A key role for the peroxisomal \textit{Abcd2} transporter in fatty acid homeostasis

Stéphane Fourcade\textsuperscript{1,2\#}, Montserrat Ruiz\textsuperscript{1,2,3\#}, Carme Camps\textsuperscript{3\#}, Agatha Schlüter\textsuperscript{1,2}, Sander M. Houten\textsuperscript{4}, Petra A.W. Mooyer\textsuperscript{4}, Teresa Pàmpols\textsuperscript{2,3}, Georges Dacremont\textsuperscript{5}, Ronald J.A. Wanders\textsuperscript{4}, Marisa Giròs\textsuperscript{2,3*} and Aurora Pujol\textsuperscript{1,2,6*@}

\textsuperscript{1}Centre de Genètica Mèdica i Molecular, Institut d’Investigació Biomèdica de Bellvitge (IDIBELL), Hospital de Llobregat, Barcelona, Spain.

\textsuperscript{2}Center for Biomedical Research on Rare Diseases (CIBERER) Barcelona, Spain.

\textsuperscript{3}Institut de Bioquimica Clinica, Hospital Clinic de Barcelona, Spain.

\textsuperscript{4}Laboratory Genetic Metabolic Diseases, Department of Clinical Chemistry and Department of Pediatrics, Emma Children’s Hospital, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands.

\textsuperscript{5}University of Ghent, Ghent, Belgium.

\textsuperscript{6}Catalan Institution of Research and Advanced Studies (ICREA) Barcelona, Spain.

@: To whom correspondence should be addressed: CGMM, IDIBELL, Hospital Duran i Reynals, Gran Via s/n, km 2.7, 08907 L’Hospitalet de Llobregat, Barcelona, Spain. Tel: +34 932607343; Fax: +34 932607414; Email: apujol@idibell.org

\#, *: these authors contributed equally

Running Title: \textit{Abcd2} in fatty acid metabolism
ABSTRACT

Peroxisomes are essential organelles exerting key functions in fatty acid metabolism such as the degradation of very long-chain fatty acids (VLCFAs). VLCFAs accumulate in X-adrenoleukodystrophy (X-ALD), a disease caused by deficiency of the Abcd1 peroxisomal transporter. Its closest homologue, Abcd2, exhibits a high degree of functional redundancy on the catabolism of VLCFAs, being able to prevent X-ALD-related neurodegeneration in the mouse. In search for specific roles of Abcd2, we screened fatty acid profiles in organs and primary neurons of mutant knockout mice lacking Abcd2 in basal conditions and under dietary challenges. Our results indicate that ABCD2 plays a role in the degradation of long-chain saturated and ω9-monounsaturated fatty acids, and in the synthesis of docosahexanoic acid (DHA). Also, we demonstrated a defective VLCFAs β-oxidation ex vivo, in brain slices of Abcd1 and Abcd2 knockouts, using radiolabeled hexacosanoic acid and the precursor of DHA as substrates. As DHA levels are inversely correlated with the incidence of Alzheimer’s and several degenerative conditions, we suggest that ABCD2 may act as modulator/modifier gene and therapeutic target in rare and common human disorders.

Key words: adrenoleukodystrophy, ABC transporter, peroxisome, PUFA, DHA
INTRODUCTION

Peroxisomes are subcellular organelles ubiquitously expressed (8). Although originally believed to be autonomous organelles like mitochondria, recent data on the ontogenetic and evolutionary origin of peroxisomes have suggested otherwise with peroxisomes being derived from the endoplasmic reticulum (21, 55). Peroxisomes are indispensable for development, morphogenesis and differentiation, and play key roles in hydrogen peroxide detoxification and lipid metabolism. In mammals, peroxisomes are involved in the degradation of purines, polyamines and amino acids, the biosynthesis of ether phospholipids and bile acids and fatty acid \( \alpha \)- and \( \beta \)-oxidation (67). The peroxisomal oxidation machinery is not able to degrade fatty acyl-CoAs to completion; the chain-shortened acyl-CoAs are shuttled to mitochondria to complete their degradation to CO2 and H2O via Krebs cycle. Defects of peroxisomal fatty acid oxidation are associated with a range of severe human disorders (66). The same peroxisomal \( \beta \)-oxidation machinery is used for the degradation of saturated and monounsaturated VLCFA and for the biosynthesis of bile acids and polyunsaturated fatty acids (PUFA) from the \( \omega 3 \) and \( \omega 6 \) series, namely docosahexaenoic acid (DHA) (C22:6\( \omega 3 \)), and docosapentaenoic acid (DPA) (C22:5\( \omega 6 \)) (11, 40, 59). DHA is the predominant \( \omega 3 \)-PUFA in the mammalian central nervous system, reaching up to 15% concentration of the total fatty acids in brain (51). A growing body of evidence based on epidemiological and interventional studies show that low levels of DHA may play a role in age-related neurodegenerative diseases and depression (30, 39, 53). The precursors of DHA and DPA, C24:6\( \omega 3 \), and C24:5\( \omega 6 \) respectively, are synthesized in the endoplasmic reticulum (ER) and transported to peroxisomes, where they must be imported across the membrane to undergo shortening via \( \beta \)-oxidation in the peroxisomal matrix (59). Four ATP-binding cassette transporters (ABC transporters) have been identified at the peroxisomal membrane in mammals (\textit{ABCD1} to \textit{ABCD4} genes, encoding homonymous proteins). Rather than being full
transporters such as cystic fibrosis transmembrane regulator (CFTR, ABCC7) or the multidrug resistance gene (MDR1, ABCB1), the peroxisomal ABCDs are half-size transporters, and need to homo or heterodimerize to become functional. ABCDs are thought to be involved in the transport of fatty acids, based on three principal findings: i) Peroxisomal membranes are not freely permeable, the entrance and exit of metabolites requires the presence of peroxisomal transporter proteins; ii) the two peroxisomal ABC transporters of Saccharomyces cerevisiae, Pxa1 and Pxa2, form functional heterodimers that transports long-chain fatty acyl-CoAs into the peroxisome (20, 63); iii) inactivation of ABCD1 causes the human disease X-linked adrenoleukodystrophy (X-ALD), which is biochemically characterized by the pathognomonic accumulation of saturated VLCFAs, mainly hexacosanoic acid (C26:0), in plasma and tissues. Oxidation of the VLCFAs C26:0 and C24:0 is impaired in X-ALD fibroblasts, suggesting that ABCD1 is involved in the import of VLCFAs into the peroxisome for degradation (45). Thus, the observed β-oxidation defect must be secondary to the impaired transport of the substrate across the peroxisomal membrane in the absence of a functional transporter in these cells (20, 58, 64). In analogy with the work in yeast, it has been postulated that ABCD1 transports the CoA esters of VLCFAs across the peroxisomal membrane in mammals (63).

