

BIOLOGICAL SCIENCES: Medical Sciences

ACOX2 deficiency: a new disorder of bile acid synthesis with transaminase elevation, liver fibrosis, ataxia and cognitive impairment

Sílvia Vilarinho<sup>1,2,\*</sup>, Sinan Sari<sup>3</sup>, Francesca Mazzacuva<sup>4</sup>, Kaya Bilguvar<sup>2,5</sup>, Güldal Esendagli-Yilmaz<sup>6</sup>, Dhanpat Jain<sup>1,7</sup>, Gülen Akyol<sup>6</sup>, Buket Dalgıç<sup>3</sup>, Murat Günel<sup>2,5,8</sup>, Peter Clayton<sup>4</sup>, Richard P. Lifton<sup>2,5,9,\*</sup>

<sup>1</sup>Department of Internal Medicine, Section of Digestive Diseases, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06517, USA.

<sup>2</sup>Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06517, USA.

<sup>3</sup>Department of Pediatrics, Division of Gastroenterology, Gazi University, Faculty of Medicine, Besevler, Ankara, TB 06500, Turkey.

<sup>4</sup>Center for Translational Omics, University College London Institute of Child Health, 30 Guilford Street, London, UK.

<sup>5</sup>Yale Center for Mendelian Genomics, Yale University School of Medicine, New Haven, Connecticut, USA.

<sup>6</sup>Department of Pathology, Gazi University, Faculty of Medicine, Besevler, Ankara, TB 06500, Turkey.

<sup>7</sup>Department of Pathology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06517, USA.

<sup>8</sup>Department of Neurosurgery, Yale Program in Brain Tumor Research, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06517, USA.

<sup>9</sup>Department of Internal Medicine and Howard Hughes Medical Institute, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06517, USA.

\*Correspondence should be addressed to: Richard P. Lifton, M.D., Ph.D., Departments of Genetics and Internal Medicine, Howard Hughes Medical Institute, Yale University School of Medicine, 333 Cedar St., SHM I308, New Haven, CT 06510, USA.

Telephone: +1-203-737-4420, Fax: +1-203-785-7560, Email: [richard.lifton@yale.edu](mailto:richard.lifton@yale.edu); or  
Sílvia Vilarinho, M.D., Ph.D., Departments of Genetics and Internal Medicine, Section of Digestive Diseases, Yale University School of Medicine, 333 Cedar St., LMP1080, New Haven, CT 06510, USA. Telephone: +1-203-737-1833, Fax: +1-203-737-1755, Email: [silvia.vilarinho@yale.edu](mailto:silvia.vilarinho@yale.edu).

Short Title: ACOX2 deficiency: a new bile acid synthesis defect

Keywords: branched-chain acyl-CoA oxidase, bile acid metabolism, peroxisomal disorder, whole-exome sequencing

## SUMMARY

ACOX2 encodes branched-chain acyl-CoA oxidase, a peroxisomal enzyme believed to be involved in metabolism of branched-chain fatty acids and bile acid intermediates. Deficiency of this enzyme has not been described. We report an 8-year-old male with intermittently elevated transaminases, liver fibrosis, mild ataxia and cognitive impairment. Exome sequencing revealed a previously unidentified homozygous premature termination mutation (p.Y69\*) in ACOX2. Immunohistochemistry confirmed the absence of ACOX2 expression in the patient's liver, and biochemical analysis showed marked elevation of intermediate bile acids upstream of ACOX2. These findings define a novel, potentially treatable, inborn error of bile acid biosynthesis caused by ACOX2 deficiency.

## SIGNIFICANCE STATEMENT

Liver disease of unknown cause represents an unmet medical need. Using exome sequencing, we have described a new syndrome associated with homozygous loss of ACOX2 featuring elevated transaminases, liver fibrosis, ataxia and cognitive impairment. ACOX2 encodes the peroxisomal branched-chain acyl-CoA oxidase and is involved in the bile acid biosynthetic pathway. Importantly, this disorder is potentially reversible, because the bile acid synthetic pathway can be suppressed with exogenous bile acids, diminishing the production of the likely toxic metabolites causing liver and neurologic dysfunction. Furthermore, this study provides the means to diagnose ACOX2 deficiency among other patients with chronic idiopathic liver disease and/or cryptogenic cirrhosis.

## INTRODUCTION

Despite major advances in Mendelian genetics, the role in normal human biology and the impact of mutation of > 75% of the 20,000 protein-coding genes in the human genome remain to be determined(1). One example of unmet medical need is idiopathic liver disease, which remains a challenge in both pediatric and adult hepatology. We and others have shown the utility of whole-exome sequencing in the diagnosis of such patients (2-6). Children with unexplained liver disease who are the offspring of consanguineous union are excellent candidates for having recessive disease-causing mutations. Such homozygous mutations can now be readily identified by exome sequencing(7). Acyl CoA Oxidase 2 (ACOX2) encodes the branched-chain acyl-CoA oxidase believed to participate in the metabolism of branched-chain fatty acids and bile acid intermediates in the peroxisomes. While genetic disorders resulting from mutations in many other genes in this pathway have been described, mutations in ACOX2 have not previously been attributed to a phenotype in humans or other animals. We describe a previously unrecognized syndrome resulting from recessive ACOX2 deficiency.

