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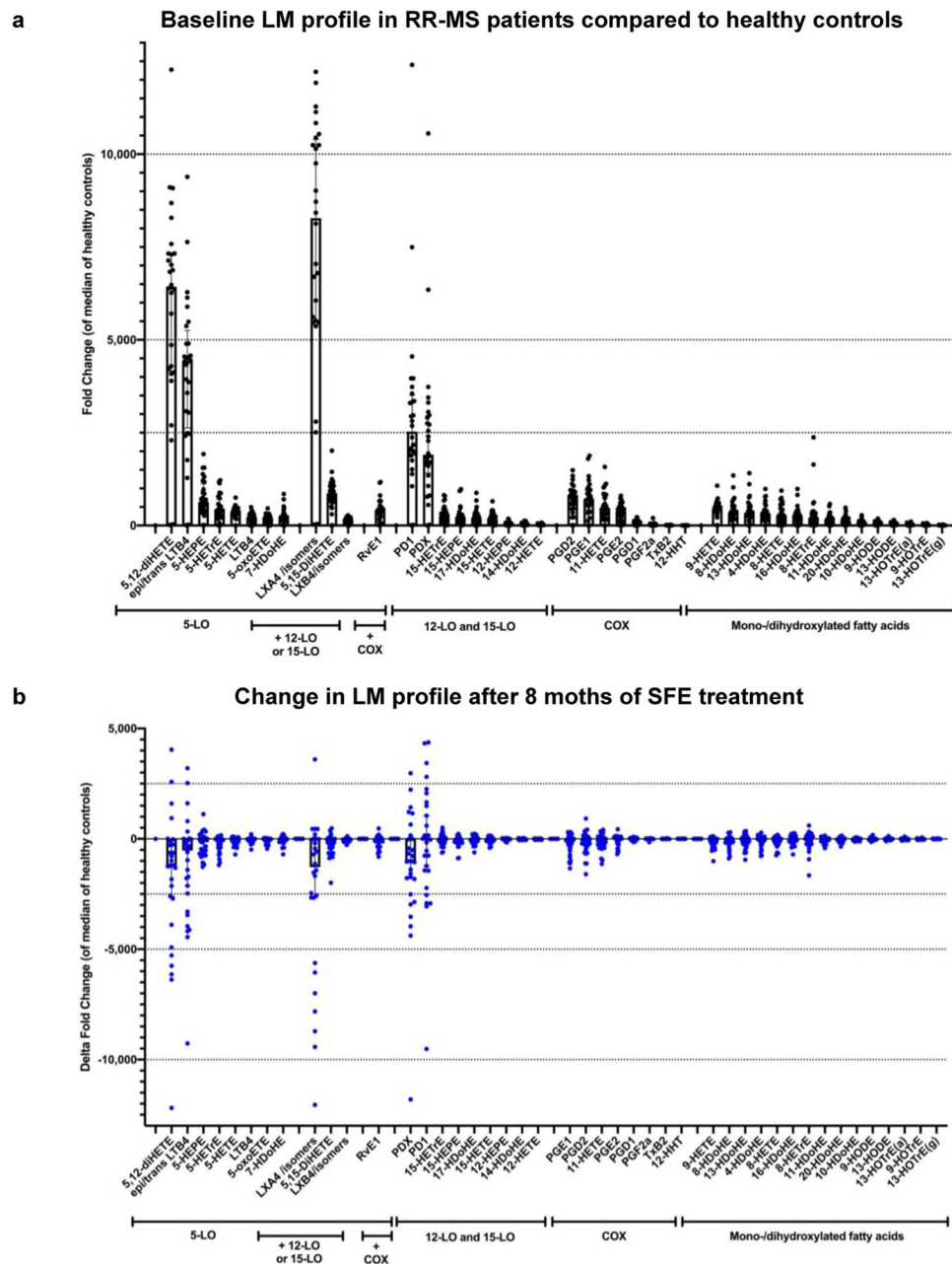
# Lipid Mediator Profiles Predict Response to Therapy with an Oral Frankincense Extract in Relapsing-Remitting Multiple Sclerosis