The four human ABCD transporters share a high sequence homology, suggesting similarity of function to a certain degree. ABCD2 shares 80%, ABCD3 60%, and ABCD4 46% of homology with ABCD1 (24, 33, 57). The function of ABCD4 has not been studied yet; mice deficient for ABCD3 have been shown to accumulate bile acid precursors (THCA (3α,7α,12α-trihydroxy-5β-cholest-26-oic acid) and DHCA (3α,7α-dihydroxy-5β-cholestan-26-oic acid)) and pristanic acid (Wanders, R.J.A., personal communication). ABCD2 and ABCD3 can compensate for β-oxidation defects in X-ALD fibroblasts when overexpressed (42). Recently, we have reported a functional overlap between ABCD1 and ABCD2 transporters on the metabolism of VLCFA in vivo, as concluded from observations in mouse
models in which either Abcd1 and/or Abcd2 was disrupted or overexpressed (12, 47). However, the differences in sequence, expression patterns (62), and phenotype of these mice suggest specific roles for ABCD2 in fatty acid homeostasis. Indeed, Abcd2-/− mice exhibit a late-onset cerebellar and sensory ataxia, areflexia, loss of cerebellar Purkinje cells and dorsal root ganglia cell degeneration (12), together with oxidative damage in adrenal gland (35). These pathological features, not shared with Abcd1 null mice, might indicate that substrate specificity occurs in spite of a certain degree of overlap on C26:0 and/or that expression patterns at the cellular level are different between both and so are the cell types most sensitive to demise upon loss of function (62). In the present study, we take advantage of the available Abcd1 and Abcd2 null mice, and set out to gain insight into ABCD2 biochemical roles related to peroxisomal metabolism. After comparative genomics analysis, we determined the composition of fatty acids of tissues with good expression of Abcd2 and subsequently, designed functional tests aimed to give strength to our observations, such as dietary manipulations and fasting. Of particular relevance, we have set up an ex-vivo peroxisomal β-oxidation assay, which has allowed to directly assessing the role of ABCD2 and ABCD1 in fatty acid oxidation in mouse brain.
MATERIAL AND METHODS

Comparative genomic analyses

BLAST (2) searches obtained all recognizable ABCD paralogues from 39 eukaryotic genome projects by using ENSEMBL genomes, NCBI genomes, Integr8 portal maintained by the European Bioinformatics Institute (EBI), Sanger Institute, Center for the Study of Biological Complexity (CSBC), The Institute for Genomic Research (TIGR), BROAD Institute, The Josephine Bay Paul Center in Comparative Molecular Biology and Evolution (JBPC), Joint Genome Institute (JGI) and Cyanidioschyzon merolae Genome Project.

Multialignment and phylogenetic analyses

ABCD proteins were analysed by ClustalX (61) and refined manually with MEGA version 3.0 (27) and GeneDOC (43). Insertions and sequence characters not alignable with confidence and incomplete sequences were removed. The final phylogenetic alignment included 87 taxa. The phylogenetic tree was created using ProtTest (1). ProtTest selects the most appropriate model of protein evolution among 64 different ones based on the smallest Akaike Information Criterion or Bayesian Information Criterion score. This software takes advantage of the PAL library (10), and of the Phyml program (16). The best-fit model of protein evolution for ABCD protein family according to ProtTest corresponds to a RtREV model with invariable site parameter (+I), different evolution rate among sites (+G) and site frequencies (+F). The ABCD1 and ABCD2 incomplete sequences from draft genomes (for instance, *P. troglodytes*, *T. rubripes*, *T. nigroviridis* and *B. taurus*) have not been included for the sake of reliability of the multialignment and phylogenetic tree.

Transmembrane predictors

Secondary structure and transmembrane predictions of ABCD1 and ABCD2 were
performed using PredictProtein (www.predictprotein.org) (50) and PHDhtm, a program to predict the location and topology of transmembrane helices from multiple sequence alignments (49).

**Mouse breeding**

The genotyping of *Abcd1* and *Abcd2* mice has been previously described (12, 36). To obtain double heterozygous mutants, we crossed Abcd1 null females (mutation is on the X chromosome), with *Abcd2* males. Double heterozygous in the F1 generation were intercrossed to obtain double knockout mice and wild type littermate controls; the offspring obeyed mendelian ratios. Mice used for quantitative-PCR experiments and fatty acid analysis in serum were on a pure C57BL/6J background (backcrossed at least 12 generations into C57BL/6J) whereas for the rest of experiments they were on a mixed C57BL/6J/129Sv background (~87% C57BL/6J and ~13% 129Sv). Only male littermates were used for all the experiments. All animals were housed under the same controlled conditions, between 22 and 25 °C, on a 12h light/dark cycle, with free access to food and water. Animals were sacrificed and tissues including brain, spinal cord, sciatic nerve, adrenal gland and liver were snap frozen in liquid Nitrogen and conserved at -80°C. All methods employed in this work are in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996).

**Mouse diets**

Animals were fed with either a standard chow ad libitum consisting of 5.5% fat, 23% protein and 50% digestible carbohydrate (D03, SAFE, Scientific Animal Food and Engineering, Villemoisson sur Orge, France), or a diet based on the standard chow enriched in saturated VLCFAs (UAR, ref: UAR9124CH, SAFE, Villemoisson sur Orge, France). For fasting experiments, animals were fed with the standard chow ad libitum or fasted for 48
hours. Only males and pure background animals were used for these studies.

