randomized population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial without lost to follow (including patients on natalizumab). A total of 61% of group A patients were on DMT at entry baseline and became 72% at the end; for PLP10 group 41% and became 53%; for group C 73% and became 74% and for placebo 53% and became 74% at study completion. At the end: for group A, four out of 13 patients on DMT transferred on natalizumab; for group B no patient was on natalizumab; for Group C two out of 14 patients on DMT transferred on natalizumab; and for placebo group four out of the 14 patients on DMT transferred on natalizumab. No significant differences measured at entry baseline between the groups.

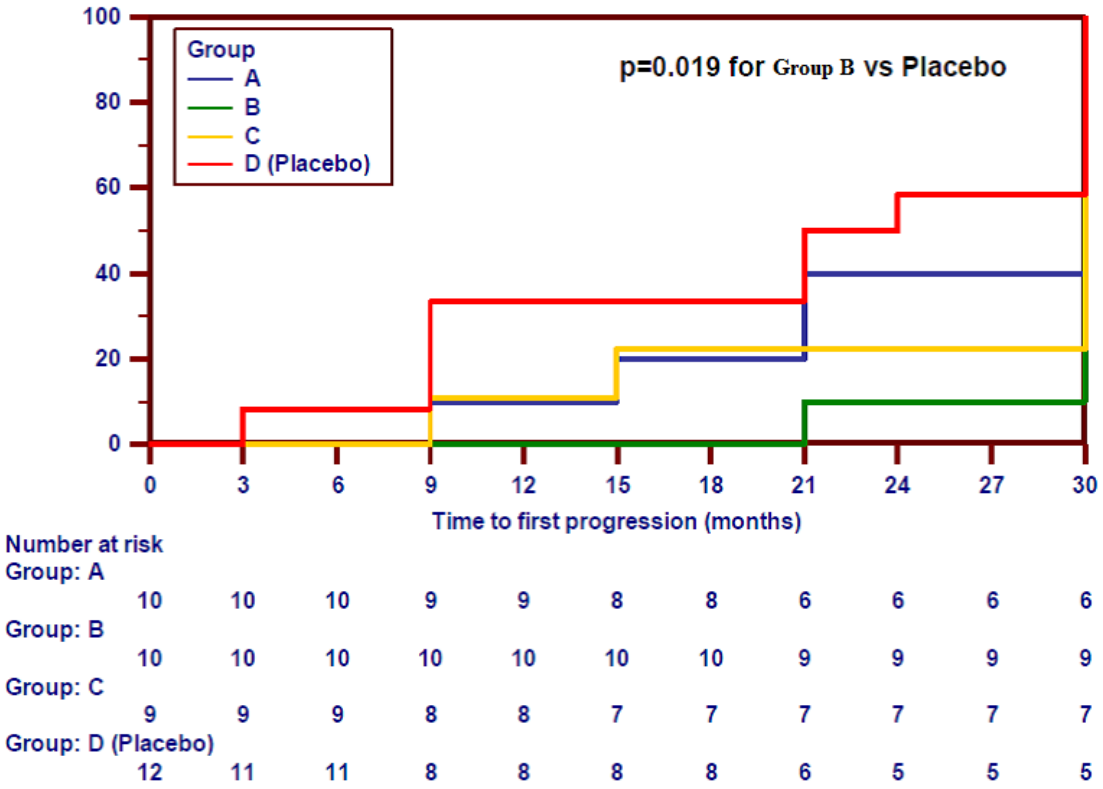

Figure 21. Kaplan–Meier estimates for the time to disability progression. Kaplan–Meier plot of the time to sustained progression of disability among all-time on study patients, including patients on natalizumab, receiving intervention A, B and C vs. placebo. Intervention Group B reduced the risk of sustained progression of disability by 83% over two years (p=0.019). The cumulative probability of progression was 10% in the intervention B group and 58% in the placebo group. Intervention formula A reduced the risk of sustained progression of disability by 32% (p=0.301) and intervention formula C by 62% (p=0.109) (From Pantzaris et al 2013).

3.4.4 Disability progression

In the per-protocol analysis, at 2 years, the time to disability progression was significantly longer only with group B. The cumulative probability of disability progression was 10% in group B and 58% in the placebo group (p=0.019); (figure 21). A statistically significant difference between group B and placebo was also observed after the exclusion of patients on natalizumab for the same analysis (p=0.006; figure 22A). For the period of 2 years, the cumulative probability of disability progression was found to be 10% in group B and 70% in the placebo group, which represents a decrease of 60% or a relative decrease of 86% for the risk of sustained progression of disability within group B (adjusted HR, 0.11; 95% CI

0.01 to 0.97, p=0.047). When patients on natalizumab were excluded, one patient out of 10 in group B and 7 out of 10 in the placebo progressed to confirmed disability. No statistically significant difference was observed for any comparison of the two other groups versus placebo (figure 22A).

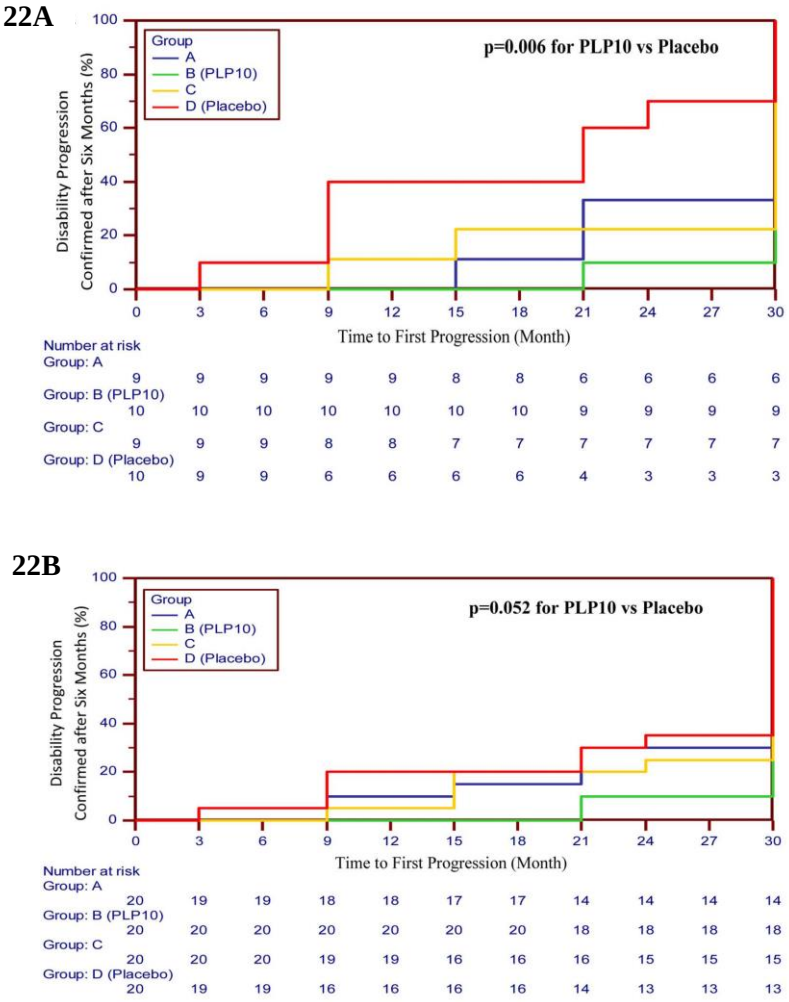

Figure 22. (A) Demonstrates the Kaplan-Meier plot of the time to sustained progression of disability among the all-time on-study patients, excluding the patients on natalizumab, receiving interventions A, B and C compared with placebo. Group B reduced the risk of the sustained progression of disability by 86% over 2 years (p=0.006). Intervention formula A reduced the risk of the sustained progression of disability by 53% (p=0.266), and intervention formula C, by 67% (p=0.061). (B) Demonstrates the Kaplan-Meier plot of the time to sustained progression of disability among the intention-to-treat population receiving interventions A, B and C compared with placebo. Group B reduced the risk of the sustained progression of disability by 71% over 2 years (p=0.052, trend). Intervention formula A reduced the risk of the sustained progression of disability by 22% (p=0.727), and intervention formula C, by 40% (p=0.447) (From Pantzaris et al 2013).

For the ITT analysis, at 2 years, the cumulative probability of progression was 10% in group B and 35% in the placebo group (p=0.052, a trend for an effect), which represents a decrease of 25% or a relative 71% decrease for group B with respect to the risk of sustained progression of disability (adjusted HR 0.22, 95% CI 0.04 to 1.07, p=0.06; figure 22B). Two patients of the total randomized patients in group B and seven in the placebo patients progressed to confirmed disability. No significant differences were observed for the groups A and C compared to placebo (figure 22B). In Figure 23 it is shown the mean change in the EDSS score per visit.

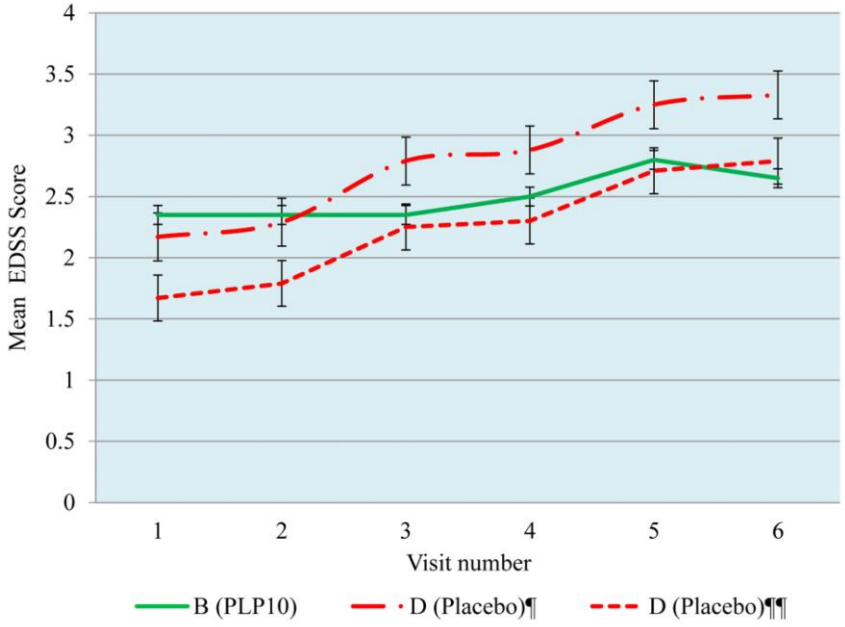

Figure 23. Mean change in the expanded disability status scale score as a function of visit number.The values are expressed as the mean±SE of the mean (s.e.m.), Including patients on natalizumab and excluding patients on natalizumab (From Pantzaris et al 2013).

3.4.5 MRI

Over 2 years, only 29% of group B and 67% of the placebo group developed new or enlarged T2 lesions (57% relative risk reduction) (see figure 24). An increased of relative risk reduction (64%) for group B versus placebo group after excluding the patients in natalizumab was observed.

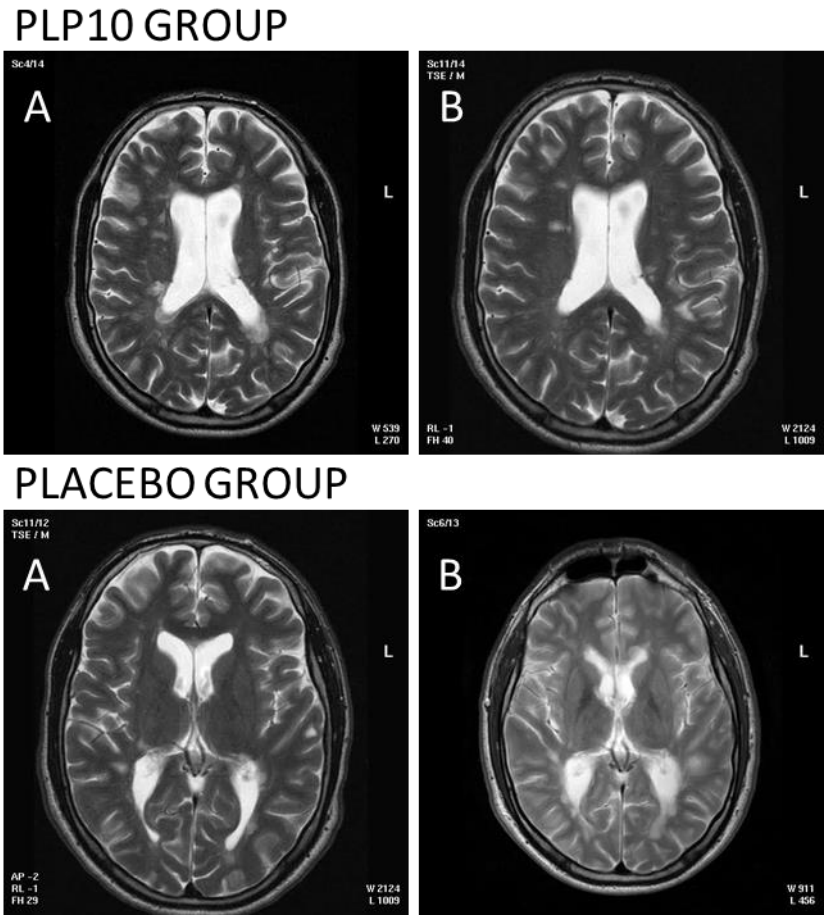

Figure 24. PLP10 group. Patient's MRI (A) 2007 before entry, and (B) 2010 at the end of the study. PLACEBO group. Patient's MRI (A) 2007 before entry, and (B) 2010 at the end of the study. It can be seen in the PLP10 group that the T2 lesions have reduced in numbers.

3.4.6 Safety

No significant adverse events were reported over the course of the study for any group. The main causes for dropouts from the study were palatability and smell of the formulas in addition to pregnancy. Only two patients reported nausea. No abnormal values were observed in any of the biochemical and haematological blood tests. No allergic reactions were reported.

3.5 Discussion

Nutrition is commonly accepted as one of the possible environmental factors involved in the pathogenesis of MS, but its role as a complementary MS treatment is unclear and largely

disregarded (Riccio 2011). It is well known that the majority of the patients suffering from MS do use dietary supplements for a variable length of time (Farinotti, Simi et al. 2007). Dietary antioxidants and fatty acids may influence the disease process in MS by reducing immune-mediated inflammation, oxidative stress and excitotoxic damage (van Meeteren, Teunissen et al. 2005). Published data have revealed that healthy dietary molecules have a pleiotropic role and are able to change cell metabolism and down regulate inflammation by interacting with enzymes, nuclear receptors and transcriptional factors (Riccio 2011). On currently available treatments are the products of reductionism, partially effective and associated with severe side effects. Interferons and glatiramer acetate, the most widely used first-line MS drugs available today, are associated with the least severe side effects among the MS therapies, but they are reported to reduce the ARR only by about one-third and with no significant effect on the progression of disability (Goldenberg 2012). Natalizumab reduces the ARR by 68% and decreases the possibility of disability progression by 43%, with 57% of patients free of new or enlarging T2 lesions on MRI scans, compared with 15% on placebo. Fingolimod is associated with a 54% ARR reduction (without a significant benefit on the progression of disability). Both natalizumab and fingolimod are second-line drugs associated with severe side effects (Kappos, Radue et al. 2010).

In this proof-of-concept, randomized, double-blind clinical trial it was sought to assess the safety and efficacy in RRMS of three different intervention treatments against a placebo. Group B intervention formula containing a balance mixture of omega-3 and omega-6 PUFAs, vitamin A (β carotene, vitamin E (alpha tocopherol) and vitamin E (gamma-tocopherol), showed, in the per-protocol analysis, a significant efficacy in the ARR and the progression of disability as compared to placebo. The results included analyses of a total period of 42 months of collected data, including a 12-month intervention-free-treatment extension period. The high drop-out rate that was observed was the result of formula

palatability, which is very common in trials using oily interventions. A statistical significant reduction in ARR was also observed in patients in group B when compare with the 24-month period prior to the study with the 24-months within the study; this became even larger when the patients who received natalizumab were excluded. The ARR in group B decreased within a year and remained stable until completion of the study. Interestingly the statistically significant difference in the ARR between group B and the placebo carried on for the 12-month extended period (persistent effect) without a significant difference on the DMT. MRI analysis showed that the patients free from new enlarging brain T2 lesions were higher in group B than in the placebo which supports the above differences in ARR.

During the first year of the treatment, the ARR was 0.80, 0.40, 0.78 and 0.83 for the four intervention groups, respectively. During the second year, the ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups, respectively. Overall, for the 2-year primary end point, eight relapses were recorded for the 10 patients in the PLP10 group (0.40 ARR) versus 25 relapses for the 12 patients on the placebo (1.04 ARR), a 64% adjusted relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% CI 0.15 to 0.87, p=0.024; tables 15A and 16 and figure 19A and C). Worth noting, as shown in table 12 section 3.2.3, there were no differences in the relapse rate, disease duration and EDSS score of patients during the 24-month period prior to enrolment, therefore the activity of the disease among all four groups was considered to bet similar. Furthermore after excluding patients due to monoclonal antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over the 2 years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016, table 15B and 16). This could be interpreted by the fact that the administered mixture of group B could be used as an adjuvant to any DMT, enhancing its effect on the disease. Pair-wise comparisons for the other two groups against the placebo did not yield significant results (Table 15 A and B). The proportion of patients with ≤1 relapse for the 2 years on-study was higher in the PLP10 group

than in the placebo group (90% vs 42%, p=0.030, table 16); This also supports the idea that the mixture administered to Group B could have an effect in efficiently reducing the relapse rate, if used as an adjuvant to the DMT. Olive oil, was used in the placebo group and in group C since there was lack of evidence to support that olive oil can modulate the function of the immune system in humans, although there has been contradicting evidence from other animal studies (Yaqoob 2002). Furthermore inconclusive evidence from animal studies, showed that the effects on the immune cells are due to oleic acid, rather than trace elements or antioxidants (Yaqoob 2002) The antioxidant present in olive oil is vitamin E as alpha tocopherol. In the intervention formulas (Groups B and C) gamma tocopherol was used instead. The inconclusiveness of the results as discussed by Yaqoob 2007 can be attributed to the higher MUFA levels used in animal studies. Also Yaqoob 2007 speculated that any effects observed in humans could be attributed to either an increased level of MUFA or a decreased consumption of SFA, since MUFA can substitute SFA in the diet (Yaqoob and Calder 2007). Olive oil consumption in Cyprus is high, approximately 3-4 tablespoons per day for several years (anecdotal evidence). The supplementation with olive oil (which will add to existing high olive oil intake) was not able to protect the patients in the placebo group to experience the significant higher rate of relapses during the study period. However the effects of MUFAs on adhesion molecules needs further investigation since their involvement in the pathology in a number of disease and probably MS.

In order to further investigate the observed difference, the relapse rate during the 24 months before the entry into the study and the 24 months on-treatment for each intervention group was compared. A significant relative reduction in the ARR (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, table 15A) was observed; within-group comparisons for the ARR reduction of the three other groups were not significant and remained not significant when the natalizumab-treated patients were further excluded from

the analysis; This despite that all groups showed no significant difference in the relapse rate or EDSS at entry (table 12). The effect of PLP10 through time at different time-windows versus placebo for all-time on-study patients is shown in figure 19A–D. Although the ARR analysis within time windows was not an assigned end point, it could help with the process of evaluating parallel information, such as the efficacy profile through time. PLP10 reached its maximum effect within 1 year on-treatment (counted from the entry baseline) and remained stable afterwards at an ARR of 0.4 with some free-relapse time-windows. Figure 19 D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) for PLP10 (n=10) versus placebo (n=12). The placebo group, in line with the existing knowledge of how the relapse history works in relation to future relapses in MS patients (contagion phenomenon), showed the expected trend of increased relapse incidences (Wang, Meyerson et al. 2009). The same phenomenon was true for groups A and C.

It is acknowledged that this study has two limitations: the small sample size and the high dropout rate. Regarding the sample size, one should bear in mind that this study is a small, proof-of-concept clinical trial assessing different interventions groups A, B, C and D. Therefore a larger randomized trial in which appropriate power calculations would be possible, taking into consideration the findings of this study could be organised. The other limitation of this study is the adherence of the participants, however it could be said that the total duration of the study that covers a total of 42 months follow-up adds power to the results(Committee for medicinal products for human use 2006). In addition the only way to deliver the intervention was by liquid form which is frequently associated with low compliance due to unpleasant taste. The high drop-out rate was therefore due to the problem of delivering the oils rather to any other site effects. The problem of taste is being address to the industry in order to improve the taste for future trials. As a direct consequence of the

low compliance and the loss of power, the performed ITT analysis was far less robust than intended, and we would then have to take into serious consideration the performed per-protocol analysis. It was focused on the per-protocol data analysis because it is the appropriate method to best provide the answer for the proof-of-concept trial-addressed question (Serhan, Arita et al. 2004). To validly incorporate the results of the per-protocol analysis into the interpretation of the overall results of the trial, it was needed to ensure that the randomization was not seriously violated due to the exclusion of the non-compliers. The comparison between the baseline characteristics of the patients included in the per-protocol analysis did show a relative balance in the compared groups for known confounders. Nevertheless, the presence of unknown confounders introducing bias to the trial results cannot be excluded despite nonsignificant differences in the baseline characteristics. As an additional safeguard towards that end, it was also performed adjusted analyses for the primary and secondary analyses for important clinical and demographic parameters, that is, relapses, EDSS, age and DMT. The present preliminary, small-size, randomised, controlled proof-of concept clinical trial provides evidence for a novel nutraceutical formula based on dietary, metabolic, immunological and neurobiological pathways possibly involved with disease progression in MS. This novel intervention showed signs of efficacy in the observed ARR and disability progression. Appropriate methodological measures were taken to control for potential sources of bias and to enable a valid interpretation to be reached. It is acknowledge that the presence of bias can only be minimised, not excluded, in any clinical research setting and also that random error is always a possible scenario in small trials.

CHAPTER FOUR

INTERVENTION WITH FATTY ACIDS/GAMMA TOCOPHEROL AND RED CELL MEMBRANE PHOSPHOLIPID FATTY ACID COMPOSITION

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4.1 Introduction

Farinotti et al in a Cochrane review reported that in the USA, Canada, the Netherlands, Germany and Australia, more than half MS patients have used complementary and alternative medicine as a treatment, mostly in the form of special diets/dietary supplements (Farinotti, Simi et al. 2007). In addition, scientists are trying to correlate diet habits and MS. A number of nutrients have been tested for efficacy on disease evolution by decreasing the number and severity of relapses and progression of disability. The focus of most studies of these is the omega-3/omega-6 PUFAs(Farinotti, Simi et al. 2007).

The mechanisms involved in the pathogenesis of MS include immune-mediated inflammation, formation of free radicals and oxidative stress. The Innate immune response in MS exhibits both neuro-protective and neuro-destructive functions against myelin components mediated by DCs, microglial, NK, NK-T and gamma delta T cells, which contribute to both effector and regulatory functions. The neuro-protective role is achieved by the secretion of neurotropic factors that help in promoting neurogenesis. The neuro-destructive functions are by direct cytotoxicity against myelin or oligodentrocytes and are mediated by perforins or reactive oxygen species (ROS) or direct contact-mediated by Fas-FasL interactions resulting in myelin disruption. The innate immune system can influence the initiation and progression of MS by influencing the effector function of T and B cells, which in turn express cytokines and activation markers that can activate innate immune cells further (Gandhi, Laroni et al. 2010).

The immune-mediated inflammation starts with increased migration of autoreactive lymphocytes across BBB. This pathological cascade of events stems from regulatory defects that allow an immune response within the brain (Compston and Coles 2008).

Furthermore Dendrou et al 2015 argue that there are different processes that drive relapses and chronic progression. This could be supported by the fact that progression of the disease is not greatly affected by the existing medications that target either the peripheral immune cell activation or entry to the CNS. It is therefore proposed that an additional inflammatory component residing in the CNS contributes to neuroaxonal loss and to the myelin-producing oligodentrocytes' death (Dendrou, Fugger et al. 2015).

Outside the CNS, autoreactive T cells are deleted in the thymus during the establishment of the central tolerance. This process is entirely perfect and as a result some autoreactive T cells are released into the periphery where, under physiological conditions peripheral, tolerance mechanisms can keep these cells under control. In pathological conditions these mechanisms can be damaged by the reduced function of T regulatory cells and/or increased resistance of effector B and T cells to these mechanisms. Molecular mimicry, novel autoantigen presentation, recognition of sequestered CNS antigen released into the periphery or bystander activation can cause activation of autoreactive B and T cells found in the periphery. The infiltration of activated $CD8^+$ T cells, differentiated $CD4^+$Th1 and Th17, B and innate immune cells into the CNS follows, which leads to inflammation and tissue damage. Furthermore B cells from the CNS will move to the periphery where they may undergo affinity maturation in the lymph nodes and then reentering the CNS causing further damage (Compston and Coles 2008; Dendrou, Fugger et al. 2015).

Peripheral, innate and adaptive immune cells, which are in the CNS from the early stages of the disease, accumulate in the perivascular spaces and may infiltrate through the meningeal

blood vessels by direct crossing of the BBB or the subarachnoid space or from the choroid plexus across the blood CSF barrier. Activated CNS-resident microglia and astrocytes, together with immune cells from the periphery, can promote demyelination and oligodendrocyte and neuroaxonal injury by the action of soluble, inflammatory and neurotoxic mediators and through direct cell contact-dependent mechanisms (Compston and Coles 2008; Dendrou, Fugger et al. 2015).