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Lipid mediators (LMs) are a unique class of immunoregulatory signalling molecules and known to be affected by frankincense extracts. We performed LM profiling by metabololipidomics in plasma samples from 28 relapsing-remitting multiple sclerosis (RR-MS) patients who took a standardised frankincense extract (SFE) daily for eight months in a clinical phase IIa trial (NCT01450124) and in 28 age- and gender-matched healthy controls. Magnetic resonance imaging, immunological outcomes and serum neurofilament light chain levels were correlated to changes in the LM profiles of the RR-MS cohort. Eight out of 44 analysed LMs were significantly reduced during an eight-month treatment period by the SFE and seven of these eight significant LM derive from the 5-lipoxygenase (5-LO) pathway. Baseline levels of 12- and 15-LO products were elevated in patients who exhibited disease activity (EDA) during SFE treatment compared to no-evidence-of-disease-activity (NEDA) patients and could predict treatment response to the SFE in a prediction model at baseline. Oral treatment with an SFE significantly reduces 5-LO-derived LMs in RR-MS patients during an eight-month treatment period. Treatment response to an SFE, however, seems to be related to 12-, 15-LO and cyclooxygenase product levels before SFE exposure. Further studies should confirm their biomarker potential in RR-MS and SFE treatment.

Multiple sclerosis is the most common debilitating chronic autoimmune disease of the central nervous system affecting more than 2 million predominantly young female adults worldwide<sup>1</sup>. Oral drugs exhibiting a favourable safety profile for the treatment of relapsing-remitting multiple sclerosis (RR-MS) are of high interest for patients and treaters because of the treatment's long-term perspective in RR-MS. Boswellic acids (BAs), the main biologically active ingredients of frankincense, are orally available and exhibit anti-inflammatory activities in combination with beneficial data on safety and tolerability<sup>2,3</sup>. Of these BA-associated anti-inflammatory responses, the best described are pharmacologically induced changes of the lipid mediator (LM) profile, in particular the inhibition of 5-lipoxygenase (5-LO)<sup>2</sup>, an enzyme which has been implicated in MS pathogenesis due to its expression in RR-MS brain lesions<sup>4</sup>. Little is known about the impact of LMs and their relevance to MS pathology, however accumulating data indicates that the importance of LMs has been underestimated in MS to date<sup>5</sup> and that LM-producing enzymes are involved in disease pathogenesis and progression<sup>6,7</sup>. Consequently, we analysed

