Research Article

Non-esterified fatty acids in blood cell membranes from patients with multiple sclerosis

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The literature on non-esterified fatty acid (NEFA) concentrations in blood cell membranes from patients with multiple sclerosis (MS) is scarce and reports on concentrations in brain tissue from these patients are inconsistent. NEFAs are needed for several biological functions, for example, as precursors for inflammatory eicosanoid synthesis. The objective of this study was therefore to compare NEFA concentrations in blood cell membranes from patients with that of healthy control subjects, and to correlate possible changes with disease outcome. NEFA C18:2*n*-6 (9,12-octadecadienoic acid) was decreased in peripheral blood mononuclear cell membranes from patients, median (quartile range): patients: 0.05 (0.02) and controls: 0.07 (0.14) μg/mg protein, $p = 0.007$. C18:2*n*-6 also showed a weaker relationship with other fatty acids: with C16:0: patients: $R = 0.40$, $p = 0.04$; controls: $R = 0.82$, $p = 0.000001$. Saturated and MUFA showed positive correlations with the Bowel and bladder Functional System Scores (FSS). In contrast, in red blood cell membranes C18:2*n*-6 and C22:0 (docosanoic acid) showed inverse correlations with the Sensory and Brainstem FSS. The decrease in NEFA C18:2*n*-6 resulted in metabolic abnormalities between itself and saturated and monounsaturated NEFAs. Altered fatty acid composition in immune cell membranes would influence immune cell functions, and could possibly have contributed to the positive correlations between these fatty acids and disease outcome.

Practical applications: Multiple sclerosis (MS) is a disease which presents with inflammation of the central nervous system. The cause of the disease is unknown and treatments such as anti-inflammatory, immunosuppressive medications, and fatty acids supplements are for the alleviation of symptoms only. The results from this study however, showed an altered relationship between polyunsaturated and saturated as well as monounsaturated non-esterified fatty acids in immune cells, which could have contributed to the inflammatory/infectious condition in these patients. The results from this study and further studies could possibly result in formulation of fatty acid supplements at correct doses or ratios for MS patients.

Keywords: C-reactive protein / Kurtzke Expanded Disability Status Scale / Multiple sclerosis / Non-esterified fatty acids

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Abbreviations: **CRP,** C-reactive protein; **EDSS,** Kurtzke Expanded Disability Status Scale; **FSS,** Functional System Scores; **MS,** multiple sclerosis; **NEFAs,** non-esterified fatty acids; **PBMC,** peripheral blood mononuclear cell; **RBC,** red blood cell

1 Introduction

Non-esterified fatty acids (NEFAs) are needed for several cellular biological functions [1–3] and are the major plasma fatty acid contributor to brain uptake [4]. They supply metabolic energy, incorporated into and form part of the cell membrane phospholipid structure [1–3] and participate in the regulation of immune responses [1, 5, 6]. During inflammatory activation, membrane PUFAs are released from membrane phospholipids as NEFAs, which then serve as

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precursors for signaling molecules, the eicosanoids [2, 3]. PUFA C20:4*n*-6 (5,8,11,14-eicosatetraenoic acid) is a major precursor for eicosanoid synthesis because of its relatively high concentration in immune cell membrane phospholipids [7]. The role of cell membrane saturated (SATS) and MUFA NEFAs in the inflammatory process is less clear, although in vitro studies have shown that SATS and MUFA NEFA could influence immune cell function and/or proliferation [5, 6]. Furthermore, studies have shown that MUFA-rich diets decrease the expression of adhesion molecules on PBMC membranes, and may therefore have specific anti-inflammatory effects [8, 9].

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system in which PUFA metabolic abnormalities have been well documented. There is scarcity of literature on NEFA concentrations in blood cell membranes from patients with MS, however, and reports on the NEFA concentrations in brain tissue from these patients are inconsistent. Craelius et al. [10] reported decreased NEFA C20:4*n*-6 and NEFA C24:4*n*-6 (9,12,15,18-tetracosatetraenoic acid; both PUFAs) in diseased white matter, while Chia et al. [11] reported slightly higher levels of NEFAs, including increases in NEFA C20:4*n*-6, and Wilson and Tocher [12] reported increases in total NEFAs in plaques from patients with MS. Increased NEFAs in plasma has been implicated in a number of diseases, such as sudden cardiac death, insulin resistance, atherosclerosis, and hypertension [13–16].

