

Supplementary Materials for

Rare variant in scavenger receptor BI raises HDL cholesterol and increases risk of coronary heart disease

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P.Z. and S.A.K. performed and analyzed data from cell and animal experiments. S.A.K. and S.D. analyzed results of Penn sequencing and genotyping studies. M.C. and D.B.L. assisted in design and recruitment of subjects for deep clinical phenotyping studies. W.F.H-C. and A.K. performed lipoprotein characterization studies and cholesterol efflux capacity assays. S.A.K. and J.S.M. performed platelet cholesterol measurement assays. P.S. D.S., S.T., J.W.J., A.D.C., P.D., N.S., I.F., C.P. A a.S.M., D.S.A., E.D.A., G.A., R.C., J.E., B.G.N., S.F.N., A.T.H., R.F.S., K.K., D.L., M.P., S.B., V.S., S.M., P.A., D.A., J.F., M.M.-N., M.F., F.K., C.J.W., N.S., H.S., A.S.B., J.M.M.H., G.M.P., N.O.S., J.D., and S.K. contributed exome array genotyping data and analysis. D.J.R. funded, conceived and designed the study. P.Z., S.A.K. and D.J.R. wrote the manuscript with input from all of the authors.

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Materials and Methods

Subject Identification and Ascertainment

Individuals with high HDL-C levels above the 95th percentile for age and sex were systematically recruited. For this study 341 individuals of European ancestry with HDL-C > 95th percentile were selected for targeted sequencing, as were 407 individuals of European ancestry with low HDL-C levels below the 25th percentile for age and sex. Briefly, using customized hybrid capture (Agilent) baits, we targeted and enriched for sequencing the exons of the ~990 genes located within 300 kb of each of the 95 loci with significant associations ($P < 5 \times 10^{-8}$) with plasma lipid levels identified by the Global Lipids Genetics Consortium as of 2010 (22). We performed next-generation sequencing using Illumina Genome Analyzers at the Broad Institute as described previously (40). Base pairs were called and sequencing reads were aligned to the human genome reference GRCh37 (hg19), and sequencing metrics were processed using the Picard pipeline with an output of Binary Alignment Map (BAM) files.

The Genome Analysis Toolkit Unified Genotyper was used to genotype all variant sites, calculate initial quality control metrics, and filter based on these values, and variants were annotated using SnpEFF (41). Variants were indexed per sample in Variant Call Format (VCF) files. Genotypes for each individual were computed at each site and tabulated.

Additionally, *SCARB1* mutation carriers were identified by genotyping subjects with high vs. low HDL-C using the Exome Chip (HumanExome BeadChip v1.0, Illumina, Inc., San Diego, CA). The Exome Chip contains >240,000 coding SNPs derived from all mutations found >2 times across >1 data set among 23 separate data sets comprising a total of >12,000 individual exome and whole genome sequences. The P376L variant is included. In total, 353 high HDL-C males (HDL-C ≥ 70 mg/dL), 747 high HDL-C females (HDL-C ≥ 80 mg/dL), 1106 low HDL-C males (HDL-C ≤ 40 mg/dL), and 573 low HDL-C females (HDL-C ≤ 50 mg/dL) were genotyped using the Exome Chip. Subject samples were drawn from previous research studies conducted in our laboratory. After removing subjects from analysis for which demographic covariates were not available, there remained 805 subjects with high HDL-C and 989 subjects with low HDL-C analyzed for association of *SCARB1* P376L allele frequency with HDL-C.

To confirm the presence of the P376L mutation in carriers, a nearby region of 995 bp of *SCARB1* was amplified by PCR using the following primers: forward: TGGTTTGGTTGGTCAGTGGCG, reverse: AGGGCTGCCTCCAGCTCACAT; and the

following PCR conditions: 95°C, 2 min; (95°C, 30 s; 62.6°C, 30 s; 72°C; 100.0 s) repeat 29 times; 72°C, 5 min; 4°C forever. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Germantown, MD, USA) and sequenced by Sanger sequencing (42) by Genewiz Inc (South Plainfield, NJ, USA). Sequences were compared with *SCARB1* NCBI reference sequence NG_028199.1 using Sequencher (Gene Codes, Ann Arbor, MI, USA).

In silico analysis

To predict the effect of the P376L mutation on SR-BI protein structure and function we used the publicly available algorithms Condel and Raptor X (43-46). Condel generated a “consensus deleteriousness” score, obtained by combining 3 prediction algorithms (PolyPhen-2, MutationAssessor, and SIFT). Raptor X was used to predict effects on local secondary structure of the SR-BI due to the P376L variant. Protein alignment data were generated by MacVector (MacVector, Inc., Cary, NC) using a Gonnet similarity matrix with open gap penalty = 10 and extended gap penalty = 0.1.

DNA cloning and adeno-associated virus generation

The coding sequence of SR-BI (CCDS 9259.1) was obtained from Thermo Scientific (cDNA clone MGC:120767 IMAGE:7939577) in a pCR4-TOPO cloning vector (Invitrogen). The P376L and the P297S mutations were inserted in the sequence by site directed mutagenesis using the Quickchange II site directed mutagenesis kit (Agilent technologies) and following primers:

P376L-F 5'-cctggacatccacctgggtcacgggaatcc-3'

P376L-R 5'-ggattcccgtgaccaggtggatgtccagg-3'

P297S-F 5'-gggtgttgaaggcatctccacatatcgcttcgt-3'

P297S-R 5'-acgaagcgataggtggagatgccttcaaacacc-3'

The wild type and the mutated coding sequences were then amplified by PCR and subcloned into a pcDNA3.1/V5-His TOPO expression plasmid (Invitrogen) according to manufacturer's instructions. For *in vivo* studies, *SCARB1* WT and P376L cDNA sequences were amplified by PCR using the following primers containing KpnI and NotI sites, and then subcloned by KpnI/NotI double digestion followed by ligation into adeno-associated virus serotype 8 vector (AAV8) containing the liver-specific thyroxine-binding globulin (TBG) promoter provided by the University of Pennsylvania Vector Core (47-52). Virus production, purification and quantification were carried out by the core facility.

