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Plasma non-esterified fatty acids in patients with multiple sclerosis

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Abstract

Objective: The purpose of this study was to investigate the levels of non-esterified fatty acids in plasma from patients with multiple sclerosis and further to correlate these findings with the neurological profile as measured by the Kurtzke Expanded Disability Status Scale. Methods: Plasma non-esterified fatty acids and esterified fatty acids from 30 control subjects and 31 patients with multiple sclerosis were measured by gas chromatography.
Results: Non-esterified fatty acids C18:2n-6, C20:4n-6, C16:1n-7, C18:1n-7, C18:1n-9, C14:0, C16:0 and C18:0 were significantly increased in plasma from patients with multiple sclerosis, $P \leq 0.01$, while esterified fatty acid C18:2n-6 was decreased, $P = 0.003$. Fatty acid PC C16:1n-7 and non-esterified fatty acids C16:1n-7, C18:1n-7 and C18:1n-9 showed positive and fatty acids C18:1n-9, C20:0, C22:0 and C24:0 showed inverse correlations with the Functional System Scores.
Conclusions: We have identified increased monounsaturated non-esterified fatty acids in plasma from patients with multiple sclerosis as indicative of a worse disease outcome. Further, the decrease in fatty acid C18:2n-6, with increases in non-esterified fatty acids C18:2n-6 and C20:4n-6, suggested a role for these eicosanoid precursor fatty acids in the inflammatory condition experienced by these patients.

INTRODUCTION

Fatty acid metabolic abnormalities have been reported in brain tissue[1], plasma[2,3] and peripheral blood cells[4] in patients with multiple sclerosis (MS). However, reports on polyunsaturated fatty acid abnormalities in plasma/serum from patients with MS are inconsistent. Cherayil[5] reported reduced C18:2n-6 and C20:4n-6, Holman *et al*.[3] and Cunnane *et al*.[6] found the n-3 fatty acids decreased, while Cumings *et al*.[1] did not find any significant abnormalities in the blood fatty acid profile from patients with multiple sclerosis. Abnormalities in non-esterified fatty acid (NEFA) concentrations have also been reported in brain tissue from these patients.[7-9] No information is available on NEFA concentrations in plasma from these patients.

Plasma fatty acids may be esterified in the form of neutral triglycerides and phospholipids, or non-esterified (NEFAs). Fatty acids may be saturated or mono- and polyunsaturated. Polyunsaturated fatty acids are metabolized respectively from the essential parent fatty acids, C18:2n-6 (Linoleic acid) and C18:3n-3 (Alpha-linolenic acid), which cannot be synthesized in the body and which must be ingested from food.[10,11] In contrast, the body can synthesize both saturated and monounsaturated fatty acids from carbohydrates, if sufficient dietary fat is unavailable.[12,13] Furthermore, when essential fatty acids are unavailable, they will be replaced by the nonessential fatty acids, saturated and monounsaturated fatty acids[12], and in patients with MS, the subnormal concentrations of polyunsaturated fatty acids were reported to be compensated for by an increase in saturated and monounsaturated fatty acids in plasma.[3,5,6]

Non-esterified fatty acids may influence immune cell functions such as chemotaxis, phagocytosis and bactericidal ability.[14,15] However, NEFAs are toxic to surrounding cells[16] and increased NEFAs in plasma have been implicated in a number of diseases, such as sudden cardiac death, insulin resistance, atherosclerosis and

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hypertension.[17,18] The concentration and possible effects of NEFAs in plasma from patients with MS are not well documented; therefore the aim of this study was to determine the levels of NEFAs in plasma from patients with multiple sclerosis and to correlate with these with disease outcome as measured by the Kurtzke Expanded Disability Status Scale (EDSS).[19] Both the patients and control subjects were specifically screened to exclude the use of interferon, cortisone and/or fatty acid supplements. "Blood sampling was done early each day after an overnight fast to minimize the possible effects of diurnal variation on the plasma fatty acid profile."

