Lipotoxicity in adrenoleukodystrophy

Size matters!

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Citation for published version (APA):
van de Beek, M.-C. (2018). Lipotoxicity in adrenoleukodystrophy: Size matters!

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Download date: 25 Jan 2020
Chapter 6

Redirecting very long-chain fatty acid synthesis in adrenoleukodystrophy

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Abstract

X-linked adrenoleukodystrophy (ALD) is a progressive neurodegenerative disease caused by mutations in \textit{ABCD1}. ALD is characterized by the accumulation of very long-chain fatty acids (VLCFA, ≥C22:0) in plasma and tissues due to impaired peroxisomal beta-oxidation. The majority of the male patients develop adrenal insufficiency and myelopathy (adrenomyeloneuropathy) but patients are at risk to develop a cerebral inflammation known as cerebral ALD. Female patients do not develop adrenal insufficiency or cerebral ALD but can develop a myelopathy. Saturated VLCFA are toxic to ALD cells, whereas mono-unsaturated VLCFA are not. Therefore, a redirection of the synthesis of saturated VLCFA towards mono-unsaturated VLCFA could alleviate the toxic effect of VLCFA and be beneficial for ALD. In this study we used human primary fibroblasts and show that liver X receptor (LXR) agonists activate stearoyl-CoA desaturase-1 (SCD1), and as a consequence, reroute the VLCFA synthesis towards the mono-unsaturated VLCFA. As a consequence, exposure of ALD fibroblasts to LXR agonists results in a complete normalization of C26:0 levels in ALD fibroblasts.
1. Introduction

X-linked adrenoleukodystrophy (ALD, MIM 300100) is the most common of the leukodystrophies with a birth incidence of 1:14,700. ALD is characterized by impaired peroxisomal beta-oxidation of very long-chain fatty acids (VLCFA, ≥C22:0). This is the result of a defect in the peroxisomal transmembrane protein ABCD1 that transports VLCFA as CoA-esters across the peroxisomal membrane into the peroxisomal matrix where the VLCFA are degraded via beta-oxidation. As a result of ABCD1 protein deficiency, VLCFA accumulate in plasma and tissues, including the spinal cord, white matter of the brain and the adrenal cortex. ALD is characterized by a striking and unpredictable variation in clinical outcomes, even within families. Almost all male patients develop adrenal insufficiency and myelopathy (adrenomyeloneuropathy). Female patients may also develop myelopathy, but adrenal insufficiency or leukodystrophy are very rare. Between 30-40% of male patients develop cerebral demyelination (cerebral ALD) between the age of 3 and 18 years, but they can still develop cerebral ALD as adults. If diagnosed in a very early stage, cerebral ALD can be halted or reversed by hematopoietic stem cell transplantation (HSCT). However, the clinical outcome is very poor if HSCT is not performed in the earliest stage of the cerebral demyelination. No disease-modifying therapy is available for the myelopathy that affects virtually all male patients and 80% of the women with ALD. Furthermore, the finding that patients who received a HSCT during childhood can still develop a myelopathy in adulthood, indicates that HSCT only stops the inflammatory component of the disease without addressing the underlying biochemical defect. This is supported by data that shows that VLCFA levels were not reduced after HSCT. For this reason we hypothesized that chronic exposure to VLCFA causes the progressive myelopathy and that additional environmental triggers and/or genetic factors are required for the initiation of cerebral demyelination. This finding highlights the need to develop effective treatments aimed at the normalization of VLCFA levels in the CNS. We anticipate that a normalization of the VLCFA levels, prevents ALD patients from developing AMN and the development of cerebral demyelination.

Only a small part of the VLCFA that accumulate in ALD is of dietary origin. The majority of VLCFA are synthesized endogenously from long-chain fatty acids, via a 4-step elongation system composed of multiple enzymes including “elongation of very long chain fatty acids protein 1” (ELOVL1), the human VLCFA-specific elongase. In the 1980’s it was shown that C26:0 levels in ALD fibroblasts can be reduced when cultured in the presence of oleic acid (C18:1) and erucic acid (C22:1). This is most likely due to the fact that the elongation enzymes show higher affinity for mono-unsaturated fatty acids. Based on these findings, Lorenzo’s oil, a 4:1 mixture of the triglyceride form of oleic acid and erucic acid, was developed. Indeed, treatment with Lorenzo’s oil normalized C26:0 levels at least in plasma of ALD patients but unfortunately neurological progression was not halted. Most likely this is due to a limited effect of Lorenzo’s oil on C26:0 levels in the brain.
The enzyme stearoyl-CoA desaturase-1 (SCD1) plays a central role in VLCFA metabolism. SCD1 is the key enzyme in the formation of mono-unsaturated fatty acids. The enzyme introduces a cis-double bond at the ω-9 position of palmitic acid (C16:0) and stearic acid (C18:0), resulting in palmitoleic acid (C16:1) and oleic acid (C18:1), respectively.29 Previously, we have shown that saturated VLCFA induce endoplasmic reticulum (ER) stress in fibroblasts from ALD patients and that exposure of ALD fibroblasts to C26:0 resulted in lipoapoptosis30. Interestingly, exposure of ALD cells to C26:1 did not induce ER stress30. This is in line with earlier reports showing that addition of oleic acid can even halt ER stress that was induced by high levels of palmitic acid31. Taken together, these results show that mono-unsaturated fatty acids are less toxic than saturated fatty acids. We hypothesize that if one would be able to increase the activity of SCD1, this would result in a shift in the fatty acid synthesis from saturated VLCFA towards mono-unsaturated VLCFA, and consequently decreased levels of saturated VLCFA. If successful, this rerouting towards the mono-unsaturated fatty acids, would open a new option towards a therapy for ALD and other peroxisomal disorders. An additional advantage of SCD1 activation with respect to Lorenzo’s oil would be that the total amount of VLCFA should not be increased by SCD1 activation. In this study, we investigated whether SCD1 is indeed a therapeutic target for ALD.

