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ORIGINAL ARTICLE

Beneficial effect of ursodeoxycholic acid in patients with acyl-CoA oxidase 2 (ACOX2) deficiency–associated hypertransaminasemia

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Abbreviations: ACOX, acyl-CoA oxidase; ADAH, ACOX2 deficiency-associated hypertransaminasemia; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; CHO, Chinese hamster ovary; DCA, deoxycholic acid; ER, endoplasmic reticulum; GCA, glycocholic acid; HPLC-MS/MS, high-performance liquid chromatography-mass spectrometry; MAF, minor allele frequency; NTCP, Na+-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; ROS, reactive oxygen species; RT-qPCR, reverse transcription followed by quantitative polymerase chain reaction; TCA, taurocholic acid; THCA, trihydroxycholestanoic acid; UDCA, ursodeoxycholic acid.

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Abstract

Background and Aims: A variant (p.Arg225Trp) of peroxisomal acyl-CoA oxidase 2 (ACOX2), involved in bile acid (BA) side-chain shortening, has been associated with unexplained persistent hypertransaminasemia and accumulation of C27-BAs, mainly 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA). We aimed to investigate the prevalence of ACOX2 deficiency-associated hypertransaminasemia (ADAH), its response to ursodeoxycholic acid (UDCA), elucidate its pathophysiological mechanism and identify other inborn errors that could cause this alteration.

Methods and Results: Among 33 patients with unexplained hypertransaminasemia from 11 hospitals and 13 of their relatives, seven individuals with abnormally high C27-BA levels (>50% of total BAs) were identified by high-performance liquid chromatography-mass spectrometry. The p.Arg225Trp variant was found in homozygosity (exon amplification/sequencing) in two patients and three family members. Two additional nonrelated patients were heterozygous carriers of different alleles: c.673C>T (p.Arg225Trp) and c.456_459del (p.Thr154fs). In patients with ADAH, impaired liver expression of ACOX2, but not ACOX3, was found (immunohistochemistry). Treatment with UDCA normalized aminotransferase levels. Incubation of HuH-7 hepatoma cells with THCA, which was efficiently taken up, but not through BA transporters, increased reactive oxygen species production (flow cytometry), endoplasmic reticulum stress biomarkers (*GRP78*, *CHOP*, and *XBP1-S/XBP1-U* ratio), and *BAXα* expression (reverse transcription followed by quantitative polymerase chain reaction and immunoblot), whereas cell viability was decreased (tetrazolium salt-based cell viability test). THCA-induced cell toxicity was higher than that of major C24-BAs and was not prevented by UDCA. Fourteen predicted *ACOX2* variants were generated (site-directed mutagenesis) and expressed in HuH-7 cells. Functional tests to determine their ability to metabolize THCA identified six with the potential to cause ADAH.

Conclusions: Dysfunctional ACOX2 has been found in several patients with unexplained hypertransaminasemia. This condition can be accurately identified by a noninvasive diagnostic strategy based on plasma BA profiling and *ACOX2* sequencing. Moreover, UDCA treatment can efficiently attenuate liver damage in these patients.

INTRODUCTION

Hypertransaminasemia is a common condition detected in primary care practice[1] whose most frequent etiologies in adults are nonalcoholic fatty liver disease, viral infections, consumption of toxic substances (mainly alcohol), autoimmune liver disease, celiac disease, iron overload, and Wilson disease.[2] Up to 15% of persistent hypertransaminasemia cases in adults are idiopathic.[3] Biopsy permits a diagnosis in most (87%) but not all cases.[4] Information on asymptomatic hypertransaminasemia in childhood is scarce, and identifying its cause is particularly difficult in pediatric patients due to the lack of accompanying clinical manifestations.[2] Thus, up to 13% of persistent hypertransaminasemia found in children remains cryptogenic.[5]

Acyl-CoA oxidase 2 (ACOX2) is a peroxisomal enzyme involved in the shortening of the cholesterol side-chain during bile acid (BA) biosynthesis. Two cases of ACOX2 deficiency were reported in 2016.[6,7] The first case, reported at the International Liver Congress-EASL (April 2016; Barcelona, Spain),[6] involved a 16-year-old man with unexplained hypertransaminasemia (2-5-fold upper normal limit) and no other associated

symptoms. In this individual, a homozygous variant (NM_003500.4:c.673C>T) in *ACOX2*, resulting in the amino acid change p.Arg225Trp, caused a decrease in enzymatic activity, together with reduced plasma levels of C24-BAs and the accumulation of C27-BAs.[8] The minor allele frequency (MAF) for c.673C>T in the Genome Aggregation Database (gnomAD v3.1), including more than 76,000 whole genomes, is 0.03%, which suggested that this is not a frequent variant found in the general population. However, data from the Medical Genome Project obtained recently in a Spanish cohort of healthy individuals (approximately 2000 genomes) showed an MAF of 0.7% for the c.673C>T allele,[9] suggesting that ACOX2 deficiency may not be an extremely rare condition. Indeed, in July 2016, a second case was reported by Vilarinho et al.,[7] which was followed by a third case identified 2 years later by Ferdinandusse et al.[10] Both children were born from consanguineous parents and died during childhood suffering from severe hepatic and neurological alterations. One of these patients reported carried a homozygous variant (c.207T>A) encoding truncated ACOX2 at codon 69 [NM_003500.4:c.207T>A (p.Tyr69Ter)],[7] whereas the other was homozygous for a four nucleotide deletion (NM_003500.4:c.461_464del), leading to a premature stop codon (p.Thr154fs).[10] Although elevated serum C27-BA levels were found, these patients did not present a significant reduction in C24-BA levels. Both cases showed a markedly higher severity in comparison with that caused by c.673C>T.[8]

BA accumulation can lead to severe hepatocellular damage, which is often accompanied by inflammatory processes.[11] We have previously shown that the most abundant C27-BAs in the plasma of these patients were 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and its conjugated derivatives, which induces oxidative stress and cell death in cells overexpressing the c.673C>T *ACOX2* variant.[8] Moreover, some BAs are capable of inducing endoplasmic reticulum (ER) stress,[12] releasing Ca^{2+} from the ER of hepatocytes, triggering Ca^{2+}-dependent apoptosis[12] and activating the generation of reactive oxygen species (ROS) by mitochondria,[13] resulting in cell death,[14] which plays a pivotal role in the hypercholanemia-induced hepatocellular damage observed in several liver diseases.

To elucidate the prevalence of ACOX2 deficiency-associated hypertransaminasemia (ADAH), we analyzed a group of 33 such patients from different hospitals. We have also explored the mechanisms underlying THCA-induced toxicity. Furthermore, because several variants that may affect ACOX2 function have been predicted,[15] we selected those with the highest likelihood of impairing ACOX2 function, whose ability to affect BA biosynthesis was then evaluated in vitro. Finally, and more importantly, we have evaluated the clinical response of patients with ADAH to ursodeoxycholic acid (UDCA) treatment.

MATERIALS AND METHODS

Reagents and cell lines

BA-related compounds were from Sigma-Aldrich (Merck, Madrid, Spain), except THCA and 7α-hydroxy-4-cholesten-3-one (C4), which were from Avanti Polar Lipids (Alabaster, AL). Cells used in this study were human embryonic kidney (HEK) 293T, HuH-7 (human hepatocellular carcinoma), HepG2 and HuH-6 (human hepatoblastoma), IHH (immortalized human hepatocytes),[16] and stably transfected Chinese hamster ovary (CHO),[17] whose origin and culture conditions, as well as the rest of detailed information regarding the materials and methods, including statistical analysis, is available in the Supporting Information.

