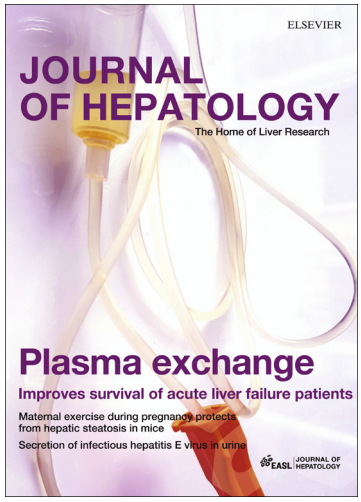

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PII: S0168-8278(16)30652-3
DOI: http://dx.doi.org/10.1016/j.jhep.2016.11.005
Reference: JHEPAT 6326

To appear in: *Journal of Hepatology*

Received Date: 1 August 2016
Revised Date: 27 October 2016
Accepted Date: 2 November 2016

Please cite this article as: Monte, M.J., Alonso-Peña, M., Briz, O., Herraez, E., Berasain, C., Argemi, J., Prieto, J., Marin, J.J.G., ACOX2 deficiency: An inborn error of bile acid synthesis identified in an adolescent with persistent hypertransaminasemia, *Journal of Hepatology* (2016), doi: http://dx.doi.org/10.1016/j.jhep.2016.11.005

ACOX2 deficiency: An inborn error of bile acid synthesis identified in an adolescent with persistent hypertransaminasemia

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Short Title: *ACOX2* mutation impairing bile acid synthesis

Key Words: BILE ACID METABOLISM; CHOLESTATIC LIVER DISEASES; DRUG INDUCED HEPATOTOXICITY; GENETIC DISORDER

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Abbreviations:
ACOX2, acyl-CoA oxidase 2; CA, cholic acid; CDCA, chenodeoxycholic acid; DBP, D-bifunctional protein; DCA, deoxycholic acid; DHCA, dihydroxycholestanoic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid; LCA, lithocholic acid; SCPx, sterol carrier protein X; SNP, single nucleotide polymorphism; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; THCA, trihydroxycholestanoic acid; TLCA, taurolithocholic acid; TSLCA, taurosulfolithocholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

Electronic word count: 5955
Number of Figures: 4
Number of Tables: 2
Number of Supplementary Figures: 1
Number of Supplementary Tables: 6

CONFLICT OF INTEREST

There is no conflict of financial, professional, or personal interest for any author.

FINANCIAL SUPPORT

This study was supported by the Spanish "Instituto de Salud Carlos III" (Grants FIS PI11/00337 and PI15/00179), Ministerio de Ciencia e Innovación (SAF2013-40620-R), "Junta de Castilla y Leon" (SA015U13 and BIO/SA52/15), Fundacion Mutua Madrileña (Call 2015), and Fundacion Samuel Solórzano Barruso (FS/10-2014). Marta Alonso-Peña is recipient of predoctoral fellowship from "Ministerio de Educación, Cultura y Deporte" (BOE-A-2015-9456).

AUTHOR CONTRIBUTIONS

J.P. and J.J.G.M. initiated the study; J.P., J.J.G.M., M.J.M., O.B. and E.H. contributed to the study design; M.J.M. run HPLC-MS/MS analyses; O.B., E.H. and M.A. carried out sequencing and *in vitro* experiments; C.B. and J.A. supplied human samples; J.P. and J.J.G.M. coordinated the work; M.J.M., J.P. and J.J.G.M. with feedback from all other authors analyzed and interpreted the data, and prepared the manuscript.

Abstract

Background: Acyl-CoA oxidase (ACOX2) is involved in the shortening of C27 cholesterol derivatives to generate C24 bile acids. Inborn errors affecting the rest of peroxisomal enzymes involved in bile acid biosynthesis have been described. These conditions are usually characterized by serious hepatic and/or neurologic manifestations.

Aims: We have investigated bile acid profile and enzymes involved in their biosynthesis in the first reported case of ACOX2 deficiency identified in an adolescent boy with persistent unexplained hypertransaminasemia and his family.

Methods & Results: In this patient's serum and urine HPLC-MS/MS and HPLC-TOF analyses showed negligible amounts of C24 bile acids, but augmented levels of C27 intermediates, mainly tauroconjugated trihydroxycholestanoic acid (THCA). Exons amplification/sequencing of enzymes potentially involved revealed a homozygous missense mutation (c.673C>T; R225W) in *ACOX2*. His only sister was also homozygous for this mutation and exhibited similar alterations in bile acid profiles. Both parents were heterozygous and presented normal C24 and C27 bile acid levels. Wild type or mutated (mutACOX2) variants were over-expressed in human hepatoblastoma HepG2 cells. Western blot and immunofluorescence studies demonstrated similar size and peroxisomal localization for both variants. THCA biotransformation into cholic acid was enhanced in cells over-expressing ACOX2, but not in those over-expressing mutACOX2. Both cells showed similar sensitivity to oxidative stress caused by C24 bile acids. In contrast, THCA-induced oxidative stress and cell death were reduced by over-expression of ACOX2, but not mutACOX2.

Conclusion*:* ACOX2 deficiency, a condition characterized by accumulation of toxic C27 bile acid intermediates, is a novel cause of isolated persistent hypertransaminasemia.

INTRODUCTION

Bile acids play essential digestive functions and are critical signaling molecules that modulate intermediate metabolism and liver tissue homeostasis. They are synthetized by hepatocytes from cholesterol [1, 2] through two main pathways involving enzymes located at the endoplasmic reticulum, cytosol, mitochondria and peroxisomes. These enzymes are responsible for modifying the sterol nucleus of cholesterol and shortening its side-chain [3, 4]. Inborn errors of bile acid synthesis are rare autosomal recessive diseases accounting for 1-2% of cholestatic disorders in children [5]. Although progressive cholestasis of infancy is the most common clinical presentation, neonatal hepatitis or the development of liver disease in later childhood can also occur [5]. However, certain cholestatic liver diseases in adults have been also associated to an inherited defect on bile acid synthesis [6].

