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Original Research

Sequencing and functional characterization of *SCARB1* variants in subjects with extreme HDL cholesterol levels

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KEYWORDS
Extreme HDL-C;
SCARB1 sequencing;
SR-B1;
ASCVD

BACKGROUND: Rare variants in *SCARB1*, which encodes the high-density lipoprotein (HDL) receptor scavenger receptor class B type 1 (SR-B1), are hypothesized to drive unexplained extreme levels of plasma HDL cholesterol (HDL-C).
OBJECTIVE: We sequenced and phenotypically correlated *SCARB1* by analyzing individuals with extreme HDL-C levels and characterizing the functional consequences of rare identified variants.
METHODS: *SCARB1* was Sanger-sequenced in 96 unrelated participants with extreme HDL-C levels. Clinical, biochemical, and anthropometric data were compared between groups. Bioinformatic tools were used to predict the functional impact of all detected variants. Familial analyses of predicted damaging *in silico* or not previously described variants was assessed, and HDL uptake was quantified by flow cytometry in HEK293 cells expressing rare *SCARB1* variants showing a suggestive pattern of familial segregation.
RESULTS: Compared with the high-HDL-C group, low-HDL-C subjects exhibited lower low-density lipoprotein cholesterol and total cholesterol but higher triglycerides, higher body mass index, and a greater frequency of atherosclerotic cardiovascular disease events. Twenty-five *SCARB1* variants were identified; 4 of them, c.-177G>T, p.(Thr118Ser), c.843-982G>A and p.(Thr378Met), were predicted to be deleterious. The missense changes p.(Thr118Ser) and p.(Thr378Met) showed a suggestive pattern of segregation with high HDL-C in available pedigrees. Cells expressing p.(Thr378Met) *SCARB1* variant showed a reduction in HDL uptake vs wild-type.

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CONCLUSION: Rare predicted damaging *in silico* variant in *SCARB1*, p.(Thr378Met), impairs SR-B1-mediated HDL uptake and associates with high HDL-C levels, highlighting *SCARB1* as a candidate gene for genetic screening in dyslipidemic patients.

Introduction

Numerous studies have demonstrated that levels of cholesterol carried by high-density lipoprotein (HDL-C) in plasma are inversely correlated with the risk of atherosclerotic cardiovascular disease (ASCVD).[1] However, Mendelian randomization studies[2] and randomized clinical trials involving HDL-C-raising drugs[3] do not support the notion that HDL-C concentration is, by itself, an independent risk factor for ASCVD.[4] Moreover, retrospective analyses of large epidemiological studies have shown that elevated plasma HDL-C levels may be associated with an increased risk of ASCVD.[5] Taken together, these observations indicate that HDL-C is an imperfect surrogate for HDL biology. Accordingly, it has been proposed that qualitative aspects of HDL (eg, particle composition and cholesterol efflux capacity) could be more informative than HDL-C levels; nevertheless, any causal role for specific HDL functions in ASCVD risk remains uncertain and is not supported by Mendelian randomization.[6]

The antiatherogenic properties of HDL include its involvement in reverse cholesterol transport (RCT) from peripheral tissues—including macrophages in the arterial wall—back to the liver through receptor-mediated mechanisms.[7] The scavenger receptor class B type 1 (SR-B1), encoded by the *SCARB1* gene,[8] mediates the selective hepatic uptake of cholesteryl esters from HDL and serves as a receptor for the proatherogenic low-density lipoprotein (LDL) and very low-density lipoproteins (VLDL), and lipoprotein(a) (Lp(a)), underscoring its central role in lipid metabolism.[9]

The role of SR-B1 in the development of atherosclerosis is complex and not yet fully understood. Several studies have shown that SR-B1 facilitates the uptake of modified lipoproteins by macrophages and endothelial cells, contributing to the progression of atherosclerosis.[5,6,8,9] However, genetic variations in *SCARB1*, including both loss- and gain-of-function mutations in humans, have strongly implicated SR-B1 as an antiatherogenic factor.[10] Given the conflicting evidence, further research is needed to clarify the impact of *SCARB1* polymorphisms on HDL-C levels and their influence in the ASCVD susceptibility.

This study aimed to identify variants in the *SCARB1* gene in individuals with extremely high or low HDL-C levels of unknown origin, perform family analyses, functionally characterize any notable genetic variants identified, and assess their allele frequencies in subjects with HDL-C above the 90th percentile from the Lipid Clinic at Hospital Universitario Miguel Servet cohort.

Material and methods

Subjects

All unrelated subjects with isolated primary HDL-C below 40 mg/dL of undetermined etiology (approximately the 5th percentile in our population) ($n = 37$) adjusted for age and sex were recruited from the Lipid Clinic at Hospital Universitario Miguel Servet (HUMS) in Zaragoza, Spain. Also, high HDL-C participants ($n = 59$), were randomly selected from individuals whose HDL-C values were above the 95th percentile, with sex frequency-matching to the low HDL-C group.

Exclusion criteria were triglyceride (TG) levels > 200 mg/dL and secondary causes of dyslipidemia, including: overweight or obesity (body mass index [BMI] > 27.5 kg/m^2), poorly controlled type 2 diabetes (glycosylated hemoglobin [HbA1c] > 7%), renal disease (glomerular filtration rate < 30 mL/min and/or macroalbuminuria), liver disease (alanine transaminase > 3 times the upper normal limit), hypothyroidism (thyroid stimulating hormone > 6 mIU/L), pregnancy, inflammatory disease (C-reactive protein > 5 mg/L), alcohol intake > 30 g/d, estrogen-replacement therapy, cholestasis (direct bilirubin > 1 mg/dL) and treatment with protease inhibitors or any drug affecting lipid metabolism.

Additionally, to estimate the allele frequency of a rare genetic variant described in this study, a supplementary group of unrelated individuals from HUMS with HDL-C values above the 90th percentile was selected ($n = 331$).

All participants provided written informed consent under a protocol approved by the Ethics and Scientific Committees of our institution. Biological specimens from patients at HUMS were provided by the Aragón Health System Biobank (PT17/0015/0039), a member of the Spanish National Biobanks Network, and processed following standard operating procedures.

Anthropometric and clinical parameters

Body weight was measured in subjects without shoes to the nearest 0.1 kg with a calibrated scale (Seca 813, Seca Deutschland). Height was assessed to the nearest 0.1 cm with a wall-mounted stadiometer (Seca 217, Seca Deutschland). BMI was calculated as weight in kilograms divided by the square of height in meters. Blood pressure was measured in triplicate with a validated semiautomatic oscillometer (Omron M3, Omron Cop).

