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Association of Arachidonic Acid-Derived Lipid Mediators With Disease Severity in Patients With Relapsing and Progressive Multiple Sclerosis

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Abstract

Background and objectives: Excessive activation of certain lipid mediator (LM) pathways play a role in the complex pathogenesis of multiple sclerosis (MS). However, the relation between bioactive LMs and different aspects of CNS-related pathophysiological processes remains largely unknown. Therefore, we here assessed the association of bioactive LMs

belonging to the ω-3 / ω-6 lipid classes with clinical, biochemical (serum neurofilament light (sNfL) and serum glial fibrillary acidic protein (sGFAP)) parameters and MRI-based brain volumes in patients with MS (PwMS) and healthy controls (HC).

Methods: A targeted high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) approach was used on plasma samples of PwMS and HC of the Project Y cohort, a cross-sectional population based cohort that contains PwMS all born in 1966 in the Netherlands and age-matched HCs. LMs were compared between PwMS and HC and were correlated with levels of sNfL, sGFAP, disability (EDSS) and brain volumes. Finally, significant correlates were included in a backward multivariate regression model to identify which LMs best related to disability.

Results: The study sample consisted of 170 patients with relapsing remitting MS (RRMS), 115 patients with progressive MS (PMS) and 125 HC. LM profiles of patients with PMS significantly differed from RRMS and HC, in particular, patients with PMS showed elevated levels of several arachidonic acid (AA) derivatives. In particular 15-HETE (r = 0.24, $p <$ 0.001), positively correlated (average r = 0.2, $p < 0.05$) with clinical and biochemical parameters such as EDSS and sNfL. In addition, higher 15-HETE levels were related to lower total brain (r = -0.24, $p = 0.04$) and deep gray matter volumes (r = -0.27, $p = 0.02$) in patients with PMS and higher lesion volume (r = 0.15, $p = 0.03$) in all PwMS.

Discussion: In PwMS of the same birth year, we show that ω-3 and -6 LMs are associated with disability, biochemical (sNfL, GFAP) and MRI measures. Furthermore, our findings indicate that particularly in patients with PMS, elevated levels of specific products of the AA pathway, such as 15-HETE, associate with neurodegenerative processes. Our findings highlight the potential relevance of ω-6 LMs in the pathogenesis of MS.

Introduction

Fatty acid-derived lipid mediators (LMs) play a fundamental role in inflammatory and immune responses as they are able to modulate innate and adaptive immune cells.[1] Given the central role of LMs in induction, resolution, and chronicity of immune responses, LMs are implicated in several inflammatory disorders.[1,2] Towards personalized treatment, assessing LMs can provide valuable clues for explaining phenotype variability and possible underlying pathophysiological mechanisms.

Recent evidence suggests that excessive activation of certain LM pathways, for instance the arachidonic acid (AA) pathway, play a role in the complex pathogenesis of multiple sclerosis (MS).[3] LMs, such as the AA-derivate 15-HETE and the linoleic acid (LA) derivative 13-HoDE, have been appreciated for their involvement in inflammatory responses. Both LMs promote lipid/debris uptake via the nuclear peroxisome proliferator-activated receptor gamma (PPARy) in macrophages, thereby enabling tissue repair.[4] Other key players in LM pathways and pivotal initiators of inflammatory responses are prostaglandins (PG) and leukotrienes (LT) (**eFigure 1**). We have previously shown that bio-active LM plasma levels are altered in different MS subtypes (e.g. patients with relapsing remitting (RRMS) during relapses or in remission and patients with progressive MS (PMS)) as compared to healthy controls (HC).[6] These findings suggest that altered bio-active LM levels may be associated with impaired resolution responses, thereby potentially contributing to the (chronic) neuro-inflammatory process as seen in MS.[6,7]

Despite emerging interest in the role of LMs in MS pathogenesis, the association between LMs and established (bio)markers of neuro-inflammation and neurodegeneration remains elusive. An earlier study, consisting of 46 PwMS and their siblings, could not detect correlations of prostaglandin E_2 (PGE$_2$) and 15(S)-HETE with neurofilament light (NfL) and glial fibrillary acidic protein (GFAP)[8], markers of neuro-axonal damage and astrocyte

activation, respectively. Inflammation-activated astrocytes have been shown to increase the production and secretion of polyunsaturated fatty acids (PUFAs) in the brain and GFAP is therefore expected to show relations with LMs.[9,10] Absent correlations of the aforementioned study, however, might be due to the study design and warrant further exploration, also including other derivatives of ω-3 and ω-6 LM pathways. Furthermore, the relation between LMs and MRI-derived brain volumes, which are known to strongly relate to clinical progression and progressive MS[11], remains unknown.

To better understand the relation between bioactive LMs and different aspects of CNS related pathophysiological processes in MS, we here investigated bioactive LM levels belonging to the ω-3 / ω-6 lipid classes in plasma of different MS phenotypes and we determined whether and how these levels relate to disability outcome measures, brain volumes and the blood biomarkers sNfL and sGFAP. LM levels were determined by HPLC-MS/MS on plasma samples from a large population based cohort of PwMS all born in 1966 and age-matched HC (Project Y).[12] By using a cohort of PwMS and HC of the same age, we have limited the confounding effect of age, as age is known to affect LM concentrations.[13,14]

Methods

Study population

PwMS and HC were selected from the cohort study Project Y. Project Y is a population-based cross sectional birth year cohort which aimed to include all PwMS (as defined by the 2017 McDonald Criteria)[15] born in 1966 in the Netherlands and age and sex matched HC born in between 1965 and 1967 in the Netherlands. Potential participants were excluded if they were unable to undergo minimal data collection and/or were not currently living in the Netherlands. Patients diagnosed with MS before study participation and HC were subjected to comprehensive examinations during a one-day study visit (single time point) between

December 2017 and January 2021 at the Amsterdam University Medical Center (Amsterdam UMC), location VUmc. Details of the study design and total study population have been described previously.[12]

Standard Protocol Approvals, Registrations, and Patient Consents

The Project Y protocol was approved by the Medical Ethical Committee of the Amsterdam UMC, location VUMC, and all participants gave written informed consent prior to participation.