Radioactive labeling of HDL

Total human HDL ($1.063 < d < 1.21$ g/ml) and HDL₃ ($1.125 < d < 1.21$ g/ml) were obtained by sequential ultracentrifugation as described before (53). HDL was labeled with ¹²⁵I by a modification of the iodine monochloride method for the binding experiment at 4°C and they were labeled with ¹²⁵I-tyramine-cellobiose (¹²⁵I-TCB) for all the other experiments (54). To further label ¹²⁵I-TCB-HDL with ³H-cholesteryl-ether (³H-CE), 500 µCi of ³H-CE resuspended in 50 µl of ethanol were added to a solution containing heat-inactivated human lipoprotein deficient serum (200 mg protein) and iodinated HDL (6 mg protein). The solution was incubated overnight at 37°C with gentle shaking, followed by reisolation of the dual-labeled HDL by sequential ultracentrifugation.

Cell culture and in vitro assays

COS7 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO₂ incubator and were passaged using trypsin. For selective cholesterol uptake COS7 cells were plated at a density of 3×10^4 cells/cm² in 6 well plates. One day following plating (Day 1), cells were transfected using Lipofectamine 2000 (4 µg DNA/well, 3:1 Lipofectamine:DNA ratio) with a pcDNA3.1/V5-His-TOPO plasmid encoding either *SCARB1* WT, *SCARB1* P376L, or *SCARB1* P297S cDNAs. One group received a mock transfection with plasmid containing no cDNA. Each experimental group was tested in quintuplicate. On Day 2, culture medium was changed to DMEM 0.5% BSA containing 20 µg/ml of ³H-cholesteryl hexadecyl ether/¹²⁵I-TCB-labeled HDL₃ and incubated for 3 hours at 37°C. In one well per plate a 40-fold excess of nonradiolabeled (“cold”) HDL was added to a final concentration of 800 µg/ml. After incubation cells were washed with PBS and lipids were extracted in two consecutive steps using a 3:2 hexane-isopropanol mixture. After drying, proteins were solubilized using 2 ml of 0.1N NaOH. The hexane/isopropanol fraction was dried under nitrogen, resuspended in 600 µl of toluene, and counted separately in scintillation and gamma counters. The NaOH fraction was counted in the gamma counter and measured for protein concentration using a Lowry protein assay (55). Counts from wells with 40-fold excess of cold HDL (non receptor-specific counts) were subtracted from counts from other wells to calculate the receptor-specific activity internalized. Data analysis was performed as previously described (56), with the following modification: ¹²⁵I activity (counts per minute) in the NaOH fraction were considered as cell-surface associated lipoproteins (it has been shown previously that when

performing this experiment under similar conditions, the fraction of cell associated counts that is trichloroacetic acid-soluble is approximately 5%) (57). A negligible amount of ^{125}I counts were found in the hexane-isopropanol fraction. SR-BI protein expression was determined by western blotting. 10 μg of protein from cellular lysates determined by Lowry assay from each sample was loaded in NuPAGE gels (Life Technologies) using denaturing and reducing conditions for one-dimensional SDS-PAGE using MOPS running buffer. Proteins were separated by electrophoresis for approx. 1 hour, transferred to nitrocellulose membranes, blocked for 2 hours at room temperature using 5% fat-free milk in PBS (0.05% tween 20). Membranes were then incubated with anti-SR-BI antibody (NB400-131, Novus, 1:500 dilution in 5% milk-PBS-tween) at room temperature for 1 hour, washed three times with PBS-tween, and then with anti-goat IgG-HRP conjugate (700-035-147, Jackson ImmunoResearch, 1:2500 dilution in 5% milk-PBS-tween) at room temperature for 1 hour. Proteins were visualized after washing again after secondary antibody incubation using Luminata Crescendo chemiluminescent agent (Millipore). Films were incubated with membranes after chemiluminescent reagent in the dark typically for 30 s, 1 min, 2 min, and 5 min exposures for each membrane. Human β -actin was used as a loading control and was visualized after incubation with mouse anti-actin primary antibody (sc-81178, Santa Cruz, 1:20 dilution in 5% milk-PBS-tween) and then goat anti-mouse IgG HRP (sc-2302, Santa Cruz, 1:1000 in 5% milk-PBS-tween).

The binding of ^{125}I -HDL₃ at 4°C to COS7 cells was measured using a modification of the method from Nieland *et al* (58). Briefly, COS7 cells were seeded at a concentration of 3×10^4 cells/cm² in 12 well plates. The next day, cells were transfected using Lipofectamine 2000 (1.6 μg DNA/well, 3:1 Lipofectamine:DNA ratio) with a pcDNA3.1/V5-His-TOPO plasmid encoding either *SCARB1* WT, *SCARB1* P376L, or *SCARB1* P297S cDNAs. An additional plate of cells was transfected with a plasmid encoding GFP (pAAV-CB-EGFP), which was used as a control. On the following day, cell plates were precooled for 30 min on ice, washed with cold DMEM and then exposed for 2 hours to the assay medium (DMEM, 1% P/S, 0.5%BSA + lipoproteins) at 4°C. After the incubation, cells were washed twice with ice-cold PBS containing 2 mg/ml of bovine serum albumin (BSA) and a third time with PBS without BSA. Cells were then lysed in 0.1N NaOH. Lysates were used for ^{125}I counting and for measuring protein content using a Lowry assay. In parallel to this, cells were seeded in 10 cm plates, transfected as

described above and lysed after 24 hours in RIPA buffer to check SR-BI protein expression by immunoblotting for SR-BI as described above using 10 µg of protein from cellular lysates.

Cell surface biotinylation assay

COS7 cells were seeded and transfected as described above in 175 cm² flasks. Twenty-four hours after transfection, cell-surface-associated proteins were isolated using the Pierce Cell Surface Protein Isolation Kit (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer's instructions. After lysis, protein concentration was determined by bicinchoninic acid (BCA) assay and the same amount of proteins was loaded on NeutrAvidin Agarose beads to isolate biotinylated proteins. After multiple washing steps, proteins were eluted from the beads and loaded on a 10% Bis-Tris polyacrylamide gels for western blotting. Blots for β-actin and Na⁺/K⁺-ATPase were used as intracellular and surface-associated controls, respectively.

We generated induced pluripotent stem cells (iPSC) from peripheral blood mononuclear cells (PBMCs) and differentiated them into hepatocyte-like cells as described elsewhere (24-26, 59, 60) After complete differentiation of iPSCs to hepatocyte-like cells (approx. 20–22 days from initiation of differentiation), we performed selective cholesterol uptake as described above.