METHODS

Ethics approval

Ethics approval for the study was obtained from the Health Sciences Research Ethics Committee (HSREC) of the Cape Peninsula University of Technology (CPUT). Patients with MS were contacted and recruited through the MS Society, Western Cape Branch, South Africa. Informed, written consent was obtained from all participants. The study was conducted according to the guiding principles of the Declaration of Helsinki.

Study population

Venous blood from 31 female patients and 30 age- race- and gender-matched control subjects was collected into EDTA tubes (Beckman Coulter, Cape Town, South Africa), immediately separated and stored at -80°C. The patients recruited were diagnosed by a neurologist based on clinical, laboratory and magnetic resonance imaging findings. The exclusion criteria used in this study included the use of fatty acid supplements, interferon and cortisone.

Measurement of the disability status of patients

The functional disability status of each patient was measured by a trained clinician using the Kurtzke Expanded Disability Status Scale (EDSS) and Functional System Scores (FSS).[19] The Functional Systems are Pyramidal, Cerebellar, Brainstem, Sensory, Bowel and bladder, Visual and Cerebral, in which higher values indicate greater disability. The scales for the EDSS is from 0 to 10, in which the 0 score indicates no disability and 10 indicates death due to MS.

Blood sample processing and analysis

Plasma NEFAs and phosphatidylcholine (PC) and sphingomyelin (SM) esterified fatty acids were measured by gas chromatography as previously described.[20,21] The plasma samples were extracted with 18 ml chloroform/methanol (CM) (ratio 2:1 v/v) according to a modified method of Folch *et al*.[20] All the extraction solvents contained 0.01 % butylated hydroxytoluene (BHT, Sigma-Aldrich, South Africa) as an antioxidant. Plasma samples were extracted and then resuspended in 80 ul CM for separation on thin layer chromatography (TLC). Individual phospholipid classes containing esterified fatty acids (PC and PE), as well as NEFAs were separated on pre-coated silica gel 60 plates (10 x 10 cm) using chloroform/ petroleum benzene/methanol/acetic acid/boric acid (ratio 40:30:20:10:1.8; v/v/v/v/w) as solvent [21]. The lipid bands were visualized with long wave ultraviolet light after spraying the plates with chloroform/methanol (ratio 1:1; v/v) containing 10 mg/100 ml BBOT (2,5-bis-(5´-tert-butylbenzoxazolyl-[2´])thiophene; Sigma Chemical Company, South Africa). These fractions were scraped off the TLC plates and transmethylated using 5 % sulfuric acid (H_2SO_4)/ methanol at 70 °C for 2 hours (SM; 18 hours). After cooling, the resulting fatty acid methyl esters (FAME) were extracted with 1 ml of distilled water and 2 ml of n-hexane. The top hexane layer was removed and evaporated to dryness, re-dissolved in carbon disulphide (CS_2) and analyzed by GC (Finnigan Focus GC, Thermo Electron Corporation, USA, equipped with flame ionization detection), using 30 meter BPX 70 capillary columns of 0.32 millimetre (mm) internal diameter (SGE International Pty Ltd, Australia). Gas flow rates were as follows: Nitrogen (make up gas), 25 ml/minute; air, 250 ml/minute; and hydrogen (carrier gas), 25 ml/minute and split ratio of 20:1. Temperature programming was linear at 5 °C/minute, initial temperature 140 °C, final temperature 220 °C, injector temperature 220 °C, and detector temperature 250 °C. The FAME were identified by comparison of the retention times to those of a standard FAME mixture (Nu-Chek-Prep Inc., Elysian, Minnesota), using an internal standard (C17:0, Sigma-Aldrich, South Africa). Fatty acids from both PC and SM phospholipids as well as NEFAs were quantified in µg per ml plasma. Esterified fatty acids and non-esterified fatty acids (NEFAs) with more than 20 % non-detectable values were not included in this study.

