Bile Acid Precursors Compete with very Long Chain Fatty Acids for Translocation into Peroxisomes

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CONCLUSION: Our study suggests that VLCFA- and bile acid-CoA esters make use of the same peroxisomal import route, with ALDP affecting this pathway.

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Key words: Peroxisomes; Bile acid biosynthesis; Bile acid precursor; ESI-MS/MS; VLCFA; X-ALD; Abcd1-null mice

INTRODUCTION

Bile acids are the end products of cholesterol metabolism, representing the principal form in which cholesterol is excreted[1]. Conversion of cholesterol to the primary bile acids cholic (CA) and chenodeoxycholic (CDCA) acid in the liver is accomplished in consecutive steps comprising modifications of the ring structure of the sterol, oxidation and shortening of its side chain and finally conjugation of the end products with either taurine or glycine[2]. A plethora of enzymes, housed in the endoplasmic reticulum (ER), the mitochondria and peroxisomes (PO) of hepatocytes, are involved in the classic pathway of bile acid biosynthesis, which contributes to ~90% in humans and ~75% in mice of total de novo synthesis[3].

Peroxisomal enzymes catalyze the final steps of this sequence, i.e. the β-oxidation and side-chain cleavage of tri- and dihydroxycholestanolic acids (THCA and DHCA), the C27-precursors of CA and CDCA[4], respectively. Before leaving PO to be excreted into bile canaliculi, de novo synthesized C24-bile acids are conjugated to the corresponding taurine- and glycine-bile acids: TCDCA, TCA, GCDCA, GCA, which is mediated by the peroxisomal bile acyl-CoA:amino acid-N-acyltransferase[5,6].

Rigorous experiments with isolated rat hepatic PO have revealed that the PO membrane is permeable to substrates of low molecular weight, but not to more bulky ones[7]. Hence, the assumption of metabolite carrier proteins in the peroxisomal membrane as already

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predicted by van Roermund et al.[21] is logical, and is supported by the present study. Indeed, a growing number of PO transport proteins have been identified[23], the best characterized being the adenine nucleotide translocator of S. cerevisiae[14], and the ATP-binding cassette (ABC) transporters[15]. The adrenoleukodystrophy protein (ALDP, termed ABCD1) has gained the most attention in this respect due to its role in X-ALD (gene: ABCD1, locus: Xq28, OMIM 300100)[16], and is suggested to mediate the import of the CoA-esters of VLCFA into PO[17]. The accumulation of VLCFA in plasma and tissues of X-ALD patients is the biochemical hallmark of this peroxisomal disorder, in contrast to pristanic and phytanic acid as well as plasmalogen and T/DHCA levels, which remain normal[17,22].

In the present study we have investigated the import of the C27 precursors into PO for their further oxidative conversion. In our experiments highly purified PO isolated from the livers of rats and mice[18] were studied, and the bile acid conjugates formed were assayed by ESI-MS/MS. This latter technique has been introduced by Mills et al.[20] and then modified by Bootma et al.[21] for the rapid analysis of conjugated bile acids in plasma or urine[22] and was shown to be a suitable method for the quantitative measurement of bile acid CoA esters in isolated PO[23] and C27 precursors in dried blood spots and serum[24].

In PO, isolated from Abcd1null mice were included in the study[25]. These mice constitute a model for a late-onset, non-inflammatory trait of X-ALD termed adrenomyeloneuropathy[26-28]. Referring to the proposed loss of ALDP-function in X-ALD, we also studied free and activated tetracosanoic acid (C24:0 or lignoceric acid) as well as plasmalogen and T/DHCA levels, which remain normal.[17,23].

MATERIALS AND METHODS

Materials

DHCA were synthesized according to a published protocol[29]. The CoA ester of DHCA and D4-CA were prepared as described previously[30], following the protocol originated by Webster and Killenberg[31]. The D4-GCA was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Free and activated lignoceric acids were kind gifts of Prof. R.J.A. Wanders (Amsterdam, The Netherlands). The taurine conjugate of CDCA, protocate, K, histone, BSA, NAD, ATP, CoA, and FAD were purchased from Sigma-Aldrich (Taufkirchen, Germany). Analytical-grade methanol, 2-propanol, formic acid and acetonitrile were obtained from Merck (Darmstadt, Germany).