For Endoglycosidase H sensitivity assays, 100 µg of liver homogenates from mice transduced with human *SCARB1* or Null AAV8 vectors were treated with Endoglycosidase H (New England Biolabs, Ipswich, MA), Sialidase A, or a combination of Sialidase A and PNGase F (Prozyme, Hayward, CA) according to manufacturer's instructions. 30 µg of digestion products were then loaded on a 10% Bis-Tris polyacrylamide gel for western blotting as described above. Non-treated homogenates were used as controls. Endoglycosidase H sensitivity assays were also performed using lysates from transfected COS7 cells (10 µg each lysate) and from iPSC-derived hepatocyte-like cells (50 µg each lysate). In these experiments, cell lysate in RIPA buffer plus Complete Protease Inhibitor Cocktail (Roche) was used.

AAV-mediated overexpression of SCARB1 WT and P376L in Scarb1 KO mice

AAV serotype 8 vectors expressing *SCARB1* WT or P376L were expressed in *Scarb1* KO mice, which were subsequently studied for effects on lipoprotein metabolism. Male *Scarb1* KO mice (6 per group) were weighed, bled through retro-orbital bleeding and injected with 10^{12} genome copies (GC) of AAV-*SCARB1*-WT, AAV-*SCARB1*-P376L, or Null vector via intraperitoneal injection. Mice were fasted for 5 hours, weighed, and bled again at 12 days after injection. Plasma lipid levels were determined as described above. At 2 weeks after injection, HDL kinetics using radiolabeled human HDL was measured (61). Briefly, mice were injected via tail-vein injection with a mixture of ^{125}I -tyramine-cellobiose-HDL (3×10^6 cpm/mouse) and ^3H -cholesteryl-ether (CE)-HDL (3×10^6 cpm/mouse) and bled at 2 min post-injection and 1, 3, 6, 9, and 24 hours. Plasma ^3H and ^{125}I activity at each time point were determined by using scintillation counting and gamma counting, respectively. At 24 hours post-injection of radiolabeled HDLs, mice were weighed, anesthetized, terminally bled, and sacrificed. Livers were lysed in phosphate buffered saline (PBS), and ^3H and ^{125}I were counted in the lysates to determine liver-associated counts. Liver-associated counts were then expressed as micrograms of HDL component/mg of liver to allow a direct comparison between liver associated ^3H and ^{125}I counts. Selective cholesterol uptake was calculated as the difference between liver-associated ^3H counts and liver associated ^{125}I counts expressed as micrograms of HDL component/mg of liver. SR-BI protein expression levels in liver homogenates were determined by western blot as described above using 30 μg of protein from tissue lysates followed by densitometric analysis performed with ImageJ (U.S. National Institutes of Health, Bethesda, MD) (62).

Subject selection and study visit

Carriers of the P376L variant identified through next-generation sequencing were recruited through a comprehensive recall protocol approved by the institutional review board of the Perelman School of Medicine at the University of Pennsylvania, Philadelphia. Additionally, control subjects were chosen from a database of previous participants in studies conducted in our laboratory. All subjects recruited gave informed consent. Two groups of controls were selected: those having HDL-C levels between the 25th and the 75th percentile for age and sex (normal HDL-C control group) and those with HDL-C levels greater than the 75th percentile for age and sex but confirmed to lack the P376L variant (high HDL-C control group). The two control groups were selected to match on aggregate the age, gender and race of the carrier group. Study

visits were performed in the Clinical and Translational Research Center (CTRC) facility at the Perelman School of Medicine at the University of Pennsylvania. Venous blood after overnight fasting was drawn from each subject to measure a comprehensive metabolic panel, complete blood count, standard urinalysis, reticulocyte count, T- and B-cell counts, antinuclear antibody screen, anti double-stranded DNA antibody screen, and anti-neutrophil cytoplasmic antibody screen, which were measured by the William Pepper Laboratory of the Hospital of the University of Pennsylvania. A comprehensive lipid panel {total cholesterol [TC], VLDL cholesterol [VLDL-C], LDL cholesterol [LDL-C], HDL-C, triglycerides [TG], lipoprotein(a) [Lp(a)], and apolipoproteins A-I, A-II, C-II, C-III, and E [ApoA-1, ApoA-II, ApoC-II, ApoC-III, ApoE]} was also performed by the Lipid Research Laboratory of the Hospital of the University of Pennsylvania per standard protocols as described previously (63). HDL-C and LDL-C levels were measured both directly and after precipitation with phosphotungstic acid (PTA). Lipoproteins were separated by fast protein liquid chromatography on a Superose 6 column as described previously (64).

Blood was also collected in BD Vacutainer CPT Cell Preparation tubes (BD, Franklin Lakes, NJ, USA) for PBMC isolation and storage and in sodium citrate tubes for platelet isolation and testing. Platelet aggregation studies (PAS) were performed at the Translational Core Laboratory (TCL) using a photometric aggregometer (Biodata Corp, Horsham, PA).

To induce platelet aggregation the following stimuli were used at the concentrations reported in brackets: arachidonic acid (200, 250, 300, 400, 500 mg/ml), collagen (0.04, 0.08, 0.12, 0.16, 0.2 mg/ml), ADP (0.625, 1.25, 2.5, 5, 10 mM), TRAP (1, 2, 3, 4, 5 μ M). Arachidonic acid, collagen, and ADP were obtained from Biodata Corp. (Horsham, PA); TRAP was obtained from Sigma Chemical Corp (St. Louis, MO). To correct for possibly unreported intake of drugs with antiaggregant action, subjects whose platelets failed to reach 95% aggregation with 500 mg/ml of arachidonic acid were excluded from the analysis. Additional blood was also drawn and frozen for later batch measurement of adrenocorticotrophic hormone (ACTH), cortisol, estradiol, progesterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone also by the TCL using standard radioimmunoassay methods ("Coat-A-Count") from SIEMENS Healthcare Diagnostics. Subjects taking exogenous steroids were excluded from the analysis. Study subjects were also given the option of performing a 24-hour urine collection according to standard methods the day before their visit or on a later date. Collected urine was

frozen in single-use aliquots for batch measurement of cortisol by the Translational Core Laboratory as per standard protocols. The acquisition of carotid intima-media thickness (IMT) ultrasound images was performed according to a standardized protocol, adopted from the Atherosclerosis Risk in Communities (ARIC) study (65) and as per American Society of Echocardiography and Society for Vascular Medicine Guidelines for IMT analysis (66). The scanner used was a Siemens Sequoia (Mountain View, CA, USA) with a 9 Linear probe and a custom designed carotid IMT preset. One heterozygous subject who was unable to travel to the study site had blood drawn locally. These samples were shipped to the study site for the lipid panel and autoimmune tests; the subject also sent the results of a recent comprehensive metabolic panel and complete blood count. Data from clinical phenotyping studies were collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted at the Perelman School of Medicine at the University of Pennsylvania (67).