2. Material and methods

2.1 Chemicals

Methyl esters of C16:0 (meC16:0) and C18:0 (meC18:0) were obtained from Sigma Aldrich. Methyl esters of C20:0 (meC20:0), C22:0 (meC22:0), C24:0 (meC24:0), C16:1 (meC16:1), C18:1 (meC18:1), C20:1 (meC20:1), C22:1 (meC22:1) and C24:1 (meC24:1) were obtained from Larodan Fine Chemicals. Methyl esters of C26:0 (meC26:0) and C26:1 (meC26:1) were prepared as described previously30. Tunicamycin (TM) was obtained from Sigma Aldrich. Hexadecanoic-16,16,16-d3 acid (D3-C16:0) and octadecanoic-17,17,18,18,18-d5 acid (D5-C18:0) were obtained from CDN isotopes. TO901317 was obtained from Cayman Chemical. GW3965 and LXR623 were obtained from Sigma Aldrich. SCD1 inhibitor 1716 was obtained from BioVision. All chemicals were dissolved in DMSO (Sigma Aldrich). Antibodies against SCD1 (M38 #2438) and XBPIs (D2C1F) were obtained from Cell Signaling Technology. Antibody against beta-actin (A5441) was obtained from Sigma Aldrich and IRDye secondary antibodies were obtained from LI-COR Inc.

2.2 Cell culture

Human primary skin fibroblast cell lines were obtained from ALD patients through the Neurology Outpatient Clinic of the Academic Medical Center. All ALD patients are currently participating in a prospective natural history study with annual follow-up (IRB: METC
Material from these patients was obtained from the peroxisomal biobank from the Academic Medical Center (IRB: METC 2015_066). The diagnosis ALD was confirmed via VLCFA and ABCD1 mutation analysis (p.Met1Val; p.Asp194His; p.Glu278*; p.Ser284*; p.Arg389His; p.Gln472Argfs*83; p.Pro480Thr; p.Leu654Pro). Written informed consent was received from each patient. Control fibroblasts were obtained from anonymous male volunteers with written informed consent. Cells were cultured in Dulbecco’s modified eagle medium DMEM (Lonza) with high glucose (4.5 g/L) and L-glutamine supplemented with 10% v/v fetal bovine serum (FBS; Bodinco), 25 mM HEPES (Lonza), 100 U/ml penicillin (Life Technology), 100 µg/ml streptomycin (Life Technology) and 0.25 µg/ml fungizone (Life Technology). Cells were cultured at 37°C/5% CO2/20% O2 in a humidified environment. To standardize tissue culture conditions, cells were kept at 100% confluency for one week, trypsinized and seeded at approximately 40% confluency at the start of each experiment. Cells were allowed to attach and recover for at least 8 hours before compounds were added. In each experiment, the final concentration of DMSO in culture medium was equal (≤ 1%) in all conditions.

2.3 VLCFA analysis

2.3.1 Desaturation assay and de novo fatty acid synthesis

Cells were cultured as described in section 2.2. To inhibit SCD1, cells were pre-incubated overnight with 100 nM SCD1 inhibitor (BioVision 1716). The next day, the culture medium was replaced by culture medium with 100 µM D5-C18:0 or 100 µM D3-C16:0 combined with the different compounds as indicated in the figure legends. After 24 hours (D5-C18:0, desaturation assay) or 72 hours (D3-C16:0, de novo synthesis assay), cells were harvested by trypsinization, rinsed twice with phosphate-buffered saline (PBS) and pellets were homogenized in deionized water via sonication on ice with a needle (12 seconds at 7-8 Watt). Pierce BCA protein assay was used to determine the protein concentration. 150 µg protein was used for the desaturation assay as described previously32, 33 or the de novo synthesis assay as described previously34, 35. VLCFA levels were analyzed as described previously (Valianpour, et al., 2003).