Therefore, the objective of this study was to investigate whether there were changes in the NEFA concentrations in peripheral blood mononuclear cell (PBMC) (lymphocytes and monocytes) and red blood cell (RBC) membranes from patients with MS as compared to a control study group, as well as to investigate possible metabolic abnormalities within membrane NEFAs. Furthermore, to correlate possible abnormalities with disease outcome as measured by the Kurtzke Expanded Disability Status Scale (EDSS) and Functional System Scores (FSS) [17] and the inflammatory status as measured by C-reactive protein (CRP). RBCs were included in the study as a non-immune cell, to investigate whether possible changes were specific to the immune cells or more wide-spread.

2 Materials and methods

2.1 Study population

Ethical approval for the study was obtained from the Health Sciences Research Ethics Committee (HSREC) of the Cape Peninsula University of Technology (CPUT), approval number CPUT/HW-REC 2011/H04. MS patients were contacted and recruited through the MS Society, Western Cape Branch, South Africa. Written informed consent was obtained from all participants. The study population consisted of 26 female patients and 25 age-matched female control subjects, with median (quartile range) 51 (20) years for patients and 52 (20) years for controls. Only two male patients responded to the study and were excluded from the study as the numbers were insufficient for meaningful statistical analysis. The patients recruited were diagnosed by a neurologist based on clinical, laboratory, and magnetic resonance imaging (MRI) findings. Participants using fatty acid supplements, or were on interferon or cortisone treatment, were excluded from the study.

2.2 Blood sample processing and analysis

Venous blood was collected into anti-coagulant ethylenediaminetetraacetic acid tubes (Beckman Coulter, Cape Town, South Africa). Blood was separated into its different components using histopaque-1077 separation medium as per manufacturer's instructions (Sigma–Aldrich, Cape Town, South Africa). Solvent mixtures and thin-layer chromatography was used for the extraction and separation of fatty acids, respectively, with further conversion to FAME for quantification by GC [18, 19]. The RBCs were extracted and resuspended in 80 μL chloroform/methanol (ratio 2:1 v/v). Of this 40 μL was used for NEFA separation on thin-layer chromatography. The PBMCs were resuspended in 70 μL chloroform/methanol and 30 μL was used for NEFA analysis. Neutral lipids were separated from the total phospholipid fraction by thin-layer chromatography on pre-coated silica gel plates (10×10 cm^2), using the solvent system petroleum benzene (boiling point 40–60°C)/diethyl ether (peroxide free)/acetic acid (90:30:1 by volume; Sigma–Aldrich, Cape Town, South Africa) as previously described [19]. These bands were scraped off the thin-layer chromatography plates and were transmethylated, using 5% sulfuric acid/methanol at 70°C for 2 h. After cooling, the resulting FAME were extracted with 1 mL of distilled water and 2 mL of *n*-hexane. The top hexane layer was removed and evaporated to dryness in a waterbath at 37°C under nitrogen gas, re-dissolved in carbon disulfide and analyzed by GC (Finnigan Focus GC, Thermo Electron Corporation, USA, equipped with flame ionization detection). The FAME were identified by comparison of the retention times to those of a standard fatty acid methyl ester mixture (Nu-Chek-Prep Inc., Elysian, Minnesota). The individual FAME were quantified against an internal standard with known concentration (C17:0, Sigma–Aldrich, South Africa). PBMC NEFAs were quantified against membrane proteins present in μg NEFA/mg protein, and RBC membrane NEFAs in μg NEFA/mL packed RBC analyzed. A bicinchoninic acid protein determination assay was used to determine the protein content in the PBMC membranes [20]. The plasma CRP determination was done on a Beckman nephelometer auto-analyzer (reagents from Beckman, South Africa).

