Sex-specific perturbation of complex lipids in response to medium-chain fatty acids in very long-chain acyl-CoA dehydrogenase deficiency

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Very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD) is the most common defect of long-chain fatty acid β-oxidation. The recommended treatment includes the application of medium-chain triacylglycerols (MCTs). However, long-term treatment of VLCAD⁻/⁻ mice resulted in the development of a sex-specific metabolic syndrome due to the selective activation of the ERK/mTORc1 signalling in females and ERK/peroxisome proliferator-activated receptor gamma pathway in males. In order to investigate a subsequent sex-specific effect of MCT on the lipid composition of the cellular membranes, we performed lipidomic analysis, SILAC-based quantitative proteomics and gene expression in fibroblasts from WT and VLCAD⁻/⁻ mice of both sexes. Treatment with octanoate (C8) affected the composition of complex lipids resulting in a sex-specific signature of the molecular profile. The content of ceramides and sphingomyelins in particular differed significantly under control conditions and increased markedly in cells from mutant female mice but remained unchanged in cells from mutant males. Moreover, we observed a specific upregulation of biosynthesis of plasmalogens only in male mice, whereas in females C8 led to the accumulation of higher concentration of phosphatidylecholines and lysophosphatidylecholines. Our data on membrane lipids in VLCAD after supplementation with C8 provide evidence of a sex-specific lipid perturbation. We hypothesize a likely C8-induced pro-inflammatory response contributing to the development of a severe metabolic syndrome in female VLCAD⁻/⁻ mice on long-term MCT supplementation.

Abbreviations
C8, octanoate; Cer, ceramide; CHOL, cholesterol; CL, cardiolipin; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; FA, fatty acid; FAOD, fatty acid oxidation disorders; HexCer, hexosylceramide; LPA, lysophosphatidate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; MCT, Medium-chain triacylglycerols; MS, mass spectrometry; MSMS, tandem mass spectrometry; mTOR, mechanistic target of rapamycin; PA, phosphatidate; PC (O-), phosphatidylcholine (-ether); PE (O-), phosphatidylethanolamine (-ether); PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TAG, triacylglycerol; VLCAD, very-long-chain acyl-CoA dehydrogenase deficiency; VLCAD⁻/⁻, VLCAD knockout mouse.
**Introduction**

Fatty acid oxidation disorders (FAODs) are inherited metabolic diseases resulting in defective mitochondrial β-oxidation and belong to the expanded newborn screening programmes in many countries worldwide [1]. The pathophysiology includes (a) a direct decrease in reducing equivalents required by oxidative phosphorylation; (b) a reduced production of acetyl-CoA as essential intermediate of the tricarboxylic acid cycle leading to further decrease in reducing equivalents; and (c) a reduced biosynthesis of ketone bodies especially during situations of increased energy demand [2]. The very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD) is the most common defect of the mitochondrial oxidation of long-chain fatty acids (FA) with an incidence of 1 : 50 000–1 : 100 000 [3–5]. VLCAD may present with different phenotypes as well as severity and age at onset [6,7].

To date, the recommended therapy includes the avoidance of fasting and the replacement of long-chain triacylglycerols (TAGs) by medium-chain TAGs (MCTs) [8,9]. A large body of literature confirms the effectiveness and safety of MCT application in the treatment of cardiomyopathy in long-chain FAOD [10–13]. Previous studies on the VLCAD knockout mouse (VLCAD−/−) have shown the beneficial effects of MCT supplementation if this is applied during the situation of increased energy demand [14]. However, long-term supplementation of MCT induced de novo biosynthesis and elongation of FAs leading to the alteration of the FA profiles in several tissues from both wild-type (WT) and VLCAD−/− mice characterized by a strong reduction in polyunsaturated FAs and the marked increase in monounsaturated species [15,16]. Of particular interest was the sex-specific development of a severe metabolic syndrome in female VLCAD−/− mice [17]. This phenotype was associated with steatohepatitis (grade 3) and systemic oxidative stress [17]. Very recent studies have demonstrated that WT and VLCAD−/− mice showed strong sex-dependent differences in basal metabolism enhanced by MCT [18]. Indeed, MCT is responsible for the selective activation of the extracellular signal-regulated kinase (ERK)/mTORc1 signalling pathway leading to an increased biosynthesis and elongation of FAs in VLCAD−/− females. In contrast, MCT induces the activation of ERK/peroxisome proliferator-activated receptor gamma pathway and the subsequent upregulation of peroxisomal β-oxidation in males [18]. Because of the sex-specific metabolic response induced by MCT [18] accompanied by the alteration of FA profiles in tissues [17], we hypothesized a subsequent sex-specific effect of MCT on the lipid composition of the cellular membranes. This effect would be of great relevance since changes in the lipid composition confer different properties to the plasma membrane acting in cell signalling and homeostasis [19–25] suggesting the need of personalized sex-based therapy concept for the treatment of long-chain FAOD.