Targeted lipid mediator analysis

Blood samples were collected via standard venipuncture during the study visit and were stored at -80°C until further analysis. Plasma samples (400μL) were quenched by adding 1.2 mL methanol (MeOH) and 4 μl internal standard solution consisting of LTB_4-d4, 15-HETE-d8, PGE_2-d4, DHA-d5 and 9(S)-HoDE-d4 (50 ng/mL in MeOH). In addition, 40μL of every sample was pooled and used as quality control (QC) once every ten samples to ensure the quality of the experiment. Samples were placed at −20 °C for 20 minutes and spun down for 10 min at 16.200 g at 4 °C. Supernatants were then diluted in 6 mL H_2O and pH was corrected to 3.5 using formic acid (99%) after which solid-phase extraction (SPE) was used to further isolate the LMs. For SPE, C-18 Cartridges (Sep-Pak, Vac3 3cc (200 mg)) were used, pre-washed with MeOH and H_2O. After sample loading, the C-18 cartridges were washed with H_2O and n-hexane after which the extracted LMs were eluted using methylformate and collected in glass tubes. The elutes were dried at 40 °C for 1 hour using a N2 flow, reconstituted in 100 μL MeOH (40%) and transferred to deactivated glass inserts.Ω-3/6 LM content of the samples was measured using a targeted HPLC-MS/MS method.[16,17] LMs were detected using their relative retention times (RRTs) together with characteristic mass transitions and these and other individually optimized parameters can be found in **eTable 1**.

Before statistical analyses, all LM data was assessed first and LMs were excluded from the dataset if missing values were found in >150 samples. Hereafter, the still remaining missing values were imputed with 1/5 of the lowest value for each LM and the data was then normalized using total area (TA) normalization to obtain relative values as previously described.[18] In total 39 LMs were used for statistical analyses, which were performed in Rstudio (version 1.4.1106).

Serum NfL and GFAP

Serum NfL (sNfL) and GFAP (sGFAP) were measured using a single molecule array assay (SIMOA) on a HD-X analyser, according to the manufacturer's instructions (Quanterix, Billerica, USA). Analyses were performed at the Neurochemistry lab of the Department of Clinical Chemistry (Amsterdam UMC, location VUmc). Serum NfL and GFAP measurements are described in detail elsewhere.[19] Quality controls showed an intra assay coefficient of variation (CV) of sGFAP below the accepted threshold of <20%.[20]

Imaging

All participants were scanned on a 3-Tesla whole body MRI scanner; the MRI protocol has been previously described.[12] Briefly, the protocol included 3D-T1 weighted images (1mm isotropic) for volumetric measurements (using FSL, https://fsl.fmrib.ox.ac.uk) and a 3D- Fluid Attenuation Inversion Recovery (3D-FLAIR) sequence (also 1mm isotropic) for white matter lesion segmentation. Normalized total brain volume (NBV) and white matter volume (NMWV) were calculated on lesion-filled T1 images using SIENAX. FAST (part of SIENAX) and FIRST were used to segment total gray matter volume and normalized deep gray matter volume (NDGMV) also including thalamic volume; normalized cortical gray matter volume (NCGMV) was obtained subtracting deep grey matter segmentations from total gray matter segmentations using FSLmaths. All volumetric measures, except lesion volumes (LV), were normalized for head size using SIENAX-derived V-scaling.

Clinical assessment

All participants were subjected to comprehensive examinations during a one-day study visit, including a detailed interview regarding (MS) disease history. PwMS were asked about date of onset and diagnosis, MS phenotype, use of disease modifying therapy (DMT) and general medical history. The patient's reported medical history was corroborated by medical records. Overall MS-related physical disability was measured using the Expanded Disability Status Scale (EDSS).

Data handling and statistical analysis

Statistical analysis was performed using Rstudio (version 1.4.1106). Normality of the data was checked by visual inspection of histograms combined with Kolmogorov-Smirnov testing. A *p*-value ≤ 0.05 was considered as statistically significant. As our research is hypothesis generating, data were unadjusted for multiple testing, however, FDR corrected data is added in supplementary tables (eTables 1-2). Both primary and secondary progressive PwMS were grouped as progressive MS (PMS) to increase statistical power. Our statistical analysis consisted of four stages.

1. **Partial Least Squares – Discriminative Analysis (PLS-DA)**

First, we performed a partial least squares discriminative analysis (PLS-DA) using the mixOmics package in R. For this, we included all 39 LMs to optimize the separation of our three cohort groups (**eFigure 2A**) and to identify which LMs contributed most to this separation as indicated by the VIP score (**eFigure 2B**). Based on this, we selected only those 14 LMs with a VIP score > 1 for further analyses, as these LMs were believed to be the most contributory variables for cohort group discrimination.

2. Cluster analysis and group comparisons

Hereafter, we performed a two-way hierarchical cluster analysis using the `ComplexHeatmap` package in R.[21] Three sample cluster groups, matching the three cohort groups and two LM groups, matching the two used LM pathways, were given to the algorithm as input and data was clustered using the Euclidean distance method of the R package. A Chi-square test was performed on the quantitative output of the three sample clusters. Second, mean LM levels were compared between all subgroups (HC; patients with RRMS; patients with PMS) using volcano plots where the log2 fold-change was plotted against the – log10 p-value. Group comparisons of individual LM's were performed using non-parametric tests (Mann-Whitney U). Also, LM levels were compared between PwMS using first-line DMT (dimethyl fumarate, interferon, teriflunomide and glatiramer acetate) and second-line DMT (natalizumab, ocrelizumab, fingolimod).

3. Correlation analyses

Next, non-normally distributed parameters (e.g. sNFL, GFAP, lesion volume and all LMs values) were transformed using a Box-Cox transformation and used in partial correlation analyses together with EDSS and the other MRI volumetric measures. As initial screening approach and for variable reduction, the relation of LMs with disability (EDSS), sNfL, GFAP and MRI volumetric measures was analyzed using Pearson correlations in which sex, disease duration, smoking, BMI and disease modifying therapy (DMTs) use at moment of sampling (present/absent) were used as covariates.

4. Linear regression analysis

To further reduce the number of tests for LM analyses, significant correlates of the EDSS (5-HETE, 8-HETE, 15-HETE, DGLA and AdA) were fed into a multivariate linear regression model using a backward selection procedure with a removal p-value of >0.10. BMI, DMT use (present vs. absent), disease duration, MS subtype (progressive vs. relapsing), smoking (ever vs. never) and sex (female vs. male) were included as covariates. The outcome of this

model identified LMs which best related to disability (as indicated with EDSS). Finally, to assess whether LMs, sNfL and sGFAP were independently related to disability, the aforementioned final model was repeated forcing sNfL and sGFAP in the final model.