2.3 Statistical analysis

A statistics programme, STATISTICA (STATISTICA 7, StatSoft Inc 1984–2004) was used to perform all statistical

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analyses. Descriptive data are presented as median (interquartile range). For asymmetrical data Mann Whitney U was used to compare distributions between the cases and control subjects. Correlations were calculated using Spearman's Rank correlation coefficient. In view of the small sample size, *p*-values were corrected for multiple testing by Bonferroni. For differences between patients with MS and control subjects: *n*-6 NEFAs: *p*-value<0.025, for correlations between the NEFAs: PUFAs versus PUFAs: PBMCs: *p*-value<0.025, SATS versus SATS: PBMCs: *p*-value<0.008, RBCs: *p*-value<0.005, MUFAs versus PUFAs: PBMCs: *p*-value<0.013, RBCs: *p*-value<0.025, SATS versus PUFAs: PBMCs and RBCs: *p*-value<0.008, SATS versus MUFAs: PBMC: *p*-value<0.013, RBCs: *p*-value<0.008, for correlations with the EDSS and NEFAs: *n*-6 NEFAs: *p*-value<0.005, MUFA NEFAs: *p*-value<0.006, SATS NEFAs: *p*-value<0.004, total NEFAs: *p*-value<0.004, were considered as statistically significant.

3 Results

Differences between NEFAs in PBMC and RBC membranes from patients with MS and control subjects are summarized in Table 1. NEFAs with more than 20% zero values were not included in this study, however, all results were used when total NEFA concentrations were calculated. NEFA C18:2*n*-6 was significantly decreased in PBMC membranes from patients with MS after *p*-values were corrected for multiple testing by Bonferroni.

The metabolic relationship between NEFAs within PBMC membranes and within RBC membranes from patients with MS and control subjects are summarized in Table 2. Correlation studies showed metabolic abnormalities between NEFAs in both PBMC and RBC cell membranes from patients with MS. In PBMC membranes: NEFAs C18:2*n*-6 (linoleic acid) and C20:4*n*-6 (AA), C16:0 (hexadecanoic acid), C18:0 (octadecanoic acid), C18:1*n*-9 (1-enyl-1,9-octadecadienoic acid); as well as between NEFAs C17:0 (heptadecanoic acid) and C16:0, C18:0. In RBC membranes: NEFAs C18:2*n*-6 and C16:0, C18:0, C20:0 (eicosanoic acid), C18:1*n*-9, as well as between NEFAs C16:0 and C20:0, C24:0 (tetracosanoic acid); and between NEFAs C20:0 and C22:0, C18:1*n*-9.

Correlations between NEFAs in PBMC and RBC membranes from patients with MS and the EDSS and FSS are summarized in Table 3. There were no significant correlations between NEFAs and the EDSS and/or FSS after *p*-values were corrected for multiple testing by Bonferroni. NEFAs C18:1*n*-9, C16:0, C17:0, C18:0, total MUFA NEFAs, total SATS NEFAs, and total NEFAs in PBMC

Table 1. Differences between NEFAs in PBMC and RBC membranes from patients with MS and control subjects

	Median (quartile range)					
	RBC			PBMC		
	Controls, N25	MS, N26	*p*-value	Controls, N25	MS, N26	*p*-value
PUFA NEFAs						
n-6 NEFAs						
C18:2*n*-6	0.20 (0.11)	0.23 (0.23)	0.17	0.07 (0.14)	0.05 (0.02)	0.007[a]
C20:4*n*-6	ND	ND	ND	0.04 (0.05)	0.05 (0.07)	0.66
n-3 NEFAs	ND	ND	ND	ND	ND	ND
MUFA NEFAs						
C18:1*n*-9	0.80 (0.73)	1.08 (0.76)	0.07	0.20 (0.32)	0.19 (0.06)	0.22
SATS NEFAs						
C16:0	3.07 (1.61)	3.09 (1.50)	0.63	0.47 (0.30)	0.43 (0.19)	0.14
C17:0	ND	ND	ND	0.27 (0.10)	0.28 (0.07)	0.98
C18:0	9.74 (7.18)	10.89 (8.01)	0.73	0.36 (0.18)	0.37 (0.10)	0.89
C20:0	0.33 (0.17)	0.32 (0.16)	0.65	ND	ND	ND
C22:0	0.32 (0.13)	0.37 (0.21)	0.44	ND	ND	ND
C24:0	0.44 (0.23)	0.49 (0.20)	0.82	ND	ND	ND
Total PUFA NEFAs	0.27 (0.24)	0.30 (0.34)	0.30	0.15 (0.16)	0.12 (0.08)	0.09
Total MUFA NEFAs	0.80 (0.73)	1.08 (0.76)	0.07	0.21 (0.32)	0.19 (0.06)	0.17
Total SATS NEFAs	13.32 (9.34)	14.88 (8.95)	0.65	1.17 (0.64)	1.11 (0.42)	0.35
Total NEFAs	14.03 (9.95)	17.00 (8.87)	0.61	1.55 (0.99)	1.48 (0.46)	0.27

