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Title:

Oxylipins in cerebrospinal fluid in clinically isolated syndrome and relapsing remitting multiple sclerosis

Authors

Irene Håkansson¹, Sandra Gouveia-Figueira², Jan Ernerudh³, Magnus Vrethem¹, Nazdar Ghafouri⁴, Bijar Ghafouri^{4,*}, Malin Nording^{2,5,*}

¹ Department of Neurology and Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

² Department of Chemistry, Umeå University, Umeå, Sweden

³ Department of Clinical Immunology and Transfusion Medicine, and Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

⁴ Pain and Rehabilitation Centre and Department of Medical and Health Sciences, Linköping University, Linköping, Sweden

⁵ Department of Entomology and Nematology, University of California, Davis, USA

* These authors contributed equally to this work.

Corresponding author:

Irene Håkansson

Department of Neurology

University Hospital in Linköping

581 85 Linköping

Sweden

Phone: +46-10-1030000

Fax: +46 10 103 44 38

E-mail: irene.hakansson@regionostergotland.se

Highlights:

- Oxylipin levels in CSF from 41 patients with CIS or RRMS and 22 controls are given.
- CSF levels of 9-HODE and 13-HODE were significantly increased in patients.
- Oxylipin levels were not associated with disease activity during follow-up.

ABSTRACT

Although oxylipins are involved in inflammation, data on these lipid mediators in multiple sclerosis are sparse. In this study, a panel of oxylipins were analysed with liquid chromatography tandem mass spectrometry in cerebrospinal fluid (CSF) from 41 treatment naïve patients with clinically isolated syndrome (CIS) or relapsing remitting MS (RRMS) and 22 healthy controls. CSF levels of 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) were significantly higher in patients than in healthy controls (9-HODE median 380 nM (interquartile range 330-450 nM) in patients and 290 nM (interquartile range 250-340 nM) in controls, 13-HODE median 930 nM (interquartile range 810-1080 nM) in patients and 690 nM (interquartile range 570-760 nM) in controls, $p < 0.001$ in Mann-Whitney U-tests). 9-HODE and 13-HODE performed well for separation of patients and healthy controls (AUC 0.85 and 0.88, respectively, in ROC curve analysis). However, baseline CSF levels of the oxylipins did not differ between patients with signs of disease activity during one, two and four years of follow-up and patients without. In conclusion, this study indicates that 9-HODE and 13-HODE levels are increased in CSF from CIS and RRMS patients compared with healthy controls, but does not support 9-HODE or 13-HODE as prognostic biomarkers of disease activity in patients during follow-up.

Keywords: oxylipins; hydroxyoctadecadienoic acid; mass spectrometry; multiple sclerosis; clinically isolated syndrome

INTRODUCTION

Relapsing remitting multiple sclerosis (RRMS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS). Pathology, clinical presentation and disease progression in RRMS is highly variable. Clinically isolated syndrome (CIS) denotes a first episode of neurological symptoms consistent with demyelinating inflammation in the CNS, but where the criteria for multiple sclerosis (MS) diagnosis, which require dissemination in time and space, are not fulfilled. Many patients with CIS later convert to MS, but some do not.

Oxylipins is an umbrella term for fatty acid metabolites involved in maintaining homeostasis. Prostaglandins belong to the eicosanoids, a sub-category of oxylipins derived from arachidonic acid (AA). Other oxylipins are derived from linoleic acid or various other polyunsaturated fatty acids by non-enzymatic oxidation, or cyclooxygenase, lipoxygenase and cytochrome P450 catalyzed reactions. The vast network of bioactive lipid mediators covered by the oxylipin metabolome includes molecules with both pro- and anti-inflammatory properties and several of them have been extensively studied in relation to disease states with an inflammatory component¹. Mapping this network in a physiological context is challenging, but a better understanding of how altered levels associate with disease may facilitate novel preventive and therapeutic strategies and reveal potential disease biomarkers.