Data availability

Anonymized data, not published in the article, will be shared upon reasonable request from a qualified investigator.

Results

Patient characteristics

Of the 367 PwMS and 125 HC who participated in the Project Y cohort, a total of 285 PwMS (170 patients with RRMS and 115 patients with PMS) and 125 age-matched HC had available serum samples and were selected for the aim of this study. Main characteristics of the study populations are depicted in **Table 1**. In short, we observed higher median EDSS scores in patients with PMS (EDSS=5.5, IQR: 4.0–6.5) versus patients with RRMS (EDSS =3.0, IQR: 2.0–4.0) (p<0.001). Moreover, sNfL and sGFAP levels were significantly higher in PwMS versus HC. When comparing subgroups, patients with PMS had significantly higher sNfL and sGFAP levels compared to patients with RRMS. All brain volumes were significantly lower in PwMS versus HC (all p<0.001). In particular, all gray matter volumes were lower in patients with PMS versus patients with RRMS.

Hierarchical clustering shows LM profile differences between HC and progressive MS

To determine whether PwMS show altered ω-3/6 LM plasma levels, a PLS-DA was first performed to assess and optimize the separation of the three subgroups (HC; RRMS; PMS) (**eFigure 2, A and B**). 14 LMs (VIP score>1) contributed to this separation; therefore, only

these LMs were used for subsequent analyses. A two-way hierarchical clustering method resulted in three sample clusters (vertical) and two LM clusters (horizontal, **Figure 1**). The horizontal LM clusters were formed based on their origin in the ω -3/6 pathways (**eFigure 3, A and B**); one LM cluster consisted of derivatives of the upstream PUFA linoleic acid (LA) (e.g. HODE's) while the other LM cluster consisted of LMs associated with the downstream PUFAs arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (e.g. HETEs, DiHETEs and 19,20-DiHDPA, respectively). The three vertical sample clusters were formed based on the individual LM profiles. The first sample cluster (SC1) contained participants with low to average levels of the HoDE's and average to high levels of the other LMs. The second sample cluster (SC2) displayed relatively high levels of both 9-, and 13- HODE and relatively low to average levels of DGLA, AA, HETEs, DiHETEs and 19,20-DiHDPA. Sample cluster three (SC3) showed low levels of the HoDE's and HoTrE's, but average to high levels of all the other LMs. After this initial clustering, we determined the distribution of the three subgroups (HC, RRMS and PMS) over these three sample clusters using a Chi-square test (**Figure 1**). The distribution was different than what could be expected based on the number of participants per subgroup ($χ^2 < 0.0008$). Sample cluster three (SC3) appears to be mainly responsible for this, as this cluster contains relatively few HC and patients with RRMS and a relatively large amount of patients with PMS.

Mean LM levels show a distinct LM profile in patients with PMS

To confirm that the PMS lipid profile indeed differed from that of HC and patients with RRMS, mean LM levels were compared between these subgroups (**Figure 2, A-C**). Significant differences in LM levels were seen between patients with PMS and RRMS and between patients with PMS and HC (**Figure 2, B and C**), whereas no significant differences were observed between patients with RRMS and HC (**Figure 2A**). Twelve LMs, predominantly associated with AA (e.g. 5-, 8-,15-HETE), were significantly higher in the PMS group compared to HC, while two LMs, 9- and 13-HoDE, associated with the more upstream LA, were significantly decreased in patients with PMS compared to HC (**Figure 2B**). Importantly,

all of the observed differences in LM levels between patients with PMS and HC survived FDR correction (**eTable 2**). Five of these 12 significantly elevated LMs were also significantly higher in the PMS group compared to patients with RRMS (**Figure 2C**), although part of this significance was lost after FDR correction (**eTable 2**). Furthermore, no significant changes in LM levels were found between PwMS using first-line DMT and second-line DMT. Taken together, we here demonstrate an altered LM profile in patients with PMS compared to patients with RRMS and HC which is predominantly linked to the ω -6 lipid pathway. In addition, this altered LM profile is characterized by increased levels of AA derivatives such as 8- and 15-HETE and decreased levels of LA derivatives such as 9- and 13-HoDE.

Ω-6 and ω-3 LMs derived LMs correlate with disability, sNfL and sGFAP

To assess whether the observed LM differences associate with clinical measures and pathological processes such as axonal damage and astrocyte reactivity, LM levels were correlated with the EDSS, sNfL and sGFAP. Both positive and negative correlations were observed between clinical parameters and ω -6-associated LMs (**Table 2** and **Figure 3**). In the PMS group, higher levels of the AA derivatives 8- and 15-HETE correlated with worse EDSS scores. Moreover, in patients with PMS, high sNfL levels were related to elevated 15-HETE levels. The LA derivative 13-HoDE negatively correlated with sGFAP in both the RRMS group and in the whole MS group (**Table 2** and **Figure 3**). In the whole MS group, similarly to patients with PMS, worse EDSS scores as well as higher sNfL levels correlated with increased levels of several AA-derived HETEs (e.g. 5-, 8-, 15-HETE), but also with increased levels of the PUFAs DGLA and AdA.

Correlations with clinical parameters and ω -3 LMs were also assessed (**Table 2** and **Figure 3**). Here, only negative correlations with sGFAP levels and the EPA-derivative 14,15-DiHETE and DHA-derivative 19,20-DiHDPA were found in patients with PMS. No correlations were observed in patients with RRMS and in the whole MS group. In summary, these findings

show that EDSS and sNFL seem to be predominantly associated with AA-derivatives, whereas sGFAP levels may be more associated with both LA derivatives and ω -3 associated LMs.

Ω-6 and ω -3 LMs correlate with MRI-derived volumetric measures

To further assess the potential link between LMs and neurodegenerative processes, we next correlated LM levels with brain volumes (**Table 3**). In general, we observed that in patients with PMS increased levels of the AA-derivative 15-HETE were related with worse atrophic changes. Specifically, higher levels of 15-HETE correlated with both lower normalized total brain volume (NTBV) and normalized deep gray matter volume (NDGMV) in this subgroup. Higher levels of the ω -6 PUFA AdA correlated with higher lesion volume (LV). Furthermore, lower levels of both EPA derivatives 11,12- and 14,15-diHETE correlated with lower thalamic volume in patients with PMS.

