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ORIGINAL ARTICLE

Attenuation of autoimmune responses to oxidative specific epitopes, but not nitroso-adducts, is associated with a better clinical outcome in Myalgic Encephalomyelitis/chronic fatigue syndrome

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Abstract

OBJECTIVES: There is evidence that inflammatory, oxidative and nitrosative stress (IO&NS) pathways participate in the pathophysiology of a subgroup of patients with Myalgic Encephalomyelitis/chronic fatigue syndrome (ME/CFS). Increased IgM-related autoimmune responses to oxidative specific epitopes (OSEs), including malondialdehyde (MDA), oleic acid and phosphatidyl inositol (Pi), and nitroso-(NO)-adducts, including NO-tryptophan (NOW), NO-arginine and NO-cysteinyl, are frequently observed in ME/CFS. Autoimmune responses in ME/CFS may be driven by increased bacterial translocation as measured by IgM and IgA responses to LPS of gram negative bacteria. **METHODS:** The aim of this study is to examine whether IgM responses to OSEs and NO-adducts are related to a better outcome as measured by the Fibromyalgia and Fatigue Rating Scale (FF). 76 ME/CFS patients with initially abnormal autoimmune responses were treated with care-as-usual, including nutraceuticals with anti-IO&NS effects (NAIOS), such as L-carnitine, coenzyme Q10, taurine + lipoic acid, with or without curcumine + quercitine or N-acetyl-cysteine, zinc + glutamine. **RESULTS:** We found that use of these NAIOS was associated with highly significant reductions in initially increased IgM-mediated autoimmune responses to OSEs and NO-adducts. A greater reduction in autoimmune responses to OSEs during intake of these NAIOS was associated with a lower FF score. Reductions in IgM responses to oleic acid, MDA and Pi, but not in any of the NO-adducts, were associated with reductions in severity of illness. These associations remained significant after adjusting for possible effects of increased bacterial translocation (leaky gut). **CONCLUSIONS:** Our results show that autoimmune responses to OSEs are involved in the pathophysiology of ME/CFS and that these pathways are a new drug target in a subgroup of ME/CFS patients. Although hypernitrosylation and nitrosative stress play a role in ME/CFS, reductions in these pathways are not associated with lowered severity of illness. Randomized controlled trials with NAIOS should be carried out in the subgroup of ME/CFS patients with initially increased autoimmune responses to OSEs.

INTRODUCTION

There is now evidence that subgroups of patients with Myalgic Encephalomyelitis / Chronic Fatigue Syndrome (ME/CFS) suffer from a neuro-immune disorder characterized by chronic mild activation of immune-inflammatory, oxidative and nitrosative stress (IO&NS) pathways (Maes & Twisk 2010). We have reviewed that ME/CFS is accompanied by an increased production of pro-inflammatory cytokines, including tumor necrosis factor (TNF)α and interleukin-(IL)1β, acute phase proteins, lowered antioxidant defenses, e.g. coenzyme Q10 and zinc; signs of O&NS, including damage to lipids, proteins and DNA; mitochondrial dysfunctions; increased translocation of gram-negative bacteria; dysfunctions in cell signaling transduction pathways; and specific brain disorders as assessed by different brain imaging techniques indicating central hypometabolism and neuroinflammation (Maes & Twisk 2010; Morris & Maes 2013a; 2013b; 2014; Morris *et al.* 2013b; Nakatomi *et al.* 2014).

Increased levels of thiobarbituric acid reactive substances, isoprostane, protein carbonyl levels and 8-OH-deoxyguanosine were found indicating damage by O&NS to fatty acids, proteins and DNA (Vecchiet *et al.* 2003; Kennedy *et al.* 2005; Smirnova & Pall 2003; Maes *et al.* 2009b). Jammes *et al.* (2005) reported that O&NS pathways may be a causal factor in chronic fatigue. Also, animal models of chronic fatigue show that O&NS are involved in its pathophysiology. In rodent models of chronic fatigue, e.g. administration of LPS or the forced swimming test, increases in O&NS, lowered antioxidant defences and O&NS damage, especially lipid peroxidation, are observed in the periphery and brain (Singal *et al.* 2005; Singh *et al.* 2002a; 2002b; Sachdeva *et al.* 2009).