The structural similarities and low abundance of most of the constituents in the oxylipin metabolome have previously been obstacles for quantification in complex human body fluids and tissues. However, recent technological and laboratory advances in lipidomics now enable accurate quantification of these compounds with liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) methods^{2, 3}. The physiological roles, including pro-inflammatory and anti-inflammatory functions, of all the constituents in the oxylipin metabolome have not yet been fully elucidated. Regarding oxylipins in MS patients, there are reports of increased levels of 13-hydroxyoctadecadienoic acid (HODE) in plasma⁴ and increased levels of prostaglandin E₂ (PGE₂) and 15-hydroxyeicosatetraenoic acid (HETE) in cerebrospinal fluid (CSF) from MS patients compared to controls⁵. Increased levels of PGE₂ and 15-HETE in CSF were also reported in active MS in a

study comparing more active MS patients to less active MS patients⁶, whereas CSF levels of 4-, 7-, 14- and 17-hydroxydocosahexaenoic acid (HDHA), 5- and 12-HETE, PGD₂, PGF₂, lipoxin A4 and B4 (LXA₄, LXB₄), leukotriene B4 (LTB₄), thromboxane B2 (TXB₂), AA, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) did not differ significantly between these groups⁶.

The aim of this study was to compare levels of a panel of constituents in the CSF oxylipin metabolome in treatment free patients with CIS or RRMS and healthy controls. Furthermore, we explored whether levels of these metabolites correlated to clinical parameters, magnetic resonance imaging (MRI) parameters and a panel of protein markers of neuroinflammation and neurodegeneration. Finally, to assess prognostic biomarker potential, we investigated whether baseline metabolite levels differed between patients that showed signs of disease activity during follow-up and patients that did not.

MATERIAL AND METHODS

Patients and controls

41 patients with CIS or RRMS were consecutively enrolled in a prospective longitudinal cohort study of CIS and newly diagnosed MS at the Department of Neurology, University Hospital of Linköping, Sweden. All patients fulfilled the revised McDonald criteria from 2010⁷ for CIS or MS. Patients underwent clinical neurological examination including expanded disability status scale (EDSS), peripheral blood and CSF sampling and MRI at baseline and at one, two and four years of follow-up. Only CSF from baseline, when all patients were untreated, was analysed in this study. Patient characteristics are presented in Table 1. For CSF, 22 age- and sex-matched healthy controls were recruited from healthy blood donors. Patients were included in the study from 2009 to 2012 and healthy controls from 2011 to 2014. Healthy controls were free from past and current neurological and autoimmune disease and their clinical neurological examinations were normal as were routine findings in CSF (Table 1). No medication, except oral contraceptives, was allowed in healthy controls.

Table 1 Patient and healthy control characteristics

Clinical and laboratory data	Patients n=41	Healthy controls n=22	p
Women/men (% women)	32/9 (78 %)	17/5 (77 %)	0.9
Age ^a (years)	31 (24-36)	32 (26-41)	0.3
Diagnosis (CIS/RRMS)	19/22	NA	
Relapse within last 2 months (yes/no)	23/18	NA	
Mean disease duration ^b (months)	11.8	NA	
Median disease duration ^b (months)	3.5	NA	
Disease duration ^b (number of subjects)		NA	
0-1 months	10		
1-3 months	10		
3-12 months	13		
13-36 months	5		
37-120 months	3		
Median EDSS	2.0	NA	
EDSS (number of subjects)			
0	6	22	
1.0	12		
1.5	2		
2.0-2.5	15		
3.0-4.5	6		
Chronic pain (number of subjects)	2 ^c	0	
CSF mononuclear cell count ^a	4.6x10 ⁶ /L (1.8-9.2)	2.1x10 ⁶ /L (0.9-2.7)	0.001
Albumin ratio ^a	4.8 (3.4-6.0)	4.7 (3.6-5.3)	0.5
IgG index ^a	0.7 (0.5-1.1)	0.5 (0.5-0.5)	<0.001
Oligoclonal CSF IgG bands (pos/neg)	33/8	0/22	<0.001
Subjects on DMT	0	NA	

P-values from Chi-square test for sex distribution and oligoclonal bands and from Mann-Whitney U test for independent samples for age and CSF data.