Biological Materials

Female Sprague-Dawley rats weighing about 250 g were obtained from the Zentrale Versuchstieranlage, University of Heidelberg, where they were kept in accordance with the guidelines of the humane care and use of laboratory animals of Germany. In order to induce an enhanced expression of peroxisomal proteins known to contribute to the oxidative degradation of lipid derivatives, each of the 20 animals was fed for 12 days with 20 g of Altromin 1324 (Altromin International, Lage, Germany) containing 1 mg/g bezafibrate (Roche Diagnostics, Mannheim, Germany), equating to a daily dose of ~75 mg/kg bodyweight. The same amount of Altromin 1324 was given to the control group of 20 animals. After the feeding period animals were starved overnight and anaesthetized with 1.5 mL of 10% chlorohydrate prior to the excision of the liver.

Mice lacking ALDP (genetic symbol Abcd1null) were bred for 10 generations into the C57BL/6 strain background. Wild type littermates served as controls. Genotyping was performed as described[31]. The mice were kept in compliance with the animal policies of the Max-Planck-Institute of Experimental Medicine, approved by the German federal state of Niedersachsen.

Liver excision

Livers of rats and mice were drained of blood by perfusion via the portal vein with physiological saline for 5 min, subsequently excised, dried by filter paper and weighed. After removing the connective tissue, the parenchyma was minced in an ice-cold homogenization buffer: 250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% ethanol (v/v), supplemented with 2 mM PMSF, 1 mM DTT, and 1 mM epsilon-aminocaproic acid, pH 7.2.

Isolation of peroxisomes from rat and mouse livers, and preparation of corresponding matrix fractions

Homogenization of the livers and isolation of peroxisomes by differential and density gradient centrifugation was performed according to an established protocol[19]. Purified PO were washed once with homogenization buffer (HB): 250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% ethanol (v/v), and immediately processed or stored at -80°C.

For subfractionation to obtain the matrix fraction, purified organelles were thawed and peroxisomal membranes were disrupted by N-cavitnation at a pressure of 1.034×105 Pa. The preparation was centrifuged at 145 000×gmax for 30 min, the pellet containing cores and the PO membrane resuspended in HB, and the resulting supernatant representing the matrix fraction collected and concentrated using VivaSpin-columns (Sartorius, Göttingen, Germany) with a size exclusion limit of 5 kDa. Protein concentrations in total PO and corresponding matrix fractions were determined according to standard protocols as described in[19].

Biochemical characterization of control and Abcd1null mice

VLCFA in plasma and plasmalogens in erythrocytes of wild type and Abcd1null mice were determined according to Moser & Moser[21] by gas chromatography-mass spectrometry. For GC-MS analysis, the quadrupole mass spectrometer MSD 5972A (Agilent, Santa Rosa, California, USA) was run in the selective ion-monitoring mode. Gas chromatography separation was achieved on a capillary column (DB-5MS, 30 m × 0.25 mm; film thickness: 0.25; J&W Scientific, Folsom, California, USA) using helium as a carrier gas. A volume of 1 µL of the derivatised sample was injected in splitless mode.

Analysis of bile acid conjugates in urine of control and Abcd1null mice was performed as described previously using solid phase extraction of the urine and subsequent determination of bile acid conjugates by electrospray ionization-tandem mass spectrometry measurement[22,23]. Bile acid conjugate contents were normalized to creatinine[22].

Bile acid loading test

Isolated peroxisomes or matrix fraction (100 µg total protein) were incubated for one hour with the free acid DHCA or the corresponding DHCA-CoA ester (2 or 20 nmol) at 37°C in 1 mL of 5 mM MOPS buffer, pH 8.0, supplemented with and without different cofactors (ATP, CoA, MgCl2, NAD, FAD, and taurine). After one hour the import and beta-oxidation of the intermediates and subsequent conjugation of the bile acids were stopped by 20 µL of 6 N KOH, and 6 N HCl was added for neutralization after hydrolysis at 50°C for 30 min. The taurine conjugate TCDDCA formed was either extracted immediately or the mixture was stored at -20°C.

