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Red Blood Cell Membrane Fluidity in the Etiology of Multiple Sclerosis

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Abstract Organisms adjust the order, or fluidity, of their cellular membranes in response to changes in their physiochemical environment by adjusting the lipid composition of their membranes. We investigated membrane fluidity using the phospholipid, fatty acid and cholesterol content of red blood cells (RBCs) from multiple sclerosis (MS) patients and correlated this with C-reactive protein (CRP) as well as with the severity of neurological outcome as measured by the Kurtzke Expanded Disability Status Scale (EDSS) and its Functional System Scores. The study group consisted of 31 patients with MS and 30 healthy control subjects. Phospholipids were determined using a colorimetric assay, fatty acids by gas chromatography, cholesterol by an enzymatic assay and CRP by a Beckman nephelometer. Cell membrane fluidity was calculated according to previously established formulae. RBC membrane fluidity as measured by the saturated to polyunsaturated fatty acid ratio was higher in patients than in controls ($P = 0.04$). The phosphatidylethanolamine saturated to polyunsaturated fatty acid ratio showed highly significant positive correlations with the EDSS and CRP < 5 μg/ml. CRP showed significant inverse correlations with the saturated nature but positive correlations with the ordered-crystalline-phase to liquid-crystalline-phase lipid ratio. In this study we show that membrane fluidity as measured by the relationship between membrane fatty acids, phospholipids and cholesterol is closely interrelated with inflammation and disease outcome in patients with MS. In conclusion, our findings suggest that the membrane lipid composition of patients with MS and, consequently, membrane fluidity are altered, which seems to be influenced by the inflammatory status.

Keywords Erythrocyte membrane · Cytoskeleton · Membrane · Fatty acid binding through membranes · Biochemistry · Structure

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Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (Ohler et al. 2004; Brück 2005), in which a decreased lipid to protein ratio has been reported (Göpfert et al. 1980; Wilson and Tocher 1991). Loss of polyunsaturated fatty acids (PUFAs) from plasma and blood cell membranes has also been reported, especially that of the n-6 fatty acids C18:2n-6 and C20:4n-6 (Harbige and Sharief 2007). These fatty acids are precursors for the inflammatory messengers eicosanoids, and because MS is an inflammatory disease, the loss of membrane PUFAs could be part of the pathogenesis of the

disease. Although the membrane fatty acid composition has been studied in the brain and blood cells from patients with MS, there is a scarcity of literature on the fluidity status of blood cell membranes, including that of red blood cells (RBCs). Decreases in PUFAs are replaced by saturated fatty acids (SATS) (Horrobin 1999) and could therefore be expected to contribute to changes in membrane fluidity in patients with MS. However, studies have shown little difference in membrane fluidity in patients with MS. Chia et al. (1984) found no significant differences in the SATS to PUFA ratios in myelin from patients with MS and normal myelin, although the authors reported some changes in fatty acid composition. Furthermore, using different techniques, such as electron spin resonance (ESR) and fluorescence polarization spectroscopy, no significant differences were observed between the fluidity or deformability of RBC membranes from patients with MS and control subjects (Boggs and Moscarello 1980; Kurantsin-Mills et al. 1982; Pollock et al. 1982). Using wide-angle X-ray diffraction, Chia et al. (1984) was able to show significant differences in the physical organization of the myelin lipid bilayer. They could detect liquid-crystalline-phase lipids as well as ordered-crystalline-phase lipids in membranes, which were not discernible by ESR and which showed a changed transition temperature in patients with MS.

Biological cell membranes are composed of lipids and protein (Caret et al. 1997) and are involved in a variety of cellular functions (Williams 1998). Membrane phospholipids differ in their headgroups and hydrocarbon chains (Hazel and Williams 1990; Williams 1998; Barenholz 2002). Their polar headgroups can be choline, ethanolamine, serine, inositol, inositol phosphates or glycerol (Koay and Walmsley 1999). The choline-containing phospholipid phosphatidylcholine (PC) is the most abundant phospholipid in animal cell membranes, and phosphatidylethanolamine (PE) is the second most (Williams 1998), with sphingomyelin (SM) also contributing significantly to the membrane phospholipid composition (Barenholz and Thompson 1999). Phospholipids PC and SM are contained on the outer leaflet of the membrane, while PE and phosphatidylserine (PS) are on the inside (Williams 1998; Koay and Walmsley 1999). PE phospholipids are ordered-crystalline-phase lipids and can pack closely in membranes, while PC phospholipids are liquid-crystalline-phase lipids and do not pack close in the membrane (Harlos and Eibl 1981; Williams 1998; Hamai et al. 2006). A combination of ordered-crystalline-phase lipids and liquid-crystalline-phase lipids is needed in cell membranes to regulate membrane fluidity.