Human samples

Plasma and white blood cells samples were collected between 2016 and 2022 in 10 hospitals in Spain and one in Germany (Figure S1). The research protocol conformed to ethical guidelines of the 1975 Declaration of Helsinki. The use of genetic information was approved by the Human Ethical Committees of the University of Salamanca, Marqués de Valdecilla University Hospital, and Vall d'Hebron University Hospital (Spain). Informed consent was obtained from patients and relatives entering this study.

Analytical methods

Plasma samples were collected after overnight fasting. BA[8,18,19] and C4[20,21] concentrations were measured by high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS). Plasma activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltranspeptidase and alkaline phosphatase, as well as total and direct bilirubin concentrations, were measured using standard clinical automatic analyzers.

Genetic analysis and gene expression

DNA was obtained from blood samples to amplify the coding sequence of *ACOX2* by high-fidelity polymerase chain reaction (PCR). The amplified fragments containing both the exons and the exon-intron boundaries from at least two reactions of PCR per exon were purified by agarose gel electrophoresis. Then, the amplicons were sequenced in both directions using forward and reverse primers as previously described.[8]

Total RNA extraction from cells, reverse transcription (RT), and quantitative PCR (qPCR) were performed

as previously reported[22] using the appropriate primers (Table S1). The results of mRNA abundance of target genes in each sample were normalized using hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) expression.

Histological assessment

Liver biopsies were processed routinely.[23] They were fixed with 10% neutral formalin and embedded in paraffin. Serial sections (4 μm) or mirror-image sections were stained with hematoxylin and eosin or Masson's trichrome (Figure S2). Histopathological findings and the fibrosis stage were graded by an expert pathologist using Knodell's and Scheuer's scores.

Immunohistochemistry, immunoblotting, and immunofluorescence

Immunostaining of ACOX2 and ACOX3 in 4-μm sections of paraffin-embedded liver biopsies was performed using Protein Atlas[24] validated antibodies anti-ACOX2 (HPA064845, Merck, Madrid) and anti-ACOX3 (HPA035840, Merck, Madrid), whose specificity was tested elsewhere.[10] Immunoblotting analyses of cell lysates were carried out with primary antibodies diluted in phosphate-buffered saline-Tween using blocking agents as appropriate (Table S2). glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a loading control.

For immunofluorescence studies, cells were fixed with paraformaldehyde and permeabilized with Triton X-100. After blocking with fetal bovine serum, cells were incubated for 1 h with anti-ACOX2 antibody (PA5-50297, Invitrogen, Thermo Fisher) and anticatalase antibody (LF-MA0004, Invitrogen, Thermo Fisher). Finally, samples were incubated with the appropriate secondary antibodies, mouse or rabbit anti-IgG conjugated with Alexa Fluor-488 or Alexa Fluor-594 (Invitrogen, Thermo Fisher) and 4,6-diamidino-2-phenylindole (Invitrogen, Thermo Fisher). Visualization of the labeling was performed under a confocal microscope (TCS SP5, Leica, Barcelona).

Functional study of ACOX2 variants

The *ACOX2* open reading frame (ORF) was amplified from human liver RNA and cloned into the pGEM-T Easy vector (Promega, Madrid), which was used to generate vectors containing different *ACOX2* variants by site-directed mutagenesis (Table S3). The mutant ORF was then transferred to lentiviral vectors containing a V5-tag.[8] Recombinant lentiviruses were produced in host HEK293T cells[25] and viral titers were determined by analyzing Enhnaced Green Fluorescent Potein (EGFP)-positive cells in a FACSCalibur flow cytometer (BD Biosciences, Madrid).[8] HuH-7 hepatoma target cells were transduced with lentiviral vectors. Overexpression of ACOX2 variants was assessed by RT-qPCR and immunoblotting. Double limiting-dilution was performed to obtain monoclonal populations, which were selected according to ACOX2 mRNA and protein expression, as determined by RT-qPCR, immunoblot, immunofluorescence, and BA metabolism studies. ACOX2 activity was assessed by studying the conversion of THCA into cholic acid (CA) in HuH-7 cells expressing each variant. Cells were incubated with 2 μM THCA for 72 h. Biotransformation of THCA into CA was measured by analyzing them in cells and culture medium.[8]

Toxicity and cell stress studies

Cell viability was determined by the tetrazolium salt-based cell viability test using thiazolyl blue tetrazolium bromide (Sigma-Aldrich, Merck). Oxidative stress was determined by flow cytometry using 2′,7′-dichlorofluorescein diacetate (Sigma-Aldrich, Merck). ER stress and apoptosis were evaluated by analyzing atypical *XBP1* splicing by RT-qPCR by combining specific primers (Table S1) for total (*XBP1*-total), short (*XBP1*-S) and long (*XBP1*-U) mRNA. Up-regulation of genes involved in ER stress (*CHOP* and *GRP78*) and apoptosis activation (*BAX* and *BCL2*) was determined by RT-qPCR (Table S1) and/or immunoblot (Table S2) after incubation with assayed agents for 24 h. Thapsigargin (2 μM) and sorafenib (5 μM) were used as a positive controls of ER stress and apoptosis activation, respectively.

In silico prediction of dysfunctional ACOX2 variants

The gnomAD v3.1 (exomes) (https://gnomad.broadinstitute.org) was searched to identify *ACOX2* variants described in the population that might result in a dysfunctional protein. Inclusion criteria for the study of *ACOX2* genetic variants were (i) single nucleotide polymorphism; (ii) MAF <1%; and (iii) generation of a missense variant. The selected variants were classified according to the prediction of the effect of each variant in ACOX2 activity, using SIFT and PolyPhen-2 algorithms. The predicted functional impact score was calculated as follows: SIFT and PolyPhen-2 predictions of the effect of each variant in ACOX2 activity was given values from 0 to 2, depending on their predicted effect by each algorithm (null = 0, probable = 1, or highly probable = 2). The sum of both scores resulted in a scale from 0 to 4 of which 0 indicated the variant was

most likely benign and 4 indicated a high probability of deleterious effects.

Transport assays

BA uptake by HuH-7 cells and transporter-mediated uptake by CHO cells was determined as previously described.[26] HuH-7 and CHO cells, either wild-type or stably expressing Na+-taurocholate cotransporting polypepetide (NTCP), organic anion transporting polypeptide-1B1 (OATP1B1), or OATP1B3,[17,27–29] were incubated with uptake medium containing 50 μM THCA, glycocholic acid (GCA), or CA with or without 250 μM taurocholic acid (TCA) for 60 min. Uptake was stopped by rinsing the wells twice with cold uptake medium and twice with cold PBS. Cells were lysed with ultra-pure water containing 5 μM of taurochenodeoxycholic acid (TCDA; internal standard), and BA concentrations were determined by HPLC-MS/MS as described above.

RESULTS

Patients with idiopathic hypertransaminasemia

Thirty-three patients with persistent hypertransaminasemia of unknown origin entered this study after ruling out common causes of elevated serum aminotransferases (viral hepatitis, alcoholic or metabolic liver damage, autoimmune hepatitis, hemochromatosis, Wilson's disease, alpha 1 antitrypsin deficiency, and obstructive cholestasis). Fourteen of these subjects showed hypercholanemia (≥10 μM), suggesting some degree of cholestasis. Among the rest of normocholanemic individuals, a C27-BAs/C24-BAs ratio >1 was found in four subjects, who were considered as potential ADAH cases. Thirteen relatives from these patients were also analyzed. Among them, three individuals had enhanced C27-BAs levels together with genetic data consistent with dysfunctional ACOX2 (Figure 1). Plasma BA profiles of the individuals with confirmed ADAH are shown in Table 1. No significant alteration was detected in some cases in which serum C4 levels were measured (Table 1).