RBC NEFAs quantified in μg/mL packed RBC.
PBMC NEFAs quantified in μg/mg protein.
ND, not detected at all, or more than 20% of values were too low to measure when analyzed.
[a] Significant *p*-values corrected for multiple testing by Bonferroni.

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Table 2. The metabolic relationship between NEFAs within PBMC membranes and within RBC membranes from patients with MS and control subjects

	RBC				PBMC			
	Controls		MS		Controls		MS	
NEFAs	*R*	*p*-value	*R*	*p*-value	*R*	*p*-value	*R*	*p*-value
PUFA vs. PUFA								
C18:2*n*-6 vs. C20:4*n*-6	ND	ND	ND	ND	0.28	0.18	0.43	0.03
SATS vs. SATS								
C16:0 vs. C17:0	ND	ND	ND	ND	0.31	0.13	0.65	0.0003[a)]
C16:0 vs. C18:0	0.76	0.00002[a)]	0.87	<0.000001[a)]	0.85	<0.000001[a)]	0.87	<0.000001[a)]
C16:0 vs. C20:0	0.49	0.01	0.65	0.0003[a)]	ND	ND	ND	ND
C16:0 vs. C22:0	0.34	0.10	0.36	0.07	ND	ND	ND	ND
C16:0 vs. C24:0	0.44	0.03	0.35	0.08	ND	ND	ND	ND
C17:0 vs. C18:0	ND	ND	ND	ND	0.39	0.05	0.61	0.001[a)]
C18:0 vs. C20:0	0.67	0.0002[a)]	0.81	0.000001[a)]	ND	ND	ND	ND
C18:0 vs. C22:0	0.45	0.03	0.49	0.01	ND	ND	ND	ND
C18:0 vs. C24:0	0.52	0.01	0.46	0.02	ND	ND	ND	ND
C20:0 vs. C22:0	0.52	0.01	0.78	0.000003[a)]	ND	ND	ND	ND
C20:0 vs. C24:0	0.66	0.0002[a)]	0.76	0.00001[a)]	ND	ND	ND	ND
C22:0 vs. C24:0	0.83	<0.000001[a)]	0.93	<0.000001[a)]	ND	ND	ND	ND
MUFA vs. PUFA								
C18:1*n*-9 vs. C18:2*n*-6	0.38	0.06	0.56	0.003[a)]	0.92	<0.000001[a)]	0.59	0.002[a)]
C18:1*n*-9 vs. C20:4*n*-6	ND	ND	ND	ND	0.39	0.06	0.13	0.54
SATS vs. PUFA								
C16:0 vs. C18:2*n*-6	0.28	0.18	0.47	0.02	0.82	0.000001[a)]	0.40	0.04
C17:0 vs. C18:2*n*-6	ND	ND	ND	ND	0.17	0.41	0.37	0.07
C18:0 vs. C18:2*n*-6	0.08	0.69	0.50	0.01	0.84	<0.000001[a)]	0.46	0.02
C20:0 vs. C18:2*n*-6	0.30	0.14	0.60	0.001[a)]	ND	ND	ND	ND
C22:0 vs. C18:2*n*-6	−0.18	0.38	0.28	0.16	ND	ND	ND	ND
C24:0 vs. C18:2*n*-6	0.02	0.92	0.25	0.21	ND	ND	ND	ND
C16:0 vs. C20:4*n*-6	ND	ND	ND	ND	0.01	0.97	−0.09	0.67
C17:0 vs. C20:4*n*-6	ND	ND	ND	ND	0.00	0.99	−0.17	0.40
C18:0 vs. C20:4*n*-6	ND	ND	ND	ND	0.28	0.17	0.09	0.65
SATS vs. MUFA								
C16:0 vs. C18:1*n*-9	0.73	0.00004[a)]	0.72	0.00003[a)]	0.82	0.000001[a)]	0.75	0.00001[a)]
C17:0 vs. C18:1*n*-9	ND	ND	ND	ND	0.17	0.41	0.34	0.09
C18:0 vs. C18:1*n*-9	0.42	0.04	0.52	0.01	0.91	<0.000001[a)]	0.83	<0.000001[a)]
C20:0 vs. C18:1*n*-9	0.19	0.36	0.43	0.03	ND	ND	ND	ND
C22:0 vs. C18:1*n*-9	−0.12	0.57	0.15	0.15	ND	ND	ND	ND
C24:0 vs. C18:1*n*-9	0.08	0.71	0.18	0.38	ND	ND	ND	ND

