

BRIEF COMMUNICATION

Plasma lipidomics of monozygotic twins discordant for multiple sclerosis

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Abstract

Blood biomarkers of multiple sclerosis (MS) can provide a better understanding of pathophysiology and enable disease monitoring. Here, we performed quantitative shotgun lipidomics on the plasma of a unique cohort of 73 monozygotic twins discordant for MS. We analyzed 243 lipid species, evaluated lipid features such as fatty acyl chain length and number of acyl chain double bonds, and detected phospholipids that were significantly altered in the plasma of co-twins with MS compared to their non-affected siblings. Strikingly, changes were most prominent in ether phosphatidylethanolamines and ether phosphatidylcholines, suggesting a role for altered lipid signaling in the disease.

Introduction

Multiple sclerosis (MS) exhibits high interpersonal variance and our capability to predict individual disease courses is limited.¹ Thus, there is an unmet need for easily accessible biomarkers to support the diagnosis and monitor disease activity as well as treatment response.² Single-molecule arrays have uncovered serum neurofilament light chain as a protein-marker for axonal damage in MS and other diseases.³ However, in MS the inflammatory process also results in extensive damage to myelin sheaths, which are multilayered membrane stacks, highly enriched in lipids such as galactosylceramides, sulfogalactosylceramides, and ether-linked phospholipids.⁴ To date, the lipidome of MS-patients is not well defined and unbiased studies with an open, untargeted approach to lipidomics are scarce.^{5–7} While so far the reproducible quantification of entire lipid profiles in multiple samples has been challenging, the recent development of high throughput and quantitative shotgun lipidomics has overcome this limitation.^{8,9} We used this technology to obtain a detailed and quantitative assessment of the plasma lipidome of MS patients including features such as fatty acyl chain length and number of acyl chain double bonds. To eliminate genetic heterogeneity and reduce environmental influences, we focused our analysis on 73 monozygotic twin pairs, discordant for MS.

Subjects/Materials And Methods

Study Cohort

Twins were recruited nation-wide in Germany. Inclusion criteria for study participation were met for monozygotic twins with an MS diagnosis according to the 2010 revised McDonald criteria or clinically isolated syndrome in one twin only.¹⁰ In total, we included 73 monozygotic twin pairs (see Table 1 for cohort description) as previously described.¹¹ Blood sampling was always performed in the morning in fasting condition. The local ethics committee of the Ludwig Maximilians University of Munich approved the study and all participants gave written informed consent.

Lipidomics

Lipid species and subspecies are annotated according to their molecular composition as described previously and lipid identifiers are provided¹² (Table S1, Table 1).

One μL of plasma was analyzed using Shotgun Lipidomics platform by Lipotype GmbH (Dresden, Germany), as described previously.⁸ Lipidomic data were analyzed as

Table 1. Cohort description.

	Healthy	Multiple Sclerosis	Wilcoxon matched-pairs signed rank test
Number of twins	73		
Age, yrs. [mean \pm SD]	40.8 \pm 12.1		
Female [%]	75.3 %		
BMI [mean \pm SD]	24.7 \pm 4.8	24.3 \pm 4.9	$P = 0.23$
Disease course [%]			
RRMS	75 %		
SPMS	19 %		
PPMS	3 %		
CIS	3 %		
Patients without treatment, no. (%)	20 (27%)		
Disease duration, yrs. [mean \pm SD]	-	11.4 \pm 9.6	
EDSS	0.3 \pm 0.5	2.8 \pm 2.0	$P < 0.0001$

BMI body mass index defined as body mass divided by the square of the body height, expressed in kg/m^2 . RRMS relapsing-remitting multiple sclerosis (MS), SPMS secondary progressive MS, PPMS primary progressive MS, CIS clinically isolated syndrome. EDSS Expanded Disability Status Scale is a method of quantifying disability in multiple sclerosis only. Scale range from 0 to 10, where 0 comprises a normal neurological exam and 10 is defined as death due to MS. In the healthy co-twins abnormal neurological signs were documented using the EDSS as well for better comparison, even though the etiology of these slight abnormal neurological findings remains unknown.

described,⁸ with criteria on lipid identification, batch- and drift correction as applied earlier¹³ using R version 3.6.1 (2019-07-05) and tidyverse packages (version 1.2.1). A 66% occupational threshold was applied to the data, that is, we only considered lipids, which occur in at least 66% of the samples in either healthy co-twins or in co-twins with MS, for further analysis. Data were mostly normally distributed and no bias of missing data was detectable before thresholding (Table S2, Figure S1). Threshold application leads to a reduction from 722 measured lipids to 243 lipids for further comparison. If not stated otherwise lipids are measured in molar amounts normalized by the total lipid amount (mol%) per person.