ME/CFS is accompanied by other specific disorders in IO&NS pathways, i.e. increased IgM-mediated autoimmune responses directed against oxidative specific epitopes (OSEs) (Maes *et al.* 2006; 2007a; 2012a). For example, the IgM-mediated responses directed against malondialdehyde (MDA) and azelaic acid, conjugated phosphatidyl inositol (Pi) and oleic acid, and anchorage molecules, including palmitic and myristic acid and S-farnesyl-L-cysteine, are significantly higher in patients with ME/CFS than in healthy controls (Maes *et al.* 2006; 2012a). These IgM-mediated autoimmune responses to immunogenic OSEs are probably the consequence of oxidative damage or breakdown of membrane components (e.g. anchorage molecules, MDA, azelaic acid) or intracellular structures (Maes *et al.* 2006; 2007a; 2012a; Morris *et al.* 2014a).

ME/CFS is also characterized by significantly increased IgM responses directed against nitroso-(NO)-adducts, including NO-tryptophan (NOW), NO-arginine, NO-cysteinyl, etc (Maes *et al.* 2006; 2012a). The IgM-mediated responses to conjugated NO-adducts indicate autoimmune responses to nitrosylated molecules (nitrosyl or nitroso-derivatives) following hypernitrosylation and nitrosative damage (Bodet *et al.* 2004; Maes *et al.* 2006; Moylan *et al.* 2014). All in all, these results not only indicate that there is O&NS damage to lipids and proteins, but also that IgM-mediated autoimmune responses are mounted against OSEs and NO-adducts. These responses may result in beneficial natural autoimmune responses, which aim to remove damaged or apoptotic cells, or detrimental effects, including neurotoxicity or demyelination, e.g. increased IgM responses to NO-cysteinyl (Boullerne *et al.* 1995; 1996; 2002; Moylan *et al.* 2014).

There is also evidence that ME/CFS is characterized by increased bacterial translocation as indicated by increased IgA/IgM responses directed to lipopolysaccharides (LPS) of gram negative, commensal gut bacteria (Maes *et al.* 2007b; 2012b; 2013). There results indicate that the integrity of the gut wall or the tight junctions barrier has been jeopardized allowing gram negative gut bacteria to translocate from the gut into the mesenteric lymph nodes or the systemic blood (Maes *et al.* 2007b; 2012b). This process may cause peripheral activation of systemic IO&NS pathways and neuroinflammation and autoimmune responses as well (Maes *et al.* 2012b; 2013; Garate *et al.* 2011; 2013). For example, in ME/CFS significant associations are found between indices of bacterial translocation and inflammatory biomarkers and increased autoimmunity to serotonin (Maes *et al.* 2013).

There are however no data whether these IgM-mediated autoimmune responses to OSEs and NO-adducts are responsive to treatment with antioxidants or are associated with the clinical outcome of ME/CFS. The present study has been carried out in order to examine the relationships between the autoimmune responses to OSEs and NO-adducts and the clinical variables in ME/CFS.

SUBJECTS AND METHODS

Subjects

In this study, 76 ME/CFS patients participated who were admitted to the Maes Outpatient Clinics, Belgium. The ME/CFS subjects were selected on the basis of increased (> 2 SD) IgM responses to 3 OSEs (MDA, Pi, oleic acid) or 3 NO-adducts (NOW, NO-arginine, NO-cysteinyl). The diagnosis ME/CFS was made using CDC criteria (Fukuda *et al.* 1994). Exclusion criteria for patients were: major medical illness, including epilepsia, inflammatory bowel disease, diabetes; a life-time diagnosis of axis I DSM IV-TR disorders, e.g. psychotic disorders, substance use disorders, organic mental disorders, bipolar depression; neuroinflammatory disorders, e.g. Parkinson's and Alzheimer's disorder, stroke and multiple sclerosis; abnormal routine blood tests including alanine aminotransferase, alkaline phosphatase, blood urea nitrogen, creatinine, and basal thyroid stimulating hormone. Patients gave written informed consent after the study was explained. The study was approved by the local IRB.

The Fibromyalgia and Chronic Fatigue Syndrome Rating Scale (FF scale) was used to measure severity of ME/CFS (Zachrisson *et al.* 2002). This FF scale contains 12 ME/CFS items, i.e. 1. pain, 2. muscular tension, 3. fatigue, 4. concentration difficulties, 5. failing memory, 6. irritability, 7. sadness, 8. sleep disturbances, 9. autonomic disturbances, 10. irritable bowel, 11. headache and 12. subjective experience of infection. The total sum of these 12 items was used as an index for severity of illness.