2.3.2 Endogenous fatty acid levels

Cells were cultured as described in section 2.2. Cells were incubated with compounds or vehicle DMSO. Concentrations and incubation times are indicated in the figure legends. Culture medium and compounds were refreshed every 5 days. After incubation, the cells were harvested by trypsinization as described in section 2.3.1. 150 µg protein was used for fatty acid analysis as described previously16.
2.3.3 Lipidomics
Cells were cultured as described in section 2.2. Cells were incubated with 5µM TO901317 or vehicle (DMSO) for 3 weeks after which the cells were harvested by typsinization as described in section 2.3.1. Lipidomics analysis was performed following the method described previously with minor modifications. Phospholipids were extracted using a single-phase extraction. To this end, to 1 mg fibroblast protein homogenate a defined amount of internal standards dissolved in 120 µL of chloroform/methanol (1:1, v/v), and 1.5 mL of chloroform/methanol (1:1, v/v) was added. The internal standards mixture consisted of: 0.1 nmol of CL(14:0), 0.2 nmol of BMP(14:0), 2.0 nmol of PC(14:0), 0.1 nmol of PG(14:0), 5.0 nmol of PS(14:0), 0.5 nmol of PE(14:0), 0.5 nmol of PA(14:0), 0.1 nmol of PI(14:0), 2.0 nmol of SM(d18:1/12:0), 0.02 nmol of LPG(14:0), 0.1 nmol of LPE(14:0), 0.5 nmol of LPC(14:0) and 0.1 nmol of LPA(14:0) (Avanti Polar Lipids). The mixture was sonicated in a water bath for 5 min, followed by centrifugation at 4 °C (16,000xg for 5 min). The liquid phase was transferred to a glass vial and evaporated under a steam of nitrogen at 60 °C. Subsequently, the residue was dissolved in 150 µL of chloroform/methanol (9:1, v/v), and 2 µL of the solution was injected into the high-performance liquid chromatography-mass spectrometry (HPLC-MS) system.

2.3.4 HPLC-MS
The HPLC system consisted of an Ultimate 3000 binary HPLC pump, a vacuum degasser, a column temperature controller, and an auto sampler (Thermo Scientific). The column temperature was maintained at 25 °C. The lipid extract was injected onto a LiChrospher 2*250-mm silica-60 column, 5 µm particle diameter (Merck). The phospholipids were separated from interfering compounds by a linear gradient consisting of solution A (methanol/water, 85:15, v/v) and solution B (chloroform/methanol, 97:3, v/v). Solutions A and B contained 5 and 0.2 ml of 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient (0.3 ml/min) was as follows: T = 0 - 1 min: 10% A; T = 1 - 4 min: 10% A–20% A; T = 4 - 12 min: 20% A–85% A; T = 12 - 12.1 min: 85% A–100% A; T = 12.1 - 14.0 min: 100% A; T = 14 - 14.1 min: 100% A–10% A and T = 14.1 - 15 min: equilibration with 10% A. All gradient steps were linear and the total analysis time, including the equilibration, was 15 min. A Thermo Scientific Q Exactive Plus Orbitrap Mass Spectrometer was used in the negative and positive electrospray ionization mode. Nitrogen was used as the nebulizing gas. The spray voltage used was 2500 V, and the capillary temperature was 256 °C. S-lens RF level: 50, auxiliary gas: 11, auxiliary gas temperature 300°C, sheath gas: 48, sweep cone gas: 2. In both the negative and positive mode, mass spectra of phospholipid molecular species were obtained by continuous scanning from m/z 150 to m/z 2000 with a resolving power of 280,000 FWHM.
2.4 Immunoblotting

XBP1s: fibroblasts were incubated with fatty acids as described previously \(^{10}\). After 4 days of incubation with 60 µM fatty acids or 1 day with 10 µg/ml tunicamycin (positive control for XBP1s), pellets were prepared as described in section 2.3.1. Pellets were resuspended in PBS with protease inhibitor cocktail (Complete mini protease inhibitor cocktail, Roche) homogenized by sonication with a needle on ice for 12 seconds at 7-8 Watt. Protein concentration was determined with the bicinchoninic acid assay using humane serum albumin as standard. The protein samples were diluted in 5x Laemmli sample buffer and incubated at 95°C for 5 minutes. Twenty-five µg protein was loaded on a 10% SDS-PAGE running gel. After protein separation, proteins were transferred to a 0.45 µM pore size nitrocellulose membrane. The membrane was incubated for one hour with 5% w/v non-fat dried milk powder-TRIS buffered saline + 0.1% Tween-20 (TBST), followed by 2 hours of incubation with anti-XBP1s (D2C1F Cell Signaling Technology) 1:1,000 in 5% w/v non-fat dried milk powder-TBST. Next, the membrane was incubated for 1 hour with IRDye goat-anti-rabbit cw800 (LI-COR Inc.) 1:10,000 in 5% w/v non-fat dried milk powder-TBST + 0.01% w/v SDS.