[a)] Significant *p*-values corrected for multiple testing by Bonferroni.

membranes from patients with MS showed non-significant positive correlations with the Bowel and bladder FSS. NEFAs C18:2*n*-6 and C22:0 in RBC membranes from patients with MS showed non-significant inverse correlations with the Sensory and Brainstem FSS, respectively.

Correlations between CRP and NEFAs in PBMC and RBC membranes from patients with MS and control subjects are summarized in Table 4. CRP showed a non-significant positive correlation with NEFA C20:4*n*-6 in PBMC membranes from patients with MS.

4 Discussion and conclusions

There is scarcity of literature regarding NEFAs in PBMC and RBC membranes from patients with MS, and both decreases [10]) and increases [11] have been reported in NEFA C20:4*n*-6 in the brain tissue from these patients. However, previous studies have shown decreases in esterified C18:2*n*-6 and/or C20:4*n*-6 in PBMC [21, 22] and RBC [23–25] membrane phospholipids. MS is an inflammatory disease of the central nervous system, in which immune cell involvement has been shown [26, 27]. Immune cell esterified PUFAs are

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Table 3. Correlations between NEFAs in PBMC and RBC membranes from patients with MS and the EDSS and FSS

		RBC MS			PBMC MS	
	EDSS/FSS	*R*	*p*-value	EDSS/FSS	*R*	*p*-value
PUFA NEFAs						
n-6 NEFAs						
C18:2*n*-6	Sensory	−0.52	0.006	Sensory	0.12	0.57
MUFA NEFAs						
C18:1*n*-9	Bowel and bladder	−0.19	0.36	Bowel and bladder	0.41	0.039
SATS NEFAs						
C16:0	Bowel and bladder	−0.16	0.43	Bowel and bladder	0.40	0.042
C17:0	ND	ND	ND	Bowel and bladder	0.41	0.037
C18:0	Bowel and bladder	−0.20	0.33	Bowel and bladder	0.47	0.016
C22:0	Brainstem	−0.40	0.040	ND	ND	ND
C22:0	Cerebral	−0.38	0.06	ND	ND	ND
C24:0	Brainstem	−0.35	0.08	ND	ND	ND
Total PUFA NEFAs	Sensory	−0.40	0.044	Sensory	−0.20	0.32
Total MUFA NEFAs	Bowel and bladder	−0.19	0.36	Bowel and bladder	0.42	0.034
Total SATS NEFAs	Bowel and bladder	−0.23	0.26	Bowel and bladder	0.49	0.010
Total NEFAs	Bowel and bladder	−0.21	0.29	Bowel and bladder	0.50	0.009

released from membrane phospholipids as NEFAs during inflammatory activation to serve as precursors for signaling molecules, the eicosanoids [1–3]. Esterified C18:2*n*-6 is the parent fatty acid of C20:4*n*-6, which is high in immune cell membrane phospholipids, and a major precursor for eicosanoid synthesis [7]. C18:2*n*-6 is an essential fatty acid, and must be ingested from food. Therefore a decrease of this NEFA in patients with MS could be either due to insufficient dietary intake or may be due to an increased turnover to its elongation product, C20:4*n*-6. In view of the inflammatory nature of MS, the decreases reported by this group and others in esterified C18:2*n*-6 and C20:4*n*-6 [21–25] as well as the decrease reported in this study in non-esterified C18:2*n*-6 in immune cell membranes from patients, suggested a possible increased turnover of these fatty acids to eicosanoid synthesis, possibly contributing to the inflammatory condition experienced by patients. Future studies should therefore include measuring eicosanoids such as prostaglandin E, known to be