In order to further investigate whether the sex-specific responses to MCT treatment in VLCAD are translated to a shift in the lipid composition of plasma membranes, we performed a comprehensive shotgun lipidomic analysis. Expression of enzymes and proteins involved in pathways responsible for the biosynthesis of several complex lipids was evaluated using a quantitative SILAC-based proteomic approach. The resulting data were corroborated by gene expression analysis to support and elucidate the regulation of biosynthesis pathway affected by MCT in the different sexes. We here provide evidence that MCT affects the lipid composition of the plasma membrane in a sex-specific manner.

**Results**

**Sex-specific alteration of lipid composition in response to octanoate**

Previous reports have shown that medium-chain FAs alter the FA composition in tissue from VLCAD−/− mice supplemented long term with an MCT-based diet [16]. As we observed a sex-specific response with regard to the metabolic adaptation to octanoate (C8) in fibroblasts from WT and VLCAD−/− mice, we investigated whether C8 is able to affect the lipid composition in VLCAD in a sex-specific manner. In this regard, we performed shotgun lipidomic analysis in cell lines from WT and VLCAD−/− mice of both sexes under control conditions and after incubation with 300 µM C8. In total, more than 1400 lipid species of 23 classes could be identified. As shown in Fig. 1A, we could clearly observe sex-specific differences in the composition of phospholipids which were further accentuated by the treatment with C8. First of all, the content of phosphatidylcholine (PC) did not differ between WT and VLCAD−/− male mice, whereas it was significantly increased in VLCAD−/− females compared with their WT counterparts (Fig. 1B). Moreover, incubation with C8 induced a significant reduction in PC in male mice of both genotypes. In strong contrast, we observed a further significant increase in PC in treated VLCAD−/− females compared with mutants on control conditions (47.7 mol% sample ± 0.08 vs 44.5 mol% sample ± 0.07). In a similar manner, the concentration of
phosphatidylethanolamine (PE) was significantly lower in cells from VLCAD<sup>−/−</sup> males, whereas no differences were detected in female cells (Fig. 1C). Additionally, incubation with C8 in male mutants induced a significant reduction in this lipid class. However, in parallel to the reduction in PC and PE in male mice, C8 induced a remarkable increase in ether phospholipids PC (-ether) (PC-O) and PE (-ether) (PE-O) suggesting a sex-specific redirection in the biosynthesis pathway to plasmalogens in these cells. On the other hand, this effect did not appear in fibroblasts from female mice of both genotypes. In accordance with the change in the total concentration of the measured phospholipids, we also calculated the PC/PE ratio as the alteration of this value in mitochondrial membranes may be responsible for disturbed energy metabolism [26]. As shown in Table 1, we indeed observed sex- and genotype-specific changes in PC/PE ratio. Fibroblasts from VLCAD<sup>−/−</sup> males displayed a markedly higher PC/PE ratio compared with their WT counterparts (3.6 vs 3.1). Treatment with C8 led in both genotypes to the same proportional increase in this ratio. In contrast, in female cells we did not observe a difference due to the genotype. However, C8 contributed to an increase in...
the PC/PE ratio in VLCAD\textsuperscript{-/-} cells (4.6 vs 3.7), whereas in fibroblasts from WT mice this value was slightly reduced (Table 2).

Although the total concentration of phosphatidylinositol (PI) and phosphatidylserine (PS) differed between the sexes (Fig. 1C,D), the effects of sex, genotype and diet were similar in both sexes. PI was significantly reduced in cells from VLCAD\textsuperscript{-/-} mice, whereas no alterations were reported for PS. Incubation with C8 induced in WT cell lines an increase in PS but simultaneously a reduction in the PS content of mutant cells (Fig. 1C,D).