Statistics

If not stated otherwise, features between healthy co-twins (H) and co-twins with MS are analyzed by matched comparison using a paired ratio t-test with significant results defined as $P < 0.05$. Adjustment for multiple testing is performed with the method of Benjamini and Hochberg (BH) ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Results

We performed shotgun lipidomics in plasma samples of 73 monozygotic twins discordant for MS (see Table 1 for cohort description) and analyzed a total of 243 lipid species after application of an occupational threshold of 66% (Table S1, Table S2). Total lipid amount did not differ between healthy co-twins and MS co-twins and principal component analysis revealed no separation between groups (Figure S1A and Figure S2A). Surprisingly, we found a significant reduction in ether phosphatidylcholines (PC O-), ether phosphatidylethanolamines (PE O-) and phosphatidylcholines (PC) in MS co-twins, of which the first two persist after correction for multiple comparison (Fig 1A; Table S3, Table S1). Of note, both PC O- and PE O- represent ether lipids, in which an ether bond attaches the hydrocarbon chain, instead of an ester bond as in the more common diacyl phospholipids. Detailed analysis of these lipid classes revealed that the reduction in PC O- species is accompanied by a relative reduction in certain acyl chains (i.e., bound fatty acids) within this lipid class. Namely, docosapentaenoic acid (DPA, C22:5), other polyunsaturated fatty acids (C22:4, C20:3, C20:4) and ether bound saturated/ monounsaturated alkyls (O-16:0;0, O-16:1;0 and O-18:1;0) were significantly reduced and the reduction in DPA maintained significance after correction for multiple testing ($P = 0.00047$, $q = 0.011$, mean reduction (MS/H) = 0.838).

On lipid species level, we detected 43 differentially concentrated lipid species between healthy and MS co-twins, thereby constituting 18% of all measured lipid species (Fig. 1B and C; Table S3, Table S2). In detail, 47% of PE O-, 44% of PC O-, 21% of PC, 13% of PI, and 9% of phosphatidylethanolamine (PEs) species were altered (7 PE O-, 18 PC O-, 15 PC, 2 PI, 1 PE species in absolute numbers). After correction for multiple comparison 1 lipid subspecies, namely PC O-16:1;0/20:3;0 remained as true discovery (0.0162 ± 0.00437 mol% (H) vs. 0.0143 ± 0.00511 mol% (MS), $P = 0.00006$, $q = 0.015$, mean of fold change (MS/H) 0.898). Of note, top hits at the species level are dominantly PC O- species with polyunsaturated fatty acids and the vast majority of significantly changed species, which we define by a nominal P -value < 0.05 for this report, are slightly decreased in MS co-twins (Figure 1C and D). Of note, paired multivariate analyses reveal a small separation between healthy co-twins and co-twins with MS and discriminating features comprise the PC O- species from the top hits from univariate testing (Figure S2). To explore if the observed alterations are rather due to MS pathology or to a side effect of disease-modifying treatment we analyzed the significantly altered lipid species in the subgroup of untreated twin pairs. Reassuringly, this led to an increase

in changes for most species indicating a genuine link to MS pathology (median of fold change MS/H_{all} = 0.96 vs MS/H_{untreated} = 0.93, $P = 0.0005$, Wilcoxon matched-pairs signed-rank test).

Discussion

We performed comprehensive, untargeted lipidomics in plasma samples of a unique cohort of 73 monozygotic twins discordant for MS. We measured 243 lipid species, and found that PC O- containing polyunsaturated fatty acids are decreased in MS. This observation was mirrored on a class level by a decrease in the ether lipid classes PC O- and PE O- and was accompanied by a reduction in specific polyunsaturated acyls and ether-bound saturated/ monounsaturated alkyls.