SCD1: control and ALD fibroblasts were seeded at approximately 40% confluence. The next day, culture medium was replaced by culture medium containing LXR agonists or vehicle DMSO. After 3 days of incubation, cell pellets were made as described under 2.3.1. Protein samples were prepared as described for the detection of XBP1s. The protein samples were diluted in 2x Laemmli sample buffer with 8 M urea. 40 µg of protein was loaded on a 12.5% SDS-PAGE running gel. After protein separation, proteins were transferred to a nitrocellulose membrane with a pore size of 0.45 µM. The membrane was incubated for 1 hour with 5% w/v BSA in TBST followed by 2 hours of incubation with anti-SCD1 (M38 #2438 Cell Signaling Technology) 1:1,000 in 5% w/v BSA in TBST. Next, the membrane was incubated for 1 hour with IRDye goat-anti-rabbit cw800 (LI-COR Inc.) 1:10,000 in 5% w/v BSA-TBST + 0.01% w/v SDS.

To control for equal protein loading, the membranes were incubated with anti-beta-actin 1:20,000 (A5441 Sigma-Aldrich) in Odyssey blocking buffer (LI-COR);phosphate buffered saline + 0.1% Tween-20 (PBST) 1:1 for 30 minutes followed by incubation with IRDye donkey-anti-mouse CW680 1:10,000 (LI-COR Biosciences) in Odyssey blocking buffer (LI-COR);PBST + 0.01% w/v SDS for 1 hour.

All incubations were performed at room temperature. Fluorescent labeled proteins were detected by LI-COR Odyssey.

2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.0. Statistical comparisons were made using one-way ANOVA followed by Bonferroni’s or Dunnett’s
multiple comparisons test as indicated in figure legends. P<0.05 was used as the criterion for statistical significance. * P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Bioinformatics and statistical analysis of the lipidomics data was performed as described previously37.

3. Results

3.1 Lipid-induced ER stress depends on the chain-length and saturation/desaturation status of fatty acids

Previously we validated the use of VLCFA methyl esters to study VLCFA metabolism in whole cells and showed that the lipid-induced ER stress response in ALD fibroblasts is chain-length dependent with the strongest effect with meC26:056. To compare the toxicity of mono-unsaturated fatty acids with saturated fatty acids, we cultured control and ALD fibroblasts with equimolar concentrations of saturated long-chain (meC16:0, meC18:0 and meC20:0) and very long-chain (meC22:0, meC24:0 and meC26:0) fatty acids and mono-unsaturated long-chain (meC16:1, meC18:1 and meC20:1) and very long-chain (meC22:1, meC24:1 and meC26:1) fatty acids. As a marker for lipid-induced ER stress XBP1s protein levels were measured. As a positive control for ER stress, control and ALD fibroblasts were exposed to tunicamycin, an inhibitor of N-linked glycosylation and well-known to induce ER stress. As expected, treatment of both control and ALD fibroblasts with tunicamycin caused increased XBP1s protein levels (Fig 1). Exposure of control fibroblasts to either long-chain or very long-chain saturated or mono-unsaturated fatty acids did not affect the expression of the XBP1s

![Figure 1](image.png)

**Figure 1.** Protein analysis of XBP1s protein after incubation with saturated and mono-unsaturated fatty acids.

Control (C) and ALD (A) fibroblasts were incubated for 1 day with 10 µg/mL tunicamycin (TM) or for 4 days with 60 µM of long-chain and very long-chain fatty acids. The effect of treatment on XBP1s protein was determined in control (n=3) and ALD (n=3) fibroblasts. Beta-actin (B) was used to control for equal amount of protein loading.
protein (Fig 1). Exposure of ALD fibroblasts to saturated and mono-unsaturated long-chain fatty acids (meC16 – meC20), meC22:0 or mono-unsaturated fatty acids did not result in increased XBP1s protein levels (Fig 1). Only when ALD cells were incubated with the saturated VLCFA meC24:0 and meC26:0 levels of XBP1s protein were markedly increased (Fig 1). These results show that the lipid-induced ER stress response in ALD fibroblasts is both chain-length dependent and specific for saturated VLCFA. Therefore, a shift in synthesis from the saturated VLCFA towards mono-unsaturated VLCFA may alleviate fatty acid toxicity in ALD.

3.2 Increased SCD1 protein and activity in human fibroblasts by LXR agonists

Treatment of mice with the liver X receptor (LXR) a/b pan agonist TO901317 resulted in increased levels of SCD1 mRNA in mouse kidney. To study whether TO901317 increases SCD1 protein levels in human fibroblasts, we cultured control and ALD fibroblasts with DMSO or TO901317 (Fig. 2A). After incubation with DMSO no SCD1 protein was detectable in control and ALD fibroblasts. In contrast, when control and ALD fibroblasts were incubated with 5 µM TO901317, SCD1 protein levels were markedly increased (Fig 2A). To study if this effect on SCD1 is specific for TO901317 or an effect of LXR agonists in general, we cultured control and ALD fibroblasts with GW3965, another LXR pan agonist and LXR623, a partial LXR agonist. SCD1 protein was markedly increased after incubation with GW3965 (Fig. 2B) and LXR623 (Fig. 2C) in both control and ALD fibroblasts. Taken together, these results show that LXR agonists increase SCD1 protein levels in human fibroblasts.