**C8 induced sex-specific upregulation of the biosynthesis of plasmalogens**

Because only cells from male VLCAD\textsuperscript{-/-} accumulated higher concentrations of plasmalogens either under control conditions or after incubation with C8, we hypothesized a sex-specific upregulation of the biosynthesis of this lipid class. As depicted in Fig. 2A, this pathway was indeed upregulated only in male cells. In particular, the expression of three out of the four analysed genes glycerone phosphate \(\text{O-acyltransferase} \) (GNPAT), alkyl-glycerone phosphate synthase (AGPS) and phosphatidic acid phosphatase (PAP2) was significantly higher in mutants compared with WT. In line with the sex-specific upregulated peroxisomal \(\beta\)-oxidation after incubation with C8 \[18\], the expression of the peroxisomal genes GNPAT and AGPS was further stimulated, whereas this effect was not observed in PAP2 which is localized in the endoplasmic reticulum (Fig. 2D) \[27\]. The expression of diacylglycerol (DAG) choline/ethanolaminephosphotransferase (Cept1) increased significantly in both genotypes after incubation with C8. In cells from female mice, the biosynthesis of plasmalogens was fully unaffected either under control conditions or on C8 (Fig. 2B).

Male mice display higher concentration of lipid species with longer total fatty acid chain length

In order to corroborate our results on the increased concentration of plasmalogens and by the expression of genes involved in the biosynthesis, we evaluated the concentration of the total FA chain length. Indeed, \textit{de novo} FA biosynthesis generates compounds with a chain length of 16–18 carbons \[28\].

The evaluation of total chain length in PC-O, PE and PE-O clearly suggested a strong upregulation of the biosynthesis of FAs and plasmalogens in fibroblasts from male mice. Indeed, male mice displayed an overall increase in longer chain length of plasmalogens, whereas this effect was not observed in PE or PC (Fig. 3). Specifically, we observed a significantly higher concentration of species with a total chain length of C34–C38 in PC-O and C36–C40 in PE-O in fibroblasts from VLCAD\textsuperscript{-/-} male mice under control conditions. These findings are strongly indicative of an increment of the FAs C16–C20. In contrast, cells from

### Table 1. Primers used in this study

| Gene       | Forward 5'-3'                 | Reverse 3'-5'                 | Accession number |
|------------|-------------------------------|-------------------------------|------------------|
| GNPAT      | GGTGGAGAAGACTCTGTGCG          | GAGGAGGCCGATATGTCGG           | NM_010322        |
| AGPS       | GGTTCGTTGCGTTCTCCCCTAA       | ACGGTCCTCCTCGAACAGTA          | NM_172666.3      |
| PAP        | ATGTTGAGGCTCTTCTCGAGTG        | CTGCCGTTTTCTGTGGAGAGT         | D84376.1         |
| Cept1      | CTGGCGAGAGGGGAGGCCGACC       | TCGTATGTCGAACACACCA           | NM_133869.4      |

### Table 2. PE-to-PC ratio measured in fibroblasts of WT and VLCAD\textsuperscript{-/-} mice of both sexes either under control diet or after incubation with C8

|        | WT-WT-C8 | VLCAD\textsuperscript{-/-} | VLCAD\textsuperscript{-/-}-C8 |
|--------|----------|-----------------|-----------------------------|
| Male   | 3.1      | 3.6             | 4.1                         |
| Female | 3.5      | 3.7             | 4.5                         |
VLCAD<sup>−/−</sup> females showed a marked reduction in longer species in PC-O, whereas the concentration of C36–C40 increased significantly in PE-O (Fig. 3). Incubation with C8 led to a further significant increase in longer FA species in fibroblasts from mutant males, while no changes were observed in PE-O from mutant females. Moreover, we observed a significant reduction in C34 PC-O species accompanied by the parallel significant increase in C38 and C40, however, without effect on the total PC-O concentration (Fig. 3).