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Biochemical analysis

EDTA plasma and serum samples were collected from all participants after at least 10 hours fasting, without lipid-lowering drugs for >5 weeks, to obtain baseline biochemical characteristics. Total cholesterol, TG, HDL-C, gamma-glutamyl transferase (GGT), and glutamic-pyruvic transaminase (GPT) were measured with standard enzymatic methods. LDL cholesterol (LDL-C) was calculated with the Friedewald formula when serum TGs were < 400 mg/dL. Apolipoprotein (apo) AI, apo B, and Lp(a) were determined by IMMAGE kinetic nephelometry (Beckman Coulter Inc.). Blood glucose concentration was measured with the glucose-oxidase method. Whole-blood HbA1c was measured by reverse-phase cationic exchange chromatography with double wave-length colorimetry quantification (Analyzer ADAMS A1c HA-810, Arkray Factory).

Genetic analysis

Whole blood genomic DNA was isolated by the commercial product Flexigene DNA (Qiagen) for all subjects. Promoter, exons, and intron-exon boundaries of the *SCARB1* gene (NM_005505.5) were amplified by polymerase chain reaction, following the temperatures and primers described in Supplementary Table 1. After purification with ExoSap-IT (USB), amplified fragments were sequenced by Sanger method using the BigDye 3.1 sequencing kit (Applied Biosystems) in an automated ABI 3500xL sequencer (Applied Biosystems). DNA sequences were analyzed using Variant Reporter software (Applied Biosystems).

To evaluate the pathogenicity of new identified genetic variants, PredictSNP2 was used.[11] To compare the frequency of identified variants with that of the general population, the allele frequencies of identified variants from the Genome Aggregation Database were collected.[12] Finally, information about microRNAs was obtained from the PolymiRTS Database 3.0.[13]

Familial analyses of rare variant predicted to be damaging in silico

All available relatives of individuals carrying rare *SCARB1* variants classified as deleterious by bioinformatic analysis or not previously reported were selected for lipid and genetic analysis. Functional assays were carried out only in those *SCARB1* variants showing a suggestive pattern of segregation with HDL-C levels within the available family members. In addition, we performed a comprehensive clinical characterization including comorbidities, history of ASCVD, and exposure to lipid-lowering and other relevant medications.

Functional characterization

HDL labelling

HDL was labelled with fluorescein isothiocyanate (FITC) as previously described.[14] Briefly, HDL (1 mg/mL) in 0.1 M $NaHCO_3$ (pH 9.0) were mixed with 10 µl/mL of FITC (2 mg/mL in dimethyl sulfoxide). The mixture was gently mixed by slow rocking at room temperature for 2 hours. The unreacted dye was removed by gel filtration on a sephadex G-25 column equilibrated with phosphate buffered saline (PBS) EDTA-free buffer. All fractions were assayed for protein content with bovine serum albumin (BSA) as standard (DC protein assay, Bio-Rad).

Cell transfection

HEK293 cells were cultured in DMEM medium supplemented with 5% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. HEK293 cells were seeded into 24-well culture plates, and transfected with plasmids carrying the *SCARB1* genetic variants using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were maintained in culture for 48 hours to achieve maximal SR-B1 expression

Quantification of SR-B1 expression by flow cytometry

To determine cell surface expression of SR-B1 by flow cytometry, transfected HEK293 cells grown during 48 hours were incubated with a mouse primary antibody anti-SR-B1 (1∶100; Anti-Scavenging Receptor SR-B1 antibody [EPR20190], abcam; Cat. No. ab217318) for 1 hour, at room temperature, then, washed twice with PBS-1%BSA and incubated with a secondary antibody Alexa Fluor 488-conjugated goat antirabbit IgG (1∶200; Molecular Probes; Cat. No. A-11,008). As negative controls, nontransfected HEK293 cells or transfected with the wild-type SR-B1 were stained with the same primary anti-SR-B1 antibody. For each sample, fluorescence of 5000 events was acquired for data analysis. All measurements have been performed, at least, in triplicate.[14]

SR-B1 activity

Transfected HEK293 cells were grown in 24-well culture plates. Forty-eight hours after transfection, cells were incubated at 37 °C for 4 hours with 20 µg/mL FITC-HDL to determine SR-B1 activity. After incubation with FITC-HDL, HEK293 cells were washed twice in PBS-1%BSA, fixed on 4% formaldehyde for 10 minutes and washed again twice with PBS-1%BSA. The quenching of external fluorescence, which distinguishes internalized from surface-adherent FITC-HDL particles, can be accomplished with the use of vital dyes such as Trypan blue, which is not able to penetrate intact cell membranes. This procedure allows removal of extracellular fluorescence by quenching and determination of the intensity of the remaining fluorescent particles inside the cells that is not affected by the external quencher. Therefore, to determine the amount of internalized HDL, Trypan blue solution (Sigma-Aldrich) was added to a

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final concentration of 0.2% directly to the samples, eliminating the extracellular signal due to the noninternalized SRB1-HDL complexes. Fluorescence intensities were measured by flow cytometry, in a CytoFlex cytometer according to the manufacturer instructions. For each sample, fluorescence of 5000 events was acquired for data analysis. All measurements have been performed, at least, in triplicate.[14]

Allele frequency

Subjects from HUMS (excluding those that had been previously studied) with HDL-C > 90th percentile (adjusted by age and sex) were screened for the presence of the p.(Thr378Met) variant by a real-time polymerase chain reaction (PCR)-based methodology. A multiplexed qPCR was carried out using TaqMan probes for the p.(Thr378Met) variant in *SCARB1* gene and an endogenous single-copy control gene, RNase P. Thermocycler conditions were: 95 °C hot-start for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute. Population comparisons were performed against gnomAD v4 and the 1000 Genomes Project, restricting to non-Finnish Europeans in both resources to approximate our cohort's ancestry. Variant frequencies are reported using standardized allele count/allele number (AC/AN) notation.

Statistical analysis

Analyses were performed using statistical computing software R version 3.5.0.[15] The level of significance was set at p-value < .05. The distribution of the clinical and biochemical variables was analyzed by the Shapiro test. Quantitative variables with a normal distribution were expressed as mean ± SD and were analyzed by the Student t-test. Variables with a skewed distribution were expressed as medians and IQRs and were analyzed with the Mann-Whitney U test. Qualitative variables were expressed as percentages and were analyzed by the Chi-squared test.

Regarding functional experiments, all measurements were performed at least 3 times, with $n = 3$ unless otherwise stated, and results are presented as mean ± SD. Levels of significance were determined by a 2-tailed Student's t-test, and a confidence level of greater than 95% ($P < .05$) was used to establish statistical significance.

To assess differences in allele frequencies between cohorts, we used Fisher's exact test (one-sided, cohort > reference, unless otherwise specified). All contrasts are presented as descriptive, in view of extreme-phenotype ascertainment, potential population stratification, and incomplete penetrance.