## RESULTS

### CASE REPORT

An 8 year-old boy of Turkish ancestry, who was the offspring of a second-cousin union, was evaluated in liver clinic. He was born at full term without complications, and had no family history of liver or neurologic disease in his parents or 10 year-old sibling. At 8 months of age he presented with vomiting presumed to be secondary to acute

gastroenteritis, and was found to have elevated transaminases. Liver and spleen were not enlarged. Over subsequent years, transaminase levels were intermittently elevated (aspartate aminotransferase 30-131 U/L, normal range 10-30 U/L); alanine aminotransferase 19-297 U/L, normal range 6-29 U/L), with normal gamma-glutamyl transpeptidase (GGT) levels and preserved liver synthetic function as indicated by normal albumin, bilirubin and INR levels. He also had hypolipidemia (total cholesterol less than 100mg/dL or low-density lipoprotein (LDL) less than 50mg/dL), with his total serum cholesterol ranging from 75-96 mg/dL and LDL-cholesterol 14-44 mg/dL; he also had vitamin D deficiency (serum level ranging from 12-17 ng/mL, normal range=20-50ng/mL), but normal levels of liposoluble vitamins A and E. Additionally, he had an elevated steatocrit (a semiquantitative measure of fat content in fecal samples), indicating steatorrhea and fat malabsorption. Serologies for viral hepatitis, TORCH (Toxoplasma gondii; Other: coxsackievirus, chickenpox, chlamydia, HIV, HTLV, syphilis; Rubella; CMV; HSV-2), and EBV, tests for autoimmunity (immunoglobulin G level; anti-smooth muscle, anti-nuclear, anti-double stranded DNA, anti-liver kidney microsomal, anti-soluble liver pancreas, anti-cytosolic, anti-tissue transglutaminase and anti-thyroglobulin antibodies), and metabolic tests (alpha-1-antitrypsin, ceruloplasmin, urine copper levels, urine organic acid analysis, sweat chloride test), were all normal. Abdominal ultrasound with doppler study was unremarkable.

The proband underwent a liver biopsy at 6 years of age; in contrast to a normal liver, the patient's liver showed many thin fibrous septa, swollen hepatocytes, glycogenated nuclei and focal acinar transformation, consistent with hepatocellular injury and

regeneration (Figure 1, Supplementary Figure 1A). There was no obvious cholestasis, cholate stasis or steatosis. The fibrous septa and portal areas showed sparse lymphocytes without any interface activity and there was no lobular inflammatory activity or evident acidophil bodies. Trichrome stain showed thin fibrous septa and some nodularity (“incomplete septal cirrhosis”), with no features of well-established cirrhosis (Supplementary Figure 1B).

The proband’s growth has been normal, with weight, height and head circumference ranging between 5<sup>th</sup>-90<sup>th</sup>, 50<sup>th</sup>-75<sup>th</sup> and 25<sup>th</sup>-50<sup>th</sup> percentile, respectively. Specifically, patient’s weight, height and head circumference were 2500 g (5<sup>th</sup> percentile), 49 cm (50<sup>th</sup> percentile) and 34 cm (25<sup>th</sup> percentile) at birth; and 33.8 Kg (90<sup>th</sup> percentile), 133 cm (75<sup>th</sup> percentile) and 53 cm (50<sup>th</sup> percentile) at age 8 on his last clinic visit in June 2016.

The proband also had neurologic abnormalities. Developmental milestones included social smile at 3 months (mild delay), sitting at 6 months and walking at 14 months of age (within normal limits). He demonstrated mild delay in language development, producing several single words by 2.5 years and sentences by 5 years of age. At 6.5 years of age, his parents and elementary school teachers noted mild intellectual disability. Neurological examination was remarkable for slurred speech, vertical gaze palsy, slight dysmetria, and mild gait ataxia. The ocular fundus exam was normal bilaterally. Wechsler Intelligence Scale for Children (WISC)-Revised test showed a total

score of 66 points (average range 90-110), supporting the diagnosis of mild intellectual disability. Brain magnetic resonance imaging (MRI) was reported as normal.

