Targeted metabolomics revealed changes in phospholipids during the development of neuroinflammation in Abcd1\textsuperscript{tm1Kds} mice and X-linked adrenoleukodystrophy patients

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Abstract
X-linked adrenoleukodystrophy (X-ALD) is the most common leukodystrophy. Despite intensive research in recent years, it remains unclear, what drives the different clinical disease courses. Due to this missing pathophysiological link, therapy for the childhood cerebral disease course of X-ALD (CCALD) remains symptomatic; the allogenic hematopoietic stem cell transplantation or hematopoietic stem-cell gene therapy is an option for early disease stages. The inclusion of dried blood spot (DBS) C26:0-lysophosphatidylcholine to newborn screening in an increasing number of countries is leading to an increasing number of X-ALD patients diagnosed at risk for CCALD. Current follow-up in asymptomatic boys with X-ALD requires repetitive cerebral MRIs under sedation. A reliable and easily accessible biomarker that predicts CCALD would therefore be of great value. Here we report the application of targeted metabolomics by AbsoluteIDQ p180-Kit from Biocrates to search for suitable biomarkers in X-ALD. LysoPC a C20:3 and lysoPC a C20:4 were identified as metabolites that indicate neuroinflammation after induction of experimental autoimmune encephalitis in the serum of Abcd1\textsuperscript{tm1Kds} mice. Analysis of serum from X-ALD patients also revealed different concentrations of these lipids at different disease stages. Further studies in a larger cohort of X-ALD patients are needed to confirm these promising results.

Abbreviations: AA, arachidonic acid; ACTH, adrenocorticotropic hormone; ALDP, adrenoleukodystrophy protein; AMN, adrenomyeloneuropathy; CCALD, childhood cerebral X-ALD; CFA, complete Freund’s adjuvant; CNS, central nervous system; CSF, cerebrospinal fluid; DHGLA, dihomo-gamma-linolenic acid; EAE, experimental autoimmune encephalomyelitis; HPETE, hydroperoxyeicosatetraenoic acid; HSCT, hematopoietic stem cell transplantation; LCAT, lecithin-cholesterol acyltransferase; lysoPC, lysophosphatidylcholine; MOG, myelin oligodendrocyte glycoprotein; PC, phosphatidylcholine; PLA2, phospholipase A2; PTX, pertussis toxin; TXB2, thromboxane B2; VLCFA, very long-chain fatty acids; X-ALD, X-linked adrenoleukodystrophy.

Hendrik Rosewich and Jutta Gärtner contributed equally to this work.

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patient sera are needed to prove the diagnostic value of these lipids for use as early biomarkers for neuroinflammation in CCALD patients.

**KEYWORDS**

ABCD1, ALPD-deficient mouse, biomarker, lysophosphatidylcholine, neuroinflammation, targeted metabolomics, X-linked adrenoleukodystrophy

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**1 | INTRODUCTION**

X-linked adrenoleukodystrophy (X-ALD; OMIM #300100) has an incidence of 1:15,000 and is therefore the most common monogenetic neuroinflammatory disorder that mainly affects the central and peripheral nervous system as well as the adrenal cortex and testis.¹⁻³ The disease is caused by mutations in the *ABCD1* gene encoding the adrenoleukodystrophy protein (ALDP), a peroxisomal ATP binding cassette transporter at the organelle membrane, resulting in the accumulation of very long-chain fatty acids (VLCFA) in organs and plasma. Human phenotypes range from asymptomatic carriers to severe childhood cerebral X-ALD (CCALD). The inflammatory nature of the acute occipital or frontal lesions in CCALD, which appear in the normal white matter and precede neurodegeneration, is poorly understood.⁴

It is currently unclear what triggers the inflammatory phenotype of cerebral demyelination in CCALD. The type of *ABCD1* mutation, namely missense, nonsense, frameshift, deletion, or insertion, does not predict the phenotype, disease course, or response to treatment.⁵

So far, cell transplantation by allogenic hematopoietic stem cell transplantation (HSCT) or autologous hematopoietic stem-cell gene therapy is the most effective therapeutic intervention that can arrest and even reverse the progression of X-ALD in early disease stages.⁶⁻⁸ In patients with advanced disease, the loss of myelin and axons is insidiously progressive and leads to the death of the children within a few years. Unfortunately, the temporally narrow therapeutic window is often missed.