**Sex-specific increase in the concentration of lysophosphatidylcholines after incubation with C8**

Because lysophosphatidylcholines (LPCs) are bioactive pro-inflammatory lipids believed to play an important role in atherosclerosis and inflammatory diseases by altering signalling cascades [29–31], we investigated the concentration of LPC16 and LPC18 species. As depicted in Fig. 4, no difference was observed in cells

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**Fig. 2.** C8 significantly upregulates biosynthesis of plasmalogen in fibroblasts from male VLCAD<sup>−/−</sup> mice. Relative expression of genes involved in the biosynthesis of plasmalogen in fibroblasts from (A) male and (B) female mice under control conditions and after incubation with C8. Reported data are presented as means ± standard deviation (SD). n denotes the number of biological replicates (n = 3). (C) Schematic representation of the biosynthesis of plasmalogen modified from Ref. [64]. Alkyl-G-3-P, alkyl-glycerol-3-phosphate; AGPAT, 1-Acyl-sn-glycerol-3-phosphate O-acyltransferase; GmP, dihydroxyacetone phosphate; lcFA, long-chain FAs. Values denoted by * and # were considered significant if P < 0.05 (two-way ANOVA; Student’s t-test and Tukey’s Test). * indicates significant differences between WT and VLCAD<sup>−/−</sup> mice under the same dietary regimen. # indicates significant differences between mice of the same genotype under different dietary regimen.
Fig. 3. Change in total FA chain length in PC, PE and their corresponding plasmalogens. (A–D) represents FA composition from male mice. (E–H) represents FAs from female mice. Concentrations of the measured FA chain length are represented as percentage. Values expressed in mol% sample are reported in detail in Table S1. Mol% sample indicates the moles of the lipid species extracted from one sample calculated as percentage of the total amount of lipid extract.
from male mice either under control diet or after incubation with C8. In contrast, a significant increase in the concentration of LPC16:0, LPC16:1, LPC18:0 and LPC18:1 was observed exclusively in fibroblasts from female mice of both genotypes suggesting that the C8-induced activation of mechanistic target of rapamycin (mTOR) may lead to a parallel stimulation of pro-inflammatory pathways [32].

Enhanced biosynthesis of ceramides and hexosylceramides in VLCAD<sup>−/−</sup> mice

Very recently, it has been shown that treatment with C8 upregulates FA biosynthesis in both sexes [18]. In order to evaluate the effect of this process, we investigated ceramides (Cer), sphingomyelins (SMs), DAG and TAG in fibroblasts of both genotypes and sexes either under control conditions or after incubation with C8. As depicted in Fig. 5A, the most marked differences in WT lines between the two sexes were observed in the concentration of SMs. On the other hand, VLCAD<sup>−/−</sup> cells differed mostly in the concentration of DAG and TAG as well as in Cer and hexosylceramides (HexCer). The full quantification of the lipid concentration is summarized in Fig. 5B,C. The concentration of SMs in WT fibroblasts from male mice was 2.6-fold higher compared with females of the same genotype (7.34 mol% sample ± 0.35 vs 2.84 mol% sample ± 0.16). With regard to the mutants, male VLCAD<sup>−/−</sup> cells showed significantly lower levels of SMs compared with their WT counterparts. However, fibroblasts from female VLCAD<sup>−/−</sup> mice increased their concentration significantly (Fig. 5C). The same situation was also observed for Cer and HexCer. Incubation with C8 had no effects on the concentration of any of these lipid classes including cardiolipins (CLs). However, although the total concentration of these lipids did not differ, the total chain length was shifted to longer chain (data not shown) suggesting that the composition of mitochondrial lipids is also sensitive to dietary interventions. Based on the observed differences between the sexes, we investigated the biosynthesis of Cer and SMs. In accordance with higher Cer and SMs in cells from VLCAD<sup>−/−</sup> male mice, the genes involved in this pathway were significantly upregulated. In that, serine palmitoyltransferase long chain base subunit 1, serine palmitoyltransferase long chain base subunit 2, 3-keto,3-dihydrosphingosine reductase, ceramide synthase 5 (Cers5) and ceramide synthase 6 (Cers6) were significantly upregulated (Fig. 5D). Incubation with C8 had only an effect on the expression of ceramide synthase 2 (Cers2) and DAG acyltransferase (DGAT) which is in line with the enhanced intracellular accumulation of DAG and TAG as shown in Fig. 5A. On the other hand, in fibroblasts from VLCAD<sup>−/−</sup> female mice, we could detect a significantly upregulated gene expression only in the genes Cers5, Cers6 and DGAT (Fig. 5E) in accordance with the observed increase in the concentration of PC in these cells, which are also synthesized via DGAT [33]. These data were supported by quantitative SILAC-based proteomic analysis. In that, as depicted in Fig. 6A, the expression of enzymes encoding SM and Cer biosynthesis was upregulated in line with the shown data at mRNA level.