Case 1

A 17-year-old boy was first seen in 2010 in the Hepatology Unit of Marqués de Valdecilla University Hospital (Santander, Spain) due to fatigue symptoms and unexplained hypertransaminasemia (Table S4). Abdominal ultrasound showed normal liver and biliary tract. A liver biopsy (2010) revealed the presence of a single portal fibrous enlargement with focal porto-portal bridging fibrosis and minimal portal inflammatory component, which suggested a preliminary diagnosis of seronegative autoimmune hepatitis. Accordingly, the patient received immunosuppressive drugs for 10 years, but biochemical remission was not achieved. Lack of response and persistent hypertransaminasemia during this time prompted new clinical evaluation. Again, all common causes of liver and biliary tract disorder were ruled out. A new liver biopsy (2019) showed no additional information (Figure S2). Finally, as the first episode of hypertransaminasemia had been recorded after treating a testicular torsion with nonsteroidal anti-inflammatory drugs, which resembled the first case of ADAH previously described,[6,8] following an empiric approach, UDCA therapy was started. After 3 weeks, plasma aminotransferases reverted to normal (Table S4), and fatigue symptoms improved. Further analysis revealed that the patient showed an increased proportion of C27-BAs (Figure 1, Table 1) and the presence in homozygosis of the previously described[8] *ACOX2* variant c.673C>T (p.Arg225Trp) (Figure 1). The study of his family revealed that his brother, mother, and uncle carried this variant in homozygosis and accumulated C27-BAs in serum (Figure 1, Table 1). Interestingly, all had undergone a cholecystectomy because of complicated cholelithiasis. Biochemical analysis of the uncle revealed hypertransaminasemia (ALT/AST: 131/68 U/l), which led his physician to prescribe him UDCA therapy. This restored his aminotransferase levels to normal values after 3 weeks (ALT/AST: 27/19 U/l). Serum aminotransferase levels were still low after 6 months of treatment (ALT/AST: 17/20 U/l), even though the abnormal proportion of C27-BAs was not corrected (Figure 1, Table 1). In contrast, the father, who was heterozygous for the same variant, and the aunt, who did not carry any *ACOX2* variant, were negative for C27-BA accumulation (Figure 1, Table S8).

Case 2

A 17-year-old boy with persistent and unexplained hypertransaminasemia was first seen in the Hepatology Unit of Marqués de Valdecilla University Hospital in 2020. He had previously been diagnosed with growth hormone deficiency, treated with somatropin, and followed up by his pediatrician in another center. Hypertransaminasemia had been recorded since his first year of life (Table S5). Usual and unusual causes of persisting hypertransaminasemia were discarded, including lysosomal acid lipase deficiency. Abdominal ultrasound and MRCP showed no relevant findings. Liver biopsy showed no remarkable signs: slight sinusoidal dilatation and minimal central vein fibrosis (Figure S2). The analysis of plasma BAs revealed an increased proportion of C27-BAs (Figure 1, Table 1). The genetic studies showed that he was homozygous for the *ACOX2* variant c.673C>T (p.Arg225Trp) (Figure 1). UDCA treatment was initiated, achieving normalization of aminotransferases (Table S5) and

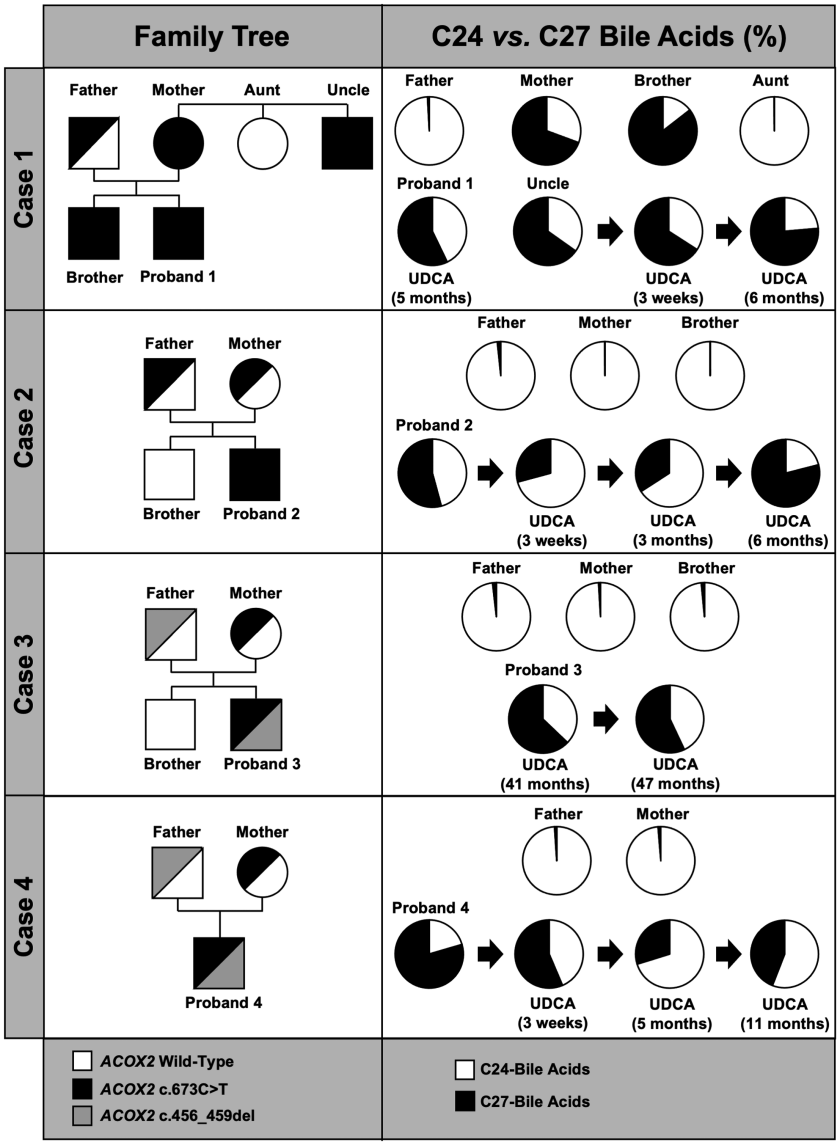

FIGURE 1 Genealogical tree of families with members showing acyl-CoA oxidase (ACOX) 2 deficiency-associated hypertransaminasemia (ADAH) (left panels) and the proportion of plasma concentrations of endogenous bile acids (BAs) with short side-chain (C24-BAs) and immature BAs with unshorten side-chain (C27-BAs) (right panels). In individuals treated with ursodeoxycholic acid (UDCA; 12 or 15 mg/kg/day), this BA and its conjugates were not considered for calculating plasma endogenous C24-BA concentrations.

reduction in the proportion of C27-BAs (Figure 1) after 3 weeks. Nevertheless, the follow-up for 6 months of treatment revealed abnormal abundance of serum C27-BAs (Figure 1, Table 1), despite the correction of serum aminotransferase levels was maintained (Table S5). The analysis of his family showed that his younger brother was homozygous for wild-type *ACOX2*, whereas the parents were heterozygotic carriers for c.673C>T (p.Arg225Trp) (Figure 1). The plasma BA profiles in the family members analyzed were normal. C24-BA plasma levels were within the normal range in the mother and brother, whereas the father presented hypercholanemia (22 μM total BAs) with general enhanced levels of all BA molecular species but without an elevated proportion of C27-BAs *versus* C24-BAs (Figure 1; Table S9).

