ORIGINAL ARTICLE

In chronic fatigue syndrome, the decreased levels of omega-3 poly-unsaturated fatty acids are related to lowered serum zinc and defects in T cell activation

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Abstract

There is now evidence that major depression is accompanied by decreased levels of ω3 poly-unsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). There is a strong comorbidity between major depression and chronic fatigue syndrome (CFS). The present study has been carried out in order to examine PUFA levels in CFS. In twenty-two CFS patients and 12 normal controls we measured serum PUFA levels using gas chromatography and mass spectrometry. We found that CFS was accompanied by increased levels of ω6 PUFAs, i.e. linoleic acid and arachidonic acid (AA), and mono-unsaturated fatty acids (MUFAs), i.e. oleic acid. The EPA/AA and total ω3/ω6 ratios were significantly lower in CFS patients than in normal controls. The ω3/ω6 ratio was significantly and negatively correlated to the severity of illness and some items of the FibroFatigue scale, i.e. aches and pain, fatigue and failing memory. The severity of illness was significantly and positively correlated to linoleic and arachidonic acid, oleic acid, ω9 fatty acids and one of the saturated fatty acids, i.e. palmitic acid. In CFS subjects, we found significant positive correlations between the ω3/ω6 ratio and lowered serum zinc levels and the lowered mitogen-stimulated CD69 expression on CD3+, CD3+CD4+, and CD3+CD8+ T cells, which indicate defects in early T cell activation. The results of this study show that a decreased availability of ω3 PUFAs plays a role in the pathophysiology of CFS and is related to the immune pathophysiology of CFS. The results suggest that patients with CFS should respond favourably to treatment with – amongst other things – ω3 PUFAs, such as EPA and DHA.

Introduction

There is now evidence that a depletion in ω3 polyunsaturated fatty acids (PUFAs) takes part in the pathophysiology of major depression [1,2]. The main ω3 PUFAs, i.e. linolenic acid (LNA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to be reduced in depressed patients, either in serum phospholipids [1,3]; the red blood cell membrane [4–6]; or in adipose tissue [7,8]. Four out of six double blind clinical trials have reported therapeutical effects for ω3 PUFAs in the treatment of major depression [2]. Epidemiological studies have shown that a reduced dietary intake of fish oil, containing EPA and DHA, is related to an increased risk for depression, postpartum depression, and suicide [9,10].

The involvement of ω3 PUFAs in depression revolves around their role in modulating the metabolism of serotonin (5-HT) and the inflammatory response system, two systems which participate in the pathophysiology of depression [3]. Thus, lowered ω3 PUFA is accompanied by a decreased fluidity of cell membranes, which in turn can affect the release and reuptake of 5-HT as well as the expression of postsynaptic 5-HT receptors [3]. Since ω3 PUFAs have anti-inflammatory effects, whereas ω6 PUFAs are proinflammatory [11], lowered ω3 and/or increased ω6 levels predispose towards inflammatory responses, which are frequently observed in depression [11,12]. Moreover, one of the inflammatory markers of depression, i.e. lowered serum zinc, is significantly and positively related to ω3 PUFA levels and the ω3/ω6 ratio [3]. Since zinc is an important cofactor in the synthesis of the long-chain ω3 PUFAs, we have suggested that lowered zinc, which is a marker of inflammation in depression, is one of the causes of lowered EPA and DHA in that illness [3].

Chronic fatigue syndrome (CFS) shows a high degree of comorbidity with depression; many CFS patients develop depression during their illness, while fatigue is one of the key symptoms of major depression [13]. As depression, CFS is characterized by disorders in the immune system, the metabolism of 5-HT and by lowered serum zinc [13]. CFS is accompanied by an activation of the inflammatory response system (IRS) with impaired levels of circulating cytokines [14–16]; signs of chronic mild inflammation [17], defects in the early activation of T and natural killer cells (NKC) [18]; and a decreased NKC activity [19,20]. Disorders in the metabolism of 5-HT which may occur in both disorders are decreased 5-HT1A receptor density, significant alterations in 5-HT transporter gene promoter polymorphism, a decreased availability of tryptophan (the precursor of 5-HT) and an increased incidence of 5-HT antibodies [21–25].