Peroxisomes play a crucial role in bile acid synthesis [7]. In the classic biosynthetic pathway, the C27-bile acid intermediates 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA), precursors of C24 mature bile acids chenodeoxycholic acid (CDCA) and cholic acid (CA), respectively, undergo β-oxidation of their side-chain in a process mediated by four peroxisomal enzymes: alpha methylacyl-CoA racemase (AMACR), ACOX2, D-bifunctional protein (DBP) and sterol carrier protein X (SCPx) (Fig. 1A). Finally, the newly formed bile acids are amidated with glycine or taurine by bile acid-CoA:amino acid N-acyltransferase (BAAT) also located at peroxisomes (Fig. 1A). Inborn errors affecting peroxisomal enzymes accounting for bile acid synthesis are usually characterized by serious hepatic and/or neurologic alterations [8, 9]. In the present paper we identified ACOX2 deficiency as a novel cause of sustained elevation of serum transaminases without any other accompanying symptomatology.

MATERIALS AND METHODS

Reagents and cell lines

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), as well as their tauroconjugate (TCA, TCDCA, TDCA, TLCA, TUDCA) and glycoconjugate (GCA, GCDCA, GDCA, GLCA, GUDCA) forms, and taurosulfolithocholic acid (TSLCA) were from Sigma-Aldrich (Madrid) and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA), as a mixture of (*25R*)- and (*25S*)-isomers, was from Instruchemie (Groningen, The Netherlands). Cell lines HEK293T (human embryonic kidney) and HepG2 (human hepatoblastoma) were obtained from the American Type Culture Collection -ATCC- (Rockville, MD), tested to guarantying the absence of mycoplasma contamination (Mycoplasma Gel Form Kit, Biotools B&M Labs, Madrid, Spain) and cultured according to the supplier instructions.

Analytical methods

Serum and urine samples were collected after overnight fasting from the patient and his parents and sister and from 5 healthy male volunteers of similar age. An adaptation [10] of a previously described method [11] for bile acid measurement by HPLC-MS/MS was used in a 6410 Triple Quad LC/MS (Agilent Technologies, Santa Clara, CA). To investigate the presence of intermediate metabolites of bile acid synthesis both Scan and Precursor Ion Modes were used. In Scan Mode, 300-800 m/z ions were allowed to pass through MS1 and MS2 quadrupoles. In the Precursor Ion Mode, MS1 was set to scan a mass range of 300-800 m/z while MS2 was fixed to scan ions 80.2 m/z, 74.0 m/z and 97.0 m/z for taurine-, glycine- and sulphate-conjugated BAs, respectively [12]. For confirmation of structure, Exact Mass determination was performed in a liquid chromatography-quadrupole time-of-flight (LC/TOF) apparatus (QSTAR XL, Applied Biosystems, Thermo Fisher Scientific, Barcelona).

Genetic analysis

DNA was obtained from samples (two per individual) of oral epithelial cells collected with sterile buccal swabs (Omni Swabs, Whatman, Sigma-Aldrich) by using the DNeasy Blood & Tissue kit from Qiagen (Izasa, Madrid). The coding sequences of DBP, SCPx and ACOX2 were amplified by high-fidelity PCR by using AccuPrime Pfx DNA polymerase (Thermo Fisher Scientific). The amplified fragments containing both the exons and the exon-intron boundaries from at least two reactions (three if changes appeared) of PCR per exon were purified by agarose gel electrophoresis followed by extraction of DNA from the gel bands using the illustra GFX PCR DNA and Gel Band

Purification kit (GE Healthcare, Barcelona). Then, the amplicons were sequenced in both directions using forward and reverse primers (Supplementary tables 1-3).

Stable expression of ACOX2 variants

The human ACOX2 ORF was amplified from total RNA isolated from healthy liver using RNeasy spin columns from GE Healthcare, followed by reverse transcription (SuperScript VILO cDNA Synthesis kit,, Invitrogen, Thermo Fisher Scientific) and by high-fidelity PCR using specific primers (Forward: GCC ACC ATG GGC AGC CCA GTG CA; Reverse: TAG CTT GGA TCT CCA ACT TTG TAA AAG TGG T). The sequence of the wild type ACOX2 cDNA was confirmed by sequencing and cloned into a pGEM-T vector (Promega, Madrid), which was used to generate a pGEM-T vector containing the c.673C>T (R225W) ACOX2 variant by site-directed mutagenesis [13]. Both cDNA variants were cloned into the *PacI* site of the pWPI lentiviral vector, which had previously been modified by site-directed mutagenesis to include the C-terminal V5-tag. Recombinant lentiviruses were produced in host HEK293T cells as previously described [14]. The cells were transfected using a standard polyethylenimine (PEI) protocol with the transfer vectors pWPI-ACOX2-V5, encoding both the desired ACOX2-V5 variant and EGFP or simply pWPI-V5 to generate Mock vectors, and the packaging plasmids psPAX2 and pMD2.G. Viral titer was determined by infection of HEK293T cells with serial dilutions of the viral suspension, and analysis of EGFP-positive cells was carried out with a FACSCalibur flow cytometer (BD Biosciences, Madrid). Lentiviral vectors were added to HepG2 target cells at a multiplicity of infection (MOI) of 10 infectious particles per cell in the presence of polybrene, and the infected cells were incubated for 4 days prior to analyses.

Determination of ACOX2 expression in cell lines

cDNA synthesized from total RNA was used as template to determine ACOX2 expression by real-time quantitative PCR (QPCR) by using gene-specific primers spanning exon-exon junctions in the target mRNA (Forward: GAC AGA GTT GGG ACA TGG GAC ATA TCT T; Reverse: GCG TGG GGC TGT GTA TCA CAA A) and AmpliTaq Gold DNA polymerase in a 7300 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The thermal cycling conditions were as follows: single cycles at 50ºC for 2 min and at 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s and at 60ºC for 60 s. Detection of amplification products was carried out using SYBR Green I. The mRNA abundance of ACOX2 in each sample was normalized on the basis of its GAPDH content.