Platelet separation and cholesterol measurement

Approximately 4.5 ml of blood was drawn from each subject into tubes containing 25 g sodium citrate, 8 g citric acid and 500 ml H₂O₂. Tubes were centrifuged using a tabletop centrifuge at 200xg for 15 min. at room temperature. Platelet-rich plasma (PRP) was collected as supernatant from this spin and was centrifuged at 900g for 5 min. The pellet from this spin was resuspended in 8 ml 1xPBS and centrifuged again at 900g for 5 min. Platelet pellets were collected and resuspended in 200 µl 1 × PBS and stored at –20°C. For measurement of cholesterol, platelet extracts were dried using a centrifugal evaporator (Genevac). D7-cholesterol (Cat. No. 700041; Avanti Polar Lipids, Inc.) was added to each sample as an internal standard. Lipids were extracted by addition of 1 ml chloroform:methanol (2:1) at 4°C for 2 hours. The nonpolar phase from the platelet extracts was collected and dried through centrifugal evaporation along with cholesterol standards (1.0–100.0 nmol) containing equivalent amounts of D7-cholesterol internal standard. All samples were derivativized using pentafluorobenzoyl chloride and extracted with petroleum ether before measurement by gas chromatography-mass spectrometry using negative chemical ionization. Peak areas for cholesterol and D7-cholesterol from platelet extracts were compared to that from the standard curve generated from cholesterol standards to yield cholesterol content in each sample. For platelet protein measurement, aqueous extracts from chloroform:methanol extraction of platelet samples were spun to remove excess methanol and dried using centrifugal evaporation and then resuspended in 200 µl RIPA buffer

containing 0.1 N NaOH and heated at 55°C for approximately 24 hours with periodic vortexing. Protein concentrations from these extracts were measured by a Lowry assay (55) (Pierce). Cholesterol measurements for each sample were then normalized to the corresponding protein concentrations.

Fractionation of plasma lipoproteins from study subjects

VLDL, LDL, and HDL subfractions were isolated from EDTA plasma by single step, isopycnic non-denaturing density gradient ultracentrifugation based on a modification of the method developed by Chapman et al. as previously described(68, 69). Using this procedure, five subfractions of LDL (LDL₁, d 1.019–1.023 g/ml; LDL₂, d 1.023–1.029 g/ml; LDL₃, d 1.029–1.039 g/ml; LDL₄, d 1.039–1.050 g/ml; and LDL₅, d 1.050–1.063 g/ml) were isolated, followed by two subfractions of large, light HDL₂ (HDL_{2b}, d 1.063–1.087 g/ml and HDL_{2a}, d 1.088–1.110 g/ml), and three subfractions of small, dense HDL₃ (HDL_{3a}, d 1.110–1.129 g/ml; HDL_{3b}, d 1.129–1.154 g/ml; and HDL_{3c}, d 1.154–1.170 g/ml). Total HDL was prepared as a mixture of HDL_{2b}, 2a, 3a, 3b and 3c subfractions at their equivalent plasma concentrations.

Protein quantification and chemical composition of HDL

Overall chemical composition of HDL subfractions was assessed using commercially available enzymatic kits (total protein: Thermo Scientific, Villebon-sur-Yvette, France; total cholesterol, free cholesterol, phospholipids: DiaSys, Holzheim, Germany; triglycerides: Biomérieux, Marcy l'Etoile, France). Cholesteryl ester (CE) concentration was calculated by multiplying the difference between total and free cholesterol concentrations by 1.67. Total lipoprotein mass was calculated as the sum of total protein, CE, FC, PL and TG and expressed as plasma concentrations (mg/dl). HDL apolipoproteins (ApoA-I, ApoA-II, ApoC-III) were quantified using automated enzymatic methods (Konelab, Thermo Scientific, Waltham, MA, USA).

Cellular cholesterol efflux capacity of HDL

The cholesterol efflux capacity of total HDL was assessed in a human THP-1 monocytic cell line (ATCC, Manassas, VA, USA) as previously reported(70). THP-1 monocytes were cultured at 37°C and 5% CO₂ in RPMI 1640 media with 10% FBS, 2 mM glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin (complete media) using standard cell culture procedures and differentiated into macrophage-like cells using 50 ng/ml phorbol 12-myristate 13-acetate (PMA). Cells were loaded for 24 h with [³H] cholesterol-labeled acetylated LDL (acLDL, 1

μCi/ml) in serum-free RPMI 1640 culture medium supplemented with 50 mM glucose, 2 mM glutamine, 0.2% BSA, 100 μg/ml penicillin and 100 μg/ml streptomycin (serum-free media). After equilibration in serum-free media, cells were incubated for 4 h with HDL from subjects. Efflux capacity was normalized to HDL-phospholipid content because PL has been shown to represent the key component in determining cholesterol efflux capacity of HDL (15 μg/ml HDL-PL in serum-free media, total volume 250 μL) (27). Cholesterol efflux capacity was measured as the percent of radioactivity counts in the media over counts in the cell lysate, after adjustment for non-specific diffusion to non-HDL containing media.

Statistical analysis

Data analysis was conducted using Excel (Microsoft Corp.) and GraphPad Prism 6.0 (GraphPad Software Inc.). *In vitro* and *in vivo* data were compared by Student's unpaired T-test. A p-value of less than 0.05 was considered statistically significant. The same test was employed to compare HDL subclasses and composition between controls and heterozygotes. For data regarding clinical samples, Chi-square testing (for categorical variables) and one-way ANOVA (for continuous variables) were used to determine the effect of *SCARB1* P376L carrier status on a number of variables. A P value less than 0.05 was considered statistically significant. Tukey's multiple comparison test ($\alpha=0.05$) was used following ANOVA where appropriate to determine which groups were responsible for statistically significant differences among groups. Data are reported as mean \pm SD. The association of P376L and CHD was tested among CHD cases and controls of European ancestry assembled from the CARDIoGRAM Exome Consortium and the CHD Exome+ Consortium. The summary OR of CHD for P376L carriers was calculated using a Mantel-Haenszel fixed-effects meta-analysis without continuity correction, a method that is well suited to low (and even zero) counts and resultant ORs. The association of the P376L variant with HDL-C, LDL-C and TG in the Global Lipids Genetics Consortium Exome Chip Genotyping study was assessed through meta-analysis of single variant score statistics in up to 301,025 individuals (71).