Although the above suggests that ω3 PUFAs may play a role in CFS, there are only a few studies reporting PUFA levels in CFS. Liu et al. [26] found significantly lower DHA and AA (one of the ω6 PUFAs) in CFS patients, while the levels of oleic acid (one of the MUFAs or monounsaturated fatty acids) and palmitic acid (one of the saturated fatty acids) were increased in CFS patients. Warren et al. [27], on the other hand, found no differences in red blood cell membrane lipids between subjects with CFS and normal volunteers. Some studies reported beneficial effects for PUFAs in the post-viral fatigue syndrome and in CFS [28–30].

The present study has been performed in order to examine serum ω3, ω6, ω9 PUFAs, MUFAs and saturated fatty acids in CFS and to examine the relationships between the ω3 PUFA levels and immune dysfunctions in CFS.

Subjects and Methods

Subjects

Thirty-four subjects participated in the present study, 22 patients with CFS and 12 unrelated controls (staff or their family members). The patients were admitted to the M-Care4U Outpatient Clinics. We made the diagnosis of CFS by means of the Centers for Disease Control and Prevention (CDC) criteria [31]: a) the patient must have a severe chronic fatigue of six months or longer, while there is no other known medical condition which can explain the fatigue; and b) the patient must have four or more of the following symptoms: substantial impairment in short-term memory or concentration, sore throat, tender lymph nodes, muscle pain, multi-joint pain without swelling or redness, headache of a new type, pattern or severity, unrefreshing sleep, and post-exertional malaise lasting more than 24 hours. We have measured the severity of CFS and its symptoms by means of the FibroFatigue scale, i.e. the Fibromyalgia and Chronic Fatigue Syndrome Rating Scale [32,33]. This scale measures 12 items reminiscent for CFS (and fibromyalgia): pain, muscular tension, fatigue, concentration difficulties, failing memory, irritability, sadness, sleep disturbances, autonomic disturbances, irritable bowel, headache, and subjective experience of infection [34].

We have excluded:

a) subjects with life-time diagnosis of psychiatric DSM IV disorders, such as (bipolar) depression, anxiety disorders, schizophrenia, substance use disorders and organic mental disorders;
b) subjects who ever had been treated with neuroleptics, anticonvulsants or mood stabilizers;
c) subjects who had been taking psychotropic drugs during the last year prior to the studies;
d) subjects who ever had been treated with dehydroepiandrosterone, zinc, growth hormone, acclydine, or fatty acids including ω3 and ω6 fatty acids;
e) subjects with abnormal values for routine blood tests, such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), calcium, creatinine, electrolytes, thyroid stimulating hormone (TSH), total protein, and iron or transferrin saturation;
f) subjects with other medical illness than CFS, e.g. diabetes, inflammatory bowel disease, essential hypertension, and arteriosclerosis;

g) subjects with acute infectious or allergic reactions for at least 2 months prior to the study;
h) subjects on a low fat diet or cholesterol-lowering drugs;
i) subjects with a a body mass index outside the normal limits; j) heavy smokers (> 15 cigarettes daily); and
k) subjects not consuming the normal Belgian diet (the mean P S ratio of a Belgian diet is 0.54 ± 0.43).

Patients and controls gave written informed consent after the study protocol was fully explained. The study has been approved by the local ethical committee.

Methods

Fasting blood was sampled during the morning hours for the determination of serum PUFAs, serum zinc, the serum alpha-2 protein fraction and CD69 expression on T lymphocytes (CD3+), T helper (CD3+CD4+), and T suppressor (C3+CD8+) cells. Plasma free fatty acids were determined by means of gas chromatography and mass spectrometry (laboratory Ategis, Waver, Belgium) [34]. We have determined the concentrations in mg/L of the following free fatty acids:

1) polyunsaturated fatty acids (PUFAs); the latter are further divided in the omega-3 PUFAs, e.g. LNA (α-linolenic acid; C18:3ω3), EPA (eicosapentaenoic acid, C20:5n-3); and DHA (docosahexaenoic acid, C22:6n-3); omega-6 PUFAs, e.g. LA (linoleic acid, C18:2ω6); GLA (gamma-linolenic acid; C18:3ω6) and AA (arachidonic acid, C20:4ω6); and omega-9 PUFAs;
2) saturated fatty acids (SFAs), e.g. myristic acid (C14:0); palmitic acid (C:16:0) and stearic acid (C18:0);
3) monounsaturated fatty acis (MUFAs), such as palmitoleic acid (C16:1) and oleic-elaidic (C18:1).

Serum zinc levels were determined by means of an atomic absorption method (PerkinElmer Analyst 200, Brussels, Belgium). The analytical inter-assay CV value of our assay was <10%. The serum alpha-2 protein fraction was measured by means of electrophoresis (SEBIA Benelux, Raketstraat, Brussels). The inter-assay CV value was < 2.0%. The CD69 expression on the CD3+, CD3+CD4+, and CD3+CD8+T cells after mitogen stimulation of whole blood was measured by means of the BD FastImmune™ assay system. This method monitors the expression of the early activation marker, CD69, in whole blood after stimulation with mitogenic stimuli [18]. Blood samples for the CD69 assays were collected in lithium heparinized tubes. 200 µl of whole blood was stimulated with pokeweed mitogen (PWM) 10 µg/ml (Sigma) at a final concentration of 5 µg/ml. Mitogen solutions were prepared in RPMI 1640 medium with L–glutamin (300 mg/ml, Gibko). All samples, including 200 µl unstimulated blood were incubated 18 hours in humidified atmosphere at 37 °C with 5% CO2. Fifty microliters of stimulated and unstimulated samples were triple stained with the immunofluorescent mABs (FastImmune Becton Dickinson), specific for CD3, CD4, CD8 and CD69. All samples were proceeded on the multi Q-prep workstation (Coulter) and analyzed on a Coulter Epics XL flow cytometer using a tree color immunofluorescence staining protocol. The analysis determined the percent of cells expressing CD3+CD69+, CD3+CD4+CD69+ and CD3+CD8+CD69+.

Statistics

Relationships between variables were assessed by means of Pearson's product moment or by means of automatic multiple regression analysis. The diagnostic performance of the PUFAs for CFS was checked by means of the ROC (receiver operating characteristics) analysis with computation of the area under the ROC curve (AUC), sensitivity, specificity and predictive value of a positive test result (PV+) and with kappa statistics [35]. Group mean differences were examined by means of analysis of variance (ANOVA) or covariance (ANCOVA) and by means of linear discriminant analysis. The significance was set at α=0.05 (two tailed) except for the contrasts between the fatty acids. Since we examined 5 subclasses of fatty acids (ω3, ω6 and ω9 PUFAs, MUFAs and saturated fatty acids) and since the fatty acids in each subclass are highly correlated, we used a p-correction, i.e. all separate fatty acid levels were tested at p=0.01. The ratios EPA/AA and total ω3/ω6 were tested at p=0.025.

Results

Table 1 shows the measurements of the fatty acid levels in CFS patients and in normal controls. We found that CFS patients had significantly higher levels of oleic acid, LA and AA than normal controls. The ω3/ω6 ratio and the EPA/AA ratio were significantly lower in CFS patients than in normal controls. The above differences between both study groups remained significant after covarying for age and gender. There were no significant correlations between any of the fatty acids and age. There were no significant differences in any of the fatty acids between males and females. The ratios and some of the fatty acids showed a good diagnostic performance for CFS: the AUCs were highly significant for oleic acid (82.2%), LA (88.8%), AA (82.4%), the ω3/ω6 ratio (87.5%) and the EPA/AA ratio (79.7%). For example, at a cut-off value for LA > 65 mg/l we found a sensitivity of 81.8%, specificity=100%, PV+=100% (κ=0.76, t=6.76, $p<10^{-3}$). At a cut-off value for the ω3/ω6 ratio < 0.15, we found a sensitivity=72.7%, specificity=91.7%, and PV+=94.0% (κ=0.59, t=4.24, $p<10^{-3}$). By means of linear discriminant analysis (an automatic stepdown method) with CFS and controls as study groups and the fatty acids as discriminatory variables, we found that LA and palmitic acid were the best discriminatory

variables between both study groups (Wilks λ=0.,61, χ^2=15.3, df=2, p=0.0004).