#### Homozygous loss-of-function mutation in ACOX2

The proband's exome was sequenced to a mean depth of 60 independent reads per targeted base, with 96% of targeted bases having more than eight independent reads, providing high confidence calling of homozygous and heterozygous variants across the exome (Supplementary Table 1). Since the affected child is the offspring of second-cousin union (Figure 1D), we sought rare alleles (allele frequency  $\leq 0.01$  in databases) that were likely to result in loss of function of the encoded protein (i.e., premature termination, frameshift and splice site variants) that were homozygous in the proband. Consistent with consanguinity, this analysis identified five rare homozygous genotypes in the proband. Four of these resulted in missense variants that were predicted to be tolerated variants by MetaSVM and unlikely to be pathogenic in this patient (Supplementary Table 2). The other homozygous variant encoded premature termination at codon 69 (NM\_003500, p. Y69\*) in ACOX2, which encodes a branched-chain acyl-CoA oxidase, a peroxisomal enzyme expressed in the liver and kidney and believed to be involved in bile acid biosynthesis(8). This variant allele was absent among >100,000 alleles in the ExAC database, and >1500 alleles in people of Turkish ancestry, and was not found in any other database examined. Moreover, no homozygous loss-of-function genotypes in this gene were found among approximately 61,000 exomes in ExAC or 894 people of Turkish ancestry. Sanger sequencing

confirmed the homozygous variant in the proband and showed that both parents and his brother were heterozygous for this variant (Figure 1E).

#### ACOX2 is absent in proband's liver

The livers of normal control subjects and the proband were stained with antibodies specific for ACOX2. While normal liver showed intense granular staining in the pericentral (zone 3) hepatocytes (Figure 2A-C), along with faint hepatocellular cytoplasmic staining, the proband's liver showed complete absence of staining, consistent with the mutation resulting in absence of this protein (Figure 2D-F).

#### Elevated levels of bile acid intermediates

The primary bile acids, cholic acid and chenodeoxycholic acid, are derived from cholesterol in the liver by the action of at least 14 different enzymes(9). The final steps of bile acid synthesis occur in peroxisomes; ACOX2, a peroxisomal branched-chain acyl-CoA oxidase, is believed to mediate the first step in the beta-oxidation of 27 carbon (C27) bile acid intermediates into 24 carbon (C24) bile acids(8, 10), resulting in the CoA esters of cholic and chenodeoxycholic acid (Figure 3). We consequently examined the proband's plasma and urine for elevated levels of C27-bile acids upstream of ACOX2 activity. Consistent with loss of ACOX2 function, the proband's plasma and urine showed striking elevation of the C27 intermediate bile acids 3,7-dihydroxy-5 $\alpha$ -cholestanoic acid (DHCA) and 3,7,12-trihydroxy-5 $\alpha$ -cholestanoic acid (THCA), principally as the taurine conjugates (Table 1). For example, the proband's plasma Tauro-THCA level was 7.94  $\mu$ M, 25-fold higher than the upper limit of normal (0.31  $\mu$ M),

and urinary Tauro-THCA was 66.19  $\mu\text{mol/mol}$  creatinine; this metabolite is normally absent in urine. The proband shows low normal levels of cholic acid and conjugates, as seen in other bile acid biosynthesis disorders(11). The proband's heterozygous parents showed no elevation of C27 intermediate bile acids. These findings indicate that homozygous loss of ACOX2 results in defective peroxisomal beta-oxidation of DHCA-CoA and THCA-CoA.

ACOX2 has also been thought to be involved in the degradation of long branched fatty acids (phytanic and pristanic acids)(8). Interestingly, however, the proband's branched-chain fatty acids (phytanic and pristanic acids) were both within the normal range (Table 1). As expected, there was no elevation of very long chain fatty acids in the plasma, consistent with their metabolism by straight-chain acyl-CoA oxidase (ACOX1).

## DISCUSSION

Our findings define a new inborn error of bile acid synthesis in a child with intermittent elevated transaminases, liver fibrosis, and mild neurological impairment, and implicate homozygous loss of ACOX2 in its pathogenesis. The evidence implicating ACOX2 is strong. The proband has the first reported homozygous loss of function mutation in ACOX2, no evidence of ACOX2 protein in his liver, and biochemical analysis shows the elevation of C27 bile acid intermediates predicted to result from loss of ACOX2 function, along with low or low-normal levels of downstream primary bile acids. His hypocholesterolemia, fat malabsorption and vitamin D deficiency are expected findings owing to decreased intestinal absorption of fat and lipids with reduced bile acid

secretion into the gut. Of note, the proband's heterozygous 10-year old brother (total cholesterol, TC, 162 mg/dl, LDL cholesterol 85 mg/dl), mother (TC 159, LDL 78) and father (TC 204, LDL 97) all had normal lipids, providing no evidence of a phenotype in the heterozygous state.

Additionally, intermediate bile acids are believed to be hepato- and neurotoxic(12, 13) providing likely explanations for the proband's elevated transaminases, liver histology and neurologic defects. Indeed, mild to severe hepatocellular damage and/or neurologic impairment, including ataxia and intellectual disability, is seen in other defects in bile acid synthesis(9). Alternatively, deficiency of ACOX2 enzymatic activity and its downstream metabolites, and/or an independent function of ACOX2 could contribute to the proband's neurological and cognitive impairment. No other variants were identified that were likely to explain aspects of the clinical features seen in the proband. The full spectrum of clinical features attributable to loss of ACOX2 will need to be determined by the study of additional patients with ACOX2 deficiency; this report should stimulate study of other children with unexplained elevation in transaminases and/or neurologic features.