In the RRMS group, higher levels of the AA-derivative 11,12-DiHET were significantly related with higher NBV (**Table 3**). In addition, both higher 11,12-DiHET levels as well as higher 8-HETE levels correlated with higher normalized cortical gray matter volume (NCGMV), whereas higher ω -3 PUFA DPAn-3 correlated with lower NCGCV. Moreover, in the RRMS group, higher DPAn-3 showed a positive correlation with higher LV. In the whole MS group, higher DGLA, AdA, 15-HETE and DPAn-3 levels correlated with higher LV. In addition, higher 14,15-diHETE and 19,20-DiHDPA levels were significantly related to normalized white matter volumes (NWMV) in this group. High levels of DPAn-3, on the other hand, correlated with lower NCGMV. Taken together, these findings demonstrate that several ω-6 and ω-3 derivatives, such as AdA, 15-HETE, DPAn-3, 11,12-DiHET and 8-HETE, relate to LV or brain atrophy as determined by MRI.

Linear regression: 15-HETE best related to disability

Finally, to identify which LMs best related to disability (EDSS), a linear regression was

performed. The backward selection linear regression model included sex, disease duration, BMI, DMT use, MS subtype, smoking status and all LMs which significantly correlated with the EDSS. The final model (**Table 4**) indicated that worse disability was associated with higher 15-HETE, longer disease duration and progressive disease (R^2=0.456). Additionally, the final model was repeated after also adding sNfL and sGFAP as fixed variables, showing significant associations of 15-HETE, disease duration, MS subtype, sNfL and sGFAP with disability in all PwMS (R^2=0.482, *p*<0.001).

Discussion

In our cohort of PwMS and HC of the same age, we found that patients with PMS in particular show a different LM profile compared to both patients with RRMS and HC, characterized predominantly by an increase of AA and its derivatives (e.g. 5-, 8-,15-HETE). We found several correlations between ω-3/6 LM plasma levels and disability (EDSS), biochemical (sNfL, sGFAP) and MRI measures in both patients with RRMS and PMS. The AA-derivative 15-HETE was of particular interest, as higher 15-HETE levels were related to higher sNfL concentrations, disability (EDSS), but also with lower NTBV and NDGMV in the PMS group. Finally, worse disability was best explained by higher 15-HETE levels and longer disease duration. Taken together, these findings indicate that in particular in patients with PMS, specific parts of the AA pathway are associated with neurodegenerative processes, reflected by disability.

As previous studies on the role of LMs are affected by age, the main strength of this study is the inclusion of patients and HC of the same age. Age is known to affect LM concentrations[13] and aging is, amongst other, associated with a deficiency of bio-active LMs like SPMs.[14] By using a cohort of PwMS and HC of the same age, we have limited the confounding effect of age. Besides, analyses were adjusted for sex given the different results seen in male and female individuals in earlier studies, such as increased 11,12-DHET in CSF of male PwMS

versus female PwMS.[22] We were able to observe correlations between higher 15-HETE levels, lower NTBV and NDGMV and higher LV, suggesting a potential link between 15-HETE and CNS-associated pathological processes in MS. This is further supported by the positive correlations we observed between 15-HETE and both sNfL and disability in PMS, indicating that 15-HETE is associated with neurodegeneration.

To our knowledge, no direct correlations between MRI measures and 15-HETE have been described before. A possible explanation for the detrimental effects of 15-HETE can be found in its ability to bind to several receptors, including leukotriene B_4 receptor 2 (BLT2) and peroxisome proliferator-activated receptor gamma (PPAR-y)), through which it can regulate several cellular processes including apoptosis.[23,24] In addition, 15-HETE can display additional detrimental effects as it is able to induce reactive oxygen species (ROS) production[25], which might be involved in neurodegenerative processes.[26] Moreover, 15-HETE may promote foam cell formation, a process also observed in MS in which CNS infiltrated macrophages become oversaturated with oxidized lipids.[16,17,27] An earlier study demonstrated that silencing of human ALOX15-B in human primary macrophages, one of the enzymes responsible for the biosynthesis of the isoform 15(S)-HETE, led to a decrease in lipid accumulation and inflammatory markers.[28] In addition, by using a knockdown of the mouse Alox15b gene in LDL-receptor deficient (Ldlr (-/-) mice, it was shown that ALOX15-B is linked to increased foam cell size and plaque lipid content in atherosclerotic plaques.[29] These findings suggest that ALOX15-B, and presumably its LM product 15(S)-HETE, are associated with lipid uptake which is a critical process in MS. Myelin internalization by foamy phagocytes is not only a disease-promoting process by presenting brain-derived autoantigens and adopting an inflammatory phenotype, but also a prerequisite for CNS repair as damaged myelin clearance is essential to facilitate remyelination.[27] However, additional experiments are required to substantiate this hypothesis and to uncover the cellular source and function of 15-HETE in the context of MS. Of note, both 8- and 15-HETE are produced by the enzyme ALOX-15B, which could explain the similar trend observed in their relation

with disability.[30] Also, the backward selection procedure, which was used to create final linear regression models for disability, might have resulted in the removal of 8-HETE in these models, because of the similar effect of 15-HETE and 8-HETE on the variance in EDSS.

In line with our results, previous studies have documented elevated levels of both 15-HETE and 15(S)-HETE in PwMS compared to controls[6,8,31], as well as higher 15-HETE CSF levels in patients with Alzheimer's disease compared to patients with mild cognitive impairment and subjective impairment.[32] In our previous work, significantly increased levels of 15-HETE were already found in the plasma of progressive PwMS compared to HC, yet no correlation with clinical data was observed, presumably due to the small sample size.[6] Moreover, in the Gothenburg MS registry, elevated CSF levels of both 15(S)-HETE and prostaglandin E_2 (PGE_2) were found in PwMS compared to healthy siblings and controls.[8] However, no correlations with either EDSS, sNFL or sGFAP were observed here, which might be explained by the small cohort size (n = 46), less advanced measuring techniques or by the fact that both progressive (n = 27) and RRMS (n = 19) patients were grouped together. While we observed significant correlations between 15-HETE and sNfL in PMS and in the whole MS group, there was no evidence of a significant correlation between 15-HETE (or other AA derivatives) and sGFAP in our cohort. Finally, we report independent associations of 15-HETE, sNfL and sGFAP with EDSS in all PwMS.