**Fatty acid determination**

Tissues from 3 to 8 mice of each genotype were dissected and stored frozen at -80°C. After chloroform/methanol extraction (2.1, v/v), upper phase is removed and lower phase is dried under Nitrogen and weighted for total lipid weight. A volume of 0.1ml of lower phase fatty acids were subjected to one-step transmethylation after the Lepage and Roy method (31), dissolved in hexane to a concentration of 0.1 g/ml and quantified by gas-liquid chromatography. These occurred as free fatty acids in cytoplasm, as acyl-CoA fatty acids or as lateral chains of membrane complex lipids. Equipment used was a high-resolution gas chromatograph 8000 Mega series (Fisons Instruments) equipped with split/splitless injector system and flame ionization detector. The column used was a DB-1 (Agilent Technologies) (30m x 0.25mm) with 0.25µm film thickness for saturated fatty acids and BPX-70 (Agilent) (60 m x 0.25mm) with 0.25µm film thickness) for mono- and polyunsaturated fatty acids. Quantification and identification of each compound was made with the respective fatty acid methyl ester standarization curve using C19:0 and C27:0 as internal standards. Detection limit or sensitivity of the assay is 0.5 nmol. The values of each fatty acid are expressed as nmol/mg of total lipid and as percentage of total fatty acids identified in Supplementary Table 1, following the method used in Heinzer et al, 2003 (19). For the sake of clarity, figures show the percentage of total fatty acids quantified only. We measured C16:0, C18:0, C20:0, C22:0, C24:0 and C26:0; C18:1, C20:1, C22:1, C24:1, C18:2, C20:3, C20:4, C22:4, C22:5ω6, C18:3, C20:5, C22:5, C22:6ω3. Unless otherwise stated, significant differences have been determined by ANOVA followed by post-hoc Tukey HSD (Honestly Significantly Different) test (p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***)) or by Wilcoxon test (p≤0.05 (§)).

**Hippocampal-Cortical primary neurons culture**
Hippocampi and cerebral cortices were dissected from E16 mice embryos of pure C57BL/6J background, the blood vessels and meninges were removed under microscope and placed in ice-cold Hank’s balanced salt solution (HBSS). Tissue was incubated for 15–20 min in a solution of trypsin and DNAse I to dissociate cells and the reactions were terminated by adding heat-inactivated horse serum. After, the cell suspensions were centrifuged at 500 g and the pellet was resuspended in Neurobasal medium supplemented with L-glutamine (1 mM), the serum-free B-27 Supplement from Gibco and 5% horse serum. Cells were plated onto poly-D-lysine-coated Petri-dishes at a seeding density of 2x10^5 cells/cm^2, and incubated at 37°C in a humidified incubator with 5% CO₂. Three hours after plating, the medium was replaced and horse serum removed. After 4 days of culture, neurons were harvested and fatty acid levels were quantified.

**Cortical slices preparation and peroxisomal fatty acid β-oxidation**

The brain from 7 weeks old male mice on C57BL/6/J was immediately removed; the cortex dissected and chopped into 350 µm slices in the paraxial plane using a McIlwain tissue chopper. The resulting slices were immediately washed three times in a Grey’s balanced salt solution (GBSS) using a modification of procedures described previously (60). Cultures were maintained in a serum-based medium (consisting of 100 ml advanced minimum essential medium (A-MEM), 50 ml heat inactivated horse serum, 50 ml Hank’s balanced salt solution (HBSS), 2 ml 30% glucose, and 1 ml 200 mM L-glutamine). [1-^{14}C]26:0 and [1-^{14}C]24:6ω3 were incubated on cortical brain slices for 3 hours. C26:0 and C24:6ω3 β-oxidation was measured as previously described (65). Activity was expressed as nmol of radiolabeled acetate made in 1h per mg protein. The β-oxidation value is expressed as percentage of activity relative to wt of 4 to 6 independent samples. Each sample has been assayed in triplicates, results shown are the pool of two independent experiments. Significant differences have been determined by ANOVA followed by Tukey HSD post-test (p≤0.05 (*), p≤0.01
Synthesis of [1-$^{14}$C]$6,9,12,15,18,21$-C24:6$\omega 3$

[1-$^{14}$C]$6,9,12,15,18,21$-C24:6$-3 was prepared from the methane sulphonate of 5,8,11,14,17,20-C23:6-3 obtained after the esterification, reduction, and mesylation of C23:6-3. This sulphonate (9.5 mg) was reacted with [1$^{14}$C]KCN (1 mCi, 55 mCi/mmole) in dimethylsulphoxide at 70° for 32 h, and the resulting 1-$^{14}$C-labeled nitrile was extracted with hexane. After hydrolysis of the nitrile to its corresponding carboxylic acid, [1-$^{14}$C]24:6-3 was purified on a small silica gel column with hexane-ether-acetic acid (60:40:1; v/v/v) as eluent. Thin-layer chromatography of the purified acid showed only one labeled band, and GC-MS analysis of the methyl ester showed one single peak with a molecular ion at $m/z$ 367 ([C$^{14}$]$M^+$ 4.5%) and 365 ([C$^{12}$]$M^+$ 0.5%). The synthesized [1-$^{14}$C]24:6-3 had a specific activity of 50 mCi/mmole (11).