^a Median and within brackets interquartile range.

^b Disease duration refers to time from first symptom suggestive of demyelinating disease.

^c One patient had osteoarthritis since several years, with low grade chronic pain in hands, feet and one hip. Another patient had chronic tension type headache and chronic neck and shoulder pain.

NA, not applicable

DMT, disease-modifying treatment for CIS and RRMS

Ethics Statement

The study was approved by The Regional Ethics Committee in Linköping and written informed consent was obtained from all participants.

Cerebrospinal fluid sample collection

All CSF sampling was carried out by the same neurologist (IH) and all lumbar punctures were performed 8 – 12 a.m. One aliquot of the CSF sample was used for cell counting, CSF/serum albumin ratio, IgG index, IgG synthesis index and isoelectric focusing, all according to clinical routines performed at the Department of Clinical Chemistry. Within one hour, the remaining CSF was centrifuged (300 x g for 10 min.) and the supernatant was aliquoted and immediately frozen and stored at -70°C.

MRI protocol and analyses of protein markers of neuroinflammation and neurodegeneration

The MRI protocol, as well as the multiplex bead array and the enzyme-linked immunosorbent assays used to determine levels of the neuroinflammatory and neurodegenerative markers, have been described in detail previously⁸. Mean time from lumbar puncture to MRI was 26 days (range -4 to 64 days).

Measurements of oxylipins in CSF

Oxylipin levels were determined with liquid chromatography coupled to tandem mass spectrometry in accordance with previously published protocols³. The researchers performing the laboratory work were blinded to clinical information. Detailed protocol information is provided in supplementary materials.

Statistics

Statistical analyses were performed using SPSS for Windows, version 23. Non-parametric tests were mostly used due to the non-Gaussian distribution of some parameters. Values below the detection limit were assigned half the value of the detection limit and included in statistical tests. The Mann-Whitney U-test was used to compare two groups. Non-parametric bivariate correlation analysis (Spearman) was used when investigating association between lipids and clinical parameters (including EDSS), MRI parameters and protein markers of neuroinflammation and neurodegeneration. Receiver operating characteristic (ROC) curves were derived from logistic regression to investigate the discriminatory power of oxylipins between patients and controls. Because of multiple testing, a stringent p-value of ≤ 0.01 was considered to be significant in the Mann-Whitney U-tests and only correlations with Spearman's $\rho \geq 0.5$ were considered to be significant. ANCOVA analysis was used to examine whether significant differences between HODE levels remained when storage time was controlled for. In this ANCOVA, one patient, a strong outlier, was omitted in order to obtain a more parametric distribution of the data, and a significance level of 0.05 was used. All p-values were based on two-tailed statistical tests.

To investigate the multivariate correlations between the oxylipins and group membership, orthogonal partial least squares discriminant analysis (OPLS-DA) was applied using SIMCA-P+ v.14.0 (UMETRICS, Umeå, Sweden)⁹. Principal component analysis (PCA) was used prior to the OPLS-DA analysis. PCA is an unsupervised analysis that can be used to extract and display systematic variation in the data matrix. A cross validation (CV) technique was used to identify nontrivial components. In the OPLS-DA, variables (regressors) were considered important if they had regression coefficients with a jack-knifed 95% confidence interval not including 0 and the variable of importance (VIP) value was greater than 1. R^2 describes the goodness of fit – the fraction of sum of squares of all the variables explained by a principal component. Q^2 describes the goodness of prediction – the fraction of the total variation of the variables that can be predicted by a principal component using cross validation methods. R^2 should not be considerably higher than Q^2 . A difference greater than 0.2-0.3 implies overfitting, meaning that the robustness of the model is poor⁹. To validate the model, obtained cross validated analysis of variance (CV-ANOVA) was used. The multivariate regressions were considered of significant importance if the CV-ANOVA had $p < 0.05$. The

procedure to compute multivariate correlation models is in accordance with Wheelock and Wheelock¹⁰.