The severity of the FibroFatigue scale was significantly and positively correlated to serum palmitic acid (r=0.45, p=0.02), oleic acid (r=0.44, p=0.03), LA (r=057, p=0.005) and AA (r=0.43, p=0.038) and the ω9 fatty acids (r=0.46, p=0.02). There was a significant negative correlation between the severity of illness and the ω3/ω6 ratio (r=–0.46, p=0.027). There were also significant and negative correlations between the ω3/ω6 ratio and the following symptoms of the FibroFatigue scale: aches and pain (r=–0.44, p=0.03), fatigue (r=–0.49, p=0.01) and failing memory (r=–0.49, p=0.01). We examined the symptom profiles of the increased oleic acid, LA and AA levels in CFS by means of automatic multiple regression analysis (with a p-to-enter of p=0.05) whereby the fatty acids were entered as dependent variables and the symptoms as explanatory variables. Up to 51.5% of the variance (F=10.7, df=2/20, p=0.0009) in oleic acid could be explained by irritability (F=11.7, p=0.003) and failing memory (F=10.0, p=0.005). Up to 55.5% of the variance in the LA levels (F=12.5, df=2/22, p=0.0005) could be explained by the regression on muscular tension (F=7.9, p=0.01) and failing memory (F=8.8, p=0.008). Up to 26.7% of the variance in the AA levels (F=7.6, df=1/21, p=0.01) could be explained by the regression on failing memory. All symptoms were always positively loaded in these regressions.

In order to examine the relationships between the decreases in the ω3 PUFAs and the immune-inflammatory variables, we examined the correlations between the EPA/AA and the ω3/ω6 ratios, on the one hand, and serum zinc, the alpha–2 protein fraction and the PWM-stimulated CD69 expression within the CD3+, CD3+CD4+, and CD3+CD8+ cells, on the other. By means of ANOVA we found significantly (F=16.6, df=1/18, p=0.0009) lower serum zinc in CFS (mean ± SD=71.7 ± 5.5 μg/dL) than in normal controls (86.0 ± 9.3 μg/dL) and significantly higher (F=7.4, df=1/23, p=0.01) serum α2-protein fraction in CFS (8.9 ± 1.8%) than in normal controls (7.1 ± 1.1%). By means of ANOVAs, we found a significantly lower expression of CD69+ in CFS than in normal controls within the CD3+ (mean ± SD= 2.80 ± 1.45% versus 7.02 ± 5.41%, F=6.2, df=1/17, p=0.02); CD3+CD4+ (1.33 ± 0.90% versus 2.47 ± 1.36%, F=4.5, df=1/17, p=0.04); and CD3+CD8+ cells (0.22 ± 0.18% versus 1.07 ± 0.75%, F=13.6, df=1/17, p=0.002).

We found significant and positive correlations between serum zinc and the ω3/ω6 ratio (r=0.56, p=0.009); the PWM–induced CD69+ expression on the CD3+ (r=0.61, p=0.005), CD3+CD4+ (r=0.46, p=0.04), and CD3+CD8+ (r=0.54, p=0.01) cells. There were no significant correlations between the ω3/ω6 ratio and the alpha–2 protein fraction (r=–0.04, p= 0.8). There were significant and positive correlations between the EPA/AA ratio and the PWM-induced CD69 expression on the CD3+ (r=0.50, p=0.03), CD3+CD4+ (r=0.67, p=0.002), and CD3+CD8+ (r=0.51, p=0.02) cells. There were no significant correlations between the EPA/AA ratio and serum zinc (r=0.31, p=0.2) or the alpha-2 fraction (r=0.03, p=0.9).

