CSF isoprostane levels are a biomarker of oxidative stress in multiple sclerosis

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ABSTRACT

Objective: To investigate the potential of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) as a biomarker for disease activity and oxidative stress in the CSF of patients with multiple sclerosis (MS).

Methods: The isoprostane 8-iso-$PGF_{2\alpha}$ is an established biomarker for in vivo oxidative stress and lipid peroxidation. We measured CSF 8-iso$PGF_{2\alpha}$ levels in 231 patients with MS (74 with relapsing-remitting MS, 67 with primary progressive MS, and 90 with secondary progressive MS [SPMS]) and 40 controls using a competition ELISA.

Results: We found increased CSF levels of 8-iso-$PGF_{2\alpha}$ in patients with MS compared to controls, with the most striking values in a subgroup of patients with SPMS. Furthermore, the increase in 8-iso-$PGF_{2\alpha}$ correlated with other parameters of lipid peroxidation as well as with a decrease in the total antioxidant status in the MS CSF samples.

Conclusions: Our study demonstrates that CSF levels of 8-iso-$PGF_{2\alpha}$ may serve as a biomarker of oxidative stress in MS. Further investigation will help establish the pathologic and clinical significance of our preliminary findings. ***Neurol Neuroimmunol Neuroinflammation* 2014;1:e21; doi: 10.1212/NXI.0000000000000021**

GLOSSARY

8-iso-$PGF_{2\alpha}$ = 8-iso-prostaglandin $F_{2\alpha}$; **AAPH** = 2,2′-azobis-2-methyl-propanimidamide dihydrochloride; **DMF** = dimethyl fumarate; **EAE** = experimental allergic encephalitis; **EDSS** = Expanded Disability Status Scale; **GSSG** = oxidized glutathione; **IMSMP** = International Multiple Sclerosis Management Practice; **IRB** = institutional review board; **MDA** = malondialdehyde; **MS** = multiple sclerosis; **OND** = other neurologic disorder; **PPMS** = primary progressive MS; **ROS** = reactive oxygen species; **RRMS** = relapsing-remitting MS; **SOD** = superoxide dismutase; **SPMS** = secondary progressive MS; **TAS** = total antioxidant status; **TBARS** = thiobarbituric acid reactive substances.

Multiple sclerosis (MS) is a disease of unknown cause with multiple factors implicated in its pathophysiology.[1–3] There is growing evidence of the involvement of oxidative stress in neural damage in MS.[3–6] Because of the high lipid content in neural tissue, oxidative stress, with its associated increased free radical production, leads to lipid peroxidation.[7] At present there is no established biomarker for investigating lipid peroxidation and oxidative stress in MS.

Isoprostanes are a class of lipid peroxidation products that are generated when free radicals attack the arachidonic acid esterified in phospholipid pools of cell membranes.[7–11] 8-Iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is one of the most abundant and well-recognized isoprostanes and is now recognized as a "gold-standard biomarker for in vivo oxidative stress and lipid peroxidation."[10–13]

Oxidative stress may contribute to the disease mechanisms in both the relapsing-remitting and progressive phases of MS through its involvement in inflammation and axonal degeneration, respectively. Increased lipid peroxidation in MS leads to an increase in the markers of oxidative stress, but there is also a depletion of the antioxidant reserves as reported in a study of patients with MS.[14] There have also been some reports of evaluation of the relative production of 8-iso-$PGF_{2\alpha}$ in biological fluids, mostly in serum and urine in patients with MS.[15–20] However, there have been no studies to determine levels of isoprostanes in the CSF of patients with MS that also

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investigate the association with other indicators of oxidative stress (including antioxidant levels) and correlate with disease severity or progression.

The present study aims to evaluate 8-iso-$PGF_{2\alpha}$ concentrations in the CSF of patients with MS as a marker for oxidative stress and to investigate its association with other oxidative stress parameters.