Results

Clinical and biochemical characteristics

The main clinical and biochemical characteristics of both studied groups (59 subjects with high levels of HDL-C and 37 subjects with low levels of HDL-C) are presented in Table 1.

Table 1 Clinical and biochemical characteristics according to HDL-C levels.

	Subjects with high HDL-C levels ($n = 59$)	Subjects with low HDL-C levels ($n = 37$)	*P*
Age, years	54 (46.5–61)	37 (26–55)	**<.001**
Men, n (%)	29 (49.2%)	17 (45.9%)	.923
Body mass index, kg/m^2	24.1 (22.2–25.0)	25.5 (23.1–26.5)	**.007**
Triglycerides, mg/dL	83.0 (69.0–109)	101 (76.0–145)	**.037**
Total cholesterol, mg/dL	300 ± 49.8	207 ± 59.0	**<.001**
HDL-C, mg/dL	92.0 (82.5–101)	33.0 (30.0–37.0)	**<.001**
LDL-C, mg/dL	188 ± 41.4	151 ± 55.7	**<.001**
GGT, UI/L	22.0 (15.0 27.5)	17.0 (13.0–27.5)	.244
GPT, UI/L	19.0 (16.5–25.0)	20.0 (14.5–28.5)	.762
Apolipoprotein A1, mg/dL	215 (197–247)	110 (104–120)	**<.001**
Apolipoprotein B, mg/dL	127 (109–149)	106 (89.2–141)	.056
Lipoprotein(a), mg/dL	29.0 (15.8–101)	24.2 (7.93–44.7)	.106
Glucose, mg/dL	89.1 ± 10.8	90.7 ± 16.4	.611
HbA1c, %	5.41 ± 0.36	5.35 ± 0.45	.479
Hypertension, n (%)	7 (11.8%)	4 (10.8%)	.985
Diabetes, n (%)	1 (1.69%)	4 (10.8%)	.137
Cardiovascular disease, n (%)	0 (0%)	6 (16.2%)	**.004**
Current smokers, n (%)	4 (6.8%)	6 (17.7%)	.200

Abbreviations: GGT, gamma-glutamyltransferase; GPT, glutamic-pyruvic transaminase; HbA1c, glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Quantitative continuous variables are expressed as mean ± SD or median [percentile 25-75].

Student's t or Wilcoxon tests were used to assess differences between 2 groups.

Quantitative categorical variables are expressed as n (%) and statistical differences were assessed by Chi-squared.

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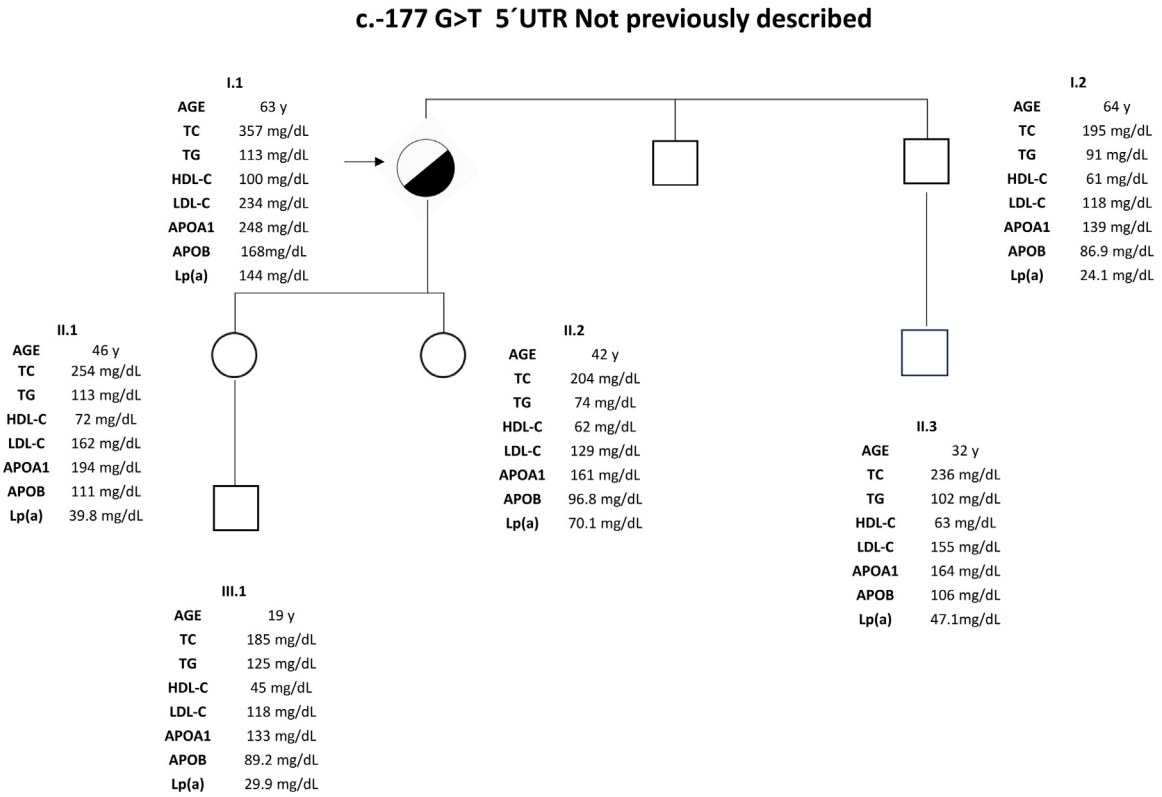

Figure 1 Family carrying the *SCARB1* variant c.-177 G>T 5ÚTR (Not previously described). Symbols used in the figure ○ Female; □ Male; ◐/◧ Heterozygous carrier (half-shaded); → Proband (index case). Abbreviations: APOA1, apolipoprotein A1; APOB, apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); TC, total cholesterol; TG, triglycerides; y, years old.

Compared with subjects with high levels of HDL-C, subjects with low levels of HDL-C were significantly younger, had significantly higher values of BMI, TG, and higher prevalence of ASCVD ($P < .001$, $P = .007$, $P = .037$, and $P = .004$, respectively). As expected, subjects with low levels of HDL-C had lower levels of total cholesterol, HDL-C, LDL-C, and apoA1 than subjects with high levels of HDL-C ($P < .001$, $P < .001$, $P < .001$, and $P < .001$, respectively). No differences were found in the other parameters evaluated.