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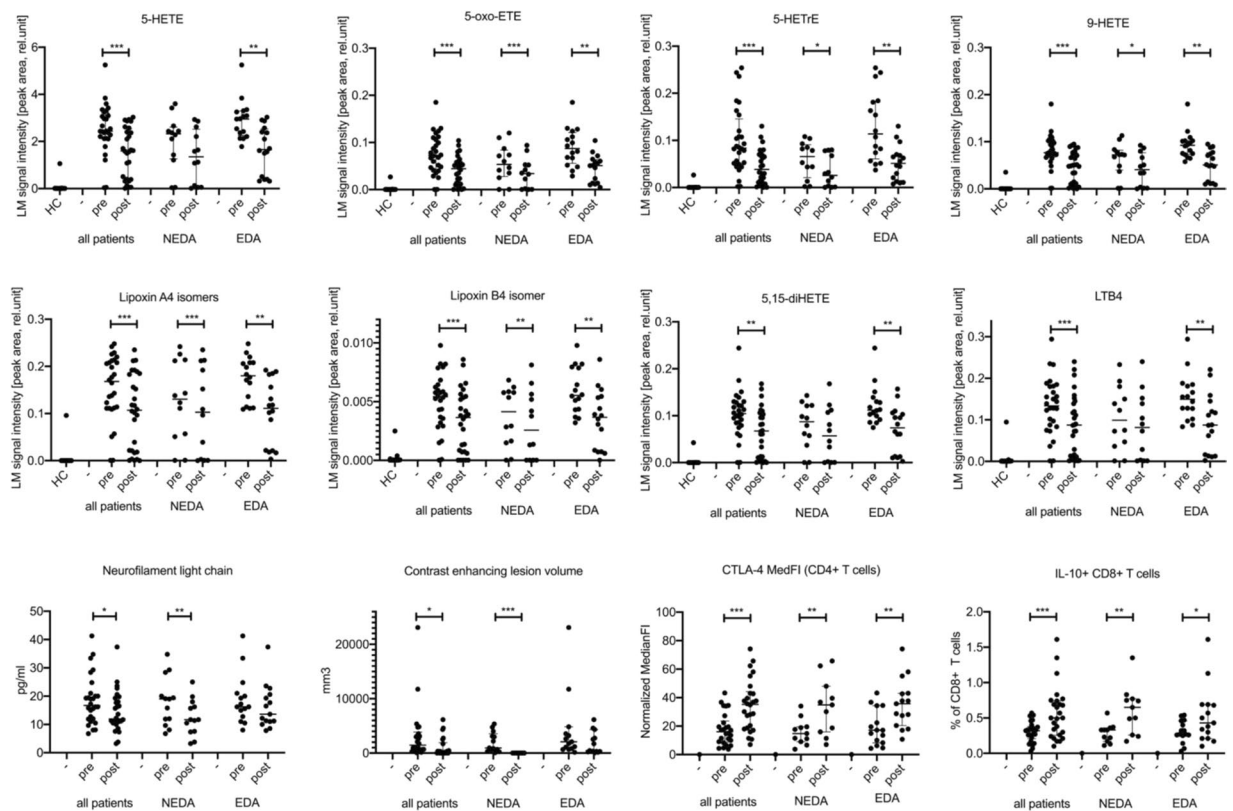


**Figure 1.** Lipid mediator profile in 28 untreated RR-MS patients (black, upper panel a) and changes (delta values) in the identical patient cohort after 8 months of treatment with a standardised frankincense extract (blue, lower panel b). Individual data are plotted as dots with median and interquartile range, in the lower panel data are presented as median delta values from baseline with interquartile range.

the LM profile in RR-MS patients before and after oral BA-treatment with an SFE for eight months in a clinical phase IIa trial.

## Material and Methods

We performed a bicentric, phase IIa, open-label, baseline-to-treatment trial between 2011 and 2017 with an orally available SFE produced by Alpinia Institute for Life Sciences AG (Walenstadt, Switzerland) at two German tertiary academic MS centers (SABA Trial<sup>8</sup>, NCT01450124). The study was approved by the German Federal Institute for Drugs and Medical Devices (BfArM, Approval No. 4036771), and by the local ethics committees (Ethik-Kommission der Ärztekammer Hamburg, Approval No. PVN3389 and PVN2758 and Ethikkommission der Charité – Universitätsmedizin Berlin, Approval No. ZS EK 13572/09). All participants gave their written informed consent at screening and the study and all experiments were conducted in accordance with the Declaration of Helsinki and with good clinical practice. Plasma and serum samples were collected before the start of treatment and at month 8, respectively, and stored at  $-80^{\circ}\text{C}$ , as described previously<sup>8</sup>. Samples originated



**Figure 2.** Treatment effect of a standardised frankincense extract (SFE) on selected lipid mediators, neurofilament light chain, contrast enhancing lesion volume, and immunological outcomes in the SABA trial ( $n = 28$  patients;  $n = 12$  NEDA and  $n = 16$  EDA patients). HC: healthy controls. Pre: baseline values before start of treatment, post: values after 8 months of continuous SFE treatment. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ .

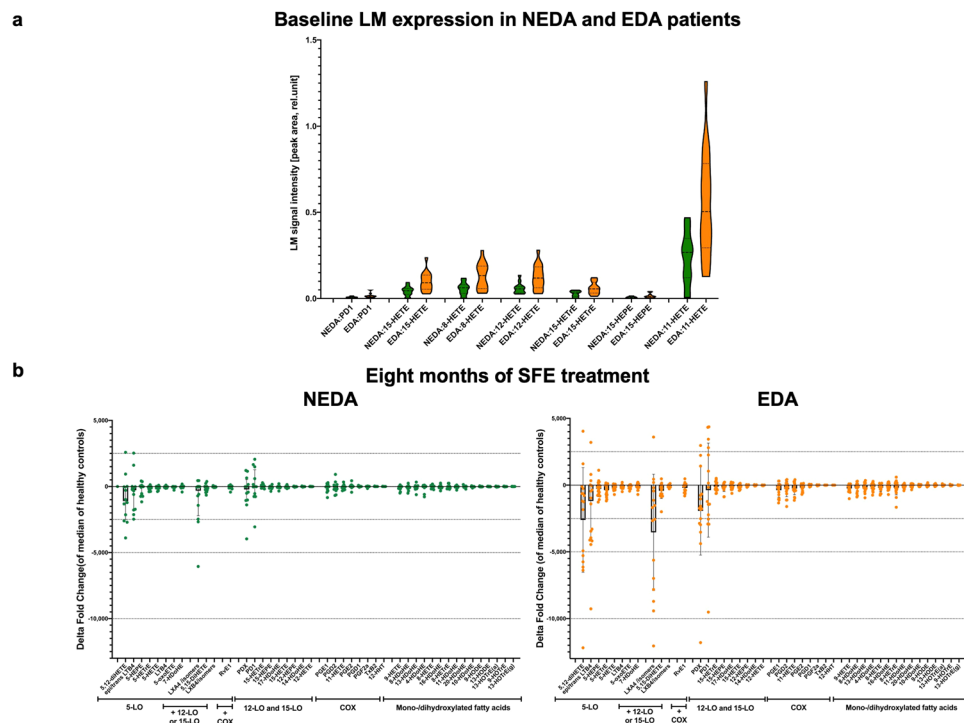
from  $n = 28$  RR-MS patients who took 3600 to 4800 mg of an SFE daily and from 28 age- and gender-matched healthy controls. LM profiling by metabololipidomics was performed by analysing a panel of 44 LMs using ultra-performance liquid chromatography ESI tandem mass spectrometry (UPLC-MS-MS)<sup>9</sup>. Signal intensities (peak areas) were normalized to the internal standard PGB1 to correct for differences in sample preparation.

Statistics were carried out using R<sup>10</sup> and the packages ggplot2<sup>11</sup> and corrram<sup>12</sup>. For calculating the fold-increase in RR-MS patients versus healthy controls the median value for each LM of the healthy control cohort was used as normalization value. LM analysis of longitudinal samples was performed using paired Wilcoxon tests (baseline vs. follow-up) with Bonferroni adjustment. LMs were also correlated with magnetic resonance imaging (MRI), immunological, biomarker outcomes (collected as described previously<sup>8</sup>) and serum neurofilament light chain (NfL) concentrations. NfL and Glial fibrillary acidic protein (GFAP) were measured using the Simoa NF-light<sup>®</sup> assay (Quanterix, USA). For analysing correlations, descriptive statistics with a Spearman's of  $p < 0.05$  were used. For further analysis, patients in the SABA trial were subdivided according to no-evidence-of-disease activity (NEDA) criteria (defined by lack of a relapse, lack of new T2 lesions and new contrast-enhancing lesions (CEL) and no confirmed disease progression (NEDA3)) into a NEDA group and an evidence of disease activity (EDA) patient group. EDA patients had at least either a relapse, a new T2 lesion or CEL or a confirmed disease progression in EDSS during the treatment phase of the trial. Combined analysis for prediction of NEDA status using LM expression was performed using the Combirc Online Tool<sup>13</sup>.

## Results

**Lipid mediator expression differs between healthy controls and MS patients.** Except for 12-HHT, measured LMs were significantly higher in RR-MS patients compared to healthy age- and gender-matched controls with the topmost fold-increase ( $>1000$ -fold) in five LMs being: the three 5-LO-derived LM lipoxin A<sub>4</sub> and/or isomers, 5,12-di-hydroxyeicosatetraenoic acid (5,12-diHETE), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), and the two 12- or 15-LO-derived LM protectin D1 (PD1) and protectin DX (PDX). Thirty-one LMs (i.e. 70%) were  $>100$ -fold more abundant in RR-MS than in healthy donors (Fig. 1a, Supplemental Table S1).