Immunofluorescence and immunoblotting

Immunostaining of V5-tagged ACOX2 and mutACOX2 was carried out in cells fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.01% Triton X-100 for 10 min. Preparations were incubated, for 1h, with mouse monoclonal primary antibody against V5 conjugated to DyLight 650 (Invitrogen, Thermo Fisher Scientific) diluted 1:65. To co-localize ACOX2 or mutACOX2 with a peroxisome marker, immunostaining was carried out, in cells fixed and permeabilized in ice-cold methanol, by using the V5 DyLight 650-conjugated antibody together with a primary antibody against PMP70 (1:250) (Abcam, Cambridge, UK) and Alexa Fluor-594 anti-mouse secondary antibody (1:1000) (Molecular Probes, Thermo Fisher Scientific). Nuclei were counter-stained with 4,6-diamidino-2-phenylindole (Dapi). Confocal laser scanning microscopy was performed using a Leica TCS SP2 confocal microscope. Immunoblotting analyses of boiled cell lysates were carried out with a primary monoclonal antibody against V5 (1:4000) (Invitrogen, Thermo Fisher Scientific).

Functional in vitro studies

ACOX2 activity was assessed by studying the conversion of THCA into CA in HepG2 cells expressing ACOX2 wild type or its R225W variant (mutACOX2). These cells were seeded in 3-cm diameter plates at 60% confluence and incubated with culture medium (MEM supplemented with sodium pyruvate and 10% fetal calf serum) containing 10 µM THCA for 48 h. Then, culture medium was collected and cells were lysed by incubation with water at 4°C for 4 h, scrapped and sonicated. Both media and cell lysates were pooled together before bile acids were extracted with Sep-Pak Plus C18 cartridges (Waters, Barcelona). Biotransformation of THCA into CA was analyzed by HPLC-MS/MS. Protein content was determined as previously described [15].

The production of reactive oxygen species (ROS) was analyzed after exposure of cells to THCA for 72 h, CellROX® Orange Reagent (Molecular Probes) was added to cells cultured in 96-well plates at a concentration of 5 µM and incubated for 30 min at 37°C. Then cells were trypsinized and fluorescence was measured by flow cytometry. Cell viability - determined by the MTT test by using thiazolyl blue tetrazolium bromide (Sigma-Aldrich) - was studied after incubating HepG2 cells with increasing concentrations of THCA for 72 h. To compare the ability of THCA to induce oxidative stress with that of two major C24 bile acids with strong (DCA) and mild (CA) pro-oxidant activity, the cells were incubated in suspension with serum-free culture medium containing 2 µM 2',7'-dichlorofluorescein diacetate (Sigma-Aldrich) at 37°C for 15 min and then exposed to 250 µM bile acids for 30 min before measuring ROS production

by flow cytometry. Previous experiments were carried out to set up the optimal 2',7'-dichlorofluorescein diacetate concentration and incubation time needed to obtain steady-state cell fluorescence (data not shown).

Statistical methods and tertiary structure prediction

Data are presented as means±SD. The Bonferroni method of multiple-range testing was used to calculate the statistical significance of differences among groups. The position in ACOX2 protein tertiary structure of the active site, defined as this region of the protein that includes the residues interacting with FAD, the catalytic residues and their surrounding residues, was deduced from the crystal structure of rat acyl-CoA oxydase-II or ACO-II [16]. Sequences of human ACOX2 (NP_003491) and rat ACO-II (2DDH_A) were aligned using protein blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the conserved amino acids were depicted in the structure of ACOX2 as predicted by the Phyre2 software [17].

Ethics

The research protocol conformed ethical guidelines of the 1975 Declaration of Helsinki, and it was reviewed and approved by the Human Ethical Committee of the Clinica Universidad de Navarra (Spain). Informed consent was obtained from the patient and family.

RESULTS

Case report

Although most known inborn errors of bile acid synthesis are pediatric diseases [5], here we present the case of a 16 years-old male who was first seen in 2009 because of unexplained hypertransaminasemia (2-5-fold upper normal limit, without any other symptoms) persisting for 2 years following a transient episode of acute ibuprofen/diclofenac-induced hepatitis. Hypertransaminasemia was moderate but showed occasional fluctuations with rises coinciding with the ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) and D-penicillamine (Supplementary table 4). Known causes of persisting hypertransaminasemia were excluded. Abdominal ultrasound demonstrated normal liver size and morphology and normal echogenicity. The gallbladder and the biliary tree were normal and cholelithiasis was excluded. A liver biopsy, performed in 2010, showed normal histology (Supplementary Fig. 1A) with only signs of mild intracellular cholestasis (Supplementary Fig. 1B). In 2012, serum transaminases reverted to normal coinciding with cholestyramine administration (Supplementary table 4), which prompted analysis of bile acid profile.

Bile acid profile

Serum (table 1) and urinary (table 2) levels of C24 bile acid species were markedly lower in the patient than in 5 healthy controls. The most abundant bile acid in the patient´s serum was GCDCA followed by GCA and TCDCA. Other major bile acids, such as DCA, TCA, CA and CDCA were also present, whereas the rest of species were below detection limit (<1 nM) (table 1). In patient´s urine TSLCA accounted for 70% of total bile acids, which also included GCA, TCA, CA, GCDCA, TCDCA and DCA (table 2). The ratio of amidated to non-amidated bile acids was higher in the patient than in controls (serum: 5.7 vs. 1.8; urine: 11.5 vs. 0.5).

HPLC-MS/MS analyses of patient's serum and urine combining Scan and Precursor Ion Modes revealed that: i) in Scan Mode a double peak of 556.3 *m/z* (8.9-9.3 min retention time) was detected in serum and urine of the patient but not in controls; ii) in Precursor Ion Mode, both peaks produced ion 80.2 *m/z* corresponding to sulfonate functional group (SO_3^-), which suggested that the compounds accounting for these two peaks were (25*R*)- and (25*S*)-stereoisomers of the taurine conjugated of trihydroxycholestanoic acid (tauro-THCA) (556.3 *m/z*). HPLC-TOF revealed a peak with Exact Mass of 556.3357, matching the mass (556.331349) of the $[M-H]^{-1}$ ion of tauro-THCA. Additional studies using MRM Mode, searching for the predicted *m/z* transitions

556.3 to 80.2 for tauro-THCA, 540.3 to 80.2 for tauro-dihydroxycholestanoic acid (tauro-DHCA), 449.6 to 449.6 for THCA and 433.6 to 433.6 for DHCA, showed double peaks in patient´s serum and urine seemingly corresponding to the stereoisomers of tauro-THCA (8.9-9.3 min retention time), tauro-DHCA (10.7-11.2 min retention time) and unconjugated THCA (13.5-13.7 min retention time).