Variants in SCARB1 *gene*

All genetic variants identified in *SCARB1* in both groups are presented in Table 2. A total of 25 genetic variants were identified by sequencing analysis, 2 of them not previously described. No genetic variant presented significantly different allele frequency between groups (data not shown). Nine variants were detected exclusively in the high HDL-C group (c.-1371T>C, c.-177G>T, p.(Pro60=), p.(Thr118Ser), p.(Ser129Leu), p.(Thr378Met), c.1203-138C>T, c.1401+164G>A, and c.*49C>T) and 5 in the low HDL-C group (p.(Ile158Met), c.843-173C>T, c.843-982G>A, c.*1530+786G>A, and c.*94G>A). Five variants were located in the promotor region: c.-1371T>C, c.-1253C>T, c.-1214C>T, c.-1108A>G, and c.-177G>T. Eight variants were located in the coding region, 6 of them were missense variants, and 2 genetic variants were synonymous. Nine variants were located in intronic regions, c.285-170G>A, c.726+54C>T, c.843-173C>T, c.843-982G>A, c.1203-138C>T, c.1255-41C>G, c.1401+130T>C, c.1401+164G>A, and c.*1530+786G>A. Two variants were located in the 3′UTR: c.*49C>T and c.*94G>A. In addition, c.-177G>T and p.(Thr118Ser) were not previously described. Finally, bioinformatic analysis classified 3 variants as deleterious: c.-177G>T and p.(Thr378Met), which were found exclusively in the high-HDL-C group, and c.843-982G>A, which was observed only in the low-HDL-C group.

Familial analyses of variant segregation

Familial analyses were carried out in subjects carrying rare *SCARB1* variants predicted damaging *in silico* or not previously described.

The rare variant c.-177G>T located in the 5ÚTR region has not been previously described, and the bioinformatic analysis predicts it to be deleterious (Table 2). One subject from high HDL-C group presented this rare variant, who exhibited high levels of total cholesterol, LDL-C, and HDL-C as well as elevated Lp(a) concentration. However, this variant was not identified in any of the relatives studied (Fig 1). Most family members exhibited high-normal HDL-C levels; however, none had HDL-C values above the 90th percentile.

Table 2 Frequency and bioinformatic clinical significance of identified variants in *SCARB1* in subjects with extreme levels of HDL-C.

Variant	Location	Genomic location (GRCh38/hg38)	HGVS cDNA	HGVS protein	Bioinformatic analysis	Allele frequency in the general population compared and population of our study			MicroRNAs[3]
					PredictSNP2[1] (probability)	High HDL-C subjects $n = 59$	Low HDL-C subjects $n = 37$	gnomAD[2]	
rs1227265812	5ÚTR	chr12:124,865,091	c.-1371T>C	NA	Neutral (88%)	0.017	0.000	0.00007	
rs36226544	5ÚTR	chr12:124,864,973	c.-1253C>T	NA	Neutral (88%)	0.347	0.230	0.351	
rs36226283	5ÚTR	chr12:124,864,934	c.-1214C>T	NA	Neutral (88%)	0.331	0.257	0.351	
rs112822039	5ÚTR	chr12:124,864,828	c.-1108A>G	NA	Neutral (88%)	0.017	0.140	0.013	
rs551998063	5ÚTR	chr12:124,864,123	c.-408 C>G	NA	Neutral (74%)	0.008	0.001	0.005	
Not previously described	5ÚTR	chr12:124,863,895	c.-177G>T	NA	Deleterious (97%)	0.008	0.000	NR	
rs4238001	Exon 1	chr12:124,863,717	c.4G>A	p.Gly2Ser	Neutral (65%)	0.195	0.257	0.115	
rs143093152	Exon 2	chr12:124,817,654	c.180T>C	p.Pro60=	Neutral (96%)	0.008	0.000	0.0003	
rs4765615	Intron 2	chr12:124,815,284	c.285-170G>A	NA	Neutral (73%)	0.576	0.541	0.683	
Not previously described	Exon 3	chr12:124,815,046	c.353C>G	p.Thr118Ser	Neutral (89%)	0.008	0.000	NR	
rs150222965	Exon 3	chr12:124,815,013	c.386C>T	p.Ser129Leu	Neutral (89%)	0.008	0.000	0.0001	
rs5891	Exon 3	chr12:124,814,996	c.403G>A	p.Val135Ile	Neutral (89%)	0.034	0.027	0.011	
rs371778051	Exon 4	chr12:124,814,358	c.474C>G	p.Ile158Met	Neutral (89%)	0.000	0.014	0.00002	
rs61932577	Intron 5	chr12:124,811,816	c.726+54C>T	NA	Neutral (74%)	0.093	0.162	0.110	
rs182346713	Intron 6	chr12:124,808,103	c.843-173C>T	NA	Neutral (77%)	0.000	0.027	0.001	
rs1302998758	Intron 6	chr12:124,808,909	c.843-982G>A	NA	Deleterious (91%)	0.000	0.014	NR	

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Table 2 (*continued*)

Variant	Location	Genomic location (GRCh38/hg38)	HGVS cDNA	HGVS protein	Bioinformatic analysis	Allele frequency in the general population compared and population of our study			MicroRNAs[3]
					PredictSNP2[1] (probability)	High HDL-C subjects $n = 59$	Low HDL-C subjects $n = 37$	gnomAD[2]	
rs5888	Exon 8	chr12:124,800,202	c.1050T>C	p.Ala350=	Neutral (96%)	0.593	0.608	0.351	
rs748231262	Exon 9	chr12:124,795,264	c.1133C>T	p.Thr378Met	Deleterious (82%)	0.008	0.000	0.00003	
rs573706452	Intron 9	chr12:124,787,595	c.1203-138C>T	NA	Neutral (88%)	0.001	0.000	0.0005	
rs838897	Intron 10	chr12:124,786,544	c.1255-41C>G	NA	Neutral (74%)	0.110	0.054	0.042	
rs2293440	Intron 11	chr12:124,786,227	c.1401+130T>C	NA	Neutral (88%)	0.059	0.027	0.032	
rs756206185	Intron 11	chr12:124,786,193	c.1401+164G>A	NA	Neutral (73%)	0.008	0.000	0.0001	
rs75446635	Intron 12	chr12:124,782,605	c.*1530+786G>A	NA	Neutral (88%)	0.000	0.014	0.0005	
rs10396214	3ÚTR	chr12:124,778,538	c.*49C>T	NA	Neutral (89%)	0.001	0.000	0.008	hsa-miR-4640-5p hsa-miR-4726-5p hsa-miR-6762-5p hsa-miR-6845-5p
rs701103	3ÚTR	chr12:124,778,493	c.*94G>A	NA	Neutral (89%)	0.000	0.014	0.0006	hsa-miR-3120-5p hsa-miR-346 hsa-miR-3692-5p hsa-miR-4288 hsa-miR-4731-5p hsa-miR-5589-5p hsa-miR-6071 hsa-miR-626 hsa-miR-632 hsa-miR-6876-3p hsa-miR-93-3p

Abbreviations: cDNA, complementary DNA; HDL-C, high-density lipoprotein cholesterol; HGVS, Human Genome Variation Society; NA, not applicable; NR, not reported.