Figure 2. SCD1 protein levels after incubation with LXR agonists.
Control (n=3) and ALD (n=5) were cultured for 3 days with 0.1% v/v DMSO (-) or different LXR agonists (+): TO901317 (A, 5 µM), GW3965 (B, 1.5 µM) and LXR623 (C, 1.5 µM) and the effect of treatment on SCD1 protein was assessed. Beta-actin was used to control for equal protein loading.
To study whether LXR agonist treatment results in increased SCD1 enzyme activity, we incubated control and ALD fibroblasts for 24 hours with D5-C18:0 combined with DMSO (untreated), TO901317, GW3965 or LXR623 and measured the amount of D5-C18:1 formed from D5-C18:0. Figure 3 shows that incubation with LXR agonists results in a significant (P<0.0001) increase in D5-C18:1 levels in both control and ALD fibroblasts. To demonstrate that the increased levels of D5-C18:1 are the result of SCD1 enzyme activity, a SCD1 inhibitor (SCD1i) was added. In the presence of SCD1i no significant increase in D5-C18:1 levels was measured. These results show that LXR agonists increase SCD1 protein and fatty acid desaturation activity in ALD and control fibroblasts.

Figure 3. Effect of LXR agonists on SCD1 desaturase activity.
ALD (n=5) and control (n=5) fibroblasts were cultured for 1 day with 100 µM D5-C18:0 without and with SCD1 inhibitor (SCD1i) combined with DMSO or with 5 µM TO901317, 1.5 µM GW3965 or 1.5 µM LXR623. SCD1 desaturase activity was assessed by measuring the amount of D5-C18:1 synthesized from D5-C18:0. Final concentration DMSO in culture medium was 1% v/v. **** P<0.0001 by one-way ANOVA followed by Bonferroni’s multiple comparison test.

3.3 LXR agonists lower saturated VLCFA levels by shifting synthesis towards mono-unsaturated VLCFA
Next, we studied if incubation with LXR agonists and subsequent increased fatty acid desaturation activity would lead to decreased levels of de novo synthesized C26:0 and increased levels of de novo synthesized C26:1. To this end, we incubated control and ALD fibroblasts for 3 days with deuterium labeled C16:0 (D5-C16:0) combined with the LXR agonists and measured the levels of the deuterium labeled C26:0 (D5-C26:0) and C26:1 (D5-C26:1) synthesized from D5-C16:0. In comparison with controls, ALD fibroblasts showed enhanced C26:0 synthesis (Fig 4A). In ALD fibroblasts, the levels of D5-C26:0 were significantly reduced after incubation with TO901317 (P<0.0001), GW3965 (P<0.0001) and LXR623 (P<0.0001). In control fibroblasts, no significant differences in the levels of D5-C26:0 after incubation with the LXR agonists were found. This is probably due to the already low levels of D5-C26:0 formed in control fibroblasts. In addition, the D5-C26:0 levels in ALD fibroblasts after treatment with LXR agonists were comparable with the levels of D5-C26:0 in control fibroblasts without
LXR agonists. This indicates that the de novo synthesis of D₃-C26:0 was normalized in ALD fibroblasts upon incubation with LXR agonists. As anticipated, the decrease in de novo D₃-C26:0 was accompanied by a shift towards the increased de novo synthesis of monounsaturated fatty acids. A significant increase in deuterium labeled C26:1 (D₃-C26:1) levels in ALD fibroblasts was found upon incubation with TO901317 (P<0.0001), GW3965 (P<0.01) and LXR623 (P<0.0001) (Fig 4B). In control fibroblasts, increased levels of D₃-C26:1 were observed after incubation with LXR agonists, but this trend was not statistically significant. Taken together, these results show that LXR agonists affect de novo VLCFA synthesis by shifting the synthesis from saturated VLCFA towards mono-unsaturated VLCFA.

Figure 4. Effect of LXR agonists on de novo VLCFA synthesis.
ALD (n=5) and control (n=5) fibroblasts were cultured for 3 days with 100 µM D₃-C16:0 combined with either DMSO, 5 µM TO901317, 1.5 µM GW3965 or 1.5 µM LXR623 and the levels of D₃-C26:0 (A) and D₃-C26:1 (B) were measured. Final concentration DMSO in culture medium was 1% v/v. ** P<0.01, ****P<0.0001 by one-way ANOVA followed by Bonferroni’s multiple comparisons test.
To study whether prolonged exposure of ALD fibroblasts to LXR agonists would affect endogenous C26:0 levels, we cultured ALD fibroblasts for up to 3 weeks in the presence of 5 µM TO901317. After 1 week of incubation, C26:0 levels in ALD fibroblasts were reduced by 50% (P<0.0001) and after 2 and 3 weeks of incubation, the endogenous C26:0 levels were reduced to the levels observed in untreated control fibroblasts (P<0.0001) (Fig. 5).