METHODS

Standard protocol approvals, registrations, and patient consents. All CSF and plasma samples were obtained with informed consent and institutional review board (IRB) approval from patient volunteers at the International Multiple Sclerosis Management Practice (IMSMP). All 231 patients with MS included in the study had clinically definite MS as assessed by board-certified neurologists at the IMSMP.[21] Patient selection was a passive rather than an active process, meaning that the first 231 patients whose CSF samples were serially collected for analysis at the start of the study were included in the study. Controls included 24 normal healthy volunteers and 16 patients with other neurologic disorders (ONDs). Although the controls were not perfectly age-matched to the MS subgroups, the mean age for each subgroup was within ±6 years of the mean age for the control group. All patients with MS were defined as having relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), or primary progressive MS (PPMS), and they were further characterized as having active or stable disease. Active disease was defined by the presence of any one of the following criteria in the 6 months preceding CSF sample collection: (1) one or more relapses documented by neurologist examination; (2) change of 0.5 points or greater in Expanded Disability Status Scale (EDSS) score; and (3) change in MRI, specifically a change in the number or size of lesions or the presence of gadolinium-enhancing lesions.

CSF collection. CSF samples were obtained by standard lumbar puncture or via aspiration of the access port of surgically implanted baclofen pumps with informed consent and IRB approval. CSF was collected from a total of 74 patients with RRMS, 67 patients with PPMS, 90 patients with SPMS, and 40 controls (table). The controls consisted of 24 normal healthy controls and 16 patients with ONDs as follows: 6 patients with CNS inflammatory diseases, 3 with spinal cord injury, 2 with transverse myelitis, 2 with spinal stenosis, and 1 patient each with human T lymphotropic virus type I–associated myelopathy, sarcoidosis, and stiff person syndrome. Serum samples were also collected from 39 patients with MS as a subset for comparison.

CSF was examined to ensure absence of red blood cell contamination and centrifuged at 200*g* for 15 minutes to remove the cells. Aliquots of CSF were analyzed immediately or stored at −80°C until use.

All the CSF analysis was carried out by researchers blinded to the disease status of the patient.

8-iso-$PGF_{2\alpha}$ assay by ELISA. 8-iso-$PGF_{2\alpha}$ levels in the CSF samples were determined using a specific competitive EIA kit from Cayman Chemical (Ann Arbor, MI) as per the vendor's instructions. Briefly, 50 μL of each sample was assayed in duplicate and each assay was repeated at least twice. We also analyzed the respective serum samples of 39 patients with MS for comparison of 8-iso-$PGF_{2\alpha}$ levels between the serum and CSF.

Thiobarbituric acid reactive substances assay. Lipid peroxidation in the CSF samples was also measured using the thiobarbituric acid reactive substances (TBARS) assay from Cayman Chemical, which measures the formation of malondialdehyde (MDA) from the decomposition of the unstable peroxides derived from polyunsaturated fatty acids. For this assay CSF was concentrated 4 times by overnight lyophilization. Samples were reconstituted in ultrapure water. MDA was assayed in 100 μL of each CSF sample by a fluorescence assay as per the manufacturer's protocol.

Oxidized glutathione. Oxidized glutathione (GSSG) was monitored in the 4 times concentrated CSF samples using a fluorescent assay from BioVision, Inc. (Milpitas, CA).

Superoxide dismutase. Superoxide dismutase (SOD) levels in the CSF samples were analyzed using the colorimetric assay from Cayman Chemical in 10 μL of undiluted CSF samples in duplicate wells. Each assay was repeated at least twice.

Total antioxidant status. The combined nonenzymatic antioxidant capacity—total antioxidant status [TAS]—of CSF samples (concentrated 4 times) was determined using the TAS assay kit from Abcam (Cambridge, UK) using Trilox as a standard as per the manufacturer's protocol.

Experimental allergic encephalitis induction. All animal experiments were approved by the St. Luke's Roosevelt Hospital Center Institutional Animal Care and Use Committee. Experimental allergic encephalitis (EAE) was induced in six 8-week-old female wild-type C57BL/6 mice using MOG35-55 peptide as described previously.[22,23] Mice were weighed and evaluated for neurologic disability daily by a blinded scorer. Disability was scored using a 0–13 EAE scale as previously described.[23,24] Mice were sacrificed at disease peak, i.e., on day 15, and total brain lysates were analyzed for 8-iso-$PGF_{2\alpha}$ levels. Data analysis is representative of 3 individual experiments.

Table Patient demographics

	Control	RRMS	SPMS	PPMS	Comments
Average age, y	46.5	43.18	52.8	51.4	RRMS vs SPMS/PPMS: $p < 0.001$
Female/male	27/13	58/16	66/24	44/63	RRMS vs PPMS: $p < 0.001$
Disease duration, y	0	12.8	13.5	18.9	RRMS vs SPMS: $p < 0.001$
EDSS	0	2	6.7	7.6	RRMS vs SPMS/PPMS: $p < 0.001$
N	40	74	90	67	

Abbreviations: EDSS = Expanded Disability Status Scale; PPMS = primary progressive multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive MS.