**An eight-month treatment with an SFE changes the LM profile in RR-MS patients.** After receiving a SFE for eight months, a general decrease of LMs was observed (Figs. 1b and 2). However, only eight of 44 analysed LMs were significantly reduced in the 28 RR-MS patients. These were: 9-HETE, lipoxin A<sub>4</sub> and/or isomers, lipoxin B<sub>4</sub> and/or isomers, 5,15-diHETE, LTB<sub>4</sub>, 5-HETE and its metabolites 5-oxo-eicosatetraenoic acid (5-oxo-EETE), and 5S-hydroxy-6E,8Z,11Z-eicosatrienoic acid, (5-HETrE). Seven of these eight LMs are generated



**Figure 3.** Upper Panel a: Baseline expression of selected LMs ( $p \leq 0.01$ ) in NEDA (in green,  $n = 12$ ) versus EDA MS patients (orange,  $n = 16$ ) of the SABA trial. LM expression is plotted as violin plots indicating median (dashed line) and interquartile range (dotted line) of the LM signal intensity measured as peak area. Lower Panel b: Changes (delta values) in NEDA ( $n = 12$ , green) versus EDA ( $n = 16$ , orange) SABA patients after eight months of treatment with a standardised frankincense extract. Individual data are plotted as median of delta values from individual baseline values of the fold increase versus healthy controls with interquartile range.

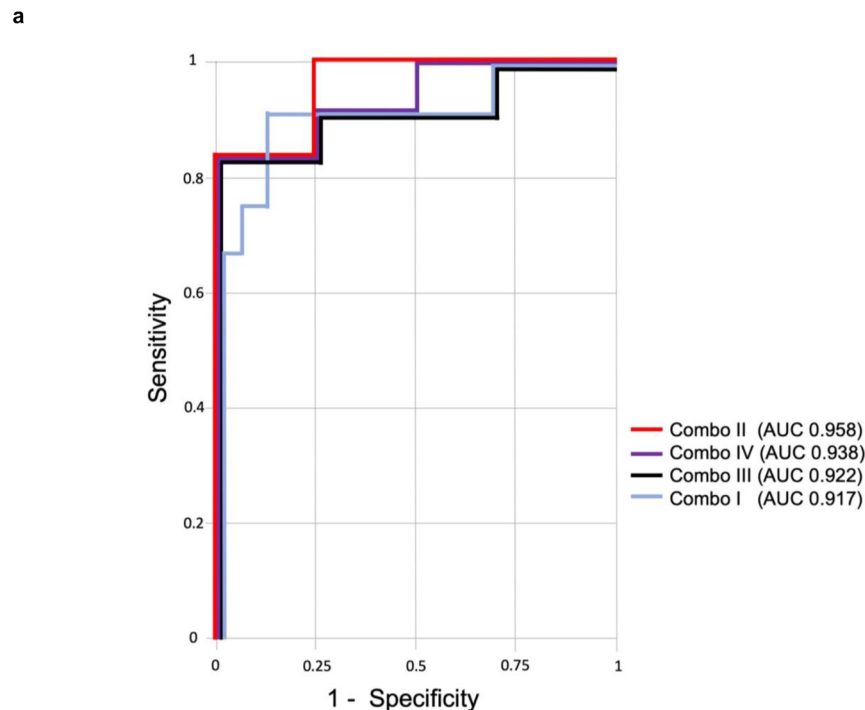
by the 5-LO pathway, while 9-HETE is predominantly produced by non-enzymatic oxidation of arachidonic acid. For seven of these LMs the reduction was more pronounced in EDA patients when compared to MS patients fulfilling NEDA criteria during SFE treatment (Figs. 2, 3b and Supplemental Table S2) thereby triggering further analyses of the NEDA and EDA subgroups. Only for 5-oxo-EETE, a bioactive metabolite of 5-HETE, the reduction during SFE treatment was more pronounced in NEDA patients (Fig. 2 and Supplemental Table 2).