Table 4. Correlations between CRP and NEFAs in PBMC and RBC membranes from patients with MS and control subjects

	RBC				PBMC			
	Controls		MS		Controls		MS	
	R	*p*-value	*R*	*p*-value	*R*	*p*-value	*R*	*p*-value
PUFA NEFAs								
n-6 NEFAs								
C18:2*n*-6	−0.29	0.20	0.10	0.64	−0.29	0.19	0.15	0.48
C20:4*n*-6	ND	ND	ND	ND	−0.01	0.95	0.38	0.07
MUFA NEFAs								
C18:1*n*-9	0.22	0.33	−0.27	0.19	−0.29	0.19	−0.15	0.47
SATS NEFAs								
C16:0	0.15	0.50	−0.18	0.41	−0.10	0.65	−0.06	0.78
C17:0	ND	ND	ND	ND	−0.30	0.18	−0.09	0.68
C18:0	0.14	0.54	−0.08	0.72	−0.32	0.14	−0.13	0.54
C20:0	0.01	0.95	−0.00	0.99	ND	ND	ND	ND
C22:0	−0.32	0.15	0.03	0.88	ND	ND	ND	ND
C24:0	−0.20	0.37	0.04	0.85	ND	ND	ND	ND
Total PUFA NEFAs	−0.32	0.15	0.20	0.35	−0.28	0.20	0.30	0.15
Total MUFA NEFAs	0.22	0.33	−0.27	0.19	−0.28	0.20	−0.15	0.48
Total SATS NEFAs	0.09	0.68	−0.09	0.69	−0.31	0.16	−0.10	0.64
Total NEFAs	0.10	0.66	−0.10	0.63	−0.34	0.12	−0.08	0.69

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synthesized from precursor fatty acid C20:4*n*-6 during inflammatory activation [2, 3, 7].

Furthermore, correlation studies showed metabolic abnormalities between the decreased NEFA C18:2*n*-6 and SATS and/or MUFA NEFAs in PBMC membranes from patients. This could possibly have contributed to the positive correlations between SATS and MUFA NEFAs and disease outcome, specifically as measured by the Bowel and Bladder FSS. SATS and MUFAs are known to replace insufficient concentrations of PUFAs in cell membranes [28]. SATS and MUFA NEFAs have been shown in vitro and/or plasma to suppress immune cell function/proliferation [5, 6]. Changes in PBMC membrane NEFAs could therefore presumably contribute to an abnormal immune condition in patients with MS, thereby contributing to disease outcome. The underlying cause of MS remains unknown, but both an autoimmune and an infectious etiology are suspected, contributing to the disease etiology [29, 30] and relapses in patients with MS frequently follow bladder, gastrointestinal, or upper respiratory tract infections [31]. Miller et al. [32] and Hennessey et al. [33] reported the prevalence of bladder dysfunction in patients with MS as 78 and 82%, respectively, with infection an additional aggravator of symptoms [32]. In contrast to the positive relationships between NEFAs in PBMC membranes and disease outcome, SATS NEFA C22:0 and PUFA NEFA C18:2*n*-6 in RBCs from patients with MS showed inverse correlations with the FSS. These results are of interest because they highlighted the difference in membrane NEFA functions between these cells, and that it was immune cells that showed fatty acid metabolic abnormalities specifically.

In conclusion, results from this and previous studies showed that both esterified and non-esterified PUFAs in PBMC membranes from patients with MS are decreased as compared to that of control subjects, suggesting either insufficient dietary intake or a possible increased turnover to its elongation product, C20:4*n*-6. However, SATS and MUFA NEFAs, which are known to suppress immune cell function in plasma, showed positive correlations with the Bowel and Bladder FSS specifically, suggesting that suppression of immune cell function per se should be carefully considered in these patients. In contrast to these, both PUFA and SATS NEFAs in RBC membranes from patients with MS showed protective effects on disease outcome, highlighting the difference in membrane NEFAs functions between these cells.

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