Oxidative stress in cell culture. The glial cell CG4 was grown in culture. The cells were subjected to oxidative stress by adding hydrogen peroxide (H_2O_2) or the reactive oxygen generator 2,2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH). The 8-iso-$PGF_{2\alpha}$ levels were assayed in the culture medium after 24 hours. The reactive oxygen species (ROS) scavenger EUK134 was added to quench the ROS increase after oxidant addition.

Statistical analysis. GraphPad Prism 5 was used for statistical analysis. Statistical significance was set at p values <0.05. Differences between disease groups in CSF were analyzed by analysis of variance.

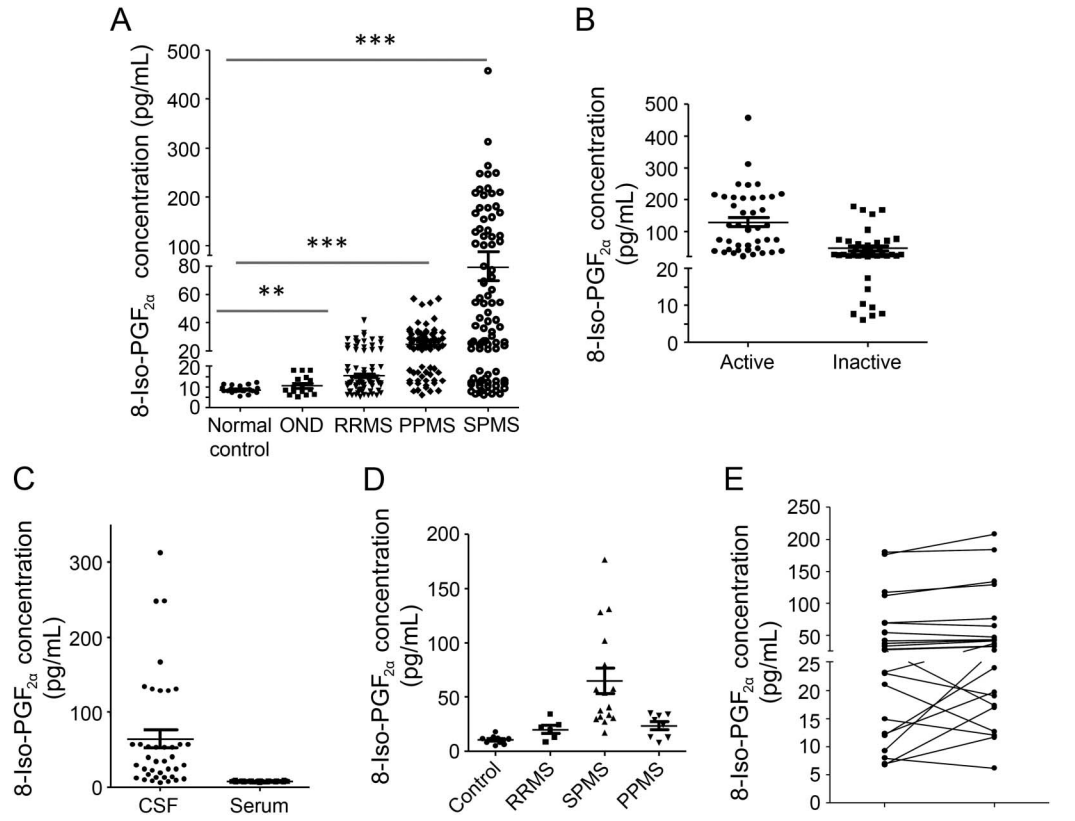
RESULTS CSF levels of 8-iso-$PGF_{2\alpha}$ are specifically elevated in MS. 8-Iso-$PGF_{2\alpha}$ levels in the CSF were measured in 231 patients with MS and 40 controls (table) using a competitive ELISA. As a group, the mean value of 8-iso-$PGF_{2\alpha}$ levels in the CSF of the patients with MS (43 pg/mL; RRMS: 15.5 ± 7.9, PPMS: 25 ± 11.8, SPMS: 79 ± 86.9) was higher (p value <0.0001) than the mean value of healthy control samples (8.7 ± 1.6 pg/mL) and the OND control group (10.6 ± 4.5 pg/mL). The range of 8-iso-$PGF_{2\alpha}$ CSF levels varied greatly among patients with MS, with several patients with RRMS, SPMS, and PPMS having levels greater than 20 pg/mL, a level not exceeded by any control (figure 1A). As a group, patients with progressive disease had higher values than those with RRMS, and only patients with SPMS had values greater than 100 pg/mL. This difference, however, is compounded by the inherent differences between the 2 groups in terms of age, disease duration and severity (table), and disease-modifying therapies.