MS historically was considered a $CD4^+$ T-helper 1 (T_h1)-mediated disease. Studies have shown that proteins such as the myelin basic protein (MBP), the proteolipid protein. the myelin oligodendrocyte glycoprotein (MOG), the myelin-associated glycoprotein (MAG), 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase), the myelin-associated oligodendrocytic basic protein (MOBP), the oligodendrocyte-specific glycoprotein (OSP), the S100β protein, transaldolase-H (Tal-H), immunoglobulins and lipid components as antigens for $CD4^+$ T cells, are the main autoreactive targets that are recognized by circulating $CD4^+$ T_h1 and were found to be in increased levels of activation in MS patients as compared with controls. (Hollfield, Harbige et al. 2003; Harbige and Sharief 2007; Dendrou, Fugger et al. 2015). These T_h1 cells differentiate in response to the IL-12 cytokine (Tzartos, Friese et al. 2008). The activation of $CD4^+$ T_h1 that is directed against CNS antigens in the periphery, results to the upregulation of surface cell adhesion molecules and cytokine receptors, as well as the secretion of pro-inflammatory cytokines such as IL-2, interferon gamma (IFN-γ) and the tumor necrosis factor alpha (TNF-α), as well as chemokines and matrix metalloproteinases (MMPs). All these will cause changes in endothelial cells with adherence of the T_h1 into the endothelium and the subsequent facilitation of migration across the BBB (Milo and Miller 2014). After entering the CNS, T_h1 are reactivated by resident antigen presenting cells (APCs). This will result in the activation of resident microglia and astrocytes, B-cell, myeloid cell, natural killer (NK) cell recruitment and activation in the

areas of inflammation, the secretion of various cytokines, chemokines and MMPs as well as other mediators. Furthermore $CD8^+$ cytotoxic T-cells that recognize myelin proteins are present in the inflammatory area and perivascular regions. This results in the amplification of the damage on myelin, oligodendrocytes and axons by both antigen and non-antigen specific injury (Milo and Miller 2014). It has been shown that in MS patients, the increased number of $CD8^+$ T cells correlates with axonal damage. Moreover these cells are found in larger numbers in the white matter and in grey matter cortical demyelinating lesions than $CD4^+$ T_h1. (Dendrou, Fugger et al. 2015). Furthermore in the active lesions of MS patients, up to a quarter of $CD8^+$ T cells can produce IL-17 and are classed as mucosa-associated invariant T cells (MAIT cells), a type of $CD8^+$ T cells that are enriched at mucosal sites (Dendrou, Fugger et al. 2015).

On the other hand together B cells and their complement infiltrate the CNS and can serve as APCs, secreting cytokines and regulating T-cells Thus B-cells are contributing to further myelin injury through complement fixation or antibody-dependent cytotoxicity. Further damage can also be mediated through a non-antigen specific way by infiltrated macrophages, mast cells or reactive microglia. NK-cells can also cause injury to supporting astrocytes indirectly (Milo and Miller 2014). B cells vary in number along the progression of the disease. The Ig number is increased in CSF of MS patients, but not in serum, indicating local production of B cells (Sospedra and Martin 2005)). Clonally selected B cells are found in the meninges, parenchyma and CSF, and B cells that cross into the subarachnoid space produce antibodies that are detectable in the CSF. Furthermore the numbers of antibody-secreting plasma cells was found to increase with age in patients with primary or secondary progressive multiple sclerosis (Dendrou, Fugger et al. 2015).

Other T-cells, such as T_h2 cells, are recruited into the sites of inflammation and secrete anti-inflammatory cytokines hence downregulating activated T_h1 effector cells. T_h2 cells secrete neurotrophic factors which contribute to repair and neuroprotection (Milo and Miller 2014).

However it is now known that inflammation can also be driven by another T-lymphocyte subtype, (T_h17) which secretes IL-17, IL-6, TNF-α and IL-22 under IL-23 control (Compston and Coles 2008; Milo and Miller 2014). The penetration of T_h17 into the brain occurs due to the secretion of IL-17 and IL-22 which disrupt the BBB, resulting to neuronal death (Compston and Coles 2008). IL-23, IL-6 and the TGFβ help to enhance the effect of T_h17 (Tzartos, Friese et al. 2008). New evidence shows that IL-17 acts as a proinflammatory cytokine, supporting a pathogenic role for IL-17 in MS. Studies by Tzartos et al 2008, show that IL-17 is not only expressed by T-cells but also by astrocytes and oligodendrocytes in MS brains. It was also been shown that IL-17 mRNA expression and protein production is primarily restricted to the active areas of MS lesions, where higher densities of IL-17 T cells, $CD8^+$, $CD4^+$ T cells that express IL-17 are also found (Tzartos, Friese et al. 2008).

The antigen specificity of these immune responses is thought to be a result of a protein that mimics myelin proteins (van Rensburg, Kotze et al. 2012).

Cytokines are important molecules in all phases of the immune response. It is important that the homeostasis between pro- and anti- inflammatory cytokines is maintained in a dynamic way. Harbige et al 2007 demonstrated that the balance between pro- (TNF-α, IL-1β and IFN-γ) and anti (TGF-β1) inflammatory cytokines is dysregulated during the MS relapse-remission phase (Harbige and Sharief 2007). Proinflammatory cytokines, such as IFN-*γ*, TNF-*α*, IL-12, and IL-17 play a very important role in the pathogenesis of MS at different points via immune system activation in both the periphery and in the CNS, resulting to damaging of oligodentrocytes and myelin. Furthermore there is strong evidence that

demonstrates the implication of cytokines from activated T cells and macrophages in the pathogenesis of MS (Sospedra and Martin 2005; Harbige and Sharief 2007). For instance TNF-α, IL-1b, IFN-γ can control immunopathogenic events such as the up-regulation of adhesion molecules on endothelial cells and the subsequent infiltration of activated T-cell into the CNS (Harbige and Sharief 2007). These cytokines are found to be increased in number within the relapse phase (Harbige and Sharief 2007). These cytokines are myelinotoxic and can prolong the disease process as shown in EAE and are all shown to be present in the CNS active lesions of MS and found to be secreted by the peripheral blood mononuclear cells of MS patients (Harbige and Sharief 2007). Anti-inflammatory cytokines like IL-4, IL-10 have been considered beneficial (Sospedra and Martin 2005). For instance the anti-inflammatory and immunosupressant TGF-β1 is reduced in the relapse phase but increases as MS patients enter remission (Harbige and Sharief 2007).

Chemokines and their receptors play an important role for the recruitment of leukocytes and other cell types of the inflammation process. Chemokines induce and activate leukocyte adhesion molecules, establish a chemotactic concentration gradient across the endothelium, facilitate rolling and stopping on the endothelium and diapedesis across the BBB. Retention of leukocytes in the CNS is further maintained by chemokines. Among the various chemokines that have been found in the blood, CSF and lesions in MS are CCR5 and CXCR3 (as key receptors on Th1 cells) and CCR3 and CCR4 (on Th2 cells). CCR7, is an important marker for the capacity of mononuclear cells to migrate to secondary lymphoid organs (Sospedra and Martin 2005; Dendrou, Fugger et al. 2015).

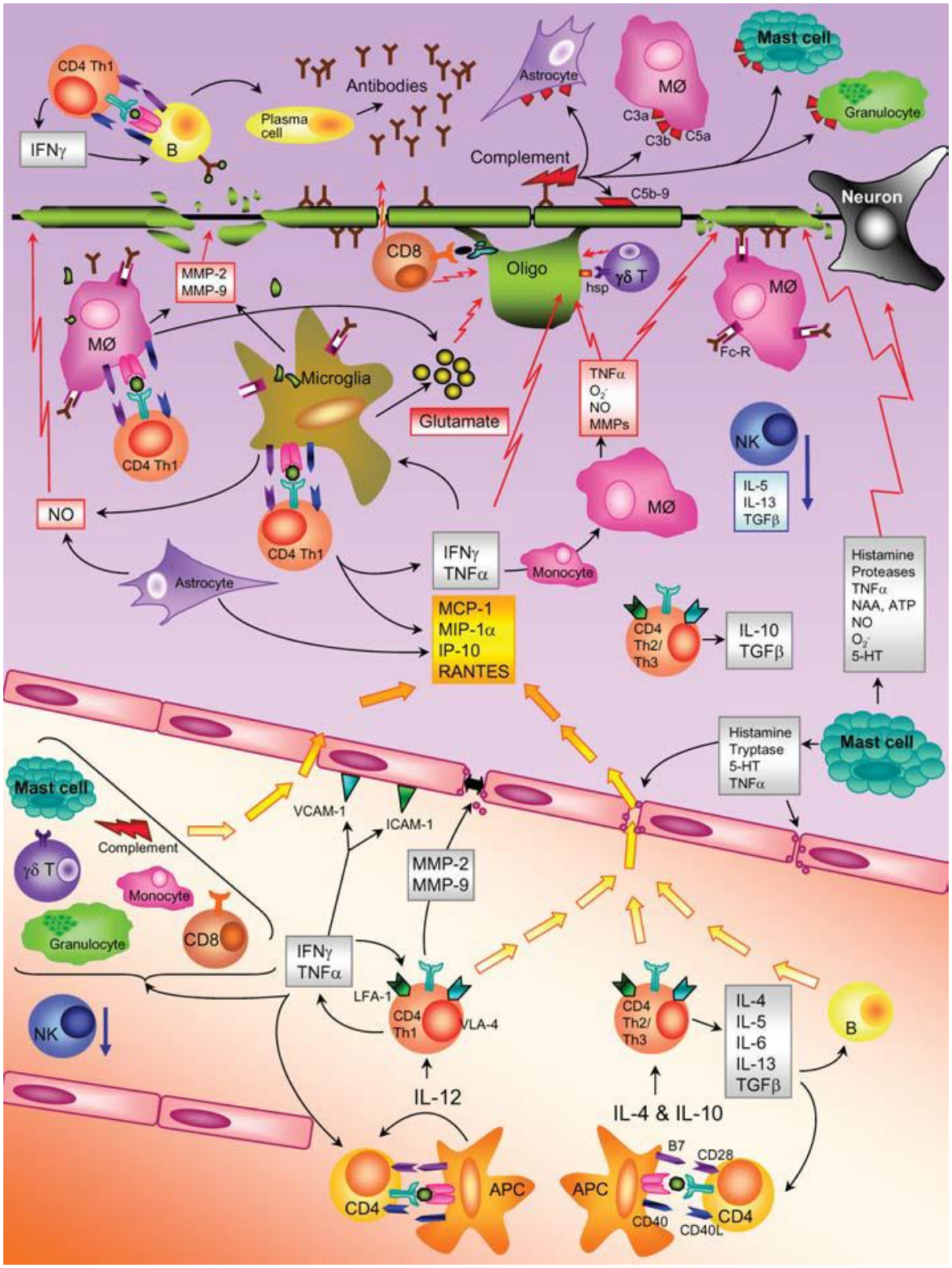


Figure 25. Summary of the most important immunological events in MS (Sospedra et al., 2005).

The products of oxidative stress, such as peroxynitrite and superoxide (reactive oxygen species (ROS)) have been reported as toxic to neurons (Farinotti, Simi et al. 2007). Free

radical formation as well as cellular injury from free radical formation may be decreased by the antioxidant activity of vitamins A, C and E. Hence these vitamins have been used in many trials in order to be tested for their effectiveness in the course of MS (Gilgun-Sherki, Melamed et al. 2004; Ferretti, Bacchetti et al. 2005; Koch, Ramsaransing et al. 2006).

Animal studies by Meade in 1978 and later Hughes in 1980, showed that LA could reduce the clinical severity of EAE in rats when given before induction of the EAE (Meade, Mertin et al. 1978; Hughes, Keith et al. 1980). Harbige in 1995 showed that induction of EAE can be partially suppressed by LA, whilst GLA exhibited full protection in a dose dependent manner (Harbige, Yeatman et al. 1995). Reduction of the severity of the acute EAE and the relapsing phase was observed by Harbige et al in 2000 after supplementing with GLA (Harbige, Layward et al. 2000). Harbige et al also observed that the reduction in the inflammatory response was associated with an increase in production of the transforming growth factor beta-1 (TGFβ-1) and prostaglandin E_2 (Hughes, Keith et al. 1980; Harbige, Layward et al. 2000).

4.1.1 Clinical trials

Non-controlled trials

Swank in a 35 year retrospective and nonrandomized study in 144 MS patients supplemented with cod liver oil or vegetable oils and fed low animal fat diet, concluded that there are long term benefits on mortality, relapse severity and disability, particularly if supplementation started early in the disease (Swank and Dugan 1990; Swank and Goodwin 2003). Cendrowski in 1986 in a non-controlled trial supplemented 12 MS patients (MSP n=12) with a combination of EPA and DHA omega-3 PUFAs for a period of 4 months (Cendrowski 1986) and showed a minimal change in disability. However the subgroup of MSP with the RR type showed a decrease in their mean EDSS score (Cendrowski 1986). Worth mentioning here is an observation by Simopoulos that any PUFA supplementation needs 4

months in order to show beneficial effects (Simopoulos 2002). Furthermore Fitzgerald et al 1987 investigated the effects of nutritional counselling on blood fatty acids and they also discussed the relation of a nutrient scoring system with neurological results. The results of 83 MS patients that completed at that time 34 months in the study out of 200 recruited were presented. The aim of the counselling was to decrease saturated fat and increase both ω-3 and ω-6 fatty acids in the diet. The results from this study showed a correlation between the EFA content of the diet and an increased blood plasma EFA status specifically LA, EPA, DHA with AA remained unchanged. The investigators also developed a scoring technique which took into account 5 parameters such as requirements for trace elements, involvement of vitamin B in the CNS, antioxidant protection for both water and fat soluble vitamins, requirements for both families of EFA. The results from the scoring system showed that patients in the top 24% remained neurologically stable and those achieving poor score i.e. on the lower 29% experienced a decline in neurological status (Fitzgerald, Harbige et al. 1987). In the Nordvik et al. 2000 study, 16 newly diagnosed patients were supplemented with EPA, DHA, vitamin E, vitamin A, D, C and B-complex for two years and at the same time advised patients on their diet in order to reduce saturated fats, increase fish, fruit and vegetable consumption, use wholegrain bread and cereals, reduce sugar and alcohol intake and stop smoking (Nordvik, Myhr et al. 2000). At the end of the 2 years there was a significant reduction in the mean and annual relapse rate and the mean EDSS score as compared with the baseline. They suggested from the results obtained that newly diagnosed MS patients can benefit from dietary advice and supplementation (Nordvik, Myhr et al. 2000).

Several clinical trials with the use of PUFAs that have been conducted in order to investigate their effect on the disease are inconclusive (Mehta, Dworkin et al. 2009).

Controlled trials

Millar et al in 1973, conducted a 2 year double blind controlled dietary study supplementing MS patients with the remitting relapsing (RR) form with LA reported that although there were no evidence that the treatment affected the overall rate of clinical deterioration there were clinical benefits with shorter and less severe relapses compared with the placebo group, receiving oleic acid, in the form of olive oil, an omega-9 fatty acid with no known immunomodulatory effects. (Millar, Zilkha et al. 1973; Bates, Fawcett et al. 1978). The same authors were also reported a non-significant trend in the ARR and the disability in both groups worsened to similar degree in the Kurtzke Disability Status Scale (DDS) and no differences were observed between the two groups (Millar, Zilkha et al. 1973).

In one other study that was conducted by Bates et al in1977 152 patients with chronic progressive MS were randomly allocated into 4 groups. One group received a combination of LA and GLA, a second group received placebo, olive oil, and two other groups received spreads, one group received LA spread and the other oleic acid. No significant differences were observed on disability, as measured by the DDS, or in the relapse rate or the relapse severity (Bates, Fawcett et al. 1977). Bates et al in a second trial, in 1978, randomly allocated 116 MS patients with the RR form into four groups (Bates, Fawcett et al. 1978). One group receiving LA and GLA, placebo group receiving oleic acid, and two groups receiving spreads one with LA and the other with oleic acid. They reported a shorter, less severe relapses in the LA spread group than the oleic group. The LA-GLA group had no effect on the relapse rate, severity and duration as compared to placebo furthermore more patients from the LA-GLA group showed more progression of disability than the placebo group. (Bates, Fawcett et al. 1978).

Paty et al in 1978 failed to show any effect on the progression of the disease (Paty, Cousin et al. 1978), after randomly assigning 96 patients with RR or progressive MS to LA or oleic

acid for 30 months (Paty, Cousin et al. 1978). Dworkin et al after reanalyzing the results of the three studies (Millar, Zilkha et al. 1973; Bates, Fawcett et al. 1978; Paty, Cousin et al. 1978) and after testing a hypothesis that the LA supplementation has more pronounced effect in patients that have s shorter disease duration and minimal disability at base line they concluded that LA has a beneficial effect in the reduction of severity of relapses and the rate of disease progression in MS patients (Dworkin, Bates et al. 1984).

Bates et al in 1989 in a 2 year double-blind study with 312 MS RR patients were randomly assigned to receive either a combination of EPA and DHA or the placebo, receiving oleic acid (Bates, Cartlidge et al. 1989). Both groups were also received advice how to decrease saturated fat and increase omega-6 PUFAs in their diet. In this study no beneficial trends were observed i.e. decreasing duration, frequency, and severity of MS relapse as compared to placebo (Bates, Cartlidge et al. 1989).

Weinstock-Guttman et al. in 2005 allocated 31 MS patients to a group receiving omega-3 PUFAs (EPA 1.98 g and DHA 1.32 g/day) or to placebo containing olive oil. Both groups received instructions how to decrease saturated fat in their diet. No differences were observed after 12 months in the trial between the two groups (Weinstock-Guttman, Baier et al. 2005).

In another study Harbige et al. in 2008 supplemented 28 MSRR for a period of 18 months with different dosages of BGC20-884, a GLA rich oil from borage oil. The high-dose GLA as compared to placebo and low-dose GLA significantly reduced relapse rates, and a reduced disability progression as measured with EDSS (Harbige, Pinto et al. 2008).

Gallai et al in 1995 supplemented with n-3 PUFA MS patients for a period of six months where they observed that n-3 PUFA decreased the secretion of pro-inflammatory cytokines, as well as reducing secretion of inflammatory eicosanoids as compared with placebo. This

study showed a beneficial effect from n-3 PUFA on the immune function of these patients by both modifying the immune cell membrane composition and the metabolic pathways involved in immune activation. N-3 PUFA may also play a role in inhibiting leukocyte/endothelium interaction, by lowering LTB4 production which in turn is reducing the production of adhesion receptors, which is an important event in the induction and maintenance of brain tissue damage in demyelinating diseases (Gallai, Sarchielli et al. 1995). In a more recent randomized, double-blind, placebo-controlled trial by Torkildsen et al 2012 (OFAMS study), 92 MS-RR patients received daily either 1350 mg of EPA and 850 mg of DHA or placebo for a total period of 24 months. It was found no difference in the relapse rate or the cumulative number of gadolinium-enhancing MRI lesions between the two groups with the conclusion that supplementation with ω-3 has no beneficial effects on disease activity (Torkildsen Ø and et al. 2012). Therefore the need of conclusive results is of major importance. Mehta et al 2009, placed first in the list "the question of appropriate clinical trial design". Either there were problems of selection bias, or disease activity differences among enrolled patients, or a miscellaneous number of patients that were in a different stage of the disease (relapsing remitting, primary or secondary progressive) or as a result of a small number of patients in the study. Duration of the trial was another issue that needed to be taken in consideration as reported by Mehta et al, 2009. Moreover, the different trials used different kind of PUFAs or used different concentrations as well as different ratios between the different kinds of lipids (Omega-3 vs. Omega-6). Therefore the need of an accepted clinical design/protocol according to the international standards for outcome measures that can detect differences in the disease progression are pivotal for conclusive results (Mehta, Dworkin et al. 2009).

In order to contribute on resolving this PUFA versus MS issue, a proof-of-concept clinical trial was carried out in order to investigate the incorporation and the changes that might

occur on the lipid composition of the red blood cell membrane before and after the intervention and correlate the efficacy of the different interventions with the disease progression (The IFNB Multiple Sclerosis Study Group and The UBC MS/MRI Study Group, Paty et al. 1993; Committee for medicinal products for human use 2006).

4.2 Laboratory methods

4.2.1 Gas Chromatography (GC)

Gas chromatography was first proposed as an analytical tool by A.J.P. Martin and R.L.M. Synge in article in 1941 (Martin and Synge 1941). Later in 1951 they developed their idea describing the first gas chromatograph and its use in the separation of volatile fatty acids (James and Martin 1952).

With Gas chromatography separation of the volatile organic compounds is dependent on the different partitioning behaviour between the mobile phase and the stationary phase in the column. GS consists of a flowing mobile phase (an inert gas), an injection point, the stationary phase (the column), a detector, and a data recording point.

The sample to be analysed is placed by a syringe in the injection port into the mobile phase before it meets with the stationary phase. The separation of the components is by elution and is detected at different times, depending on their retention time or the time that it takes them to travel through the column. The areas under the chromatographic peaks are proportional to the amount of the components (fatty acids) present in the sample, and are calculated by a computer linked to the detector.

4.2.2 Analysis of lipids and fatty acids

Lipid extraction

1ml of the sample was added to 15ml of methanol, 30ml of chloroform was then added (figure 26). Extraction tubes were stoppered and then shaken well, the samples in the

extraction tubes were flushed using nitrogen for approximately 20 seconds, stoppered and placed in the fridge at 4^0C overnight.

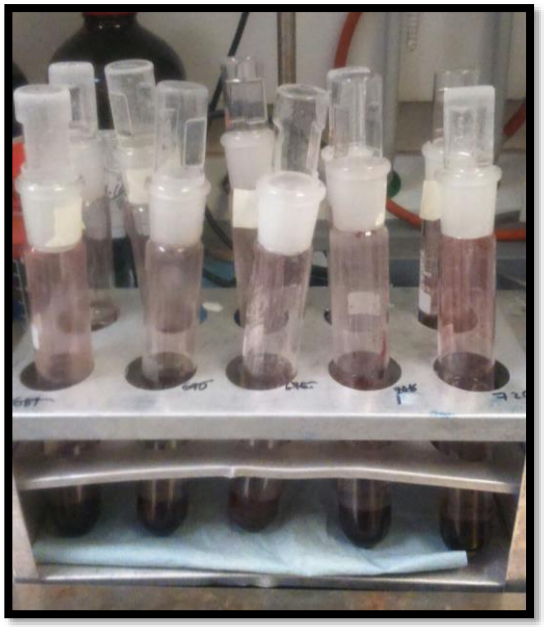


Figure 26. Extraction tubes.

Partitioning-phase separation of lipid and non-lipid components

The extraction tubes were allowed to equilibrate at room temperature. A five clamp stand was set up in the fume cupboard with labelled 100 ml partitioning funnels (with well-fitting opening valves), and silver filter funnels lined with one filter paper (figure 27). Samples were transferred into the silver filter funnels in the extraction tubes. Extraction tubes were washed with 15ml of solvent to remove the RBCs. 25% by volume of 0.85% saline was added to the samples in the partitioning funnels after the filter funnels were removed. The solution in the funnels was flushed with nitrogen for 1 minute and then stoppered. The funnels were stored at 4^o C overnight.

Figure 27. Partitioning funnels

Recovery of total lipids

The funnels were allowed to equilibrate in a dark room for 30 minutes. The organic layer at the bottom was drained out. The extracting solvent mixture was evaporated off using a rotary evaporator (BuCHI UK Ltd, Chadderton, Oldham, UK) (see figure 28) under low pressure at 37^{o} C. 4ml of methanol were added to each sample and evaporated off again. This step was repeated twice. The dried lipid was dissolved after rinsing the sides of the flask with 2-3ml chloroform:methanol solvent mixture; this step was repeated 3 times. The lipids were then placed in storage vials were dried down with nitrogen to approximately 1 ml, flashed with nitrogen and stored at 4^{o} C prior to thin layer-Chromatography (TLC).

Figure 28. Rotary evaporation.

4.2.3 Thin -Layer Chromatography (TLC)-Separation of lipids

Thin-layer chromatography plates

TLC plates (20 cm x 20 cm and 25 mm thickness) coated with silica gel were used for TLC experiments. They were conditioned (dried) at 120^{o} C for 1 hour in an oven and stored in desiccators until use.

Separation of phospholipids

a) Equilibration of developing chambers

A rectangular tank with thick heavy glass and a close fitting lid, suitable for 2 plates, was used for TLC. The tank was washed, rinsed with the solvent and allowed to dry before use. The inner walls of the tank were lined with filter paper. The solvent for phospholipid separation contained chloroform / methanol /methylamine (65:35:15 v/v/v) and contained 0.01% BHT (100 mg/l). (Butylated hydroxytoluene (BHT) is an antioxidant compound routinely added to extracting solvents and methylating reagent at 0.01% in fatty acid analysis. This compound is added to prevent the peroxidation of unsaturated fatty acids which are very labile/easily decomposed). The tank was filled with 150 - 200 ml of solvent

and allowed to equilibrate with the lid on for 30 minutes before use (the solvent should have reached the top of the filter paper).

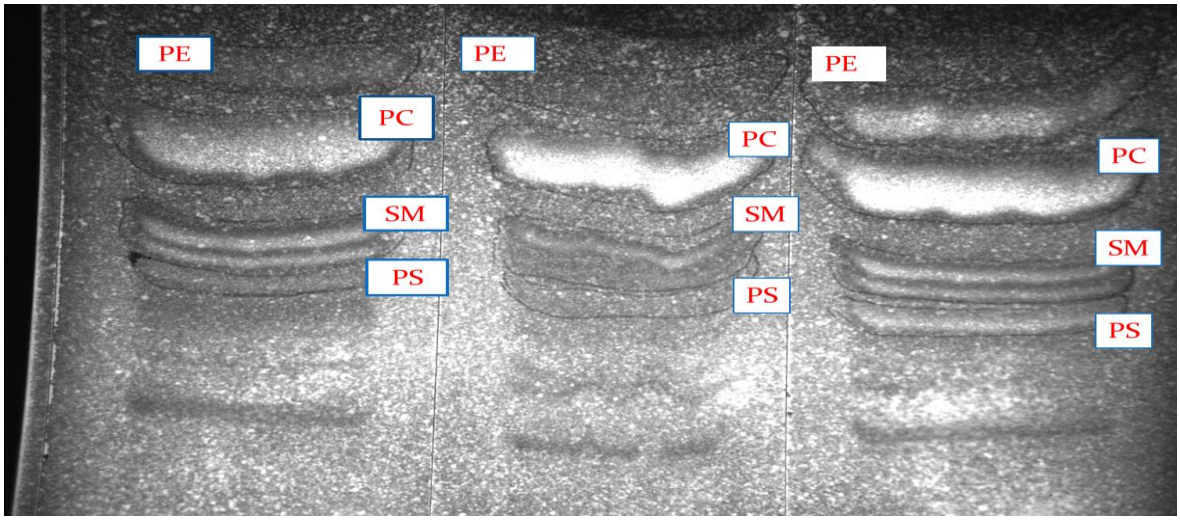
b) Application of sample to TLC plates

Once cooled, using a plastic guide plate, the silica was scored from top to bottom, approximately 3 cm from each side and from left to right at the top of the plate. Using a soft pencil, pencil lines were drawn from top to bottom, of the plate to separate the samples. Each plate held 2 samples and 1 standard. The total lipid extract after was dried down was dissolved in approximately 4 drops of CM solvent. The samples were carefully applied to a narrow and evenly distributed line on the bottom of the plate with a 2 µl Microcap pipettes. 2-3 more drops of CM solution were used to rinse the sides of the vial, and were then applied to plate.