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

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

Membrane lipid changes can also result in changes in RBC membrane fluidity and deformability, which is important to these cells when passing through small capillaries (Allen et al. 2006; Labrouche et al. 1996). Changes in RBC membrane deformability could compromise oxygen delivery and could therefore contribute to disease outcome. RBC deformability correlates with the phospholipid PE to PS ratio (Allen et al. 2006). Maintaining a balanced degree of membrane fluidity is important to RBCs because these cells are vulnerable to hydrolysis by serum phospholipase A_2 ($sPLA_2$) and a target for prostaglandin action with diminished fluidity and deformability (Allen and Rasmussen 1971; Harris et al. 2001). These changes in membrane lipid composition may all therefore contribute to changes in membrane fluidity (Allen et al. 2006).

Therefore, the aim of the present study was to investigate whether there would be differences in RBC membrane

fluidity and permeability, as measured by the relationship between membrane phospholipids, fatty acids and cholesterol, between patients with MS and healthy control subjects. Furthermore, we correlated these parameters against the disability status of the patients as measured by the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke 1983). C-reactive protein (CRP) concentrations were used as a marker for inflammation and studied for correlation with both possible membrane lipid changes in MS and disease progression.

Materials and Methods

Ethical Approval

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

Study Population

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

Measurement of the Disability Status of Patients

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

Blood Sample Processing and Analysis

Venous blood from consenting participants was collected into anticoagulant EDTA tubes (Beckman Coulter, Johannesburg, South Africa), and separated using histopaque-1077 separation medium per the manufacturer's instructions (Sigma–Aldrich, Johannesburg, South Africa). RBCs were washed in 0.85% saline solution and stored at −80°C. Plasma CRP was determined using a Beckman nephelometer autoanalyzer, using reagents from Beckman Coulter. The diagnostic values of this laboratory are considered positive for CRP values $\geq$ 5 μg/ml.

Membrane phospholipids PC, PE, PS and SM were determined using a colorimetric assay as previously described (Smuts et al. 1994; Itaya and Ui 1966). Phospholipids were quantified according to their phosphate (Pi) content and reported as membrane phospholipids in micrograms Pi per milliliter packed RBCs.

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

Factors Used as Possible Contributors to Changes in Membrane Fluidity

Membrane SATS to PUFA Ratio

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

Membrane Saturated Nature

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

Cholesterol and Cholesterol to Total Phospholipid Ratio

The membrane cholesterol and cholesterol to total phospholipid ratio were calculated and used as an

indication of membrane fluidity and permeability (Voet and Voet 1995; Barenholz 2002; Allen et al. 2006).

Membrane Ordered-Crystalline-Phase Lipid to Liquid-Crystalline-Phase Lipid Ratio

The composition of membrane ordered-crystalline-phase lipids to liquid-crystalline-phase lipids was measured by the PE (ordered-crystalline-phase lipids) to PC (liquid-crystalline-phase lipids) ratio (Chia et al. 1984; Williams 1998).

Rheologic Property (Deformability)

The membrane PE to PS ratio was calculated and used as an indication of the deformability of RBCs (Allen et al. 2006).

Statistical Analysis

A statistics program, STATISTICA 7 (StatSoft, Tulsa, OK; 1984–2004), was used to perform all statistical analyses. Descriptive data are presented as median (quartile range). For asymmetrical data the Mann–Whitney *U*-test was used to compare distributions between cases and control subjects. Correlations were calculated using Spearman's rank correlation coefficient. Because the study population consisted of patients who had relapsed at different time periods and CRP levels have been shown to correlate with infectious episodes and clinical relapses in MS patients (Giovannoni et al. 1996), all statistical analyses were done on all patients as a group and on a subgroup comprised of patients with CRP < 5 μg/ml. Meaningful statistics could not be done on the subgroup with CRP > 5 μg/ml as we did not ascertain whether this was due to the inflammatory aspect of MS or other conditions that increase CRP levels, such as an infection.