Case 3

A 15-year-old boy in follow-up at Santa Creu i Sant Pau Hospital (Barcelona, Spain) since the first years of life due to abdominal distension, steatorrhea, and hypertransaminasemia was first seen in the Pediatric Hepatology and Liver Transplantation Unit from Vall d'Hebron Hospital (Barcelona, Spain) in 2017. All clinical studies performed were negative, including the analysis of classic inborn errors of BA biosynthesis. An abdominal ultrasound revealed discrete hepatic hyperechogenicity, and a liver biopsy showed slight fibrosis (Figure S2). UDCA was empirically administered before ADAH was diagnosed. The treatment resulted in the normalization of plasma aminotransferases (Table S6) and symptoms relief. Then, BA profiling (Table 1) and

TABLE 1 Bile acid species in serum collected from patients with acyl-CoA oxidase (ACOX) 2 deficiency–associated hypertransaminasemia (ADAH)

	Controls	Case 1	Uncle case 1			Case 2				Case 3		Case 4			
UDCA treatment (months)	**None**	**5**	**None**	**0.75**	**6**	**None**	**0.75**	**3**	**6**	**41**	**47**	**None**	**0.75**	**5**	**11**
7α-OH-4-cholesten-3-one (C4) (nM)	17 ± 3	16	12	11	38	9	16	6	11	ND	ND	ND	ND	ND	ND
Endogenous C24-bile acids (nM)	2290 ± 500	980	610	1137	1895	620	13,545	295	1336	960	600	703	1454	656	649
Endogenous C27-bile acids (nM)	20 ± 3	1304	1334	2201	6122	791	5581	153	5014	2315	795	3245	2092	307	512
C27-bile acids (%)	0.9 ± 0.1	57.1	68.6	65.9	76.4	56.1	29.2	34.1	79.0	70.7	57.0	82.2	59.0	31.9	44.1
C24-bile acid species (nM)															
Glyco-cholic	117 ± 40	43	66	152	615	18	607	13	227	163	30	102	134	27	15
Glyco-chenodeoxycholic	421 ± 90	256	201	662	1018	40	2289	84	676	461	129	284	664	460	67
Glyco-deoxycholic	180 ± 44	27	2	74	31	11	450	19	144	5	8	101	71	2	33
Glyco-lithocholic	9 ± 2	4	<1	46	7	2	4	22	14	1	5	1	6	1	18
Glyco-ursodeoxycholic	51 ± 17	5287*	6	7998*	4232*	16	13,065*	1667*	6264*	9949*	2524*	107	9281*	3305*	1477*
Tauro-cholic	18 ± 7	1	5	11	57	<1	24	<1	21	1	5	14	11	1	4
Tauro-chenodeoxycholic	53 ± 11	14	22	50	88	4	60	2	75	44	12	57	83	29	7
Tauro-deoxycholic	23 ± 6	3	1	9	7	1	6	1	13	2	1	26	8	1	2
Tauro-lithocholic	1 ± 1	<1	<1	3	2	<1	<1	<1	2	<1	<1	<1	1	<1	1
Tauro-ursodeoxycholic	2 ± 1	97*	<1	117*	201*	<1	189*	15*	230*	235*	63*	**7**	263*	64*	33*
Tauro-sulfo-lithocholic	103 ± 15	3	4	78	3	8	18	10	72	31	205	14	11	1	189
Cholic	403 ± 92	122	276	5	47	504	6546	63	27	93	56	6	33	18	153
Chenodeoxycholic	330 ± 180	445	14	20	11	6	2755	32	19	140	119	41	311	110	46
Deoxycholic	417 ± 124	59	4	13	5	7	781	34	29	8	16	45	114	4	67
Lithocholic	93 ± 27	1	1	14	4	1	5	17	16	1	13	9	10	1	48
Ursodeoxycholic	65 ± 21	6737*	2	7645*	96*	3	105,873*	1276*	223*	3197*	2725*	23	2812*	2555*	1296*
C27-bile acid species (nM)															
Glyco-trihydroxycholestanoic	<1	118	156	189	763	38	1133	24	521	290	59	153	167	27	54
Glyco-dihydroxycholestanoic	<1	15	39	127	117	19	79	8	61	30	7	18	33	<1	3
Tauro-trihydroxycholestanoic	16 ± 3	706	902	1348	4492	553	3199	71	3443	1575	597	2125	1432	194	291
Tauro-dihydroxycholestanoic	<1	87	204	472	708	164	364	14	959	333	77	936	438	69	118
Trihydroxycholestanoic	4 ± 1	379	32	63	42	17	806	36	30	87	55	14	21	16	46

Note: Control samples were collected from 18 healthy individuals.

UDCA-associated species were marked with an asterisk (*) when the patient was treated with UDCA (12 or 15 mg/kg/day).

Abbreviations: ND, not determined; UDCA, ursodeoxycholic acid.

directed genetic studies were performed. The patient showed an increased proportion of plasma C27-BAs (Figure 1) and two heterozygous variants, presumably on two different alleles of *ACOX2*, the c.673C>T variant and a deletion of 4 nucleotides (c.456_459del) (Figure 1). This was predicted to generate the same protein change (p.Thr154fs) that had been previously described for a similar variant identified in *ACOX2* by Ferdinandusse et al.[10] Liver function tests and analysis of BA profile after another 6 months of UDCA treatment revealed maintained normalization of aminotransferases besides a persistently altered BA profile (Figure 1, Table 1, and Table S6). The analysis of the proband's family showed that the younger brother was homozygous for wild-type *ACOX2*, whereas the father was heterozygous for c.456_459del (p.Thr154fs) and the mother showed the c.673C>T (p.Arg225Trp) variant in heterozygosis (Figure 1). Their plasma BA profiles were normal (Figure 1, Table S10), with C27-BAs and C24-BAs within normal ranges.

Case 4

A 7-year-old boy presented a history of congenital heart disease (mild mitral stenosis, and moderate-severe aortic insufficiency) with slight developmental delay and oscillating hypertransaminasemia (Table S7). He was referred to the Pediatric Hepatology and Liver Transplantation Unit from Vall d'Hebron Hospital, where common causes of liver damage were ruled out. Abdominal ultrasound showed slight hepatic hyperechogenicity. Liver biopsy presented subtle polygonal morphology of the hepatocytes with no other remarkable findings (Figure S2). Due to the multiorgan affectation, a complete exome sequencing study was indicated. This showed the same double heterozygous variant of *ACOX2* as in Case 3; i.e., variants c.673C>T (p.Arg225Trp) and c.456_459del (p.Thr154fs) (Figure 1). Plasma BA profiling (Table 1) confirmed enhanced C27-BA proportion (Figure 1). Diagnosis of ADAH prompted UDCA treatment, resulting in the normalization of aminotransferase levels (Table S7) and a partial reduction in the proportion of C27-BAs in plasma (Figure 1, Table 1). The analysis of the proband's family members showed that the father carried the c.456_459del (p.Thr154fs) variant in heterozygosis and the mother presented the c.673C>T (p.Arg225Trp) variant in heterozygosis (Figure 1). In both parents, plasma C27-BA and C24-BA levels were within normal ranges (Figure 1; Table S11).