Other patients previously described with increases in C27 bile acid intermediates without general peroxisomal defects have proven distinct from ours. A patient with cirrhosis and liver failure at age four had recessive loss of ABCD3(14), which transports branched-chain acyl CoAs into peroxisomes; like our patient, this patient had normal levels of phytanic and pristanic acid. Other patients have been described with

sensorimotor neuropathy with recessive loss of alpha-methyl-acyl-CoA racemase (AMACR)(15), the enzymatic step in bile acid synthesis prior to ACOX2. These patients have elevated levels of phytanic and pristanic acid.

While ACOX2 has also been proposed to be involved in the oxidation of long branched fatty acids(8, 10), normal levels of phytanic and pristanic acids were measured in the proband's plasma. In humans, phytanic and pristanic acids are dietary in origin, predominantly from dairy products, beef and lamb(16). The proband is not vegetarian and ingests dairy products and beef regularly, providing no support for low dietary intake. These findings suggest that another pathway (e.g. omega-oxidation) can achieve full catabolism of these branched-chain fatty acids in children. The fact that phytanic and pristanic levels are elevated in patients without AMACR, which converts 2R pristanoyl-CoA to the S enantiomer, suggests that this alternate pathway can only metabolize S enantiomers. We infer that as a consequence of ACOX2 deficiency, C27-intermediate bile acids accumulate in peroxisomes as CoA esters and are readily converted into taurine conjugates (by bile acyl-CoA amino acid acyltransferase, BAAT), and much smaller amounts are converted to glycine conjugates. This is consistent with the observation that taurine conjugates predominate in peroxisome biogenesis disorders(17).

Importantly, the clinical consequences of defects in bile acid synthesis can be mitigated using primary bile acid therapy. Oral bile acid replacement with cholic acid reduces endogenous bile acid synthesis, thereby decreasing the accumulation of toxic bile acid

intermediates and correcting fat malabsorption(18, 19). This therapy has also proved effective in normalizing transaminases and improving neurologic findings(20). Since the proband shows low normal levels of cholic acid and conjugates, cholic acid therapy has the potential for clinical impact; a trial with close monitoring of liver function tests and bile acid intermediates levels is planned.

To date, most peroxisomal disorders have been diagnosed via the identification of probands with severe phenotypes with marked biochemical abnormalities, such as increased levels of substrates normally handled by peroxisomes (VLCFAs, pristanic acid, phytanic acid, DHCA, THCA) and decreased levels of end products of peroxisomal metabolism (cholic and chenodeoxycholic acid), followed by investigation leading to identification of the genetic defect. It is noteworthy that our patient presented with a relatively non-specific clinical presentation, and that a primary defect in bile acid biosynthesis was not considered prior to identification of the ACOX2 mutation.

Particularly because of the potential for mitigation of the clinical consequences of ACOX2 deficiency, this diagnosis should be considered in children with unexplained transaminase elevations and neurologic abnormalities, and raise the question of whether ACOX2 mutation might also contribute to cases of cryptogenic cirrhosis later in life.

## MATERIALS AND METHODS

DNA isolation, exome capture and sequencing. Genomic DNA of the proband, his unaffected parents, and brother was isolated from peripheral blood leukocytes

using standard procedures. Exome sequencing of the proband was performed using the Roche/Nimblegen SeqCap EZ Human Exome Library v2, with 74 base paired-end sequencing on the Illumina HiSeq platform as previously described(21).

Exome Analysis. Exome sequence data was aligned to the reference human genome (build 19) and variants were called using GATK; the impact of missense variants was predicted using MetaSVM(22). Allele frequencies of identified variants were determined in the NHLBI Exome Variant Server (4,300 European and 2,203 African-American subjects; last accessed March 2015), 1000 Genomes (1,094 subjects of various ethnicities), the Exome Aggregation Consortium (ExAC; 61,000 subjects of various ethnicities; January 2015 release), and a Yale database of 894 Turkish exomes, predominantly of consanguineous union.

Sanger sequencing of genomic DNA. Sanger sequencing of the identified ACOX2 mutation (p.Y69\*) was performed by PCR amplification of genomic DNA of the proband, his parents and his sibling using forward primer: 5'-TCTTCTAACCAACCCAGGCG-3', and reverse primer: 5'-CAGAAACCTCACCCAGGTCC-3'. Nomenclature of the ACOX2 variant is based on NCBI reference sequence NM\_003500.

ACOX2 liver immunohistochemistry. Anti-ACOX2 antibody from Sigma-Aldrich (HPA038280) at 1:200 dilution was used to perform immunohistochemistry in normal and ACOX2-mutant liver sections using standard techniques.