**Baseline expression of 12- and 15-LO derived lipid mediators predicts treatment response to an SFE.** When we analysed the LM profile of NEDA and EDA patients of the trial cohort at baseline, certain LMs were already found to be increased in the trial subjects, later defined as EDA RR-MS patients (Fig. 3a). When analysing the 7 LMs with the lowest p-values (all  $p \leq 0.01$ : PD1, 11-HETE, 12-HETE, 15-HETE, 8-HETE, 15-HETrE, 15-HEPE) for their performance in predicting the response to SFE treatment, the ROC curve yielded results of 83% for sensitivity and 100% for specificity (AUC 0.958, Fig. 4), indicating that these LMs could be useful as biomarkers to predict therapy response to an SFE prior to treatment. For the majority of these markers, 12- and 15-LOs are involved in their biosynthesis.

**LM correlations with biomarkers of MS are dependent on treatment response to an SFE.** Correlation analysis (Fig. 5, Suppl. Tables S3–5) between LM and MRI, immunological and NfL outcomes in the complete SFE-treated patient cohort revealed that LMs showed only moderate correlations in the total patient cohort. However, when the SFE-treated RR-MS patient cohort was split according to NEDA criteria, the correlation matrices revealed differing patterns in the NEDA versus EDA groups (Fig. 5, middle and right panel).

For NEDA patients, three strong negative correlations involving LMs were apparent (Fig. 6a and Suppl. Table 4). These were: 1)  $PGE_1$  and the proportion of IL-10 positive CD8+ T cells ( $r = -0.754$ ), 2)  $PGE_1$  and the proportion of IL-17+ CD4+ T cells ( $r = -0.736$ ) and 3) CD86 expression on myeloid dendritic cells and the 5-LO-derived LM resolvin E1 (RvE1,  $r = -0.645$ ). The significant strong positive correlations involving LMs in NEDA patients were changes in: 1) CTLA-4 expression and 12-HHT ( $r = 0.728$ ) and 2)  $PGD_2$  and the proportion of Foxp3+ CD4+ T cells ( $r = 0.727$ ). Intra-individual NfL concentration changes during the trial showed a strong negative correlation with the significantly decreased 5-LO products 5-HETrE ( $r = -0.637$ ) and 5-oxo-EETE ( $r = -0.648$ ) and additionally with 12-HHT ( $r = -0.714$ ). These negative correlations between NfL and LMs were only seen in the NEDA patient group.

Contrastingly, positive correlations were found in EDA patients for TGF- $\beta$  and 12-HHT and for several 12/15-LO products and CD86 surface expression on myeloid dendritic cells (Fig. 6b and Suppl. Table 5). Strong



**Figure 4.** (a) Overlay of multiple ROC curves for the LMs of Fig. 3a in the CombiROC analysis. (b) Results from the CombiROC analysis for the prediction of EDA status/Non-Response to SFE treatment from baseline LM levels.