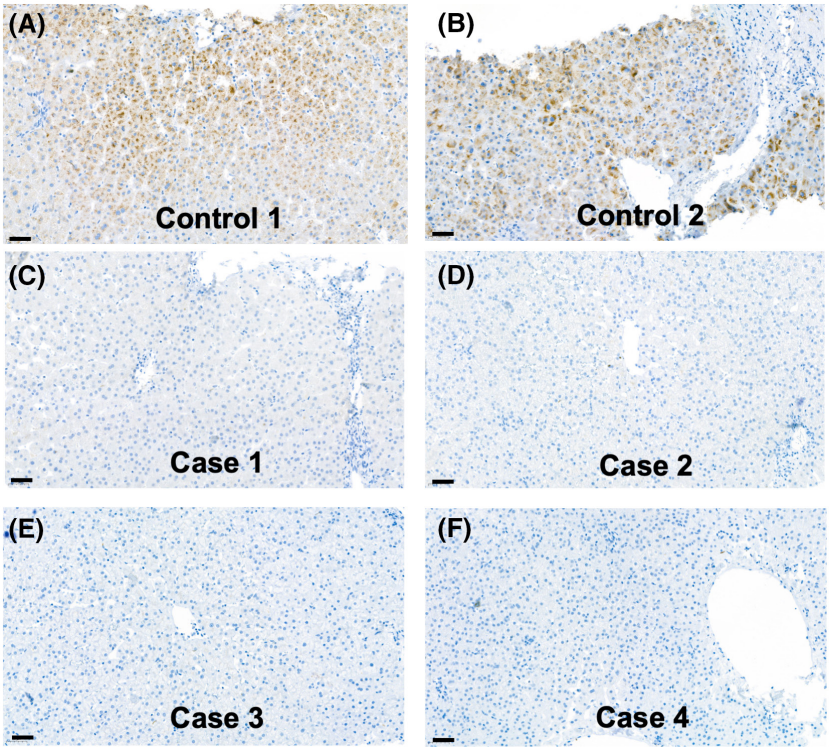

FIGURE 2 Immunohistochemistry analysis of acyl-CoA oxidase (ACOX) 2 expression in liver biopsies collected from patients with ACOX2 deficiency–associated hypertransaminasemia (ADAH). Two samples from patients with hypertransaminasemia but not ADAH, namely, NAFLD F1 and alcoholic cirrhosis used as Control 1 (A) and Control 2 (B), respectively. When expressed, ACOX2 was detected as intense granular staining inside the hepatocytes. Negative staining for ACOX2 was seen in liver biopsies from probands of Case 1 (C), Case 2 (D), Case 3 (E), and Case 4 (F). All images were obtained at 20× magnification. Scale bar indicates 50 μm.

Immunohistochemistry studies

Immunohistochemistry of liver biopsies showed a complete absence of ACOX2 staining in patients with ADAH (Figure 2). In contrast, staining was positive in a series of biopsy samples collected from adult patients with different liver diseases (data not shown), two of which have been included in Figure 2 as controls. In contrast, immunohistochemistry for ACOX3, another acyl-CoA oxidase that does not metabolize C27-BA,[10] showed positive signal (with different intensity) in hepatocytes in all cases (Figure S3).

C27-BA–induced toxicity

To analyze the cytotoxic effects of THCA, the main unconjugated C27-BA synthesized in ADAH cases, we first analyzed the ability of THCA to enter liver cells. CA and GCA uptake by HuH-7 cells was inhibited by TCA, which was also taken up by these cells. THCA uptake was higher and not affected by TCA (Figure 3A). CA, GCA, and TCA uptake by CHO cells was markedly enhanced by NTCP expression (Figure 3B) but very moderately by OATP1B1 (Figure 3C) and OATP1B3 (Figure 3D) expression. In all circumstances, a substantial THCA uptake was found, which was only slightly increased by NTCP expression and was not inhibited by TCA; THCA ability to induce oxidative stress was tested in liver cells. THCA-induced ROS production was higher than that induced by C24-BAs, such as CA, chenodeoxycholic acid (CDCA), and deoxycholic acid (DCA) in HuH-7 and HuH-6 cells, whereas HepG2 and IHH cells showed resistance to BA-induced oxidative stress (Figures 4A–D).

To study the effect on ER stress, cells sensitive (HuH-7) and resistant (HepG2) to BA-induced oxidative stress were selected. THCA induced a strong effect, which in most tests was stronger than that caused by C24-BAs (Figure 4E–K).

The reduction in viability of HepG2 and HuH-7 cells after incubation with THCA was concentration-dependent in both cell lines, with HepG2 being less sensitive than HuH-7 to THCA-induced cell death (Figure 4L,M). To confirm the link between THCA-toxicity and the presence of the p.Arg225Trp variant in ACOX2, monoclonal HuH-7 cell sublines overexpressing wild-type ACOX2 (ACOX2-WT) or the p.Arg225Trp mutated variant (ACOX2-V1) were generated (Figure 5). ACOX2-WT overexpression partly protected cells from THCA-induced cytotoxicity, whereas this C27-BA caused a similar reduction in cell viability in control (Mock) and ACOX2-V1 cells (Figure 4N). THCA-induced oxidative stress was not prevented by coincubation with UDCA (Figure 4O). Moreover, THCA markedly upregulated *BAX* mRNA, which was not reduced by UDCA (Figure 4P), whereas *BCL2* was scarcely expressed by HuH-7 cells (Figure 4Q). Consistently, THCA-induced cell death was not inhibited by UDCA (Figure 4R).

Potentially harmful ACOX2 variants

The search for variants that could cause ADAH, carried out in the databases, led to the selection of 46 variants, of which most (32 variants) had an extremely low MAF. Hence, a maximum contribution of 1.56% of all predicted cases of dysfunctional ACOX2 could be expected. Accordingly, this set was discarded from the study and attention was focused on the functional analysis of the remaining 14 variants (Table 2). This specific set included the c.673C>T (p.Arg225Trp) variant initially identified (ACOX2-V1). According to SIFT and PolyPhen-2 predictions, only 11 of these variants are expected to have a functional impact on ACOX2 enzymatic activity. These predictions were further validated by measuring the ability of the variants, when expressed in HuH-7 cells (Figure 5A,B), to enhance THCA biotransformation into CA. Monoclonal HuH-7 cells stably overexpressing ACOX2-WT or ACOX2-V1 were used as positive and negative controls, respectively (Figure 5C,D). The activity of variants V11 and V14 was similar to that of ACOX2-WT, suggesting that these variants do not have a functional impact on enzyme activity, as was predicted by in silico analysis. In contrast, variants V5 and V8 showed a moderately decreased activity, whereas variants V2, V4, V6, and V10 were significantly less functional (Figure 5E).