[1]PredictSNP2 uses CADD, DANN, FATHMM y Funseq2 as predictors.

[2]Karczewski, K.J., Francioli, L.C., Tiao, G. et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature 2020, 581, 434–443. https://gnomad.broadinstitute.org/ using as reference the European population (non-Finnish).

[3]PolymiRTS Database 3.0: http://compbio.uthsc.edu/miRSNP/

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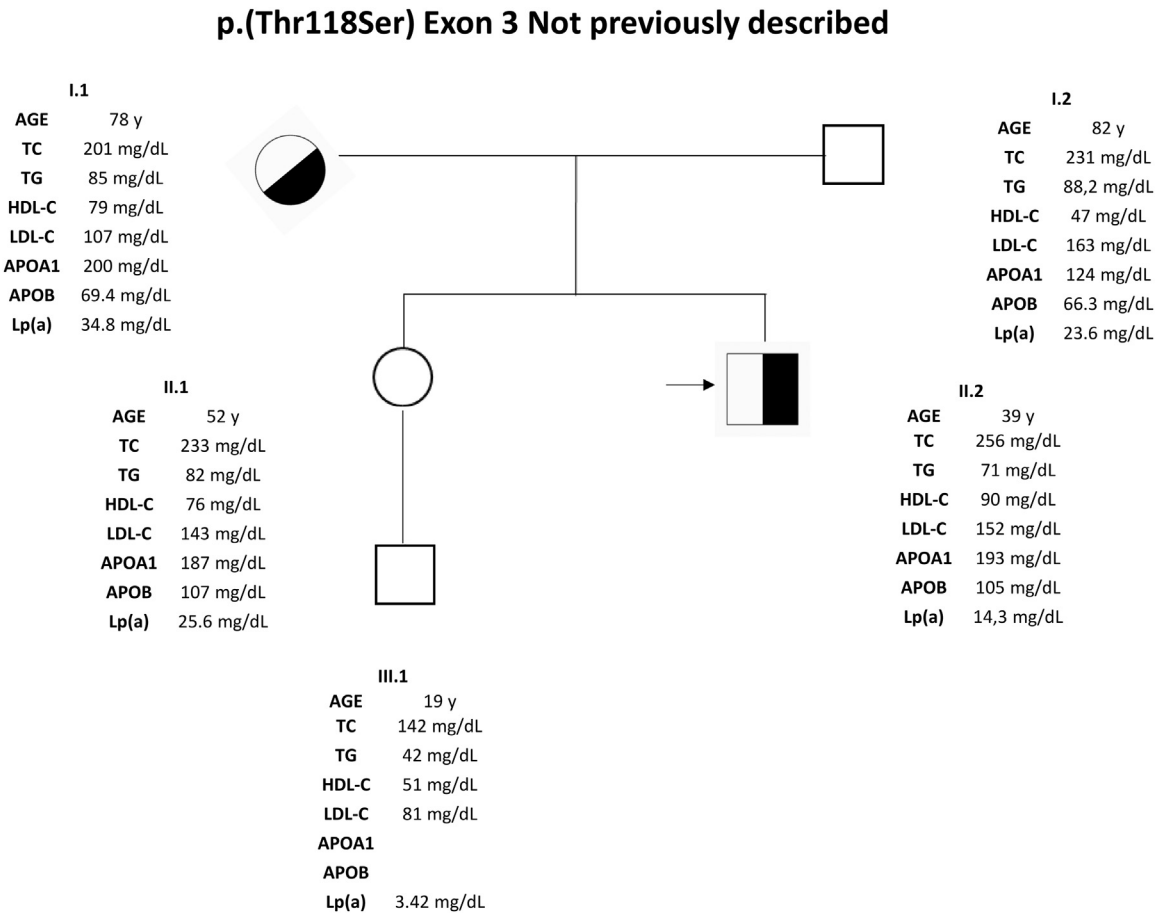

Figure 2 Family carrying the S*CARB1* variant p.(Thr118Ser) Exon 3 (Not previously described). Symbols used in the figure ○ Female; □ Male; ◐/◧ Heterozygous carrier (half-shaded); → Proband (index case). Abbreviations: APOA1, apolipoprotein A1; APOB, apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); TC, total cholesterol; TG, triglycerides; y, years old.

The rare variant c.353C>G located in exon 3 of the *SCARB1* gene is predicted to cause a substitution of threonine by serine at amino acid 118 of SR-B1, p.(Thr118Ser). Although this genetic variant is novel, computational predictions consistently classify it as neutral. One patient from the high HDL-C group carried this rare variant and presented high levels of total cholesterol and HDL-C. His mother was also identified as a carrier of the same variant and showed similarly high levels of HDL-C and apoA1 (Fig 2).

The rare variant c.843-982G>A is located in intron 6 and the bioinformatic analyses classified this genetic variant as deleterious (Table 2). A patient from the low HDL-C group exhibited this rare variant, who presented low levels of HDL-C and LDL-C. The variant was identified in both his wife and their son. His wife exhibited normal HDL-C levels, whereas his son exhibited a lipid profile comparable to that of the proband (Fig 3).

The rare variant c.1133C>T is located in exon 9 and is predicted to induce a substitution of threonine by methionine at amino acid 378 of SR-B1, p.(Thr378Met). This amino acid change has not been previously reported in patients with high HDL-C, although is described as deleterious by bioinformatic analysis. In this study, 1 patient from the high HDL-C group carried this rare variant and presented high levels of total cholesterol, HDL-C, and Lp(a). Her mother and 2 sisters were also carriers of the same rare variant and showed high levels of total cholesterol and HDL-C (Fig 4).

Familiar analyses seem to indicate that the p.(Thr118Ser) and p.(Thr378Met) variants showed a suggestive pattern of segregation with the clinical phenotype in the available pedigrees, whereas with the c.-177G>T and c.843-982G>A variants, the evidence of familial segregation was not demonstrated.

Clinical characteristics, including comorbidities, history of ASCVD, and exposure to lipid-lowering and other relevant medications, were assessed in all participants. No variable analyzed showed a significant association with increased ASCVD prevalence or comorbidity burden among carriers compared with noncarriers. Furthermore, none of the lipid-modifying medications used by participants had a significant impact on HDL-C concentrations, which represents the main outcome of the study.