Development of TLC plates

The plates were placed and sealed in the TLC tanks and left to develop for 90 minutes or until the solvent front had reached the line scored at the top of the plate. After reaching the top the plates were left in the tank for another 10 minutes to improve the definition of the bands. The plates were then removed and allowed to dry in a darkened fume cupboard.

Visualisation and identification



PE: phosphatidylethanolamine, PC: phosphatidylcholine, SM: Sphingomyeline, PS: Phosphatidylserine

Figure 29. Visualisation of bands.

The plates were removed from the tank, placed in the fume cupboard, dried in a stream of cold air, and sprayed with 2,7-dichloroflurescein (0.01% w/v) in methanol until the plate was lightly and evenly covered. The plates were left to dry under a stream of cold air. The visualisation of the bands was made under UV light (figure 29). The visualised bands were marked with soft pencil. The bands were identified using the phospholipid standards. The bands were scraped onto filter paper using a flat, blunt spatula, and the dust and flakes transferred to methylating tubes via a filter funnel.

4.2.4 Preparation of fatty acid methyl ester

4.2.4.1 Methylation (Methyl Esterification)

Transmethylation

The methylating reagent15% acetyl chloride in methanol, was prepared fresh (always less than 2 days old). 15 ml of acetyl chloride was added drop-wise to 100 ml of dry methanol in a 500 ml conical flask, while swirling the flask in the fume cupboard, being careful not to let it boil by keeping it in ice. The mixture was transferred to a stoppered bottle. 4 ml of the

methylating reagent was transferred using a Pasteur pipette to the bands scraped into the methylating tubes. The solution in the methylating tubes was flashed with nitrogen, vortexed well and the volume of the methylating mixture was monitored. Tubes were placed in the oven for 3 hours at 70^{o}C. The tubes were removed from the oven; the level of the methylating mixture was checked to ensure it had not decreased (in the case that the level decreased then methylating reagent was added up to the checked level) vortexed and returned to the oven. This was repeated once more. At the end of the 3 hours, the tubes were removed from the oven and allowed to cool to room temperature.

Extraction of fatty methyl esters

4 ml of 5% saline and 2 ml of petrol spirit and BHT were added to each sample (figure 30). Methylated tubes were capped and shaken vigorously. A few drops of methanol were added to dissolve emulsions. The upper petrol layer was carefully removed from the methylating tube to a second test tube containing 2 ml of 2% potassium bicarbonate in order to neutralise any acid transferred. 1 ml of petrol was added to methylating tube, mixed and shaken vigorously and the transferred to the test tube containing the potassium bicarbonate. This step was repeated one more time to obtain a total extract of 4 ml. The petrol/potassium bicarbonate suspension extract was vortexed and the upper layer transferred to a test- tube containing 100-200 mg (depending on the amount of methanol added) dried granular sodium sulphate, to remove any traces of water. The solution of fatty acid methyl esters in petrol was then transferred to a 3 ml vial and the solvent was removed under a stream of nitrogen gas and an aliquote was taken up in 1 ml heptane and BHT. The sample was sealed in nitrogen gas and stored at -20 ^{0}C until further use.

Figure 30. Extraction of fatty methyl esters

4.2.5 Analysis of fatty acid methyl esters (FAMEs)

FAMEs were separated by a gas liquid chromatograph (HRGC MEGA 2 Series, Fisons Instruments, Italy) fitted with a BP 20 capillary column (30m x 0.32mm ID, 0.25 m film, SGE (UK) Ltd., Potters Lane, Kiln Farm, Milton Keynes, UK). Hydrogen was used as a carrier gas. The injector, oven and detector temperatures were 240, 210 and 260 ^{o}C respectively. The oven temperature was programmed to start at 150 ^{o}C for 1 minute and to gradual increase to 220^{0}C. Flame ionization detector signals were evaluated with computer software (EZChrom Chromatography Data System, Scientific Software Inc., San Ramon, CA). The FAME peaks were identified by comparison with authentic fatty acids, analytical standard fatty acids purchased from Sigma-Aldrich Cillingham, UK. They were characterised and quantified with the use of gas chromatograph mass spectrometer by the company. FAMEs were identified by comparison of retention times with authentic standards (the authentic standards included, saturates (C10:0 to C24:0), monounsaturated (C14:1 to

C24:1), ω-6 (C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6 and C22:4n-6) and ω-3 (C18:3n-3, C18:4n-3, C20:5n-3,C 22:5n-3, C22:6n-3)); and interpretation of equivalent chain length (the logarithm of the relative retention time of saturated fatty acids (retention time of C10:0 to C24:0 divided by the retention time of C18:0) was plotted against the chain length of the fatty acids (C10:0 to C24:0). The resulting graph which is linear is used to identify an unknown fatty acid from its relative retention time. An internal or external standard was not added to the samples before extraction or the lipid extracts of the samples for quantification purposes.

The fatty acids data is expressed as a relative area percent (% total fatty acids). Eg. Percent of C16:0 = Peak area of C16:0 divided by the sum of the peak areas of all the fatty acids commonly found in biological samples multiplied by 100.

4.3 Statistical analysis

Statistical analysis for PUFA comparison between groups was performed with one-way Analysis of Variance (ANOVA) with a Bonferonni correction. A two-tailed t-test statistical analysis was also performed with a Bonferonni correction for comparison of PUFA concentration between time points. SPSS Statistics V. 20 (IBM) statistical analysis was used for both the above.

4.4 Results

Among the 80 patients, 20 were randomly assigned to each of the three groups to receive the interventions, and 20 to receive the placebo. Five patients were lost to follow-up before their first scheduled visit. Two other patients who dropped out before their first scheduled visit progressed to secondary progressive MS. Fifteen patients dropped out by the first six months of the trial, including five pregnancies. Another 17 patients dropped out early after the six months period. Seven patients who dropped out were given monoclonal antibody treatment

(natalizumab). Overall, a total of 41 (51%) patients completed the 24-month study, one patient from group A and two from the placebo group transferred to natalizumab, and 39 (49%) patients either withdrew (dropped out) or were lost to follow-up.

Data are available at 0, 6, 18 and 24 months. However due to the similarities of the results between 18 and 24 months the data from 24 months were used for simplicity for this thesis.

The data collected in this study was analysed to detect differences between time 0 and 6 months and between time 0 and 24 months (end of the study). Several interesting statistically significant results were obtained as follows: (Note, p=0.05 was used to indicate significance).

At time zero, significant differences were observed for LA (C18:2n-6), C22:4 n-6 and C22:5 n-6 fatty acids in the PS fraction of the RBC lipid membrane between the different groups (Table 17). The concentration of C18:2n-6 fatty acid was higher in group A as compared to groups B, C and D. Between groups B and C the concentration is higher in C ($p=<0.001$). The concentration of the C22:4 n-6 fatty acid was higher in B as compared to A ($p=0.01$). The concentration of the C22:5 n-6 fatty acid was higher in group D as compared to A ($p=0.01$).

Table 17. Showing the Fatty acid profile of the phosphatidylserine (PS) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at time zero**.**

RBC PS Time Zero	Group A n=10		Group B n=10		Group C n=9		Group D n=12	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14:0	0.03	±0.01	0.05	±0.02	0.03	±0.02	0.04	±0.02
C16:0	6.43	±1.39	5.84	±0.76	6.63	±1.74	5.57	±1.07
C18:0	41.44	±1.00	40.57	±1.63	41.59	±1.90	40.15	±3.17
C20:0	0.25	±0.14	0.23	±0.08	0.27	±0.12	0.23	±0.09
C22:0	0.23	±0.15	0.21	±0.07	0.31	±0.22	0.22	±0.08
C24:0	0.19	±0.17	0.21	±0.18	0.30	±0.33	0.19	±0.11
Σ Saturates	48.57	±2.87	47.12	±2.72	49.13	±4.33	46.40	±4.53
C16:1n-7	0.01	±0.01	0.03	±0.02	0.04	±0.03	0.04	±0.03
C18:1n-7	0.32	±0.14	0.38	±0.19	0.32	±0.24	0.41	±0.18
C18:1n-9	6.28	±0.93	6.44	±0.45	6.72	±0.69	7.08	±0.92
C20:1n-9	0.28	±0.13	0.32	±0.10	0.30	±0.11	0.28	±0.15
C22:1n-9	0.33	±0.12	0.30	±0.28	0.18	±0.07	0.25	±0.14
C24:1n-9	0.35	±0.25	0.27	±0.16	0.39	±0.36	0.28	±0.26
Σ Monoenes	7.58	±1.58	7.73	±1.20	7.97	±1.50	8.33	±1.68
C20:3n-9	0.23	±0.28	0.16	±0.11	0.09	±0.04	0.18	±0.18
[a]C18:2n-6	5.90	±0.54	4.35	±0.63	5.81	±1.01	5.09	±0.61
C18:3n-6	0.07	±0.08	0.07	±0.07	0.10	±0.07	0.11	±0.06
C20:2n-6	0.25	±0.23	0.27	±0.12	0.29	±0.12	0.25	±0.10
C20:3n-6	2.17	±0.49	1.80	±0.51	2.36	±0.78	2.22	±0.53
C20:4n-6	20.03	±4.76	23.17	±3.38	19.09	±4.19	22.46	±3.11
[b]C22:4n-6	2.44	±0.58	3.96	±0.94	3.08	±1.37	3.38	±1.08
[b]C22:5n-6	0.77	±0.22	1.05	±0.27	0.94	±0.35	1.25	±0.43
N-6 Metabolites	25.73	±6.35	30.32	±5.29	25.85	±6.88	29.68	±5.30
Σ N-6	31.63	±6.90	34.67	±5.92	31.66	±7.90	34.77	±5.91
C18:3n3	0.10	±0.09	0.07	±0.04	0.13	±0.22	0.16	±0.20
C20:5n3	0.56	±0.09	0.57	±0.05	0.56	±0.09	0.57	±0.06
C22:5n3	2.69	±0.70	2.23	±0.66	2.33	±0.54	2.31	±0.61
C22:6n3	6.40	±0.86	6.54	±0.27	6.07	±0.50	6.25	±0.69
N-3 Index	6.96	±0.95	7.11	±0.33	6.63	±0.59	6.82	±0.75
N-3 Metabolites	9.65	±1.65	9.34	±0.99	8.97	±1.13	9.13	±1.35
Σ N-3	9.75	±1.74	9.41	±1.03	9.10	±1.35	9.30	±1.55
16:0 DMA	0.07	±0.03	0.36	±0.80	0.06	±0.07	0.08	±0.05

[a]p=<0.001 between groups A and B, groups C and B, [a]p=0.05 between groups A and D
[b]p=0.01 between groups A and B, A and D

Significant differences were observed in the SM fraction of the RBC lipid membrane at time zero (Table 18) regarding the concentrations of C16:0, C24:0, C18:1n-9, C20:2n-6, C20:4n-6, C22:5n-3, C22:6n-3. For C16:0 a higher concentration was observed in C as compared to D (p=0.029). For C24:0 a higher concentration was detected in group C when compared to B (p=0.004) and D (p=0.023). For C18:1n-9 a higher concentration was detected in group D as compared to A (p=0.037) For C20:2n-6, a higher concentration was detected in group A as compared to B(p=0.008) and D (p=0.043). For C20:4n-6 a higher concentration was observed in group A as compared to C and B (p=<0.001 for both comparisons) and higher in D when compared to C (p=<0.001)and B (p=0.032). For C22:5n-3 a higher concentration in B was observed when compared to A, C and D (p=<0.001 for all comparisons). For C22:6n-3 a higher concentration was observed in B as compared to C (p=<0.001), and D (p=0.043).

Table 18. Showing the Fatty acid profile of the sphingomyelin (SM) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at time zero.

RBC SM Time Zero	Group A n=10		Group B n=10		Group C n=9		Group D n=12	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
C14:0	0.19	±0.10	0.33	±0.46	0.32	±0.24	0.19	±0.09
[a]C16:0	25.32	±2.57	26.18	±3.24	26.86	±1.69	23.97	±2.02
C18:0	13.51	±1.99	13.21	±1.61	12.19	±1.57	12.80	±0.95
C20:0	1.52	±0.23	1.40	±0.29	1.56	±0.23	1.56	±0.26
C22:0	4.32	±0.37	4.26	±0.76	4.39	±0.67	4.30	±0.55
[b]C24:0	17.03	±0.89	16.33	±0.91	18.00	±1.12	16.76	±0.98
Σ Saturates	61.87	±6.15	61.71	±7.26	63.31	±5.52	59.58	±4.84
C16:1n-7	0.16	±0.02	0.17	±0.03	0.17	±0.04	0.19	±0.06
C18:1n-7	0.28	±0.08	0.27	±0.14	0.23	±0.08	0.30	±0.09
[c]C18:1n-9	5.77	±1.57	6.30	±1.23	6.40	±1.34	7.27	±0.86
C20:1n-9	0.22	±0.05	0.19	±0.05	0.20	±0.04	0.19	±0.03
C22:1n-9	0.43	±0.08	0.45	±0.07	0.45	±0.09	0.44	±0.07
C24:1n-9	17.51	±1.37	16.67	±2.19	16.72	±1.08	17.92	±1.77
Σ Monoenes	24.37	±3.17	24.04	±3.72	24.17	±2.67	26.31	±2.88
C20:3n-9	0.09	±0.10	0.10	±0.08	0.09	±0.08	0.15	±0.13

C18:2n-6	3.19	±0.70	3.56	±0.87	3.59	±0.72	3.98	±0.54
C18:3n-6	0.11	±0.15	0.08	±0.06	0.18	±0.16	0.20	±0.23
[d]C20:2n-6	0.57	±0.11	0.41	±0.12	0.50	±0.07	0.45	±0.10
C20:3n-6	0.48	±0.15	0.43	±0.22	0.35	±0.16	0.35	±0.22
[e]C20:4n-6	5.63	±0.39	4.58	±0.25	4.14	±0.63	5.33	±0.72
C22:4n-6	0.50	±0.20	0.64	±0.16	0.50	±0.17	0.50	±0.19
C22:5n-6								
N-6 Metabolites	7.29	±1.00	6.14	±0.81	5.67	±1.19	6.83	±1.46
Σ N-6	10.48	±1.71	9.70	±1.68	9.26	±1.91	10.81	±2.01
C18:3n3	0.04	±0.02	0.04	±0.03	0.04	±0.02	0.05	±0.02
C20:5n3	0.22	±0.09	0.16	±0.04	0.22	±0.08	0.24	±0.13
[f]C22:5n3	0.87	±0.08	1.62	±0.25	0.86	±0.08	0.85	±0.08
[g]C22:6n3	1.44	±0.30	1.79	±0.75	1.08	±0.05	1.32	±0.20
N-3 Index	1.66	±0.39	1.95	±0.78	1.30	±0.13	1.56	±0.33
N-3 Metabolites	2.53	±0.47	3.57	±1.03	2.16	±0.21	2.41	±0.41
Σ N-3	2.57	±0.95	7.18	±2.10	4.35	±0.44	4.87	±0.85
16:0 DMA	0.17	±0.10	0.06	±0.02	0.14	±0.13	0.21	±0.13

[a]p=0.029 between groups: C and D; [b]p=0.004 between groups: C and B; p=0.023 between groups: C and D
[c]p=0.037 between groups: A and D
[d]p=0.008 between groups: A and B; p=0.043 between groups: A and D
[Te]p=<0.001 between groups: A and B, A and C, C and D; p=0.032 between groups: B and D
[f]p=<0.001 between groups: A and B, C and B, B and D
[g]p=<0.001 between groups: C and B; p=0.043 between groups: B and D

Significant differences were observed in the PE fraction of the RBC lipid membrane at time zero (Table 19) regarding the concentrations of C16:0, C18:0, C18:1n-7, C20:2n-6, C20:5n-3. For C16:0 a higher concentration was observed in A as compared to C (p=0.002) and B (p=0.007). For C18:0 a higher concentration was detected in B (p=0.016) and D (p=0.03) as both compared to A. For C18:1n-7 a higher concentration was detected in group D as compared to A (p=0.03) and C (p=0.04). For C20:2n-6 a higher concentration was observed in group C as compared to D (p=0.04). For C20:5n-3 a higher concentration was observed in group A as compared to B (p=<0.001), C (p=<0.001) and D (p=<0.001). For the same fatty acid a higher concentration was also observed in group B as compared to D (p=<0.001).

Table 19. Showing the Fatty acid profile of the phosphatidylethanolamine (PE) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at time zero.

RBC PE Time Zero	Group A n=10		Group B n=10		Group C n=9		Group D n=12	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14:0	0.11	±0.10	0.23	±0.15	0.16	±0.20	0.14	±0.09
[a]C16:0	14.94	±0.59	13.67	±0.72	13.59	±0.93	14.19	±0.84
[b]C18:0	7.54	±0.96	8.67	±0.83	7.98	±0.47	8.50	±0.77
C20:0	0.06	±0.02	0.07	±0.01	0.07	±0.01	0.11	±0.11
C22:0	0.14	±0.03	0.12	±0.05	0.15	±0.03	0.16	±0.02
C24:0	0.15	±0.03	0.16	±0.03	0.15	±0.03	0.15	±0.03
Σ Saturates	22.94	±1.72	22.93	±1.79	22.09	±1.67	23.24	±1.86
C16:1n-7	0.23	±0.21	0.24	±0.20	0.24	±0.20	0.25	±0.20
[c]C18:1n-7	0.83	±0.33	0.93	±0.26	0.86	±0.26	1.24	±0.38
C18:1n-9	15.88	±1.60	15.39	±1.28	16.74	±0.63	15.47	±1.46
C20:1n-9	0.51	±0.42	0.45	±0.31	0.50	±0.39	0.56	±0.33
C22:1n-9	0.03	±0.01	0.03	±0.02	0.02	±0.02	0.07	±0.06
C24:1n-9	0.14	±0.15	0.09	±0.06	0.13	±0.15	0.13	±0.10
Σ Monoenes	17.62	±2.71	17.13	±2.12	18.49	±1.65	17.73	±2.52
C20:3n-9	0.06	±0.03	0.06	±0.10	0.06	±0.05	0.12	±0.16
C18:2n-6	6.27	±1.04	6.50	±1.26	6.83	±1.00	6.32	±1.23
C18:3n-6	0.10	±0.06	0.15	±0.11	0.16	±0.19	0.12	±0.10
[d]C20:2n-6	0.25	±0.09	0.23	±0.09	0.31	±0.06	0.22	±0.07
C20:3n-6	1.13	±0.23	1.00	±0.22	1.23	±0.26	1.09	±0.29
C20:4n-6	18.79	±0.67	19.29	±0.88	19.11	±0.89	18.97	±1.09
C22:4n-6	6.66	±0.58	6.49	±1.00	6.19	±1.45	6.81	±0.70
C22:5n-6	0.85	±0.23	0.62	±0.18	0.72	±0.24	0.82	±0.58
N-6 Metabolites	27.77	±1.87	27.77	±2.48	27.72	±3.09	28.03	±2.82
Σ N-6	34.04	±2.91	34.27	±3.74	34.55	±4.09	34.36	±4.05
C18:3n3	0.35	±0.51	0.29	±0.29	0.31	±0.40	0.46	±0.53
[e]C20:5n3	2.42	±0.35	1.97	±0.08	1.98	±0.09	1.54	±0.29
C22:5n3	3.24	±0.60	3.09	±0.49	3.10	±0.59	3.16	±0.72
C22:6n3	5.93	±0.54	6.67	±0.84	6.37	±0.70	6.54	±0.50
N-3 Index	8.34	±0.88	8.63	±0.91	8.35	±0.79	8.08	±0.80
N-3 Metabolites	11.58	±1.49	11.73	±1.40	11.45	±1.38	11.24	±1.51
Σ N-3	11.93	±2.00	12.01	±1.69	11.76	±1.78	22.94	±3.55
[f]16:0 DMA	3.45	±0.74	2.99	±0.65	3.06	±0.67	2.62	±0.57
18:0 DMA	7.07	±0.59	6.55	±1.00	6.59	±0.62	6.95	±0.77
18:1 DMA	1.23	±0.19	1.44	±0.32	1.38	±0.30	1.32	±0.23
18:2 DMA	0.15	±0.05	0.10	±0.04	0.07	±0.05	0.12	±0.07

[a]p=0.002 between groups: A and C; p=0.007 between groups: A and B
[b]p=0.016 between groups: A and B; p=0.03 between groups: A and D
[c]p=0.03 between groups: A and D; p=0.04 between groups: C and D
[d]p=0.04 between groups: C and D
[e]p=<0.001 between groups: A and C, A and B, A and D, C and D, B and D
[f]p=0.03 between groups: A and D

At time zero, significant differences were observed for C18:1n-9 and C20:4n-6 fatty acids in the PC fraction of the RBC lipid membrane between the different groups (Table 20). The concentration of C18:1n-9 fatty acid was higher in group D as compared to B (p=0.045). The concentration of C20:4n-6 was higher in group C as compared to A (p=<0.001) and D (p= 0.026).

Table 20. Showing the Fatty acid profile of the phosphatidylcholine (PC) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at time zero.

RBC PC Time Zero	Group A n=10		Group B n=10		Group C n=9		Group D n=12	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
C14:0	0.35	±0.07	0.30	±0.08	0.33	±0.08	0.27	±0.07
C16:0	33.13	±1.59	33.84	±1.86	32.85	±1.65	32.86	±2.04
C18:0	14.54	±1.55	14.94	±1.15	15.05	±1.49	15.35	±1.15
C20:0	0.05	±0.03	0.06	±0.03	0.07	±0.02	0.07	±0.03
C24:0	0.04	±0.01	0.04	±0.01	0.07	±0.14	0.03	±0.01
Σ Saturates	48.11	±3.24	49.19	±3.12	48.43	±3.38	48.64	±3.34
C16:1n-7	0.18	±0.07	0.21	±0.08	0.22	±0.06	0.22	±0.08
C18:1n-7	1.57	±0.28	1.37	±0.30	1.52	±0.23	1.46	±0.08
[a]C18:1n-9	14.35	±0.72	13.61	±1.09	13.74	±1.13	14.87	±1.13
C20:1n-9	0.23	±0.03	0.24	±0.02	0.26	±0.03	0.25	±0.03
C22:1n-9	0.02	±0.02	0.03	±0.02	0.03	±0.02	0.03	±0.02
Σ Monoenes	16.38	±1.11	15.48	±1.51	15.85	±1.54	16.89	±1.42
C20:3n-9	0.04	±0.02	0.03	±0.01	0.03	±0.01	0.03	±0.02
C18:2n-6	22.74	2.71	21.66	±1.83	21.17	±1.70	21.25	±1.59
C18:3n-6	0.16	0.02	0.17	±0.01	0.16	±0.05	0.15	±0.03
C20:2n-6	0.22	0.04	0.20	±0.06	0.21	±0.07	0.21	±0.07
C20:3n-6	2.12	0.55	1.73	±0.57	1.97	±0.61	2.03	±0.54
[b]C20:4n-6	6.64	1.05	7.44	±1.09	8.29	±0.59	7.19	±0.80
C22:4n-6	0.44	0.15	0.43	±0.24	0.40	±0.17	0.44	±0.23
C22:5n-6	0.17	0.07	0.21	±0.08	0.24	±0.09	0.23	±0.10
N-6 Metabolites	9.76	±1.87	10.19	±2.05	11.28	±1.58	10.25	±1.77
Σ N-6	32.50	±4.58	31.84	±3.88	32.45	±3.29	31.50	±3.35
C18:3n3	0.36	±0.03	0.31	±0.07	0.32	±0.05	0.34	±0.06
C20:5n3	0.20	±0.06	0.23	±0.10	0.22	±0.11	0.26	±0.13
C22:5n3	0.38	±0.11	0.33	±0.11	0.39	±0.19	0.34	±0.11
C22:6n3	1.34	±0.46	1.42	±0.43	1.54	±0.50	1.52	±0.62
N-3 Index	1.55	±0.51	1.66	±0.53	1.76	±0.61	1.79	±0.74

N-3 Metabolites	1.93	±0.62	1.99	±0.64	2.15	±0.80	2.13	±0.85
Σ N-3	2.28	±0.65	2.30	±0.71	2.47	±0.84	2.46	±0.91
16:0 DMA	0.18	±0.11	0.18	±0.11	0.19	±0.12	0.16	±0.08
18:0 DMA	0.12	±0.14	0.09	±0.03	0.11	±0.05	0.07	±0.04
18:1 DMA	0.07	±0.03	0.05	±0.02	0.06	±0.02	0.07	±0.03

[a]p=0.045 between groups: B and D
[b]p=<0.001 between groups: A and C; p= 0.026 between groups: C and D

Significant differences were observed in the PS fraction of the RBC lipid membrane at six months' time (Table 21) regarding the concentration of C18:1n-9, C22:1n-9, C18:2n-6, C20:4n-6, C22:4n-6, C22:5n-6, C20:5n-3, C22:5n-3, C22:6n-3. For C18:1n-9 a higher concentration was observed in group C as compared to A (p=<0.001), and B (p=<0.001). For the same fatty acid a higher concentration was detected in D as compared to A (p=0.003) and B (p=<0.001). For C22:1n-9 a higher concentration was observed in group B as compared to C (p=0.036). For C18:2n-6 a higher concentration was observed in groups B (p =0.03), C (p=<0.001) and D (p=0.05) as all compared to A. For C20:4n-6 a higher concentration was detected in group D as compared to A (p=0.01), B (p=<0.001) and C (p=0.003). For C22:4n-6 a higher concentration was observed in group C as compared to A (p=<0.001), B (p=<0.001) and D (p=0.008). For the same fatty acid a higher concentration was observed in group D as compared to A (p=<0.001) and B (p=<0.001). For C22:5n-6 a higher concentration was detected in group C as compared to A (p=0.03). For the same fatty acid a higher concentration was observed in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=0.01). For C20:5n-3 a higher concentration was observed in group A as compared to C (p=<0.001 and D (p=<0.001). For the same fatty acid a higher concentration was detected in group B as compared to C (p=<0.001) and D (p=<0.001), a higher concentration was also observed in group D as compared to C (p=0.004). For C22:5n-3 a higher concentration was observed in group A as compared to C (p=0.002) and D (p=0.002). For C22:6n-3 a higher concentration was observed in group A as compared to C

(p=<0.001) and D (p=<0.001. For the same fatty acid a higher concentration was detected in group B as compared to C (p=<0.001) and D (p=<0.001).