Discussion

The main findings of the present study are that the ω3/ω6 and EPA/AA ratios are significantly reduced in patients with CFS and that the latter show increased levels of ω6 PUFAs, e.g. LA and AA, and MUFAs, i.e. oleic acid.

The findings of the present study only in part concur with those of Liu et al. [26] who reported lower DHA and increased oleic and palmitic acid in CFS. These authors, however, found lower AA in CFS. No significant changes in PUFA contents were detected by Warren et al. [27] in the red cell membrane of CFS patients. In the present study we report that a lowered ω3 status is related to the key symptoms of CFS, i.e. aches and pain, fatigue and failing memory, and that the increases in the ω6 PUFAs, MUFAs and saturated fatty acids are positively related to CFS symptoms, such as irritability, failing memory, and muscular tension. Phrased differently, changes in the relationships between the ω3 PUFAs, on the one hand, and ω6 PUFAs, MUFAs and saturated fatty acids, on the other, appear to play a role in the pathophysiology of CFS and to determine the severity of some key symptoms of CFS.

Previously, Behan et al. [28] have shown that a preparation of fatty acids containing linoleic acid + GLA + EPA + DHA may provide an effective treatment for the post–viral fatigue syndrome. Up to 85% of the fatigue patients had a clinically significant improvement of their symptoms. However, using the current research criteria for CFS, Warren et al. [27] found no significant treatment effects of Efamol Marine, which contains linoleic acid: 58%, GLA: 7.2%, EPA: 3.6%, and DHA: 2.4%. We think that the contradictory results as well as the lack of a therapeutic response in Warren's study can be attributed to the high contents of ω6 PUFAs in Efamol Marine. Indeed, as shown in the present study there is a highly specific decrease in EPA/AA and the ω3/ω6 ratios and a highly significant increase in LA in CFS patients. Puri et al. [30] found that patients with CFS when treated solely with a high-EPA containing supplement showed an improvement in their symptomatology within eight to 12 weeks. Given this, we may hypothesize that the most appropriate treatment of CFS would consist of – amongst other things – ω3 PUFAs without any addition of ω6 PUFAs, MUFAs or saturated fatty acids.

The second main finding of this study revolves around the relationships between the ω3 PUFAs and the immune-inflammatory response in CFS. Firstly, we found a significant positive correlation between the ω3/ω6 ratio and serum zinc. These findings are in agreement with those of a previous report showing significant correlations between serum zinc and ω3 PUFAs in major depression [3]. In both studies, serum zinc has been used to study the relationships between

Table 1. Measurements of the fatty acids in patient with chronic fatigue syndrome (CFS) and the control subjects.

Fatty acids	mean (± SD) controls in mg/L	mean (± SD) CFS in mg/L	F	p
LNA	1.74 (0.16)	2.35 (1.45)	2.0	0.2
EPA	4.25 (0.73)	4.88 (1.77)	1.4	0.2
DHA	6.94 (2.32)	9.77 (4.23)	4.5	0.04
LA	52.8 (6.8)	100.5 (39.3)	17.1	0.0004
GLA	3.13 (0.42)	5.32 (2.85)	6.9	0.012
AA	9.54 (3.61)	17.9 (9.6)	8.4	0.007
myristic	2.73 (1.06)	5.93 (4.13)	6.8	0.012
palmitic	72.4 (15.4)	110.2 (47.2)	7.2	0.011
stearic	27.8 (8.5)	40.4 (19.1)	4.7	0.04
palmitoleic	5.89 (2.54)	12.0 (7.7)	7.0	0.014
oleic	53.9 (11.4)	96.7 (42.8)	11.4	0.002
w9	4.85 (0.49)	6.97 (2.98)	5.9	0.02
w3/w6	0.198 (0.030)	0.144 (0.036)	19.2	0.0003
EPA/AA	0.523 (0.256)	0.332 (0.207)	5.6	0.02

This Table shows the measurements of three families of fatty acids:

1. Polyunsaturated fatty acids (PUFAs); the latter are further divided in the omega-3 PUFAs, e.g. LNA (-linolenic acid; C18:3ω3), EPA (eicosapentaenoic acid, C20:5n-3); and DHA (Docosahexaenoic acid, C22:6n-3); omega-6 PUFAs, e.g. LA (linoleic acid, C18:2ω6); GLA (gamma-linolenic acid; C18:3ω6) and AA (arachidonic acid, C20:4ω6); and omega-9 PUFAs.
2. Saturated fatty acids (SFAs), e.g. myristic acid (C14:0); palmitic acid (C:16:0) and stearic acid (C18:0).
3. Monounsaturated fatty acis (MUFAs), such as palmitoleic acid (C16:1) and oleic-elaidic (C18:1).

All results of ANOVAs (df=1/32).

the inflammatory response and serum PUFAs. Firstly, zinc plays an important role in the desaturase enzymes, which are responsible for the conversion of the essential precursors to the longer chain PUFAs [36]. Secondly, decreases in serum zinc in depression and CFS are hallmarks for the inflammation in those disorders [3]. Indeed, lowered serum zinc is secondary to sequestration of the intracellular heavy metal binding protein metallothionein in the liver, which is induced by an increased activity of interleukin-1 (IL−1) and IL-6 [37,38]. By interference, lowered serum zinc in CFS, which is an indicator of the inflammatory response, could – in part – explain the depletion of the long chain ω3 PUFAs. However, the results show that in CFS, but not depression, increases in ω6 PUFAs, MUFAs and saturated fatty acids play a role as well.

Of course, it is also possible that lowered ω3 PUFAs levels in CFS (and depression) are in part induced by other inflammatory-related mechanisms, such as increases in oxidative stress. There is now evidence for severe oxidative stress and oxidative damage to fatty acids in CFS [13]. As in depression, increased oxidative stress may have caused damage to ω3 PUFAs, thus decreasing the levels of the long chain PUFAs [4]. It is well known that cytokines and reactive oxygen species released by activated neutrophils and monocytes during inflammation may increase lipid peroxiation [39].

To make the picture even more complex we should add that ω3 PUFAs have anti-inlammatory effects, whereas ω6 PUFAs such as LA and AA, but not GLA, have pro−inflammatory effects. There are now preclinical and clinical studies showing that ω3 PUFAs have anti-inflammatory effects [40,41]. Dietary ω3 PUFAs may reduce the production of pro-inflammatory cytokines, e.g. IL−1, IL-6 and tumor necrosis factor-α (TNFα), whilst ω6 PUFAs are precursors of the pro-inflammatory prostaglandins and stimulate the production of the pro-inflammatory cytokines, e.g. TNFα [42–44]. By inference, the lowered ω3 PUFA status in CFS – which is partly caused by inflammation through lowered zinc and oxidative stress – could further increase the inflammatory response in CFS.

Another major finding of this study is that there was a significant correlation between decreased ω3 PUFAs and the expression of the CD69 molecule on activated T cells. Since the latter indicates a defect in the early activation of T cells [18], our results suggest that ω3 PUFAs may be involved in the immune disorders in CFS. Since on T cells, the expression and function of CD69 is dependent on the activation of PKC [45], we have suggested that a defect in PKC activation may underlie the decreased expression of the CD69 molecule in CFS. There are now results that ω3 PUFAs affect PKC translocation and activity. However, there are not enough data to support the hypothesis that the decreased ω3 PUFA status in CFS may have affected PKC-dependent CD69 expression. Indeed, ω3 PUFA have been shown to attenuate T-cell-mediated inflammation, in part, by suppressing T-cell activation and proliferation [46], whilst EPA and DHA induce immunosuppression in

part through PKC-dependent pathways [47]. There is more evidence to hypothesize that the defects in early T cell activation in CFS are related to the inflammatory response (lowered zinc and increased oxidative stress; [17]), which is partly induced by a lowered ω3 status. The results suggest that multiple and complex intertwined relationships between ω3 PUFAs, serum zinc and the inflammatory response system participate in the pathophysiology of CFS and depression. Thus, the similarities in pathophysiology between both CFS and depression may explain the strong comorbidity between both disorders.

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