Genetic studies

The observed low levels of C24 bile acids together with accumulation of C27 bile acid intermediate THCA and to a much lesser extent of DHCA (tables 1 and 2) led us to sequence the coding exonic and proximal intronic regions of *ACOX2*, *DBP* and *SCP-X* as potentially involved in blockade of CA biosynthesis at this level (Fig. 1A). Defects of other peroxisomal enzymes seemed unlikely as (25S)-isomers and amidated bile acids were detected, which excluded AMACR and BAAT deficiencies, respectively. The analysis of variants present in the three sequenced genes (Supplementary table 5) revealed only a critical genetic alteration in *ACOX2*. This was a homozygous mutation (c.673C>T) in exon 6 (Fig 2B), leading to arginine substitution by tryptophan at position 225 (R225W) (Fig 2C). This SNP (rs150832314) has been reported by the NCBI SNP database to have a Global Minor Allele Frequency (MAF) of 0.04% with not known phenotypic consequences.

Family studies

Both parents, born in two neighbor valleys of northern Spain, were found to be heterozygous for R225W ACOX2, whereas the patient's only sister (3 years younger) was also homozygous from the same mutation (figure 2D). Serum bile acid profile was comparable in the parents and controls, while it was similar in both siblings. Thus, as observed in our patient, his sister exhibited markedly low concentrations of major C24 bile acids (Fig. 1C) and abnormally elevated levels of C27 bile acids (Fig 1D). In the proband and his sister the proportion of THCA species with respect total bile acids was respectively 47.4 and 26.6 %, while it was below 0.3 % in both parents and controls. Interestingly, for the last 4 years of follow-up the sister showed normal serum ALT levels, except for a rise of 2-fold UNL upon administration of NSAIDs during a flu-like episode (Supplementary table 6). She also showed, similarly to her brother, a mild subclinical elevation of serum bilirubin (Supplementary tables 4 and 6).

Functional evaluation of mutACOX2

Recombinant lentiviruses containing the wild type cDNA of ACOX2 or its R225W variant (mutACOX2) were generated and employed to transduce human

hepatoblastoma HepG2 cells, a cell line competent for bile acid synthesis [18, 19]. The cells expressed similar amounts of wild type and mutated ACOX2 mRNA (figure 3A) and protein (figure 3B) that were of similar molecular size as assessed by Western blot (figure 3B). Immunofluorescence studies showed that both forms of the enzyme equally localized at the peroxisome (figure 3C-3E).

HPLC-MS/MS was used to evaluate bile acid biosynthesis by the transduced cells under basal conditions and upon incubation with THCA for 48 h. In the absence of any exogenous bile acid, control (Mock) HepG2 cells synthesized mainly CA, a process that was markedly increased (3-fold) in cells over-expressing ACOX2, while it was decreased in those transduced with mutACOX2 (figure 4A). These differences became more marked when THCA was added as substrate. Under these conditions, CA synthesis did not increase in mutACOX2 transduced cells while it was enhanced in Mock cells and even more in cells over-expressing ACOX2 (figure 4B).

Incubation of HepG2 cells with THCA for 72h caused oxidative stress and cell death in a dose-dependent manner (Fig 4C-E). These two effects were efficiently prevented by transduction of HepG2 cells with ACOX2 but not with mutACOX2. In contrast to THCA, the effect of C24 bile acids deoxycholic acid (DCA) or CA was similar in cells over-expressing ACOX2 or mutACOX2 (figure 4C).

DISCUSSION

We have identified a defect in ACOX2 activity, a peroxisomal enzyme involved in bile acid biosynthesis, in an adolescent boy with mild elevation of serum transaminases persisting 5 years after an episode of severe liver damage upon exposure to NSAIDs. The patient was found to be homozygous for a missense mutation in *ACOX2* gene resulting in R225W substitution. The same mutation was found in homozygosity in his only sister and in heterozygosity in both parents.

Whereas peroxisomal acyl-CoA oxidase 1 (ACOX1) is involved in oxidizing CoA esters of straight chain fatty acids and prostaglandins, ACOX2 oxidizes CoA esters of 2-methyl-branched fatty acids as well as those of the bile acid intermediates DHCA and THCA [20]. In addition, a gene encoding prystanoil-CoA oxidase (ACOX3) has been identified in human genome [21]. Consistent with the role of ACOX2 in the shortening of the cholesterol side chain, both the proband and his sister showed a marked decrease of mature C24 bile acids in association with accumulation of the C27 intermediates THCA and DHCA and their taurine conjugates. While C27 bile acids are abundant and play a major physiological role in reptiles and early evolving birds, they are efficiently removed by transformation into C24 bile acids in humans [22].

Human ACOX2 is a 76 kDa flavoprotein expressed in the liver but also in extrahepatic tissues, such as heart, kidney, skeletal muscle and pancreas [23]. Among the orthologs and paralogs in rat [24] of human ACOX2, only rat acyl-CoA oxidase-II or ACO-II crystal structure has been resolved and it has been shown that the enzyme works as a homodimer that contains one molecule of a noncovalently-bound FAD per subunit as the prosthetic group. The dimer formation is essential for FAD binding [16]. Based on the alignment of human ACOX2 and rat ACO-II amino acid sequences and the predicted three-dimensional structure of human ACOX2 (figure 2C) it could be hypothesized that the residue affected by the R225W substitution is located in a highly conserved β-domain in the neighborhood of the predicted FAD recognition site and the predicted catalytic region [16] (figure 2C). Indeed, although R225W does not modify ACOX2 molecular size and the subcellular targeting to the peroxisomes, it does interfere with its catalytic activity, resulting in impaired CA production from THCA in cells expressing the mutated ACOX2 and presumably accounting for the altered bile acid profile in homozygous individuals. We found that endogenous ability of HepG2 cells to biotransform THCA into CA was reduced by overexpressing mutACOX2. These findings could be due either to the displacement of the endogenous enzyme by the

over-expressed mutated one in the peroxisomes, or, it might reflect a dominant-negative effect of mutACOX2. However, although the later cannot be ruled out, the fact that bile acid profile was not altered in heterozygous individuals argues against mutACOX2 acting as a dominant negative of normal ACOX2 *in vivo*.