Table 21. Showing the Fatty acid profile of the phosphatidylserine (PS) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at six months.

RBC PS Time 6 months	**Group A n=10**		**Group B n=10**		**Group C n=9**		**Group D n=12**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
C14:0	0.05	±0.02	0.05	0.02	0.05	±0.02	0.04	±0.02
C16:0	5.98	±1.06	4.94	0.59	5.71	±0.65	5.26	±1.06
C18:0	40.65	±1.33	40.23	0.87	40.64	±0.63	40.19	±1.44
C20:0	0.31	±0.12	0.22	0.08	0.23	±0.14	0.30	±0.26
C22:0	0.26	±0.14	0.35	0.43	0.22	±0.17	0.19	±0.09
C24:0	0.25	±0.12	0.41	0.40	0.22	±0.16	0.22	±0.09
Σ Saturates	47.51	±2.78	46.21	2.38	47.07	±1.77	46.19	±2.97
C16:1n-7	0.02	±0.01	0.05	±0.05	0.04	±0.03	0.03	±0.01
C18:1n-7	0.27	±0.11	0.37	±0.16	0.39	±0.37	0.32	±0.12
[a]C18:1n-9	6.58	±0.81	6.42	±0.58	8.48	±0.82	7.71	±0.69
C20:1n-9	0.32	±0.24	0.30	±0.12	0.26	±0.15	0.24	±0.11
[b]C22:1n-9	0.45	±0.41	0.54	±0.33	0.24	±0.17	0.21	±0.10
C24:1n-9	0.20	±0.08	0.22	±0.17	0.20	±0.20	0.17	±0.09
Σ Monoenes	7.84	±1.66	7.90	±1.42	9.60	±1.74	8.69	±1.13
C20:3n-9	0.09	±0.05	0.21	±0.29	0.22	±0.22	0.15	±0.18
[c]C18:2n-6	2.51	±0.55	4.25	±0.86	4.90	±1.68	4.02	±1.62
C18:3n-6	0.08	±0.03	0.06	±0.03	0.13	±0.12	0.06	±0.05
C20:2n-6	0.23	±0.12	0.25	±0.08	0.22	±0.06	0.20	±0.08
C20:3n-6	1.65	±0.25	1.72	±0.52	2.05	±0.61	1.95	±0.39
[d]C20:4n-6	20.81	±1.74	19.12	±2.64	20.50	±3.01	23.99	±1.77
[e]C22:4n-6	1.52	±0.26	2.15	±0.72	4.38	±0.74	3.45	v0.82
[f]C22:5n-6	0.48	±0.11	0.56	±0.19	0.79	±0.31	1.12	±0.30
N-6 Metabolites	24.76	±2.51	23.85	±4.19	28.07	±4.85	30.77	±3.41
Σ N-6	27.28	±3.06	28.09	±5.06	32.97	±6.53	34.79	±5.03
C18:3n3	0.09	±0.13	0.10	±0.13	0.24	±0.27	0.20	±0.23
[g]C20:5n3	0.84	±0.03	0.86	±0.02	0.65	±0.03	0.73	±0.09
[h]C22:5n3	3.24	±0.68	2.67	±0.64	2.26	±0.68	2.24	±0.41
[i]C22:6n3	12.29	±0.59	12.51	±1.53	6.10	±0.51	6.21	±0.50
N-3 Index	13.12	±0.63	13.36	±1.55	6.76	±0.54	6.95	±0.58
N-3 Metabolites	16.36	±1.31	16.03	±2.19	9.01	±1.22	9.19	±1.00
Σ N-3	16.44	±1.43	16.14	±2.33	9.25	±1.49	9.38	±1.23
16:0 DMA	0.03	±0.04	0.11	±0.07	0.04	±0.01	0.07	±0.06

[a] p=<0.001 between groups: A and C, B and C, B and D; p=0.003 between groups: A and D
[b]p=0.036 between groups: B and D
[c]p=<0.001 between groups: A and C; p =0.03 between groups: A and B; p=0.05 between groups: A and D
[d]p=0.01 between groups: A and D; p=<0.001 between groups: B and D; p=0.003 between groups: C and D
[e]p=<0.001 between groups: A and C, A and D, C and B, B and D; p=0.008 between groups: C and D
[f]p=0.03 between groups: A and C; p=<0.001 between groups: A and D, B and D; p=0.01 between groups: C and D
[g] p=<0.001 between groups: A and C, A and D , C and B, B and D p=0.004 between groups: C and D
[h] p= 0.002 between groups: A and C, A and D
[i] p=<0.001 between groups: A and C, A and D, C and B, B and D

Significant differences were observed in the SM fraction of the RBC lipid membrane at six months' time (Table 22) regarding the concentration of C16:0, C18:0, C22:0, C24:0, C24:1n-9, C18:2n-6, C20:4n-6, C22:4n-6, C20:5n-3, C22:6n-3. For C16:0 a higher concentration was observed in groups A (p=<0.001), B (p=<0.001) and C (p=0.002) when all compared to D. For C18:0 a higher concentration was observed in B when compared to A (p=<0.001), C (p=<0.001) and D (p=<0.001). For the same fatty acid a higher concentration was detected in group A as compared to D (p=<0.001), and a higher concentration was observed in group C as compared to D (p=<0.001). For C22:0 a higher concentration was observed in group B as compared to A (p=<0.002) and C (p=<0.005). For C24:0 a higher concentration was detected in group A as compared to B (p=0.037), a higher concentration was also observed in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=<0.001). For C24:1n-9 a higher concentration was observed in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=<0.001). For C18:2n-6 a higher concentration was observed in group C as compared to A (p=0.005), higher in D as compared to A (p=0.036). For C20:4n-6 a higher concentration was observed in group D as compared to A (p=0.011) and B (p=<0.001), a higher concentration was also detected in C as compared to B (p=<0.001). For C22:4n-6 a higher concentration was observed in group C as compared to A (p=<0.001), B (p=<0.001) and D (p=<0.001), a higher concentration was also detected in group D as compared to A (p=<0.001). For C20:5n-3 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001), a higher concentration was also

detected in group B as compared to C (p=0.003) and D (p=<0.001), a higher concentration was observed in group C as compared to D (p=0.032). For C22:6n-3 a higher concentration was observed in group A as compared to B (p=<0.001), C (p=<0.001) and D (p=<0.001), a higher concentration was detected in group B as compared to C (p=<0.001), a higher concentration was also observed in group D as compared to C (p=<0.001).

Table 22. Showing the Fatty acid profile of the sphingomyelin (SM) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at six months.

RBC SM Time 6 months	**Group A n=10**		**Group B n=10**		**Group C n=9**		**Group D n=12**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
C14:0	0.22	±0.09	0.33	±0.25	0.29	±0.14	0.22	±0.08
[a]C16:0	29.98	±1.21	29.58	±2.41	29.08	±1.29	25.89	±2.62
[b]C18:0	14.04	±1.18	16.41	±0.69	14.37	±0.99	11.69	±1.43
C20:0	1.46	±0.26	1.67	±0.23	2.19	±2.45	1.56	±0.28
[c]C22:0	4.71	±0.59	5.72	±0.40	4.81	±0.59	5.09	±0.71
[d]C24:0	13.78	±0.81	12.45	±1.31	14.59	±1.05	16.66	±1.02
Σ Saturates	64.19	±4.15	66.17	±5.28	65.33	±6.51	61.10	±6.13
C16:1n-7	0.21	±0.05	0.22	±0.08	0.20	±0.05	0.18	±0.04
C18:1n-7	0.25	±0.12	0.25	±0.14	0.28	±0.10	0.26	±0.10
C18:1n-9	8.00	±0.80	8.03	±0.63	7.45	±0.97	7.83	±0.77
C20:1n-9	0.18	±0.04	0.17	±0.05	0.20	±0.06	0.19	±0.04
C22:1n-9	0.44	±0.08	0.44	±0.08	0.46	±0.08	0.43	±0.07
[e]C24:1n-9	14.11	±1.04	13.64	±0.97	14.10	±1.40	18.48	±1.74
Σ Monoenes	23.18	±2.13	22.76	±1.95	22.69	±2.66	27.38	±2.77
C20:3n-9	0.12	±0.16	0.18	±0.21	0.10	±0.09	0.10	±0.10
[f]C18:2n-6	2.17	±0.38	2.69	±1.02	3.33	±0.90	3.10	±0.69
C18:3n-6	0.13	±0.13	0.09	±0.14	0.14	±0.11	0.25	±0.20
C20:2n-6	0.17	±0.24	0.17	±0.17	0.15	±0.09	0.14	±0.12
C20:3n-6	0.34	±0.15	0.31	±0.08	0.34	±0.19	0.31	±0.13
[g]C20:4n-6	3.58	±0.31	2.94	±0.62	4.14	±0.68	4.40	±0.69
[h]C22:4n-6	0.25	±0.03	0.25	±0.03	0.52	±0.09	0.42	±0.06
C22:5n-6								
N-6 Metabolites	4.47	±0.86	3.75	±1.04	5.16	±1.06	5.52	±1.19
Σ N-6	6.63	±1.25	6.44	±2.07	8.63	±2.07	8.62	±1.89
C18:3n3	0.06	±0.06	0.15	±0.25	0.05	±0.02	0.07	±0.05
[i]C20:5n3	0.47	±0.08	0.40	±0.14	0.27	±0.03	0.17	±0.04
C22:5n3	0.53	±0.26	0.36	±0.19	0.32	±0.20	0.27	±0.23
[j]C22:6n3	3.31	±0.43	1.93	±0.24	1.21	±0.13	2.00	±0.48
N-3 Index	3.78	±0.51	2.33	±0.38	1.47	±0.17	2.18	±0.52

N-3 Metabolites	4.31	±0.77	2.69	±0.57	1.80	±0.37	2.44	±0.75
Σ N-3	4.37	±0.82	2.84	±0.82	1.85	±0.39	2.51	±0.80

[a]p=<0.001 between groups: A and D, B and D; p=0.002 between groups: C and D
[b]p=<0.001 between groups: A and B, A and D, C and D, B and D, C and B
[c]p=0.002 between groups: A and B; p=0.005 between groups: C and B
[d]p=0.037 between groups: A and B; p=<0.001 between groups: A and D, C and B, C and D, B and D
[e]p=<0.001 between groups: D and A, D and C, D and B
[f]p= 0.005 between groups: A and C; p=0.036 between groups: A and D
[g]p=0.011 between groups: A and D; p=<0.001 between groups: C and B, D and B
[h]p=<0.001 between groups: A and C, A and D, C and B, B and C, C and D
[i]p=<0.001 between groups: A and C, A and D, B and D; p=0.003 between groups C and B; p=0.032 between groups: C and D
[j]p=<0.001 between groups: A and C, A and B, A and D, C and B, C and D

Significant differences were observed in the PE fraction of the RBC lipid membrane at six months' time (Table 23) regarding the concentrations of C18:0, C18:1n-9, C20:4n-6, C22:5n-6, C20:5n-3, C22:5n-3, C22:6n-3. For C18:0 a higher concentration was observed in group D as compared to A (p=0.004) and B (p=0.03). For C18:1n-9 a higher concentration was observed in group C as compared to A (p=<0.001) and B (p=<0.001), a higher concentration was also observed in group D as compared to A (p=<0.001) and B (p=<0.001). For C22:5n-6 a higher concentration was detected in group D as compared to A (p=0.03) and B (p=0.006). For C20:5n-3 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001), a higher concentration was detected in group B as compared to C (p=<0.001) and D (p=<0.001). For C22:5n-3, a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001), a higher concentration was also detected in group B as compared to C (p=<0.001) and D (p=<0.001). For C22:6n-3 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001), a higher concentration was also detected in group B as compared to C (p=<0.001) and D (p=<0.001).

Table 23. Showing the Fatty acid profile of the phosphatidylethanolamine (PE) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at six months.

RBC PE Time 6 months	Group A n=10		Group B n=10		Group C n=9		Group D n=12	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14:0	0.15	±0.03	0.15	±0.03	0.14	±0.03	0.13	±0.03
C16:0	13.75	±0.90	13.66	±0.71	13.39	±0.91	14.12	±0.63
[a]C18:0	6.66	±0.72	6.90	±0.83	7.49	±0.87	7.92	±0.79
C20:0	0.04	±0.02	0.05	±0.03	0.05	±0.02	0.06	±0.02
C22:0	0.30	±0.44	0.13	±0.01	0.13	±0.04	0.15	±0.03
C24:0	0.14	±0.02	0.13	±0.02	0.16	±0.03	0.16	±0.03
Σ Saturates	21.05	±2.14	21.02	±1.63	21.36	±1.91	22.53	±1.53
C16:1n-7	0.30	±0.06	0.34	±0.09	0.33	±0.06	0.31	±0.07
C18:1n-7	1.00	±0.13	1.10	±0.24	0.99	±0.09	1.01	±0.10
[b]C18:1n-9	14.23	±0.85	14.94	±0.42	18.85	±0.74	18.97	±0.70
C20:1n-9	0.40	±0.18	0.39	±0.21	0.39	±0.18	0.41	±0.15
C22:1n-9	0.11	±0.13	0.06	±0.08	0.03	±0.01	0.03	±0.01
C24:1n-9	0.14	±0.17	0.11	±0.13	0.21	±0.19	0.13	±0.07
Σ Monoenes	16.19	±1.51	16.94	±1.16	20.80	±1.27	20.86	±1.09
C20:3n-9	0.08	±0.11	0.02	±0.01	0.07	±0.06	0.04	±0.02
C18:2n-6	5.07	±0.96	5.83	±1.37	5.25	±0.78	5.16	±0.79
C18:3n-6	0.15	±0.03	0.15	±0.03	0.16	±0.03	0.15	±0.03
C20:2n-6	0.26	±0.13	0.24	±0.10	0.27	±0.13	0.26	±0.05
C20:3n-6	1.00	±0.35	0.99	±0.17	1.04	±0.21	1.03	±0.16
[c]C20:4n-6	14.13	±0.54	14.94	±0.87	18.93	±1.17	18.59	±0.64
C22:4n-6	4.45	±0.55	4.42	±0.82	4.80	±0.55	4.79	±0.49
[d]C22:5n-6	0.34	±0.08	0.28	±0.05	0.59	±0.20	0.75	±0.30
N-6 Metabolites	20.33	±1.68	21.02	±2.02	25.79	±2.30	25.57	±1.67
Σ N-6	25.40	±2.65	26.85	±3.39	31.04	±3.08	30.73	±2.46
C18:3n3	0.27	±0.10	0.26	±0.08	0.28	±0.10	0.23	±0.08
[e]C20:5n3	4.63	±0.57	4.47	±0.37	2.34	±0.17	2.45	±0.42
[f]C22:5n3	4.69	±0.27	4.36	±0.49	3.20	±0.61	3.19	±0.47
[g]C22:6n3	11.93	±0.53	11.64	±0.80	6.44	±0.94	6.39	±0.58
N-3 Index	16.56	±1.09	16.12	±1.18	8.78	±1.11	8.84	±1.00
N-3 Metabolites	21.25	±1.37	20.48	±1.67	11.98	±1.72	12.03	±1.46
Σ N-3	21.51	±1.47	20.74	±1.75	12.26	±1.82	12.26	±1.55
[h]16:0 DMA	4.46	±0.25	4.18	±0.71	4.05	±0.62	3.10	±0.49
[i]18:0 DMA	8.23	±0.79	7.53	±0.85	7.12	±0.91	6.57	±0.66
18:1 DMA	1.77	±0.41	2.15	±0.63	1.55	±0.51	2.10	±0.64
18:2 DMA	0.14	±0.06	0.12	±0.03	0.09	±0.04	0.14	±0.07

[a]p=0.004 between groups: A and D; p=0.03 between groups: B and D
[b]p=<0.001 between groups: A and C, A and D, C and B, B and D
[c]p=<0.001 between groups: A and C, A and D, C and B, B and D
[d]p=0.03 between groups: A and D; p=0.01 between groups: B and D
[e]p=<0.001 between groups: A and C, A and D, C and B, B and D

[f]p=<0.001 between groups: A and C, A and D, C and B, B and D
[g]p=<0.001 between groups: A and C, A and D, C and B, B and D
[h]p=<0.001 between groups: A and D, C and D, B and D
[i]p=0.02 between groups: A and C; p=<0.001 between groups: A and D; p=0.05 between groups: B and D

Significant differences were observed for the PC fraction of the RBC lipid membrane at six months' time (Table 24) regarding the concentrations of C16:0, C18:0, C18:1n-9, C22:1n-9, C24:1n9, C18:2n-6, C20:2n-6, C20:4n-6, C22:5n-6, C18:3n-3, C20:5n-3, C22:5n-3, C22:6n-3. For C16:0 a higher concentration was observed in groups A (p=<0.001), B (p=<0.001) and C (p=<0.001) as all compared to D. For C18:0 a higher concentration was detected in groups A (p=<0.001), B (p=<0.001), and C (p=<0.001) as all compared to D. For C18:1n-9 a higher concentration was observed in group C as compared to A (p=<0.001), for the same fatty acid a higher concentration was also detected in group B as compared to C (p=<0.001). For C22:1n-9 a higher concentration was observed in group B as compared to A (p=0.032). For C24:1n9 a higher concentration was detected in group B as compared to A (p=0.003), C (p=0.002) and D (p=0.002). For C18:2n-6 a higher concentration was observed in group A as compared to C (p=0.005), for the same fatty acid a higher concentration was detected in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=<0.001). For C20:2n-6 a higher concentration was observed in group A as compared to C (p=0.041). For C20:4n-6 a higher concentration was detected in group B as compared to A (p=<0.001) and C (p=<0.001), for the same fatty acid a higher concentration was detected in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=<0.001). For C22:5n-6 a higher concentration was observed in group A as compared to B (p=<0.001), C (p=<0.001) and D (p=<0.001). For C18:3n-3 a higher concentration was observed in group C as compared to A (p=<0.001) and B (p=<0.001), for the same fatty acid a higher concentration was also observed in group C as compared to A (p=<0.001) and B (p=<0.001). For the same fatty acid a higher concentration was detected in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=<0.001). For C20:5n-3 a higher concentration was observed in group A as

compared to C (p=<0.001) and D (p=<0.001), for the same fatty acid a higher concentration was also detected in group B as compare to C (p=<0.001) and D (p=<0.001). For C22:5n-3 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001), for the same fatty acid a higher concentration was also detected in group B as compared to A (p=0.026), C (p=<0.001) and D (p=<0.001). For C22:6n-3 a higher concentration was observed in group A as compared to C (p=0.023) and D (p=0.023), for the same fatty acid a higher concentration was also detected in group D as compared to B (p=0.023).

Table 24. Showing the Fatty acid profile of the phosphatidylcholine (PC) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at six months.

RBC PC Time 6 months	Group A n=10		Group B n=10		Group C n=9		Group D n=12	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14:0	0.31	±0.12	0.29	±0.09	0.32	±0.14	0.28	±0.08
[a]C16:0	36.70	±1.19	36.20	±1.02	37.34	±1.24	34.55	±1.07
[b]C18:0	15.62	±1.20	16.35	±0.80	15.57	±0.43	14.42	±0.82
C20:0	0.06	±0.03	0.05	±0.02	0.04	±0.03	0.04	±0.02
C24:0	0.03	±0.01	0.04	±0.01	0.07	±0.13	0.03	±0.01
Σ Saturates	52.72	±2.55	53.09	±2.00	53.72	±1.97	49.32	±2.00
C16:1n-7	0.45	±0.18	0.47	±0.20	0.51	±0.14	0.55	±0.15
C18:1n-7	1.47	±0.25	1.69	±0.24	1.65	±0.19	1.40	±0.31
[C]C18:1n-9	15.21	±0.97	15.05	±0.93	18.72	±0.71	18.35	±0.89
C20:1n-9	0.25	±0.04	0.24	±0.02	0.27	±0.02	0.27	±0.04
[d]C22:1n-9	0.02	±0.01	0.03	±0.02	0.03	±0.02	0.03	±0.01
[e]C24:1n-9	0.04	±0.01	0.19	±0.11	0.05	±0.03	0.04	±0.03
Σ Monoenes	17.42	±1.47	17.69	±1.52	21.23	±1.11	20.63	±1.43
C20:3n-9	0.01	±0.01	0.02	±0.02	0.03	±0.02	0.03	±0.02
[f]C18:2n-6	16.91	±2.01	15.88	±0.80	14.94	±0.91	19.43	±1.09
C18:3n-6	0.17	±0.02	0.16	±0.02	0.14	±0.04	0.16	±0.02
[g]C20:2n-6	0.21	±0.05	0.16	±0.03	0.16	±0.04	0.19	±0.05
C20:3n-6	1.54	±0.30	1.62	±0.21	1.70	±0.18	1.58	±0.33
[h]C20:4n-6	3.62	±0.40	4.44	±0.30	3.69	±0.34	5.69	±0.54
C22:4n-6	0.20	±0.07	0.32	±0.14	0.27	±0.09	0.33	±0.15
[i]C22:5n-6	0.26	±0.02	0.15	±0.03	0.15	±0.03	0.15	±0.03
N-6 Metabolites	6.00	±0.87	6.85	±0.72	6.11	±0.72	8.09	±1.12
Σ N-6	22.90	±2.88	22.72	±1.52	21.05	±1.63	27.52	±2.21

[j]C18:3n3	0.16	±0.03	0.16	±0.03	0.24	±0.03	0.32	±0.05
[k]C20:5n3	1.16	±0.08	1.19	±0.11	0.22	±0.10	0.24	±0.09
[l]C22:5n3	1.24	±0.14	1.45	±0.27	0.25	±0.09	0.29	±0.09
[m]C22:6n3	2.46	±0.81	1.96	±0.68	1.48	±0.34	1.22	±0.34
N-3 Index	3.62	±0.89	3.15	±0.79	1.71	±0.44	1.47	±0.43
N-3 Metabolites	4.86	±1.03	4.60	±1.06	1.96	±0.53	1.76	±0.52
Σ N-3	5.01	±1.06	4.77	±1.09	2.20	±0.56	2.08	±0.57
16:0 DMA	0.17	±0.13	0.17	±0.08	0.13	±0.05	0.18	±0.08
18:0 DMA	0.11	±0.08	0.14	±0.06	0.07	±0.03	0.10	±0.05
18:1 DMA	0.06	±0.02	0.06	±0.03	0.04	±0.02	0.06	±0.02

[a]p=<0.001 between groups: D and A, D and C; p=0.01 between groups: D and B
[b]p=0.01 between groups: D and A and D and C; p=<0.001 between groups: D and B
[c]p= <0.001 between groups: A and C, A and D, C and B, B and D
[d]p= 0.032 between groups: A and B
[e]p=0.003 between groups: B and A; p=0.002 between groups: C and B, B and D
[f]p=<0.001 between groups: D and A, D and B, D and C; p=0.005 between groups: A and C
[g]p=0.041 between groups: A and C
[h]p=<0.001 between groups: B and A, B and D, D and A, D and C; p=0.001 between groups: C and B
[i]p=< 0.001 between groups: A and B, A and C, A and D
[j]p=<0.001 between groups: C and A, B and C, D and A, D and B, D and C
[k]p=<0.001 between groups: A and C, A and D, C and B, B and D
[l]p=<0.001 between groups: A and C, A and D, C and B, B and D; p=0.026 between groups: A and B
[m]p= <0.001 between groups: A and C, D and A; p=0.023 between groups: D and B

Significant differences were observed for the PS fraction of the RBC lipid membrane at twenty four months' time (Table 25) regarding the concentrations of C16:0, C18:1n-9, C22:1n-9, C18:2n-6, C20:4n-6, C22:4n-6, C22:5n-6, C20:5n-3, C22:5n-3, C22:6n-3. For C16:0 a higher concentration was observed in group C as compared to A (p=0.04). For C18:1n-9 a higher concentration was observed in group C as compared to A (p=<0.001) and B (p=<0.001), for the same fatty acid a higher concentration was also detected in group D as compared to A (p=<0.001) and B (p=<0.001). For C22:1n-9 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001), for the same fatty acid a higher concentration was also detected in group B as compare to C (p=0.044) and D (p=0.027). For C18:2n-6 a higher concentration was observed in group C as compared to A (p=<0.001), B (p=<0.001) and D (p=<0.001), for the same fatty acid a higher concentration was also observed in group A as compared to B (p=<0.001) and D (p=<0.001). For C20:4n-

6 a higher concentration was observed in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=<0.001). For C22:4n-6 a higher concentration was observed in group C as compared to A (p=<0.001) and B (p=<0.001), for the same fatty acid a higher concentration was also detected in group D as compared to A (p=<0.001) and B (p=<0.001). For C22:5n-6 a higher concentration was observed in group C as compared to A (p=0.002) and B (p=0.019), for the same fatty acid a higher concentration was also observed in group D as compared to A (p=<0.001) and B (p=<0.001). For C20:5n-3 a higher concentration was observed in group A as compared to C (p=0.008) and D (p=<0.001), for the same fatty acid a higher concentration was also detected in group B as compared to C (p=<0.001) and D (p=<0.001).For the same fatty acid a higher concentration was observed in group C as compared to D p=0.005). For C22:5n-3 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001), for the same fatty acid a higher concentration was detected in group B as compared to C (p=<0.001) and D (p=<0.001). For C22:6n-3 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001), for the same fatty acid a higher concentration was also observed in group B as compared to C (p=<0.001) and D (p=<0.001). For the same fatty acid a higher concentration was observed in group D as compared to C (p=<0.001).