Mass spectrometry. Analysis of cholanoic acids (normal bile acids) and their taurine and glycine conjugates was performed by targeted UPLC-MS/MS essentially as described by(23). The following modifications were used to include analysis of cholestanolic acids (C27 bile acids): THCA (3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy-5 $\beta$ -cholestanolic acid) and [27,27,27-D<sub>3</sub>]-THCA were purchased from Dr. Herman Ten Brink, VU Medical Centre, Amsterdam, Netherlands. UPLC retention times and mass spectrometry transitions for the C27 bile acids are shown in Supplementary Table 3. Quantitation of glycine and taurine conjugates was achieved by measuring the ratio of the peak area produced by the C27 bile acid in the patient sample to the corresponding deuterated C24 bile acid standard e.g. tauro-THCA/D<sub>5</sub>-taurocholic acid. Some compounds were identified by comparison to samples from previous patients diagnosed with peroxisomal disorders.

## ACKNOWLEDGMENTS

We thank the proband and his family for their invaluable contribution to this work; the staff of the Yale Center for Genome Analysis for exome sequencing; and Lynn Boyden, Weizhen Ji and Samir Zaidi for helpful discussions and critically reading the manuscript. This work was supported by the US National Institutes of Health (NIH) Centers for Mendelian Genomics (U54 HG006504) and in part by National Institutes of Health - Yale Liver Center P30DK034989. S.V. is an AASLDF Sheila Sherlock Clinical and

Translational Research Award in Liver Diseases recipient. R.P.L is an Investigator of the Howard Hughes Medical Institute.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest with respect to this study.

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## FIGURE LEGENDS

Figure 1. Liver histology and ACOX2 mutation in a subject with a novel bile acid synthesis disorder. (A) Liver parenchyma of proband shows many thin fibrous septa (black arrows) and some nodularity (“incomplete septal cirrhosis”). Features of well-established cirrhosis are not seen. (B) Details of the lobular parenchyma show swollen hepatocytes (asterisks), glycogenated nuclei (black arrows) and very thin incomplete fibrous-septa (blue arrow). (C) Liver parenchyma from age and gender matched patient showing normal-appearing portal tracts and a central venule (asterisk). The lobular architecture is preserved with no fibrosis. (D) The affected proband and unaffected subjects are shown with black and white symbols, respectively. Consanguineous union is shown with a double line. ACOX2 alleles are denoted WT (wild-type) or Mut (p. Y69\*). (E) Sanger sequencing chromatograms of the proband, his unaffected parents and brother. The ACOX2 p.Y69\* mutation is homozygous in the proband and heterozygous in the unaffected parents and sibling. Scale bars, 50 $\mu$ m.

Figure 2. Absence of ACOX2 in proband's liver. (A, B, C) Immunohistochemistry for ACOX2 in normal liver shows intense granular staining in the pericentral (zone 3) hepatocytes. The staining in remaining hepatocytes, including periportal hepatocytes (zone 1) is faint. (D, E, F) Immunohistochemistry for ACOX2 in the proband's liver biopsy, showing complete absence of staining. Scale bars, 50 $\mu$ m.

Figure 3. Schematic representation of  $\beta$ -oxidation of trihydroxycholestanoyl-CoA by ACOX2. 'CA' denote cholic acid.