DISCUSSION

This study identified seven individuals from four unrelated families carrying *ACOX2* variants involved in ADAH. Among them, five individuals showed persistent oscillating hypertransaminasemia. Three patients were homozygous carriers of c.673C>T (p.Arg225Trp), the same variant found in the first ADAH case reported,[8] whereas the other two carried heterozygous c.673C>T and c.456_459del *ACOX2* variants in different alleles. Interestingly, the latter generates p.Thr154fs, which results in a premature stop codon and a truncated protein. This change is also caused by another similar deletion (c.461_464del) in *ACOX2* mRNA described.[10] In contrast to our patients, the subject of that study carried the deletion in homozygosity and presented a more severe condition, with neurological symptoms and affection of lung, liver, heart, and muscle functions, and only survived 6 months.[10] The presence of other congenital alterations responsible for the multiorgan dysfunction of this case was not ruled out. The clinical conditions of the six patients with ADAH we have described so far

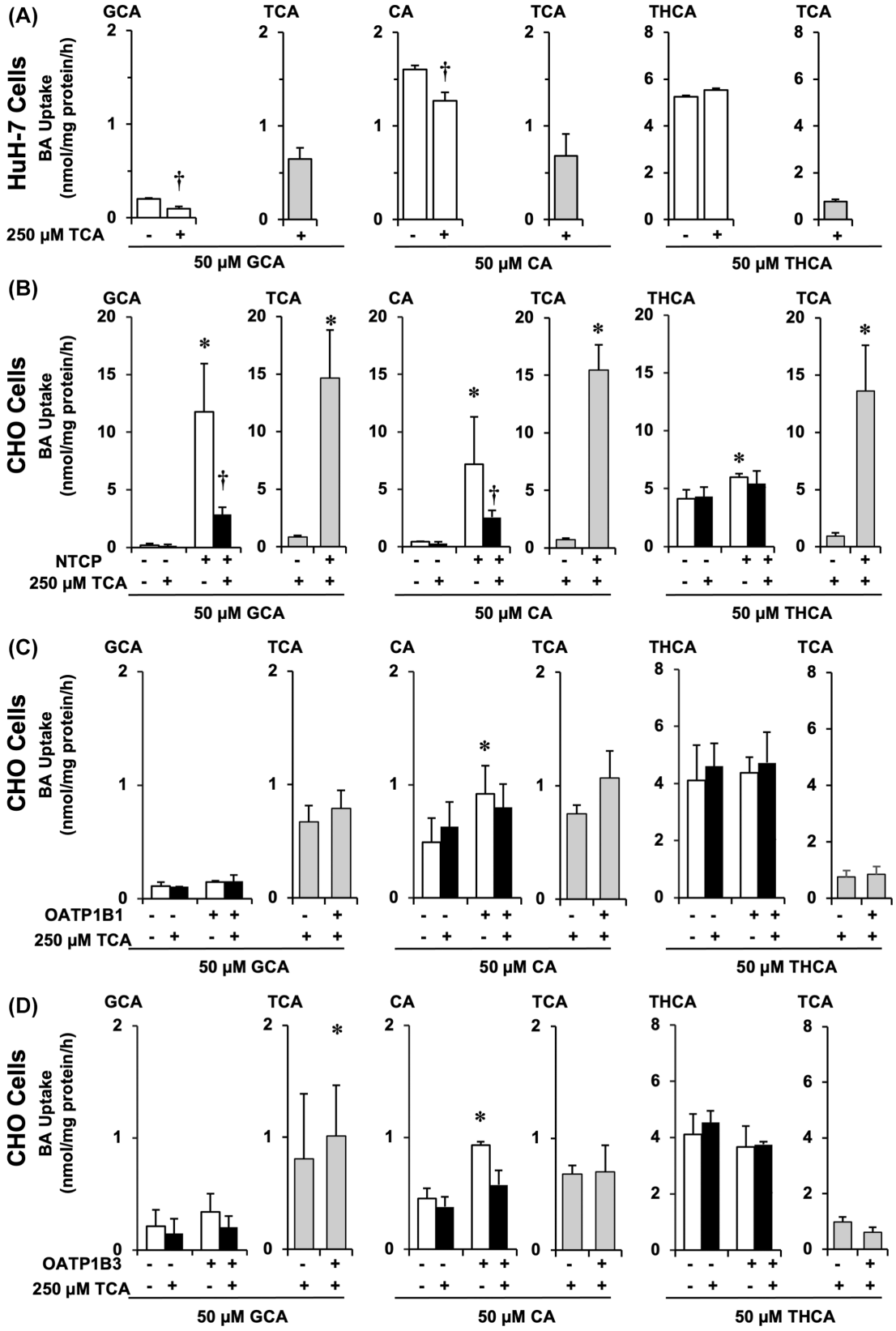

FIGURE 3 Uptake of glycocholic (GCA), taurocholic acid (TCA), cholic (CA), and trihydroxycholestanoic (THCA) acid by wild-type HuH-7 hepatoma cells (A) and Chinese hamster ovary (CHO) cells (Control) or CHO cells stably expressing NTCP (B), OATP1B1 (C), or OATP1B3 (D). Cells were incubated with 50 µM GCA, CA, or THCA, without or with 250 µM TCA for 1 h. Bile acid (BA) concentrations in cell lysates were determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS). Values are mean ± SD from eight measurements per condition, carried out in four different cultures. *$p < 0.05$ by comparing with and without transporter expression; †$p < 0.05$ by comparing uptake in the presence and the absence of TCA, using paired *t* test.