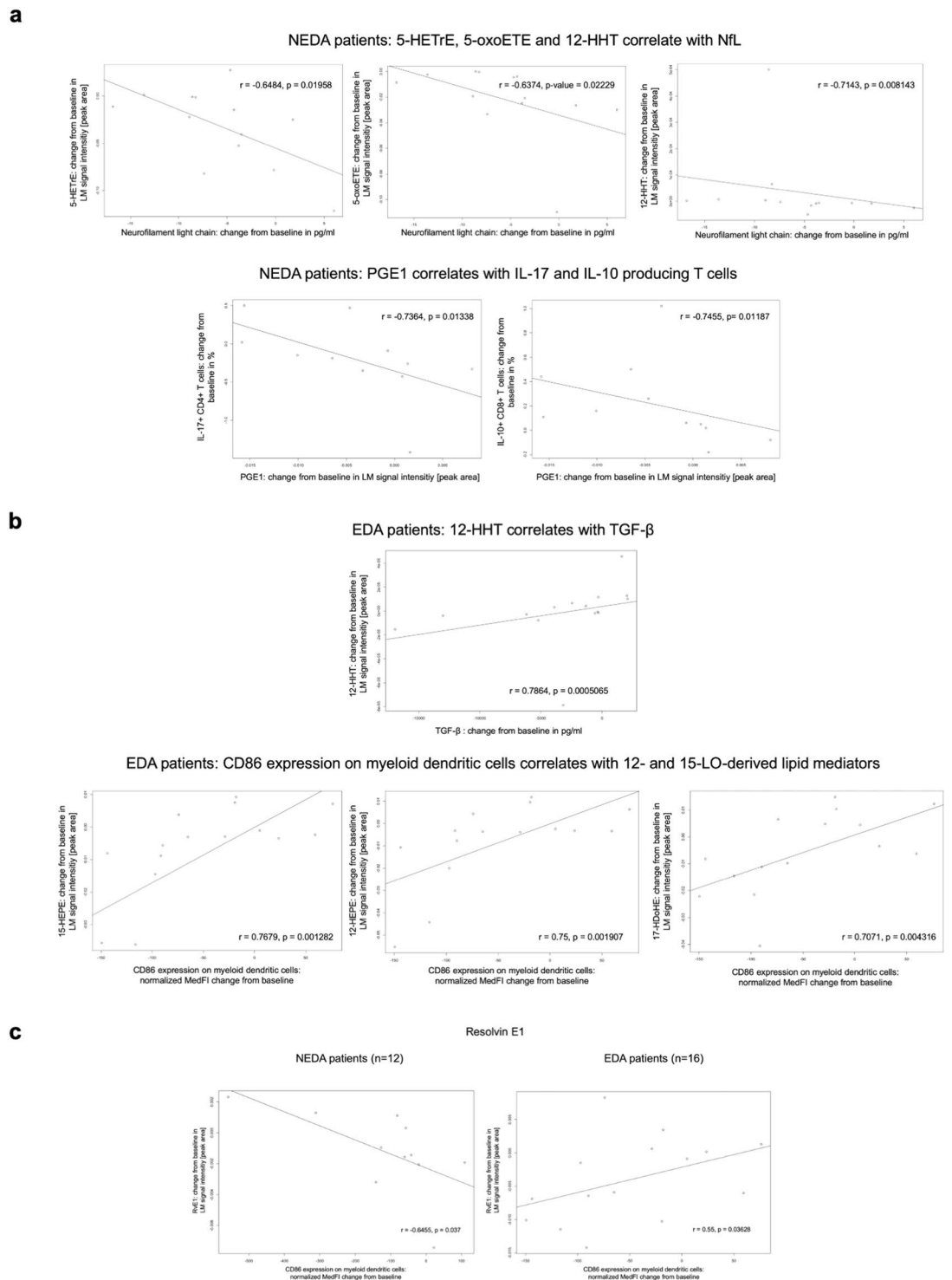
negative correlations were evident in EDA patients for serum IL-17 levels and CTLA-4 ( $r = -0.696$ ),  $\text{PGD}_2$  ( $r = -0.615$ ) and  $\text{PGE}_2$  ( $r = -0.604$ ), respectively.

When comparing the correlation matrices of NEDA and EDA patients, the most prominent difference indicated that COX-derived LMs correlated with other LMs only in EDA patients and failed to do so in the NEDA patient cohort (Fig. 5). The most striking single difference between the NEDA and EDA subgroups during SFE treatment was a correlation between CD86 expression on myeloid dendritic cells and the 5-LO-derived LM RvE1 (Fig. 6C), which was moderate and positive in EDA RR-MS patients ( $r = 0.55$ ) and strong and negative in NEDA patients ( $r = -0.645$ ).