Methods

We measured FF values, the IgM responses to 3 OSEs and 3 NO-adducts and IgA/IgM responses to LPS in ME/CFS patients who had initially increased FF (FF>20) values and IgM responses to OSEs or NO-adducts, defined as any of the 6 IgM values to OSEs or NO-adducts >2 SDs. Patients participated irrespective of care-as-usual, i.e. treatments initiated before admission to our policlinic and continued thereafter, e.g. cognitive behavioural treatment (CBT, n=13), graded exercise therapy (GET, n<5), selective serotonin (and noradrenaline) reuptake inhibitors (SSRIs/SNRIs, n=17), vitamin or antioxidant supplements, etc.) and treatments initiated by our policlinic, e.g. emotional freedom therapy (EFT, n=9), neurofeedback (n<5), cranio-electro stimulation (n<5), exclusion diets (n=19) and nutraceuticals with anti-IO&NS effects (NAIOS). All patients took a combination of NAIOS, i.e. L-carnitine, coenzyme Q10, taurine + lipoic acid, with or without curcumine + quercitine or N-acetyl-cysteine, zinc + glutamine. These combined approaches were continued for 14.3 (±4.2) months (mean ± SD) when blood was sampled and the FF scores measured. In this non-interventional study we did not target to examine the effects of a specific treatment or combinatorial treatments, but rather the relationships between the IgM responses to 3 OSEs (oleic acid, Pi and MDA) and to 3 NO-adducts (NOW, NO-arginine, NO-cysteinyl) at baseline (called "baseline") or some months later (called "endpoint").

In baseline and endpoint conditions fasting blood was collected during the morning hours and analysed for serum IgM directed against 3 OSEs and 3 NO-adducts and IgA/IgM directed against the LPS of 6 different commensal bacteria, i.e. Hafnei Alvei, Pseudomanes Aeruginosa, Morganella Morganii, Pseudomanus Putida, Citrobacter Koseri and Klebsiella Pneumoniae. The analyses were performed as explained before (Maes *et al.* 2006, 2007b; 2012a; 2012b). Briefly, we employed an indirect ELISA method performed according to the methods outlined by Gemacbio (The Ultimate Biopharmaceuticals, France). Each serum sample was measured in duplicate and tested simultaneously with three standard solutions. The optical densities (OD) of the three standards are expressed as Z values and from this the reference linear curve is calculated as Z = f(OD) with Z = a OD + b. Thus, the Z value of the lowest standard can be negative. This curve allows to deduce the mean values of the duplicate measurements of the OD values. The biological interassay CV values were <10%. In order to have a measure of IgM autoimmune responses against OSEs or NO-adducts we made the sum of the response values of the 3 OSEs and 3 NO-adducts, respectively. In order to have an index of autoimmunity directed against all neoepitopes (OSEs and NO-adducts) we made the sum of the 6 OD values, i.e. oleic acid, Pi, MDA, NOW, NO-arginine and NO-cysteinyl. In order to assess the "total LPS translocation load" we have employed the total sum of the 6 IgM and 6 IgA levels (either at baseline or at endpoint).

Statistics

Differences between group means were assessed with analysis of variance (ANOVA). Associations between classification systems was checked using analyses of contingency Tables (χ^2-test). In order to assess correlations between two variables we used Pearson's product moment correlation coefficients. Automatic stepwise binary logistic regression analysis with the dichotomized endpoint FF value as dependent variable and O&NS biomarkers, duration of illness and treatment, etc., as explanatory variables was used to assess the best prediction of a good response. Multivariate repeated measurement (RM) general linear model (GLM) analyses were used to examine the differences between baseline and endpoint values. We used univariate and multivariate GLM analyses to examine the associations between endpoint clinical status and different explanatory variables, including O&NS biomarkers, duration of illness and treatment, age, gender, etc. Levene's test was employed to check for homogeneity of variance. All tests were two-tailed and a p-value of 0.05 was used for statistical significance.