RNA extraction and quantitative real-time PCR

Total RNA was extracted using RNeasy Kit (Qiagen) and Q-PCR experiments were performed according to manufacturer’s instructions (LightCycler, Roche Diagnostics). PCR were carried out with 36b4 (also called Rpl0) used as a standard gene. The nucleotide sequences of primers are available (Supplementary figures Table. 2). Data are given as mean+/-sem.
RESULTS

The origin of ABCD1 and ABCD2

Phylogenetic analysis of ABCD family members of 39 eukaryotic full sequenced genomes indicated that ABCD1 and ABCD2 are close paralogues which diverged rather late in evolution, at the vertebrate lineage (Fig 1). Indeed, the ascidian Ciona intestinalis, a sea squirt and the only chordate invertebrate whose genome is available, contains only one bona fide ABCD, similar to both ABCD1 and ABCD2. The same is true for Worms and Insectae; only one ABCD half way between ABCD1 and ABCD2 can be found in their genomes. Plants, here represented by O. sativa and A. thaliana, do not have any ABCD1-ABCD2 homologue; their closest ABCD, the comatose protein, is more similar to ABCD3 than to ABCD1 in contrast to previous reports (13). Fungi have two ABCD related proteins: Pxa2p and Pxa1p, which appear closer to ABCD1-2 than to ABCD3 or ABCD4, although they are more similar to one another than to any metazoan ABCD (Fig. 1 and Mr Bayes phylogenetic tree in www.peroxisomeDB.org (54)). As compared to model organisms such as yeasts, drosophila or C. elegans, the mouse thus appears as a suitable model to resolve the biological function of ABCD1 and ABCD2.

Non-conserved domains between ABCD1 and ABCD2

The human ABCD1 and ABCD2 proteins share an overall 80% homology. The most conserved part encompasses the ABC domain (92% of homology), whereas the N-terminal part (1-496 in ABCD2), exhibits only 74% of homology (Supplemental Fig. 1). In order to get a better view on the non-conserved domains between ABCD1 and ABCD2, we performed a multi-alignment by CLUSTALW, excluding the ABC domain. This revealed three main non-conserved regions: i) the largest region of divergence is located in the first 100 N-terminal residues ii) the second region is encompassed between amino acids 380 and 395 with an extra
motif for ABCD1; iii) the third region is located between amino acids 460 to 480. Based on transmembrane predictors (see Material and Methods), these three regions should be located outside the intramembrane domains, suggesting involvement in the conformation of the ligand binding domain and thus, might account for substrate specificities between ABCD1 and ABCD2 (Supplemental Fig. 1).

Changes in the fatty acid profile of sciatic nerve, adrenal gland and spinal cord from Abcd2\(^{+/−}\) and Abcd1\(^{−/−}\)/Abcd2\(^{−/−}\) mice

Fatty acids are delivered to the cells either complexed into lipoproteins or as non-esterified fatty acids, which enter the cells via fatty acid transporters (17). Once in cells, the non-sterified fatty acids are rapidly converted to fatty acyl-CoA thioesters by acyl-CoA synthetases (ACSs), specific for carbon length. Four types of fatty acids are most commonly found in tissues: saturated, \(ω9\) monounsaturated, \(ω3\) and \(ω6\) polyunsaturated fatty acids. We have previously reported on the levels of saturated VLCFAs in Abcd1\(^{−}\), Abcd2\(^{−/−}\) and Abcd1\(^{−/−}\)/Abcd2\(^{−/−}\) mice (46). Briefly, C26:0 accumulated in adrenal gland, sciatic nerve and spinal cord from Abcd1\(^{−/−}\) and Abcd1\(^{−/−}\)/Abcd2\(^{−/−}\) mice, whereas Abcd2\(^{−/−}\) mice had normal levels of this fatty acid in the same organs (46). Interestingly, in the dorsal root ganglia, not Abcd1 mutants but Abcd2 accumulate C26:0 (12). This suggests that ABCD2 can carry out at least some of the function(s) assigned to ABCD1 without requiring overexpression, provided that its endogenous expression levels are high enough in the given organ or cell type. A cooperative function of both transporters could be seen in serum, because only double mutants significantly accumulated C26:0. A similar scenario applies to C24:0, which is found increased in spinal cords of double mutants only (46). In contrast, the adrenal glands of Abcd2\(^{−/−}\) and double knockouts, showed raised levels of C22:0, a metabolite never found increased in Abcd1\(^{−}\) mice (46) or X-ALD patients, indicating that deficiencies of either Abcd1 or Abcd2 are not equivalent if the metabolism of C22:0 is concerned.
In search for specific substrates putatively transported by ABCD2, we analyzed the total fatty acid profile in adrenal gland, sciatic nerve, spinal cord, brain and liver of the different mouse models (Abcd1, Abcd2+/−, Abcd1/Abcd2−/− and wild type controls) at 8 months of age. It is worth noting that the analysis of total fatty acids encompasses the fatty acids found as lateral chains of complex lipids in membranes, the fatty acids as acyl-CoA esters and the free fatty acids, the latter in little amounts. We have shown the altered profiles in Fig 2. The obtained values are indicated as relative proportions of the specific fatty acids measured per tissue, and also as nmol/mg of total lipid in Supplemental Table 1 as a reference. In addition to the above mentioned changes in saturated VLCFAs, we also found an accumulation of C20:0 in adrenal gland and sciatic nerves from Abcd2+/− and Abcd1/Abcd2−/− mice (Fig. 2A). Further, we detected increased levels of the ω9-monounsaturated fatty acids C20:1ω9 and C22:1ω9 in sciatic nerves and adrenal gland from Abcd2−/− and Abcd1/Abcd2−/− mice (Fig. 2B and C). A marked increase of C22:1ω9 level was also noticed in spinal cord from Abcd1/Abcd2−/− mice (Fig. 2D), although no alteration of any ω9-MUFA could be observed in brain or liver (not shown). Abcd1− mice exhibited ω9-MUFA levels similar to control mice. These results point to a specific role for ABCD2 in the metabolism of ω9-MUFAs.

As peroxisomes play an indispensable function in the biosynthesis of the PUFAs DHA (C22:6ω3) and DPA (C22:5ω6), we have analysed long and very-long chain ω3 and ω6 PUFAs in the above mentioned organs. We observed a decrease of C22:6ω3 in adrenal gland from Abcd1/Abcd2−/− mice (Fig. 2E). C22:5ω6 levels were not affected in whole organs of our mouse models (Fig. 2F). As C22:5ω6 is synthesized by β-oxidation of C24:5ω6, the C22:5ω6/C22:4ω6 ratio can be used as an indirect indicator of the C22:5ω6 synthesis. Interestingly, the ratio C22:5ω6/C22:4ω6 was decreased in tissues from Abcd1/Abcd2−/− mice (Fig. 2G).