To determine whether the elevated levels in patients with SPMS correlated with disease activity, we analyzed CSF levels of 8-iso-$PGF_{2\alpha}$ and found a strong correlation with active disease, as depicted in figure 1B ($n = 41$ and $p < 0.0001$).

The increase in CSF levels of 8-iso-$PGF_{2\alpha}$ appeared to be CNS-specific, as no correlation was seen in 39 patients with MS who had CSF and serum samples analyzed simultaneously (figure 1C).

To determine whether CSF 8-iso-$PGF_{2\alpha}$ level elevations were a secondary effect of treatment, we analyzed 31 untreated patients with MS (6 RRMS, 17 SPMS, and 8 PPMS) and 10 controls and found

Figure 1 8-Iso-$PGF_{2\alpha}$ levels in CSF of patients with MS and controls



CSF 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) levels were estimated using an ELISA. (A) 8-Iso-$PGF_{2\alpha}$ levels in the multiple sclerosis (MS) subgroups compared to normal healthy controls and other neurologic disease controls. (B) Isoprostane levels as a function of disease activity in active vs inactive patients with secondary progressive MS. (C) Comparison of 8-iso-$PGF_{2\alpha}$ levels in CSF with serum from the patients with MS. (D) Levels of 8-iso-$PGF_{2\alpha}$ in CSF from untreated patients with MS. (E) Changes in 8-iso-$PGF_{2\alpha}$ levels over an 18-month period in a group of 23 patients with MS. Groups were statistically compared using GraphPad Prism 5.0. * indicates statistical significance with a p value of <0.05.

results comparable to our initial cohort (figure 1D). This analysis suggests that our findings were not a treatment-induced phenomenon.

A longitudinal analysis of the repeat samples collected from 23 patients over a period of a year or more was also done. As can be seen in figure 1E, 8-iso-$PGF_{2\alpha}$ levels in the CSF of 18 individual patients were found to vary over time ($p < 0.005$), even though some patient samples (n = 5) did not show any significant variation over this period of time.

CSF 8-iso-$PGF_{2\alpha}$ levels correlate with other indicators of oxidative stress. In order to establish oxidative stress, we also measured other parameters of oxidative stress/lipid peroxidation, namely MDA via TBARS, GSSG, and SOD, in the CSF samples of patients with MS. In the 124 samples assayed for all the different parameters, CSF 8-iso-$PGF_{2\alpha}$ levels showed a high correlation with TBARS ($r = 0.78$) and GSSG ($r = 0.63$) but did not correlate with SOD ($r = 0.016$), as shown in figure 2.

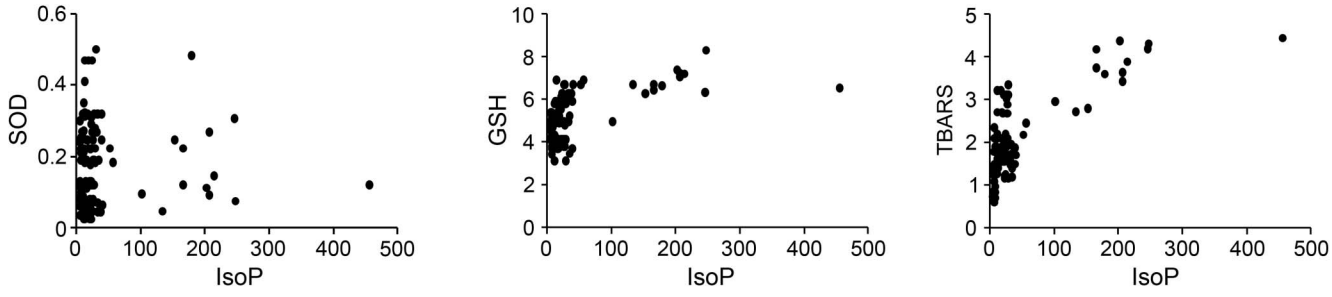
Total antioxidant status of CSF is decreased in patients with MS. The antioxidant status of patients with MS was also investigated. TAS as determined by the overall nonenzymatic antioxidant capacity samples showed a decrease ($p < 0.0001$) in the CSF of patients with MS (n = 231; mean ± SD = 121.8 ± 45.3 mMol) compared to the control CSF samples (n = 40; 258.3 ± 113.3 mMol), as shown in figure 3. The TAS was most uniformly reduced in progressive forms of MS, with the most pronounced decrease observed in the SPMS group.