RESULTS

Table 1 shows the results of univariate tests, i.e ANOVAs and analysis of contingency Tables (without p-correction for multiple testing), examining the differences between individuals with lower and higher endpoint FF values (dichotomized as FF<12). Table 1 shows that there are no significant differences in age, gender, duration of illness or treatment, and any of the baseline or endpoint O&NS or LPS biomarkers between both groups. Individuals with a higher endpoint FF value had higher baseline FF values. There were significant positive correlations between baseline IgM responses to OSEs and NO-adducts: r=0.613, p<0.001; endpoint OSEs and NO-adducts: r=0.593, p<0.001; and the residual changes in OSEs and NO-adducts (the residual values were obtained by regression of the endpoint on baseline values): r=0.504, p<0.001.

Table 2 shows the results of two multivariate RM GLM analyses examining the effects of treatment (time) and the interaction time by FF status (low versus high endpoint FF values) on the O&NS biomarkers.

Tab. 1. Socio-demographic and oxidative and nitrosative stress data of the ME/CFS patients dichotomized into two groups according to the endpoint values of the Fibromyalgia and Fatigue (FF) Rating Scale.

Variables		Endpoint FF<12	Endpoint FF≥12	F or X^2	df	*p*-value
Age (years)		40.7 (±16.1)	40.6 (±10.4)	0.00	1 / 74	0.96
Gender (male / female)		9 / 29	4 / 34	2.32	1	0.128
duration of illness (years)		5.9 (±7.6)	8.8 (±5.8)	3.68	1 / 74	0.059
duration of treatment (months)		15.2 (±4.8)	13.5 (±3.5)	3.16	1 / 74	0.080
Baseline FF		35.9 (±7.7)	41.6 (±7.8)	10.50	1 / 74	0.002
IgM / Oleic acid (SDs)	Basal	1.69 (±2.14)	1.69 (±1.63)	0.00	1/ 68	0.999
	End	0.95 (±1.45)	1.92 (±2.12)	3.69	1 / 51	0.060
IgM / MDA (SDs)	Basal	1.75 (±2.40)	2.33 (±2.67)	0.89	1 / 68	0.348
	End	0.82 (±1.58)	1.81 (±2.80)	2.50	1 / 51	0.120
IgM / Pi (SDs)	Basal	2.01 (±2.55)	1.88 (±2.16)	0.05	1 / 68	0.818
	End	0.80 (±1.58)	1.82 (±2.41)	3.30	1 / 51	0.075
IgM / sum 3 OSEs (SDs)	Basal	5.46 (±6.21)	5.90 (±5.27)	0.10	1 / 68	0.751
	End	2.57 (±4.10)	5.55 (±6.67)	3.80	1 / 51	0.057
IgM / NO-Cysteinyl (SDs)	Basal	1.72 (±2.55)	1.98 (±2.68)	0.16	1 / 68	0.689
	End	0.98 (±2.10)	1.96 (±2.45)	2.45	1 / 51	0.123
IgM / NO-Arginine (SDs)	Basal	1.55 (±1.85)	1.81 (±2.54)	0.23	1 / 68	0.630
	End	0.93 (±1.63)	1.93 (±2.68)	2.64	1 / 51	0.111
IgM / NOW (SDs)	Basal	1.52 (±1.69)	2.00 (±2.26)	1.02	1 / 68	0.316
	End	1.04 (±1.86)	1.93 (±2.41)	2.26	1 / 51	0.139
IgM / sum 3 NO-adducts (SDs)	Basal	4.79 (±5.52)	5.78 (±6.99)	0.43	1 / 68	0.513
	End	2.95 (±5.39)	5.82 (±6.88)	2.84	1 / 51	0.098
IgA / sum LPS (SDs)	Basal	3.07 (±11.58)	5.76 (±15.51)	0.12	1 / 69	0.725
	End	0.31 (±6.80)	3.35 (±9.54)	3.58	1 / 58	0.064
IgM / sum LPS (SDs)	Basal	14.26 (±11.08)	14.98 (±12.33)	1.06	1 / 69	0.306
	End	4.77 (±7.69)	9.36 (±10.63)	1.97	1 / 58	0.166

Results are shown as mean (±SD); Basal: measurements at baseline; End: at endpoint; MDA: malondialdehyde; Pi: phosphatidyl inositol; NO: nitroso-adducts; NOW: NO-tryptophan; Sum 3 OSEs: sum of OD values of the autoimmune responses to oleic acid, MDA and Pi; Sum 3 NO-adducts: sum of OD values of the autoimmune responses to NOW, NO-arginine and NO-cysteinyl; IgA / sum LPS (SD s): sum of OD values of the IgA responses to the LPS of 6 gram negative bacteria; IgM / sum LPS (Sds): sum of OD values of the IgM responses to the LPS of 6 gram negative bacteria.