The AA-derivative 11,12-DiHET (ω-6) was also identified as an interesting target in our cohort as higher levels of this LM correlated with higher NBV and NCGMV. An earlier study reported increased levels of 11,12-DiHET in relapsing PwMS compared to non-relapsing PwMS.[22] Originally, DiHETs are believed to be non-functional degradation products of the EETs[33], which possess anti-inflammatory and neuroprotective properties under pathophysiological circumstances such as cerebral ischemia.[34,35] The positive correlation between 11,12-DiHET and brain volumes in our study could therefore be the result of higher levels of the functional, but relatively unstable precursor 11,12-EET. However, some studies

have proposed that several DiHETs, including 11,12-DiHET, may have some biological functions similar to their EET precursor and therefore 11,12-DiHET itself may be crucial in protecting the CNS against cellular damage[30]. However, these findings warrant further exploration.

In our cohort, plasma levels of the LA-derivatives 9- and 13-HoDE were significantly lower in PMS compared to HC. This decrease in LA-derivatives in PMS, together with elevated levels of the more downstream AA-derivatives, could be indicative of a LM switch in the ω-6 lipid pathway. We therefore speculate that changes in enzyme expression and/or activity required for the synthesis of ω-3/6 PUFA derivatives (e.g. ALOX-5,15-1/B, COX-1/2) may result in higher levels of 15-HETE and lower levels of 13-HoDE, presumably due to higher expression of ALOX15-B.[36] This is based on the fact that ALOX15-1, the main producer of 13-HoDE, more efficiently metabolizes LA than AA, whereas ALOX15-B, the main producer of 15-HETE, metabolizes LA very poorly, but in turn very efficiently metabolizes AA.[37] Decreased 13-HoDE levels were also (weakly) related to increased sGFAP levels in patients with RRMS and in the whole MS group. In the CNS, astrocytes are a major source of fatty-acid synthesis and we therefore speculate that astrocyte activation is linked to changes in the LM profile of PwMS.[9,10] However, additional studies are necessary to further assess this hypothesis.

In contrast to other studies, pro-inflammatory LMs like PGE_2 and leukotriene B_4 and D_4 (LTB_4, LTD_4), which levels are generally elevated and linked to severe inflammation[5], were insufficiently detectable in our study to include them in our data analysis. This limitation has been previously described[22] and may arise from the fact that PGE_2 has a rapid turnover rate *in vivo*.[38] Another possible explanation could be the relatively high age of our cohort combined with a long disease duration and its associated decrease of inflammatory activity compared to younger equivalents, thus showing reduced levels of these pro-inflammatory LMs. We therefore speculate that the correlations between other AA-derivatives and our clinical, biochemical and MRI parameters do not reflect acute inflammation per se, but may

represent the neurodegenerative processes in the CNS.

Several limitations of this study need to be addressed. Due to the cross-sectional nature of this study, conclusions about causality cannot be made. Furthermore, as our study design did not include gadolinium administration, we were not able to differentiate between active and inactive inflammatory CNS lesions. Third, we only assessed the EDSS as clinical measure. Although the EDSS is the most frequent used outcome measure in MS, the relation between LMs and other clinical (disability) measures might have revealed more detailed information. Based on our results, LMs could be useful in clinical trials addressing PMS at group-level, however, considering the overlap between patients with PMS and RRMS, LMs might be less valuable to inform clinical decisions at the individual level. A possible explanation for this overlap could be the higher age of the patients and the associated decrease in inflammation. Moreover, as the observed correlations between LMs and study outcome measures were weak, our results need confirmation by replication studies. Large longitudinal studies are warranted to study plasma and CSF LM profiles in PwMS with acute inflammation, during remission and during the more progressive phase of the disease, in order to define the exact LM profiles during disease pathogenesis and their potential contribution to neuropathological events in MS. These studies should control for all DMT types, as we considered DMT as present or absent.

In conclusion, in a cohort of patients of the same age, we show that several of the ω-3/6 associated LMs are related to CNS-related pathophysiological processes in MS. We demonstrate that patients with PMS in particular display an altered LM profile compared to RRMS and HC. This altered LM profile in PMS is mainly driven by increased levels of AA and its derivatives, which correlated to volumetric MRI measures, sNfL and disability. The AA-derivative 15-HETE best related to the EDSS, indicating that this AA-derivative may mark

neurodegeneration, reflected by disability. In general, these findings highlight the potential

relevance of LMs in the pathogenesis of MS.

SDC -- http://links.lww.com/WNL/C884

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Tables

Table 1. Demographic and clinical characteristics of patients with multiple sclerosis and healthy controls

	Healthy controls (n = 125)	All patients with MS (n = 285)	Patients with RRMS (n = 170)	Patients with PMS (n = 115)
Demographic and clinical parameters				
Age, y (SD)	52.9 ± 1.2	52.9 ± 0.9	52.9 ± 0.9	53.0 ±0.9
Female (%)	92 (74%)	204 (71.6%)	139 (81.8%)	65 (56.6%)[†]
BMI (SD)	25.6 ± 3.7	26.2 ± 4.9	26.6 ± 5.2	25.5 ± 4.2
NfL (pg/mL) (median, IQR)	8.8 (7.0 – 10.5)	9.8 (7.8 – 12.1)*	9.3 (7.4 – 11.5)	10.5 (8.8 – 14.0)[†]
Unknown	-	1 (0.4%)	1 (0.6%)	-
GFAP (pg/mL) (median, IQR)	51.7 (40.1 – 68.3)	63.7 (48.7 – 84.5)*	59.8 (45.8 – 77.4)	71.9 (50.1 – 100.6)[†]
Unknown		1 (0.4%)	-	1 (0.9%)
EDSS, (median, IQR)	-	3.5 (2.5 – 4.5)	3.0 (2.0 – 4.0)	5.5 (4.0 – 6.5)[†]
Age at MS diagnosis	-	40.8 (34.7 – 47.3)	41.1 (36.3 – 47.2)	40.5 (34.3 – 47.6)
Disease duration since symptom onset (median, IQR)	-	15.5 (8.8 – 24.5)	14.2 (8.1 – 23.3)	18.5 (10.3 – 25.7)
Current DMT, n (%)	-			
First line DMT[‡]		89 (31.2%)	68 (40.0%)	21 (18.3%)[†]
Second line DMT[§]		45 (15.8%)	26 (15.3%)	19 (16.5%)
DMT total duration (median, IQR)	-	6.2 (2.6 – 11.5)	6.4 (2.8 – 11.8)	6.0 (2.4 – 11.2)
Number or prior DMT used (mean, SD)	-	1.6 (0.9)	1.6 (0.9)	1.8 (0.9)
Smoking ever, n (%)	67 (53.5%)	180 (63.2%)	101 (59.4%)	79 (68.7%)
MRI volumes	**(n = 113)**	**(n = 229)**	**(n = 144)**	**(n =85)**
Normalized brain volume, L (mean, SD)	1.54 ± 0.077	1.48 ± 0.078*	1.49 ± 0.077	1.48 ± 0.078
Normalized cortical gray matter volume, L (mean, SD)	0.79 ± 0.051	0.76 ± 0.052*	0.76 ± 0.052	0.75 ± 0.052[†]
Normalized deep gray matter volume, mL (mean, SD)	63.53 ± 4.89	59.06 ± 5.39*	59.58 ± 4.86	58.18 ± 6.08[†]
Thalamic volume, mL (mean, SD)	21.36 ± 1.67	19.57 ± 2.01*	19.76 ± 1.88	19.25 ± 2.2[†]
Normalized white matter volume, L (mean, SD)	0.71 ± 0.043	0.69 ± 0.043*	0.69 ± 0.041	0.70 ± 0.045
Lesion volume, mL (median, IQR)	2.79 (1.97 – 4.40)	10.82 (5.97 – 20.05)*	9.78 (5.74 – 16.20)	13.85 (7.18 – 23.39)[†]