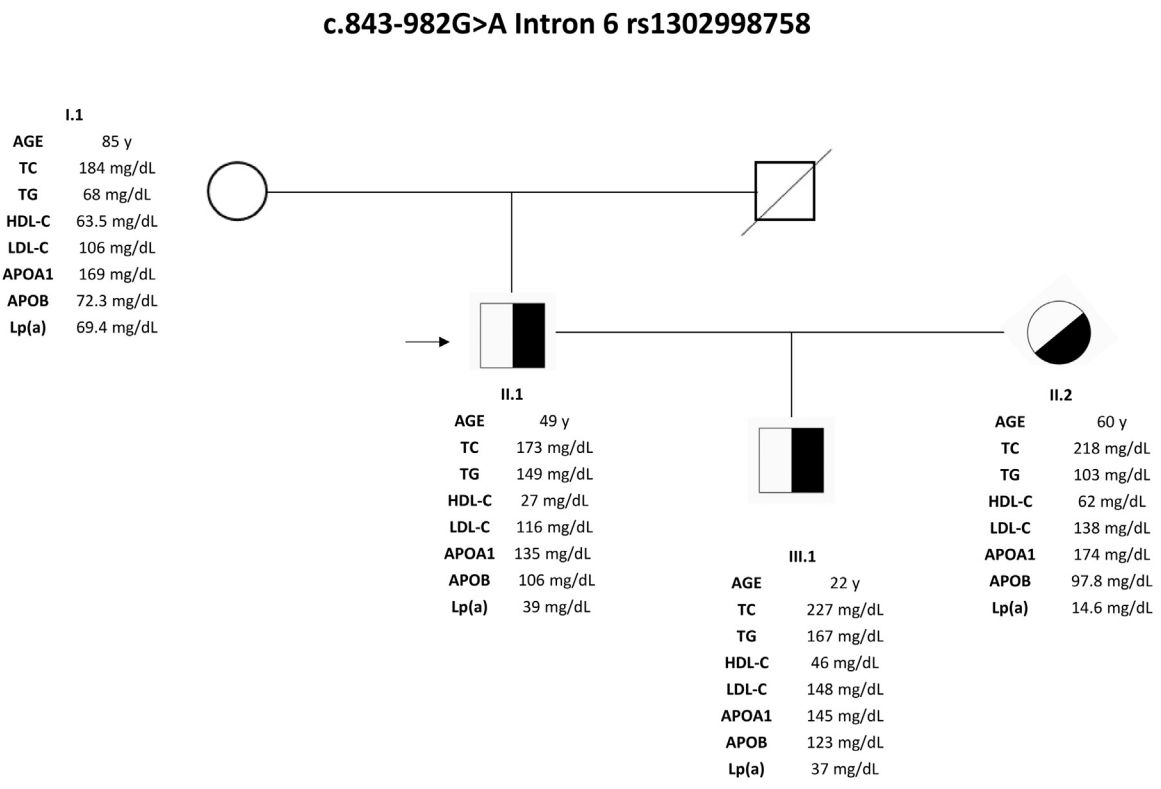

Figure 3 Family carrying the S*CARB1* variant c.843-982G>A Intron 6 rs1302998758. Symbols used in the figure ○ Female; □ Male; ◐ / ◧ Heterozygous carrier (half-shaded); → Proband (index case). Abbreviations: APOA1, apolipoprotein A1; APOB, apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); TC, total cholesterol; TG, triglycerides; y, years old.

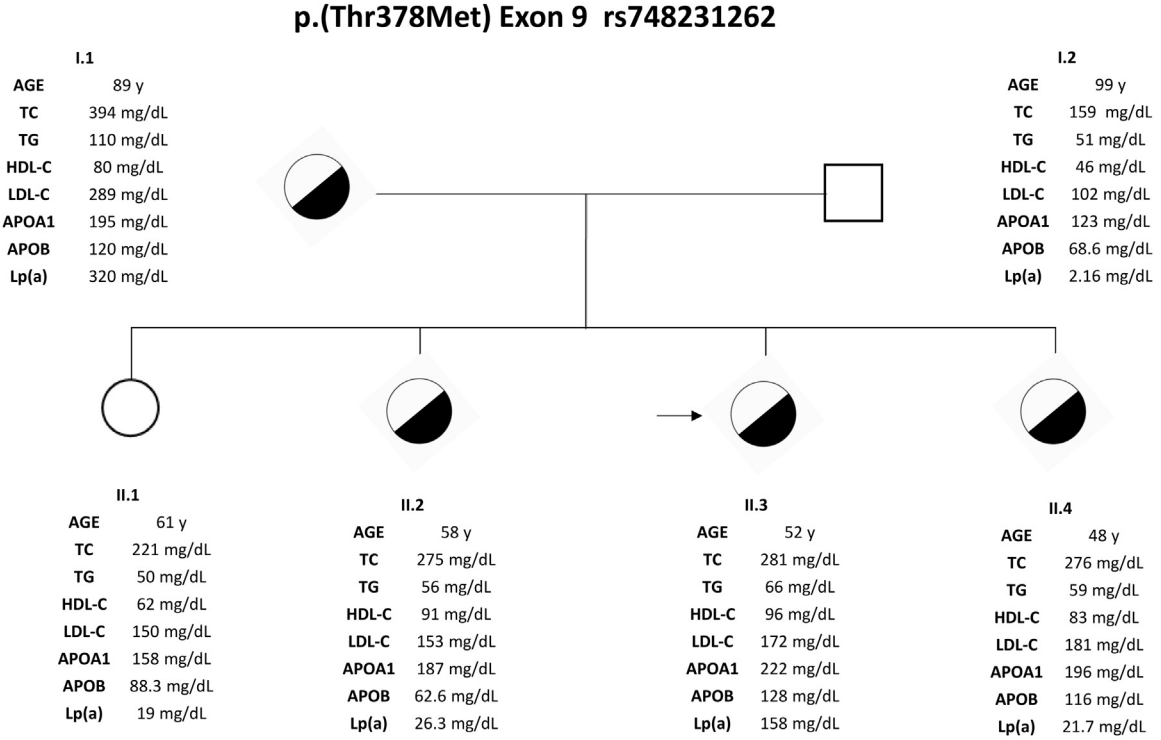

Figure 4 Family carrying the S*CARB1* variant p.(Thr378Met) Exon 9 rs748231262. Symbols used in the figure ○ Female; □ Male; ◐ / ◧ Heterozygous carrier (half-shaded); → Proband (index case). Abbreviations: APOA1, apolipoprotein A1; APOB, apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); TC, total cholesterol; TG, triglycerides; y, years old.