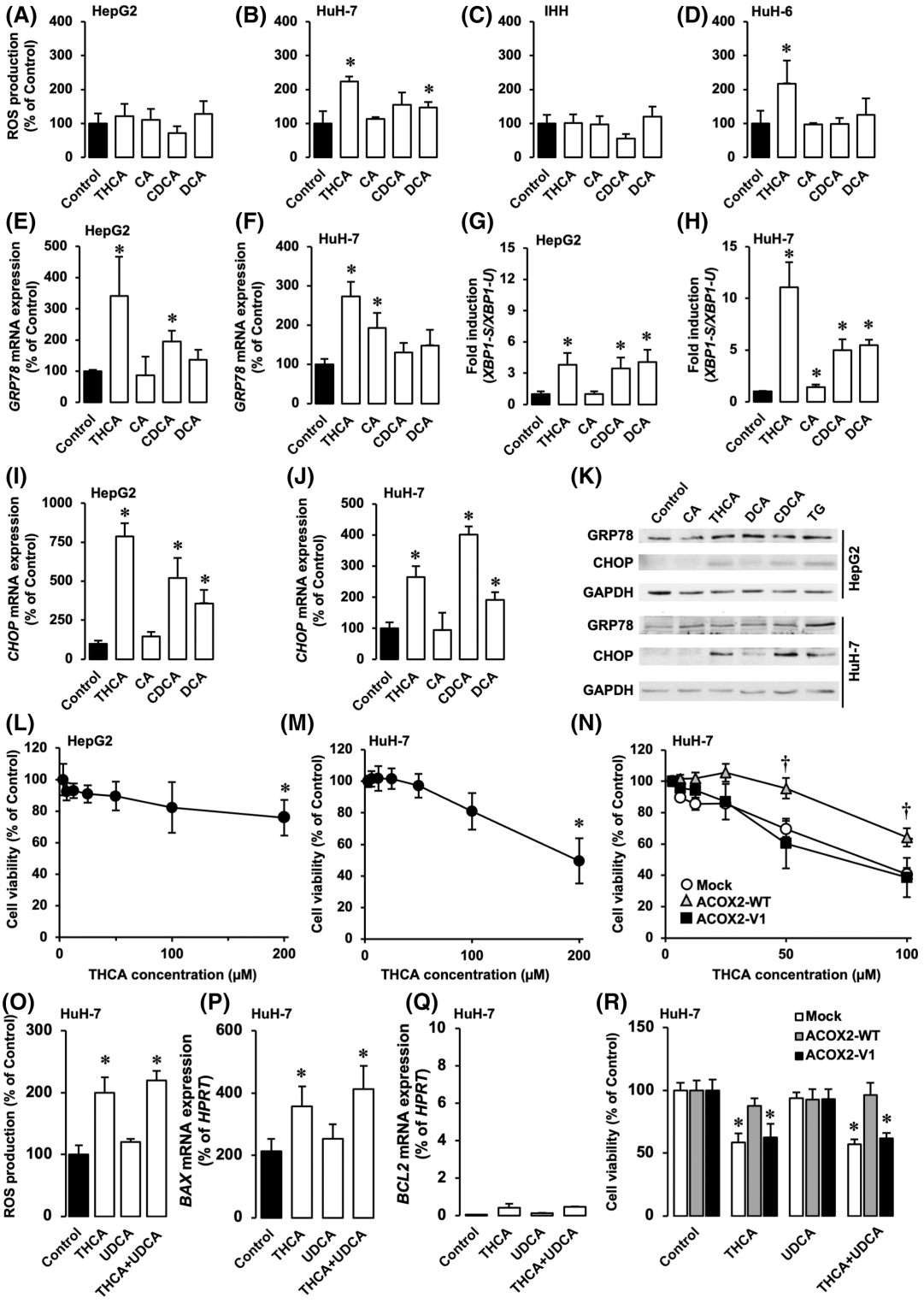

FIGURE 4 Effect of trihydroxycholestanoic (THCA), cholic (CA), chenodeoxycholic (CDCA), and deoxycholic (DCA) acids on the generation of reactive oxygen species (ROS) in HepG2 (A), HuH-7 (B), IHH (C), and HuH-6 (D) cells. Effect of THCA, CA, CDCA and DCA on the expression (mRNA) of endoplasmic reticulum (ER) stress markers: *GRP78* (E,F); *XBP1* (unspliced form *XBP1-U* and spliced form *XBP1-S*) (G,H); and *CHOP* (I,J). Changes in the expression of GRP78 and CHOP were confirmed by immunoblot (K). ER stress induced by 2 μM thapsigargin (TG) was used as a positive control. Effect of THCA on the viability of HepG2 (L), and HuH-7 (M) cells. Effect of THCA on the viability of monoclonal HuH-7 cells overexpressing wild-type acyl-CoA oxidase (ACOX) 2 (ACOX2-WT), the p.Arg225Trp variant of ACOX2 (ACOX2-V1) or transduced with empty lentiviral vectors (Mock) (N). Effect of ursodeoxycholic acid (UDCA) on THCA-induced oxidative stress (O), *BAX* (P), and *BCL2* (Q) mRNA levels in wild-type HuH-7 cells and on cell viability reduction (R) in HuH-7 without (Mock) or with stable expression of ACOX2-WT or ACOX2-V1. Values (mean ± SD from 6 to 12 determinations performed in 3–6 different cultures) are expressed as the percentage of data determined in cells treated with the vehicle alone (Control). *$p < 0.05$, compared to Control; †$p < 0.05$, comparing to Mock by paired *t* test.

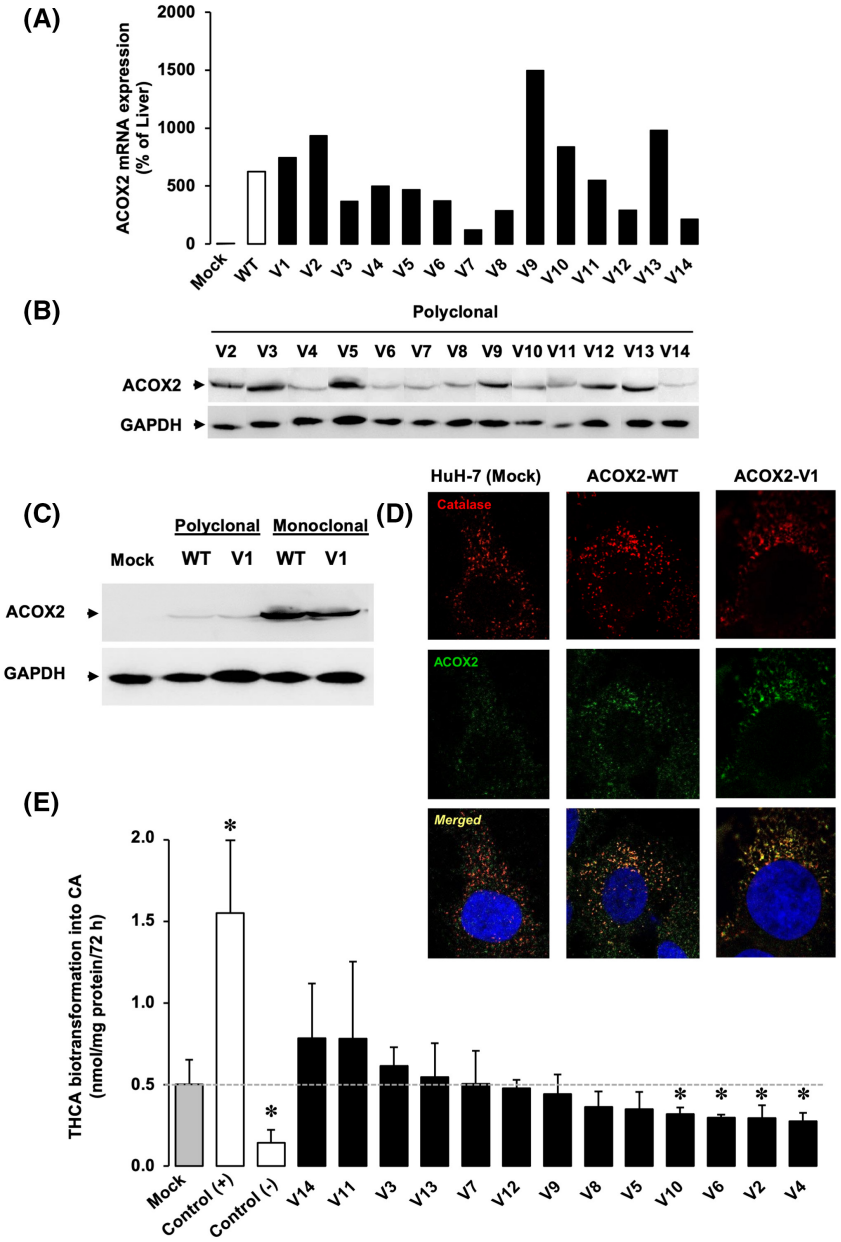

FIGURE 5 Acyl-CoA oxidase (ACOX) 2 mRNA expression determined by reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) in HuH-7 control cells transduced with empty lentiviral vector (Mock) and different polyclonal subpopulations overexpressing wild-type (WT) or ACOX2 variants (V1-V14) (A). ACOX2 protein expression determined by Western-Blot using anti-V5 antibody in lysates from Mock cells and polyclonal cells overexpressing ACOX2 variants from V2 to V14 (B), and monoclonal cells overexpressing WT or V1 variant. GAPDH was used as loading control (C). Representative immunofluorescence images of HuH-7 cells without (Mock) or with overexpression of ACOX2 WT or V1 variant, showing the subcellular localization of peroxisomal catalase (red), ACOX2 (green, using an anti-ACOX2 antibody) and its colocalization (merged, yellow). Nuclei were labeled with 4,6-diamidino-2-phenylindole (blue) (D). Change in the ability of HuH-7 cells expressing *ACOX2* variants to biotransform trihydroxycholestanoic acid (THCA) into cholic acid (CA) (E). Six weeks after transduction, the cells were incubated with 2 μM THCA for 72 h. CA concentrations in the culture medium plus cell lysate were determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS). Monoclonal sublines stably expressing ACOX2 WT or V1 were used as positive (+) and negative (-) controls, respectively. Values are mean ± SD from at least six determinations performed in three different cultures. *$p < 0.05$, compared with control (Mock) using the Bonferroni multiple comparison test.

markedly differ from that case, showing severe symptoms unrelated to liver function only in Case 4. Besides hypertransaminasemia, no symptoms were observed in several of our patients, as in the first reported case of ADAH.[8] Other defects in the peroxisomal pathway of BA biosynthesis also show great interindividual variability, as in the case of alpha-methylacyl-CoA racemase (AMACR)[30] and bile acid-CoA:amino acid N-acyltransferase (BAAT)[31] deficiencies.