Statistical analysis

A statistics programme, STATISTICA (STATISTICA 7, StatSoft Inc 1984 – 2004) was used to perform statistical analyses. Because data was skewed, descriptive data are presented as median (quartile range). 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 fatty acids in plasma from patients with MS and control subjects: polyunsaturated fatty acids: P-value < 0.0071, polyunsaturated fatty acid NEFAs: P-value < 0.0125, monounsaturated fatty acid NEFAs: P-value < 0.0167, saturated fatty acid NEFAs: P-value < 0.0167 were considered as statistically significant.

RESULTS

Quantified fatty acids in plasma from patients with MS and control subjects are summarized in Table 1. SM C18:2n-6 was significantly decreased and NEFAs C18:2n-6, C20:4n-6, C16:1n-7, C18:1n-7, C18:1n-9, C14:0, C16:0, C18:0 were significantly increased in plasma from patients with MS after P-values were corrected for multiple testing by Bonferroni.

There were no significant correlations between plasma fatty acids and the EDSS or FSS after correction for multiple testing by Bonferroni, but monounsaturated fatty acids PC C16:1n-7, NEFAs C16:1n-7, C18:1n-7 and C18:1n-9 showed non-significant positive correlations with the Cerebellar FSS, while monounsaturated fatty acid SM C18:1n-9 showed an inverse correlation with the Sensory FSS. Saturated fatty acids SM C20:0, SM C22:0, SM C24:0 showed inverse correlations with the Brainstem FSS (Table 2).

DISCUSSION

Results from this study showed increases in non-esterified fatty acids (NEFAs), including C18:2n-6 and C20:4n-6 in plasma from patients with multiple sclerosis. Similarly, Chia *et al*.[8] reported increases in NEFA C20:4n-6 in diseased myelin, while Craelius *et al*.[7] reported decreases in NEFAs C20:4n-6 in diseased white matter. Similar to previous reports[5] results showed that in contrast to the increase in NEFAs, esterified C18:2n-6 and C20:4n-6 were decreased in plasma from patients. We hypothesise that the increased polyunsaturated fatty acids NEFAs in plasma from patients could have been sourced from esterified polyunsaturated fatty acids, possibly indicating an increased turnover for eicosanoid production. Polyunsaturated fatty acids are precursors for mediators of inflammation, the eicosanoids and MS is characterized by inflammation of the central nervous system. The n-6 polyunsaturated fatty acids s, C20:3n-6 and C20:4n-6, and the n-3 polyunsaturated fatty acids, C20:5n-3 and C20:6n-3, are precursors for cell-signalling eicosanoids, which, together with cytokines mediate the immune response.[10,22,23] Eicosanoids derived from the metabolism of the n-6 polyunsaturated fatty acids are inflammatory substances, while those derived from the metabolism of the n-3 polyunsaturated fatty acids show anti-inflammatory effects.[23-25] The production of eicosanoids, including that of prostaglandin E2 (PGE2), is markedly increased during inflammation. Because membrane phospholipids contain high concentrations of C20:4n-6, which is used as a major precursor for eicosanoid production[22,25], it has been implicated in brain pathologies such as multiple sclerosis.[26,27] Furthermore, an increase in phospholipase A2 activity, the enzyme which releases C20:4n-6 from membrane phospholipids, is also associated with neurological disorders such as multiple sclerosis.[28]

Non-esterified monounsaturated fatty acids were also increased in plasma from patients and furthermore showed positive correlations with the Functional System Scores (FSS). There is scarcity of literature regarding NEFAs in plasma from patients with MS, but increased plasma NEFAs are toxic to surrounding cells[16] and has been implicated in a number of diseases.[17,18] In contrast, saturated fatty acids SM C20:0, SM C22:0 and SM C24:0 showed inverse correlations with the Functional System Scores. Myelin phospholipids contain a high percentage of saturated and monounsaturated fatty acids[29] and in MS the lipid to protein ratio has been reported to be severely decreased, fully due to a decrease in lipids.[9,30] Esterified n-7 monounsaturated fatty acids showed a positive and n-9 monounsaturated fatty acids showed an inverse correlation with the FSS. Although the role of monounsaturated fatty acids in inflammation has not been as well defined as that of the n-6 and n-3 polyunsaturated fatty acids s[31] they may have specific anti-inflammatory effects.[31-32] Therefore these results suggested that the n-7 and n-9 monounsaturated fatty acids may have different functions in this process, similar to