Table 25. Showing the Fatty acid profile of the phosphatidylserine (PS) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at twenty-four months.

RBC PS Time 24 months	**Group A n=10**		**Group B n=10**		**Group C n=9**		**Group D n=12**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
C14:0	0.03	±0.02	0.04	0.02	0.04	±0.01	0.03	±0.02
[a]C16:0	4.08	±0.60	5.17	±1.36	5.40	±1.54	5.03	±0.70
C18:0	40.77	±1.83	42.16	±1.86	41.49	±2.18	40.77	±0.71
C20:0	0.17	±0.08	0.16	±0.02	0.16	±0.05	0.16	±0.03
C22:0	0.14	±0.09	0.14	±0.08	0.18	±0.11	0.18	±0.10
C24:0	0.18	±0.10	0.13	±0.04	0.17	±0.10	0.12	±0.03
Σ Saturates	45.38	±2.72	47.79	±3.37	47.44	±3.99	46.29	±1.60
C16:1n-7	0.02	±0.01	0.02	±0.02	0.02	±0.01	0.02	±0.02

C18:1n-7	0.16	±0.11	0.25	±0.10	0.24	±0.12	0.18	±0.12
[b]C18:1n-9	5.76	±0.67	5.94	±0.81	8.27	±0.98	8.25	±0.75
C20:1n-9	0.29	±0.20	0.39	±0.22	0.36	±0.15	0.32	±0.17
[c]C22:1n-9	0.43	±0.27	0.34	±0.20	0.14	±0.06	0.13	±0.04
C24:1n-9	0.12	±0.05	0.12	±0.04	0.12	±0.06	0.14	±0.07
Σ Monoenes	6.78	±1.31	7.06	±1.38	9.15	±1.37	9.04	±1.17
C20:3n-9	0.07	±0.05	0.15	±0.21	0.16	±0.27	0.21	±0.34
[d]C18:2n-6	3.69	±0.83	2.44	±0.27	5.37	±0.94	2.38	±0.20
C18:3n-6	0.03	±0.02	0.03	±0.02	0.03	±0.02	0.03	±0.02
C20:2n-6	0.15	±0.04	0.16	±0.04	0.20	±0.05	0.16	±0.07
C20:3n-6	1.89	±0.63	1.72	±0.27	2.16	±0.66	1.79	±0.46
[e]C20:4n-6	20.71	±2.53	19.34	±3.64	20.67	±3.21	25.49	±1.61
[f]C22:4n-6	2.06	±1.02	2.08	±0.70	4.33	±0.75	4.20	±0.93
[g]C22:5n-6	0.62	±0.22	0.72	±0.39	1.23	±0.33	1.39	±0.49
N-6 Metabolites	25.47	±4.46	24.05	±5.06	28.63	±5.02	33.06	±3.57
Σ N-6	29.16	±5.28	26.50	±5.33	33.99	±5.96	35.44	±3.77
C18:3n3	0.01	±0.01	0.03	±0.03	0.04	±0.07	0.01	±0.01
[h]C20:5n3	0.67	±0.05	0.71	±0.06	0.60	±0.05	0.52	±0.05
[i]C22:5n3	4.23	±0.60	4.03	±0.86	2.00	±0.69	2.01	±0.72
[j]C22:6n3	12.45	±0.50	13.23	±0.84	5.36	±0.96	7.42	±0.37
N-3 Index	13.13	±0.55	13.94	±0.90	5.96	±1.02	7.94	±0.42
N-3 Metabolites	17.36	±1.15	17.96	±1.76	7.96	±1.71	9.94	±1.14
Σ N-3	17.37	±1.15	18.00	±1.79	8.00	±1.78	9.96	±1.15
16:0 DMA	0.06	±0.02	0.08	±0.05	0.07	±0.05	0.06	±0.04

[a]p=0.04 between groups: A and C
[b]p=<0.001 between groups: A and C, A and D, B and C, B and D
[c]p=<0.001 between groups: A and C, A and D; p=0.044 between groups: C and B; p=0.027 between groups: B and D
[d]p=<0.001 between groups: A and B, A and C, A and D, C and B, C and D
[e]p=<0.001 between groups: D and A, D and C, D and B
[f]p=<0.001 between groups: A and C, A and D, B and C, B and D
[g]p=0.002 between groups: A and C; p=<0.001 between groups: A and D, B and D; p=0.019 between groups: C and B
[h]p=0.008 between groups: A and C; p=0.005 between groups: C and D; p=<0.001 between: A and D, B and C, B and D
[i]p=<0.001 between groups: A and C, A and D, B and C, B and D
[j]p=<0.001 between groups: A and C, A and D, C and B, C and D, B and D

Significant differences were observed for the SM fraction of the RBC lipid membrane at twenty four months' time (Table 26) regarding the concentrations of C16:0, C18:0, C20:0, C24:0, C18:1n-9, C24:1n-9, C22:6n-3, C22:4n-6, C20:5n-3. For C16:0 a higher concentration was observed in group A as compared to D (p=<0.001), for the same fatty acid a higher concentration was observed in group C as compared to D (p=<0.001). For the same

fatty acid a higher concentration was also detected in group B as compared to D (p=<0.001). For C18:0 a higher concentration was observed in group A as compared to D (p=<0.001), for the same fatty acid a higher concentration was observed in group C as compared to D (p=0.015). For the same fatty acid a higher concentration was detected in group B as compare to D (p=<0.001). For C20:0 a higher concentration was observed in group A as compared to C (p=0.034). For C24:0 ahigher concentration was observed in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=<0.001). For C18:1n-9 a higher concentration was detected in group C as compared to A (p=<0.001) and B (p=<0.001), for the same fatty acid a higher concentration was observed in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=0.013). For C24:1n-9 a higher concentration was observed in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=<0.001), for the same fatty acid a higher concentration was observed in group C as compared to B (p=0.02). For C22:4n-6 a higher concentration was observed in group C as compared to A(p=<0.001) and B (p=<0.001). For the same fatty acid ahigher concentration was observed in group D as compared to A (p=<0.001) and B (p=<0.001). For C20:5n-3a higher concentration was observed in group A as compared to B (p=<0.001), C (p=0.003) and D (p=<0.001). For C22:6n-3 a higher concentration was observe in group B as compared to A (p=0.003), C (p=0.003) and D (p=<0.001). For the same fatty acid a higher concentration was detected in group A as to C (p=0.003) -and D (p=0.003), a higher concentration was also detected in group D as compared to C (p=0.009).

Table 26. Showing the Fatty acid profile of the sphingomyelin (SM) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at twenty-four months.

RBC SM Time 24 months	Group A n=10		Group B n=10		Group C n=9		Group D n=12	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14:0	0.21	±0.03	0.21	±0.08	0.27	±0.10	0.25	±0.10
[a]C16:0	29.22	±1.10	29.70	±0.96	28.90	±1.21	25.14	±1.69
[b]C18:0	15.65	±1.43	16.07	±1.56	14.50	±1.71	12.53	±1.37
[c]C20:0	1.58	±0.26	1.45	±0.25	1.30	±0.17	1.43	±0.25

C22:0	5.03	±0.31	5.15	±0.58	5.30	±0.43	4.88	±0.70
[d]C24:0	13.50	±0.58	12.95	±0.74	13.81	±0.88	16.44	±0.73
Σ Saturates	65.18	±3.71	65.53	±4.17	64.07	±4.51	60.67	±4.83
C16:1n-7	0.19	±0.04	0.21	±0.04	0.18	±0.04	0.20	±0.05
C18:1n-7	0.27	±0.14	0.30	±0.13	0.30	±0.12	0.31	±0.15
[e]C18:1n-9	5.94	±1.30	5.07	±0.65	7.53	±0.45	8.57	±0.53
C20:1n-9	0.18	±0.05	0.18	±0.04	0.18	±0.05	0.19	±0.03
C22:1n-9	0.43	±0.05	0.41	±0.05	0.47	±0.04	0.44	±0.08
[f]C24:1n-9	14.01	±0.80	13.68	±1.31	15.36	±1.22	18.71	±1.54
Σ Monoenes	21.02	±2.38	19.84	±2.21	24.01	±1.92	28.41	±2.39
C20:3n-9	0.04	±0.03	0.07	±0.08	0.06	±0.09	0.04	±0.04
C18:2n-6	2.35	±0.46	2.24	±0.82	2.87	±0.93	2.25	±0.64
C18:3n-6	0.08	±0.08	0.04	±0.02	0.11	±0.11	0.11	±0.17
C20:2n-6	0.11	±0.08	0.10	±0.09	0.12	±0.18	0.08	±0.07
C20:3n-6	0.41	±0.13	0.28	±0.11	0.37	±0.15	0.34	±0.10
C20:4n-6	4.98	±0.58	4.47	±0.85	4.58	±1.00	5.07	±0.87
[g]C22:4n-6	0.24	±0.03	0.25	±0.03	0.43	±0.10	0.49	±0.11
C22:5n-6								
N-6 Metabolites	5.82	±0.89	5.15	±1.10	5.62	±1.54	6.08	±1.31
Σ N-6	8.18	±1.35	7.39	±1.92	8.49	±2.47	8.33	±1.96
C18:3n3	0.08	±0.12	0.04	±0.02	0.11	±0.22	0.04	±0.03
[h]C20:5n3	0.36	±0.03	0.27	±0.03	0.30	±0.04	0.27	±0.04
C22:5n3	0.25	±0.29	0.35	±0.41	0.21	±0.17	0.21	±0.21
[i]C22:6n3	3.46	±0.88	4.91	±0.33	1.09	±0.04	1.78	±0.43
N-3 Index	3.82	±0.90	5.17	±0.36	1.39	±0.08	2.05	±0.47
N-3 Metabolites	4.07	±1.19	5.52	±0.77	1.60	±0.25	2.25	±0.69
Σ N-3	4.14	±2.51	11.08	±1.56	3.31	±0.72	4.55	±1.40

[a]p=<0.001 between groups: D and A, D and C, D and B.
[b]p=<0.001 between groups: A and D, B and D; p=0.015 between groups: C and D
[c]p=0.034 between groups: A and C
[d]p=<0.001 between groups: A and D, C and D, B and D
[e]p=<0.001 between groups: A and C, A and D, C and B, B and D; p=0.013 between groups: C and D
[f]p=<0.001 between groups: A and D, C and D, B and D; p=0.02 between groups: C and B
[g]p=<0.001 between groups: A and C, A and D, C and B, B and D
[h]p=0.003 between groups: A and C; p=<0.001 between groups: A and B, A and D
[i]p=<0.001 between groups: A and C, A and B, A and D, B and D; p=0.009 between groups: C and D

Significant differences were observed for the PE fraction of the RBC lipid membrane at twenty four months' time (Table 27) regarding the concentrations of C16:0, C16:1n-9, C18:1n-9, C18:1n-7, C20:3n-9, C18:2n-6, C20:2n-6, C20:4n-6, C22:4n-6, C18:3n-3, C20:5n-3, C22:5n-3, C22:6n-3. For C16:0 a higher concentration was observed in group D

as compared to D (p=<0.001). For the same fatty acid a higher concentration was detected in group A as compared to B (p=0.003), a higher concentration was also detected in group C as compared to B p=0.05). For C16:1n-9 a higher concentration was observed in group A as compared to B (p=0.003). For the same fatty acid a higher concentration was detected in group C as compared to B (p=0.05). For C18:1n-9 a higher concentration was observed in group C as compared to A (p=<0.001) and B (p=<0.001). For the same fatty acid a higher concentration was detected in group D as compared to A (p=<0.001) and B (p=<0.001). For C18:1n-7 a higher concentration was observed in group C as compared to A (p=<0.001), B (p=<0.001) and D (p=<0.001). For C20:3n-9 a higher concentration was observed in group C as compared to A (p=0.04) and B (p=0.004). For the same fatty acid a higher concentration was also detected in group D as compared to B (p=0.009). For C18:2n-6 a higher concentration was observed in group C as compared to D (p=0.009). For C20:2n-6 a higher concentration was observed in group C as compared to A (p=<0.001). For C20:4n-6 a higher concentration was observed in group C as compared to A (p=<0.001) and B (p=<0.001). For the same fatty acid a higher concentration was also detected in group B as compared to A (p=<0.001), a higher concentration was also detected in group D as compared to A (p=<0.001) and B (p=<0.001). For C22:4n-6 a higher concentration was observed in group C as compared to A (p=<0.001) and B (p=<0.001). For the same fatty acid a higher concentration was also observed in group B as compare to A (p=<0.001), a higher concentration was observed in group D as compared to A (p=<0.001) and B (p=<0.001). For C18:3n-3 a higher concentration was observed in group C as compared to A (p=0.002) and B (p=<0.001). For C20:5n-3 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001). For the same fatty acid a higher concentration was also detected in group B as compared to C (p=<0.001) and D (p=<0.001), a higher concentration was also observed in group C as compared to D (p=<0.001). For C22:5n-3 a higher

concentration was observed in group A as compared to C (p=0.01). For C22:6n-3 a higher concentration was observed in group A ascompared to C (p=<0.001) and D (p=<0.001). For the same fatty acid a higher concentration was observed in group B as compared to C (p=<0.001) and D (p=<0.001).

Table 27. Showing the Fatty acid profile of the phosphatidylethanolamine (PE) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at twenty-four months.

RBC PE Time 24 months	Group A n=10		Group B n=10		Group C n=9		Group D n=12	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14:0	0.14	±0.03	0.16	±0.04	0.15	±0.03	0.13	±0.03
[a]C16:0	13.64	±1.21	14.30	±0.64	14.54	±0.77	15.05	±0.50
C18:0	6.77	±0.38	6.67	±0.50	6.84	±0.97	6.98	±0.98
C20:0	0.07	±0.02	0.05	±0.03	0.07	±0.02	0.06	±0.02
C22:0	0.14	±0.02	0.15	±0.02	0.15	±0.02	0.14	±0.03
C24:0	0.15	±0.02	0.15	±0.03	0.16	±0.03	0.16	±0.03
Σ Saturates	20.91	±1.68	21.48	±1.26	21.91	±1.83	22.52	±1.59
[b]C16:1n-7	0.41	±0.07	0.27	±0.06	0.37	±0.11	0.35	±0.09
[c]C18:1n-7	0.94	±0.04	0.90	±0.08	1.18	±0.12	0.94	±0.07
[d]C18:1n-9	14.29	±0.91	15.17	±0.85	18.09	±1.24	19.16	±0.85
C20:1n-9	0.38	±0.09	0.41	±0.06	0.43	±0.10	0.42	±0.09
C24:1n-9	0.07	±0.02	0.08	±0.03	0.06	±0.03	0.08	±0.03
Σ Monoenes	16.10	±1.12	16.89	±1.11	20.15	±1.59	20.95	±1.12
[e]C20:3n-9	0.04	±0.03	0.03	±0.02	0.06	±0.02	0.06	±0.02
[f]C18:2n-6	5.19	±0.68	4.95	±0.57	5.86	±1.16	4.77	±0.61
C18:3n-6	0.15	±0.02	0.17	±0.02	0.16	±0.02	0.15	±0.02
[g]C20:2n-6	0.18	±0.06	0.23	±0.04	0.28	±0.04	0.23	±0.05
C20:3n-6	1.13	±0.15	1.04	±0.19	1.16	±0.34	1.04	±0.15
[h]C20:4n-6	13.73	±0.37	15.09	±0.55	18.78	±1.00	18.69	±0.73
[i]C22:4n-6	4.11	±0.71	4.39	±0.61	4.58	±0.61	5.26	±0.65
N-6 Metabolites	19.30	±1.30	21.28	±1.51	25.83	±2.01	25.36	±1.60
Σ N-6	24.49	±1.98	26.23	±2.08	31.69	±3.17	30.13	±2.22
[j]C18:3n3	0.16	±0.01	0.16	±0.05	0.25	±0.06	0.22	±0.07
[k]C20:5n3	4.99	±0.86	4.70	±0.43	2.08	±0.27	0.80	±0.24
[l]C22:5n3	4.38	±0.70	3.66	±0.76	3.44	±0.70	3.88	±0.53
[m]C22:6n3	11.75	±0.95	11.74	±0.99	6.52	±0.87	6.48	±0.85
N-3 Index	16.73	±1.81	16.44	±1.41	8.59	±1.14	7.28	±1.09
N-3 Metabolites	21.12	±2.50	20.10	±2.17	12.03	±1.84	11.16	±1.61
Σ N-3	21.28	±2.52	20.25	±2.22	12.28	±1.90	11.37	±1.68

[n]16:0 DMA	5.28	±0.49	5.11	±0.36	4.13	±0.66	3.81	±0.79
[o]18:0 DMA	7.89	±0.48	7.90	±0.60	6.77	±0.61	6.91	±0.75
18:1 DMA	1.91	±0.47	1.73	±0.59	1.98	±0.40	2.16	±0.47

[a]p=<0.001 between groups: A and D
[b]p=0.003 between groups: A and B; p=0.05 between groups C and B
[c]p=<0.001 between groups: A and C, C and B, A and D
[d]p=<0.001 between groups: A and C, A and D, C and B, B and D
[e]p=0.04 between groups: C and A; p=0.004 between groups: C and B, p=0.009 between groups: B and D
[f]p=0.009 between groups: C and D
[g]p=<0.001 between groups: A and C
[h]p=<0.001 between groups: A and C, A and B, A and D, C and B, B and D
[i]p=<0.001 between groups: D and A; p=0.02 between groups: D and B
[j]p=<0.001 between groups: C and B; p=0.002 between groups A and C
[k]p=<0.001 between groups: A and C, A and D, C and B, C and D, B and D
[l]p=0.01 between groups: A and C
[m]p=<0.001 between groups: C and A, C and B, D and A, D and B
[n]p=<0.001 between groups: A and C, A and D, B and D; p=0.004 between groups: C and B
[o]p=<0.001 between groups: A and C, C and B; p=0.004 between groups: A and D; p=0.003 between groups: B and D

Significant differences were observed for the PC fraction of the RBC lipid membrane at twenty four months' time (Table 28) regarding the concentrations of C16:0, C18:1n-9, C18:2n-6, C20:4n-6, C22:5n-6, C18:3n-3, C20:5n-3, C22:5n-3, C22:6n-3. For C16:0 a higher concentration was observed in group B as compared to D p=0.006). For C18:1n-9 a higher concentration was observed in group C as compared to A (p=<0.001) and B (p=<0.001). For the same fatty acid a higher concentration was observed in group B as compared to A (p=0.016), a higher concentration was also detected in group D as compared to A (p=<0.001) and B (p=<0.001). For C18:2n-6 a higher concentration was observed in group A as compared to B (p=<0.001). For the same fatty acid a higher concentration was observed in group C as compared to B (p=<0.001), a higher concentration was also detected in group D as compared to A (p=<0.001), B (p=<0.001) and C (p=<0.001). For C20:4n-6 a higher concentration was observed in group A as compared to B (p=<0.001). For the same fatty acid a higher concentration was detected in group C as compare to B (p=<0.001), a higher concentration was also observed in group D as compared to B (p=<0.001). For C22:5n-6 higher concentration was observed in group A as compared to B (p=<0.001), C (p=<0.001) and D (p=<0.001). For C18:3n-3 a higher concentration was observed in group

C as compared to A (p=<0.001). or the same fatty acid a higher concentration was observed in group B as compared to A (p=<0.001) and C (p=<0.001), a higher concentration was also detected in group D as compared to A (p=<0.001) and C (p=<0.001). For C20:5n-3 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001). For the same fatty acids higher concentration was also detected in group B as compared to C (p=<0.001) and D (p=<0.001). For C22:5n-3 a higher concentration was observed in group A as compared to C (p=<0.001) and D (p=<0.001). For the same fatty acid a higher concentration was observed in group B as compared to A (p=<0.001), C (p=<0.001) and D (p=<0.001). For C22:6n-3 a higher concentration was observed in group A as compared to B (p=0.04), C (p=<0.001) and D (p=<0.001). For the same fatty acid a higher concentration was observed in group B as compared to C (p=<0.001) and D (p=0.006).

Table 28. Showing the Fatty acid profile of the phosphatidylcholine (PC) fraction of the red blood cells' membrane of the on-treatment patients, groups A, B, C and D at twenty-four months.

RBC PC Time 24 months	Group A n=10		Group B n=10		Group C n=9		Group D n=12	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14:0	0.27	±0.14	0.26	±0.09	0.35	±0.09	0.24	±0.10
[a]C16:0	35.56	±0.76	36.23	±1.17	35.18	±0.99	34.85	±0.73
C18:0	16.20	±1.28	16.32	±0.81	15.68	±1.06	15.13	±0.96
C20:0	0.06	±0.02	0.06	±0.02	0.07	±0.02	0.06	±0.02
C24:0	0.10	±0.15	0.05	±0.02	0.06	±0.02	0.07	±0.02
Σ Saturates	52.33	±2.40	55.30	±3.34	51.44	±2.18	50.57	±1.88
C16:1n-7	0.51	±0.23	0.45	±0.25	0.38	±0.23	0.39	±0.17
C18:1n-7	1.50	±0.29	1.68	±0.23	1.49	±0.31	1.33	±0.37
[b]C18:1n-9	14.60	±0.72	15.55	±0.72	18.49	±0.63	18.63	±0.60
C20:1n-9	0.27	±0.05	0.25	±0.03	0.26	±0.03	0.26	±0.05
C22:1n-9	0.04	±0.02	0.04	±0.02	0.04	±0.03	0.05	±0.02
C24:1n-9	0.14	±0.13	0.07	±0.04	0.09	±0.06	0.08	±0.05
Σ Monoenes	17.08	±1.44	18.04	±1.29	20.74	±1.30	20.74	±1.25
C20:3n-9	0.03	±0.02	0.04	±0.03	0.03	±0.02	0.03	±0.02
[c]C18:2n-6	17.36	±0.50	14.58	±0.53	17.62	±1.03	19.22	±0.97
C18:3n-6	0.21	±0.16	0.15	±0.02	0.14	±0.03	0.17	±0.02
C20:2n-6	0.18	±0.06	0.14	±0.02	0.17	±0.08	0.15	±0.04
C20:3n-6	1.57	±0.31	1.29	±0.27	1.59	±0.43	1.55	±0.24

[d]C20:4n-6	4.35	±0.25	3.44	±0.29	4.49	±0.37	4.63	±0.42
C22:4n-6	0.23	±0.08	0.20	±0.08	0.23	±0.08	0.24	±0.05
[e]C22:5n-6	0.24	±0.03	0.14	±0.04	0.15	±0.02	0.14	±0.02
N-6 Metabolites	6.78	±0.89	5.36	±0.71	6.77	±1.02	6.89	±0.79
Σ N-6	24.13	±1.39	19.95	±1.24	24.39	±2.05	26.12	±1.76
[f]C18:3n3	0.15	±0.03	0.36	±0.02	0.28	±0.03	0.36	±0.03
[g]C20:5n3	1.13	±0.09	1.20	±0.20	0.24	±0.17	0.22	±0.10
[h]C22:5n3	1.23	±0.13	1.58	±0.36	0.25	±0.11	0.27	±0.08
[i]C22:6n3	2.61	±0.79	2.02	±0.44	1.07	±0.22	1.33	±0.25
N-3 Index	3.74	±0.89	3.22	±0.64	1.31	±0.39	1.55	±0.35
N-3 Metabolites	4.97	±1.02	4.80	±1.00	1.56	±0.50	1.82	±0.43
Σ N-3	5.12	±1.05	5.16	±1.02	1.84	±0.53	4.00	±0.89
[j]16:0 DMA	0.21	±0.11	0.58	±0.61	0.22	±0.10	0.19	±0.07

[a]p=0.006 between groups: B and D
[b]p=<0.001 between groups: A and C, A and D, C and B, B and D; p=0.016 between groups: A and B
[c]p=<0.001 between groups: B and A, C and B, B and D, D and A , D and C
[d]p=<0.001 between groups: B and A, C and B, B and D
[e]p=<0.001 between groups: A and B, A and C, A and D
[f]p=<0.001 between groups: A and B, A and C, A and D, B and C, C and D
[g]p=<0.001 between groups: A and C, A and D, C and B, B and D
[h]p=<0.001 between groups: A and C, A and D, C and B; p=0.006 between groups: B and D; p=0.04 between groups: B and A
[i]p=<0.001 between groups: A and C, A and D, B and C; p=0.039 between groups: A and B; p= 0.006 between groups: B and D
[j]p=0.036 between groups: B and D

Table 29. Group A showing the significant differences in concentration of the Fatty acids of phosphatidylcholine (PC) fraction in time (zero time, six months and twenty-four months).

Group A (n=10)				
PC	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0	T0	[a] T06	±3.57	±0.55
		[a] T24	±2.43	±0.55
C16:1n-7	T0	[b] T06	±0.27	±0.08
		[a] T24	±0.33	±0.08
C18:0	T0	[c] T24	±1.67	±0.60
C18:2n-6	T0	[a] T06	±5.83	±0.88
		[a] T24	±5.38	±0.88
C18:3n-3	T0	[a] T06	±0.20	±0.01
		[a] T24	±0.21	±0.01
C20:1n-9	T0	[d] T24	±0.04	±0.02
C20:3n-9	T0	[e] T06	±0.02	±0.01
C20:3n-6	T0	[f] T06	±0.58	±0.18
		[f] T24	±0.54	±0.18
C20:4n-6	T0	[a] T06	±3.02	±0.30

		[a] T24	±2.29	±0.30
C20:3n-3	T06	[g] T24	±0.04	±0.01
C22:1n-9	T06	[g] T24	±0.03	±0.01
C20:5n-3	T0	[a] T06	±0.96	±0.04
		[a] T24	±0.93	±0.04
C22:4n-6	T0	[a] T06	±0.24	±0.05
		[a] T24	±0.21	±0.05
C22:5n-6	T0	[a] T06	±0.09	±0.02
		[f] T24	±0.06	±0.02
C22:5n-3	T0	[a] T06	±0.86	±0.06
		[a] T24	±0.85	±0.06
C22:6n-3	T0	[h] T06	±1.11	±0.32
		[a] T24	±1.27	±0.32

[a]p=<0.001, [b]p=0.006, [c]p=0.03, [d]p=0.06, [e]p=0.04,
[f]p=0.01, [g]p=0.002, [h]p=0.005
T=Time, T0= Time zero, T06= Time six months, T24=Time24 months

Table 29 shows the concentration of the fatty acids of the PC fraction in group A that significantly changed during the duration of the study. For the following fatty acids their concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C16:0, C18:0 (no significant change observed from zero to six months), C16:1n-7, C20:1n-9 (no significant change observed from zero to six months), C22:1n-9, C20:3n-3 (significant change observed only after the six months), C20:5n-3, C22:5n-3, C22:6n-3, C22:5n-6. For the following fatty acids there concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty four months: C20:3n-9 (only between zero time and six months), C18:3n-3, C18:2n-6, C20:3n-9 (significant change only observed for the first six months), C20:4n-6, C22:4n-6.