One of the unique features of our study cohort is increased test power due to matched comparison among genetically identical twins. Thus, unmatched comparison results in a reduction in significant hits and maintains a total of seven differentially concentrated lipids, which all belong to the PC O- and PC class and include PC O-16:1;0/20:3;0. On class level, reduction in PC O- persists, if an unmatched test is performed.

Ethers, as PC O- and PE O-, do not only effect biophysical properties of membranes (e.g., lipid packing), but do also play an important role in lipid signaling and inflammation.¹⁴ Ether lipids are enriched in polyunsaturated fatty acids as arachidonic acid (AA) and docosahexaenoic acid (DHA), which can be released upon activation of phospholipase A2. Eicosanoids, their oxygenated products, are major signaling compounds in inflammation.¹⁵ Furthermore, ethers are thought to be protective against oxidative stress by acting as sacrificial antioxidants and are potential ligands of peroxisome proliferator-activated receptor gamma, a transcription factor with metabolic and anti-inflammatory functions.^{14,16,17} Ether lipids and polyunsaturated fatty acids are also decreased in autoimmune diseases like systemic lupus erythematosus.¹⁸ Therefore, it seems more likely that the observed reduction in ethers is a general consequence of prolonged inflammation, even if a MS-specific pathophysiological role cannot be excluded. In this study, we used monozygotic twin pairs with discordant health status as a genetically matched case-control cohort for a quantitative plasma lipidome analysis. As alterations increase in the untreated subgroup of twin pairs, the observed lipidome variations seem to be genuinely linked to disease rather than to treatment effects. Further studies are required to validate our results in larger, genetically heterogenous MS cohorts, to understand the specificity and pathophysiological role of lipidome alterations in MS and to explore if