The multivariate tests showed no significant differences between the groups, but significant effects of time and time X FF status. The consequent univariate analyses showed significant effects on time decreasing (see Table 1 for mean values) the autoimmune responses to oleic acid, MDA, Pi, NOW, NO-arginine and NO-cysteinyl. Univariate anlyses also showed significant interaction effects of time X FF status on oleic acid and Pi. IgM-mediated autoimmune responses to both oleic acid and Pi were significantly suppressed from baseline to endpoint in FF responders (FF<12), while no differences in these values could be found in subjects with higher endpoint FF values (see Table 1 for mean values).

Table 2 shows the results of multivariate GLM analyses with the autoimmune responses to the sums of the 3 OSEs and 3 NO-adducts as dependent variables. There were significant suppressant effects of treatments on both the 3 OSEs and 3 NO-adducts (see Table 1 for values) and significant interaction effects of time X FF status on the 3 OSEs, but not the NO-adducts (although there was a trend towards significance). Table 1 shows that treatment significantly suppressed the sum of the 3 OSE values in patients with a lower endpoint FF value but not in those with higher values.

Table 3 shows the results of GLM analysis with the endpoint FF values as the dependent variable and age, gender, duration of illness, duration of treatment, and the actual changes in the 6 O&NS biomarkers as explanatory variables. The actual changes in the auto-

Tab. 2. Results of two multivariate repeated measurements GLM analyses examining the effects of treatment (time) and the interaction time by responder status (FF status) on oxidative and nitrosative stress (O&NS) biomarkers.

Tests	Effect	variables	F	df	*p*-value
Between-subject multivariate tests	FF status	All 6 O&NS markers	0.40	6 / 46	0.878
Within-subject multivariate tests	time	All 6 O&NS markers	4.24	6 / 46	0.002
	time X FF status	All 6 O&NS markers	2.72	6 / 46	0.024
Univariate tests	time	**IgM / Oleic acid**	9.03	1 / 51	**0.004**
		IgM / MDA	13.12	1 / 51	**0.001**
		IgM / Pi	20.28	1 / 51	**<0.001**
		IgM / NO-Cysteinyl	10.03	1 / 51	**0.003**
		IgM / NO-Arginine	6.99	1 / 51	**0.011**
		IgM / NOW	6.19	1 / 51	**0.016**
	time X FF status	**IgM / Oleic acid**	6.44	1 / 51	**0.014**
		IgM / MDA	0.81	1 / 51	0.372
		IgM / Pi	10.29	1 / 51	**0.002**
		IgM / NO-Cysteinyl	3.30	1 / 51	0.075
		IgM / NO-Arginine	3.40	1 / 51	0.071
		IgM / NOW	0.99	1 / 51	0.323
Between-subject multivariate tests	FF status	sums 3 OSEs and 3 NO-adducts	0.54	1 / 50	0.585
Within-subject multivariate tests	time	**sums 3 OSEs and 3**	11.36	2 / 50	<0.001
	time X FF status	**NO-adducts**	3.49	2 / 50	0.038
Univariate tests	time	**sum 3 OSEs**	22.35	1 / 51	**<0.001**
		sum 3 NO-adducts	10.91	1 / 51	**0.002**
	time X FF status	**sum 3 OSEs**	6.85	1 / 51	**0.012**
		sum 3 NS markers	3.39	1 / 51	0.071

MDA: malondialdehyde; Pi: phosphatidyl inositol; NO: nitroso-adducts; NOW: NO-tryptophan; Sum 3 OSEs: sum of OD values of the autoimmune responses to oleic acid, MDA and Pi; Sum 3 NO-adducts: sum of OD values of the autoimmune responses to NOW, NO-arginine and NO-cysteinyl; All 6 O&NS markers: sum of OD values of the autoimmune responses to oleic acid, MDA, Pi, NOW, NO-arginine and NO-cysteinyl.