The chosen tissues for our analysis exhibit good expression levels of ABCD2 (28, 46), and some display histopathological features, such as the adrenals, sciatic nerve and spinal cord
Interestingly, the altered fatty acid profiles seem to correlate with the histopathologically or functionally altered tissues.

**Accumulation of C20:0 and C22:0 in liver after feeding of a VLCFAs-rich diet**

We intended to add strength to our observations by means of a dietary challenge. Since mouse chow is known to contain low lipids levels, including the VLCFAs species, we fed Abcd2<sup>−/−</sup> mice a diet enriched in saturated fatty acids (hereafter called SAT-diet, containing a higher content of C20:0, C22:0 and C24:0, Supplemental Fig. 2). Because the fatty acid content of liver parenchyma is thought to be related to the exogenous source, we analyzed hepatic fatty acid levels from both SAT-diet and standard chow-fed Abcd2<sup>−/−</sup> and control littermates. No differences were detected in the saturated fatty acid levels in livers of 5- and 12-months old chow fed Abcd2<sup>−/−</sup> mice when compared to wild type mice (data not shown). In contrast, C20:0 and C22:0 levels were increased in livers from Abcd2<sup>−/−</sup> mice on SAT-diet, reaching statistical significance at 12 months (Fig. 3A). C24:0 and C26:0 levels were raised as well, although not enough to be statistically different from controls. No effect of the diet was noticed on the ω3-, ω6- or ω9-fatty acid series, also no additional differences were observed in other organs analyzed.

**ABCD2 plays a role in fatty acid catabolism during fasting**

Fasting is a physiological stress condition that leads to lipolysis in adipose tissue and the release of free fatty acids into the circulation, which are subsequently taken up and degraded as an energy source in most organs. We investigated whether ABCD2 could have a functional role in fasting by first checking its expression levels. Interestingly, Abcd2 mRNA levels were increased 2.8-fold times in livers from mice fasted for 48h (Fig. 3B). Knowing this, we hypothesized that the fatty acids transported via Abcd2 under normal conditions, would most likely accumulate in Abcd2 knockouts in a given situation that would require
extra-work to be performed by Abcd2. We then fasted wild type and Abcd2\(^{-/-}\) mice for 48h and quantified fatty acid levels in serum (Fig. 3C). We noticed that C22:1\(_{\omega 9}\) content was higher in Abcd2\(^{-/-}\) mice under basal and fasting conditions. Moreover, while levels of circulating C20:0 decreased in wild type mice on fasting, most likely because of degradation in liver or adipose tissue obeying energetic requirements, in Abcd2 knockouts this was not the case. These results reinforce the notion that ABCD2 could be involved in the degradation of saturated VLCFAs and \(\omega 9\)-MUFAs.

**Changes in the fatty acid profile of primary hippocampal-cortical culture from Abcd2\(^{-/-}\) and Abcd1\(^{-/-}\)/Abcd2\(^{-/-}\) mice**

Because Abcd2 is highly expressed in mouse hippocampal and cortical neurons, particularly during differentiation in embryonic development (12, 62), we investigated the fatty acid profile in hippocampal-cortical primary neurons from E16 mice embryos (Abcd1\(^{+/+}\), Abcd2\(^{+/+}\), Abcd1\(^{-/-}\)/Abcd2\(^{-/-}\) and wild type controls). After 4 days of culture, levels of saturated, monounsaturated and polyunsaturated fatty acids were measured. As observed in several other tissues, C20:0 and C20:1\(_{\omega 9}\) levels were increased in Abcd2\(^{-/-}\) and Abcd1\(^{-/-}\)/Abcd2\(^{-/-}\) neurons. The PUFAs, C22:6\(_{\omega 3}\) and C22:5\(_{\omega 6}\), were lowered in Abcd1\(^{+/+}\), Abcd2\(^{-/-}\) and Abcd1\(^{-/-}\)/Abcd2\(^{-/-}\) neurons, again suggesting a role for Abcd2 (and also Abcd1) in C22:6\(_{\omega 3}\) and C22:5\(_{\omega 6}\) biosynthesis (Fig. 4A).

**Decreased C26:0 and C24:6\(_{\omega 3}\ \beta\)-oxidation rates in cortical slices from Abcd1\(^{-/-}\), Abcd2\(^{-/-}\), Abcd1\(^{-/-}\)/Abcd2\(^{-/-}\) mice**

In order to demonstrate directly a role of ABCD2 in the oxidation of VLCFAs and PUFAs, we performed \(\beta\)-oxidation assays with radiolabeled fatty acids as a measure of fatty acid transport across the peroxisomal membrane. We chose as a target tissue the brain cortex, which provides sufficient amount of material and highly expressed Abcd2. To create
conditions as close as possible to the *in vivo* situation, we used freshly chopped cortical brain slices from the different mouse models as starting material, and prepared cortical brain slices from 6 week old *Abcd1*, *Abcd2*<sup>+/−</sup>, *Abcd1*/*Abcd2*<sup>+/−</sup> mice. As we needed an internal control to validate the experiment, we sought of testing the capacity of *Abcd1* intact tissue to degrade C26:0, that we expected to be defective as it is the case in human X-ALD fibroblasts (65). We incubated cortical slices with [1-<sup>14</sup>C] radiolabeled C26:0 during 3 hours to demonstrate for the first time a marked impairment up to 50% of C26:0 β-oxidation in tissues from *Abcd1*<sup>−/−</sup> mice. The same result holds true for *Abcd2*<sup>−/−</sup> and *Abcd1*/*Abcd2*<sup>−/−</sup> mice, as a further proof of functional redundancy between both transporters (Fig 4B). This functional assay confirms that the detected accumulation of C26:0 in brain (*Abcd1* and *Abcd1*/*Abcd2*<sup>−/−</sup>) mice (14, 36, 46) is indeed due to an impaired oxidation of this fatty acid. Next we incubated the slices of the different mutants with [1-<sup>14</sup>C] radiolabeled C24:6ω3. We evidenced a 25% decrease in the capacity to β-oxidize C24:6ω3 in *Abcd2*<sup>−/−</sup> and *Abcd1*/*Abcd2*<sup>−/−</sup> mice. Interestingly, we found no deficiency of C24:6ω3 oxidation in *Abcd1*<sup>−/−</sup> mice (Fig. 4B). These results indicate a selective function of Abcd2 in the chain shortening of C24:6ω3 or synthesis of DHA in the cortex, as the underlying reason for the lowered DHA levels found in primary neurons.