**Discussion**

In the present study, we show that sex and treatment with C8 impact the composition of complex lipids in
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fibroblasts from WT and VLCAD<sup>−/−</sup> mice resulting in a sex-specific signature of the molecular profile. Especially, the content of Cer and SMs differed significantly under control conditions and increased markedly in cells from mutant female mice but remained unchanged in cells from mutant males. Treatment with C8, as one of the main components of a MCT-based diet, induces a sex-specific alteration of complex lipids only in fibroblasts from VLCAD<sup>−/−</sup> mice. Previous studies have demonstrated that incubation with C8 resulted in a sex-specific activation of MAPK/mTORC pathway leading to a different stimulation of de novo FA biosynthesis and lipogenesis [18]. This effect translates into a specific upregulation of biosynthesis of plasmalogens only in male mice, whereas in females C8 led to the accumulation of higher concentration of PCs and LPCs.

Sex difference in the FA composition of plasma, erythrocyte membrane lipids and adipose tissues has been widely described [34–36]. Especially, long-chain polyunsaturated FAs such as arachidonic acid and docosahexaenoic acid of the phospholipid fraction differ significantly between sexes [36], although this variable accounted only for &lt;2% of the variability of plasma phospholipids [35]. In a similar manner, the impact of endogenous sex hormones on FAs, TAGs and lipoproteins for metabolism and cardiovascular risk is acknowledged and has been in the past the subject of several reviews [37–41]. On the other hand, so far very little has been reported on membrane lipids in their complexity including their FA chain length, double bonds and hydroxy residues with regard to differences between sexes [35,42,43]. In this work, we pointed out that sexually dimorphic differences can be markedly accentuated by both the presence of a monogenic disease as in the case of VLCAD and the treatment applied. Of interest was the overall difference in Cer and SM contents between the sexes under control conditions. However, VLCAD ablation led to a striking increment in female mice. These data are indicative of a more generalized inflammatory response [44–46], which correlates well with our previous observation on the development of a severe metabolic syndrome in VLCAD<sup>−/−</sup> female mice on MCT [17]. Indeed, behind their structural role and their several cellular functions [47–49], these lipids are the main components of microdomains that allow membrane compartmentalization of transporter and receptors and act directly as a messenger in the inflammatory response [50–52]. In line with these findings was also the remarkable increment of LPC species in female cells – although more prominent in mutants – of both genotypes. Our results strongly suggest that the activation of the mTORC1 pathway in VLCAD<sup>−/−</sup> females modulated by C8 results not only in the upregulation of lipogenesis [18] but also in the activation of inflammatory processes either directly via TSC/mTOR cascade [53–55] or as the consequence of enhanced biosynthesis of LPC immunomodulating species [56].

A further peculiar difference was the specific higher concentration of PC depicted in female VLCAD<sup>−/−</sup> cells. Similar observations were already described in brown adipose tissue in obese mice [34]. Overall, we observed a different phospholipid content between the sexes that affected also the ratio between PC and PE. Abnormalities of this value in various tissues have been long implicated in metabolic disorders, insulin resistance and obesity [26,35]. Specifically, when the alteration involves the PC/PE ratio of the mitochondrial membrane, the mitochondrial production is impaired [26]. Mechanisms that attempt the maintenance of phospholipid homeostasis are especially known in liver associated with nonalcoholic fatty liver disease [57–59]. Moreover, an increase in the PC/PE ratio has been described in the leptin-deficient ob/ob mouse [60]. Here, in order to cope with the excessive accumulation of intrahepatic lipids, the phospholipid biosynthesis was stimulated which resulted in an increased PC/PE ratio [60]. From this point of view, we may speculate that a similar mechanism occurs also in female VLCAD<sup>−/−</sup> mice after a long-term MCT-based diet. These animals developed a severe metabolic syndrome presenting with a severe steatohepatitis, whereas male mice were protected [17]. Studies in fibroblasts demonstrated very recently that C8 was used less efficiently in cell lines from female mutants which upregulated lipogenesis via mTORC1 pathway when treated with C8 [18]. The striking increase in PC/PE ratio as shown in this study is strongly suggestive of a stimulation of the phospholipid biosynthesis to accommodate the FA and lipid biosynthesis as shown in the ob/ob mouse [60].