**Figure 5. Effect of TO901317 on endogenous C26:0 levels.**
ALD fibroblasts (n=3) were cultured in the presence of 5 µM TO901317 up to 3 weeks. Control fibroblasts (n=3) were untreated. Two biological replicates were included in each condition. **** P<0.001 by one-way ANOVA followed by Dunnett’s multiple comparisons test compared with vehicle treated ALD fibroblasts.

To study if GW3965 an LXR623 have similar effects on endogenous C26:0 levels as TO901317, ALD and control fibroblasts were cultured for 3 weeks with TO901317, GW3965 and LXR623 and the endogenous C26:0 levels were measured (Fig. 6). Compared to vehicle treated ALD fibroblasts, C26:0 levels were significantly decreased after incubation with TO901317 (P<0.0001), GW3965 (P<0.0001) and LXR 623 (P<0.0001). In control fibroblasts, the C26:0 levels were significantly decreased after exposure to TO901317 (P<0.0001), GW3965 (P<0.0001) and LXR623 (P<0.0001). Interestingly, there is no significant difference between the C26:0 levels of DMSO treated control fibroblasts and the ALD fibroblasts cultured with respectively TO901317, GW3965 or LXR623. The results of this experiment show that LXR agonists reduced endogenous C26:0 levels in ALD fibroblasts to levels found in control fibroblasts.
Redirecting VLCFA synthesis in ALD

Figure 6. Endogenous C26:0 levels in fibroblasts.
ALD (n=5) and control (n=5) fibroblasts cell lines cultured for 3 weeks with 0.1% v/v DMSO or 5 µM TO901317 or 1.5 µM GW3965 or 1.5 µM LXR623. Two biological replicates were included for each condition. **** P<0.001, ns: not significant by one-way ANOVA followed by Bonferroni’s multiple comparisons test.

3.4 Effect of TO901317 on phospholipids
The VLCFA that accumulate in ALD are present in many different lipid species, but the greatest excess has been reported in gangliosides\(^{39}\), phosphatidylcholine\(^{8}\) and cholesterol ester fractions\(^{40}\). VLCFA-enriched phosphatidylcholine species are highly increased in intact myelin in ALD patients\(^{40}\) and have been suggested to trigger the onset of disease. Indeed, injection of C24:0-lysophosphatidylcholine, but not C16:0-lysophosphatidylcholine, into wild type mouse brain resulted in widespread microglial activation and apoptosis\(^{41}\).
To investigate whether exposure of ALD fibroblasts to LXR agonists affects not only total VLCFA levels, but also the composition of phospholipids, we performed lipidomic analysis. For this, 8 control and 8 ALD fibroblasts cell lines were cultured with DMSO and 4 ALD fibroblast cell lines were cultured with 5 µM TO901317 for 3 weeks. We identified 1,061 distinct lipid species. Phospholipids are indicated as C(XX:Y), where XX indicates the total number of carbon atoms and Y the total number of double bonds in the fatty acyl chains. This analysis, without fragmentation, does not allow the specification of which fatty acid is located at the sn-1 or sn-2 position. Comparison of the lipid profiles of control and ALD cells revealed an overall increase in phosphatidylcholine (PC) species containing fatty acids with a total number of >44 carbon atoms, which indicates an enrichment in VLCFA (Fig 7).
Figure 7: Visualization of the comparison of the lipid profiles of control and ALD fibroblasts in a volcano plot.

Lipid species marked in green indicate a >4-fold change.

The x-axis shows the log2 fold-change in ALD cells and the y-axis the significance of this change represented as –log10(p) value. More in overall increase in phospholipid fatty acids (PC) species containing fatty acids with a total number of >4 carbon atoms, which indicates an enrichment in VLCFA.

Lipid species marked in green indicate a >4-fold change.
Figure 8. Visualization of the comparison of lipid profiles of control, ALD and ALD fibroblasts after incubation with TO901317.

**A:** Heat map showing the Top 50 most changed lipid species based on the variable influence on projection (VIP) score extracted from the orthogonal projections to latent structures (OPLS). The left panel indicates samples from control fibroblasts (n=8), the middle panel samples from ALD patient fibroblasts (n=8), and the right panel samples from ALD fibroblasts treated with 5 µM TO901317 for 3 weeks (n=4). Color in the heat map reflects the logarithm of the relative lipid abundance with red being higher and blue lower than the mean abundance value per lipid.

**B:** C26:0-lysoPC levels in control (n=8), ALD (n=8) and ALD fibroblasts after treatment with 5µM TO901317 for 3 weeks (n=4).
Next, we investigated if the correction in total VLCFA levels was also reflected in the ALD phospholipid profile. To this end, we compared control and ALD fibroblasts with ALD fibroblasts treated with TO901317 for 3 weeks. The heat map in Figure 8 shows the 50 lipid species that were most increased in ALD cells in comparison to control fibroblasts and the effect of TO901317 treatment on these lipid species. Treatment of ALD fibroblasts with TO901317 resulted in a complete correction in the majority of phospholipids. This is exemplified by the complete correction of C26:0-lysoPC (Figure 8B).