RESULTS

Oxylipins in patients and healthy controls

9-HODE and 13-HODE levels were significantly higher in patients than in healthy controls, both $p < 0.001$ (Table 2 and Figure 1). Excluding the two patients with chronic pain did not change this finding (both p still < 0.001). Samples from patients had longer storage times than samples from healthy controls, but ANCOVA analysis showed that there was a significant difference between HODE levels in patients and healthy controls also when storage time was controlled for. 9-HODE and 13-HODE levels correlated significantly in patients (Spearman's ρ 0.55, $p < 0.001$), whereas in healthy controls Spearman's ρ was 0.47, $p = 0.03$. All analytes were detected in CSF samples from all subjects, except PGE_2 and 15-HETE, which were not detected in any subject.

Table 2 Oxylipins in CSF from patients with CIS or RRMS and from healthy controls

	Patients (n=41)	Healthy controls (n=22)	p
11,12-DHET	24 (20-27)	22 (16-33)	0.55
14,15-DHET	52 (43-63)	52 (43-78)	0.65
15-HETE	ND	ND	
9-HODE	380 (330-450)	290 (250-340)	<0.001
13-HODE	930 (810-1080)	690 (570-760)	<0.001
9,10-DiHOME	55 (49-71)	57 (49-64)	0.59
12,13-DiHOME	91 (77-110)	84 (78-98)	0.32
9,10,13-TriHOME	190 (160-210)	180 (170-200)	0.98
9,12,13-TriHOME	580 (530-660)	580 (530-700)	0.72
PGE_2	ND	ND	
$\text{PGF}_{2\alpha}$	165 (120-240)	225 (140-350)	0.07
TXB_2	63 (56-74)	55 (47-71)	0.12

Data are expressed as median and within brackets interquartile range and concentrations of oxylipins are given in nM. P-values from Mann-Whitney U-test. ND: Not detectable. DHET: Dihydroxyeicosatrienoic acid; HETE: Hydroxyeicosatetraenoic acid; HODE: Hydroxyoctadecadienoic acid; DiHOME: Dihydroxyoctadecenoic acid; TriHOME: Trihydroxyoctadecenoic acid; PGE_2 Prostaglandin E_2 ; $\text{PGF}_{2\alpha}$: Prostaglandin $F_{2\alpha}$; TXB_2 : Thromboxane B_2

Figure 1 Levels of 9-HODE and 13-HODE in CSF from patients with CIS or RRMS and from healthy controls. Lines show median values and interquartile range.

*** $p < 0.001$ in Mann-Whitney U-test.

Oxylipins for separation of patients and healthy controls

ROC curve analysis was performed on 9-HODE and 13-HODE to investigate their ability to discriminate between patients and healthy controls. ROC curves and area under curve (AUC) values are presented in Figure 2. For comparison, the AUC value for neurofilament light chain in CSF for separation of the same patients and healthy controls was 0.85, as previously reported⁸.

Figure 2 Receiver operating characteristics (ROC) curves for 9-HODE and 13-HODE in CSF at baseline for separation of patients with CIS or RRMS (n=41) and healthy controls (n=22). Area under curve (AUC) values are given in the figure.

Oxylipins in relation to clinical, MRI and CSF parameters at baseline

11,12-DHET was higher in men than in women ($p=0.003$) in the patient group (Figure 3A). Apart from that, lipid levels did not differ significantly between women and men in patients or healthy controls. Age did not correlate significantly with lipid levels in patients or healthy controls, except for $\text{PGF}_{2\alpha}$ in healthy controls (Spearman's ρ 0.54, $p=0.01$).