For many years, the measurement of VLCFA, in particular the ratio of C26:0/C22:0, in patient plasma by means of gas-liquid chromatography has been the well-established metabolic screening method for identifying X-ALD patients.⁹ Recently, C26:0-lysoPC (C26:0-lysoPC) was evaluated as a diagnostic marker in dried blood spots (DBS) from newborns and patients with X-ALD with a sensitivity of 100%. In addition, C26:0-lysoPC can better identify female carriers of X-ALD than VLCFA. Therefore, it is recommended to use C26:0-lysoPC as a routine diagnostic procedure for X-ALD.¹⁰,¹¹

In recent years, an increasing number of countries have C26:0-lysoPC in their newborn screening program, which significantly increased the known number of patients at risk for developing CCALD.¹²⁻¹⁶ Monitoring of asymptomatic X-ALD patients includes several biennial or annual tests, including cerebral MRIs and hormone measurements, to help determine the onset of the inflammatory cerebral processes and adrenocortical insufficiency in a timely manner. Cerebral MRIs in children require anesthesia, and repeated administration of gadolinium can lead to Gd-DTPA deposits.¹⁷ In addition, these repetitive follow-up procedures are emotionally stressful and costly.¹⁸ A reliable and easily accessible biomarker that predicts CCALD in time is therefore of great value.

Thus, in the last years, there are different attempts to identify such biomarkers by omics approaches. Hama et al profiled the content of phospholipid and lysophospholipid species in *Abcd1*¹¹m¹¹md mice brain and discussed the potential of measuring phospholipids to evaluate the starting degradation of myelin.¹⁹ Very recently Richmond et al tried to identify molecular markers that may be prognostic of cerebral demyelination by performing multi-omic profiling of blood samples including genome, epigenome, transcriptome, metabolome/lipidome, and proteome profiling. In six well-characterized brother pairs affected in a discordant manner for the presence of CALD they were unable to identify valuable molecular markers.²⁰

In 1997, three independent groups generated ALDP-deficient mice in order to gain a deeper understanding of the pathophysiology of the inflammatory central nervous system (CNS) phenotype. Although these mice show the biochemical changes of X-ALD, particularly the accumulation of very long-chain fatty acids in several tissues, including the CNS, ALDP-deficient mice lack the inflammatory demyelinating phenotype of CCALD known in humans.²¹⁻²³ To investigate neuroinflammation in *Abcd1*¹¹m¹¹Kds mice, experimental autoimmune encephalomyelitis (EAE) is used, the most widely accepted model for investigating autoinflammatory changes in the CNS of rodents.²⁴,²⁵

To identify potential biomarkers of early CNS inflammation, we initially used the X-ALD EAE model due to
the limited available biosamples from X-ALD patients. We then confirmed the identified metabolites in our X-ALD patient cohort.

2 METHODS

2.1 Mice

C57BL/6 mice were obtained from Charles River Laboratories. ALDP-deficient mice on C57BL/6 background (Abcd1tm1Kds) were generated as described before and kindly provided by Kirby D. Smith. These mice were at least 10-times backcrossed to on C57BL/6 background. Mice were bred and kept in the animal facility of the University of Göttingen under SPF conditions (Göttingen, Germany) with a 12 hours light/dark cycle and two to five mice per cage. Only male X-ALD/C0/C0 mice were used for the experiments. All experiments were performed in accordance with the German animal protection law and with the permission of the Lower Saxony Federal State Office for Consumer Protection and Food Safety (LAVES).

2.2 Experimental autoimmune encephalomyelitis

Twenty to thirty-five-week-old male animals were s.c. immunized with 20 μg MOG35-55 peptide emulsified in 250 μL CFA substituted with 5 mg/mL Mycobacterium tuberculosis H37Ra (Difco, 231 141) and received 300 ng PTX i.p. EAE animals were scored as previously described for mice for at least 30 days after immunization.26

2.3 Mice sample collection

Mice 1-3 days after onset of EAE were used for blood sampling regarding metabolomic analysis. Therefore, animals were deeply terminal anesthetized with ketamine (250 mg/kg i.p.) and medetomidine (2 mg/kg i.p.) for cardiac puncture and were subsequently transcardially perfused with phosphate-buffered saline (PBS, pH 7.4). To obtain serum, whole blood samples were stored at room temperature for 1 to 2 hours before being separated by centrifugation and stored at −20°C.