The biotransformation rate of THCA by cell homogenates containing required supplements (ATP, $MgCl_2$, CoA, FAD^+, NAD^+, KCN and DTT) was too low to carry out accurate enzymatic activity analysis of ACOX2, mutACOX2 and the combination of both. Further investigations using specific substrates and purified protein variants are required to elucidate the effect of R225W mutation in ACOX2 dimerization and enzymatic activity.

The two subjects with ACOX2 deficiency here reported have in common with patients with Zellweger syndrome, who lack functional peroxisomes, and with patients with a defect of the peroxisomal enzymes AMACR, DBP or SCPx the presence of reduced levels of C24 mature bile acids and increased C27 bile acid intermediates DHCA and THCA [25]. These compounds and the metabolites originated from them, are believed to be the main hepatotoxic compounds mediating liver injury in different peroxisomal disorders [7]. Supporting this notion, it has been shown that conjugated and unconjugated C27-bile acids impaired mitochondrial respiratory function and boosted ROS production causing cell death in rat hepatoma cells [26]. Similarly, in the present study, we have observed that THCA induced in HepG2 cells a dose-related increase of ROS production with concomitant reduction of cell viability and that this effect could be efficiently prevented by over-expressing wild type ACOX2 but not mutACOX2. Consistent with this observation we found that while wild type ACOX2 accelerated CA production from THCA, the R225W variant failed to enhance CA generation in cells incubated with THCA. By contrast neither ACOX2 nor mutACOX2 modified the cell toxicity mediated by C24 bile acids.

The case of ACOX2 deficiency described here differs phenotypically from other previously reported inborn errors affecting peroxisomal enzymes involved in bile acid biosynthesis. AMACR deficiency can have two very different phenotypic manifestations, either as early onset liver failure with cholestasis and fat-soluble vitamin deficiency [27] or as late onset neuropathy with encephalopathy, epilepsy, thamalic lesions, tremor, cataract, and/or pigmentary retinopathy, but no liver failure [8]. DBP deficiency results not only in impaired bile acid metabolism but also fatty acid β-oxidation and almost all patients display a severe phenotype dying early in life [28]. On the contrary, in the single patient with SCPx deficiency that has been described so

far no apparent liver abnormalities were observed [29]. In our ACOX2 deficient patient the only clinical manifestation was a mild-to-moderate elevation in serum levels of transaminases, with no significant histological changes at the time of the study.

Likely the cause of hypertransaminasemia relates to the accumulation of C27 bile acids as resin cholestyramine reverted liver biochemistry to normal. Intriguingly in both the proband and his sister hypertransaminasemia peaked upon exposure to drugs, such as NSAIDs and D-penicillamine in the proband, and anti-flu medication in the case of the proband´s sister. Whether ACOX2 deficiency may predispose to drug-induced liver injury or whether other xenobiotics may trigger THCA-induced hepatocellular damage is an issue deserving to be explored. It should be also noted that the proband´s sister exhibited normal serum transaminases during most of the follow-up (3 years), a fact that might reflect sex-related sensitivity to toxic C27 bile acids or differences in the exposure to other hepatotoxicants.

It is somehow surprising that ACOX2 deficiency might coexist with normal transaminasemia (as in the case of the proband´s sister), whilst THCA causes *in vitro* significant oxidative stress in ACOX2-deficient cultured cells. However, it should be considered that THCA was present at a constant concentration in the culture medium of cells expressing mutACOX2, whereas the intrahepatic levels of this compound in ACOX2 deficient subjects might be substantially lower if THCA is readily secreted into bile. It seems, thus, possible that, in these patients, hepatocellular damage might be triggered by exposure to compounds that compromise THCA biliary output. On the other hand, differences in the intensity of liver damage in patients with homozygous ACOX2 mutation might also reflect inter-individual variability of the compensatory anti-oxidant defense response. In addition, although our results demonstrated reduced ACOX2 function in individuals homozygous for R225W mutation, the existence of a residual activity cannot be ruled out.

Regarding treatment perspectives, it should be noted that despite the benefit afforded by resin cholestyramine, bile acid replacement therapy, such as CDCA+UDCA combined administration, appears to be the best option for ACOX2 deficient patients as it would enrich the bile acid pool with C24 species while inhibiting endogenous bile acid synthesis hence reducing THCA generation.

After the presentation of our case at the International Liver Congress – EASL (April 2016; Barcelona, Spain) and its publication in abstract form [30], in the period between

the provisional and definitive acceptance of this paper, a second case of ACOX2 deficiency has been reported [31]. The affected individual was a Turkish boy of 8 years of age with a homozygous non-sense mutation (Y69*) in *ACOX2* causing early stop codon leading to complete absence of the protein. The patient presented a mixed phenotype with mild to moderate hypertransaminasemia accompanied by ataxia and cognitive impairment similar to these already described for other peroxisomal disorders [8, 9]. By contrast, our cases of ACOX2 deficiency due to homozygous missense mutation (R225W) showed a pure "liver phenotype" indicating that ACOX2 deficiency should be sought in cases of unexplained persistent hypertransaminasemia even in the absence of neurological symptoms. Whether the discrepancy in the severity of symptoms between the patients here reported and the Turkish child was due to complete lack of ACOX2 protein in the latter versus residual ACOX2 activity in the more benign phenotype is a matter to be settled once more cases of ACOX2 deficiency are identified.

In summary, our study has revealed ACOX2 deficiency as a novel cause of persistent elevation of serum transaminases in otherwise asymptomatic patients. This entity should be considered in the diagnostic workout of patients with hypertransaminasemia of unknown origin.