*Indicates a significant difference (p < 0.05) between all patients with MS and healthy controls (uncorrected)
[†] Indicates a significant difference (p < 0.05) between patients with RRMS and PMS (uncorrected)
[‡] Including interferon-beta, glatiramer acetate, teriflunomide, dimethyl fumarate
[§] Including ocrelizumab, natalizumab, fingolimod

Table 2. Pearson correlation matrices with clinical parameters and ω-3/6 LM's stratified by multiple sclerosis subtypes. Values depicted Pearson's R and are corrected for Body Mass Index (BMI), sex, disease modifying therapy (DMT) use (presence/absence), smoking (ever/never) and disease duration.

Substrate	LM's	Patients with PMS (n = 115)			Patients with RRMS (n = 170)			All patients with MS (n = 285)		
		EDSS	sNFL	sGFAP	EDSS	sNFL	sGFAP	EDSS	sNFL	sGFAP
LA (Ω-6)	9-HoDE	0.04	-0.16	-0.02	-0.08	0.09	-0.13	-0.08	-0.02	-0.10
	13-HoDE	0.08	-0.13	-0.08	-0.05	-0.01	-0.17*	-0.04	-0.08	-0.15*
	DGLA	0.08	0.07	0.05	0.02	0.00	0.04	0.12*	0.04	0.06
AA (Ω-6)	AA	-0.10	0.09	0.07	-0.06	-0.10	0.09	0.03	0.00	0.10
	AdA	0.13	0.18	-0.02	0.04	0.06	0.05	0.16**	0.13*	0.04
	5-HETE	0.14	0.13	-0.02	0.03	-0.02	-0.02	0.21***	0.08	0.02
	8-HETE	0.21*	0.16	-0.08	0.04	-0.08	-0.10	0.23***	0.05	-0.05
	15-HETE	0.23*	0.29**	0.03	0.12	0.07	0.01	0.24***	0.18**	0.05
	20-HETE	0.06	-0.04	-0.06	-0.01	-0.08	-0.08	0.07	-0.05	-0.05
	11,12-DiHET	0.02	0.08	-0.14	-0.05	-0.06	-0.08	0.10	0.03	-0.07
EPA (Ω-3)	11,12-DiHETE	0.01	-0.04	-0.06	0.00	-0.15	0.05	0.08	-0.06	0.03
	14,15-DiHETE	-0.04	-0.11	-0.22*	-0.05	-0.11	-0.07	0.04	-0.07	-0.11
	DPAn-3	0.00	0.06	-0.06	0.00	0.03	0.13	0.08	0.08	0.07
DHA (Ω-3)	19,20-DiHDPA	0.02	0.03	-0.20*	-0.01	-0.04	-0.08	0.09	0.02	-0.10

*p-value < 0.05; **p-value< 0.01; ***p-value < 0.001.

Table 3. Pearson correlation matrices with brain volume measurements and ω-3/6 LM's stratified by multiple sclerosis subtypes. Values depicted Pearson's r and are corrected for body mass index (BMI), sex, disease modifying therapy (DMTs) use (presence/absence), smoking (ever/never) and disease duration.