Whole brain was removed after transcardial perfusion and cut in the midline. One-half of the brain was frozen in liquid nitrogen and stored upon preparation at −80°C. For brain dissection samples were cut manually into 6-7 pieces and homogenized in 1:6 (mg tissue/μL solvent, w/v) extraction solvent (ethanol/phosphate buffer [85:15]) using Tissue Ruptor (Qiagen, Hilden, Germany) at full speed for 20 seconds at 4°C. Cellular debris was reduced by centrifugation for 10 minutes at 13 000 rpm and the supernatant was transferred into a new vial. Samples were stored at −80°C upon analysis.

Due to the well-known circadian rhythm of corticosterone release, the urine samples were always taken between 7 and 8 AM.27 Urine collection was performed according to the Fitchett’s method.28 Briefly, one mouse at a time was transferred into a clean empty cage, and after urinating the urine sample was collected from the cage by pipette and stored in 1.5 mL microtubes at −20°C upon analysis.

2.4 Corticosterone and adrenocorticotropic hormone measurement

Urine samples were diluted 40-fold with distilled water and corticosterone levels were measured using the Corticosterone ELISA kit (Enzo Life Sciences Inc., Farmingdale, New York, catalog ADI-900-097) according to the manufacturer’s instructions. The sensitivity of the assay was 26.99 pg/mL corticosterone.

Serum samples were diluted 25-fold with Calibrator A and ACTH levels were measured using the ACTH ELISA kit (Enzo Life Sciences, Farmingdale, New York, catalog ENZ-KIT138) according to the manufacturer’s instructions. The sensitivity of the assay was 0.46 pg/mL ACTH.

Sample concentrations for both ELISA kits were calculated from a standard curve using GraphPad Prism version 8.3.0 (GraphPad Software Inc., San Diego, California). All samples were assayed in duplicates during a single procedure.

2.5 Patients and samples

All human serum samples were from X-ALD patients who had their biannual routine monitoring at the Department of Pediatrics and Adolescent Medicine, University Medical Centre Göttingen, Germany. The serum samples were obtained as part of routine diagnostics and all parents provided written informed consent for storage and use of materials for research. In total, we have analyzed 40 human serum samples from 37 patients.

CCALD was defined as the presence of white matter lesions in MRI in a distribution consistent with CCALD. For metabolome analysis three groups were defined as followed: Patients without CCALD (neurologically asymptomatic), patients approximately 1 year prior to
first MRI changes (prior CCALD), and patients with maximum 12 months after first MRI changes (CCALD). With the exception for three patients, one patient's serum only is included in one of these groups. Table 1 summarizes the patient characteristics in the predefined groups.

| Patient group       | Neurologically asymptomatic | Prior CCALD | CCALD |
|---------------------|-----------------------------|-------------|-------|
| Number of serum samples/patientsa | 15                          | 4           | 21    |
| Age of patients (years) | 11.7 (5.1-18.3)             | 5.5 (4.4-7.3) | 8.3 (5.1-20.6) |
| Addison disease      | 86% (13/15)                 | 75% (3/4)   | 95% (20/21) |
| Time prior MRI changes (months) | —                          | 14.0 (12.4-15.2) | —     |
| Time after MRI changes (months) | —                          | —           | 4.1 (0.2-11.0) |
| LOES-score29,30      | —                           | —           | 12.4 (1-24) |

*aMaximum one sample of each patient per group.

Validation of the kit run was done using supplied quality control (QC) samples and MetIDQ software. The medium quality control sample (QC2) was distributed four times across the plate.

For more information about AbsoluteIDQ p180-kit see additional material (analytical specifications).

2.7 | Data analysis

Data of metabolites were exported from MetIDQ and metabolites above the lower limit of quantification were subjected to statistical analysis using MetaboAnalyst 4.0.31 Data were normalized using cube root transformation and auto-scaling.