Table 1: **Differences between esterified fatty acids and non-esterified fatty acids (µg/ml) in plasma from patients with multiple sclerosis and control subjects**

	Controls, N30	Patients with MS, N 31	P-value
	Median (quartile range)		
Polyunsaturated fatty acids			
PC C18:2n-6	218.0 (59.8)	202.2 (58.7)	0.30
PC C20:3n-6	38.3 (13.6)	33.3 (20.3)	0.47
PC C20:4n-6	103.5 (42.3)	99.2 (29.9)	0.06
PC C22:4n-6	3.47 (1.54)	3.43 (1.63)	0.48
SM C18:2n-6	1.77 (0.58)	1.21 (0.58)	**0.0006***
PC C20:5n-3	6.68 (5.06)	5.64 (4.08)	0.62
PC C22:6n-3	35.7 (17.1)	38.9 (26.2)	0.64
Monounsaturated fatty acids			
PC C16:1n-7	3.89 (1.40)	4.28 (2.63)	0.35
PC C18:1n-7	13.5 (3.9)	13.8 (5.6)	0.90
PC C18:1n-9	80.3 (24.3)	81.0 (28.3)	0.73
SM C18:1n-9	1.60 (0.92)	1.47 (0.63)	0.39
SM C24:1n-9	27.7 (8.2)	30.8 (12.0)	0.07
Saturated fatty acids			
PC C14:0	1.79 (1.14)	1.87 (1.53)	0.27
PC C16:0	247.0 (65.6)	254.9 (81.6)	0.82
PC C18:0	120.0 (31.3)	130.4 (48.6)	0.25
SM C16:0	31.4 (6.5)	33.4 (11.6)	0.35
SM C18:0	12.8 (5.1)	13.3 (4.2)	0.64
SM C20:0	7.19 (1.70)	7.93 (2.62)	0.14
SM C22:0	20.7 (6.0)	23.2 (7.0)	0.28
SM C24:0	17.0 (6.1)	19.0 (5.8)	0.24
Polyunsaturated non-esterified fatty acids			
NEFA C18:2n-6	12.9 (5.8)	19.6 (12.2)	**0.003***
NEFA C20:4n-6	0.82 (0.30)	0.92 (0.50)	**0.007***
NEFA C22:4n-6	0.70 (0.35)	0.64 (0.33)	0.97
NEFA C22:6n-3	0.60 (0.20)	0.72 (0.41)	0.10
Monounsaturated non-esterified fatty acids			
NEFA C16:1n-7	2.77 (1.71)	4.18 (2.62)	**0.0062***
NEFA C18:1n-7	1.37 (0.74)	1.99 (1.13)	**0.0059***
NEFA C18:1n-9	28.7 (14.7)	36.6 (23.9)	**0.011***
Saturated non-esterified fatty acids			
NEFA C14:0	1.04 (0.61)	1.47 (1.11)	**0.0025***
NEFA C16:0	21.6 (11.0)	28.1 (15.4)	**0.0033***
NEFA C18:0	9.50 (2.75)	11.5 (5.8)	**0.0012***

***Significant P-values: corrected for multiple testing by Bonferroni**

Table 2: Correlation between plasma fatty acids and non-esterified fatty acids, and the Kurtzke Expanded Disability Status Scale (EDSS) and Functional System Scores (FSS) in plasma from patients with multiple sclerosis (MS)