Table 30. Group B showing the significant differences in concentration of the Fatty acids of phosphatidylcholine (PC) fraction in time (zero time, six months and twenty-four months).

Group B (n=10)				
PC	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0	T0	[b]T06	±2.35	±0.63
		[b]T24	±2.39	±0.63
C16:1n-7	T0	[c]T06	±0.27	±0.08

		[d]T24	±0.25	±0.08
C18:0	T0	[g]T06	±1.41	±0.42
		[f]T24	±1.37	±0.42
C18:1n-9	T0	[h]T06	±1.44	±0.41
		[e]T24	±1.94	±0.41
C18:1n-7	T0	[d]T06	±0.33	±0.12
		[i]T24	±0.31	±0.12
C18:2n-6	T0	[e]T06	±5.78	±0.53
		[e]T24	±7.08	±0.53
C18:3n-3	T06	[e]T0	±0.14	±0.02
		[e]T24	±0.19	±0.02
C20:2n-6	T0	[c]T24	±0.06	±0.02
C20:3n-6	T0	[a]T24	±0.44	±0.17
C20:4n-6	T0	[e]T06	±3.01	±0.30
		[e]T24	±4.00	±0.30
	T24	[f]T06	±1.00	±0.30
C20:5n-3	T0	[e]T06	±0.96	±0.06
		[e]T24	±0.97	±0.06
C22:4n-6	T0	[c]T24	±0.23	±0.07
C22:5n-6	T0	[c]T24	±0.07	±0.02
C24:1n-9	T0	[d]T06	±0.16	±0.05
	T06	[i]T24	±0.12	±0.04
C22:5n-3	T0	[e]T06	±1.12	±0.12
		[e]T24	±1.25	±0.12

[a]p=0.04, [b]p=0.002, [c]p=0.01, [d]p=0.02, [e]p=<0.001, [f]p=0.008, [g]p=0.007, [h]p=0.005, [i]p=0.03

Table 30 shows the concentration of the fatty acids of the PC fraction in group B that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C16:0, C18:0, C16:1n-7, C181n-7, C18:1n-9, C24:1n-9, C18:3n-3, C20:5n-3, C22:5n-3. For the following fatty acids there concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty four months: C18:2n-6, C20:2n-6 (no significant change observed from zero to six months), C20:3n-6 (no significant change observed from zero to six months), C20:4n-6, C22:4n-6 (no significant change observed from zero to six months), C22:5n-6 (no significant change observed from zero to six months).

Table 31. Group C showing the significant differences in concentration of the Fatty acids of phosphatidylcholine (PC) fraction in time (zero time, six months and twenty-four months).

Group C (n=9)				
PC	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0_PCt0	T0	[a]T06	±4.48	±0.54
		[a]T24	±2.32	±0.54
	T24	[a]T06	±2.16	±0.54
C16:1n-7_PCt0	T0	[a]T06	±0.29	±0.07
C18:1n-9_PCt0	T0	[a]T06	±4.98	±0.35
		[a]T24	±4.74	±0.35
C18:2n-6_PCt0	T0	[a]T06	±6.23	±0.52
		[a]T24	±3.55	±0.52
	T24	[a]T06	±2.67	±0.52
C18:3n-3_PCt0	T0	[a]T06	±0.08	±0.02
		[b]T24	±0.04	±0.02
C20:0_PCt0	T06	[c]T0	±0.03	±0.01
		[b]T24	±0.03	±0.01
C20:4n-6_PCt0	T0	[a]T06	±4.60	±0.18
		[a]T24	±3.80	±0.18
	T06	[a]T24	±0.80	±0.18
C22:2n-6_PCt0	T0	[d]T24	±0.01	±0.00
C22:4n-6_PCt0	T0	[e]T06	±0.13	±0.05
		[f]T24	±0.17	±0.05
C22:5n-6_PCt0	T0	[a]T06	±0.10	±0.02
		[a]T24	±0.09	±0.02
C22:6n-3_PCt0	T24	[g]T0	±0.47	±0.15
		[b]T06	±0.42	±0.15

[a]p=<0.001, [b]p=0.02, [c]p=0.005, [d]p=0.04, [e]p=0.03, [f]p=0.006, [g]p=0.01

Table 31 shows the concentration of the fatty acids of the PC fraction in group C that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C16:0, C20:0, C16:1n-7 (only between zero time and six months), C18:1n-9, C22:2n-6 (no significant change observed from zero to six months). For the following fatty acids there concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty four months: C22:6n-3, C18:2n-6, C22:4n-6, C22:5n-6.

Interesting fatty acid C20:4n-6 concentration significantly decreased within the first six months and in 24 months significantly increased as compared to six months; however comparing its concentration between zero and 24 months there was significant decrease.

Table 32. Group D showing the significant differences in concentration of the Fatty acids of phosphatidylcholine (PC) fraction in time (zero time, six months and twenty-four months).

Group D (n=12)				
PC	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0_PCt0	T0	[a]T06	±1.70	±0.56
		[b]T24	±2.00	±0.55
C16:1n-7_PCt0	T0	[c]T06	±0.33	±0.06
		[a]T24	±0.17	±0.06
	T06	[a]T24	±0.16	±0.06
C18:1n-9_PCt0	T0	[c]T06	±3.48	±0.36
		[c]T24	±3.76	±0.36
C18:2n-6_PCt0	T0	[b]T06	±1.82	±0.51
		[c]T24	±2.03	±0.50
C20:0_PCt0	T0	[a]T06	±0.03	±0.01
C20:2n-6_PCt0	T0	[e]T24	±0.06	±0.02
C20:3n-6_PCt0	T0	[f]T06	±0.45	±0.16
		[a]T24	±0.48	±0.15
C20:4n-6_PCt0	T0	[c]T06	±1.51	±0.25
		[c]T24	±2.56	±0.24
	T06	[c]T24	±1.05	±0.24
C22:1n-9_PCt0	T24	[f]T0	±0.02	±0.01
		[a]T06	±0.02	±0.01
C22:2n-6_PCt0	T0	[a]T24	±0.01	±0.00
C22:4n-6_PCt0	T0	[a]T24	±0.20	±0.06
C22:5n-6_PCt0	T0	[e]T06	±0.07	±0.02
		[gi]T24	±0.08	±0.02
C24:0_PCt0	T24	[c]T0	±0.04	±0.01
		[c]T06	±0.04	±0.01

[a]p=0.01, [b]p=0.003, [c]p=<0.001, [d]p=0.01,[e]p=0.02,[f]p=0.045,[g]p=0.006

Table 32 shows the concentration of the fatty acids of the PC fraction in group D that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C16:0, C24:0, C16:1n-7, C18:1n-9, C22:1n-9, C22:2n-6 (no significant

change observed from zero to six months). For the following fatty acids there concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty four months: C20:0 (significant change only observed for the first six months), C18:2n-6, C20:2n-6(no significant change observed from zero to six months), C20:3n-6, C20:4n-6 (significant changes besides that observed between zero time and 24 months a significant change was also observed between six months and 24 months), C22:4n-6, C22:5n-6.

Table 33. Group A showing the significant differences in concentration of the Fatty acids of phosphatidylethanolamine (PE) fraction in time (zero time, six months and twenty-four months).

Group A (n=10)				
PE	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0	T00	[c]T06	±1.19	±0.42
		[d]T24	±1.30	±0.42
C16:1n-7	T00	[d]T24	±0.19	±0.06
C18:0	T00	[e]T06	±0.87	±0.32
C18:1n-9	T00	[d]T06	±1.65	±0.52
		[d]T24	±1.59	±0.52
C18:2n-6	T00	[d]T06	±1.20	±0.41
		[e]T24	±1.08	±0.41
C18:3n-6	T00	[c]T06	±0.06	±0.02
		[c]T24	±0.06	±0.02
C20:0	T06	[f]T24	±0.02	±0.01
C20:4n-6	T00	[a]T06	±4.66	±0.24
		[a]T24	±5.05	±0.24
C20:3n-3	T06	[g]T00	±0.09	±0.03
		[h]T24	±0.09	±0.02
C20:5n-3	T00	[a]T06	±2.22	±0.28
		[a]T24	±2.57	±0.28
C22:4n-6	T00	[a]T06	±2.21	±0.28
		[a]T24	±2.55	±0.28
C22:5n-3	T00	[a]T06	±1.45	±0.25
		[a]T24	±1.15	±0.25
C22:6n-3	T00	[a]T06	±6.00	±0.31
		[a]T24	±5.82	±0.31

[a]p=<0.001, [b]p=0.005, [c]p=0.025, [d]p=0.013,
[e]p=0.036, [f]p=0.04, [g]p=0.003, [h]p=0.002

Table 33 shows the concentration of the fatty acids of the PE fraction in group A that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C20:0 (no significant change observed from zero to six months), C16:1n-7 (no significant change observed from zero to six months), C20:5n-3, C22:5n-3, C22:6n-3, C18:3n-6. For the following fatty acids there concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty four months: C16:0, C18:0, (significant change only observed for the first six months), C18:1n-9, C18:2n-6, C20:4n-6, C22:4n-6, C20:3n-3 (interesting there was a significant increase in concentration from zero to 6 months. However the concentration was decreased significantly at 24 months as compared to zero time and six months).

Table 34. Group B showing the significant differences in concentration of the Fatty acids of phosphatidylethanolamine (PE) fraction in time (zero time, six months and twenty-four months).

Group B (n=10)				
PE	**Time**	**Time**	**Mean Difference**	**SEM**
C18:0	T00	[c]T06	±1.47	±0.39
		[b]T24	±1.76	±0.38
C18:2n-6	T00	[d]T24	±1.42	±0.51
C18:4n-3	T06	[c]T00	±0.06	±0.01
		[a]T24	±0.04	±0.01
C20:4n-6	T00	[b]T06	±4.19	±0.42
		[b]T24	±3.90	±0.41
C20:5n-3	T00	[b]T06	±2.29	±0.25
		[b]T24	±2.51	±0.24
C22:4n-6	T00	[c]T06	±1.70	±0.44
		[b]T24	±1.82	±0.43
C22:5n-6	T00	[e]T06	±0.34	±0.07
C22:5n-3	T00	[c]T06	±1.14	±0.29
C22:6n-3	T00	[b]T06	±4.41	±0.61
		[b]T24	±4.54	±0.59

[a]p=0.01, [b]p=<0.001, [c]p=0.002, [d]p=0.03, [e]p=0.003

Table 34 shows the concentration of the fatty acids of the PE fraction in group B that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C20:5n-3, C22:5n-3 (only between zero time and six months), C22:6n-3. For the following fatty acids there concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty four months: C18:0, C18:2n-6 (no significant change observed from zero to six months), C20:4n-6, C22:4n-6, C22:5n-6 (only between zero time and six months), C18:4n-3.

Table 35. Group C showing the significant differences in concentration of the Fatty acids of phosphatidylethanolamine (PE) fraction in time (zero time, six months and twenty-four months).

Group C (n=9)				
PE	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0	T24	[b]T00	±0.95	±0.36
		[c]T06	±1.15	±0.36
C18:0	T00	[e]T24	±1.15	±0.34
C18:1n-9	T00	[a]T06	±2.11	±0.39
		[d]T24	±1.35	±0.39
C18:1n-7	T24	[a]T00	±0.32	±0.07
		[f]T06	±0.18	±0.07
C18:2n-6	T00	[g]T06	±1.59	±0.43
C20:0	T06	[h]T24	±0.02	±0.01
C20:3n-3	T24	[c]T00	±0.11	±0.04
		[f]T06	±0.09	±0.04
C20:5n-3	T06	[a]T00	±0.36	±0.08
		[i]T24	±0.26	±0.08
C22:4n-6	T00	[e]T06	±1.39	±0.41
		[a]T24	±1.61	±0.40

[a]p=<0.001, [b]p=0.03, [c]p=0.01, [d]p=0.004, [e]p=0.005,
[f]p=0.04, [g]p=0.002, [h]p=0.02, [i]p=0.009

Table 35 shows the concentration of the fatty acids of the PE fraction in group C that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to

twenty four months: C16:0, C20:0 (significant change observed only after the six months), C18:1n-7, C18:1n-9, C20:5n-3(significantly increase as compared between zero and 24 months and zero and six months). For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty four months: C18:0, C18:2n-6 (significant change only observed for the first six months), C22:4n-6, C20:3n-3, C20:5n-3 (significantly decreased between 6 months and 24 months).

Table 36. Group D showing the significant differences in concentration of the Fatty acids of phosphatidylethanolamine (PE) fraction in time (zero time, six months and twenty-four months).

Group D (n=12)				
PE	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0	T24	[c]T00	±0.86	±0.27
		[d]T06	±0.93	±0.27
C18:0	T24	[a]T00	±1.52	±0.34
		[b]T06	±0.94	±0.34
C18:1n-9	T00	[a]T06	±3.49	±0.43
		[a]T24	±3.68	±0.42
C18:1n-7	T00	[c]T24	±0.30	±0.09
C18:2n-6	T00	[e]T06	±1.16	±0.37
		[a]T24	±1.55	±0.36
C18:4n-3	T00	[a]T06	±0.06	±0.01
		[a]T24	±0.05	±0.01
C20:5n-3	T00	[a]T06	±0.91	±0.13
		[a]T24	±0.75	±0.13
	T06	[a]T24	±1.66	±0.13
C22:4n-6	T00	[a]T06	±2.02	±0.25
		[a]T24	±1.55	±0.25
C22:5n-3	T24	[e]T00	±0.72	±0.23
		[e]T06	±0.69	±0.23

[a]p=<0.001, [b]p=0.02, [c]p=0.008, [d]p=0.004, [e]p=0.01

Table 36 shows the concentration of the fatty acids of the PE fraction in group D that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C16:0, C18:1n-9, C18:4n-3, C20:5n-3(significantly increased as

compared between zero and six months), C22:5n-3. For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty-four months: C18:0, C181n-7, C18:2n-6 (significant change only observed for the first six months), C22:4n-6, C20:5n-3, (significantly decreased as compared between zero and 24 months).

Table 37. Group A showing the significant differences in concentration of the Fatty acids of sphingomyelin (SM) fraction in time (zero time, six months and twenty-four months).

Group A (n=10)				
SM	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0	T00	[a]T06	±4.66	±0.75
		[a]T24	±3.90	±0.75
C16:1n-7	T00	[b]T06	±0.05	±0.02
C18:0	T00	[c]T24	±2.14	±0.67
C18:1n-9	T06	[a]T00	±2.22	±0.54
		[d]T24	±2.06	±0.54
C18:2n-6	T00	[a]T06	±1.02	±0.23
		[e]T24	±0.84	±0.23
C20:2n-6	T00	[a]T06	±0.40	±0.07
		[a]T24	±0.46	±0.07
C20:4n-6	T00	[a]T06	±2.05	±0.19
		[f]T24	±0.65	±0.19
	T06	[a]T24	±1.40	±0.19
C22:0	T00	[d]T24	±0.72	±0.19
C20:5n-3	T00	[a]T06	±0.25	±0.03
		[a]T24	±0.13	±0.03
	T06	[d]T24	±0.11	±0.03
C22:4n-6	T00	[a]T06	±0.24	±0.05
		[a]T24	±0.25	±0.05
C24:0	T00	[a]T06	±3.25	±0.33
		[a]T24	±3.53	±0.33
C24:1n-9	T00	[a]T06	±3.40	±0.47
		[a]T24	±3.50	±0.47
C22:5n-3	T00	[f]T06	±0.34	±0.10
		[a]T24	±0.62	±0.10
	T06	[b]T24	±0.28	±0.10
C22:6n-3	T00	[a]T06	±1.87	±0.25
		[a]T24	±2.02	±0.25

[a]p=<0.001, [b]p=0.03, [c]p=0.01, [d]p=0.002, [e]p=0.003, [f]p=0.005

Table 37 shows the concentration of the fatty acids of the S M fraction in group A that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C16:0, C18:0 (no significant change observed from zero to six months), C22:0 (no significant change observed from zero to six months), C16:1n-7 (only between zero time and six months), C20:4n-6 (A significant increase at 24 months was observed when compared to six months. However, a significant overall decrease was observed at 24 months when compared to zero time), C20:5n-3, C22:6n-3. For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty-four months: C24:0, C18:1n-9, C24:1n-9, C18:2n-6, C20:2n-6, C20:4n-6 (see also note above on this fatty acid), C22:4n-6, C20:5n-3 (for this fatty acid an overall significant increase at 24 months as compared to zero time. However, there was a significant decrease at 24 months when compared to six months) significantly decreased as compared between zero and 24 months) C22:5n-3.

Table 38. Group B showing the significant differences in concentration of the Fatty acids of sphingomyelin (SM) fraction in time (zero time, six months and twenty-four months).

Group B (n=10)				
SM	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0	T00	[a]T06	±3.41	±1.10
		[a]T24	±3.52	±1.10
C18:0	T00	[b]T06	±3.20	±0.63
		[b]T24	±2.86	±0.63
C18:1n-9	T00	[b]T06	±1.73	±0.40
		[a]T24	±1.24	±0.40
	T06	[b]T24	±2.97	±0.38
C20:2n-6	T00	[c]T06	±0.24	±0.06
		[b]T24	±0.31	±0.06
C20:4n-6	T06	[b]T00	±1.65	±0.31
		[b]T24	±1.54	±0.29

C22:0	T00	[b]T06	±1.46	±0.28
		[a]T24	±0.88	±0.28
C20:5n-3	T06	[b]T00	±0.24	±0.04
		[a]T24	±0.13	±0.04
C22:4n-6	T00	[b]T06	±0.39	±0.04
		[b]T24	±0.39	±0.04
C24:0	T00	[b]T06	±3.88	±0.49
		[b]T24	±3.37	±0.49
C24:1n-9	T00	[b]T06	±3.02	±0.72
		[b]T24	±2.99	±0.72
C22:5n-3	T00	[b]T06	±1.26	±0.15
		[b]T24	±1.27	±0.14
C22:6n-3	T24	[b]T00	±3.11	±0.22
		[b]T06	±2.97	±0.21

[a]p=0.01, [b]p=<0.001, [c]p=0.002

Table 38 shows the concentration of the fatty acids of the SPM fraction in group B that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C16:0, C18:0, C22:0, C18:1n-9 (significant increase at six months as compared to six months. However overall decrease was observed at 24 months as compared to zero time and six months), C20:5n-3 (see paragraph below), C22:6n-3. For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty four months: C24:0, C18:1n-9 (see paragraph above), C24:1n-9, C20:4n-6 (A significant decrease was observed for this fatty acid at six months' time as compared to zero time. However a significant increase was observed at 24 months as compared to six months. No significant changes were observed between zero time and 24 months) C22:4n-6, C20:5n-3 (significant decrease was observed at 24 months as compared to six months. No significant change was observed between zero time and 24 months; time although a non-significant increase was observed. Furthermore a significant increase was observed at six months as compared to zero time), C22:5n-3.

Table 39. Group C showing the significant differences in concentration of the Fatty acids of sphingomyelin (SM) fraction in time (zero time, six months and twenty-four months).

Group C (n=9)				
SM	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0	T00	[a]T06	±2.22	±0.59
		[b]T24	±2.04	±0.59
C18:0	T00	[c]T06	±2.19	±0.61
		[a]T24	±2.31	±0.61
C18:1n-9	T00	[d]T06	±1.05	±0.41
		[e]T24	±1.13	±0.41
C20:2n-6	T00	[f]T06	±0.35	±0.05
		[f]T24	±0.38	±0.05
C22:0	T00	[a]T24	±0.91	±0.24
C20:5n-3	T00	[g]T24	±0.08	±0.02
C24:0	T00	[f]T06	±3.41	±0.42
		[f]T24	±4.19	±0.42
C24:1n-9	T00	[f]T06	±2.61	±0.52
		[d]T24	±1.36	±0.52
C22:5n-3	T00	[f]T06	±0.53	±0.07
		[f]T24	±0.64	±0.07
C22:6n-3	T06	[b]T00	±0.13	±0.04
		[g]T24	±0.12	±0.04

[a]p= 0.002, [b]p=0.004, [c]p=0.003, [d]p=0.04, [e]p=0.02, [f]p=<0.001, [g]p=0.005

Table 39 shows the concentration of the fatty acids of the SPM fraction in group C that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty-four months: C16:0, C18:0, C22:0 (no significant change observed from zero to six months), C18:1n-9, C20:5n-3 (no significant change observed from zero to six months), C22:6n-3 (Significantly increased at six months. However there was a decrease at 24 months as compared to six months. No significant change observed between zero and six months). For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty-four months: C24:0, C24:1n-9, C20:2n-6, C22:5n-3, C22:6n-3 (significant decrease at 24 months as compared to six months. See also paragraph above).

Table 40. Group D showing the significant differences in concentration of the Fatty acids of sphingomyelin (SM) fraction in time (zero time, six months and twenty-four months).

Group D (n=12)				
SM	**Time**	**Time**	**Mean Difference**	**SEM**
C18:1n-9	T24	[a]T00	±1.30	±0.29
		[b]T06	±0.74	±0.29
C18:2n-6	T00	[c]T06	±0.88	±0.25
		[a]T24	±1.73	±0.25
	T06	[d]T24	±0.85	±0.25
C20:2n-6	T00	[a]T06	±0.31	±0.04
		[a]T24	±0.38	±0.04
C20:3n-9	T00	[e]T24	±0.10	±0.04
C20:4n-6	T00	[f]T06	±0.93	±0.30
C22:0	T00	[f]T06	±0.79	±0.26
C20:5n-3	T06	[f]T24	±0.09	±0.03
C22:5n-3	T00	[a]T06	±0.58	±0.08
		[a]T24	±0.64	±0.07
C22:6n-3	T00	[a]T06	±0.68	±0.15
		[f]T24	±0.46	±0.15

[a]p=<0.001, [b]p=0.04, [c]p=0.003, [d]p=0.005, [e]p=0.03, [f]p=0.01

Table 40 shows the concentration of the fatty acids of the SPM fraction in group D that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C22:0 (significant change only observed for the first six months), C18:1n-9, C20:5n-3 (significant change observed at 24 months as compared to six months), C22:6n-3. For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty-four months: C20:3n-9 (no significant change observed from zero to six months), C18:2n-6, C20:2n-6, C20:4n-6 (observed decrease at six months as compared to zero time, no significant change between zero time and 24 months), C22:5n-3.

Table 41. Group A showing the significant differences in concentration of the Fatty acids of phosphatidylserine (PS) fraction in time (zero time, six months and twenty-four months).

Group A (n=10)				
PS	**Time**	**Time**	**Mean Difference**	**SEM**
C16:0	T24	[a]T00	±2.36	±0.46
		[a]T06	±1.90	±0.46
C18:1n-7	T00	[b]T24	±0.16	±0.05
C18:2n-6	T00	[a]T06	±3.39	±0.28
		[a]T24	±2.21	±0.28
	T06	[a]T24	±1.18	±0.28
C20:0	T06	[c]T24	±0.14	±0.05
C20:3n-3	T06	[d]T24	±0.25	±0.10
C20:5n-3	T00	[a]T06	±0.28	±0.03
		[a]T24	±0.11	±0.03
	T06	[a]T24	±0.17	±0.03
C22:4n-6	T00	[b]T06	±0.92	±0.30
C22:5n-6	T00	[e]T06	±0.29	±0.08
C24:1n-9	T00	[e]T24	±0.23	±0.07
C22:5n-3	T24	[a]T00	±1.54	±0.28
		[e]T06	±1.00	±0.28
C22:6n-3	T00	[a]T06	±5.89	±0.29
		[a]T24	±6.05	±0.29

[a]p=<0.001, [b]p=0.01, [c]p=0.02, [d]p=0.04, [e]p=0.004

Table 41 shows the concentration of the fatty acids of the PS fraction in group A that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C16:0, C18:2n-6 significant increase at 24 months as compared to 6 months), C20:5n-3 (significant increase from zero to six months followed by a decrease at 24 months as compared to six months. Overall this fatty acid significantly increased at 24 months as compared to zero time), C22:5n-3, C22:6n-3. For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty-four months: C18:1n-7 (no significant change observed from zero to six months), C24:1n-9 (no significant change observed from zero to six months), C18:2n-6 (Overall decrease at 24 months as compared to zero time. However an increase was observed at 24

months as compared to six months), C22:4n-6 (significant change only observed for the first six months), C22:5n-6 (significant change only observed for the first six months).

Table 42. Group B showing the significant differences in concentration of the Fatty acids of phosphatidylserine (PS) fraction in time (zero time, six months and twenty-four months).