Patients in relapse (time from onset of relapse within two months before CSF collection) (n=23) had significantly higher levels of 11,12-DHET ($p=0.007$) (Figure 3B) and a trend towards higher levels of 14,15-DHET ($p=0.019$) than non-relapse patients (n=18). The percentage of persons in relapse at the time of sample collection was the same, 56%, among male and female patients. Relapse status did not affect levels of the other lipids. None of the analyzed lipids differed significantly between CIS patients (n=19) and RRMS patients (n=22) (all $p>0.1$). Baseline EDSS score, disease duration, number of T2 hyperintense lesions in brain MRI, mononuclear cell count in CSF and IgG index did not correlate significantly with any of the analyzed lipids (all Spearman's $\rho < 0.5$).

Figure 3 Levels of 11,12-DHET in CSF from male and female patients (A) and from patients in relapse and not in relapse (B) at the time of sample collection. The percentage of persons in relapse at the time of sample collection was the same (56%) among male and female patients.

Lines show median values and interquartile range.

P-values from Mann-Whitney U-test for independent samples.

Oxylipins in relation to protein neuroinflammatory and neurodegenerative markers at baseline

Baseline CSF levels of the chemokines CXCL1, CXCL8, CXCL10, CXCL13 and CCL22 from the patients and healthy controls in this study have previously been reported, as well as baseline CSF levels of neurofilament light chain (NFL), neurofilament heavy chain (NFH), matrix metalloproteinase 9 (MMP-9), glial fibrillary acidic protein (GFAP), chitinase 3 like 1 (CHI3L1) and osteopontin (OPN)⁸.

In correlation analyses based on data from all subjects, *i.e.* pooled data from patients and healthy controls, 13-HODE correlated significantly with CXCL8 and CCL22 with our strict significance level of Spearman's $\rho \geq 0.50$ and $p \leq 0.001$ (Table 3). Several correlation coefficients between 0.40 and 0.49 were noted, for 9-HODE with CXCL10 and CXCL13, CCL22 and MMP-9 and for 13-HODE with CXCL10 and NFL (Table 3). There were no significant correlations, and also no correlation coefficients between 0.40 and 0.49, between baseline CSF levels of $\text{PGF}_{2\alpha}$, TXB_2 , 11,12-DHET, 14,15-DHET, 9-HODE, 9,10-DiHOME, 12,13-DiHOME, 9,10,13-TriHOME or 9,12,13-TriHOME and the proteins. When data from patients and healthy controls were analyzed separately, no significant correlations were present in either the patient group or in the healthy control group.

Table 3 Correlations between CSF levels of protein neuroinflammatory and neurodegenerative markers and 9-HODE and 13-HODE, respectively, in all subjects (n=63).

	9-HODE	13-HODE
CXCL1	0.24	0.39
CXCL8	0.39	0.54*
CXCL10	0.40	0.47
CXCL13	0.44	0.34
CCL22	0.40	0.50*
NFL	0.33	0.44
NFH	0.12	0.17
GFAP	0.12	0.12
CHI3L1	0.22	0.23
MMP-9	0.49	0.37
OPN	0.19	0.09

Magnitude of correlation coefficients from bivariate non-parametric correlation analyses (Spearman) are shown, with correlation coefficients ≥ 0.4 in bold.

*: Spearman's $\rho \geq 0.50$ and $p \leq 0.001$

Multivariate data analysis

PCA was performed in order to identify multivariate outliers. The PCA score plots in combination with Hotelling's T² (identifies strong outliers) and distance to model in X-space (identifies moderate outliers) showed one strong outlier and that patient was excluded from further analyses. To identify the substance important for group separation of patients and healthy controls an OPLS-DA regression model was generated (figure 4). A total of 10 substances with VIP value >1 were considered important for group separation and they are presented in figure 5. These oxylipins and proteins together explained 44% (R^2) of the variation and with a prediction of 30% (Q^2). The CV-ANOVA revealed that the model was highly significant ($p=0.002$). Among the strongest discriminating substances were the oxylipins 9-HODE and 13-HODE, with VIP values of approximately 1.5, which were more abundant in patients than in healthy controls. This difference in concentration was furthermore found to be significant (using univariate statistical analysis), independently of other factors, indicating that these factors constitute some of the key differences seen between patients and controls.