Plasmalogens are essential components of several cellular membranes including endoplasmic reticulum, Golgi, nucleus and mitochondria [61]. Defects in the biosynthesis of plasmalogens have severe consequences for human health [62]. This pathway initiates in the peroxisomes and is finalized in the ER [63,64]. Therefore, the functional integrity of these organelles is also a necessary requirement for the proper biosynthesis of plasmalogens [65]. To the best of our knowledge, no reports describe sex differences in the concentration of plasmalogens with the exception of the stimulation of spermatogenesis in germ cells from male mice [66]. Interestingly, the increase in this lipid species observed in fibroblasts from male mice especially after
incubation of C8 corroborated our previous report on the upregulation of peroxisomal activity and β-oxidation in this sex [18]. We hypothesize that long-chain FAs generated from the de novo biosynthesis and elongation of C8 [15–18,67] may accumulate in part as neutral lipids or may be redirected to the biosynthesis

Fig. 5. Upregulated biosynthesis of Cer and SMs in fibroblasts from VLCAD−/− mice. (A) Heatmap of log2-transformed lipid concentration from fibroblasts of WT and VLCAD−/− mice of both sexes treated with 300 μM C8 compared to respective controls (twofold enrichment, P-value < 0.05). (B, C) Quantification of different species of phospholipids in fibroblasts from WT and VLCAD−/− mice of both sexes treated with 300 μM C8 compared to respective controls. Mol% sample indicates the moles of the lipid species extracted from one sample calculated as percentage of the total amount of lipid extract. Reported data are presented as means ± standard deviation (SD). n denotes the number of biological replicates (n = 3). (D, E) Expression of genes encoding for enzymes involved in the biosynthesis of Cer and SMs in male and females, respectively. Reported data are presented as means ± SD. n denotes the number of biological replicates (n = 3). Sptlc1: serine palmitoyltransferase long chain base subunit 1; Sptlc2: serine palmitoyltransferase long chain base subunit 2; Kdsr: 3-ketodihydrosphingosine reductase. Values denoted by * and # were considered significant if P < 0.05. Statistical parametric (two-way ANOVA; Student’s t-test and Tukey’s test) and nonparametric analyses (Mann-Whitney test and Friedman test) were conducted with LIPOTYPEZOOM Interactive Data and Visualisation software and further confirmed by GRAPHPAD PRISM 6.0 (GraphPad Software). * indicates significant differences between WT and VLCAD−/− mice under the same dietary regimen. # indicates significant differences between mice of the same genotype under different dietary regimen.
of plasmalogens. In contrast, females that did not increase their plasmalogen biosynthesis and content upon C8 incubation did not display any increment of peroxisomal activity [18]. Based on the fact that lack of plasmalogens impaired intracellular cholesterol (CHOL) distribution in several cell culture systems [68] and on our results on the sex-specific response to C8 treatment, we cannot exclude differences in CHOL trafficking after treatment with medium-chain FAs.

In summary, our data underline clearly that treatment with medium-chain FAs may have a deep impact not only on de novo biosynthesis and elongation of FAs as reported previously [15–18, 67], but also on the overall homeostasis of cellular complex lipids. Although C8 led to the stimulation of lipogenesis in both sexes, the outcome was completely different. Fibroblasts from VLCAD<sup>−/−</sup> female mice accumulated PC and LPC (Fig. 6B) which is strongly suggestive of a sex-dependent pro-inflammatory effect of C8. In contrast, the C8-induced stimulation of peroxisomal activity described in cells from VLCAD<sup>−/−</sup> male mice [18] was reflected in an enhancement of the concentration of plasmalogens and neutral lipids. Despite the remarkable differences observed between the sexes, the biological and physiological implications of alterations in the composition of membrane phospholipids are rather complex. Therefore, the clinical implication of our findings is difficult to predict and to translate to patients on long-term medium-chain FA supplementation.