Volcano-plots were generated using Python 3.6 and Matplotlib 3.0.32 Significant results with an FDR below 0.05 and a fold change greater than 1.0 or lower than −1.0 are displayed. Hierarchical clustering analysis was performed on normalized data of the 65 metabolites with the lowest P-values after Student's t-test using Euclidean distance measure and Ward clustering algorithm.

Data from single metabolites were analyzed using Prism 8.3 (GraphPad Software Inc., San Diego, California). If not otherwise mentioned, data are shown as mean ± SEM. Significance testing was done using one-way ANOVA multiple comparisons analysis with Tukey correction and significance levels are displayed as *P < .05, **P < .01, ***P < .001. Correlation analysis used Pearson correlation coefficient and Spearman rank correlation, respectively.

3 | RESULTS

3.1 | Targeted metabolomics using the AbsoluteIDQ p180 kit identified metabolomic changes in Abcd1tm1Kds mice

To determine the sensitivity and reliability of the AbsoluteIDQ p180 kit from Biocrates in different samples...
FIGURE 1  Legend on next page.
we compared serum and homogenized brain samples from Abcd1tm1Kds and control mice. Comparison of serum samples revealed more than a 2-fold up- or downregulation for the levels of seven compounds. LysoPC a C26:0, lysoPC a C26:1, PC aa C42:2 and SM C26:0 were significantly increased, while PC aa C36:5, alpha-aminoadipic acid, and PC aa C34:4 were significantly reduced in Abcd1tm1Kds mice (Figure 1A). The diagnostic marker for X-ALD, lysoPC a C26:0, was highly significantly increased in serum (0.107 μM vs 0.253 μM) (Figure 1B).

A comparative analysis of Abcd1tm1Kds and control homogenized brain samples showed that five compounds are regulated more than 1.41-fold differently between mice genotypes. PC aa C42:1, lysoPC a C26:0, and lysoPC a C26:1 levels were significantly elevated, while concentrations of some plasmalogens (PC ae C38:2, PC ae C38:3, and PC ae C36:3) were slightly but significantly decreased in Abcd1tm1Kds mice (Figure 1C). The concentration values of lysoPC a C26:0 were also significantly increased in homogenized brain samples (0.0397 μM vs 0.0624 μM) of Abcd1tm1Kds mice compared to wildtype (Figure 1D).

These results indicate that targeted metabolomics by AbsoluteIDQ p180 kit from Biocrates can measure the key metabolic findings of Abcd1tm1Kds mice as statistically significant. However, our results for lysoPC a C26:0 show an overlap between Abcd1tm1Kds and control mice, especially for the brain. These findings are in contrast with results from most human studies using LC-MS/MS method from DBS for detection of lysoPC a C26:0. Despite the lower specificity of the AbsoluteIDQ p180 kit compared to LC-MS/MS, this kit seems to be a reliable tool for the screening application of metabolic changes particularly in the serum of mice.

3.2 Abcd1tm1Kds mice develop more severe paralysis after EAE

Due to the lack of a spontaneous neuroinflammatory phenotype in the Abcd1tm1Kds mice, we induced EAE in Abcd1tm1Kds and control mice to study metabolic changes due to neuroinflammation.

Interestingly, Abcd1tm1Kds mice displayed an earlier disease onset (8 vs 13 days after immunization) and a higher mean maximum disease score in contrast to control animals (3.4 vs 2.1) (Figure 2; Table S1). In addition to the classically ascending paralysis known for EAE, Abcd1tm1Kds mice demonstrated encephalopathic symptoms such as apathy and shivering. In some animals, these additional symptoms disappeared as the disease progressed.

In summary, these results indicate that the Abcd1tm1Kds mice are more sensitive to the EAE protocol used in this study than control animals. Due to this sensitivity, the classic EAE protocols led to excessive mortality in Abcd1tm1Kds mice (data not shown). We therefore had to adapt the s.c. immunized dose of MOG35-55 peptide and the i.p. dose of PTX, which explains the relatively low mean EAE score of the control animals.