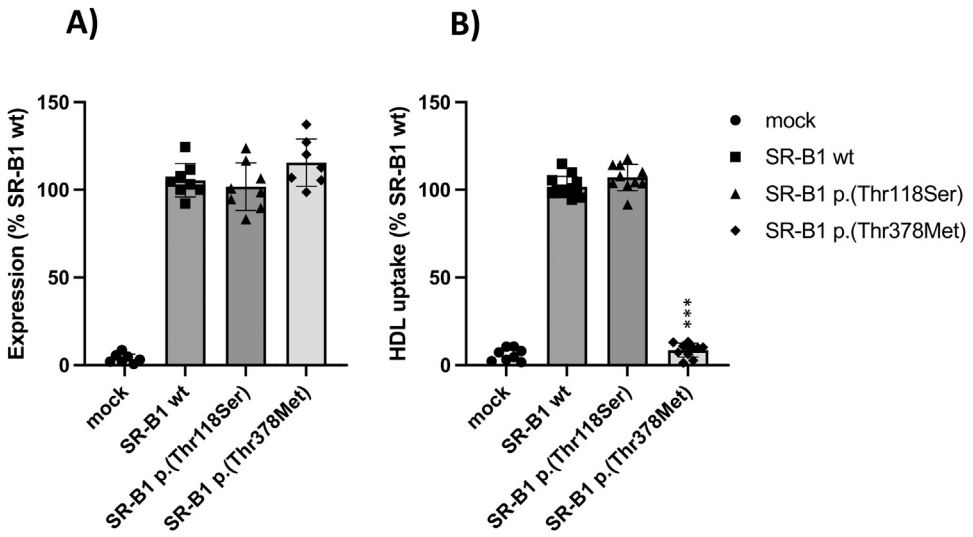

Figure 5 SR-B1 expression in HEK 293 and SR-B1 activity. Data are expressed as mean ± SD. Student's test was used to assess differences between SR-B1 wt and SR-B1 variants. $^{***}P < .001$. Abbreviations: HDL, high-density lipoprotein; SR-B1, scavenger receptor B1; wt, wild-type.

SR-B1 expression in HEK 293

Expression of the p.(Thr118Ser) and p.(Thr378Met) SR-B1 variants was analyzed by flow cytometry. Using an anti-SR-B1 antibody, the results showed that the expression levels of p.(Thr118Ser) and p.(Thr378Met) variants were similar to that of the wild type (see Fig 5A), indicating that the nucleotide substitutions did not affect protein expression in either case. Transfection efficiency was verified, confirming that SR-B1 wild-type and variants p.(Thr118Ser) and p.(Thr378Met) were expressed at comparable levels, supporting that the observed differences are not due to altered receptor expression or trafficking (see Supplementary Fig 1).

SR-B1 activity

The activity of p.(Thr118Ser) and p.(Thr378Met) SR-B1 variants was assessed in HEK293 transfected cells. Receptor activity was measured by analyzing FITC-HDL uptake using flow cytometry. As shown in Figure 5B, HDL uptake in cells expressing the p.(Thr118Ser) variant was similar to that of the wild-type SR-B1. In contrast, for the p.(Thr378Met) variant, and despite comparable SR-B1 expression levels, HDL internalization was significantly reduced compared to the wild type (approximately 85% reduction; 100 ± 14 vs 16.5 ± 14, $P < .01$).

Allelic frequency

The *SCARB1* p.(Thr378Met) variant (rs748231262) was genotyped in 331 unrelated subjects from HUMS whose HDL-C concentrations exceeded the 90th percentile, using a TaqMan allelic-discrimination assay. Two subjects presented this genetic variant in heterozygosis. Therefore, the allele frequency of the *SCARB1* p.(Thr378Met) variant (rs748231262) was 0.0030; 95% CI 0.00083-0.01095, in subjects with high HDL-C levels from our population. In the non-Finnish European subset of gnomAD v4, genotype counts (589,686 GG; 36 GA; 0 AA), being the allele frequency 3.05×10^{-5}, yielding a significantly higher frequency in our cohort (Fisher's exact $P = 2.2 \times 10^{-4}$). The variant was not detected in the 1000 Genomes non-Finnish European panels (AC = 0). These contrasts are likely influenced by extreme-phenotype ascertainment, limited precision, and residual ancestry differences, and should therefore be regarded as hypothesis-generating rather than inferential for population risk.

Discussion

In this study, we sequenced the *SCARB1* gene in individuals with idiopathic, extreme HDL-C values to determine whether variation in this gene contributes to the contrasting metabolic phenotypes of very high and very low HDL-C. Familial analyses were then performed in relatives of probands carrying variants predicted to be deleterious by bioinformatic tools or not previously described variants. Variants that showed a suggestive pattern of segregation with the lipid phenotype underwent functional evaluation in vitro. Lastly, the allele frequency of each functionally deleterious rare variant predicted by *in silico* was assessed in individuals from the HUMS cohort whose HDL-C levels were above the 90th percentile.

Although secondary causes of HDL-C impairment were part of the exclusion criteria, subjects with high HDL-C exhibited a healthier cardiometabolic profile with lower BMI, TG, and a lower frequency ASCVD. This highlights the importance of environmental factors even within the normal

range and the difficulty of fully matching subjects with high and low HDL-C.

A notable finding from the analysis of the variants found is that they are more frequent in subjects with high HDL-C, and 2 out of 3 variants suspected of being deleterious in the *in silico* analysis were found in subjects with high HDL-C levels. This fact is consistent with genome-wide association study (GWAS) of HDL-C, in which the *SCARB1* locus is closely associated with its concentration and single nucleotide variants have a globally positive effect on HDL-C concentration.[16] By sequencing the 13 exons of the *SCARB1* gene, exon-intron boundaries and promoter in selected subjects with extreme HDL-C values, 25 genetic variants were identified. Two of them had not been previously described: a variant located in the 5′UTR noncoding region (c.-177G>T) and an amino acid change in the third exon (p.(Thr118Ser)), being c.-177G>T deleterious according to the bioinformatic analysis, with a probability of 97%. In addition, 2 previously described variants, c.843-982G>A and p.(Thr378Met), were also predicted as deleterious by bioinformatic tools. Notably, these variants have only been found in patients with elevated HDL-C levels (c.-177G>T, p.(Thr118Ser) and p.(Thr378Met)) or low HDL-C (c.843-982G>A), suggesting that these genetic variants could play a role in HDL metabolism. Familial analyses suggested a possible segregation pattern of the p.(Thr118Ser) and p.(Thr378Met) variants with the clinical phenotype in the available pedigrees, whereas evidence of segregation was not observed for the c.-177G>T and c.843-982G>A variants. These results should be interpreted with caution given the limited number of relatives and the multifactorial nature of HDL-C regulation. Subsequently, the 2 variants showing a suggestive pattern of familial segregation, p.(Thr118Ser) and p.(Thr378Met), were functionally characterized in vitro. Surface expression of wild-type SR-B1 and of SR-B1 harboring each variant was quantified, and no differences were detected. Binding assays were then conducted, showing that the p.(Thr378Met) variant displayed reduced internalization of HDL, indicating impaired functionality of this genetic alteration. However, no functional alteration was found in p.(Thr118Ser). Although p.(Thr118Ser) showed a suggestive familial pattern, no effect was detected in HEK293 cells; this likely reflects tissue-specific SR-B1 biology and the limited scope and sensitivity of our uptake assay, so these negative results should be interpreted cautiously. Our results demonstrated for the first time an association between the p.(Thr378Met) variant and elevated HDL-C concentrations. In addition, our study showed a reduced binding capacity of the SR-B1 receptor for HDL. These results are in line with a previous genetic variant p.(Pro376Leu) found in subjects with high HDL-C. The p.(Pro376Leu) variant disrupts SR-B1 post-translational maturation and abolishes selective HDL-C uptake in transfected cells.[17,18] This variant is situated at the C-terminal end of SR-B1 extracellular domain, immediately upstream of the first transmembrane segment. The proline-to-leucine substitution blocks post-translational maturation of the receptor, leaving it in an Endo-H–sensitive immature form and abolishing the selective uptake of cholesterol esters, thereby highlighting the functional importance of this region.[17]