In order to unravel variables, which might stimulate or inhibit the
uptake of the bile acid precursors, bovine serum albumin (BSA) was added in variable concentrations up to 10.5 μM to the incubation medium. Moreover, isolated total peroxisomes as well as the corresponding matrix protein fractions were pretreated prior to the assay with protease K (10 μg / 100 μg total peroxisomal protein), histone 2A (100 μg / 100 μg total peroxisomal protein) or Triton X-100 (0.05% w/v). Finally, peroxisomes isolated from rats fed for 12 days with the hypolipidemic drug bezafibrate were tested in addition to organelles obtained from control animals.

The assumption that VLCFA and ALDP have an impact on the import of bile acid intermediates into peroxisomes was tested as follows: (1) the medium for the incubation of rat hepatic peroxisomes was supplemented with free and activated tetracosanoic acid (20 nmol each, dissolved in ethanol) in addition to DHCA-CoA; (2) the transfer of DHCA-CoA into peroxisomes isolated from the livers of Abcd1+/− and wild type mice was compared.

**Determination of TCDCA**

Extraction of TCDCA was performed as described previously[23]. In brief, TCDCA was extracted under vacuum by means of Waters Oasis HLB (hydrophilic-lipophilic-balance) extraction cartridges with a sorbent mass of 30 mg and a volume of 3 mL, purchased from Waters (Milford, MA, USA). The cartridges were preconditioned with 2 mL dichlormethane/methanol (2:1, v/v) and water respectively. In order to compensate for variations in sample preparation and ionization efficiency, D3-glycocholic acid (D3-GCA), an artificial species, was added as an internal standard (IS) prior to extraction. 10 μL IS solution (100 μmOL/L D3-GCA) was added to 1 mL peroxisomal incubations mixture. The sample was then applied onto the preconditioned cartridge and washed with 2 mL each of purified water and n-hexane. The bile acid conjugates attached were eluted with 350 μL of 70% aqueous methanol (v/v), and 25 μL of the prepared eluate were directly injected into ESI-MS/MS analysis.

Quantification of samples by mass spectrometry was conducted on a Sciex API 365 triple quadrupole tandem mass spectrometer (SCIEX, CA, USA) with an electrospray ionisation (ESI) source. The injection volume was 25 μL, and a PE 200 LC (Perkin Elmer, USA) was used with a constant flow of 80 μL/min. The mobile phase contained acetonitrile / H2O (1:1 v/v). The API 365 was operated in the negative ion mode, with a capillary voltage of 3.5 kV.

The mass spectrometer was operated in multiple reaction monitoring (MRM) modus monitoring the following transitions: m/z 468→74 for D3-GCA and m/z 498→106 for TCDCA.

The ratio between analyte and IS peak intensity was used for quantification. Furthermore, calibration curves were generated by the mixture of TCDCA in methanol which was quantified from 0 to 100 μM with a constant amount of D3-GCA (1 nmOL). Analyst software (Applied Biosystems, version 1.4.1) was used to calculate peak heights for the conjugate and internal standard.

**Data analysis**

Data are expressed as mean±standard deviation (SD). For each experiment at least four measurements were performed. Statistical analysis (Table 1 and Figure 2) was performed by Mann-Whitney-U and posthoc Bonferroni test using the SPSS 10.0 software for Windows. Student’s t test (two groups) was calculated using Microsoft Office Excel 2003 software for statistical analysis of the data depicted in Figure 1.

**RESULTS**

**Bile acid loading test:** Effects of substrates and cofactors

Standard conditions to study the uptake and conversion of DHCA-CoA by intact peroxisomes or matrix fraction to TCDCA were defined as follows: 100 μg protein of each fraction were preincubated at 37°C in 1 mL of 5 mM MOPS buffer, pH 8.0, supplemented with 2 mM ATP, 0.5 mM CoA, 10 mM MgCl2, 2 mM NAD, 7.5 μM FAD and 0.5 mM taurine. The same protocol was also used to analyze peroxisomes of Abcd1+/− mice.