FIGURE LEGENDS

Fig. 1. Impaired bile acid synthesis due to ACOX2 deficiency. (A) Peroxisomal steps of bile acid synthesis. (B) HPLC-TOF mass spectrum of compound present in the patient serum. The exact mass 556.3357 matches that of taurine conjugated trihydroxy-5β-cholestanoic acid (THCA). Inset: Representative chromatograms obtained by HPLC-MS/MS in MRM Mode showing double peaks of *m/z* transition 556.3 to 80.2, corresponding to taurine-conjugated (*25S*)- and (*25R*)-THCA, and 540.3 to 80.2, corresponding to taurine-conjugated (25S)- and (25R)-dihydroxy-5β-cholestanoic acid (DHCA) in serum and urine. Serum concentrations in healthy control volunteers (n=5) and the affected family of C24 bile acids (in µM) (C) and C27 bile acids (in nM) (D). ACOX2: acyl CoA oxidase 2; AMACR: alpha methylacyl-CoA racemase; BAAT: bile acid CoA: amino acid N-acyltransferase; BACS: bile acid CoA synthetase; DBP: D-bifunctional protein; CYP27A1: sterol 27-hydroxylase; SCPx: sterol carrier protein X; VLCS: very long chain acyl CoA synthetase.

Fig. 2. Genetic bases of ACOX2 deficiency. (A) *ACOX2* gene structure showing translated and untranslated exonic regions. (B) Electropherogram showing the homozygous missense mutation c.673C>T found. (C) Predicted tertiary structure of human ACOX2 showing the proximity of the replaced amino acid (R225W) as result of the mutation, and the active site, i.e., the region of the protein that includes the residues interacting with FAD, the catalytic residues and their surrounding residues. (D) Genealogical tree of the affected family.

Fig. 3. Protein expression of mutated ACOX2 gene. (A) ACOX2 mRNA abundance in HepG2 cells before and after transduction with lentiviral vectors (MOI 10) either empty (Mock) or containing the ORF of wild type ACOX2 or the mutated variant (mutACOX2). The amount of mRNA was determined by RT-QPCR and expressed as percentage of that found in human liver. Values are expressed as means±SD from 4 independent experiments performed in triplicate. *$p<0.05$ compared with HepG2 cells by the Bonferroni method of multiple-range testing. (B) Representative western blot of lysates from HepG2 cells transduced with lentiviruses containing V5-tagged ACOX2, mutACOX2 or only the V5 tag (Mock). Confocal

fluorescence microscopy of HepG2 cells expressing V5-tagged ACOX2 (C) or mutACOX2 (D and E). Peroxisomes were labeled with an antibody against the 70-kDa peroxisomal membrane protein PMP70 (E).

Fig. 4. In vitro evaluation of functional consequences of ACOX2 deficeincy. Cholic acid (CA) synthesis by HepG2 cells incubated without (A) or with (B) 10 μM THCA for 48 hours. Effect of THCA on reactive oxygen species (ROS) production (C, D) and cell viability (E). Control cultures were incubated in the absence of bile acids. Results are means±SD from at least 9 determinations using three different cultures. *$p<0.05$ compared with Mock; $^{\dagger}p<0.05$ on comparing mutACOX2 with ACOX2 by the Bonferroni method of multiple-range testing. DCA, deoxycholic acid.

ACKNOWLEDGMENTS

The authors thank N. Skinner for revision of the English spelling, grammar and style of the manuscript and Virginia Villar for sample collection.

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Table 1. Bile acid species in serum.

	Control (n=5)	Patient	Sister	Father	Mother
Total C24 bile acids (µM)	4.68±0.61	0.06±0.01	0.28	2.57	7.84
Tauroconjugated (µM)	0.67±0.08	0.01±0.01	0.04	0.10	0.34
Glycoconjugated (µM)	2.37±0.59	0.04±0.01	0.11	0.90	3.53
Non-amidated (µM)	1.65±0.33	0.01±0.01	0.13	1.56	3.97
C24 Bile acid species (nM)					
Glyco-Cholic	478±111	9±3	23	117	589
Glyco-Chenodeoxycholic	1049±278	24±1	30	399	1867
Glyco-Deoxycholic	672±199	<1	42	215	877
Glyco-Lithocholic	40±19	<1	<1	4	61
Glyco-Ursodeoxycholic	127±40	<1	17	110	134
Tauro-Cholic	136±9	3±1	3	13	49
Tauro-Chenodeoxycholic	203±53	8±4	8	38	147
Tauro-Deoxycholic	129±34	<1	7	23	68
Tauro-Lithocholic	2±1	<1	<1	1	7
Tauro-Ursodeoxycholic	23±9	<1	<1	3	2
Tauro-Sulfo-Lithocholic	96±15	<1	18	23	70
Cholic	242±157	2±1	19	586	1969
Chenodeoxycholic	604±246	1±1	9	161	709
Deoxycholic	529±151	6±3	76	691	1166
Lithocholic	145±100	<1	24	117	112
Ursodeoxycholic	42±14	<1	<1	9	16
Total C27 bile acids (nM)	3±2	52±6	100	6	14
Tauro-trihydroxycholestanoic	3±2	32±6	56	6	14
Tauro-dihydroxycholestanoic	<1	9±1	33	<1	<1
Trihydroxycholestanoic	<1	11±2	11	<1	<1

Patient values are mean±SD from measurements carried out by duplicate in two samples collected over a 6 months interval. Control samples were collected from healthy male subjects of comparable age. Result of serum concentrations of the sister, father and mother was calculated from two samples analysed in duplicate.

Table 2. Bile acid species in urine

	Control (n=5)	Patient
Total C24 bile acids (µM)	1.18±0.13	0.07±0.02
Tauroconjugated (µM)	0.28±0.11	0.06±0.02
Glycoconjugated (µM)	0.14±0.02	0.01±0.01
Non-amidated (µM)	0.76±0.18	0.01±0.01
C24 Bile acid species (nM)		
Glyco-Cholic	71±8	6±2
Glyco-Chenodeoxycholic	33±8	2±1
Glyco-Deoxycholic	23±4	<1
Glyco-Lithocholic	<1	<1
Glyco-Ursodeoxycholic	15±5	<1
Tauro-Cholic	15±4	6±5
Tauro-Chenodeoxycholic	6±2	1±1
Tauro-Deoxycholic	2±1	<1
Tauro-Lithocholic	<1	<1
Tauro-Ursodeoxycholic	<1	<1
Tauro-Sulfo-Lithocholic	247±115	52±15
Cholic	572±149	3±1
Chenodeoxycholic	28±9	<1
Deoxycholic	105±17	1±1
Lithocholic	2±1	<1
Ursodeoxycholic	<1	<1
Total C27 bile acids (nM)	<1	6±2
Tauro-trihydroxycholestanoic	<1	2±1
Tauro-dihydroxycholestanoic	<1	1±1
Trihydroxycholestanoic	<1	4±1

Patient values are means±SD from measurements carried out by duplicate in two samples collected over a 6 months interval. Control samples were collected from healthy male subjects of comparable age.