Group B (n=10)				
PS	**Time**	**Time**	**Mean Difference**	**SEM**
C18:0	T06	[a]T24	±1.93	±0.68
C18:2n-6	T24	[b]T00	±1.91	±0.28
		[b]T06	±1.80	±0.28
C20:2n-6	T00	[a]T24	±0.11	±0.04
C20:4n-6	T00	[a]T06	±4.05	±1.45
		[c]T24	±3.83	±1.45
C20:5n-3	T00	[b]T06	±0.29	±0.02
		[b]T24	±0.14	±0.02
	T06	[b]T24	±0.15	±0.02
C22:4n-6	T00	[b]T06	±1.81	±0.36
		[b]T24	±1.88	±0.36
C22:5n-6	T00	[d]T06	±0.49	±0.13
C22:5n-3	T24	[b]T00	±1.79	±0.32
		[b]T06	±1.36	±0.32
C22:6n-3	T00	[b]T06	±5.96	±0.46
		[b]T24	±6.69	±0.46

[a]p=0.02, [b]p=<0.001, [c]p=0.04, [d]p=0.003

Table 42 shows the concentration of the fatty acids of the PS fraction in group B that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C18:0 (no significant change observed from zero to six months), C20:5n-3 (significant increase from zero to six months followed by a decrease at 24 months as compared to six months. Overall this fatty acid significantly increased at 24 months as compared to zero time), C22:5n-3, C22:6n-3. For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty-four months: C18:2n-6, C20:2n-6 (no significant change observed from zero to six

months), C20:4n-6, C22:4n-6, C22:5n-6 (significant change only observed for the first six months), C20:5n-3 (significant decrease at 24 months as compared to six months. See also note paragraph above).

Table 43. Group C showing the significant differences in concentration of the Fatty acids of phosphatidylserine (PS) fraction in time (zero time, six months and twenty-four months).

Group C (n=9)				
PS	**Time**	**Time**	**Mean Difference**	**SEM**
C18:1n-9	T00	[a]T06	±1.76	±0.34
		[a]T24	±1.55	±0.34
C18:3n-6	T06	[b]T24	±0.09	±0.03
C20:2n-6	T00	[c]T24	±0.09	±0.03
C20:5n-3	T00	[d]T06	±0.09	±0.03
C22:4n-6	T00	[e]T06	±1.31	±0.41
		[f]T24	±1.25	±0.41
C22:5n-6	T06	[e]T24	±0.44	±0.13
C24:1n-9	T00	[b]T24	±0.27	±0.10
C22:6n-3	T06	[c]T24	±0.74	±0.28

[a]p=<0.001, [b]p=0.02, [c]p=0.04, [d]p=0.003, [e]p=0.008, [f]p=0.01

Table 43 shows the concentration of the fatty acids of the PS fraction in group C that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C18:1n-9, C20:5n-3 (significant increase from zero to six months no significant change at 24 months as compared to either zero time or six months), C22:4n-6, C22:5n-6 (significant changes at 24 months were observed as compared to six months). For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty-four months: C24:1n-9 (no significant change observed from zero to six months), C18:3n-6 (significant changes at 24 months were observed as compared to six months), C20:2n-6, (no significant change observed from zero to six

months), C22:6n-3 (significant changes at 24 months were observed as compared to six months).

Table 44. Group D showing the significant differences in concentration of the Fatty acids of phosphatidylserine (PS) fraction at time (zero time, six months and twenty-four months).

Group D (n=12)				
PS	Time	Time	Mean Difference	SEM
C18:1n-9	T00	[a]T24	±1.17	±0.31
C18:1n-7	T24	[b]T00	±0.23	±0.06
		[c]T06	±0.14	±0.06
C18:2n-6	T00	[d]T06	±1.07	±0.40
		[b]T24	±2.71	±0.40
	T06	[b]T24	±1.64	±0.40
C18:3n-6	T00	[e]T06	±0.05	±0.02
		[b]T24	±0.08	±0.02
C18:3n-3	T06	[d]T24	±0.18	±0.07
C20:2n-6	T00	[e]T24	±0.09	±0.03
C20:4n-6	T00	[a]T24	±3.03	±0.89
C20:3n-3	T00	[e]T24	±0.19	±0.07
C22:1n-9	T00	[e]T24	±0.11	±0.04
C20:5n-3	T06	[b]T00	±0.16	±0.03
		[b]T24	±0.21	±0.03
C24:0	T06	[e]T24	±0.10	±0.03
C22:6n-3	T24	[b]T00	±1.17	±0.21
		[b]T06	±1.20	±0.21

[a]p=0.002, [b]p=<0.001, [c]p=0.04, [d]p=0.03, [e]p=0.02

Table 44 shows the concentration of the fatty acids of the PS fraction in group D that significantly changed during the duration of the study. For the following fatty acids there concentration in the RBC cell membrane increased during the study i.e. from time zero to twenty four months: C18:1n-9 (no significant change observed from zero to six months), C22:6n-3, C20:4n-6 (no significant change observed from zero to six months). For the following fatty acids their concentration in the RBC cell membrane decreased during the study i.e. from time zero to twenty four months: C24:0 (significant changes at 24 months were observed as compared to six months), C18:1n-7, C22:1n-9 (no significant change

observed from zero to six months), C18:2n-6, C18:3n-6, C20:2n-6, (no significant change observed from zero to six months), C18:3n-3 (significant changes at 24 months were observed as compared to six months), C20:3n-3 (no significant change observed from zero to six months), C20:5n-3.

Table 45. Groups A, B, C, D. The difference in concentrations of linoleic acid (LA) of all fractions Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), and Sphingomyelin (SM) of the red blood cell (RBC) membranes analyzed for the period (a) between zero time and six months in the study (6mo.) and (b) zero time and 24 months in the study (24mo.). Paired Sample t-test for LA, of PC, PE, PS, SM from RBC membranes.

Group A	**Linoleic Acid, C18:2n-6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	22.74	16.91	17.36	0 time-6m	5.83	<0.001
				0 time-24m	5.38	<0.001
PE	6.27	5.07	5.19	0 time-6m	1.20	0.01
				0 time-24m	1.08	0.03
PS	5.9	2.51	3.69	0 time-6m	3.39	<0.001
				0 time-24m	2.21	<0.001
SM	3.19	2.17	2.35	0 time-6m	1.02	<0.001
				0 time-24m	0.84	0.003
Group B	**Linoleic Acid, C18:2n-6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	21.66	15.88	14.58	0 time-6m	5.78	<0.001
				0 time-24m	7.08	<0.001
PE	6.5	5.83	4.95	0 time-6m	0.67	No significance
				0 time-24m	1.42	0.02
PS	4.35	4.25	2.44	0 time-6m	1.80	<0.001
				0 time-24m	1.91	<0.001
SM	3.56	2.69	2.24	0 time-6m	0.87	No significance
				0 time-24m	1.32	No significance
Group C	**Linoleic Acid, C18:2n-6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	21.17	14.94	17.62	0 time-6m	6.23	<0.001
				0 time-24m	3.55	<0.001
PE	6.83	5.25	5.86	0 time-6m	1.59	0.002
				0 time-24m	0.97	No significance

PS	5.81	4.9	5.37	0 time-6m	0.91	No significance
				0 time-24m	0.44	No significance
SM	3.59	3.33	2.87	0 time-6m	0.26	No significance
				0 time-24m	0.72	No significance
Group D	**Linoleic Acid, C18:2n-6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	21.25	19.43	19.22	0 time-6m	1.82	0.003
				0 time-24m	2.03	<0.001
PE	6.32	5.16	4.77	0 time-6m	1.16	No significance
				0 time-24m	1.55	No significance
PS	5.09	4.02	2.38	0 time-6m	1.07	0.03
				0 time-24m	2.71	<0.001
SM	3.98	3.1	2.25	0 time-6m	0.88	0.005
				0 time-24m	1.73	<0.001

The C18:2n-6 (LA) concentration in the phospholipid fractions of PC, PE and SM in the RBC membrane at time zero had no significant differences between the four intervention groups. There were significant differences for the PS fraction between groups A and B, C and B (lower in B than A and C), (see also table Table 17).

There were also significant differences in the four phospholipid fractions at six months and 24 months between the intervention groups. The results were all presented for information (see tables 21-28). Although there were significant differences between the groups are only discussed the significant differences of a fatty acid in a phospholipid fraction within time in a group and the significant differences amongst the groups are only reported. Furthermore as it can be seen from table 45

1. In group A, a significant difference in concentration of LA was observed in all fractions (PC, PE, PS, SM) as compared between zero time and six months and zero time and 24 months. The difference observed is a reduction in concentration on the RBC membrane.

2. In group B, a significant reduction of the LA concentration was observed for the PC, PS fractions at 6 months and 24 months, while for PE the reduction was observed at 24 months as compared to zero time no significant changes were seen at six months. No significant changes were observed in the SM fraction.

3. In group C a significant reduction of the LA concentration was observed for the PC fraction at 6 months and 24 months while for PE fraction a reduction was observed only for six months. No other significant changes were observed.

4. In group D a significant reduction of the LA concentration was observed for the PC, SM and PS fractions at six months and 24months as compared to zero time. No significant changes were observed for the PE fraction.

Worth noting is that the significant changes in groups A and B were observed in all the fractions of the phospholipids of the cell membrane of the RBCs at 24 months as compared to zero time; furthermore a significant decrease was also observed at six months for group A for all fractions and for group B on PC and PS.

In group C there was a significant reduction of LA in 24 months as compared to zero time on the PC fraction and no significant changes were observed in fractions PE, PS, SM. In group D significant reductions were observed in PC, PS and SM fractions on the 24 months and a non-significant change was observed on the PE fraction.

Table 46. Groups A, B, C, D. The difference in concentrations of Gamma Linolenic Acid (GLA) of all fractions Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), and Sphingomyelin (SM) of the red blood cell (RBC) membranes analyzed for the period (a) between zero time and six months in the study (6mo.) and (b) zero time and 24 months in the study (24mo.). Paired Sample t-test for GLA, of PC, PE, PS, SM from RBC membranes.

Group A	**Gamma Linolenic Acid, C18:3-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	0.16	0.17	0.21	0 time-6m	-0.01	No significance

				0 time-24m	-0.05	No significance
PE	0.1	0.15	0.15	0 time-6m	-0.06	0.02
				0 time-24m	-0.06	0.02
PS	0.07	0.08	0.03	0 time-6m	-0.05	No significance
				0 time-24m	0.04	No significance
SM	0.11	0.13	0.08	0 time-6m	-0.02	No significance
				0 time-24m	0.03	No significance

Group B	**Gamma Linolenic Acid, C18:3-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	0.17	0.16	0.15	0 time-6m	0.01	No significance
				0 time-24m	0.02	No significance
PE	0.15	0.15	0.17	0 time-6m	0	No significance
				0 time-24m	-0.02	No significance
PS	0.07	0.06	0.03	0 time-6m	0.01	No significance
				0 time-24m	0.04	No significance
SM	0.08	0.09	0.04	0 time-6m	-0.01	No significance
				0 time-24m	0.04	No significance

Group C	**Gamma Linolenic Acid, C18:3-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	0.16	0.14	0.14	0 time-6m	0.02	No significance
				0 time-24m	0.02	No significance
PE	0.16	0.16	0.16	0 time-6m	0	No significance
				0 time-24m	0	No significance
PS	0.1	0.13	0.03	0 time-6m	-0.03	No significance
				0 time-24m	0.07	No significance
				6m-24m	0.09	0.02
SM	0.18	0.14	0.11	0 time-6m	0.04	No significance
				0 time-24m	0.07	No significance

Group D	**Gamma Linolenic Acid, C18:3-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	0.15	0.16	0.17	0 time-6m	-0.01	No significance
				0 time-24m	-0.02	No significance

PE	0.12	0.15	0.15	0 time-6m	-0.03	No significance
				0 time-24m	-0.03	No significance
PS	0.11	0.06	0.03	0 time-6m	0.05	0.02
				0 time-24m	0.08	<0.001
SM	0.2	0.25	0.11	0 time-6m	-0.05	No significance
				0 time-24m	0.09	No significance

There were no significant differences between the four intervention groups in the concentrations of the phospholipid fractions PS, SM, PE, PC at time zero, 6 months and 24 months for the C18:3n-6 (GLA) fatty acid (see tables 17-28). Furthermore as it can be seen from table 46, the concentrations of GLA in the PC, PE, SM and PS fractions of the four intervention groups at time zero, 6 and 24 months are as follows:

1. In Group A a significant increase in the concentration of the PE fraction at six and 24 months as compared to zero time.
2. In Group B, no significant changes were observed.
3. In Group C, no significant changes were observed for fractions PC, PE, SM, PS at 24 months as compared to zero time. However a significant reduction in the PS fraction was observed at 24 months as compared to six months.
4. In Group D a significant reduction was observed in the PS fraction at 6 and 24 months as compared to zero time.

Table 47. Groups A, B, C, D. The difference in concentrations of Di-homo-γ-linolenic acid (DGLA) of all fractions Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), and Sphingomyelin (SM) of the red blood cell (RBC) membranes analyzed for the period (a) between zero time and six months in the study (6mo.) and (b) zero time and 24 months in the study (24mo.). Paired Sample t-test for EPA, of PC, PE, PS, SM from RBC membranes.

Group A	**Di-homo-γ-linolenic Acid, C20:3-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	2.12	1.54	1.57	0 time-6m	0.58	<0.001
				0 time-24m	0.54	0.01

PE	1.13	1.00	1.13	0 time-6m	0.13	No significance
				0 time-24m	0	No significance
PS	2.17	1.65	1.89	0 time-6m	0.52	No significance
				0 time-24m	0.28	No significance
SM	0.48	0.34	0.41	0 time-6m	0.14	No significance
				0 time-24m	0.07	No significance
Group B	**Di-homo-γ-linolenic Acid, C20:3-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	1.73	1.62	1.29	0 time-6m	0.11	No significance
				0 time-24m	0.44	0.04
PE	1.00	0.99	1.04	0 time-6m	0.01	No significance
				0 time-24m	-0.04	No significance
PS	1.8	1.72	1.72	0 time-6m	0.08	No significance
				0 time-24m	0.08	No significance
SM	0.43	0.31	0.28	0 time-6m	0.12	No significance
				0 time-24m	0.15	No significance
Group C	**Di-homo-γ-linolenic Acid, C20:3-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	1.97	1.7	1.59	0 time-6m	0.27	No significance
				0 time-24m	0.38	No significance
PE	1.23	1.04	1.16	0 time-6m	0.19	No significance
				0 time-24m	0.07	No significance
PS	2.36	2.05	2.16	0 time-6m	0.31	No significance
				0 time-24m	0.2	No significance
SM	0.35	0.34	0.37	0 time-6m	-0.01	No significance
				0 time-24m	-0.02	No significance
Group D	**Di-homo-γ-linolenic Acid, C20:3-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	2.03	1.58	1.55	0 time-6m	0.45	0.01
				0 time-24m	0.48	<0.001
PE	1.09	1.03	1.04	0 time-6m	0.06	No significance

				0 time-24m	0.05	No significance
PS	2.22	1.95	1.79	0 time-6m	0.27	No significance
				0 time-24m	0.43	No significance
SM	0.35	0.31	0.34	0 time-6m	0.04	No significance
				0 time-24m	0.01	No significance

There were no significant differences in concentrations for the C20:3n-6 fatty (DGLA) acid for the PS, SM, PE,PC fractions between the four intervention groups at time zero, six months' and 24 months' time (see tables 17-28).

As shown in table 47 the concentrations of DGLA in all fractions (PC, PE, PS, SM) in all intervention groups at time zero, 6 and 24 months are as follows:

1. In Group A a significant decrease in the concentration of the PC fraction at six and 24 months as compared to zero time.
2. In Group B a significant decrease in the concentration of the PC fraction at 24 months was observed as compared to zero time.
3. In Group D a significant increase was observed in the PC fraction at six and 24 months as compared to zero time.

Table 48. Groups A, B, C, D. The difference in concentrations of Arachidonic Acid(AA) of all fractions Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), and Sphingomyelin (SM) of the red blood cell (RBC) membranes analyzed for the period (a) between zero time and six months in the study (6mo.) and (b) zero time and 24 months in the study (24mo.). Paired Sample t-test for AA, of PC, PE, PS, SM from RBC membranes.

Group A	**Arachidonic Acid, C20:4-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	6.64	3.62	4.35	0 time-6m	3.02	<0.001
				0 time-24m	2.29	<0.001
PE	18.79	14.13	13.73	0 time-6m	4.66	<0.001

				0 time-24m	5.05	<0.001
PS	20.03	20.81	20.71	0 time-6m	-0.78	No significance
				0 time-24m	-0.68	No significance
SM	5.63	3.58	4.98	0 time-6m	2.05	<0.001
				0 time-24m	0.65	0.005
				6m-24m	-1.40	<0.001
Group B	**Arachidonic Acid, C20:4-n6**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	7.44	4.44	3.44	0 time-6m	3.01	<0.001
				0 time-24m	4	<0.001
				6m-24m	1	0.008
PE	19.29	14.94	15.09	0 time-6m	4.19	<0.001
				0 time-24m	3.9	<0.001
PS	23.17	19.12	19.34	0 time-6m	4.05	0.02
				0 time-24m	3.83	0.04
SM	4.58	2.94	4.47	0 time-6m	1.65	<0.001
				0 time-24m	0.11	No significance
				6m-24m	-1.54	<0.001

Group C	Arachidonic Acid, C20:4-n6					
	Mean Concentration at:			Paired Samples Test		
	0 Time	6 months	24 months	Time Frame	Mean	Sig. (2-tailed)
PC	8.29	3.69	4.49	0 time-6m	4.6	<0.001
				0 time-24m	3.8	<0.001
				6m-24m	-0.8	<0.001
PE	19.11	18.93	18.78	0 time-6m	0.18	No significance
				0 time-24m	0.33	No significance
PS	19.09	20.5	20.67	0 time-6m	1.41	No significance
				0 time-24m	1.52	No significance
SM	4.14	4.14	4.58	0 time-6m	0	No significance
				0 time-24m	-0.44	No significance

Group D	Arachidonic Acid, C20:4-n6					
	Mean Concentration at:			Paired Samples Test		
	0 Time	6 months	24 months	Time Frame	Mean	Sig. (2-tailed)
PC	7.19	5.69	4.63	0 time-6m	1.51	<0.001
				0 time-24m	2.56	<0.001
				6m-24m	1.05	<0.001
PE	18.97	18.59	18.69	0 time-6m	0.38	No significance
				0 time-24m	0.28	No significance
PS	22.46	23.99	25.49	0 time-6m	-1.53	No significance
				0 time-24m	-3.03	0.005
SM	5.33	4.4	5.07	0 time-6m	0.93	0.01
				0 time-24m	0.26	No significance

At time zero, AA a C20:4n-6 fatty acid, showed no significant differences between the intervention groups for phospholipid fractions PE and PS (see table 17, 19); there were however significant differences in the PC fraction between groups A and C, C and D (higher in C than A and D) (see table 20); significant differences were also observed for the SM fraction between groups A and C, A and B (higher in A for both) and between D and C, D and B (higher in D for both) (see table 18). Significant differences were observed for 6 and 24 months (see tables 21-28).

As shown in table 48, the concentrations of arachidonic acid (AA) in all fractions (PC, PE, PS, SM) and in all intervention groups at time zero, 6 and 24 months are described below.

A statistical significant reduction at 24 months was observed in the concentration of AA from the membranes of RBC in both groups A (for phospholipid fractions PC, PE, SM) and B (for PC, PE, PS, and SM only the six months). A significant reduction at 24 months was also observed in groups C and D only for PC fraction; an increase in concentration for PS in group D was also observed.

Table 49. Groups A, B, C, D. The difference in concentrations of Eicosapentaenoic acid (EPA) of all fractions Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), and Sphingomyelin (SM) of the red blood cell (RBC) membranes analyzed for the period (a) between zero time and six months in the study (6mo.) and (b) zero time and 24 months in the study (24mo.). Paired Sample t-test for EPA, of PC, PE, PS, SM from RBC membranes.

Group A	**Eicosapentaenoic Acid, C20:5-n3**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	0.2	1.16	1.13	0 time-6m	-0.96	<0.001
				0 time-24m	-0.93	<0.001
PE	2.42	4.63	4.99	0 time-6m	-2.22	<0.001
				0 time-24m	-2.57	<0.001
PS	0.56	0.84	0.67	0 time-6m	-0.28	<0.001
				0 time-24m	-0.11	<0.001
				6m-24m	0.17	<0.001
SM	0.22	0.47	0.36	0 time-6m	-0.25	<0.001
				0 time-24m	-0.13	<0.001
				6m-24m	0.11	<0.001
Group B	**Eicosapentaenoic Acid, C20:5-n3**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	0.23	1.19	1.2	0 time-6m	-0.96	<0.001
				0 time-24m	-0.97	<0.001
PE	1.97	4.47	4.7	0 time-6m	-2.29	<0.001
				0 time-24m	-2.51	<0.001
PS	0.57	0.86	0.71	0 time-6m	-0.29	<0.001
				0 time-24m	-0.14	<0.001
				6m-24m	0.15	<0.001
SM	0.16	0.4	0.27	0 time-6m	-0.24	<0.001

				0 time-24m	-0.11	No significance
				6m-24m	0.13	<0.001
Group C	**Eicosapentaenoic Acid, C20:5-n3**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	0.22	0.22	0.24	0 time-6m	0	No significance
				0 time-24m	-0.02	No significance
PE	1.98	2.34	2.08	0 time-6m	-0.36	<0.001
				6m-24m	0.26	0.009
				0 time-24m	-0.1	No significance
PS	0.56	0.65	0.6	0 time-6m	-0.09	0.003
				0 time-24m	-0.04	No significance
SM	0.22	0.27	0.3	0 time-6m	-0.05	No significance
				0 time-24m	-0.08	0.005
Group D	**Eicosapentaenoic Acid, C20:5-n3**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	0.26	0.24	0.22	0 time-6m	0.02	No significance
				0 time-24m	0.04	No significance
PE	1.54	2.45	0.8	0 time-6m	-0.91	<0.001
				0 time-24m	0.75	<0.001
				6m-24m	1.66	<0.001
PS	0.57	0.73	0.52	0 time-6m	-0.16	<0.001
				0 time-24m	0.05	No significance
				6m-24m	0.21	<0.001
SM	0.24	0.17	0.27	0 time-6m	0.07	No significance
				0 time-24m	-0.03	No significance
				6m-24m	-0.09	0.01

There were no significant differences in the concentration of C20:5n-3 (EPA) fatty acid at time zero between the groups for fractions PS, SM and PC fractions (see tables 17, 18, 20). There were however significant differences in the PE fraction between groups A-B, A-C, A-D (higher in A than B, C and D), C-D (higher in C than D), B-D (higher in B than D) (see table 19). Significant differences were observed between groups for 6 and 24 months (see tables 21-28). Table 49, EPA concentration in group A showed a significant increase in the PC, PE, PS, SM fractions at 24 months as compared to zero time. Group B however showed

significant increase in the PC, PE, PS fractions at 24 months as compared to zero time; In the SM fraction, although an increase in concentration was observed in the first six months, this followed by a decrease in concentration in the 24 months as compared to six months. Overall there were no significant changes in 24 months as compared to zero time in the SM fraction. Group C overall showed no significant changes in concentration in 24 months as compared to zero time. However significant changes (decrease or increase in concentration) were observed at six months as compared to zero time and 24 months respectively. Group D showed the same pattern as group C at the exception of the PE fraction where significant reduction in concentration was observed between zero time and 24 months.

Table 50. Groups A, B, C, D. The difference in concentrations of Docosahexaenoic Acid (DHA) of all fractions Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), and Sphingomyelin (SM) of the red blood cell (RBC) membranes analyzed for the period (a) between zero time and six months in the study (6mo.) and (b) zero time and 24 months in the study (24mo.). Paired Sample t-test for DHA, of PC, PE, PS, SM from RBC membranes.

Group A	**Docosahexaenoic Acid, C22:6n-3**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	1.34	1.46	2.61	0 time-6m	-1.11	0.005
				0 time-24m	-1.27	<0.001
PE	5.93	11.93	11.75	0 time-6m	-6	<0.001
				0 time-24m	-5.82	<0.001
PS	6.4	12.29	12.45	0 time-6m	-5.89	<0.001
				0 time-24m	-6.05	<0.001
SM	1.44	3.31	3.46	0 time-6m	-1.87	<0.001
				0 time-24m	-2.02	<0.001
Group B	**Docosahexaenoic Acid, C22:6n-3**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	1.42	1.96	2.02	0 time-6m	-0.54	No significance
				0 time-24m	-0.6	No significance
PE	6.67	11.64	11.74	0 time-6m	-4.41	<0.001
				0 time-24m	-4.54	<0.001

PS	6.54	12.51	13.23	0 time-6m	-5.96	<0.001
				0 time-24m	-6.69	<0.001
SM	1.79	1.93	4.91	0 time-6m	-3.11	<0.001
				0 time-24m	-2.97	<0.001

Group C	**Docosahexaenoic Acid, C22:6n-3**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	1.54	1.48	1.07	0 time-6m	0.47	0.01
				0 time-24m	0.42	0.02
PE	6.37	6.44	6.52	0 time-6m	-0.07	No significance
				0 time-24m	-0.15	No significance
PS	6.07	6.1	5.36	0 time-6m	-0.03	No significance
				0 time-24m	0.71	No significance
				6m-24m	0.74	0.04
SM	1.08	1.21	1.09	0 time-6m	-0.13	0.04
				0 time-24m	-0.01	No significance
				6m-24m	0.13	0.04

Group D	**Docosahexaenoic Acid, C22:6n-3**					
	Mean Concentration at:			**Paired Samples Test**		
	0 Time	**6 months**	**24 months**	**Time Frame**	**Mean**	**Sig. (2-tailed)**
PC	1.52	1.22	1.33	0 time-6m	0.3	No significance
				0 time-24m	0.19	No significance
PE	6.54	6.39	6.48	0 time-6m	0.15	No significance
				0 time-24m	0.06	No significance
PS	6.25	6.21	7.42	0 time-6m	0.04	No significance
				0 time-24m	-1.17	<0.001
				6m-24m	-1.2	<0.001
SM	1.32	2	1.78	0 time-6m	-0.68	<0.001
				0 time-24m	-0.46	0.01

There were no significant differences in the per cent concentration of C22:6n-3 (DHA) fatty acid at time zero between the groups for fractions PS, PE and PC (see tables 17, 19, 20). There were however significant differences in the SM fraction between groups B-C, B-D (higher in B than C and D) (see table 18). Significant differences were observed for 6 and 24 months (see tables 21-28)

Table 50, DHA per concentration significantly increased in all fractions in the intervention group A in the 24 months as compared to zero time. The per cent concentration was significantly increased in the intervention group B in phospholipid fractions PE, PS, SM, in the 24 months as compared to zero time, no significant change was observed in the PC fraction for the same period of time. In the intervention group C a decreased in the per cent concentration was observed in the PC fraction in the 24 months as compared to zero time. Significant changes were observed between zero time and six months and between six months and 24 months however overall there were no significant changes in 24 months when compared to zero time. In the intervention group D an increase in the per cent concentration was observed in the PS and SM fractions in the 24 months as compared to zero time.