**Lack of transcriptional effects on SREBP, PPAR, LXR or HNF-4-target genes**

Fatty acyl-CoAs are metabolized in many different metabolic pathways (β-oxidation, elongation, desaturation, triglyceride or cholesterol synthesis, prostanoid or leukotriene synthesis, etc), where each intermediate metabolite or end product can be responsible for the transcriptional effect of long-chain fatty acids. For instance, it was shown that non sterified fatty acids, long chain acyl-CoAs, lipoxygenase-derived metabolite leukotriene B4, prostacyclins, 15-deoxy- 12,14-prostaglandin J2, etc, are potent regulators of transcription depending upon the gene considered and the cell-specific context, for general review see (34, 41). In particular, the manipulation of ω3-PUFA and/or the ratio of ω6-PUFA to ω3-PUFA
may influence the expression of many genes in the brain (52). Specific PUFAs might act as ligands of several nuclear receptors, such as Peroxisome Proliferator-Activated Receptors $\alpha$, $\beta$ and $\gamma$ (PPARs), Liver X Receptor $\alpha$ (LXR$\alpha$), Hepatocyte Nuclear Factor 4$\alpha$ (HNF4$\alpha$) or as modulators of nuclear transcription factors such as Sterol Regulatory Element Binding Protein (SREBP) (9, 23). To investigate whether disturbances in metabolic pathways controlled by these transcription factors might be affected in our mouse models, we analyzed the expression levels of selected target genes. We studied Acyl-CoA oxidase ($Acox1$), Fatty Acid Synthase ($Fasn$) (32), $Abca1$, $Abcg1$ (56), Stearoyl-CoA desaturase 1 ($Scd1$) (32), delta5-desaturase ($Fads1$) and delta6-desaturase ($Fads2$) (48) mRNA expression levels in spinal cord, sciatic nerve and adrenal gland from 12-months old $Abcd1^{-/-}$, $Abcd2^{-/-}$ and $Abcd1^{-/-}/Abcd2^{-/-}$ mice. We found no differences in the expression levels of the above mentioned genes in any of the mutants (data not shown), which suggests that despite significant changes in the fatty acid profiles, transcriptional dysregulation of key genes in pathways controlled by SREBP, PPARs, LXR$\alpha$ and HNF4$\alpha$ does not occur.
DISCUSSION

We have previously shown a functional overlap between Abcd1 and Abcd2 peroxisomal transporters. Overexpression of Abcd2 is able to correct the defective catabolism of VLCFA in Abcd1 mice, and even to prevent the development of a late onset neurodegenerative phenotype caused by loss of function of Abcd1 (12, 46). Phylogenetic analysis suggests that the divergence of ABCD1 and ABCD2 originated as the vertebrate lineage appeared. Both transporters are well conserved among all sequenced vertebrate genomes, strongly suggesting that they have kept specific substrates and functions. It is tantalizing to speculate that this divergence could represent a “gain of function”, belonging to an integrated evolutionary program, and necessary to allow the development of higher complexity systems, i.e, the vertebrates’ nervous system, for which compounds such as DHA are of paramount importance. In search for ABCD2-specific roles, we set out to explore fatty acid profiles in tissues of the three different mouse mutants and used dietary manipulation and fasting to give strength to our observations. We readily detected an accumulation of saturated C20:0 and ω9-monounsaturated long-chain fatty acids in an ABCD2-dependent manner, indicating that this transporter is involved in the homeostasis of these FAs. Since the different ABCDs have been suggested to act as metabolite transporters, one simple explanation would be that Abcd2 either as homo- or heterodimer, would catalyze the uptake of these FA, as CoA-ester or otherwise, across the peroxisomal membrane. A diet enriched in saturated VLCFAs strengthened this defect as a function of time. Fasting is a metabolic stress condition that results in upregulation of Abcd2 expression; analysis of serum from Abcd2/−/− fasted mice provided therefore further evidence pointing to the impaired degradation of C20:0, most likely as a mirror of the situation in liver or white adipose tissue. Concerning the ω9 series, our results are in agreement with other studies in man, suggesting that erucic acid (22:1ω9) degradation is preferentially peroxisomal (5, 7, 44). Moreover, accumulation of C20:0,
C20:1ω9, C22:1ω9 and C24:1ω9 has been noticed in Zellweger fibroblasts (3), but not in X-ALD fibroblasts. This indicates that ω9-monounsaturated VLCFAs catabolism requires functional peroxisomes, but is independent of Abcd1, at least in fibroblasts (3, 6). In conclusion, our observations underline the role of peroxisomes in the degradation of saturated and ω9-monounsaturated LCFA and VLCFAs and uncover specific functions for Abcd2, distinct from its overlapping function with Abcd1. It is worth noting that in organs such as adrenals and sciatic nerve, Abcd1 seems to function in cooperation with Abcd2, resulting in significant increases of C22:1ω9 when both transporters are missing. This is even clearer in spinal cord, where the inactivation of both transporters only, results in a significant increase of C22:1ω9 levels, providing a strong indication of functional redundancy regarding this specific metabolite. This is also seen in neuronal cultures, where significantly increased levels of C20:1ω9 are accumulated in double mutants only. This indicates that Abcd1 and Abcd2 can be functionally redundant for C22:1ω9 and C20:1ω9, in addition to C26:0 as formerly reported. The fact of having found specific accumulation of saturated and ω9-monounsaturated long- and VLCFAs in adrenal gland, spinal cord or sciatic nerve of Abcd2⁻/⁻ mice, but not in other organs, might indicate that the physiological role of Abcd2 in these tissues is more important than for instance, in liver or brain; or alternatively, that loss of ABCD2 function in these particular organs or cell types is not compensated by any of the other members of the ABCD subfamily or by means of an alternative metabolic route.