Figure 1

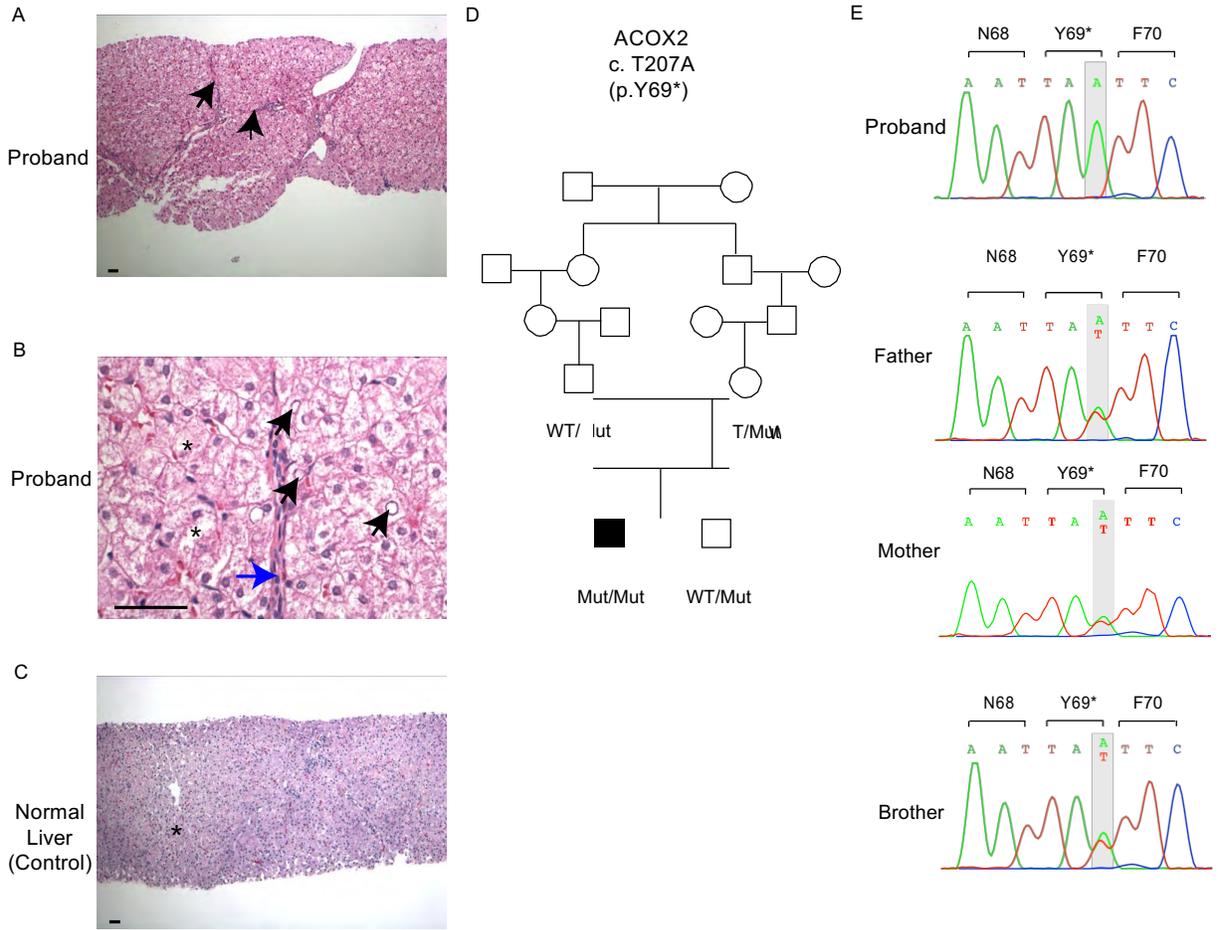


Figure 2

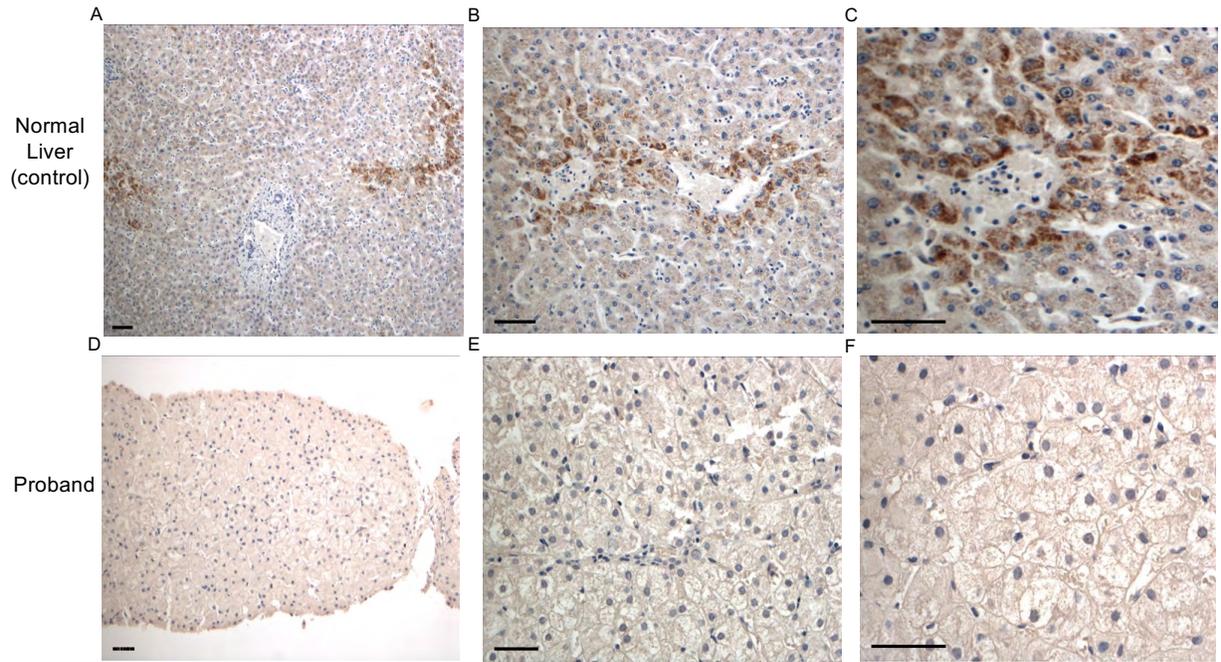


Figure 3

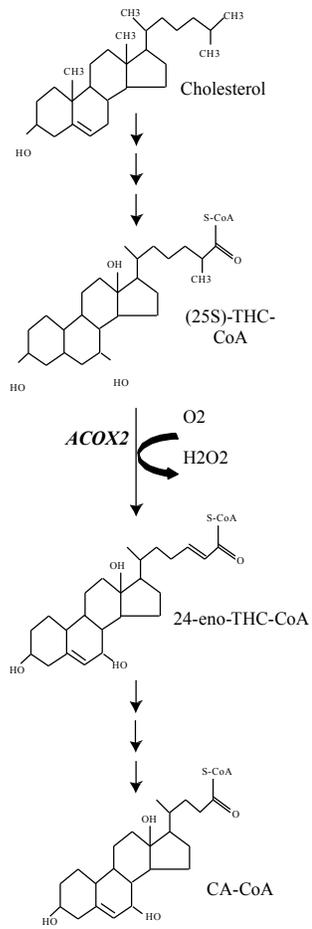
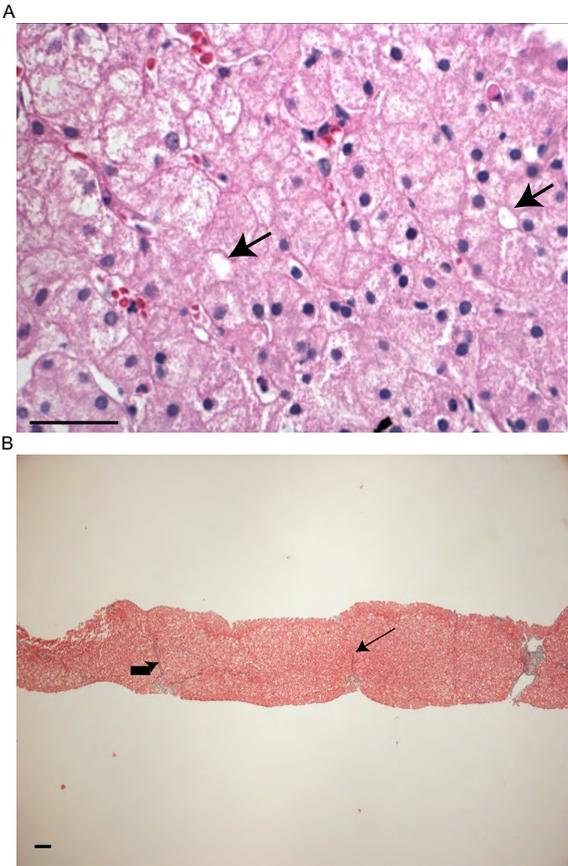


Table 1. Biochemical data from proband's plasma and urine.

Parameter	Proband	Mother	Father	Reference Values
ACOX2 genotype (p.Y69*)	Mut/Mut	Wt/Mut	Wt/Mut	n.a.
<u>Plasma</u>				
Bile acid intermediates				
Tauro-DHCA, $\mu\text{M}$	0.32 ( $\uparrow$ )	0	0	0 - 0.09
Tauro-THCA, $\mu\text{M}$	7.94 ( $\uparrow$ )	0.011	0.01	0 - 0.31
Glyco-DHCA, $\mu\text{M}$	0.06 ( $\uparrow$ )	0.01	0	0 - 0.01
Glyco-THCA, $\mu\text{M}$	0.24 ( $\uparrow$ )	0.01	0.02	0 - 0.19
DHCA, $\mu\text{M}$	0.262 ( $\uparrow$ )	0.003	0.004	0 - 0.006
THCA, $\mu\text{M}$	0.10 ( $\uparrow$ )	0.01	0.02	0 - 0.07
Bile Acids				
Glycodihydroxycholanoates, $\mu\text{M}$	0.58	0.76	1.41	0.22 - 4.16
Glycocholate, $\mu\text{M}$	0.41	0.08	0.24	0.05 - 2.52
Taurodihydroxycholanoates, $\mu\text{M}$	0.14	0.04	0.03	0.03 - 1.43
Taurocholate, $\mu\text{M}$	0.07	0.003	0.009	0.001 - 0.61