Supplemental Text

Conservation of SR-BI Proline 376 Across Species and Paralogues

The proline at position 376 of SR-BI is highly conserved down through *Drosophila* and *Anopheles*, as well as in the closely related human genes CD36 and LIMP2 and is in a sequence of highly invariant residues (**Fig. S1**). The “consensus deleteriousness” score according to Condel was 0.906 (on a scale from 0 to 1, with 1 indicating maximum deleteriousness). Raptor X, a secondary structure prediction program, predicts that substitution of proline 376 with leucine increases the probability of this region to be in a beta-sheet confirmation from 36% to 61%. This finding is in agreement with the suggestion from the recently reported structure of another scavenger receptor LIMP-2 (72) that the region containing P376 in SR-BI is a disordered linker sequence joining beta strands 15 and 16 on the extracellular loop proximal to the C-terminal transmembrane domain.

Impact of SCARB1 P376L on SR-BI processing and cholesterol ester uptake in transfected COS7 cells

We tested the functional impact of the P376L variant on HDL-CE selective uptake in transfected COS7 cells. Studies with transfected COS7 cells revealed that the P376L variant was defective in promoting selective CE uptake (**Fig. S3A**) despite similar protein expression in total cell lysates (**Fig. S3B**). Notably, the P376L variant had less activity in this assay than one previously reported P297S variant (32) (**Fig. S3A**). In studies of ¹²⁵I-labeled HDL₃ binding at 4 °C, the P376L variant abrogated cell-surface binding (**Fig. S3C**), despite equal SR-BI protein levels in total cell lysates among groups (**Fig. S3D**).

We hypothesized that the reduced CE uptake and cell-surface HDL-binding by the SR-BI P376L variant could be due to abnormal processing of the mutant protein, preventing its cell-surface localization. To study this, we isolated cell-surface proteins from COS7 cells transfected with *SCARB1* WT, P376L and P297S using biotinylation and streptavidin pull-down. Western blotting of whole cell lysates compared with purified biotinylated samples (as tested by probing for β -actin and Na/K-ATPase) indicated that cell-surface expression of SR-BI P376L was markedly reduced compared to both WT and P297S (**Fig. S3E**). This suggested that the P376L mutation causes SR-BI to be retained inside the cell after translation. We next sought to determine the molecular defect underlying loss of cell-surface localization and CE uptake function by SR-BI P376L observed in the cell and mouse models. Given that SR-BI undergoes

N-glycosylation in the endoplasmic reticulum concomitant with proper folding and localization, we hypothesized that altered post-translational modification may underlie its reduced cell-surface localization (27-29). We measured the molecular weights of SR-BI forms after endoglycosidase-H (Endo-H) treatment of transfected COS7 expressing WT or mutant SR-BI (**Fig. S3F**). In COS7 cell lysates in the absence of Endo-H, whole cell lysates from cells transfected with SR-BI WT, P376L, or the P297S variant displayed a single band of approximately 65 kDa, representing fully glycosylated mature SR-BI. For all three constructs, Endo-H treatment resulted in a major band of approximately 45 kDa, representing the fully-deglycosylated (fully Endo-H sensitive) SR-BI protein. However, WT and P297S displayed additional larger bands consistent with a pool of partially Endo-H sensitive SR-BI possessing complex N-linked glycans, whereas P376L displayed only the single, unmodified, fully-sensitive band. Because of high levels of cDNA overexpression in the COS7 transfection, we believe the majority of the SR-BI across all groups in this experiment is not fully processed, thus resulting the relatively higher proportion of the total SR-BI exhibiting complete Endo-H sensitivity (**Fig. S3F**) relative to that observed in analogous experiments in iPSC-derived hepatocyte-like cell and murine hepatocyte lysates after Endo-H treatment (**Fig. 2F-G** of main text).

Phenotyping studies of P376L homozygote and heterozygotes

The P376L homozygote and eight P376L heterozygotes were recruited for additional deep phenotyping. Two age, sex, and race matched control groups were used, one with normal HDL-C levels (25th–75th percentile for age, race and sex) and a second with high HDL-C levels (>95th percentile for age, race and sex) in which *SCARB1* mutations were excluded. All of the P376L study participants were of European ancestry, almost exclusively of Ashkenazi Jewish descent. Two P376L heterozygotes, 4 normal HDL-C controls and 4 high HDL-C controls reported an alcohol intake of more than 1 drink per day, but there was no self-reported alcohol abuse. Smokers and subjects with diabetes were excluded. The P376L homozygote reported having a seizure disorder that was not treated pharmacologically at the time of participation.

Previous *in vitro*, mouse and human genetics studies have suggested that SR-BI in platelets is necessary for proper platelet activity and thrombosis (6, 32, 73-75). To test the effects of the P376L variant on platelet activity, we isolated platelets from carriers and controls and performed light transmission aggregometry (LTA) after stimulation with known platelet activators arachidonic acid, collagen, ADP and TRAP over a range of doses. We found only a

slight decrease in ADP-induced maximal aggregation in platelets isolated from the P376L homozygote relative to heterozygotes and control subjects at a dose of 5 mmol (**Fig. S5A**). No differences in platelet activity in response to other stimulants were observed between the groups. We also extracted lipids from platelets and measured platelet cholesterol content among groups. We observed that platelet cholesterol increased in a genotype dose-dependent manner from controls (mean 122 nmol/mg protein) to heterozygotes (mean 139 nmol/mg protein) to the homozygote subject (244 nmol/mg protein) (**Fig. S5B**). However, the difference between normal HDL-C controls and heterozygotes was not significant, and these differences were reduced when the values normalized to plasma total cholesterol levels, suggesting that elevated platelet cholesterol in carriers reflects increased plasma HDL-C levels rather than a platelet SR-BI specific function (**Fig. S5C**). There was also no difference in total circulating platelet levels among groups (data not shown).

SR-BI also takes up HDL-cholesteryl esters in adrenal glands and reproductive tissues for steroid hormone production in mice and humans (6, 18, 76), so we evaluated the impact of SCARB1 loss-of-function on steroid hormones in our recruited participants. We found no difference in morning serum cortisol, ACTH and 24-hr urinary cortisol-to-creatinine (**Fig. S6**) across participants, moderately higher testosterone in male P376L heterozygotes relative to normal HDL-C controls, but no differences across groups in FSH and LH.