In view of the small sample size, *P* values were corrected for multiple testing by Bonferroni. For comparison of SATS to PUFA ratios between MS patients and controls, $P < 0.01$; for correlations between CRP (as well as EDSS) and SATS to PUFA ratios, $P < 0.01$; with phospholipids, $P < 0.01$, with phospholipid ratios, $P < 0.016$ and with cholesterol, $P < 0.025$, were considered statistically significant.

Results

Differences in Membrane Lipids Between Patients with MS and Control Subjects

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

Correlation Between CRP and Membrane Lipids in Patients with MS and Control Subjects

CRP showed inverse correlations with the RBC SM SATS to PUFA ratio in patients with CRP < 5 μg/ml (Table 2). CRP also showed inverse correlations with the saturated nature (phospholipid PC + SM/PE + PS ratio) and phospholipid phosphatidylinositol (PI), but positive correlations with the ordered-crystalline-phase to liquid-crystalline-phase lipid ratio (phospholipid PE to PC ratio). The CRP showed an inverse correlation with cholesterol in both MS patients and controls (Table 2).

Correlation Between Membrane Lipids and Kurtzke EDSS in Patients with MS

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

Figure 1 shows the decrease in RBC membrane total fatty acids in patients with MS when CRP ≥ 5.00 μg/ml: median (quartile range) when CRP < 5.00 μg/ml 881.8 (231.0) and when CRP ≥ 5.00 μg/ml 740.1 (157.6); $P = 0.036$. Both membrane total saturated and polyunsaturated fatty acids also showed a decrease in patients with CRP ≥ 5.00 μg/ml: saturated fatty acids, when CRP < 5.00 μg/ml 378.4 (93.5) and when CRP ≥ 5.00 μg/ml 326.1 (84.8) ($P = 0.053$); PUFAs, when CRP < 5.00 μg/ml 337.0 (110.7) and when CRP ≥ 5.00 μg/ml 286.1 (29.9) ($P = 0.032$) (data not shown). The membrane total phospholipids and cholesterol did not show a difference in patients with CRP < 5.00 or ≥ 5.00 μg/ml: phospholipids, when CRP < 5.00 μg/ml 1,393.4 (588.6) and when CRP ≥ 5.00 μg/ml 1,356.0

Table 1 Differences in clinical characteristics and in RBC membrane fluidity as measured by membrane lipids between patients with MS and control subjects

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

Fatty acids are quantified in micrograms per milliliter packed cells

Membrane phospholipids are quantified in micrograms Pi per milliliter packed RBCs

Membrane cholesterol is quantified in micrograms cholesterol per milliliter packed RBCs

CRP is quantified in micrograms per milliliter plasma

(154.7) ($P = 0.49$); cholesterol, when CRP < 5.00 μg/ml 422.4 (54.7) and when CRP ≥ 5.00 μg/ml 411.0 (39.1) ($P = 0.20$).

Discussion and Conclusion

In response to changes in their physiochemical environment, organisms adjust the order, or fluidity, of their cellular membranes (Williams 1998). The most commonly observed alterations are changes in the membrane ordered-crystalline-phase to liquid-crystalline-phase lipid ratio (phospholipid PE to PC ratio) (Williams 1998); the membrane saturated nature, as measured by the phospholipid PC + SM/PE + PS ratio; the cholesterol to total phospholipid ratio; and the SATS to PUFA ratio—factors which may all contribute to changes in membrane fluidity (Zamaria 2004; Allen et al. 2006). Various investigators have assessed the levels of fatty acids in a range of biological tissues, such as RBCs and peripheral blood mononuclear cell (PBMC) membranes. Generally, lower levels of PUFAs and increased SATS are reported (Cheravil

Table 2 Correlation between CRP and RBC membrane fluidity as measured by membrane lipids in patients with MS and control subjects

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

* Significant *P* values, after correction for multiple testing by Bonferroni

1984; Holman et al. 1989; Navarro and Segura 1989; Nightingale et al. 1990; Hon et al. 2009a, b, c). In this study, both total SATS and PUFAs were marginally lower in RBC membranes from patients with MS than in controls, with an insignificant decrease in RBC membrane fluidity (higher rigidity) as measured by the increase in the SATS to PUFA ratio. Previous studies have not shown significant differences between MS and control RBC membrane fluidity and deformability, using different methods, such as ESR (Kurantsin-Mills et al. 1982) and microfiltration (Pollock et al. 1982).