However, the p.(Thr378Met) variant, located 2 residues downstream of p.(Pro376Leu) shows normal cell-surface expression but a marked reduction in HDL uptake compared with the wild type, consistent with a partial loss-of-function that likely affects the uptake/transfer step rather than receptor maturation. Thus, although both variants are associated with high HDL-C, they differ in mechanism and magnitude of dysfunction. This graded functional impairment may underlie variant-specific phenotypic and risk associations, which should be interpreted cautiously given the limits of our assay and the absence of HDL quality or RCT measurements. Moreover, p.(Pro376Leu) carriers showed increased risk of developing coronary heart disease. SR-B1 has been involved in the progression of ASCVD.[5] In this sense, SR-B1 drives cholesterol efflux from macrophages to the liver and is further linked to anti-inflammatory and antioxidative effects.[5,9,10] Nonetheless, SR-B1 expressed in macrophages and endothelial cells can also promote HDL-mediated uptake of modified lipoproteins, inducing the formation of early atherosclerotic lesions.[19-21] In this study, subjects carrying the p.(Thr378Met) variant have not exhibited evidence of ASCVD. These observations are consistent with human-genetic data: large lipid GWAS identify common variation at/near *SCARB1* with robust effects on HDL-C,[16] whereas well-powered Mendelian randomization analyses do not support a causal, independent protective effect of HDL-C on coronary arterial disease (CAD) risk.[2] Moreover, gene-focused studies of *SCARB1* suggest heterogeneity: several HDL-C–raising *SCARB1* alleles show little or no increase in CAD risk,[4] while rare, more disruptive variants such as p.(Pro376Leu) have been linked to higher risk.[17] In this context, our p.(Thr378Met) carriers, who show high HDL-C with impaired HDL uptake but no ASCVD events to date, fit the view that HDL-C is an imperfect surrogate of HDL biology and that variant-specific mechanisms at *SCARB1* determine whether changes in HDL traits translate into clinical risk. Moreover, in contrast to the infertility phenotype reported in *SCARB1*-deficient mice, in vitro studies using steroidogenic cells carrying the p.(Pro376Leu) variant have not shown functional alterations, and the female homozygous carrier described to date has had healthy offspring. Similarly, individuals carrying the p.(Thr378Met) variant have also had healthy children. Taken together, these observations suggest that these variants are unlikely to cause clinically relevant alterations in steroidogenic tissues.

SCARB1 gene sequencing detected 5 missense variants that have been reported previously: p.(Gly2Ser), p.(Ser129Leu), p.(Val135Ile), p.(Ile158Met), and p.(Thr378Met). All of these appear at higher allele frequencies in our cohort than in population databases. The p.(Gly2Ser) variant, first described in a Spanish cohort,[22] was found in both the HDL-C high and HDL-C low groups, echoing the heterogeneous phenotypic associations observed elsewhere.[16,23,24] Although p.(Ser129Leu) and p.(Val135Ile)

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are predicted to be neutral *in silico*, earlier work has linked them to impaired cholesteryl-ester uptake or unfavorable lipid profiles,[24-26] highlighting the limitations of purely computational annotation.

Our analyses identified 2 synonymous substitutions, p.(Pro60=) and p.(Ala350=), with the latter previously associated with low LDL-C and modulation of cardiovascular risk.[23,27] Furthermore, five 5′UTR variants, c.-1371T>C, c.-1253C>T, c.-1214C>T, c.-1108A>G, and c.-408C>G, were identified in our cohort; notably, c.-1253C>T, c.-1214C>T, and c.-1108A>G were also reported in individuals with extremely high HDL-C in a prior resequencing study, with no significant differences vs controls.[28] Finally, 2 3′UTR changes, c.*49C>T and c.*94G>A, are located within predicted microRNA binding sites and could influence *SCARB1* expression.

There are several limitations in our study. Due to the large size of the *SCARB1* gene, we sequenced only the coding region and the exon-intron boundaries, and the number of subjects in our study was limited. It could be possible that we have missed less common functional variants present in introns and have not identified all functional variants present in the coding region. In addition, as previously discussed, the sample size in this study did not provide adequate statistical power to observe differences in allele frequencies between the high and low HDL-C groups. Moreover, the number of available relatives for the familial analyses was small, which limited our ability to demonstrate definitive cosegregation of the identified variants with the phenotype; therefore, the observed segregation patterns should be interpreted as suggestive rather than conclusive. Despite these limitations, our study is the first to demonstrate that the *SCARB1* p.(Thr378Met) variant could be associated with altered HDL-C levels and impaired SR-B1 function.

CRediT authorship contribution statement

Irene Gracia-Rubio: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Asier Benito-Vicente:** Writing – review & editing, Methodology, Investigation. **Itziar Lamiquiz-Moneo:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Ana María Bea:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Victoria Marco-Benedí:** Writing – review & editing, Methodology, Investigation. **Endika Cabrera-Antón:** Writing – review & editing, Methodology, Investigation. **César Martín:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Fernando Civeira:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Ana Cenarro:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Ethical approval

All patients provided informed consent to a protocol previously approved by the Ethics Committee of our institution. The samples from patients included in this study were provided by the Biobank of the Aragon Health System (PT17/0015/0039), integrated in the Spanish National Biobanks Network, and they were processed following standard operating procedures with the appropriate approval of the Ethics and Scientific Committees.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data statement

The data that support the findings of this study are available on request from the corresponding authors. Due to confidentiality agreements with participants, the raw data are not publicly archived; however, deidentified datasets can be obtained from the corresponding author upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jacl.2025.11.017.

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