		Patients with MS, N31	
		R	P-value
Polyunsaturated fatty acids	**EDSS FSS**		
PC C20:3n-6	Cerebellar	0.33	0.07
PC C22:4n-6	Brainstem	-0.34	0.06
PC C20:5n-3	Brainstem	0.31	0.08
PC C22:6n-3	Brainstem	0.33	0.07
Monounsaturated fatty acids			
PC C16:1n-7	Cerebellar	**0.42**	**0.0181**
SM C18:1n-9	Sensory	**-0.37**	**0.039**
Saturated fatty acids			
PC C14:0	Cerebellar	0.33	0.07
SM C18:0	Cerebellar	0.33	0.07
SM C20:0	Brainstem	**-0.40**	**0.027**
SM C22:0	Brainstem	**-0.39**	**0.031**
SM C24:0	Brainstem	**-0.45**	**0.011**
Polyunsaturated non-esterified acids			
No correlations	No correlations	No correlations	No correlations
Monounsaturated non-esterified fatty acids			
NEFA C16:1n-7	Cerebellar	**0.41**	**0.020**
NEFA C18:1n-7	Cerebellar	**0.42**	**0.019**
NEFA C18:1n-9	Cerebellar	**0.37**	**0.042**
Saturated non-esterified fatty acids			
No correlations	No correlations	No correlations	No correlations

the different roles the n-6 and n-3 polyunsaturated fatty acids s play in inflammation.

The limitation of this study was that dietary fatty acid intake was not considered. Only female patients were used in this study, as the hormonal status may have had an effect on NEFA concentrations in plasma. Fatty acids do show diurnal fluctuations[33], thus, blood sampling was done early each day after an overnight fast to minimize the possible effects of diurnal variation on the plasma fatty acid profile. In addition, for each patient, a gender and age-matched control subject was recruited on the same day further minimizing differences in blood fatty acid profile due to factors other than that caused by the diseased state. In conclusion, results from this study showed a similar increase in NEFAs in plasma from patients with MS as has been reported in diseases such as sudden cardiac death, insulin resistance, atherosclerosis and hypertension. However, these finding need to be corroborated by further studies.