Fig. S1

<i>Homo sapiens</i>	L	N	A	D	P	V	L	A	E	A	V	T	G	L	H	P	N	Q	E	A	-	H	S	L	F	L	D	I	H	P	V	T	G	I	P	M	N	C	S	V	K	L	Q	L	S	L	Y	M	K	S	V	A	G	I	G	Q	T	G	K	I
<i>Pan troglodytes</i>	L	N	A	D	P	V	L	A	E	A	V	T	G	L	H	P	N	Q	E	A	-	H	S	L	F	L	D	I	H	P	V	T	G	I	P	M	N	C	S	V	K	L	Q	L	S	L	Y	M	K	S	V	A	G	I	G	Q	T	G	K	I
<i>Macaca mulatta</i>	L	N	A	D	P	V	L	A	E	A	V	T	G	L	H	P	N	Q	E	A	-	H	S	L	F	L	D	I	H	P	V	T	G	I	P	M	N	C	S	V	K	L	Q	L	S	L	Y	M	K	S	V	A	G	I	G	Q	T	G	K	I
<i>Canis lupus familiaris</i>	Y	N	A	D	P	M	L	A	E	A	V	L	G	L	H	P	N	Q	E	E	-	H	S	L	F	L	D	I	H	P	V	T	G	I	P	M	N	C	S	V	K	L	Q	L	S	L	Y	I	K	A	I	K	G	I	G	Q	T	G	K	I
<i>Bos taurus</i>	Y	N	A	D	P	V	L	A	E	A	V	S	G	L	H	P	N	P	K	E	-	H	S	L	F	L	D	I	H	P	V	T	G	I	P	M	N	C	S	V	K	L	Q	L	S	L	F	V	K	S	V	K	G	I	G	Q	T	G	K	I
<i>Mus musculus</i>	Y	N	A	D	P	V	L	S	E	A	V	L	G	L	N	P	N	P	K	E	-	H	S	L	F	L	D	I	H	P	V	T	G	I	P	M	N	C	S	V	K	M	Q	L	S	L	Y	I	K	S	V	K	G	I	G	Q	T	G	K	I
<i>Rattus norvegicus</i>	Y	N	A	D	P	V	L	S	E	A	V	L	G	L	N	P	D	P	K	E	-	H	S	L	F	L	D	I	H	P	V	T	G	I	P	M	N	C	S	V	K	M	Q	L	S	L	Y	I	K	S	V	K	G	V	G	Q	T	G	K	I
<i>Gallus gallus</i>	Y	N	A	D	P	S	L	V	D	A	V	E	G	L	H	P	S	R	E	E	-	H	A	L	F	L	D	I	H	P	V	T	G	I	P	M	N	C	S	I	K	L	Q	L	N	Q	Y	M	K	Q	V	S	G	I	L	Q	T	G	K	I
<i>Salmo salar</i>	Y	N	A	D	P	V	L	L	D	Y	V	Q	G	L	Q	P	T	E	D	E	-	H	G	L	F	I	D	I	H	P	E	T	G	V	P	L	N	V	S	I	R	L	Q	L	N	L	Y	M	K	K	V	S	G	I	T	E	T	G	K	I
<i>Danio rerio</i>	Y	N	A	D	P	Q	L	L	D	T	V	S	G	L	S	P	S	E	D	E	-	H	G	L	F	I	D	I	H	P	E	T	G	V	P	L	N	V	S	I	R	L	Q	L	N	L	F	L	K	V	S	G	I	S	E	T	G	N	I	
<i>Drosophila melanogaster</i>	Y	L	A	D	E	S	L	R	T	Q	V	E	G	I	S	P	P	M	K	E	K	H	Q	F	F	D	V	Q	P	K	M	G	T	T	L	R	V	R	A	R	I	Q	I	N	L	A	V	S	Q	V	F	D	I	K	Q	V	A	N	F	
<i>Anopheles gambiae</i>	Y	M	A	D	Q	T	L	R	T	A	V	E	G	I	S	P	P	E	K	D	K	H	Q	L	F	I	D	V	Q	P	D	M	G	T	A	L	R	A	R	A	R	I	Q	I	N	L	A	V	S	Q	V	F	D	I	K	Q	V	A	N	F
<i>hCD36</i>	L	Y	A	S	P	D	V	S	E	P	I	D	G	L	N	P	N	E	E	E	-	H	R	T	Y	L	D	I	E	P	I	T	G	F	T	L	Q	F	A	K	R	L	Q	V	N	L	L	V	K	P	S	E	K	I	Q	V	L	K	N	L
<i>hLIMP2</i>	Y	Q	A	D	E	R	F	V	S	A	I	E	G	M	H	P	N	Q	E	D	-	H	E	T	F	V	D	I	N	P	L	T	G	I	I	L	K	A	A	K	R	F	Q	I	N	I	Y	V	K	L	D	D	F	V	E	T	G	D	I	

Fig. S1. SR-BI Protein Sequence Alignment Across Species. Amino acid sequence alignment of SR-BI across 12 species and human SR-BI paralogues CD36 and LIMP-2. Shown is the ~60 residue sequence alignment adjacent human SR-BI residue Pro376 (indicated by red box). Dark grey shading indicates full conservation of a given residue across indicated species. Light grey shading indicates a different but conservative amino acid for the given species compared to the others listed.