		LA (Ω-6)		AA (Ω-6)								EPA (Ω-3)			DHA (Ω-3)
		9-HoDE	13-HoDE	DGLA	AA	AdA	5-HETE	8-HETE	15-HETE	20-HETE	11,12-DiHET	11,12-DiHETE	14,15-DiHETE	DPAn-3	19,20-DiHDPA
PMS	Normalized total brain volume (NTBV)	0.09	0.06	-0.12	-0.13	-0.16	-0.16	-0.06	-0.24*	0.06	0.02	-0.03	0.08	-0.04	0.10
	Normalized white matter volume (NWMV)	0.00	-0.03	0.00	0.01	-0.06	-0.08	0.01	-0.16	-0.01	0.12	0.10	0.16	-0.01	0.15
	Normalized deep gray matter volume (NDGMV)	0.07	0.04	-0.19	-0.13	-0.21	-0.15	-0.15	-0.27*	-0.02	0.01	0.04	0.11	-0.07	0.05
	Normalized cortical gray matter volume (NCGMV)	0.13	0.11	-0.15	-0.19	-0.16	-0.15	-0.07	-0.20	0.11	-0.07	-0.12	-0.02	-0.05	0.03
	Thalamus volume	-0.01	-0.07	-0.09	0.06	-0.13	-0.04	-0.05	-0.17	0.03	0.14	0.23*	0.24*	0.01	0.15
	Lesion volume	0.02	-0.03	0.18	0.16	0.23*	0.20	0.06	0.21	0.12	-0.05	-0.05	-0.12	0.08	-0.11
RRMS	Normalized total brain volume (NTBV)	-0.05	0.05	-0.07	0.12	-0.08	0.06	0.14	0.12	0.07	0.20*	0.08	0.16	-0.14	0.13
	Normalized white matter volume (NWMV)	-0.06	0.01	-0.05	0.09	-0.07	-0.01	0.02	0.02	0.04	0.10	0.05	0.13	-0.03	0.12
	Normalized deep gray matter volume (NDGMV)	0.04	0.02	-0.05	0.16	-0.06	0.04	0.08	0.02	0.01	0.14	0.01	0.11	-0.15	0.06
	Normalized cortical gray matter volume (NCGMV)	-0.03	0.06	-0.05	0.09	-0.06	0.10	0.19*	0.16	0.07	0.21*	0.07	0.13	-0.17*	0.10
	Thalamus volume	0.04	0.05	-0.11	0.10	-0.10	0.03	0.06	0.00	-0.01	0.10	0.01	0.10	-0.15	0.05
	Lesion volume	-0.15	-0.14	0.14	0.00	0.16	0.07	0.01	0.11	0.04	-0.02	0.08	-0.01	0.21*	-0.03
All MS	Normalized total brain volume (NTBV)	0.01	0.05	-0.08	0.03	-0.11	-0.02	0.07	-0.01	0.07	0.12	0.03	0.12	-0.11	0.11
	Normalized white matter volume (NWMV)	-0.04	-0.01	-0.03	0.08	-0.06	-0.02	0.04	-0.02	0.02	0.12	0.08	0.15*	-0.02	0.14*
	Normalized deep gray matter volume (NDGMV)	0.06	0.04	-0.10	0.03	-0.12	-0.05	-0.02	-0.10	0.00	0.07	0.01	0.09	-0.13	0.04
	Normalized cortical gray matter volume (NCGMV)	0.04	0.09	-0.09	-0.01	-0.10	-0.01	0.08	0.01	0.08	0.09	-0.02	0.06	-0.14*	0.05
	Thalamus volume	0.03	0.01	-0.11	0.03	-0.12	-0.01	0.01	-0.07	0.01	0.09	0.09	0.13	-0.10	0.06
	Lesion volume	-0.10	-0.11	0.16*	0.07	0.18**	0.13	0.04	0.15*	0.08	-0.02	0.04	-0.03	0.17*	-0.04

*p-value < 0.05; **p-value< 0.01; ***p-value < 0.001.

Table 4. Linear regression models: EDSS. Significant LM correlates of disability (EDSS, Figure 3), sex (female vs. male), body mass index (BMI), disease modifying therapy (DMT) use (presence vs. absence), disease duration, MS subtype (progressive vs. relapsing remitting) and smoking (ever vs. never) were fed into a backward selection linear regression model (step 1) including all patients with MS. The final model was repeated after also adding serum neurofilament light (sNfL) and serum glial fibrillary acidic protein (sGFAP) (step 2) as fixed variables.

Predictors	Adjusted R^2	F value	P value	Beta	t	P value
1. LM model						
	0.46	79.66	P<0.001			
15-HETE				0.17	3.72	<0.001
MS subtype (progressive vs. relapsing)				0.58	12.98	<0.001
Disease duration				0.21	4.71	<0.001
2. Including sNfL and sGFAP						
	0.48	53.38	P<0.001			
15-HETE				0.15	3.47	<0.001
MS subtype (progressive vs. relapsing)				0.54	12.13	<0.001
Disease duration				0.19	4.34	<0.001
sNfL				0.12	2.45	0.015
sGFAP				0.10	2.18	0.030

Figure legends

Figure 1. Two-way hierarchical clustered heatmap shows differences in LM profiles related to the ω-3/6 lipid pathways. Heatmap with hierarchical cluster analysis (Euclidean distance) showing the distribution of Project Y cohort subjects (x-axis) over the three sample clusters that are based on the ω-3/6 lipid mediator plasma levels (y-axis). Table indicates the cohort group distribution over the sample clusters (chi-square test = 0.0008)
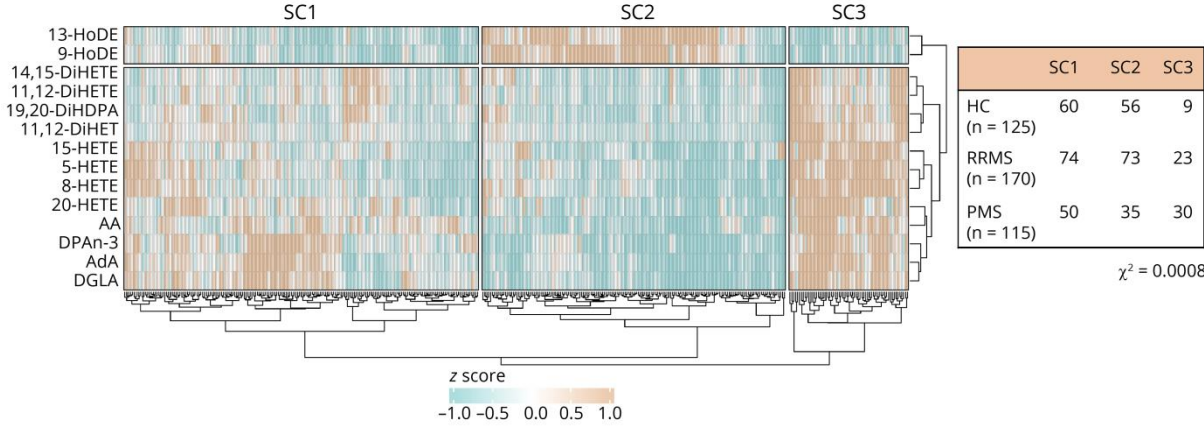