Different experimental conditions were tested. As shown in table 1, formation of TCDCA was highest when PO of rats were incubated with the CoA ester of DHCA (experiment #1). Formation of TCDCA dropped significantly when omitting NAD and FAD (experiments #2 and #4) as well as by replacing the ester by the corresponding free acid (experiment #3).

Pre-incubation of PO with protease K (0.1 mg/mg protein, experiment #5) or with Triton X-100 (0.05%, experiment #6) significantly diminished the synthesis of TCDCA. The latter was also markedly affected, albeit to a lesser extent, by pre-incubating the corresponding matrix fraction with Triton X-100 (73% of inhibition compared to control). In order to evaluate whether BSA, a well-known carrier of lipid compounds, interferes with the translocation of DHCA-CoA ester across the PO membrane, the assay mixture was supplemented with BSA in variable concentrations up to 10.5 μM. An unexpected inhibitory effect was observed in this experiment increasing progressively up to a maximum of about 60% at a BSA concentration of 10.5 μM (experiment #7).

Remarkably, omission of ATP/Mg2+ did not result in a lower formation of TCDCA (experiment #8) compared to experiment #1.

Bezafibrate is known to improve markers combined with hyperlipidemia. It is a common peroxisome proliferator-activated receptor ligand (PPAR) agonist which stimulates the expression of PPAR-responsive enzymes[24,25]. In female rats pretreated with bezafibrate, peroxisomal bile acid biosynthesis in liver was found to be stimulated about 2-fold compared to controls (experiment #9).

**Table 1** Biosynthesis of TCDCA.

<table>
<thead>
<tr>
<th>#</th>
<th>Bile intermediates</th>
<th>acid Cofactors</th>
<th>Others</th>
<th>Production of TCDCA (nmol/mg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>ATP CoA NAD/FAD</td>
<td></td>
<td>3.11±0.77</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>0.52±0.04 0.24±0.11</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.4±0.02 0.51±0.15</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.4±0.02 0.24±0.11</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.36±0.03 0.60±0.05</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>Triton X-100</td>
<td>BSA</td>
<td>1.36±0.64 1.60±0.01</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>w/o ATP Bezafibrate</td>
<td>7.02±2.70</td>
<td></td>
</tr>
</tbody>
</table>

Peroxisomes were incubated with DHCA-CoA ester or the free acid DHCA in the presence or absence of different cofactors or defined treatment conditions ("others"). # number of experiment. Production of TCDCA is given as the mean: SD of at least three independent experiments (range: 3-18). Significance levels to control experiment #1 were calculated using Mann-Whitney-U and posthoc Bonferroni test.
Bile acid formation in plasma of control and Abcd1null mice

In order to examine this assumption, isolated peroxisomes and the corresponding matrix fractions from the livers of Abcd1null and wild type mice were incubated with DHCA-CoA esters (Figure 1 B). Rates of conjugates formed by total PO from Abcd1null mice were about 57% lower than with PO of the wild type animals (p<0.003), yet differed by only 14% comparing the rates of the corresponding matrix fraction (p=0.331). This suggests that in PO of the Abcd1null mice the translocation across the peroxisomal membrane, but not the subsequent processing of the DHCA-CoA ester by the matrix enzymes, was impaired.

CoA esters of VLCFA have an impact on the biosynthesis of bile acids

While very long-chain fatty acids (VLCFA) are reportedly not oxidized by the THCA-CoA oxidase[36], and hence should not

![Figure 1](image1.png)

![Figure 2](image2.png)

Table 2: Biochemical characterization of control and Abcd1null mice.