3.3 Abcd1tm1Kds mice did not show adrenocortical insufficiency

To rule out adrenocortical insufficiency as a factor for the different disease courses, we measured the corticosterone levels in the urine and the ACTH levels in the serum. Urine corticosterone levels of untreated animals showed differences between mice genotypes (27.97 for control vs 13.66 ng/mL for Abcd1tm1Kds mice), but these results lack to be significant. Also, there were no significant differences between untreated and treated animals (27.97 vs 24.74 ng/mL for control and 16.89 ng/mL for Abcd1tm1Kds mice) (Figure S1A). For mean serum ACTH levels there were no differences between untreated animals (620 pg/mL for control vs 565 pg/mL for Abcd1tm1Kds mice), but after EAE induction mean ACTH levels of Abcd1tm1Kds mice were significantly decreased (565 pg/mL for control vs 277 pg/mL for Abcd1tm1Kds mice). (Figure S1B).

With the hormone levels measured in the animals, a chronic primary adrenocortical insufficiency could be excluded, since low corticosterone values and compensatory increased ACTH values are characteristic of adrenal insufficiency. The lowered ACTH levels in Abcd1tm1Kds mice could possibly be a sign of functional adrenocortical insufficiency in serious illness.
3.4 | Targeted metabolomics revealed distinct metabolic changes after the beginning of an EAE triggered neuroinflammation in serum of mice

To answer the question of whether metabolic changes could indicate the onset of neuroinflammation in Abcd1<sup>tm1Kds</sup> mice, we analyzed serum samples from EAE treated mice right after the beginning of EAE related symptoms using the AbsoluteIDQ p180 kit.

For the evaluation, Abcd1<sup>tm1Kds</sup> mice were divided into three groups. Animals without EAE induction were labeled not treated (NT), EAE induced animals that did not develop any paralysis (EAE score 0) were labeled treated—non-sick (Tr-NS), and EAE induced animals that developed paralysis (EAE score > 0) were labeled treated—sick (Tr-S).

Heatmap clustering was able to separate these three groups properly (Figure 3A). Comparison of Tr-NS and Tr-S animals revealed that 21 compounds were significantly different, defined as more than 2-fold change and P < .05 (Figure 3B). There were more compounds decreased in their levels instead of increased (7 vs 14). The compound showing the most pronounced difference between Tr-NS and Tr-S animals was lysoPC a C20:3 with a 6.3-fold decreased concentration in Tr-S animals (see arrow in Figure 3B).

LysoPC a C20:3 is a lysophosphatidylcholine bearing a 20 carbon-atom fatty acid chain with three double bonds. Only differing in the position of these bonds, it could represent either mead acid or dihomo-gamma-linolenic acid (DHGLA). Both are involved into eicosanoid metabolism, especially in the case of low levels of arachidonic acid (AA). We also observed a significant drop of lysoPC a C20:4, potentially representing arachidonic acid.

LysoPC a C20:3 and lysoPC a C20:4 levels were both significantly decreased in Tr-S animals. The values of these metabolites of Tr-NS animals were not different from that of NT animals (Figure 3C,D). This indicates that the decline in lysoPC a C20:3 and lysoPC a C20:4 values is related to neuroinflammation. In addition, we observed the same drop of these lipids in control mice (Figure S2A,B), outlining that the observed metabolomic changes were not Abcd1<sup>tm1Kds</sup>-specific, but rather good indicators for neuroinflammation. In this regard the more pronounced drop of lysoPC a C20:3 and lysoPC a C20:4 level in Abcd1<sup>tm1Kds</sup> mice may be associated with a higher overall EAE-score (Table S2).

3.5 | Targeted metabolomics of human blood samples confirm lysoPC a C20:3 and lysoPC a C20:4 as early indicators for neuroinflammation in CCALD

To study whether the metabolites found in Abcd1<sup>tm1Kds</sup> mice are also able to identify the onset of neuroinflammation in patients with X-ALD potentially even before the first changes on the cerebral MRI appear, the following groups were analyzed using the AbsoluteIDQ p180 kit. Patients without CCALD (neurologically asymptomatic), patients approximately 1 year before first changes in cerebral MRI (prior CCALD), and patients right after the first MRI changes (CCALD). For further details on patients’ characteristics see Section 2 including Table 1.