Dihydroxycholanoates, $\mu\text{M}$	0.03 ( $\downarrow$ )	0.53	0.18	0.09 - 2.21
Cholate, $\mu\text{M}$	0.003	0.09	0.03	0.003 - 0.19
VLCFA				
C26:0, $\mu\text{M}$	1.07	n.a.	n.a.	$\leq$ 1.30
C24:0, $\mu\text{M}$	34.45	n.a.	n.a.	$\leq$ 91.4
C22:0, $\mu\text{M}$	32.43	n.a.	n.a.	$\leq$ 96.3
C24:0/C22:0	1.06	n.a.	n.a.	$\leq$ 1.39
C26:0/C22:0	0.03	n.a.	n.a.	$\leq$ 0.023
Branched-chain fatty acids				
Phytanic acid, $\mu\text{M}$	1.40	n.a.	n.a.	$\leq$ 9.88
Pristanic acid, $\mu\text{M}$	0.14	n.a.	n.a.	$\leq$ 2.98
<u>Urine</u>				
Bile acid intermediates				
Tauro-DHCA ( $\mu\text{mol/mol creat}$ )	0.24 ( $\uparrow$ )	0	0	0
Tauro-THCA ( $\mu\text{mol/mol creat}$ )	66.19 ( $\uparrow$ )	0	0	0
Tauro-tetraOH-cholestanoic acids ( $\mu\text{mol/mol creat}$ )	371.1 ( $\uparrow$ )	0	0	0 - 0.77
Glyco-THCA ( $\mu\text{mol/mol creat}$ )	14.11 ( $\uparrow$ )	0.31	0.39	0 - 0.78
Glyco-tetraOH-cholestanoic acids ( $\mu\text{mol/mol creat}$ )	53.93 ( $\uparrow$ )	2.07	2.24	0 - 13.20
THCA ( $\mu\text{mol/mol creat}$ )	4.62 ( $\uparrow$ )	0	0	0 - 0.13