Tab. 3. Results of general linear model (GLM) analysis with endpoint values of the Fibro-Fatigue (FF) Rating Scale as dependent variable and age, gender, duration of illness, duration of treatment, and the actual changes in the oxidative and nitrosative stress markers from baseline to post-treatment.

Explanatory variables	B	SE	F	df	*p*-value	partial eta squared
Model	-	-	10.58	3 / 49	<0.001	0.393
Duration of illness	0.44	0.17	6.40	1 / 49	0.015	0.116
Duration of treatment	-0.76	0.26	8.69	1 / 49	0.005	0.151
residual IgM / MDA	2.55	0.56	21.00	1 / 49	<0.001	0.300

Residual IgM / MDA: residual IgM values to MDA obtained by the regression of endpoint on baseline values.

immune responses to the 6 O&NS biomarkers were computed as the residual values obtained by regression of the endpoint on the baseline values. We found that duration of illness and treatment and the residual MDA values were significantly and positively associated with endpoint FF values.

Table 4 shows the results of a similar GLM analysis with the endpoint FF values as dependent variable and basal FF values, age, gender, duration of illness, duration of treatment, and the residual changes in the 6 O&NS biomarkers as explanatory variables. We found that 49.8% of the variance in endpoint FF values was

explained by the baseline FF values and the residual changes in autoimmune responses to oleic acid.

Table 5 shows the results of an automatic binary logistic regression analysis with the patient group with higher endpoint FF values as dependent variable and basal FF values, age, gender, duration of illness, duration of treatment, and the actual changes in the 6 O&NS biomarkers as explanatory variables. We found that increased endpoint FF values were significantly predicted by duration of treatment and the residual Pi responses (X^2=17.07, df=2, p<0.001, Nagelkerke=0.367).

Table 6 shows the results of multivariate GLM analysis with the endpoint FF score and the residualized FF values (obtained by regression of the endpoint on baseline values) as dependent variables using the following explanatory variables: age, duration of treatment and illness, and the residual responses from baseline to endpoint in IgM responses directed against the sum of the 3 OSEs and 3 NO-adducts, and the IgA/IgM responses to LPS load as explanatory variables. Multivariate analyses showed that only the residualized sum of the 3 OSEs was significantly associated with the dependent variables. Univariate analyses showed that both the endpoint FF and residual FF values were significantly related to the residualized OSE responses. There were no correlations with the residualized sum of the 3 NO-adduct values. Also, forced entry of the LPS data did not change these results, i.e. the effects of the residual OSE values remained significant (F=11.22, df=2/31, p<0.001), while neither the residual IgM responses directed to LPS (F=0.00, df=2/31, p=0.99) nor the residual IgA responses directed to LPS (F=1.56, df=2/31, p=0.226) were significant.

Finally, we have examined the effects of other treatments on the endpoint status and O&NS biomarkers.

Tab. 4. Results of general linear model (GLM) analysis with the endpoint Fibro-Fatigue (FF) Rating Scale as dependent variable and basal FF values, age, gender, duration of illness, duration of treatment, and the actual changes in the oxidative and nitrosative stress markers from baseline to post-treatment as explanatory variables.

Explanatory variables	B	SE	F	df	*p*-value	partial eta squared
Model			24.84	2 / 50	<0.001	0.498
FF basal	22.43	5.32	21.33	1 / 50	<0.001	0.299
residual IgM / oleic acid	2.98	0.64	22.26	1 / 50	<0.001	0.308

Residual IgM / oleic acid: residual IgM values to oleic acid obtained by the regression of endpoint on baseline values.

Tab. 5. Results of binary logistic regression analysis with the patient group with higher post-treatment Fibromyalgia and Fatigue (FF) Rating Scale values as dependent variable and the group with lower FF values as reference group.

Significant explanatory variables	B	SE	Wald	df	*p*-value	Odds ratio	95% CI
duration of treatment	–1.97	0.08	5.84	1	0.016	0.82	0.70–0.96
residual IgM / Pi	0.81	0.28	8.58	1	0.003	2.24	1.31–3.85

Tab. 6. Results of multivariate GLM analysis with the endpoint scores on the Fibromyalgia and Fatigue (FF) scale and the residualized FF values from baseline to endpoint as dependent variables and age, duration of treatment and illness, and the residual responses from baseline to post-treatment in IgM responses to oxidative stress specific epitopes (OSEs), nitrosative stress specific epitopes (NSEs), IgA responses to lipopolysaccharides (LPS) and IgM resposnses to LPS as explanatory variables.