Fig. S2

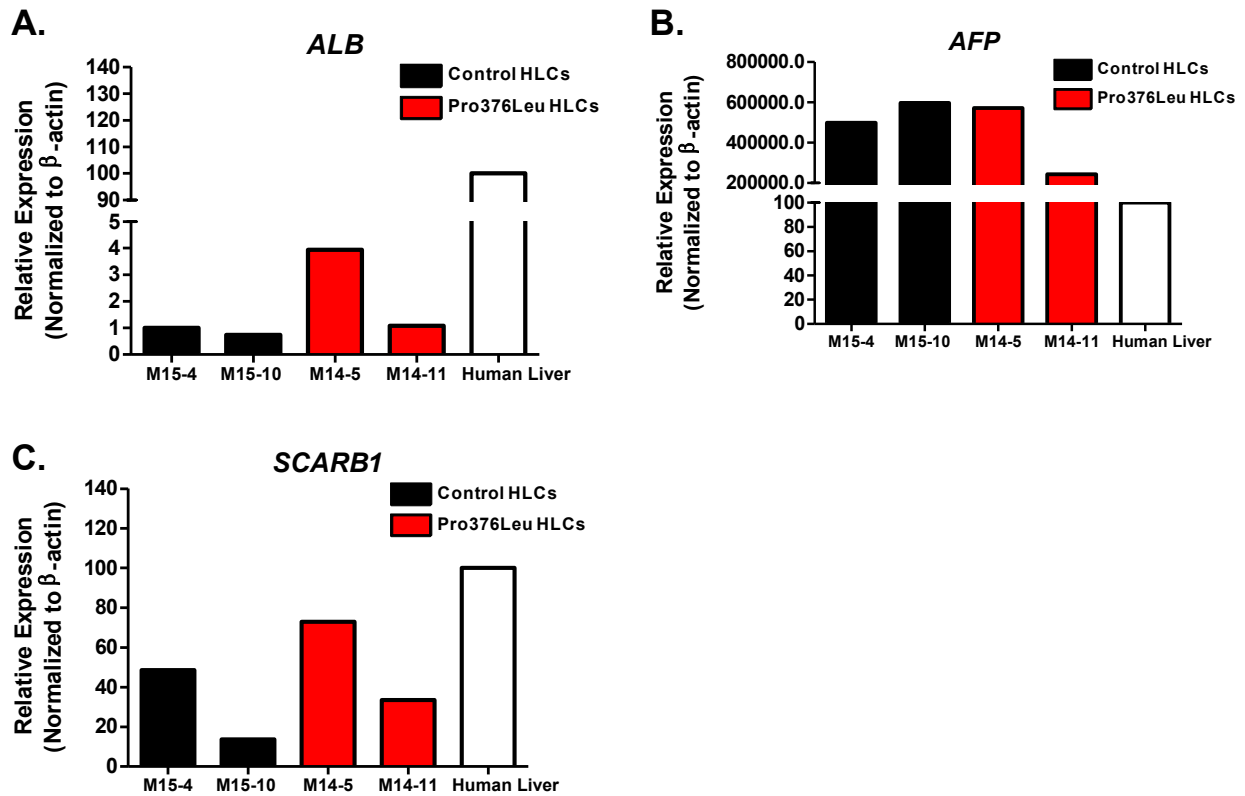


Fig. S2. Gene expression in control and *SCARB1* P376L iPSC-derived hepatocyte-like cells (HLCs). **A.** *ALB* Gene expression by quantitative RT-PCR of mRNA from control and P376L mutant iPSC-derived HLCs. Cells were differentiated 21–25 days before experiments and RNA isolation for gene expression analysis. **B.** *AFP* gene expression in iPSC-derived HLCs. **C.** *SCARB1* gene expression in iPSC-derived HLCs.

Fig. S3

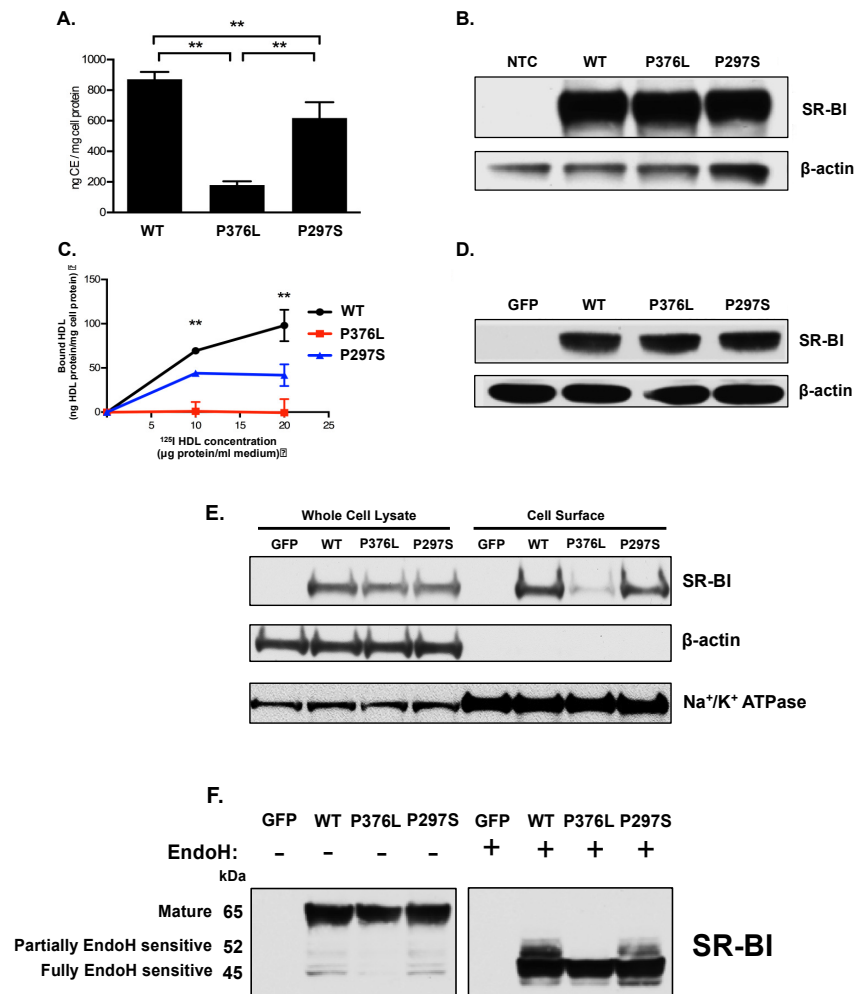


Fig S3. *SCARB1* P376L abrogates SR-BI function in transfected COS7 cells. **A.** Selective cholesterol uptake in COS7 cells expressing SR-BI WT vs. P376L. Cells were transfected with plasmids expressing WT, P376L or P297S forms of SR-BI and incubated at 37°C for 3 hours with 125 I/ 3 H-labeled HDL₃ to determine HDL cholesterol ester (CE) specific uptake. Data represent the mean of a quintuplicate determination after subtraction of the determinations done with the addition of a 40-fold excess of cold HDL. **B.** Western blot showing SR-BI expression levels in whole cell lysates from COS7 cells transfected for selective cholesterol uptake experiment in (A). **C.** Binding of HDL to SR-BI at 4°C in transfected COS7 cells. Transfected cells were exposed to 125 I-labeled HDL₃ for 2 hours at 4°C to measure HDL binding. Radioactive counts from cells were then quantified to determine the amount of cell-associated 125 I-HDL₃. Data points represent the mean +/- S.D. of a triplicate determination after subtraction of the