<table>
<thead>
<tr>
<th>VLCFA in plasma</th>
<th>control µmol/L</th>
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</tr>
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<tbody>
<tr>
<td>Docosanoic acid</td>
<td>21.61</td>
<td>17.85</td>
</tr>
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<td>Tetracosanoic acid</td>
<td>13.30</td>
<td>20.59</td>
</tr>
<tr>
<td>Hexacosanoic acid</td>
<td>0.35</td>
<td>1.01</td>
</tr>
<tr>
<td>Tetracosanoic - / Docosanoic acid</td>
<td>0.62</td>
<td>1.15</td>
</tr>
<tr>
<td>Hexacosanoic - / Docosanoic acid</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Pristanic acid in plasma</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Phytanic acid in plasma</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Plasmalogenes in erythrocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexadecanal dimethyl acetal/ Hexadecanal</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Octadecanal dimethyl acetal/ Octadecanal</td>
<td>0.06</td>
<td>0.07</td>
</tr>
<tr>
<td>Bile acids in urine mmol/mol creatinine</td>
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</tr>
<tr>
<td>TCDCA</td>
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</tr>
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Each value is a single experiment from a pool of 6 control and Abcd knock out animals. VLCFA: very long chain fatty acids; GCA and TCA: glycine and taurine conjugates of CA; GCDCA and TCDCA: glycine and taurine conjugates of CDCA.

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While very long-chain fatty acids (VLCFA) are reportedly not oxidized by the THCA-CoA oxidase[36], and hence should not
interfere with the β-oxidation of activated bile acid intermediates, they might affect their translocation across the peroxisomal membrane. To test this hypothesis, rat hepatic PO were incubated with an admixture comprising either tetracosanoic (lignoceric / C24) acid or its CoA ester, in addition to DHCA-CoA. The results presented in figure 2 show that free C24 acid did not affect the formation of TCDCA (p=1.691), while its corresponding CoA-ester significantly and dose-dependently decreased the rate of formation, when supplementing the assay mixture with 2 nmol and 20 nmol, respectively.

These findings support the concept that the translocation of DHCA-CoA across the PO membrane, rather than the formation of the corresponding conjugates by the matrix enzymes, was impaired by the C24-CoA-ester.

**DISCUSSION**

Transport of bile acids across the peroxisomal membrane is still poorly understood but it has been suggested that ABCD1-4 might play a role\[13,15,37\]. Studies on the pathophysiology of X-ALD patients who are characterized by mutations affecting the ABCD1 gene and the accumulation of VLCFA support this suggestion\[17\]. It is further substantiated by data recently published on PO from the yeast *S. cerevisiae* which indicate that homodimeric ALDP is involved in the transport of acyl-CoA esters across the peroxisomal membrane and is able to rescue, at least partially, the impaired peroxisomal import of fatty acids in the pxa1/pxa2Δ mutant\[15\].

Conceptually, the translocation of lipid derivatives across the PO membrane comprises two subsequent steps; (1) activation of a fatty acid (V/LCFA) by an acyl-CoA synthetase (V/LCS) and (2) transport across the PO membrane involving the peroxisomal ATP-binding cassette containing transporters ABCD1-4.

To study the transport of bile acids across the peroxisomal membrane, we purified peroxisomes and corresponding matrix fractions from livers of rats and mice according to an established protocol\[10\]. Since PO contain the corresponding transferase for conjugation\[48\], we monitored the formation of bile acid conjugates instead of quantification of CDCA-CoA-ester as described previously\[29\]. TCDCA formed was quantified by ESI-MS/MS.

The following observations summarize the main findings of the present study: (1) import of bile acid intermediates into PO depends on CoA-esterification but not on the presence of ATP/Mg\textsuperscript{2+}; (2) import is impaired by pre-treating the organelles with protease K, which suggests that membrane protein(s) are likely involved; (3) import is affected by disturbing the integrity of the membrane; (4) activated very-long-chain fatty acids interfere with the uptake of the intermediates; (5) translocation across PO lacking the ALDP is impaired.