In fact, the lysoPC a C20:3 and lysoPC a C20:4 concentrations also differed between the analyzed X-ALD groups in humans (Figure 4). LysoPC a C20:3 levels were significantly reduced in CCALD patients compared to neurologically asymptomatic patients (1.3-fold) (Figure 4A). LysoPC a C20:4 levels were also decreased, but not significantly in CCALD patients compared to neurologically asymptomatic patients (1.2-fold) (Figure 4B). Remarkably, this decrease is even more pronounced and significantly for values of both compounds in patients prior to CCALD (2.0-fold for lysoPC a C20:3 and 1.9-fold for lysoPC a C20:4) (Figure 4A,B). For three patients, we were able to collect serum sample prior CCALD and right after their progress to CCALD. These results were labeled by individual numbers in Figure 4A,B. In accordance with the whole
disease groups, the levels of lysoPC a C20:3 and lysoPC a C20:4 increased for each individual patient after the manifestation of CCALD on MRI. The raw data of metabolome measurement for all sera are provided in Table S3.

To rule out other parameters to influence the lysoPC a C20:3 and lysoPC a C20:4 values we correlated them with patients’ age and the storage time of the serum samples. We could not find any correlations of lysoPC a C20:3 and lysoPC a C20:4 with these parameters (Figure S3A,B).

In conclusion, these results show that lysoPC a C20:3 and lysoPC a C20:4 are metabolic indicators of neuroinflammation in X-ALD patients.

4 | DISCUSSION

Despite all efforts in several years of research and from the first analyses with omics techniques, no biomarkers have yet been identified that reliably predict the initiation of the cerebral course of X-ALD (CCALD). Due to the sole effectiveness of HSCT in the early disease stage of CCALD there is only a small therapeutic window to stop neuroinflammation and thus neurodegeneration. This group of early affected CCALD patients would benefit enormously from a reliable biomarker.
In our study, we applied for the first-time targeted metabolomics with the well-established AbsoluteIDQ p180 kit from Biocrates to samples of X-ALD in both mice and humans. In mice we could show that targeted metabolomics is able to differentiate Abcd1tm1Kds animals with or without neuroinflammation. The most noticeable difference was the lowering of lysoPC a C20:3 in the serum of Abcd1tm1Kds mice with neuroinflammation. The same metabolites were also reduced in our comparative study in patients with X-ALD, which is an indication that the metabolites found may be pathophysiologically linked to neuroinflammation. The most noticeable difference was the lowering of lysoPC a C20:3 in the serum of Abcd1tm1Kds mice with neuroinflammation. The same metabolites were also reduced in our comparative study in patients with X-ALD, which is an indication that the metabolites found may be pathophysiologically linked to neuroinflammation. The most noticeable difference was the lowering of lysoPC a C20:3 in the serum of Abcd1tm1Kds mice with neuroinflammation.

It must be noted that the time points for blood collection for metabolome measurement and the extent of neuroinflammation in mice and humans were different. It is whether unclear to what extent the sharp decrease in lysoPC a C20:3 and lysoPC a C20:4 after the beginning of a strong inflammatory reaction in the CNS in mice corresponds to the decrease seen in X-ALD patients with minimal inflammatory changes which were not yet seen in MRI (prior CCALD). Moreover, the meaning of the increase in lysoPC a C20:3 and lysoPC a C20:4 from prior CCALD to CCALD remains questionable. In this regard, only measuring additional serial serum samples from patients transitioning from neurologically asymptomatic to CCALD could help improve our understanding of the changes in lysoPC values observed in this analysis.

One potential pathophysiological explanation of the decreased lyso PCs could be the metabolism of AA. AA is a polyunsaturated omega-6 fatty acid 20:4(ω-6) and a key substance in the metabolism of inflammatory lipids. It is mainly produced by the activity of lecithin-cholesterol acyltransferase (LCAT) and phospholipase A2 (PLA2) from phospholipids in the plasma membrane. It is known that proinflammatory cytokines such as interferon α and β themselves increase the activity of PLA2 to increase the local concentration of AA in the tissue. AA is responsible for the homeostasis of neurons and is the starting substance for a large number of other hormonally active substances such as prostaglandins, leukotrienes (5-hydroperoxyeicosatetraenoic acid, short 5-HPETEs), 15-HPETEs, and 12-HPETEs. Even in very low concentrations, AA and its metabolites act.