VLCFA = very long chain fatty acids; DHCA = 3 $\langle$ ,7 $\langle$ -dihydroxy-5 $\text{\textcircled{R}}$ -cholestanoic acid; THCA = 3 $\langle$ ,7 $\langle$ ,12 $\langle$ -trihydroxy-5 $\text{\textcircled{R}}$ -cholestanoic acid; n.a. = not assessed; ( $\uparrow$ ) = above the reference values; ( $\downarrow$ ) = below the reference values.

SUPPLEMENTARY INFORMATION



Supplementary Figure 1. Additional histological findings in the proband's liver. (A) Hepatocytes are pale and swollen with focal acinar transformation (black arrows). Scale bar, 50µm. (B) Low magnification of liver biopsy showing thin fibrous septa (black arrows) and some nodularity on Trichrome stain. Scale bar, 200µm.

Supplementary Table 1. Sequencing coverage and quality metrics for the proband's exome.

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Mean independent reads per targeted base	59.8
% of bases mapping to genome	91.14
% of targeted bases with $\geq 8$ independent reads	95.9
Mean error rate (%)	0.44

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Supplementary Table 2. Rare homozygous protein-altering variants detected in the proband.

Gene Symbol	Chr: position (hg19)	AA change	MetaSVM score (Prediction)	NHLBI	1000G	Turkish DB (894 subjects)	ExAC (overall)	ExAC (highest subfrequency)
ACOX2	3:58520203	Y69*	Truncating	0	0	0	0	0
CHDH	3:53851817	A591V	-1.051 (T)	0	0	0	0	0
LRRIQ1	12:85531684	I1422M	-0.927 (T)	0.007414	0.01	0.0006	0.00861	0.02184 (South Asian)
OSBPL6	2:179255872	R817W	-0.412 (T)	0	0	0	0	0
RASEF	9:85620405	R347W	-0.118 (T)	0	0	0	4.94E-05	0.0002312 (East Asian)

None of the above genes are OMIM-related genes; Chr, chromosome; AA, amino acid; NHLBI, National Heart, Lung, and Blood Institute Exome Sequencing Project database; 1000G, 1000 Genome database; DB, database; ExAC, Exome Aggregation Consortium database; MetaSVM scores missense variants on a scale from -2 to 3, with scores < 0 predicted to be tolerated (T) and scores > 0 predicted to be damaging (D).

Supplementary Table 3. Retention times, cone voltage, collision energy, precursor and product ions used in identification of bile acids detected in patients with peroxisomal disorders.

Bile acid <sup>1</sup>	Conjugation <sup>2</sup>	Retention	Cone	Collision	Precursor	Product ion
	[Abbreviation]	time (min)	voltage (V)	energy (V)	ion (m/z)	(m/z)
Dihydroxycholestanoic, DHCA (3 $\alpha$ ,7 $\alpha$ -diOH-5 $\beta$ -C27)	Tau [Tauro-DHCA]	1.85	116	59	540.0	80.0
Trihydroxycholestanoic, THCA (3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triOH-5 $\beta$ -C27)	Tau [Tauro-THCA]	1.91	116	59	556.3	80.0
Tetrahydroxycholestanoic, hydroxy-THCA (3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ ,13 $\alpha$ - tetraOH-5 $\beta$ -C27)	Tau [Tauro-tetraOH- cholestanoics]	1.5 - 1.8	116	59	572.0	80.0
Dihydroxycholestanoic, DHCA (3 $\alpha$ ,7 $\alpha$ -diOH-5 $\beta$ -C27)	Gly [Glyco-DHCA]	1.85	88	31	490.0	74.0
Trihydroxycholestanoic, THCA (3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triOH-5 $\beta$ -C27)	Gly [Glyco-THCA]	1.78	88	31	506.0	74.0
Tetrahydroxycholestanoic, hydroxy-THCA (3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ ,13 $\alpha$ - tetraOH-5 $\beta$ -C27)	Gly [Glyco-tetraOH- cholestanoics]	1.56	88	31	522.0	74.0
Dihydroxycholestanoic, DHCA (3 $\alpha$ ,7 $\alpha$ -diOH-5 $\beta$ -C27)	None [DHCA]	1.94	86	10	433.0	433.0
Trihydroxycholestanoic, THCA (3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triOH-5 $\beta$ -C27)	None [THCA]	1.86	86	10	449.3	449.3
D <sub>3</sub> -THCA	None [D <sub>3</sub> -THCA]	1.86	86	10	452.3	452.3

<sup>1</sup>5 $\beta$ -C27, 5 $\beta$ -cholestan-26-oic acid; <sup>2</sup>Tau, taurine; Gly, glycine.