**Materials and methods**

**Animals**

All animal studies were performed with the approval of the University’s Institutional Animal Care and Use Committee in Freiburg and were in accordance with the Committees’ guidelines (approval number: 35-9185.81/G-14/20). The data presented in this research article were obtained by experiments performed in fibroblasts from male and female WT and VLCAD<sup>−/−</sup> mice. VLCAD<sup>−/−</sup> mice were generated as described by Exil et al. [69].

**Cell culture**

Experiments were performed with murine fibroblasts from WT and VLCAD<sup>−/−</sup> mice. Fibroblasts cell lines were obtained after a skin biopsy was performed from ears of WT and VLCAD<sup>−/−</sup> mice of both sexes. Primary fibroblasts cultures were obtained from mice at the age of 9–10 weeks. The experiments were performed using three biological replicates obtained by three different mice of each sex and genotype (n = 3). Skin dermal cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (x1) containing 10% FBS, 4.5 g L<sup>−1</sup> D-glucose, GlutaMAX, 20 Mm HEPES, 100 units mL<sup>−1</sup> penicillin and 100 µg mL<sup>−1</sup> streptomycin, at 37 °C in air with 5% CO<sub>2</sub>. Incubation studies with C8 were performed by adding 300 µM of C8 to the control medium until the preparation of the pellets for
the different analysis. To ensure a constant concentration of C8, the medium was changed every second day.

**SILAC-based proteomic analysis, sample preparation and measurement**

Cells used for SILAC labelling were cultured in DMEM (x1) containing 10% FBS, 1 g·L⁻¹ D-Glucose, L-glutamine, 100 units·mL⁻¹ penicillin and 100 μg·mL⁻¹ streptomycin, at 37 °C in air with 5% CO₂. For the labelling, cells were cultured in SILAC/DMEM without lysine and arginine supplemented with 10% dialysed FBS, penicillin/streptomycin (100 units·mL⁻¹, 100 μg·mL⁻¹), 2 mM L-glutamine, 82 mg·L⁻¹ proline and different isotopes of lysine and arginine.

Forty milligram per liter L-arginine HCl (Arg0) and 73 mg·L⁻¹ L-lysine HCl (Lys0) for light labelling. Arg0 and Lys0 were replaced by L-arginine-13C6-14N4 (Arg6) and L-lysine-2H4 (Lys4) for medium labelling and with L-arginine-13C6-15N4 (Arg10) and L-lysine-13C6-15N2 (Lys8) for heavy labelling. The culture media were changed every 2–3 days. Cells were kept in ‘labelled’ medium for 6 cell passages to achieve >95% labelling over a total period of 7 weeks. Sample preparation and measurement were performed as previously described [18,70].

**Lipid extraction for mass spectrometry lipidomics**

Mass spectrometry (MS)-based lipid analysis was performed by Lipotype GmbH (Dresden, Germany) as described [71]. Fibroblasts were kept in culture over 6 weeks either under control conditions or under incubation with C8 until the needed amount of cells for analysis was reached. From this culture, 3*10⁶ cells were pelleted for lipidomic analysis, while a second aliquot consisting of 1*10⁶ cells was used for RNA extraction. Lipids were extracted using a two-step chloroform/methanol procedure [72]. Samples were spiked with internal lipid standard mixture containing CL 16 : 1/15 : 0/15 : 0, Cer 18 : 1/2 : 17 : 0, DAG 17 : 0/17 : 0, HexCer 18 : 1/2/12 : 0, lysophosphatidate 17 : 0 (LPA), LPC 12 : 0, lysophosphatidyethanolamine 17 : 1 (LPE), lysophosphatidylglycerol 17 : 1, lysophosphatidylinositol 17 : 1 (LPI), lysophosphatidylserine 17 : 1 (LPS), phosphatidate 17 : 0/17 : 0 (PA), PC 17 : 0/17 : 0, PE 17 : 0/17 : 0, phosphatidylglycerol 17 : 0/17 : 0 (PG), PI 16 : 0/16 : 0, PS 17 : 0/17 : 0, CHOL ester 20 : 0 (CE), SM 18 : 1/2/12 : 0 and TAG 17 : 0/17 : 0/17 : 0. After extraction, the organic phase was transferred to an infusion plate and dried in a speed vacuum concentrator. 1st step dry extract was resuspended in 7.5 mM ammonium acetate in chloroform/methanol/propanol (1 : 2 : 4, V : V : V) and 2nd step dry extract in 33% ethanol solution of methylene in chloroform/methanol (0.003 : 5 : 1; V : V : V). All liquid handling steps were performed using Hamilton (Gräfelfing, Germany) Robotics STARlet robotic platform with the Anti Droplet Control feature for organic solvent pipetting.