The increase in the SATS to PUFA ratio in the RBC membranes from patients with MS is unlikely to be the result of adjustment by these cells to changes in their environment but more likely the result of decreases in the PUFAs C18:2n-6 and C20:4n-6, which have been shown to be reduced in the plasma, platelets, RBCs, leukocytes and cerebrospinal fluid of patients with MS (Harbige and Sharief 2007). The loss of C20:4n-6 from RBC and PBMC membranes has been shown to correlate with an increase in inflammation as measured by CRP (Hon et al. 2009a), as well as an increase in the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) (Harbige and Sharief 2007), respectively, indicating a possible association with the pathogenesis of the disease. Furthermore, these results indicated strongly that the inflammatory processes are not confined to the brain alone. This is especially important as RBCs with compromised membrane fluidity and deformability are targeted by the action of PLA_2 and PGE_2, which are released and synthesized, respectively, during the inflammatory process (Allen and Rasmussen 1971; Harris et al. 2001).

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

Table 3 Correlation between RBC membrane fluidity as measured by membrane lipids and the Kurtzke Expanded Disability Status Scale and Functional System Scores in MS patients

		All CRP values		CRP < 5.00 μg/ml	
		MS ($n = 31$)		MS ($n = 17$)	
		R	*P*	*R*	*P*
Fatty acids					
Total SATS	Visual	0.38	0.03	0.43	0.08
Total PUFA	Pyramidal	−0.31	0.09	−0.37	0.15
Fatty acid ratios					
PC SATS to PUFA	Cerebellar	0.31	0.09	0.32	0.21
PE SATS to PUFA	EDSS	0.22	0.25	0.67	0.003*
PE SATS to PUFA	Pyramidal	0.27	0.15	0.66	0.004*
PE SATS to PUFA	Cerebellar	0.45	0.012	0.85	0.00001*
PS SATS to PUFA	Sensory	0.31	0.08	0.18	0.50
PS SATS to PUFA	Bowel and Bladder	0.11	0.55	0.54	0.03
Total SATS to PUFA	EDSS	0.10	0.60	0.47	0.06
Total SATS to PUFA	Pyramidal	0.05	0.81	0.41	0.10
Total SATS to PUFA	Cerebellar	0.20	0.29	0.46	0.06
Total SATS to PUFA	Brainstem	0.28	0.13	0.46	0.07
Phospholipids					
PC	EDSS	−0.38	0.037	−0.61	0.009*
PC	Pyramidal	−0.35	0.06	−0.45	0.07
PE	EDSS	−0.45	0.011	−0.61	0.009*
SM	EDSS	−0.28	0.13	−0.57	0.018
SM	Pyramidal	−0.26	0.15	−0.45	0.07
SM	Bowel and Bladder	−0.29	0.12	−0.46	0.06
Total phospholipids	EDSS	−0.44	0.015	−0.66	0.004*
Total phospholipids	Pyramidal	−0.38	0.037	−0.45	0.07
Phospholipid ratios					
Saturated nature					
PC + SM/PE + PS	Sensory	0.30	0.11	0.30	0.24
Deformability					
PE to PS	Sensory	0.41	0.021	0.27	0.30
Ordered- to liquid-crystalline-phase lipids					
PE to PC	Bowel and Bladder	−0.21	0.26	−0.51	0.037
Cholesterol					
Cholesterol	EDSS	−0.25	0.18	−0.45	0.07
Cholesterol to phospholipids	EDSS	0.24	0.20	0.40	0.11