Figure 2A-C. Volcano plots displaying significant differences of ω-3/6 LMs between HC and MS groups. Volcano plots showing the log2Fold change of LMs between the healthy controls (HC) and A) patients with RRMS and B) patients with PMS. C) Log2Fold change of LMs between patients with RRMS and PMS. Level of significance is indicated on the y-axis as –log10(P-value).
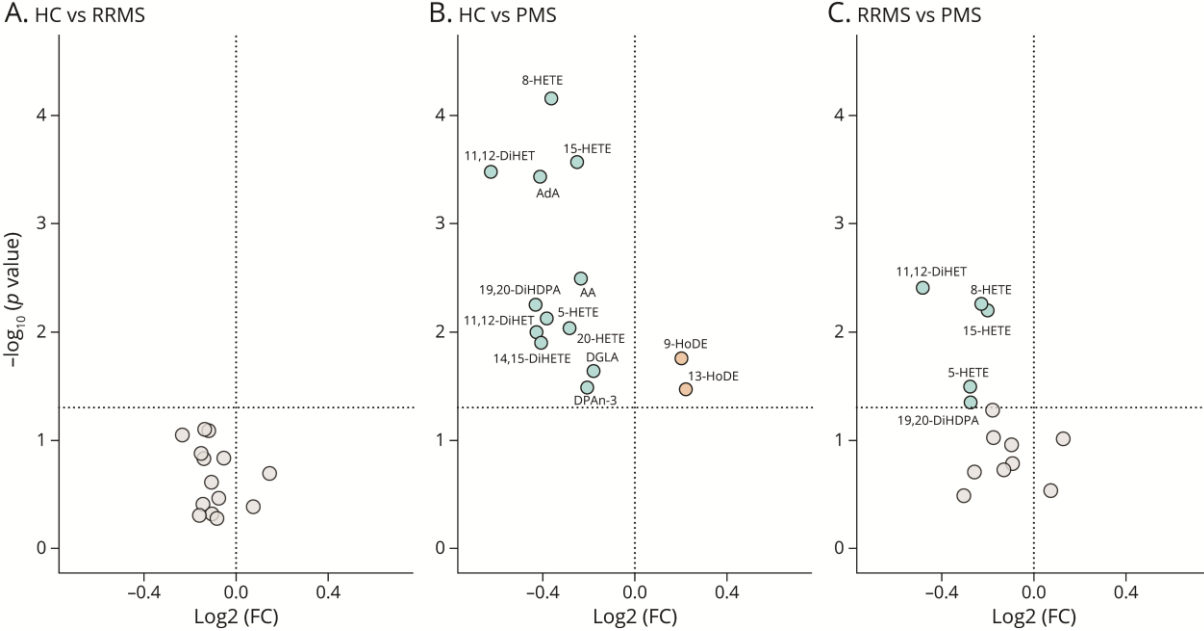
Figure 3. Schematic overview of ω-3/6 LM pathways and correlations with clinical parameters.
This figure represents all significant correlations between lipid mediators, clinical and biochemical parameters (EDSS, NfL and GFAP) in both patients with progressive MS and in the whole MS group. Symbols and colors are indicative of the direction of the correlation (orange / hexagonal = positive correlation, blue / square = negative correlation) and numbers are indicative for the clinical/biochemical correlate (1 = EDSS, 2 = sNFL, 3 = sGFAP).
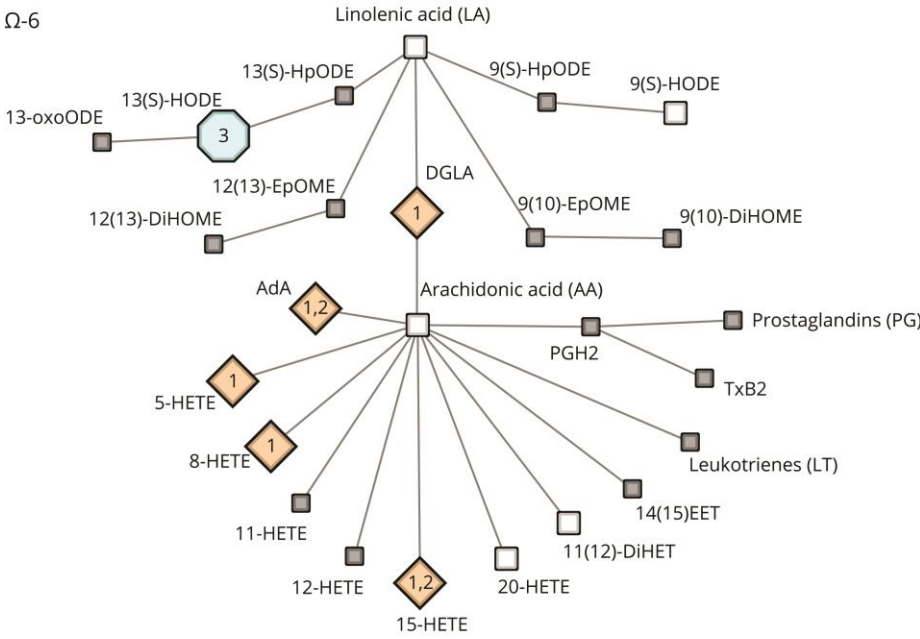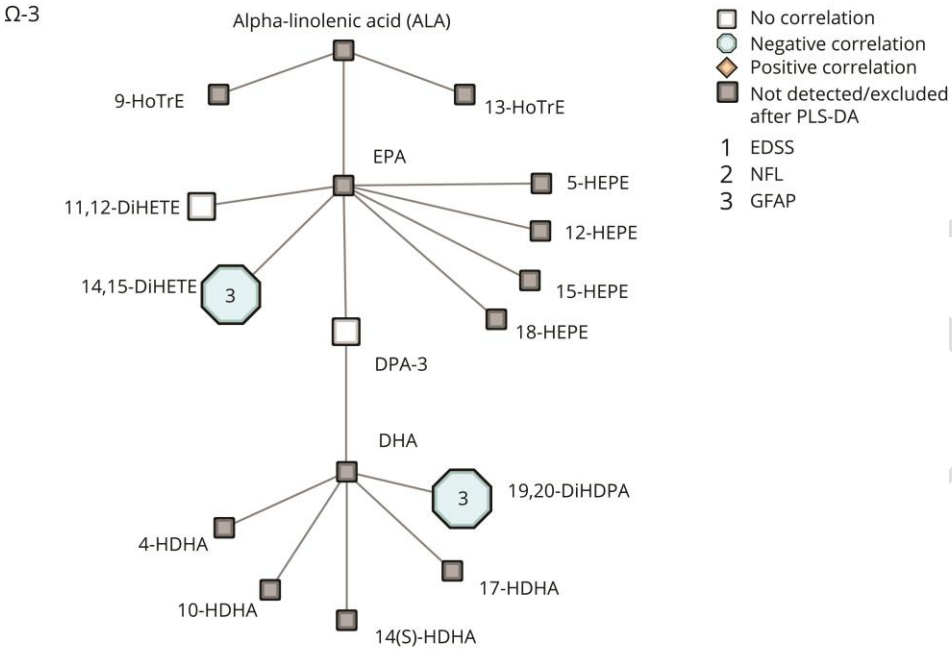
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Association of Arachidonic Acid-Derived Lipid Mediators With Disease Severity in Patients With Relapsing and Progressive Multiple Sclerosis

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