Figure 4. An OPLS-DA (first principal component t1 vs. the first orthogonal component to1) model showing the discriminant separation between the patients (green filled circles) and the controls (blue filled circles). The longitudinal dimension (X-axis) shows the interclass discrimination and the latitudinal dimension (Y-axis) shows the intraclass discrimination between patients and controls.

Figure 5. Variable importance of projection (VIP) values for oxylipins and proteins. VIP values >1.0 (red bars) were considered significant. 13-HODE and 9-HODE were the most important substances for group separation (patients vs. controls). MC: mononuclear cell count in CSF

Baseline oxylipin levels in relation to disease activity during follow-up

All 41 patients were evaluated at one year, 39 patients at two years (one patient did not undergo an MRI due to pregnancy and one patient had left the study) and 39 patients at four years (two patients had left the study). Evidence of disease activity during follow-up was defined as relapses, brain MRI activity (new or enlarging T2 lesions or Gadolinium-enhancing lesions) or sustained disability worsening (EDSS progression). There were no significant differences in CSF levels of the lipids at baseline between patients with evidence of disease activity during one (n=21), two (n=27) and four

(n=32) years of follow-up, and patients with no evidence of disease activity (NEDA-3) during follow-up (n=20 at one year, n=12 at two years and n=7 at four years).

DISCUSSION

We report that CSF levels of 9-HODE and 13-HODE were significantly higher in patients with CIS and RRMS than in healthy controls. Patients in relapse had significantly higher levels of 11,12-DHET than patients not in relapse at the time of sample collection and this lipid was also significantly higher in men compared to women in the patient group. 9-HODE and 13-HODE performed well at separating patients and healthy controls and also correlated with several protein markers of neuroinflammation and neurodegeneration when data from all subjects were analyzed. However, in the patient group, there were no significant correlations between oxylipins and protein markers at baseline and there were also no significant differences in CSF oxylipin levels at baseline between patients with and patients without evidence of disease activity during follow-up.

An essential feature of the innate immune system is maintaining cellular homeostasis by identifying and removing senescent and apoptotic cells and modified lipids on the surface of cell membranes and lipoprotein particles¹¹. As cellular membranes undergo lipid peroxidation, previously hydrophobic portions of fatty acids will move from the interior of the lipid bilayer to the aqueous exterior enabling physical contact between pattern recognition receptors and molecular pattern ligands and lipid peroxidation has been shown to induce disturbance of fine structures, alteration of integrity, fluidity, and permeability, and functional loss of biomembranes¹¹. The formation of lipid peroxidation products must be strictly controlled and programmed in order to exert physiologically important functions, such as regulation of gene expression and mediation of cellular signaling¹². Accumulation of many of the lipid peroxidation products are known to be toxic for cells and the CNS is particularly vulnerable to uncontrolled lipid peroxidation¹³. Increased lipid peroxidation in patients with MS has previously been suggested and challenged^{14, 15, 16, 17, 18, 19, 20}. HODEs are considered as nearly ideal markers for lipid peroxidation²¹. To our knowledge, there are no previous reports on 9-HODE or 13-HODE levels in CSF in CIS or RRMS patients. It has recently been reported that higher levels of 13-HODE in serum were part of a metabolomic signature associated with relapses and increase in EDSS in MS⁴. However, CSF was not analyzed in that study. It should be noted that alterations in HODE levels are not restricted to MS. As for reports on HODE levels in CSF in other neurological diseases, 9-HODE levels in children with brain tumors²² and hydrocephalus²³ did not differ from controls. Outside the

central nervous system, HODEs have been implicated in various contexts, *e.g.* in the inflammatory process in atherosclerosis²⁴, in the metabolic syndrome and some cancers²⁵, in perceived pain in individuals with localized musculoskeletal pain²⁶ and Achilles tendinopathy²⁷, and as oxidative stress biomarkers in acute exercise²⁸.