* Significant *P* values, after correction for multiple testing by Bonferroni

have used CRP as an inflammatory marker in assessing inflammation in MS patients (Giovannoni et al. 2001; Sellner et al. 2008; Hon et al. 2009b). Hon et al. (2009b) showed that the degree of inflammation may be one of the determinants of the resultant fatty acid concentrations. Similarly, in the present study the total RBC membrane fatty acids from patients with MS were decreased in patients with CRP ≥ 5.00 μg/ml. Evidence from the literature suggests that lipids, particularly fatty acids, play a role in the pathogenesis of MS (Harbige and Sharief 2007). Increased dietary SATS intake is associated with an increased risk of developing MS, while supplementation with PUFAs is thought to improve disease outcome (van Meeteren et al. 2005). In this regard, RBCs with changed membrane fluidity could be compromised and have diminished oxygen-carrying capacity, thereby affecting disease outcome. Similarly, we also found that the PE SATS to PUFA ratio correlated positively with the EDSS and FSS, especially in the subgroup with CRP < 5 μg/ml. These results highlight the importance of considering the fatty acids contained in the

different phospholipid fractions as well as the inclusion of CRP measurements in the analysis of fatty acid levels in patients with MS.

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

Although keeping RBC membrane integrity is of importance, its rheologic properties (deformability) are an important aspect when cells are passing through capillaries, and membrane lipid changes can also result in changes in deformability (Allen et al. 2006; Labrouche et al. 1996). Similar to Pollock et al. (1982), we did not find differences in the RBC/deformability between MS patients and control subjects. However, the RBC membrane PL ratio PE/PS (deformability) correlated positively with the Sensory FSS in MS patients, indicating that higher deformability (less rigidity) could have resulted in less protection to the cells.

The damaging effect of inflammation on RBCs from patients with MS is highlighted by the significant decrease in membrane saturated, polyunsaturated and total fatty acids in patients with CRP $\geq$ 5.00 μg/ml compared to the concentrations when CRP $<$ 5.00 μg/ml (Fig. 1). The membrane total phospholipids and cholesterol did not show a difference in patients with CRP $<$ 5.00 or $\geq$5.00 μg/ml. These results strongly suggest that membrane fatty acids are targeted by inflammatory processes more so than the other membrane lipids and that this can result in cell death in a disease such as MS, in which relapses may occur frequently. These results indicate that decreased lipid

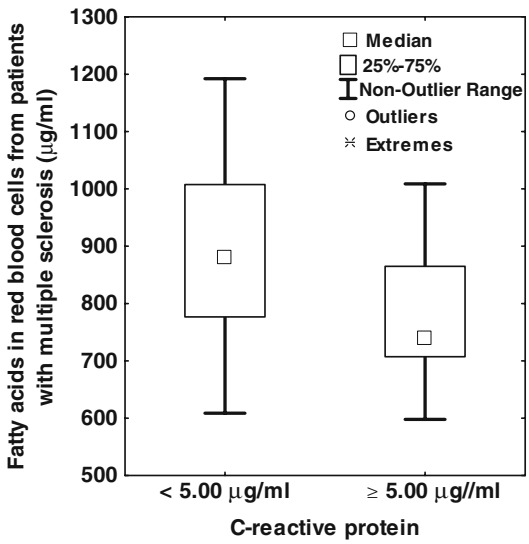

Fig. 1 Total fatty acids and CRP. Patients with MS were subgrouped according to their CRP levels. The total RBC membrane fatty acids in patients with MS when CRP $\geq$ 5.00 μg/ml was lower: median (quartile range) when CRP $<$ 5.00 μg/ml, 881.8 (231.0) and when CRP $\geq$ 5.00 μg/ml, 740.1 (157.6); $P = 0.036$

weight as shown by a lower lipid to protein ratio in myelin from patients with MS (Göpfert et al. 1980; Wilson and Tocher 1991) could be reflected in RBC membranes as well. On the other hand, it appears that membrane phospholipids and cholesterol do not differ between patients with CRP $<$ 5.00 or $\geq$5.00 μg/ml. Furthermore, membrane phospholipids showed inverse correlations with the EDSS and FSS, suggesting a protective role of phospholipids against these conditions.

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

altered, which seems to be influenced by the inflammatory status.

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