Analyses	Dependent variables	Explanatory variables	F	df	*p*-value
Multivariate	Endpoint FF and Residual FF	Age	1.45	2 / 29	0.251
		Duration treatment	0.96	2 / 29	0.393
		Duration of illness	0.83	2 / 29	0.447
		Residual sum IgM OSEs	7.59	2 / 29	**0.002**
		Residual sum IgM NO-adducts	0.09	2 / 29	0.907
		Residual sum IgA LPS	0.07	2 / 29	0.929
		Residual sum IgM LPS	0.12	2 / 29	0.312
Between-subjecteffects	Endpoint FF	Residual sum IgM OSEs	15.59	1 / 30	**<0.001**
	Residual FF	Residual sum IgM OSEs	10.92	1 / 30	**0.002**

Forced entry of the use of SSRIs/SNRIs in a multivariate GLM analysis showed that the effects of the residualized OSEs values on the endpoint FF and residual FF values remained significant (F=6.62, df=2/47, p=0.003) and that there was no significant effect of antidepressant use (F=0.17, df=2/47, p=0.843). Similar negative results were obtained for CBT, EFT and exclusion diets. Multivariate GLM analysis with the sums of the baseline and endpoint OSEs and NO-adducts as 4 dependent variables showed no significant effects of use of SSRIs/SNRIs, CBT, EFT or exclusion diets.

DISCUSSION

The first major finding of this study is that initially increased IgM-mediated autoimmune responses to all OSEs and NO-adducts were highly significantly attenuated some months later. Since there were no significant effects of other treatments on these levels, including SSRIs/SNRIs, different types of psychotherapy or diets, we conclude that these effects are probably related to the use of high dose NAIOS. Indeed, it is well known that NAIOS, including coenzyme Q10, taurine and lipoic acid, have anti-IO&NS effects and that the complementary use of curcumine, quercitine, N-acetyl-cysteine, zinc or glutamine may further attenuate initially increased IO&NS responses (Maes & Leunis 2008; Maes *et al.* 2011; https://www.consumerlab.com). In addition, this combination of NAIOS also improves mitochondrial functions, which are other drug targets in ME/CFS (Morris & Maes 2013a; 2014; Morris *et al.* 2013a). Interestingly, in this study there was no effect of antidepressants attenuating the autoimmune responses although antidepressants have anti-inflammatory and maybe antioxidative effects (Maes *et al.* 1999; Wang *et al.* 2013). This is, however, a non-interventional study using NAIOS, which (in contrast to studies with antidepressants) could not be standardized. Thus, it is well known that antioxidant quality, bio-availability and even content may vary greatly by brand (https://www.consumerlab.com). For example, little coenzyme Q10 may be found in some supplement brands, while some supplements with curcumine and lipoic acid contain less active compound than listed on the labels (https://www.consumerlab.com). Thus, differences in the quality, content and bioavailability of the NAIOS used by the patients certainly induced bias in our results. Nevertheless, our data show that there was a significant overall effect of NAIOS regardless of this bias and the use of the other care-as-usual treatments. All in all, the effects observed here may be explained by NAIOS reducing IO&NS pathways and thus the ensuing IgM-mediated autoimmune responses directed against O&NS formed neoepitopes.

The second major finding of this study is that the IgM-mediated autoimmune responses to the 3 OSEs, but not the NO-adducts, were significantly and positively associated with the endpoint FF scores or the actual changes in the FF scores from baseline to endpoint. Thus the higher the autoimmune responses to OSEs, the higher the endpoint FF scores. This may indicate that a significant reduction in the formation of OSEs is associated with a better clinical outcome in some patients with ME/CFS and therefore that increased oxidative stress is a key component in the pathophysiology of a subgroup of ME/CFS patients. These findings extent previous research results that oxidative processes are associated with ME/CFS, as indicated by oxidative damage to lipids, DNA and proteins in humans (Vecchiet *et al.* 2003; Kennedy *et al.* 2005; Smirnova & Pall 2003; Maes *et al.* 2009b; Jammes *et al.* 2005) and in animal models (Singal *et al.* 2005; Singh *et al.* 2002a; 2002b; Sachdeva *et al.* 2009). It should be underscored, however, that around 30–40% of patients with ME/CFS show moderately to highly increased OSEs or NO-adduct responses, suggesting that other subgroups of ME/CFS patients may show another pathophysiology.