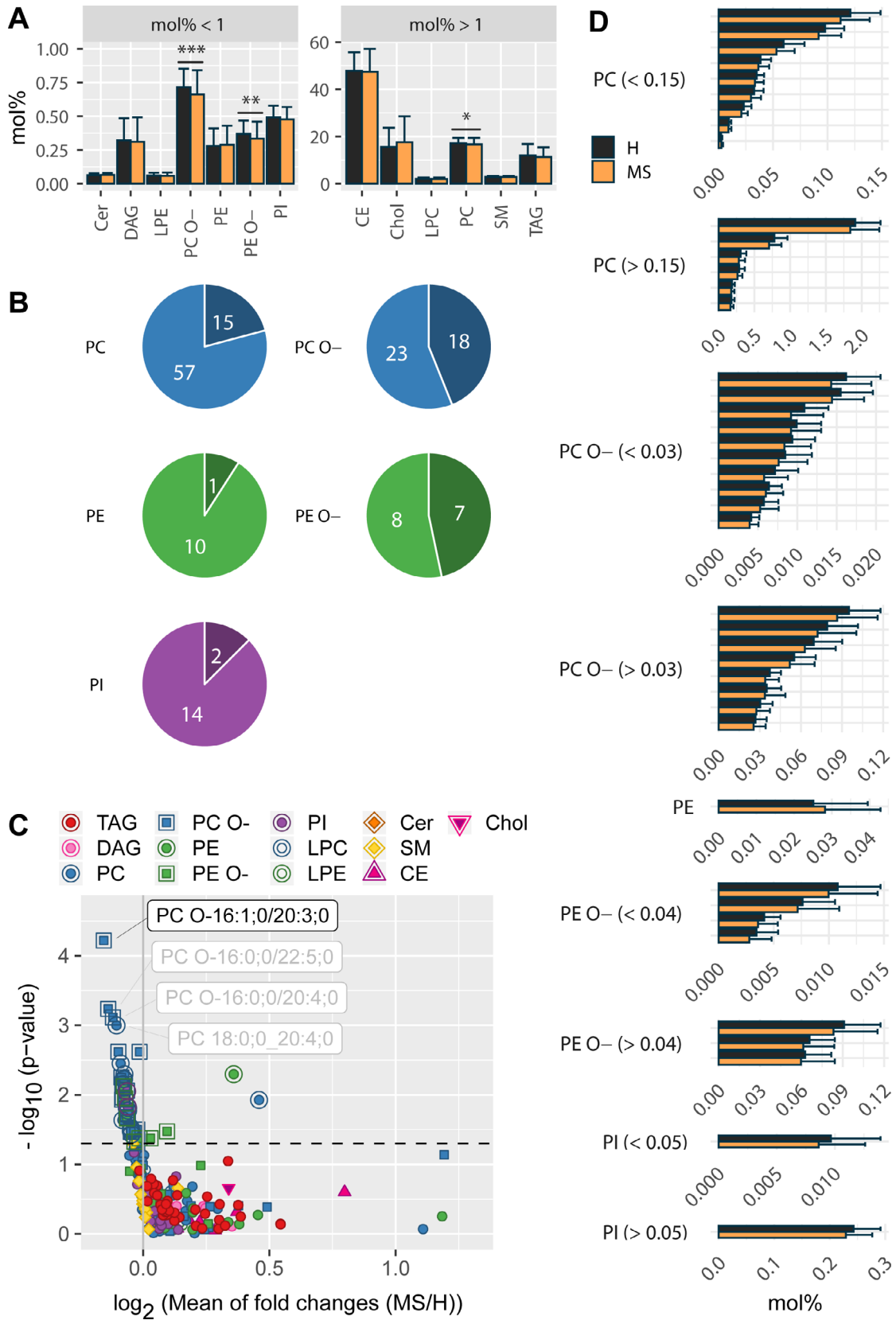


Figure 1. Altered lipid classes and species in monozygotic co-twins discordant for Multiple Sclerosis. (A) On lipid class level, PC O-, PE O-, and PC are significantly decreased in MS co-twins ($P = 0.00081$, $P = 0.0015$, $P = 0.017$, and $q = 0.0095$, $q = 0.0095$, $q = 0.074$, respectively). Y axis depicts lipids in molar amounts normalized by the total lipid amount per participant [mol%]. Paired ratio t-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (B) Number of significantly altered lipid species (darker color) as parts of whole of the corresponding lipid class. (C) A Volcano plot of all measured lipid species shows that most significantly altered lipid species are less concentrated in participants with MS and that species with the smallest p-values consist mainly of PC and PC O- species. X axis shows \log_2 of fold changes (MS/H), gray vertical line depicts $x = 0$ (MS = H), Y axis shows $-\log_{10}$ of P-values (paired ratio t-test), dotted horizontal line depicts $P = 0.05$. Significantly changed species ($P < 0.05$) are marked with an additional outline. (D) Significantly altered lipid species between healthy (black) and MS (orange) co-twins grouped per lipid class. X axis depicts lipids in molar amounts normalized by the total lipid amount per participant [mol%]. Only significant results are illustrated. (B), (C) Lipids are colored according to lipid class. TAG Triacylglycerol, DAG Diacylglycerol, PC Phosphatidylcholine, LPC Lysophosphatidylcholines, PC O- ether-phosphatidylcholine, PE Phosphatidylethanolamine, LPE Lysophosphatidylethanolamine, PE O- ether-phosphatidylethanolamine, PI Phosphatidylinositol, Cer Ceramide, SM Sphingomyelin, Chol Cholesterol, CE Cholesteryl ester. H healthy, MS Multiple Sclerosis.

lipid profiles may function as biomarkers as recently suggested¹⁹.

Acknowledgments

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Conflicts of Interest

Christian Klose is shareholder of Lipotype GmbH. Mathias Gerl, Chris Lauber, and Markus Damm are employees of Lipotype GmbH. All other authors declare no conflict of interest.

Authors' Contributions

H.P., L.G., and M.S. conceived the project and designed experiments. H.P., C.K., M.G. C.L., C.K., and D.F. carried out experiments, H.P., C.K., M.G. C.L., C.K., M.D., D.F., R.H., L.G., and M.S. analyzed the data or supervised data acquisition. H.P., M.G., C.L., and M.D. visualized the data. L.G., A.F.H., and T.K. recruited patients. M.K. and R.H. supervised the MS twin cohort. H.P. and M.S. supervised the project and wrote the manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Lipid distribution.

Figure S2. Paired multivariate analyses show a discrimination between healthy co-twins and co-twins with MS.

Table S1. Annotation of lipid species and subspecies and mol% raw data.

Table S2. Data distribution and test for normality.

Table S3. Quantification of lipid classes and species.