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DISCLOSURE

Conflict of interest: None

REFERENCES

1. Cumings J, Shortman RC, Skrbic T. Lipid studies in the blood and brain in multiple sclerosis and motor neurone disease. *J Clin Pathol* 1965; 18:641-4.
2. Baker RWR, Thompson RHS, Zilkha KJ. Serum fatty acids in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 1964;27:408-14.
3. Holman RT, Johnson SB, Kokmen E. Deficiencies of polyunsaturated fatty acids and replacement by nonessential fatty acids in plasma lipids in multiple sclerosis. *Proc Natl Acad Sci USA* 1989;86:4720-4.
4. Harbige LS, Sharief MK. Polyunsaturated fatty acids in the pathogenesis and treatment of multiple sclerosis. *Br J Nutr* 2007;98:S46-S53.
5. Cherayil GD. Sialic acid and fatty acid concentrations in lymphocytes, red blood cells and plasma from patients with multiple sclerosis. *J Neurol Sci* 1984;63:1-10.
6. Cunnane SC, Ho S-Y, Dore-Duffy P, Ells KR, Horrobin DF. Essential fatty acid and lipid profiles in plasma and erythrocytes in patients with multiple sclerosis. *Am J Clin Nutr* 1989;50:801-6.
7. Craelius W, Gurmankin RS, Rosenheck DM, Schaefer DC. Free fatty acid patterns in normal and multiple sclerosis white matter. *Acta Neurol Scand* 1981; 63:197-203.
8. Chia LS, Thompson JE and Moscarello MA. Alteration of lipid-phase behavior in multiple sclerosis myelin revealed by wide-angle x-ray diffraction. *Proc Natl Acad Sci USA* 1984;81:1871-4.
9. Wilson R, Tocher DR. Lipid and fatty acid composition is altered in plaque tissue from multiple sclerosis brain compared with normal brain white matter. *Lipids* 1991;26:9-15.
10. Horrobin DF, Manku MS. Clinical biochemistry of essential fatty acids. In: Horrobin DF, ed: Omega-6 essential fatty acids. Pathophysiology and roles in clinical medicine. New York: Alan R Liss Inc, 1990: 21-49
11. Zamaria N. Alteration of polyunsaturated fatty acid status and metabolism in health and disease. *Reprod Nutr Dev* 2004;44:273-82.
12. Horrobin DF. The phospholipid concept of psychiatric disorders and its relationship to the neurodevelopmental concept of schizophrenia. In: Peet M, Glen I, Horrobin DF, eds: Phospholipid spectrum disorder in psychiatry. Lancashire, UK: Marius Press, 1999: 3-16
13. German JB, Dillard CJ. Saturated fats: what dietary intake? *Am J Clin Nutr* 2001;80:550-9.
14. Hawley HP, Gordon GB. The effects of long chain free fatty acids on human neutrophil function and structure. *Lab Invest* 1976;34:216-22.
15. Gorjão R, Cury-Boaventura MF, De Lima TM, Curi R. Regulation of human lymphocyte proliferation by fatty acids. *Cell Biochem Funct* 2007;25:305-15.
16. Leaf A. Plasma nonesterified fatty acid concentration as a risk factor for sudden cardiac death. *Circulation* 2001;104:744-5.
17. Blaak EE. Fatty acid metabolism in obesity and type 2 diabetes mellitus. *Proc Nutr Soc* 2003;62:753-60.
18. Sarafidis PA, Bakris GL. Non-esterified fatty acids and blood pressure elevation: a mechanism for hypertension in subjects with obesity/insulin resistance? *J Hum Hypertens* 2007;21:12-9.
19. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an Expanded Disability Status Scale (EDSS). *Neurology* 1983;33:1444-52.
20. Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957;226:497-509.
21. Van Jaarsveld PJ, Smuts CM, Tichelaar HY, Kruger M, Benadé AJS. Effect of palm oil on plasma lipoprotein concentrations and plasma low-density lipoprotein composition in non-human primates. *Int J Food Sci Nutr* 2000;51:S21-S30.
22. Calder PC. Polyunsaturated fatty acids, inflammation, and immunity. *Lipids* 2001;36:1007-24.
23. Bagga D, Wang L, Farias-Eisner R, Glaspy JA, Reddy ST. Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. *Proc Natl Acad Sci USA* 2003;100:1751-6.
24. Haag M. Essential fatty acids and the brain. *Can J Psychiatry* 2003;48:195-203.
25. Calder PC. Immunomodulation by omega-3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids* 2007;77:327-35.
26. Hofman FM, Von Hanwehr RI, Dinarello CA, Mizel SB, Hinton D, Merrill JE. Immunoregulatory molecules and IL-2 receptors identified in multiple sclerosis brain. *J Immunol* 1986;136:3239-45.
27. Repovic P, Kaihong MI, Benveniste EN. Oncostatin M enhances the expression of prostaglandin E_2 and cyclooxygenase-2 in astrocytes: Synergy with interleukin-1β, tumor necrosis factor-α, and bacterial lipopolysaccharide. *Glia* 2003;42:433-46.
28. Farooqui AA, Horrocks LA, Farooqui T. Modulation of inflammation in brain: a matter of fat. *J Neurochem* 2007;101:577-99.
29. Manzoli FA, Stefoni S, Manzoli-Guidotti L, *et al*. The fatty acids of myelin phospholipids. *FEBS Lett* 1970;10:317-20.
30. Göpfert E, Pytlik S, Debuch H. 2′,3′ -Cyclic nucleotide 3′ -Phosphohydrolase and lipids of myelin from multiple sclerosis and normal brains. *J Neurochem* 1980;34:732-9.
31. Harwood JL, Yaqoob P. Nutritional and health aspects of olive oil. *Eur J Lipid Sci Technol* 2002;104:685-97.
32. Yaqoob P, Knapper JME, Webb DH, Williams E, Newsholme A, Calder PC. The effects of olive oil consumption on immune functions in middle-aged men. *Am J Clin Nutr* 1998;67:129-35.
33. Barter PJ, Carroll KF, Nestel PJ. Diurnal Fluctuations in Triglyceride, fatty acids and insulin during sucrose consumption and insulin infusion in man. *J Clin Invest* 1971;50:583-91.