As a standard feature, all individuals with ADAH showed an increased proportion of C27-BAs (56%–86% of total plasma BAs) (Figure 1, Table 1). Interestingly,

TABLE 2 Definition, nomenclature, and prediction of the functional consequence of the most frequent missense mutations in the ORF of acyl-CoA oxidase 2 (*ACOX2*) mRNA

Name	MAF	Alleles	ID	Nucleotide change	Amino acid change	Functional impact
V1	A = 0.030%	G, A	rs150832314	673C>T	Arg225Trp	4
V2	A = 0.054%	G, A	rs143133121	1840C>T	Arg614Cys	3
V3	T = 0.010%	C, T	rs529617348	674G>A	Arg225Gln	3
V4	G = 0.011%	C, G	rs142358437	1216G>C	Glu406Gln	4
V5	C = 0.001%	T, C	rs578023477	790A>G	Arg264Gly	4
V6	G = 0.301%	A, G	rs114465932	713T>C	Ile238Thr	2
V7	T = 0.035%	C, T	rs112279882	946G>A	Ala316Thr	3
V8	A = 0.026%	G, A	rs151184272	1720C>T	Arg574Cys	2
V9	T = 0.032%	C, T	rs143508132	850G>A	Gly284Ser	2
V10	A = 0.0004%	C, A	rs143508132	850G>T	Gly284Cys	2
V11	A = 0.010%	T, A	rs550491341	1087A>T	Ser363Cys	1
V12	C = 0.058%	G, C	rs143228600	1582C>G	Gln528Glu	0
V13	T = 0.002%	C, T	rs191136907	1984G>A	Glu662Lys	0
V14	T = 0.024%	C, T	rs149888394	841G>A	Val281Ile	0

Note: Data, updated in September 2021, were obtained from the Genome Aggregation Database (gnomAD v3.1, Exomes). The nucleotide position refers to the open reading frame of the wild-type sequence NM_003500 and the amino acid position is based on the protein NP_003491. The predicted functional impact score was calculated as follows: SIFT and PolyPhen-2 predictions of the effect of each variant in ACOX2 activity was given values from 0 to 2, depending on their predicted effect by each algorithm (null = 0, probable = 1, or highly probable = 2). The sum of both scores resulted in a scale from 0 to 4 in which 0 indicates that the variant is probably benign, whereas 4 indicates a high probability of deleterious effects.

Abbreviations: MAF, minor allele frequency; V, variant.

C24-BA biosynthesis was reduced but not completely abolished (Table 1, Tables S8–S11). This finding points to a certain remnant ACOX2 activity or the existence of a yet unknown mechanism capable of overcoming ACOX2 deficiency, the only recognized enzyme able to metabolize C27-BAs.[7,8,10] In this sense, Shefer et al. previously proposed, in 1976,[32] an alternative pathway in both humans and rats for BA β-oxidation, named the 25-hydroxylation pathway, that does not require the participation of peroxisomes.

UDCA has been used for the treatment of several cholestatic liver diseases[33] due to its multiple beneficial effects, which include strong choleretic activity and protection of hepatocytes and cholangiocytes from the toxic effects of more hydrophobic BA species. This has been suggested to occur owing to its antioxidant, anti-apoptotic, and immunomodulatory properties,[34] which has justified its use as an anti-inflammatory drug in treating several liver diseases.[35]

Here, we report the positive effects of UDCA in the treatment of patients with ADAH, reverting aminotransferases to normal levels and ameliorating accompanying symptoms, after 3 weeks of UDCA treatment, despite the lack of correction in the abnormal proportion of C27-BAs (Figure 1). Results from our *in vitro* studies suggest that UDCA-mediated prevention of harmful THCA-induced effects does not mainly occur at hepatocellular level but rather owing to mechanisms acting at the organ (e.g., hepatobiliary washing associated with marked UDCA-induced stimulation of bile formation) or systemic (e.g., change in BA pool composition, which is enriched in UDCA-related species, more polar and less toxic than C27-BAs) levels. Another interesting finding associated with UDCA treatment is that, as expected, based on its poor ability to activate FXR, administered UDCA cannot inhibit *de novo* synthesis of BAs and hence cannot inhibit C27-BAs production, which recommends additional pharmacological intervention for a better long-term management of patients with ADAH.

The fact that gallbladder disease was reported in some adult individuals with ADAH prompted the suspicion of a possible link between C27-BA accumulation and cholelithiasis. The low number of cases analyzed here and their occurrence in only one family preclude robust conclusions in this respect.

Although p.Arg225Trp variant affected just one amino acid, the staining of ACOX2 in liver biopsies was negative in all affected patients. The protein structure analysis revealed that the amino acid change might be relevant for ACOX2 dimerization.[8] Thus, the variant could hinder dimerization or result in altered dimers with a greater probability of being degraded by the proteasome. Recently, some studies have also described the absence of protein expression due to the presence of missense gene variants.[36,37] The mechanism accounting for enhanced mRNA degradation in these cases is still poorly understood.

In contrast with conjugated and unconjugated forms of CA, THCA is taken up by a process markedly independent of NTCP, OATP1B1, and OATP1B3. Regarding the

mechanisms accounting for C27-BAs noxious effects, our results show that THCA is more toxic than DCA and CDCA, which are primary mediators of hepatotoxicity in situations of C24-BA accumulation.[12,38] C27-BA-induced hepatocellular toxicity can be partly due to impaired mitochondrial respiratory function and increased ROS production.[39] Although intrahepatic concentrations of C27-BAs in patients with ADAH may be variable and lower than those used in our *in vitro* experiments, chronic exposure to C27-BAs and the concurrence of other deleterious factors may promote hepatocellular vulnerability and eventually result in liver damage.

Finally, we have identified six *ACOX2* variants that, when expressed in HuH-7 cells, show impaired ability to enhance THCA biotransformation into CA and therefore can be considered candidates to cause ADAH. This in vitro study has some limitations, such as measuring the final product of the biochemical pathway, CA, instead of the direct product of ACOX2 activity, the 24-enoyl-CoA derivative of THCA. This may limit the capacity to discriminate the activity of the overexpressed ACOX2 variant due to the necessary action of enzymes downstream of the pathway, i.e., D-bifunctional protein (DBP) and sterol carrier protein x (SCPx). Thus, even though our results add a valuable piece of evidence about the predicted negative impact of *ACOX2* variants, their deleterious consequences cannot be confirmed until the diagnosis of additional ADAH individuals carrying these variants could be made. Therefore, more extensive genetic studies in subjects presenting idiopathic hypertransaminasemia and other hepatic pathologies of unknown origin, should be encouraged to gather accurate data on ADAH prevalence. Still, it is foreseeable that more cases of ADAH will appear in the future among patients with unexplained hypertransaminasemia carrying *ACOX2*-V1 or other variants with predicted functional impact and high MAF, such as *ACOX2*-V2 and *ACOX2*-V6, which are expected to be present in approximately 1 and 5 out of 2000 exomes, respectively.

Finally, the two most relevant clinical contributions of the present study are that a noninvasive and effective diagnostic strategy based on plasma BA profiling and *ACOX2* sequencing can accurately identify patients with ADAH and that their liver damage can be efficiently attenuated by UDCA treatment.

AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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