4. Discussion

Saturated VLCFA are toxic to ALD cells. Exposure of saturated VLCFA to ALD cells causes oxidative stress and mitochondrial dysfunction and an increase in the ER stress response. Interestingly, mono-unsaturated VLCFA do not induce an ER stress response. In addition, it has been reported that mono-unsaturated long-chain fatty acids (C18:1) can even rescue ER stress caused by high levels of palmitic acid (C16:0). For these reasons, a shift from saturated towards mono-unsaturated VLCFA is beneficial. VLCFA are synthesized via de novo synthesis from saturated long-chain fatty acids thereby generating intrinsically toxic saturated VLCFA. SCD1 desaturates C16:0 and C18:0 resulting in C16:1 and C18:1 which are substrates for the same elongation enzymes that elongate saturated fatty acids. Therefore, we hypothesized that increased SCD1 activity causes a shift in the fatty acid synthesis from saturated fatty acids towards mono-unsaturated fatty acids and that as a consequence saturated VLCFA levels may decrease. Our results demonstrate that increased levels of XBP1s mRNA, a marker for ER stress, were only detected in ALD fibroblasts after incubation with the saturated VLCFA meC24:0 and meC26:0. When ALD fibroblasts were exposed to equal levels of the mono-unsaturated VLCFA meC24:1 and meC26:1, this did not result in ER stress. These data show that rerouting the fatty acid synthesis towards mono-unsaturated fatty acids is beneficial for ALD.

The key enzyme for the formation of mono-unsaturated fatty acids is SCD1. Zhang et al. reported that the LXR pan agonist TO901317 increases the levels of SCD1 mRNA in the kidney of mice that were treated with TO901317. Here we show that incubation of fibroblasts from ALD patients and healthy controls with TO901317 resulted in a markedly increase in the SCD1 protein levels. To test whether this result was specific for TO901317 or a result of LXR agonists in general, we included additional LXR agonists. GW3965 is also a LXR pan agonist and LXR623 is a partial LXR agonist. Treatment of control and ALD fibroblasts with GW3965 and LXR623 resulted in a comparable increase in SCD1 protein, which demonstrates that the increase in SCD1 protein is not a specific effect of TO901317 but an effect of LXR agonists in general. The increase in SCD1 protein by LXR agonists is not directly regulated via LXR but indirectly via sterol regulatory element-binding protein 1c (SREBP1c). The promotor region
of SCD1 does not contain a LXR response element but it has been shown that SREBP1c is a direct target of LXR and SCD1 is regulated via SREBP.

To demonstrate that the effect of LXR agonists on SCD1 protein levels also led to an increased SCD1 enzymatic activity, control and ALD fibroblasts were cultured in the presence of D₅-C₁₈:₀ and the produced amount of D₅-C₁₈:₁ was measured. Treatment of ALD and control cells with LXR agonists resulted in a 2 to 3-fold increase in the formation of D₅-C₁₈:₁ from D₅-C₁₈:₀. The addition of a SCD1 inhibitor completely blocked the synthesis of D₅-C₁₈:₁, which confirms that LXR agonists increase SCD1 enzyme activity.

In order to verify that an increase in SCD1 results in a shift in the de novo fatty acids synthesis from the saturated towards the mono-unsaturated VLCFA, we measured the de novo synthesis of D₃-C₂₆:₀ from D₃-C₁₆:₀. In the absence of LXR agonists D₃-C₂₆:₀ levels in ALD cells were 10-fold higher when compared to control cells, which is in line with previous results that demonstrated that ALD cells have enhanced de novo D₃-C₂₆:₀ synthesis. In the presence of LXR agonists, we found a significant decrease in D₃-C₂₆:₀ de novo synthesis in ALD fibroblasts. Interestingly, the levels of D₃-C₂₆:₀ in ALD cells were reduced to the levels measured in untreated control fibroblasts. In line with the activation of SCD1 activity, the de novo synthesis of D₃-C₂₆:₁ was significantly increased in ALD fibroblasts after incubation with the LXR agonists. In control cells, a similar trend was found, but the effect was not statistically significant. This study shows that LXR agonists activate SCD1 expression and activity which causes a shift in the de novo fatty acid synthesis of saturated VLCFA towards mono-unsaturated VLCFA. After the desaturation the mono-unsaturated fatty acid is further elongated to D₃-C₂₆:₁ by the concerted action of ELOVL6, ELOVL3 and ELOVL1, respectively.

Both saturated and mono-unsaturated fatty acids are substrates for ELOVL6, ELOVL3 and ELOVL1.