We have also investigated another major function of peroxisomes: the biosynthesis of the PUFAs docosapentaenoic acid, DPA (C22:5ω6), and docosahexaenoic acid, DHA (C22:6ω3) (68). In cultures derived from cortical neurons, where Abcd2 is highly expressed, we have observed a 30% decrease of DHA and DPA levels, comparable to the decrease found in brain of Pex5⁻/⁻ mice (22), and brain of Zellweger patients which are devoid of functional peroxisomes (37). DHA is one of the major building structures of membrane phospholipids; its deficiency can prevent proper renewal of membranes and accelerate aging. DHA has been
shown to exert positive effects on neuronal differentiation, function and neurotransmission (4, 25, 26). Given the low levels of DHA detected in our primary neuronal cultures, in depth behavioral analysis directed at exploring higher cognitive functions of ABCD2 knockouts seem appropriate. Moreover, conclusive evidence for a role of Abcd2 in PUFA biosynthesis is provided by the reduced β-oxidation capability for C24:6ω3, as seen in Abcd2<sup>−/−</sup> and double Abcd2/Abcd1 knockout tissue. These results are in favor of a central role of Abcd2 in fatty acid homeostasis, most likely by acting in the uptake of the precursors C24:6ω3 and C24:5ω6 into the peroxisome.

Indeed, a major contribution of this work derives from the set-up of the peroxisomal β-oxidation technique that allows ex vivo assessment of fatty acid oxidation in nervous tissue. Recent investigations could not find alterations of the β-oxidation of C24:0 in any of the tissue homogenates from the X-ALD mouse model tested (38), which prompted the authors to argue against a role of Abcd1 in the degradation of VLCFA. It should be noted that the authors performed C24:0 β-oxidation assays using frozen postnuclear supernatants of tissue homogenates, or purified peroxisomes from liver (19), which may well account for the different experimental results, especially since the peroxisomal membrane is notoriously fragile upon isolation. If membrane integrity is disrupted, substrates for the peroxisomal β-oxidation system can freely reach their site of oxidation in contrast to the situation in intact cells or tissue, as shown in this work. This is supported by the observation that yeast lacking one or both ABCD paralogues (Pxa1p and Pxa2p) show elevated VLCFAs levels and defective β-oxidation in intact cells, whereas oxidation is normal in cell lysates. Thus, we convincingly show that Abcd1 and Abcd2 are required for correct peroxisomal β-oxidation of C26:0 and C24:6ω3 in intact brain tissue.

Taking our findings together, we propose a model for the functioning of ABCD1 and ABCD2 in figure 5. As discussed above, these roles are based on: (i) the deficiency of C26:0 and C24:6ω3 β-oxidation and, (ii) the changes in the fatty acid profile in tissues of mutant
animals, under basal or challenged conditions. Based on the overlapping functions of Abcd1 and Abcd2 and because deficiency of C26:0 and C24:6ω3 β-oxidation is only partial in the mutant mice studied, it seems plausible that the other members of the ABCD family (ABCD3 or ABCD4), might contribute for the remaining β-oxidation activity. To further clarify this issue, the same type of experiments should be repeated in single or double Abcd3−/−Abcd4−/−/Abcd2−/− mice once they become available. Nonetheless, definitive establishment of the role of ABCDs awaits successful reconstitution of ABCDs, either as homo- or heterodimers, into liposomes followed by in vitro transport studies. Very recently, significant advances in the field (15) may eventually help to circumvent the technical complexity associated with the approach and complete our understanding of ABCDs roles in fatty acid transport.

To date, no human disease has been linked to the ABCD2 gene locus. Mice lacking Abcd2 exhibit hyperactivity, late-onset peripheral neuropathy and spinocerebellar ataxia (12), presumably as a consequence of the chronic disturbances of fatty acid metabolism as reported here. Our findings deliver specific profiles of altered fatty acids enhancing the previously described, rather broad phenotype, thus greatly facilitating associations of compatible phenotypes to the ABCD2 gene locus at 12q1.1-2. In analogy with X-ALD patients, in which we find a four to ten fold accumulation of C26:0 in plasma, in spite of a milder accumulation of C26:0 in tissues and none in plasma in the Abcd1 knockout mice, we believe that patients with mutations in ABCD2 could present with lowered levels of C22:6ω3 and C22:5ω6 in plasma, mononuclear cells or fibroblasts. The importance of our findings go well beyond the field of rare disease, as recent reports have demonstrated that lower levels of DHA in plasma or tissues are correlated with high risk for coronary heart disease (18) and Alzheimer’s disease (53). Moreover, low plasma levels of ω6 and DHA fatty acids are associated with accelerated decline of peripheral nerve function with aging (29). This suggests that ABCD2 as peroxisome gate opener, might well be a key player regulating DHA and DPA endogenous synthesis and in view of the literature, a potential candidate modifier gene and/or modulating
factor contributing to a growing list of common human disorders and aging.
ACKNOWLEDGEMENTS

We are indebted to Pr Jean Louis Mandel for fruitful scientific discussions and support for carrying out the project. We are thankful to Dr Elisabeth Metzger, the IGBMC mouse facility manager, for colony and diet supervision. Abcd1− mice were a gift of Dr Kirby D. Smith (KKI, Baltimore, MD, USA). This study was supported by funds from the European Commission (contract nº LSHM-CT2004-502987), the European Leukodystrophy Association (ELA), the Association Francaise contre les Myopathies (AFM) (Project nº 9315), the Asociación Española contra la Leucodistrofia (ALE-ELA España), the Fondo de Investigación Sanitaria (FIS, Ministerio de Sanidad Español, Instituto de Salud Carlos III, grant nº 01/1667 and PI051118) and the Spanish Research Network REDEMETH (nº G03/054). The CIBER de Enfermedades Raras is an initiative of the ISCIII. The work was developed under the COST action BM0604 (A.P.). C.C. was a fellow of the ELA, A.S. was a fellow of the AFM, Decrypton Program and a recipient of FIS (CAOS/0090) from the Instituto de Salud Carlos III. S.F. was a fellow of the ELA (grant nº ELA 2007-018F4) and the European Commission, and M.R. was a fellow of REDEMETH.
REFERENCES