**MS data acquisition**

Samples were analysed by direct infusion on a QExactive mass spectrometer (Thermo Scientific, Dreieich, Germany) equipped with a TriVersa NanoMate ion source (Advion Biosciences, Ithaca, NY, USA). Samples were analysed in both positive and negative ion modes with a resolution of R₅₅/z = 200 = 280 000 for MS and R₅₅/z = 200 = 17 500 for tandem MS (MSMS) experiments, in a single acquisition. MSMS was triggered by an inclusion list encompassing corresponding MS mass ranges scanned in 1-Da increments [73]. MS and MSMS data were combined to monitor CE, DAG and TAG ions as ammonium adducts; PC, PC O-, as acetate adducts; and CL, PA, PE, PE O-, PG, PI and PS as deprotonated anions. MS only was used to monitor LPA, LPE, LPE (-ether), LPI and LPS as deprotonated anions and Cer, HexCer, SM, LPC and LPC (-ether) as acetate adducts.

**Data analysis and postprocessing**

Data were analysed with in-house developed lipid identification software based on LIPID2FLORER [74,75]. The identified lipid molecules were quantified by normalization to a lipid class-specific internal standard. The amounts in pmol of individual lipid molecules (species of subspecies) of a given lipid class were summed to yield the total amount of the lipid class. The amounts of the lipid classes were normalized to the total lipid amount yielding mol% per total lipids. The quantities of the lipid species containing the same number of double bonds were summed, and these values were normalized to the total amount of the given lipid class. The values were thus given as mol% per total lipids. The quantities of the lipid species containing the same number of carbon atoms in the hydrocarbon moiety were summed, and these values were normalized to the total amount per total lipids. Data postprocessing and normalization were performed using an in-house developed data management system. Only lipid identifications with a signal-to-noise ratio > 5, and a signal intensity fivefold higher than in corresponding blank samples were considered for further data analysis. Statistical parametric and nonparametric analyses were conducted with LIPOTYPEZOOM Interactive Data and Visualisation software and further confirmed by the Mann–Whitney test and Friedman test via GRAPHPAD PRISM 6.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant if P < 0.05.
RT–PCR analysis

RT–PCR was performed in RNA extracted from 1 x 10^6 cells obtained from the same culture used for lipidomic analysis. Total RNA from the fibroblast pellets was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Forward and reverse primers were designed with the primer design tool from NCBI (http://www.ncbi.nlm.nih.gov/tools). RT–PCR was performed in a single-step procedure with the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad, München, Germany) on a CFX96 Touch (Bio-Rad). Gene coding for the 18S ribosomal subunit was used as reference. Primers used in this work are summarized in Table 1.

Statistical analysis

All reported data are presented as means ± standard deviation (SD). n denotes the number of biological replicates (n = 3). Analysis for the significance of differences with the exception of the lipidomic data was performed using Student’s t-tests for paired and unpaired data. To test the effects of the variables, diet and genotype two-way analysis of variance (ANOVA) with the Tukey post-test was performed (Graphpad Prism 6.0; GraphPad Software). Differences were considered significant if P < 0.05.

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Conflicts of interest

ST has received a grant by Vitafl and travel reimbursements by Dr. Schär unrelated to this study. There is no other potential conflict of interest to be declared by any of the coauthors.

Author contributions

ST conceptualized the data. ST, KIA and ZW involved in methodology. KIA and ZW investigated the data, curated the data and wrote the original draft. US and ST wrote, reviewed and edited the manuscript. ST serves as a project administrator, supervised the work and acquired funding.

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**Supporting information**

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

Table S1. Change of total fatty acid chain length in phosphatidylcholine, phosphatidylethanolamine and their corresponding plasmalogen.