These CSF results of TAS provide additional support for the occurrence of oxidative stress in MS as shown by the lipid peroxidation assays.

Neural 8-iso-$PGF_{2\alpha}$ levels are increased in an experimental model of MS and by "stressed" glial cells in culture. To determine whether 8-iso-$PGF_{2\alpha}$ levels are increased in experimental models of demyelination, we measured levels of 8-iso-$PGF_{2\alpha}$ in whole-brain lysate of mice induced with EAE and compared the values to those in the brain lysates of control mice (figure 4A). The EAE mice were sacrificed at disease peak (i.e., on day 15) and had a mean disability score of 7.8 ± 2.1 on a 0–13 scale.[22,23] In EAE, levels of 8-iso-$PGF_{2\alpha}$ (0.784 ± 0.03 pg/mg tissue) at peak of disease (day 15) were higher ($p < 0.003$ value) than the levels in control mice (0.372 ± 0.71 pg/mg tissue).

To determine whether 8-iso-$PGF_{2\alpha}$ is produced by glial cells under oxidative stress conditions, we investigated the glial cell line CG4 in culture. The addition of H_2O_2 or the reactive oxygen generator AAPH to CG4 cells in culture significantly increased the 8-iso-$PGF_{2\alpha}$ levels in an assay of culture medium. Furthermore, the levels of 8-iso-$PGF_{2\alpha}$ increased in a dose-dependent manner, and the increase was inhibited by preincubation with the ROS scavenger EUK134 (figure 4B).

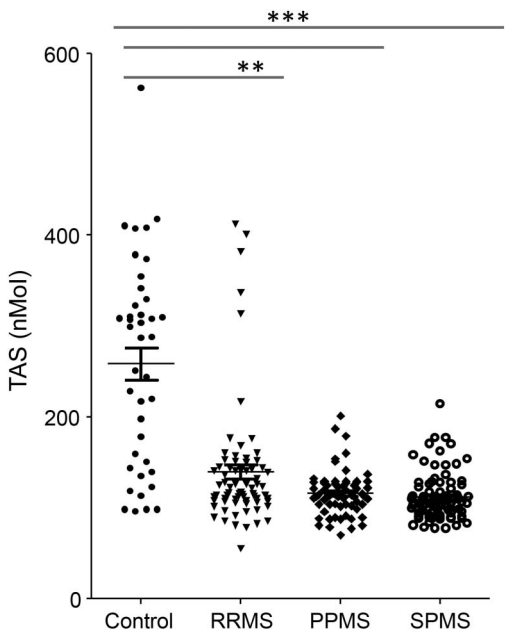
Figure 2 Correlation of CSF 8-iso-$PGF_{2\alpha}$ levels with other indicators of oxidative stress



Parameter	SOD	GSH	TBARS
Number of XY pairs	124	124	124
Pearson r	0.01822	0.5663	0.7399
95% confidence interval	-0.1586 to 0.1940	0.4332 to 0.6753	0.6481 to 0.8105
p value (two-tailed)	0.8408	< 0.0001 (***)	< 0.0001 (***)
Is the correlation significant?	No	Yes	Yes
R square	0.000332	0.3207	0.5474

Correlations of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) values with the corresponding values for superoxide dismutase (SOD), oxidized glutathione (GSH), and thiobarbituric acid reactive substances (TBARS) in the CSF samples of patients with multiple sclerosis are shown as a scatter plot. The corresponding values are given at the bottom.