Bile acid biosynthesis in rat liver peroxisomes required activation of intermediates for uptake and NAD/FAD for β-oxidation. Biosynthesis of taurine conjugates was markedly increased in the transport of acyl-CoA esters across the peroxisomal membrane and is commonly reported\[7\], but not on the conversion of DHCA to CDCA\[45\]. Activities of purified THCA-CoA oxidase are also reported to strongly depend on the presence of BSA\[39\]. A likely explanation for our controversial finding (Table 1, experiment #7) is that BSA effectively binds the DHCA-CoA and thereby decreases the amount of free DHCA-CoA which is available for transport.

The inhibitory effect observed after pre-treating PO with protease K clearly points out that translocation of the activated precursors across the PO membrane is protein-mediated. Triton X-100 was found to significantly reduce the formation of conjugates not only in intact peroxisomes (Table 1, experiment #6), but also in the matrix fraction (maximum of inhibition: 81 and 73%, respectively). In contrast, histone 2A affected conjugate formation in the matrix fraction only slightly (~16%) but markedly reduced the rate of conjugate formation in total peroxisomes (maximum of inhibition: 66%, Figure 1 A) suggesting that histone 2A directly impairs the import of DHCA-CoA. Histones not only bind tightly to DNA through ionic interactions with the negative charges along the phosphate backbone, thereby playing a central role in the compaction of DNA into nucleosomes, or permeabilize membranes such as the ER membrane, but have also been reported to induce conformational changes in easily accessible domains of transporters and other membrane proteins\[14,41,42\].

Remarkably transport of bile acids and formation of TCDCA was not dependent on ATP/Mg\textsuperscript{2+} (Table 1, experiment # 8). This unexpected result is in accordance with the report of Singh and co-workers on the transport of palmitoyl-CoA and lignoceric acid into human and rat PO\[47\], which consequently implies that the translocation of VLCFA across the PO membrane does not require ATP. Taken together these results argue against an active import of these metabolites into PO. They also contradict the concept of an energy-dependent translocation of bile acid precursors across the PO membrane, even if mediated by ABC transporters, as well as the studies of Une et al and Imanaka et al which suggested an ATP-dependency\[44,45\]. It is of interest in this context to note that a passive unidirectional transport of conjugated primary bile acids has been described\[46\]. This prompts to speculate as to whether import of precursors and export of end products of peroxisomal bile acid biosynthesis might be coupled in a kind of shuttle system. Alternatively, PO may contain enough ATP to maintain the transport of bile acid intermediates. Interestingly, studies in *Arabidopsis thaliana*\[47\] revealed that the CoA-moiety is cleaved by the corresponding transporter (PX1) when long chain fatty acid acyl-CoAs are transported across the PO membrane. Cleavage of the CoA-moiety may provide the energy needed for active transport.

CoA-activated VLCFA inhibited the translocation of bile acid intermediates across the membrane of intact peroxisomes of rats whereas the production in the matrix fraction remains unaffected (Figure 2). This observation suggests that the import of bile acid into PO but not their subsequent conversion to their conjugates in the matrix compartment is impaired by activated VLCFA.

Strikingly, Abdell\textsuperscript{null} mice revealed a strongly reduced PO import of bile acid precursors. Since ALDP does not seem to interfere with bile acid conjugate formation in the PO matrix fraction\[35\], this finding strongly suggests that ALDP is directly involved in the transport of VLCFA and bile acids across the PO membrane as reviewed by Kemp and coworkers\[49\].

Biochemical characterization of control and Abdell\textsuperscript{null} mice clearly showed that VLCFA were markedly elevated in Abdell\textsuperscript{null} mice, as expected for X-ALD. In addition, TCA was decreased in urine from Abdell\textsuperscript{null} mice. This supports the hypothesis of impaired bile acid
transport into peroxisomes of Abedlnulla mice. Furthermore, the method used here for the analysis of bile acid conjugates in urine is, in addition to its use in this basic research work, a powerful tool for monitoring bile acid biosynthesis defects and for cholestatic liver diseases because these diseases are accompanied with increased levels of glycine and taurine conjugates of cholic acid and chenodeoxycholic acid22.

In summary, based on our current observations we suggest that VLCFA- and bile acid-CoA esters share the same PO import route thereby competing for a common "translocator", most likely ALDP.

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