The increased CSF levels of 9-HODE and 13-HODE in CIS and RRMS patients compared to controls in our study may reflect inflammation in the CNS in the patient group. The HODEs have pleiotropic effects in humans. Actions that may be relevant in the context of MS inflammation are that the HODEs, as well as 15-HETE, activate the peroxisome proliferator-activated receptor gamma (PPAR γ) receptor, thereby influencing the maturation, differentiation and activation of monocytes, macrophages and dendritic cells²⁹. PPAR γ also interacts with transcription factors involved in proinflammatory signaling pathways, such as NF- κ B and STAT1, thereby repressing pro-inflammatory responses in *e.g.* activated T cells³⁰. These are potentially anti-inflammatory effects. On the other hand, HODEs are chemotactic agents for neutrophils and natural killer cells *in vitro*^{31, 32}, which could suggest pro-inflammatory effects of HODEs in MS. The effects of lipid peroxidation products might be concentration dependent since although many of them exert cytotoxicity, sublethal concentrations of such products, including HODEs, induce cellular adaptive responses and enhance tolerance against subsequent oxidative stress suggesting both deleterious and beneficial effects of lipid peroxidation *in vivo*¹².

PGE₂ and 15-HETE in CSF have been reported to be higher in highly active MS compared to less active MS⁶ and to be higher in MS patients compared to controls⁵, whereas levels of TXB₂ in CSF did not differ between highly active and less active disease⁶. TXB₂ was not associated with disease activity in our study either. Median PGE₂ was 430 ng/L in patients and 216 ng/L in controls and median 15-HETE was 646 ng/L in patients and 565 ng/L in controls in the study reporting higher levels of these analytes in MS patients than in controls⁵. PGE₂ and 15-HETE were not detectable in our patients and controls.

The overall lack of association between the baseline oxylipin levels and disease activity parameters in patients at baseline and during follow-up in our study suggests that, although levels of two of them

differ between patients and healthy controls, these oxylipins may not be central in the specific pathophysiology of MS, at least not linked to degree of disease activity. Rather, increased HODE levels in patients may be an unspecific sign of neuroinflammation. On the other hand, oxylipins are produced on demand, act locally, are metabolized differently and are more sensitive to pre-analytical influences than protein markers and therefore levels in CSF may not reflect disease activity as well as e.g. NFL. These remarks are only speculative, and it is to be recognized that this is an observational study that does not allow conclusions about causality or pathophysiology. However, we do claim that none of the lipids in our panel shows potential as a prognostic biomarker in MS, based on that we do not see an association with NEDA-3 during follow-up and the lack of significant correlations with accepted prognostic biomarkers like NFL in the patient group. While both NFL and the hydroxyoctadecadienoic acids show significant differences between patients and healthy controls, NFL also conveys prognostic information in the patient group⁸, whereas the lipids do not. In future studies, it would be of interest to measure oxylipins in MS patients repeatedly during follow-up and to evaluate possible associations with pain, spasticity and cognitive impairment in addition to NEDA-3.

Strengths of our study are the well characterized and treatment free patient group and the age- and sex-matched healthy control group, as well as availability of data on protein neuroinflammatory and neurodegenerative markers and follow-up data on disease activity. Limitations are the relatively small number of patients and the large number of comparisons made in some of the statistical analyses. We used strict significance levels to lower the risk of false positive findings, but further studies are needed to verify our findings. Also, HODE levels vary with dietary intake of linoleic acid³³ and we cannot exclude diet as a potential confounding factor.

In conclusion, this study indicates that levels of 9-HODE and 13-HODE in CSF are increased in patients with CIS and RRMS. We found no association between baseline levels of the oxylipins analyzed and disease activity during follow-up.