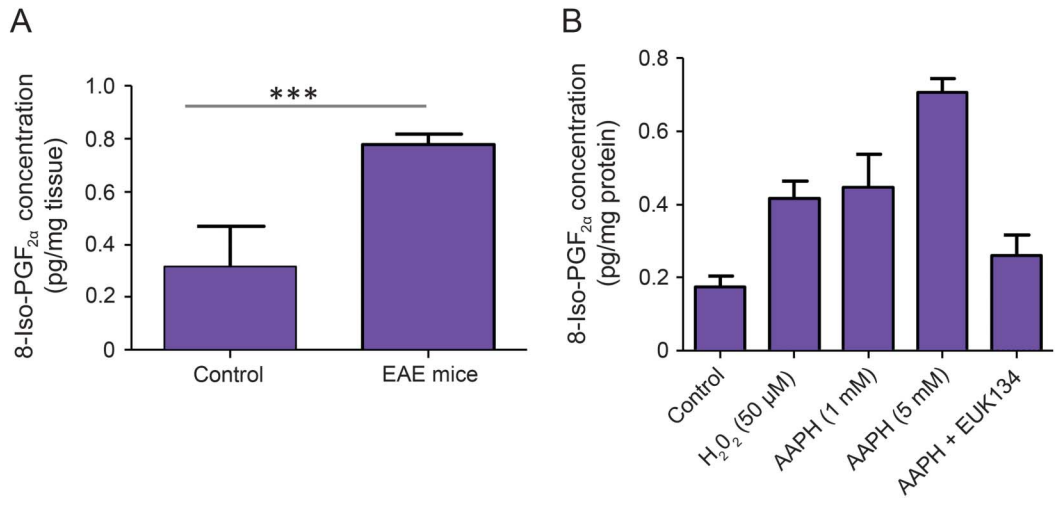
Figure 3 Total antioxidant status (TAS) (nonenzymatic)



TAS of CSF samples was determined in controls and patients with MS. Groups were statistically compared using GraphPad Prism 5.0. * indicates statistical significance with a p value of <0.05. PPMS = primary progressive multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive MS.

DISCUSSION MS is a disease of unknown etiology, although there is evidence that genetic, autoimmune, and environmental factors all contribute to the pathophysiology of the disease.[25] Despite many advances in immunotherapy, complete cessation of disease activity and progression is not possible in all cases.[3] It is thus postulated that in addition to autoimmune factors, mechanisms such as oxidative stress directly or indirectly related to inflammation contribute to disease activity and progression.[3,5] At present there is no established biomarker indicative of oxidative stress in MS, so its impact on the pathophysiology of the disease is difficult to quantify.[25–27] In this study we measured CSF isoprostane 8-iso-$PGF_{2\alpha}$ levels to determine its possible utility as a biomarker of oxidative stress in MS. Isoprostane 8-iso-$PGF_{2\alpha}$ is an established biomarker for oxidative stress and the associated lipid peroxidation in multiple human diseases including cardiovascular diseases, diabetes and obesity, cigarette smoking, and immune disorders like rheumatoid arthritis.[12,13] Previous studies on isoprostanes in MS have reported increased levels of 8-iso-$PGF_{2\alpha}$ in urine,[14] plasma,[16] and CSF[17–20] in patients with MS. However, the sample sizes were rather small, and the studies on CSF were based on early-stage MS or clinically isolated syndrome.[17–19] A later study[20] found that elevated CSF levels of 8-iso-$PGF_{2\alpha}$ did not correlate with markers of levels

Figure 4 8-Iso-$PGF_{2\alpha}$ increases in vivo during EAE and during oxidative insult in cell culture



(A) 8-Iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) levels in whole-brain lysate of mice induced with experimental allergic encephalomyelitis (EAE) was induced in 8-week-old mice. Mice were sacrificed at peak of disease (day 15) and whole-brain lysates were prepared. Shown here are the 8-iso-$PGF_{2\alpha}$ levels as determined by ELISA. *** indicates statistical significance with a p value of <0.05. (B) 8-Iso-$PGF_{2\alpha}$ is produced by glial cells under oxidative stress conditions. The glial cell line CG4 was subjected to oxidative stress in culture by the addition of 50 μM hydrogen peroxide (H_2O_2) or the reactive oxygen generator 2,2′-azobis-2-methyl-propanimidamide dihydrochloride AAPH). Reactive oxygen species (ROS) scavenger EUK134 was added to quench the ROS released. The 8-iso-$PGF_{2\alpha}$ levels were then assayed in the cell culture medium by ELISA. The experiment is a mean of n = 3 and *** indicates statistical significance with a p value of <0.05.

of inflammatory activity in an RRMS population (n = 41). They suggested that 8-iso-$PGF_{2\alpha}$ may represent a sensitive marker of degenerative phenomena, independent of inflammatory activity.[20] Our study aimed to investigate 8-iso-$PGF_{2\alpha}$ levels in the CSF of patients with MS across disease stages/progression to establish lipid peroxidation and investigate it as a marker of oxidative stress in MS.