Irrespective of the analysed LMs prominent correlations among the other analysed biomarkers in the total SABA patient cohort were: 1) changes in NfL serum concentration and the CEL volume, 2) the proportion of IL-17+ CD4+ T cells and CD40 surface expression of myeloid dendritic cells and 3) changes in NfL serum concentration and the proportion of IL-10+ CD8+ T cells (Suppl. Figures 1–3). In NEDA patients changes in CTLA-4 expression on CD4+ T cells and the volume of CEL in MRI showed a strong correlation ( $r = -0.836$ , Suppl. Figure 4). GFAP serum levels remained unaltered during treatment (Suppl. Figure 5). Most of these observations confirm previous reports<sup>8,14,15</sup> and can serve as a quality control of the data set.







**Figure 6.** Strongest LM correlations in (a) NEDA and (b) EDA RR-MS patients of the SABA trial; (c) shows the correlation plots for Resolvin E1 in NEDA (left) versus EDA (right) RR-MS patients of the SABA trial.

higher COX-controlled LM levels could also indicate the involvement of another dioxygenase in active RR-MS patients. One such dioxygenase that is known to be inhibited by COX is indoleamine 2,3-dioxygenase (IDO), a key enzyme in the kynurenine pathway, which exhibits immune checkpoint function in MS<sup>26,27</sup>. A pre-existing enhancement of COX activation in active RR-MS patients is thus likely to lower IDO expression and thereby prevent an immune therapeutic effect of BAs in these patients, while in the NEDA patients, the lower COX activity allows BAs to take effect.

## Conclusion

Oral treatment with an SFE significantly reduces bioactive 5-LO-derived LMs, MRI disease activity, and peripheral neurofilament light chain concentrations in RR-MS patients during an eight-month treatment period. However, inhibition of 5-LO *per se* is not associated with a treatment response to an SFE. In EDA patients during SFE treatment, LMs produced by 12- or 15-LOs are primarily upregulated before the start of the treatment and might be useful as predictors of an SFE-mediated treatment response in the future. When comparing correlation plots of the parameters analysed in the total SABA patient cohort, in the NEDA responder subgroup and the EDA treatment failure patient subgroup, different correlation profiles can be detected indicating a difference in the regulation of 12/15-LO- and COX-derived LM in these respective subpopulations and their associated immunological profiles. Further studies should investigate the potential of LMs as biomarkers in RR-MS and the mechanism of frankincense extracts in the suppression of disease activity in RR-MS.

## Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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### Author contributions

K.H.S., C.H., F.P. and O.W. designed the study, K.H.S., F.P., O.P. collected data. K.H.S., O.W., A.K., M.O., F.L., F.P., O.P. and C.H. analysed the data. K.H.S. and F.L. performed the statistical analysis. K.H.S., O.W., A.K., F.L., F.P. and C.H. wrote the report. K.H.S., O.W., A.K., M.O., F.L., F.P., O.P. and C.H. interpreted data and revised the report.

### Competing interests

Klarissa Hanja Stürner has received personal fees and travel grants from Bayer, Biogen, MerckSerono and Genzyme during the last 5 years. F. Leypoldt reports speaker honoraria from Bayer, Roche, Novartis, Fresenius, travel funding from Merck, Grifols and Bayer and serving on advisory boards for Roche, Biogen and Alexion. Friedemann Paul has received honoraria and research support from Alexion, Bayer, Biogen, Chugai, MerckSerono, Novartis, Genzyme, MedImmune, Shire, Teva, and serves on scientific advisory boards for Alexion, MedImmune and Novartis. He has received funding from Deutsche Forschungsgemeinschaft (DFG Exc 257), Bundesministerium für Bildung und Forschung (Competence Network Multiple Sclerosis), Guthy Jackson Charitable Foundation, EU Framework Program 7, National Multiple Sclerosis Society of the USA. Ole Pless, Oliver Werz and Andreas Koeberle have nothing to disclose and declare no potential conflict of interest. Markus Otto served as consultant for Biogen, Roche, Axon Neuroscience and Fujirebio. C. Heesen has received travel and research grants from Biogen, Genzyme, Merck, Novartis and Roche.

### Additional information

**Supplementary information** is available for this paper at <https://doi.org/10.1038/s41598-020-65215-6>.

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