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SUPPLEMENTARY MATERIAL

Measurements of oxylipins in CSF

A previously validated solid phase extraction (SPE) protocol was modified to isolate oxylipins from CSF samples³. In summary, Waters Oasis HLB cartridges (60 mg of sorbent, 30 µm particle size) were first washed with ethyl acetate (1 mL) and methanol (2x2 mL), then conditioned with 5% methanol in water (containing 0.1% acetic acid) before the CSF sample was loaded. Quantitative volumes from each sample (~0.3 mL) were spiked with a 10 µL internal standard mixture in methanol with the following concentrations, 50 ng/mL 12(13)-DiHOME-d₄ and 12(13)-EPOME-d₄, and 25 ng/mL 9-HODE-d₄, PGE₂-d₄ and TXB₂-d₄, , as well as 10 µL antioxidant solution (0.2 mg/mL BHT/EDTA in methanol/water (1:1)). After the samples were applied, the SPE cartridges were washed, dried under high vacuum and eluted with 3 mL acetonitrile, 2 mL methanol and 1 mL ethyl acetate. To trap the analytes, all three fractions were pooled into polypropylene tubes containing 6 µL of a glycerol solution (30% in methanol) and evaporated using a MiniVac system (Farmingdale, NY, U.S.A.), then reconstituted in 100 µL of methanol, spiked with 10 µL recovery standard (50 ng/mL CUDA), transferred to vials, and analyzed by LC-MS/MS. The Agilent Ultra-Performance LC system (Infinity 1290) coupled to an Agilent 6490 Triple Quadrupole mass spectrometer with an electrospray ionization source (ESI) equipped with the iFunnel Technology (Agilent Technologies, Santa Clara, CA, USA) was used for separation and detection of the analytes. From the LC vials, 10 µL was injected on a Waters BEH C18 column (2.1 mm x 150 mm, 130 Å, 1.7 µm particle size). and the mobile phase used for elution of the analytes was composed of (A) 0.1% acetic acid in deionized water and (B) acetonitrile:isopropanol (90:10) with the following gradient: 0.0-3.5 min 10-35% **B**, 3.5-5.5 min 35-40% **B**, 5.5-7.0 min 42% **B**, 7.0-9.0 min 50% **B**, 9.0-15.0 min 65% **B**, 15.0-17.0 min 75% **B**, 17.0-18.5 min 85% **B**, 18.5—19.5 min 95% **B**, 19.5-21.0 min 10% **B**, 21.0-25.0 min 10% **B**.

The ESI-MS/MS conditions for operating the ESI in negative mode were the following: capillary and nozzle voltage at 4000 V and 1500 V, drying gas temperature 230°C with a gas flow of 15 L/min, sheath gas temperature 400 °C and gas flow of 11 L/min; the nebulizer gas flow was set at 35 psi, and iFunnel high and low pressure RF were 150 and 60 V. Dynamic multiple reaction monitoring (MRM) mode was used with fixed time windows (retention time ±2 min) to profile one quantitative and one qualitative transition per analyte³. The dynamic MRM option was performed for all analytes with

optimized transitions and collision energies. The MassHunter Workstation software was used for instrument control and for subsequent manual integration of the peaks. Native standards (TXB₂, 9,12,13-TriHOME, 9,10,13-TriHOME, PGF_{2α}, PGE₂, 12,13-DiHOME, 9,10-DiHOME, 14,15-DHET, 11,12-DHET, 13-HODE, 9-HODE, and 15-HETE) and internal standards (TXB₂-d₄, PGE₂-d₄, 12,13-DiHOME-d₄, 20-HETE-d₆, 9-HODE-d₄, d₈-5-HETE, and 12(13)-EpOME-d₄) were purchased from Cayman Chemicals (Ann Arbor, USA). The limit of detection was 2.7 pM for TXB₂, 0.15 pM for 9,12,13-TriHOME, 3 pM for 9,10,13-TriHOME, 3 pM for PGE₂, 3 pM for PGF_{2α}, 3.5 pM for 12,13-DiHOME, 0.35 pM for 9,10-DiHOME, 3.25 pM for 14,15-DHET, 16 pM for 11,12-DHET, 18.5 pM for 13-HODE 0.4 pM for 9-HODE and 3.4 pM for 15-HETE.