In our analysis we found that CSF levels of 8-iso-$PGF_{2\alpha}$ less than 20 pg/mL were present in all normal controls (N = 24) and in all patients with ONDs (N = 16). Taken as a group, patients with MS (N = 231) had significantly elevated mean CSF 8-iso-$PGF_{2\alpha}$ levels compared to controls. However, 57% of patients with RRMS, 30% of patients with SPMS, and 35.8% of patients with PPMS had CSF values of 8-iso-$PGF_{2\alpha}$ in the "normal" range, suggesting that oxidative stress may not be a universal phenomenon in MS. Modest elevations of CSF isoprostane (20–80 pg/mL) were observed in 40% of patients with RRMS and in a greater proportion of patients with progressive disease (38.9% SPMS, 64.2% PPMS). CSF isoprostane levels higher than 100 pg/mL were only seen in a subset of patients with SPMS (31% of all SPMS samples [N = 90]). It may be postulated from this data that oxidative stress as indicated by CSF isoprostane levels is not a universal phenomenon in all forms of MS but rather a particular manifestation of inflammatory neurodegeneration best illustrated by a subset of patients with SPMS. This occurrence is not a medication effect because it is seen in treatment-naive patients and furthermore it correlates with other markers of oxidative stress. The levels of CSF 8-iso-$PGF_{2\alpha}$ in patients with MS were significantly higher in patients with recent relapses, changes in EDSS, or recent new brain MRI lesions compared to clinically stable patients with MS, suggesting a correlation between oxidative stress and disease activity. Furthermore, the TAS as reflected in the CSF appears compromised in patients with MS compared to controls. Our CSF clinical findings were supported by experimental data in which higher 8-iso-$PGF_{2\alpha}$ levels were found in EAE brain tissue compared to normal brain. These findings suggest that oxidative stress as measured by CSF levels of 8-iso-$PGF_{2\alpha}$ is a pathophysiologic phenomenon in MS and is a potential biomarker of disease activity related to oxidative stress in progressive forms of MS.

Oxidative stress in general and isoprostanes and other lipid peroxidation products in particular may contribute to the pathogenesis of MS by a variety of mechanisms. Oxidative stress may directly lead to neuronal and axonal loss, but oxidative stress can also regulate the function of different immune cells.[28,29] One study[30] demonstrated that oxidative stress alters B-cell function in part by increasing proteolysis within the cells. As a result, the amount of antigen presented to the specific T cells is reduced. Isoprostanes and other lipid peroxidation products can initiate signaling cascades to regulate cell viability and toxicity.[30,31] Lipid peroxidation can also lead to changes in neurotransmission and signaling by simply altering the neuronal membrane fluidity and permeability and the stereotypic presentation of the membrane receptor.[32–35]

If validated by additional studies, measurement of CSF isoprostane levels may have clinical application, such as in selecting "likely responders" and in monitoring the clinical efficacy of medications purported to affect oxidative stress in MS. Thus, it would be of interest to determine whether there is a correlation between high pretreatment CSF isoprostane levels and treatment responders with agents such as dimethyl fumarate (DMF) in patients with RRMS.[35] Also, it is possible that as treatment trials in patients with progressive disease are considered, pretreatment levels of CSF 8-iso-$PGF_{2\alpha}$ may be measured to later determine whether the patients with the highest levels responded more favorably to DMF. It is probable that other disease-modifying agents that affect inflammation also affect CSF isoprostane levels. Thus if correlation of CSF isoprostane levels with therapeutic response can be shown, oxidative stress markers would have utility as biomarkers of treatment efficacy.

These preliminary findings are exciting because they show that CSF analysis may allow determination of oxidative stress in MS. Further studies are needed to confirm this work using an independent cohort and to investigate the mechanisms linking oxidative stress and disease progression.

AUTHOR CONTRIBUTIONS

Fozia Mir: planned and performed experiments, analyzed the results, and wrote the manuscript. Donald Lee and Hetal Ray: performed many of the experiments. Saud A. Sadiq: provided supervision throughout the study and critically revised the manuscript. All authors discussed the results and commented on the manuscript.

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DISCLOSURE

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