Previously, we have shown that decreased levels of antioxidants, including coenzyme Q10, zinc and glutathione-related pathways, may play a role in the pathophysiology of ME/CFS (Maes *et al.* 2009a; Maes & Twisk 2010; Morris & Maes 2014; Morris *et al.* 2013a; 2014b). Lowered levels of antioxidants attenuate antioxidant defences and thus enhance O&NS processes (Morris & Maes 2013a; 2013b; 2014; Brucknerova *et al.* 2013). Based on our results it can tentatively be hypothesized that treatment with NAIOS targeting IO&NS pathways, including autoimmune responses to OSEs, lowered antioxidant defences and mitochondrial functions, may be associated with a better clinical outcome. These findings extent those of different animal models of CFS showing that treatment with NAIOS, including curcumine and quercitine, may reverse chronic fatigue-like behaviours (Lin *et al.* 2014; Sachdeva *et al.* 2009; 2010; 2011; Moriya *et al.* 2011; Gupta *et al.* 2009; 2010). In humans with fibromyalgia it has been shown that treatment with one of the NAIOS used here, i.e. coenzyme Q10, may improve chronic fatigue scores and specific symptoms (Miyamae *et al.* 2013; Cordero *et al.* 2012). These NAIOS not only downregulate O&NS pathways but also activated immune-inflammatory pathways, which are known to induce physio-somatic symptoms, including fatigue and pain, and depression and neurocognitive defects (Maes *et al.* 2011; Morris & Maes 2013a; Kontoangelos *et al.* 2014).

Although increased IgM-mediated autoimmune responses to NO-adducts are frequently associated with ME/CFS (Maes *et al.* 2006; 2012a), we did not find any association between these autoimmune responses and clinical outcome measurements. Increased IgM responses to NO-adducts indicate that ME/CFS may be associated with the detrimental effects of hypernitrosylation and synthesis of neurotoxic antibodies, which may induce dysfunctions in many key intracellular processes (Moylan *et al.* 2014). Also, in this and other studies we found that the IgM responses to OSEs and NO

adducts were significantly inter-correlated, suggesting that oxidative and nitrosative pathways are partly interconnected, for example via lowered antioxidant levels, immune-inflammatory pathways and common intracellular activation pathways (Moylan et a., 2014). Taken together, these results could be interpreted to indicate that targeting autoimmunity to OSEs is more important than targeting NO-adducts. However, although we selected patients who showed initial increases in the IgM responses to OSEs or NO-adducts, post-hoc inspection of our data shows that the autoimmune disorders in OSEs were more pronounced that those in NO-adducts and that the suppressant effects of NAIOS on the autoimmune responses to OSEs were more pronounced than their effects on NO-adducts. Therefore, future research should examine a) the effects of NAIOS in two subgroups of patients, i.e. those with initially increased autoimmunity against OSEs versus those with increased autoimmune responses to NO-adducts; and b) delineate other drugs/NAIOS, which more specifically target NO-adducts.

Since increased bacterial translocation may drive immune-inflammatory and O&NS pathways, including autoimmunity in ME/CFS and since treatment with specific NAIOS (N-acetyl cysteine, zinc and glutamine) may attenuate initial increased bacterial translocation in those patients (Maes & Leunis 2008) we have adjusted our data for possible effects of bacterial translocation. Forced entry of the IgA/IgM responses to LPS did not affect the previously discussed results. Thus, although leaky gut may drive autoimmune responses to OSEs, the effects of NAIOS on the autoimmune responses directed against OSEs may not be explained via possible effects on bacterial translocation.

In summary, NAIOS-induced decreases in oxidative stress and in autoimmune responses to OSEs may be associated with a better outcome in ME/CFS suggesting that these processes are involved in the pathophysiolgy of ME/CFS. These results indicate that autoimmunity against OSEs is a new drug target in a subgroup of ME/CFS patients and that randomized controlled trials with NAIOS are needed in these patients and in those with increased autoimmunity against NO-adducts.

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