After incubation with TO901317 for at least two weeks, the endogenous C₂₆:₀ levels in ALD fibroblasts decreased to control levels. After 3 weeks of incubation with TO901317 or GW3965 or LXR623, a complete normalization of the endogenous C₂₆:₀ levels in ALD fibroblasts is found. This is the first study that identifies small molecules that are able to cause a complete normalization of endogenous C₂₆:₀ levels in ALD fibroblasts. Earlier studies reported reduced endogenous C₂₆:₀ levels with other small-molecules. For example, treatment of ALD cells for 3 weeks with bezafibrate, a PPAR agonists, that was also shown to be an inhibitor of ELOVL1, reduced endogenous C₂₆:₀ levels up to 30%. 4-phenylbutyrate, which induces ABCD2 expression and causes peroxisome proliferation in human primary fibroblasts, reduced C₂₆:₀ levels in ALD fibroblasts by 47% after 3 weeks of incubation. Incubation of ALD fibroblasts with oleic acid (C₁₈:₁) resulted in a decrease in C₂₆:₀ levels of approximately 30%. However, a complete normalization of C₂₆:₀ in ALD fibroblasts to the levels found in control fibroblasts has not been reported before. It is important to mention that treatment of ALD cells with LXR agonists did not reduce the C₂₆:₀ levels below
the C26:0 levels that are found in untreated control cells. Incubation of control fibroblasts with LXR agonists, however, did result in a further reduction of the C26:0 levels. Although no increased levels of cell death were observed, it cannot be ruled out that this further lowering of C26:0 levels to levels below those observed in normal cells may be toxic in vivo. Saturated VLCFA are essential because they are part of complex lipids in cell membranes and myelin. For example, the epidermal barrier of the skin consists of approximately 50% ceramides, 25% cholesterol, and 15% long- and very long-chain fatty acids. That a further reduction in VLCFA to sub-normal levels may cause deleterious effects is clearly exemplified in the knockout mouse model for Elovl1. Elovl1 knockout mice died within 1 day after birth due to epidermal barrier defects. Furthermore, a Phase 1 clinical trial to evaluate safety and tolerability of LXR623 in healthy subjects reported central nervous system-related adverse events and neurologic- or psychiatric-related adverse events. However, the subjects in this trial did not suffer from a peroxisomal beta-oxidation disorder and therefore did not have increased VLCFA levels. Therefore, it cannot be ruled out that too low levels of VLCFA as a result of treatment might have contributed to at least some of the adverse effects that were reported in this study.

Finally, our study revealed large differences in the phospholipid profile between control and ALD fibroblasts. Comparison of the lipid profiles of control and ALD cells revealed an overall increase in phosphatidylcholine (PC) species containing fatty acids with a total number of >44 carbon atoms, which indicates an enrichment in VLCFA. These PC species are all potential new biomarkers for ALD but the data must be confirmed by repeating the analysis in a series of independent samples. In line with a general reduction in the total C26:0 level upon treatment of ALD cells with LXR agonists, we observed a correction in the phospholipid profile in ALD fibroblasts after incubation with TO901317. Almost all phospholipid species that are elevated in ALD fibroblasts were reduced to the levels that were seen in control fibroblasts. Interestingly, the levels of C26:0-lysoPC (LPC(C26:0)), the marker that is used in newborn screening to detect ALD, are completely normalized in ALD fibroblasts upon treatment with TO901317.

LXR agonists are extremely interesting compounds to study for a potential therapy for ALD for various reasons. One of the reasons is that saturated VLCFA induce lipoapoptosis in ALD fibroblasts. Therefore, increased levels of SCD1 protein might be protective against lipoapoptosis in ALD. Indeed, it has been shown that overexpression of SCD1 in renal proximal tubular epithelial cells protects against lipoapoptosis induced by palmitic acid (C16:0). Another study showed that TO901317 protects against inflammation in the spinal cord after spinal cord injury. Furthermore, LXR agonists are studied as a possible treatment option in various neurodegenerative disorders, such as Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, and multiple sclerosis. Based on the results presented here, LXR agonists are interesting small molecules to study for the development of a
Our data are highly promising, but it should be stressed that the results so far are in vitro data using human primary fibroblasts from ALD patients. We are currently collaborating with Dr. Sonja Forss-Petter and Prof. Johannes Berger of the Medical University in Vienna to evaluate the efficacy of LXR agonists of VLCFA levels in vivo by treating Abcd1−/− mice with TO901317.

**Acknowledgement**

We thank Prof A.K. Groen for valuable discussions. We greatly appreciate F. Stet, H. van Lenthe and M.A. Vervaart for excellent technical assistance. This work was supported by grants from the European Leukodystrophy Association (ELA 2011-024I1 and ELA 2014-014I1 to SK).

**Conflict of interest**

The authors state no conflict of interest.
References

49. Ohno, Y. et al. ELOVL1 production of C24 acyl-CoAs is linked to C24 sphingolipid synthesis. Proc Natl Acad Sci U S A 107, 18439-44 (2010).