The PC and the SM phospholipid fractions of the intervention group B showed no significant changes in the DHA and EPA respectively (tables 49, 50) in the 24 months' time as compared to zero time. Furthermore EPA and DHA per cent concentration increased at 24 months as compared to zero time in all the rest phospholipid fractions in the intervention groups A and B (both supplemented with PUFAs) (tables 49, 50). Interesting the increase was observed at six months as compared to zero time, and no significant increase was further observed from six months as compared to 24 months,

4.5 Discussion

Two important factors that are involved in the pathogenesis of MS are: the environmental exposure (nutrients/dietary habits) and genetic susceptibility (Compston and Coles 2008). Studies on the effects of diet as a therapy (mono/or as adjuvant) are very few and inconclusive (Mehta, Dworkin et al. 2009). At present there are no dietary recommendations for MS, since are "largely disregarded" due to the fact that is not clear clue that nutrients can have a positive effect on the course of MS (Riccio 2011). But, a growing number of MS

patients are using complementary or alternative treatments (Farinotti, Simi et al. 2007). Among other nutrients, dietary antioxidants and fatty acids, may have an effect on the disease course since these nutrients can reduce immune-mediated inflammation, oxidative stress and exitotoxicity (van Meeteren, Teunissen et al. 2005). Data that have been published showed that these nutrients can have multiple roles including change of cell metabolism, down-regulate inflammation, affect nuclear receptors and transcriptional factors (Riccio 2011). In a review paper by Mehta et al. in 2009, different clinical studies are discussed that have been using different PUFAs as intervention molecules for efficacy on MS treatment (Mehta, Dworkin et al. 2009). However there are no reports for the use of γ-tocopherol as an antioxidant as a means of treatment for MS.

The formulation and the choice of the ingredients for the study was based on the existing in vivo (both animal and human trials) and in vitro data. The 1:1 was used as the ideal and physiological ω-3 to ω-6 ratio, as previously reported by many scientific investigators (Simopoulos 2008). This PUFA ratio was also observed in populations with almost zero MS incidence among their population, like the Eskimos where their diet is based on fish (Rafat, Saame et al. 2004).

The majority of the studies regarding MS, whether being on supplementation with EPA/ DHA or GLA/LA, were investigating serum fatty acid profiles (Bates et al., 1989, Millar et al., 1973, Weinstock-Guttman et al., 2005). Furthermore the placebo group was also different as in the case of Harbige et al where polyethylene glycol was used as instead of olive oil (Harbige and Sharief, 2007). Therefore there are no reports on the effects of different supplementations on the concentration of fatty acids in red blood cell membranes of MS patients and therefore nothing that can be compared with the results obtained. Firstly from the results (tables 17-20) comparing the FA concentrations of the phospholipid fractions at time zero it can be observed that there were significant differences concentration

in different FA in all phospholipid fractions, indicating that the four intervention groups where not homogeneous at time zero as far as the FA is concerned. These differences it could be the result of different individual dietary patterns. As it was discussed elsewhere (pages 93-95) individual dietary pattern can influence membrane lipid composition especially those of ω-3, ω-6 PUFA (Hulbert, Turner et al. 2005). Furthermore the same authors argued that SFA and MUFA phospholipid membrane content remain relatively constant over a wide range of dietary variation of these FA (Hulbert, Turner et al. 2005). The presence of relatively high concentration of the ingredients in all intervention groups can help to overcome any significant differences that could be observed at time zero. On the other hand the effects of the supplementation that could be observed during the 24 months of the study on the FA of RBC membranes are more pronounced within the intervention groups. For example, a significant difference was that the EPA concentration of the PE fraction between groups A and D and B and D. Interestingly though after a 24 month period of supplementation, the EPA concentration was doubled in groups A and B and decreased in D. For instance, the effect of supplementation that have had on LA in all fractions of the intervention groups A and B was a significant decrease in concentration at 24 months as compared to zero time (table 45), an effect that could be due to the substitution of LA with DHA (Gudbjarnason and Oskarsdottir 1977; Weber 1989; Harbige, Ghebremeskel et al. 1990) supplied with the intervention formulas. Interesting in the SM fraction of group B, there was a non-significant decrease in concentration which could probably be due to gamma tocopherol when mixed with PUFAs, which could become significant upon expanding the number of participants in the study. As far as group C is concerned there was a significant reduction in concentration for LA in 24 months as compared to time zero for the PC fraction only. In group D significant reductions were observed in the PC, PS, and SM fractions. These significant reductions could probably be due to the olive oil present in the intervention

formulae in both C and D. However in group C the gamma tocopherol that was added in the olive oil of the intervention formulae could probably cause the non-significant change on PE, PS, SM which may become significant upon expanding the number of participants in the study. The GLA concentration on RBC membranes on the different phospholipid fractions didn't present any significant changes at the exception in group A (the supplemented group with the PUFAs) where significant increase in the concentration was observed for the PE fraction at six months as compared to zero time and at 24 months as compared to zero time. A significant decrease was observed for group D on the PS fraction at 24 months as compared to zero time. The non-significant changes could probably be explained specially for groups A and B that LA can be converted into GLA by the activity of Δ6 desaturase which is rapidly elongated to DGLA. However DGLA concentrations didn't increase as was expected (Harbige, Pinto et al. 2008; Wang, Lin et al. 2012). One other explanation could be that RBC is using these fatty acids as structural lipids for its cell membrane and not further metabolizing them.

The DGLA concentration in the RBC membrane showed a significant reduction in Groups A, B and D in the PC fraction at 24 months as compared to zero time. No significant changes were observed for the other fractions in the intervention groups. As previously mentioned GLA is rapidly elongated to DGLA, which is in turn is converted to AA via a Δ5 desaturase. However because of the relatively lower efficiency of Δ5 desaturase DGLA is only partially converted to AA therefore DGLA is accumulated after GLA (Wang, Lin et al. 2012). The increase in concentration of the DGLA can reduce the synthesis of AA derived eicosanoids, promoting the production of PG1 leukotrienes of series 3, hence having an anti-inflammatory effect (Rakesh and Yung-Sheng 2006; Wang, Lin et al. 2012). Furthermore it has been established from research that physiological and pathophysiological states such as aging, premenstrual syndrome, diabetes, alcoholism, atopic dermatitis, rheumatoid arthritis, cancer

and cardiovascular disease reduce the capacity to convert LA to DGLA (Rakesh and Yung-Sheng 2006). Although Groups A and B were supplemented with LA and GLA, the DGLA concentration didn't significantly increase as was expected. It can be speculated that the presence of EPA, DHA and gamma tocopherol could be interfering or may suggest that the accumulation of DGLA can occur in other cell types such as in cells of the immune system. In figure 19 (A) of chapter 3, the ARR of all-time on-study patients are presented. At six months a reduction in the ARR for all intervention groups was observed as compared to the baseline at entry. For the same period of time, the AA concentration in RBC membranes (table 48) was decreased in all intervention groups (PC, PE, SM for group A; PC, PE, PS, SM for group B; PC for group C; PC, SM for group D). The reduction in AA observed is the expected effect of supplementation with EPA and DHA that can substitute AA on the cell membrane. AA in the cytoplasm gives rise to the production of pro-inflammatory eicosanoids which in turn can increase inflammation. RBCs do not contain COX-1 (which is constitutively expressed and is found in the endothelial cell lining of the blood vessels and platelets, in stomach and in kidneys) or COX-2 (which is an inducible enzyme and is induce by cytokines as part of the inflammatory process). Therefore the current observations regarding the decrease of AA in the RBC it could have been taken place in cells that do possess COX-1 or COX-2 that will give rise to pro-inflammatory reactions. Furthermore changes that are observed in RBCs can reflect changes that could take place in other cells in the body including the cerebrospinal fluid. In addition RBCs have a short half-life, a property of RBCs that allows the rapid observation of changes in cellular functions, e.g. changes in the composition of the plasma membrane. (van de Rest, Geleijnse et al. 2008; Gadaria-Rathod, Dentone et al. 2013). On the other hand EPA and DHA are known to have anti-inflammatory effects. Therefore the decrease of ARR in the first six months in groups A and B can be attributed to the effect of EPA and DHA substituting AA. For the same six month

period, the AA in group C (PC fraction) and D (PC and SM fraction) was reduced in concentration and at the same time the ARR was reduced as compared to the entry baseline; the ARR of group C was 0.2, and of group D was 0.7 at six months. The consumption of olive oil from intervention groups C and D significantly increased the concentration of oleic acid in RBC membranes (table 48). Oleic acid consumption can normalize the fatty acid composition of cell membranes of healthy and hypertensive people by increasing the ω-3 PUFA content and reducing the ω-6 PUFA, LA and AA content (Ruiz-Gutiérrez, Muriana et al. 1996; Nagyova, Haban et al. 2003; Perona, Vögler et al. 2007). In the first six months of the present study, there was a reduction in the ω-6 PUFA concentration and an increase in the ω-3 PUFA (DHA and EPA) in the PE and SM fractions for both groups A and B, which probably accounts for the reduction of ARR in these groups. It is worth noting that the presence of γ-tocopherol in the olive oil (group C) brought about a reduction in ARR to 0.2. γ-tocopherol was also present in the mixture of PUFAs in group B that had a reduction in ARR of 0.8 for the same period of time.

In 12 months within the study, the ARR was further reduced at 0.4 for group B and remained the same for all the duration of the study. The concentration of AA on the RBC membranes for the same period of time decreased in concentration in the PC, PE and SM fractions. In group A, the AA concentration decreased in the PC, PE and SM fractions and the observed increase in the PS fraction at six months was not observed in 24 months. The ARR at the 24 month time increased to 0.9 as compared to 0.6 at six months and to1.1 at entry. The presence of γ-tocopherol in the mixture could probably be responsible for the differences in ARR observed between groups A and B in which similar reductions in AA concentrations were observed.

This is an observation that has never been seen in any other study on PUFAs and MS. Worth noting is the probable synergy between PUFAs and γ-tocopherol on the effects that they

might have on the decrease in the concentration of AA on the PS fraction of RBC in group B, which cannot be observed either on group A or group C.

Despite the presence of olive oil and the subsequent decrease in the concentration of AA and LA at 24 months for groups C and D and the increase in the concentration of EPA and DHA in the PE, SM fractions, the decrease of ARR with respect to time zero was only observed in group C (0.9 to 0.7). As it was suggested earlier, the presence of γ-tocopherol in the mixture could in some way affect the progress of the ARR. The known mechanisms involved in MS can be summarised to include immune-mediated inflammation, oxidative stress and excitotoxicity which contribute to disease progression via oligodendrocyte and neuronal damage, and even cell death. (Evans 1993; Knight 1997; Smith, Kapoor et al. 1999; Matute, Alberdi et al. 2001; Owens 2003). BBB disruption and T-cell infiltration have also been observed. Furthermore the CSF and especially the brain tissue level of antioxidant activity is decreased in MS, in addition to the high concentration of free radicals ($O_2^{-\cdot}$ and NO) that have been found within inflammatory MS lesions and are considered to play an important role in MS pathogenesis. Therefore it can be speculated that γ-tocopherol, through its antioxidant and non-antioxidant properties, can interfere with the above mechanisms and hence ameliorate the progression of the disease. The observed results of the ARR in figure 19 (A) of chapter 3 showed that at entry, group B (PUFA and γ-tocopherol) had an ARR of 1.3 and dropped to 0.4 in 24 months whereas in group A (PUFA only) the ARR started 1.1 and dropped to 0.9. For group C (γ-tocopherol and olive oil) the ARR at entry was 0.9 and dropped to 0, whereas group D (olive oil alone) had an ARR of 0.8 at entry and 1 at the end. One may perhaps conclude that the presence of γ-tocopperol has a synergistic effect which will eventually contribute to the slowing of the disease progression. Therefore this small single study produced prototype results that can be consider novel for the effects of PUFAs and γ-tocopherol on MS.

Furthermore it has been reported that ingested EPA and DHA as dietary fatty acids need 4-6 months in order to exert their beneficial effects and about the same time is needed for the AA to decline, first in the plasma and then in RBC (Marangoni, Angeli et al. 1993)

The GLA concentration on RBC membranes on the different phospholipid fractions didn't present any significant changes with the exception of group A, where a significant increase in the concentration of GLA was observed for the PE fraction at six months and 24 months as compared to zero time A significant decrease was observed for group D on the PS fraction at 24 months as compared to zero time. The non-significant changes could probably be explained specially for groups A and B that LA can be converted into GLA by the activity of Δ6 desaturase which is rapidly elongated to DGLA. However DGLA concentrations didn't increase as was expected (Harbige, Pinto et al. 2008; Wang, Lin et al. 2012). One other explanation could be that RBC is using these fatty acids as structural lipids for its cell membrane and not further metabolizing them.

The DGLA concentration in the RBC membrane showed a significant reduction in Groups A, B and D in the PC fraction at 24 months as compared to zero time. No significant changes were observed for the other fractions in the intervention groups. As previously mentioned GLA is rapidly elongated to DGLA, which is in turn is converted to AA via a Δ5 desaturase. However because of the relatively lower efficiency of Δ5 desaturase DGLA is only partially converted to AA therefore DGLA is accumulated after GLA (Wang, Lin et al. 2012). The increase in concentration of the DGLA can reduce the synthesis of AA derived eicosanoids, promoting the production of PG1 leukotrienes of series 3, hence having an anti-inflammatory effect (Rakesh and Yung-Sheng 2006; Wang, Lin et al. 2012). Furthermore it has been established from research that physiological and pathophysiological states such as aging, premenstrual syndrome, diabetes, alcoholism, atopic dermatitis, rheumatoid arthritis, cancer and cardiovascular disease reduce the capacity to convert LA to DGLA (Rakesh and

Yung-Sheng 2006). Although Groups A and B were supplemented with LA and GLA, the DGLA concentration didn't significantly increase as was expected. It can be speculated that the presence of EPA, DHA and gamma tocopherol could be interfering or may suggest that the accumulation of DGLA can occur in other cell types such as in cells of the immune system.

As far as the reduction in concentration for AA in groups A and B is concerned (table 48) this correlates with the increase in number of relapse activity occurring in the first six months on treatment; AA in groups A and B is substituted by the EPA and DHA and AA could give rise to the production of pro-inflammatory eicosanoids which in turn can increase inflammation. RBCs do not contain COX-1 (which is constitutively expressed and is found in the endothelial cell lining of the blood vessels and platelets, in stomach and in kidneys) or COX-2 (which is an inducible enzyme and is induce by cytokines as part of the inflammatory process). Therefore the current observations regarding the decrease of AA in the RBC it could have been taken place in cells that do possess COX-1 or COX-2 that will give rise to pro-inflammatory reactions. Furthermore changes that are observed in RBCs can reflect changes that could take place in other cells in the body including the cerebrospinal fluid. In addition RBCs have a short half-life, a property of RBCs that allows the rapid observation of changes in cellular functions, e.g. changes in the composition of the plasma membrane. (van de Rest, Geleijnse et al. 2008; Gadaria-Rathod, Dentone et al. 2013). In Groups C and D, AA was significantly decreased in the PC fraction, which could probably be due to the effect of olive oil, as was also observed by Nagyova et al in 2003 (Nagyova 2003).

This is an observation that has never been seen in any other study on PUFAs and MS. Worth noting is the probable synergy between PUFAs and γ-tocopherol on the effects that they

might have on the decrease in the concentration of AA on the PS fraction of RBC in group B, which cannot be observed either on group A or group C.

Therefore this small single study produced prototype results that can be consider novel for the effects of PUFAs and γ-tocopherol on MS.

It has been reported that ingested EPA and DHA as dietary fatty acids need 4-6 months in order to exert their beneficial effects and about the same time is needed for the AA to decline, first in the plasma and then in RBC (Marangoni, Angeli et al. 1993). This goes along with the findings of this study where groups A and B showed a statistically significant increase in concentration of both DHA and EPA in all phospholipid fractions that was not observed in group C (supplemented with γ-tocopherol) and in group D, the placebo, supplemented with olive oil.

CHAPTER FIVE

CONCLUSIONS AND FURTHER STUDIES

CHAPTER FIVE

5.1 Conclusions and Further Studies

Overall, this study has demonstrated that supplementation with PUFAs such as EPA, DHA, GLA and LA may cause significant changes of the AA and LA concentrations in the RBC membranes and the subsequent substitution of AA by EPA and DHA. Furthermore Group B intervention formula containing the above mixture of PUFAs as well as γ-tocopherol showed, in the per-protocol analysis, a significant efficacy in the ARR and the progression of disability as compared to placebo without any known side effects. The findings regarding the RBC membranes correlate very well with clinical observations, and are in turn correlated with the increase in number of relapse activity occurring in the first six months after treatment and later on with a prolonged period of remission. The results included analyses of a total period of 42 months of collected data, including a 12-month intervention-free-treatment extension period. In addition, MRI results support the observations in the reduction in ARR in Group B, where fewer patients presented with new or enlarged T2 lesions. These results also support observations from other researchers who have shown that supplementation with DHA can accelerate myelination. The small sample size included in this study is perhaps the first issue that needs to be addressed in future studies. However, despite the limited sample size, it was able to draw conclusions with statistical significance. Furthermore many trends were observed in the data that had a non-significant p-value and it is possible that a larger sample size may indicate that there is a significance. As the sample size available in Cyprus is inherently smaller due to the population size, reducing the drop-out number can be a way to increase the study sample size. A major issue for the high drop-out rate resulted from the unpleasant taste of the intervention formulas. Although it was sought to musk the taste of fish and olive oil with citrus flavour, the resulted product was

still an unpalatable mixture to take. Special effort was made and strategies were proposed to the patients in order to facilitate the oral administration of the intervention formulas such as mixing the formulas with yoghurt, juices, smoothies and ice-cream. The problem couldn't be rectified resulting in the high drop-out. Therefore future studies should first concentrate in the development of pleasantly tasting product, in order to increase adherence.

The use of fish oils, specially DHA and EPA, bypeople is increasing andcompanies are now following the trend and the science behind the use of fish oils and their benefits for health. The fishing industry worldwide, however, is becoming unable to satisfy the growing demand for these PUFAs. This issue should be seriously taken into account and alternative sources of EPA and DHA must be sought and then need to be tested for efficacy. Although plant ALA is available, its conversion to DHA in the body is not efficient. The effort is to supply DHA and EPA from vegetable oil in order to produce sustainable terrestrial plant-based sources of these long chain fatty acids (Ruiz-Lopez, Usher et al. 2014). Other forms for the production of DHA and EPA are sought within the marine kingdom such as those of phytoplancton (Bianchi, Olazábal et al. 2014; Ianora, Hamilton et al. 2015). However gut absorption, blood transportation and usage by human cells should be further investigated. Furthermore, their efficacy on human health should be thoroughly investigated.

α- and γ-tocopherol, and vitamin D measurements in the plasma should be consider in any future work in order to establish plasma levels in the Cypriot population in relation to MS development and progress of the disease. Furthermore a correlation between levels of EPA and DHA and vitamin D should also be examined.

Future studies can test the supplementation mixtures in other neurodegenerative diseases that share common pathways with MS, such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS).

Other autoimmune diseases could also be included when studying this supplementation mixture in order to establish any potential benefits in ameliorating symptoms or disability.

Furthermore future studies should try to establish standardised dietary guidelines for the consumption of these PUFAs in order to prevent diseases and determine the stage of the disease in which these supplements are most effective. These could only be achieved with a good experimental study design, paying special attention to the choice of stage of the disease and duration of the study.

Measurements of the levels of different cells of the immune system, such as T and B cells and pro-inflammatory cytokines, such as TGF-b1, TNF-α, interlukin-1-b, IFN-γ in the peripheral blood mononuclear cells during the relapse and remission phases in combination with MRI are also important.

The use of a different placebo that shows no effect on the immune system like polyethylene glycol (that unlike olive oil it has no anti-inflammatory properties, contains no fatty acids, phenolic and antioxidant compounds and is routinely used as a carrier in pharmaceutical formulations) could be used especially for populations that olive oil is not part of their diet.

From the present study few things have emerged, for instance it is shown that the presence of γ-tocopherol in the mixture of PUFAs probably can act not only as an antioxidant for PUFAs and as an NO quencher, but can also, in synergy with PUFAs, cause a significant decrease of AA in RBCs and aid the substitution of these fatty acids with DHA and EPA. Moreover a few speculations could be brought forward as a result of the large volume of bibliographic references which have been also presented either in the introduction or in the different chapters of this thesis such as the knowledge that:

1. PUFAs modulate the MHC conformation in cell membranes and also incorporates and controls the lipid-protein rafts which can inhibit downstream signalling that results in suppression of T cell activation and proliferation via T cell receptors (Shaikh and Edidin 2006).
2. PUFAs affect pro-inflammatory and anti-inflammatory pathways in favor of modulating or reducing inflammation. Furthermore PUFAs competitively inhibit AA which is the initiator of the inflammatory process (Calder 2006).
3. Protectins and resolvins, are molecules that are derived from PUFAs and can promote resolution of inflammation by reducing neutrophil activity and macrophage proinflammatory cytokine production, and stimulate the uptake of apoptotic polymorphonuclear leukocytes (Serhan, Chiang et al. 2008).
4. PUFAs stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of anti-inflammatory (Th1/Th7 phenotype suppression, Th2 phenotype induction and decreased expression of proinflammatory cytokines, e.g. IL-6), neuroprotective and antioxidant related genes (Edwards and O'Flaherty 2008; van Horssen, Drexhage et al. 2010).
5. PUFAs can also act as PPARγ agonists therefore decreasing the inflammatory response (Dyall, Michael et al. 2010).
6. MMP9, along with elastase, appears to be a regulatory factor in neutrophil migration across the basement membrane and PUFAs have been shown to interfere with the production of certain matrix metalloproteinases, especially MMP9 (Harris, Hansen et al. 2001).
7. PUFAs indirectly regulate genes involved in lipid metabolism, in the anti-inflammatory response, neuroprotection and neurogenesis (Dyall, Michael et al. 2010).

8. PUFAs directly and indirectly affect mitochondrial function by reducing the oxidative stress response (Dias, Junn et al. 2013; Chang, Khatchadourian et al. 2015).
9. PUFAs affect T-cell proliferation, differentiation, migration and function (Denys, Hichami et al. 2005).
10. PUFAs reduce TNF production (Simopoulos 2002; Trebble, Arden et al. 2003)
11. PUFAS prevent oligodendrocyte cell death as a result of ER stress (Chaudhari, Talwar et al. 2014).
12. PUFAs are ligands of the retinol X receptor (RXR)γ, a regulator of endogenous oligodendrocyte precursor cell differentiation, remyelination and myelinogenesis (Lengqvist, Mata de Urquiza et al. 2004; Jolanda Münzel and Williams 2013)
13. PUFAs affect expression of adhesion molecules and other factors like iron induced endothelial damage at the level of the BBB and iron-deposition related inflammation and neurodegeneration (Calder 2006; Freeman, Hibbeln et al. 2006; Hong, Khoutorova et al. 2015).
14. PUFAs have been shown to be neuroprotective against excitotoxicity, inflammation and oxidative stress with neurotrophic properties (Gamze, Yasemin et al. 2010)
15. Vitamin E, in the form of γ-T can also protect neuronal cells against glutamate induced excitotoxicity (Christen, Woodall et al. 1997; Saito, Nishio et al. 2010; Kostic, Zivkovic et al. 2013; Jiang 2014).
16. γ-T is also a strong metalloprotein quencher, possibly able to protect BBB disruption (Sanches, Santos et al. 2013).
17. Furthermore NF-κB inhibition in immune cells of peripheral and CNS cells can be protective against the development of EAE and can have therapeutic effect in MS (Yan and Greer 2008).

18. Vitamin E (γ-and α-T) exhibit non-antioxidant properties, including modulation of cell signaling and immune function, regulation of specific gene transcription and induction of apoptosis (Christen, Woodall et al. 1997; Glauert 2007).
19. Vitamin E (γ-and α-T) due to its strong free radical scavenging, metal chelation and radical chain reaction-breaking properties, it is important in prevention of oxidative stress from free radicals (Nimse and Pal 2015).

The known mechanisms involved in MS can be summarised to include immune-mediated inflammation, oxidative stress and excitotoxicity which contribute to disease progression via oligodendrocyte and neuronal damage, and even cell death. (Evans 1993; Knight 1997; Smith, Kapoor et al. 1999; Matute, Alberdi et al. 2001; Owens 2003). BBB disruption and T-cell infiltration has also been observed. Furthermore the CSF and especially the brain tissue level of antioxidant activity is decreased in MS, in addition to the high concentration of free radicals (O2-. and NO) that have been found within inflammatory MS lesions and are considered to play an important role in MS pathogenesis. Other metabolic disturbances that have been observed in MS patients are those of PUFA metabolism and antioxidants, along with decreased cellular antioxidant defence mechanisms (van Meeteren, Teunissen et al. 2005; Harbige and Sharief 2007).

The proposition of nervonic acid as a biomarker for CNS myelin damage in MS as suggested by Jones and Harbige in 1987, or lignoceric acidsuggested by Homa et al 1981 (Homa, Conroy et al. 1981), as well as otherpossible biomarkers need to be investigated further in order to establish if are specific only fto MS and at what stage of the course of the disease they appear.

Taking into account the above speculative assumptions- and the results obtained from this proof-of-concept study, a larger study on MS using this nutraceutical formula could be

designed in order to investigate and eventually prove true or otherwise all the above mentioned speculations and results.

CHAPTER SIX

BIBLIOGRAPHY

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6.1 Bibliography

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