**FIGURE 4** Serum of X-ALD patients also revealed a drop of lysoPC a C20:3 and lysoPC a C20:4 values regarding beginning neuroinflammation. A, Scatter plot of lysoPC a C20:3 revealed significant decreased values in serum of patients prior childhood cerebral X-ALD (CCALD) disease (n = 4) and with early CCALD disease (n = 21) compared to neurologically asymptomatic patients (n = 15). B, In contrast to lysoPC a C20:3, values of lysoPC a C20:4 were not significantly decreased in CCALD disease vs control. Therefore, the results showed a significant difference between patients prior to CCALD disease and patients with early CCALD disease. P values of one-way ANOVA are depicted as **P < .01, *P < .05, and ns P ≥ .05. Serum of individual patients, who were included in more than one disease group were labeled with numbers (1-3).
locally in the tissue and have a short half-life. Thus, the measurement of the AA itself is usually not possible.36 LysoPC a C20:4 in serum mainly results from the cleavage by LCAT, transforming PCs and cholesterol to LPCs and cholesterol-esters, therefore linking it closely to the AA content in membrane lipids. As a result, one could indirectly deduce an increased consumption of AA from the lowering of lysoPC a C20:4. LysoPC a 20:4 could thus be an indirect indicator of ongoing inflammatory reactions.

LysoPC a C20:3 can similarly result from the cleavage by LCAT and this could then be linked to a higher consumption of its fatty acid in different cells. Especially in a state of essential fatty acid deprivation, mead acid is preferably used, as it can be synthesized by de novo pathways.37 Additionally, mead acid as well as DHGLA plays an important role as regulatory substances for eicosanoid metabolism. As they are associated with downregulation of proinflammatory cytokines such as leukotrienes from AA, they may also be signs for a counter-regulation of the immune-system.34,38

There is already good evidence about the importance of inflammatory lipids for leukodystrophies in general as well as AA-dependent metabolites in X-ALD. The hypothesis regarding the connections between disturbed lipid metabolism due to the primary defect in the peroxisome on the one hand and an increased PLA2 activity with the consecutive increase in AA in tissue as a driving effect in inflammatory leukodystrophy on the other hand was already mentioned before.39 This thesis is mainly supported by observations such as increased levels of eicosanoids in many neurodegenerative diseases and leukodystrophies as well as by the measurement of increased levels of some leukotrienes in the cerebrospinal fluid (CSF) of patients with X-ALD.40,41 In addition, it could first be shown by metabolomic/lipidomic analysis on PBMC and plasma obtained from patients with adrenomyeloneuropathy (AMN), a slowly progressive disease course of X-ALD, that lipid-driven inflammation-associated pathways such as ceramide degradation and sphingomyelin metabolism were increased. Later the same group could demonstrate that patients with AMN exhibited significantly higher plasma levels of lipid mediators of inflammation (12S-HETE, 15S-HETE, and TXB2), all of which are derivatives of AA.42,43 In this context, experimental attempts with PLA2 in EAE mouse models appear to be particularly interesting, because they suggest that AA influences susceptibility to EAE.44,45 In our case, this could even explain the different EAE courses seen in Abcd1tm1Kds mice as well as of patients with CCALD. Further research is needed to determine whether these metabolites are suitable early biomarkers for CCALD.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Matthias Kettwig, Ralph Krätzner, Hendrik Rosewich, and Jutta Gärtnert designed and supervised the project. Matthias Kettwig, Henry Klemp, and Stefan Nessler performed and analyzed all experiments. Frank Streit supported Henry Klemp in setting up the mass spectrometer and supported us performing the AbsoluteIDQ p180-Kit. Matthias Kettwig and Henry Klemp drafted the manuscript and the figures. All authors read, critically revised, and approved the manuscript.

ETHICS STATEMENT
All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all parents for patients being included in the study. All institutional and national guidelines for the care and use of laboratory animals were followed.

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**SUPPORTING INFORMATION**

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

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