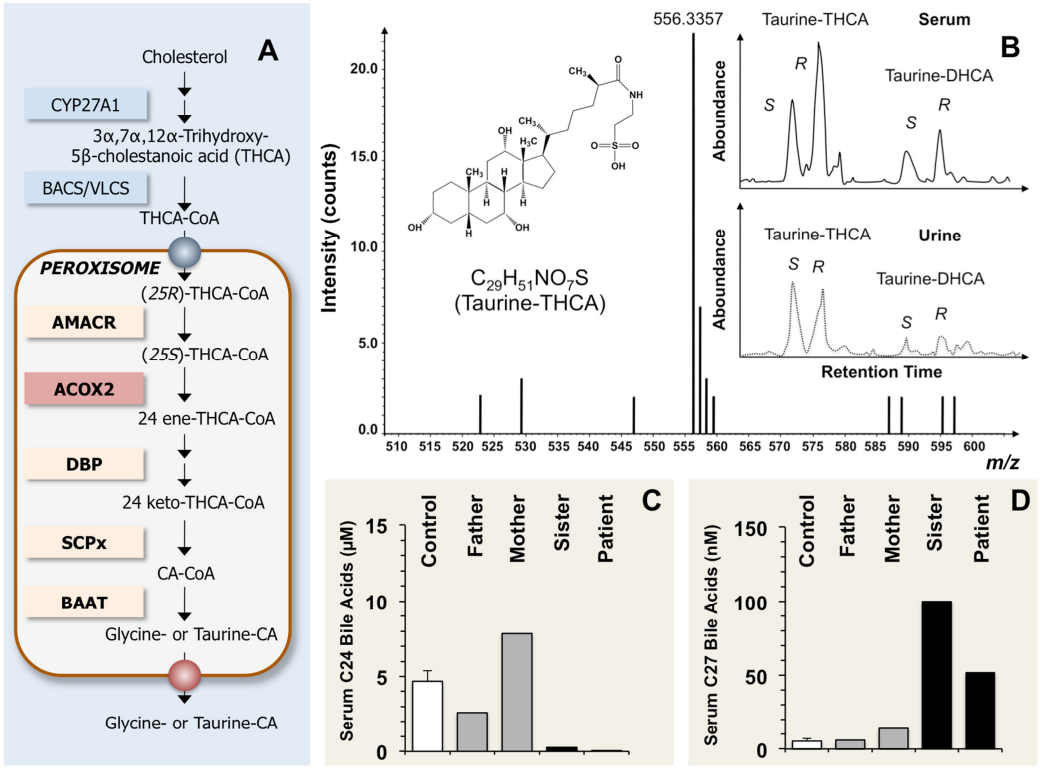

Figure 1

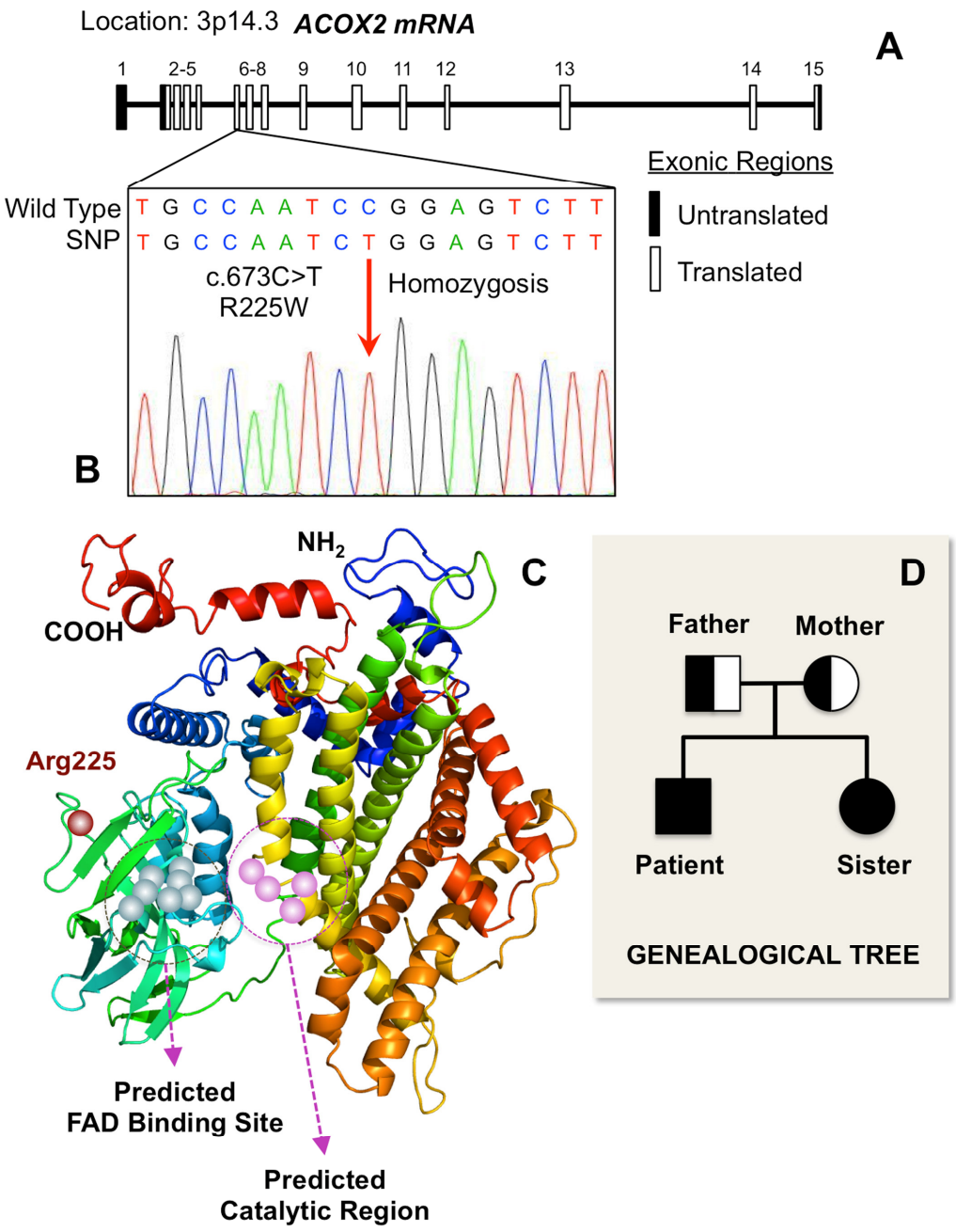

Figure 2

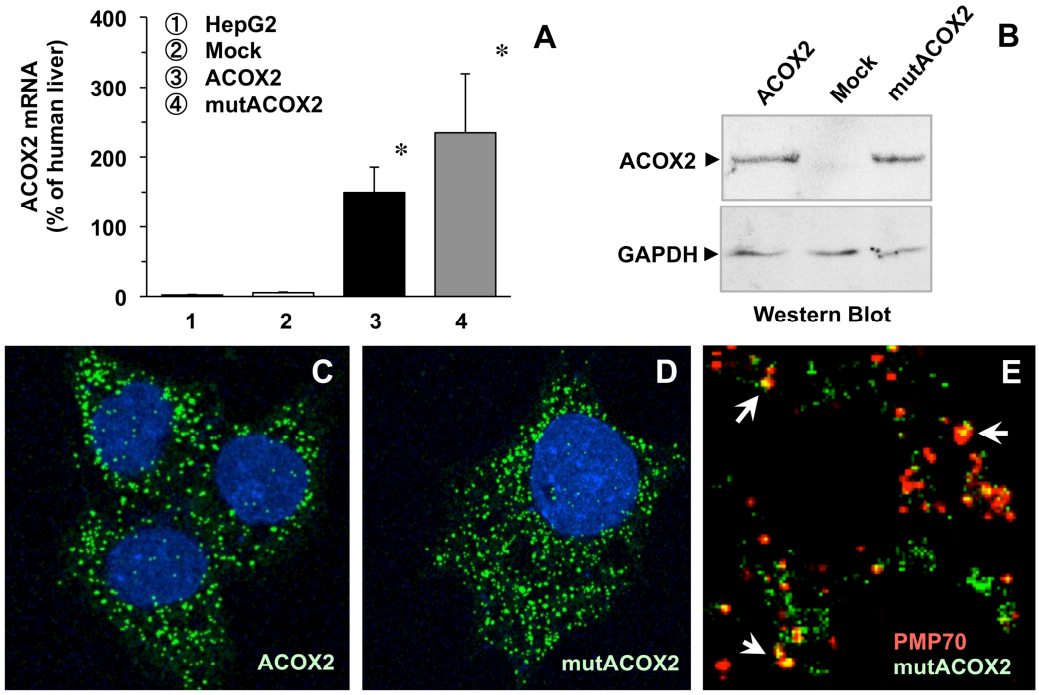

Figure 3

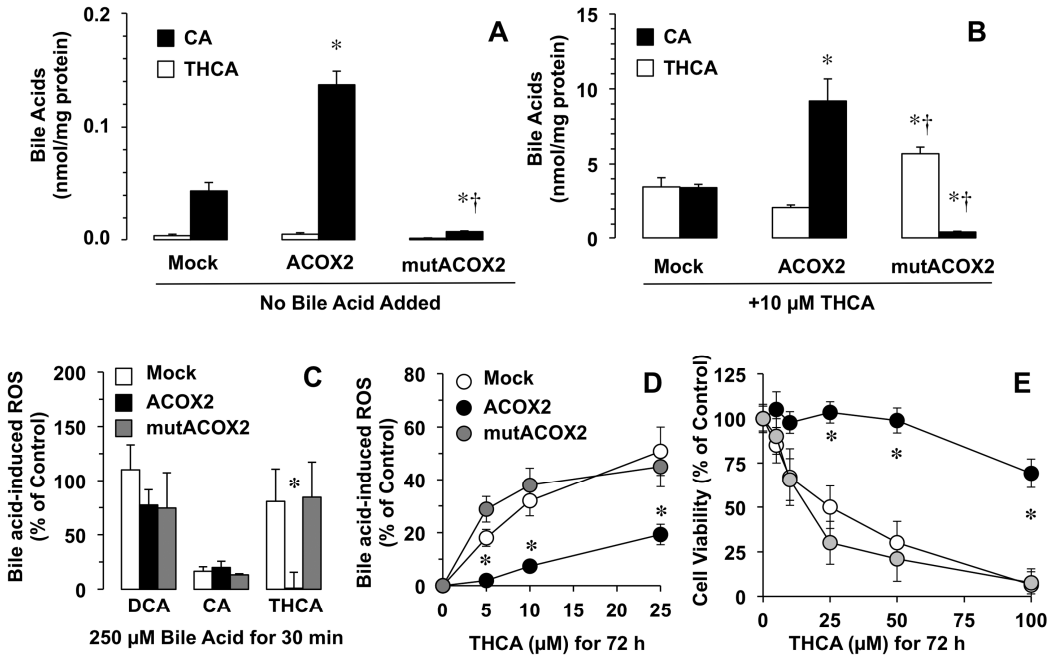

Figure 4

LAY SUMMARY

Elevation of serum transaminases is a biochemical sign of liver damage due to multiplicity of causes (viruses, toxins, autoimmunity, metabolic disorders). In rare cases the origin of this alteration remains unknown. We have identified by the first time in a young patient and his only sister a familiar genetic defect of an enzyme called ACOX2, which participates in the transformation of cholesterol into bile acids as a cause of increased serum transaminases in the absence of any other symptomatology. This treatable condition should be considered in the diagnosis of those patients where the cause of elevated transaminases remains obscure.