11. Ferdinandusse S, Denis S, Dacremont G, and Wanders RJ. Studies on the


20. **Hettema EH, van Roermund CW, Distel B, van den Berg M, Vilela C, Rodrigues-Pousada C, Wanders RJ, and Tabak HF.** The ABC transporter proteins Pat1 and Pat2 are


29. **Lauretani F, Bandinelli S, Bartali B, Cherubini A, Iorio AD, Ble A, Giacomini V, Corsi AM, Guralnik JM, and Ferrucci L.** Omega-6 and omega-3 fatty acids predict


37. **Martinez M.** Abnormal profiles of polyunsaturated fatty acids in the brain, liver,


43. **Nicholas KB, Nicholas HB, and Deerfield II DW.** GeneDOC. *EMBnet* 4: 1-4, 1997.


47. **Pujol A, Hindelang C, Callizot N, Bartsch U, Schachner M, and Mandel JL.** Late onset neurological phenotype of the X-ALD gene inactivation in mice: a mouse model for


56. **Schmitz G and Langmann T.** Transcriptional regulatory networks in lipid


ABBREVIATIONS

VLCFAs: Very long-chain fatty acids; PUFA: Polyunsaturated fatty acids; MUFA: Monounsaturated fatty acids; PBDs: Peroxisome Biogenesis Disorders; DRG: Dorsal root ganglion cells; HNF4α: Hepatocyte Nuclear Factor 4α; LXR: Liver X Receptor; PPARs: Peroxisome Proliferator Activated Receptors; SREBP: Sterol Regulatory Element Binding Protein.
FIGURE LEGENDS


**Figure 2.** C20:0 levels in adrenal gland, sciatic nerve and spinal cord from 8-months old Wt, *Abcd1*, *Abcd2* and *Abcd1*/*Abcd2* mice (A). ω9-monounsaturated fatty acids levels in adrenal gland (B), in sciatic nerve (C) and in spinal cord (D) from 8-months old mice. C22:6ω3 levels (E), C22:5ω6 levels (F) and C22:5ω6/C22:4ω6 ratio (G) in adrenal gland, in sciatic nerve and in spinal cord from 8-months old mice. Concentration of fatty acid in total tissue lipids is expressed as percentage of total fatty acids relative to wt of 3 to 8 samples. For adrenal gland and sciatic nerve, a given value results from pooling the organs of two or four animals, respectively. A minimum of three independent pools were used for lipid analysis. Significant differences have been determined by ANOVA followed by Tukey HSD post-hoc (p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***)).

**Figure 3.** Saturated fatty acid levels in liver from 5-and 12-months old Wt, *Abcd2* mice fed with SAT-diet (A). Analysis of *Abcd2* mice under fasting conditions. *Abcd2* (*Aldr*)
expression levels. Two and half months old Wt mice (n=5) were fasted for 2 days and hepatic levels of Abcd2 were compared to those of fed animals (n=6) by quantitative RT-PCR and normalized to 36b4 (B). C20:0 and C22:1ω9 levels in serum from 2.5-months old Abcd2+/− (n=3) and Wt (n=6) mice fasted for 2 days compared to Abcd2−/− (n=4) and Wt (n=6) fed mice (C). Significant differences have been determined by ANOVA followed by Tukey HSD post-hoc (p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***)).

**Figure 4.** C20:0, C20:1ω9, C22:6ω3 and C22:5ω6 levels in hippocampal-cortical primary neurons of Wt (n=8), Abcd1−/− (n=5), Abcd2−/− (n=4) and Abcd1−/Abcd2−/− (n=9) mice (A). β-oxidation assays in cortical brain slices from Wt, Abcd1−, Abcd2−/− and Abcd1−/Abcd2−/− mice, for C26:0 and C24:6ω3 radiolabeled fatty acids. Activity was expressed as nmol of radiolabeled acetate made in 1h per mg protein. The β-oxidation value is expressed as percentage of activity relative to wt of 4 to 8 samples per genotype, out of three independent experiments (B). Significant differences have been determined by ANOVA followed by Tukey HSD post-test (p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***)).

**Figure 5.** Proposed model for the roles of ABCD1 and ABCD2 transporters in fatty acid homeostasis. Activated saturated, MUFA or PUFA are translocated into peroxisomes via ABCD1, ABCD2 or both for degradative or biosynthetic (retroconversion) β-oxidation.

**Supplemental figure legends:**

**Table 1. Fatty acid composition in Adrenal Gland, Sciatic Nerve and Spinal cord from 8-months old Wt mice.** Each fatty acid is expressed as nmol/mg of total lipid and as percentage of total fatty acids quantified.
Table 2. List of primers used to quantify gene expression by Q-PCR.

Figure 1. ABCD1 and ABCD2 sequence alignment. The alignment was performed only with ABCD1 and ABCD2 from full sequenced genomes (except for zebrafish ABCD2), excluding the conserved ABC domain. We represented in black the conserved residues, in red and in blue the conserved residues restricted to ABCD2 and ABCD1 protein, respectively. Major differences between ABCD1 and ABCD2 are indicated in green.

Figure 2. Saturated fatty acids composition of standard chow and SAT-diet. Fatty acids are expressed as percentage of total saturated fatty acids analysed.
Figure 1.
Figure 2
A Saturated fatty acid composition in liver from Wt and Abcd2<sup>−/−</sup> mice fed with SAT-diet

B Abcd2 expression in liver from Wt fasted for 2 days

C C20:0, C22:1ω9 levels in serum from Wt and Abcd2<sup>−/−</sup> mice fed or fasted for 2 days

Figure 3
A C20:0, C20:1ω9, C22:6ω3 and C22:5ω6 in primary neurons

B C26:0 and C24:6ω3 β-oxidation in cortical slices

Figure 4
Figure 5