determinations done with the addition of a 40-fold excess of unlabeled HDL₃. ** P<0.01, one-way ANOVA. **D.** Western blot showing SR-BI expression levels in whole cell lysates from COS7 cells transfected in panel (C). **E.** Immunoblot of SR-BI after cell-surface biotinylation in transfected COS7 cells. Cells were transfected with GFP, SR-BI WT, P376L or P297S plasmids and biotinylated before collection of whole cell lysate (left) or incubation with NuetrAvidin beads and elution of cell-surface localized proteins (right). Whole cell lysates (lanes 1–4) and cell-surface proteins (lanes 5–8) were separated by SDS-PAGE and immunoblotted for human SR-BI. Actin and Na/K-ATPase were used respectively as intracellular and surface-associated controls. **F.** Endo-H sensitivity of SR-BI from transfected COS7 cells. Cells were transfected with plasmids encoding GFP or different forms of SR-BI (WT, P376L, P297S) and cell lysates were treated with Endo-H to release complex N-linked glycans and molecular forms of SR-BI were monitored by immunoblotting. For A & C, data represent mean Error bars indicate mean values \pm S.D. ** P<0.01, Student's unpaired T-test.

Fig. S4

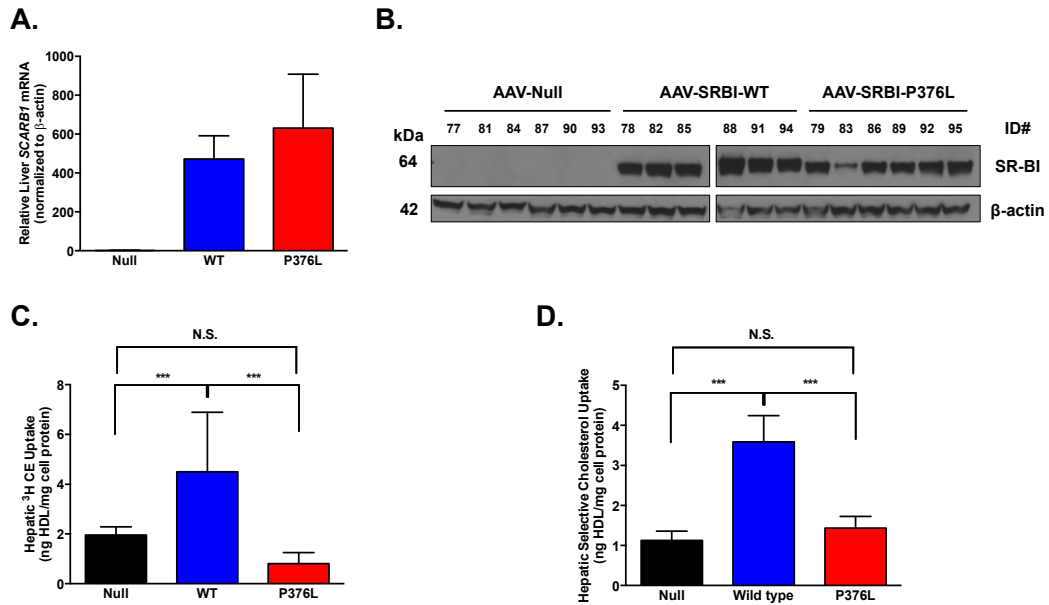


Fig. S4. Hepatic *SCARB1* expression and impact on selective cholesterol uptake from HDL in mice. **A.** Human *SCARB1* transcript expression levels measured by quantitative RT-PCR from livers of mice expressing Null or SR-BI AAV vectors. Gene expression was measured from total hepatic RNA after reverse transcription and normalized to expression levels of actin. **B.** SR-BI immunoblot (right) from livers of *Scarb1* KO mice expressing Null, SR-BI WT, and SR-BI P376L 2 weeks after AAV administration. **C.** Liver ^3H CE uptake from dual-labeled HDL administration in mice expressing Null or SR-BI AAVs. **D.** Hepatic selective cholesterol uptake measured by relative difference of hepatic ^3H CE and ^{125}I TC uptake in livers of mice expressing Null or SR-BI AAVs after dual-labeled HDL administration. All data represent mean values \pm S.D. for each of the 3 groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Unpaired T-test.

Fig. S5

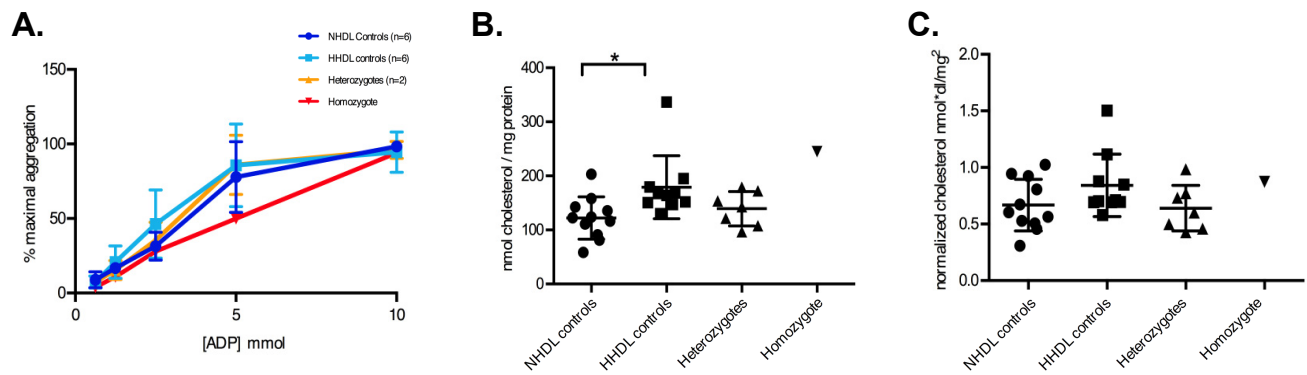


Fig. S5. Platelet aggregation and cholesterol content. **A.** Platelet aggregation measured by light transmission aggregometry after stimulation with increasing doses of ADP. Data represent the percentage maximal aggregation. **B.** Platelet cholesterol content measured by LC/MS. **C.** Platelet cholesterol content after normalization for plasma total cholesterol levels. Bars represent mean values \pm S.D.. * $P<0.05$, one-way ANOVA followed by Tukey's multiple comparisons test.

Fig. S6

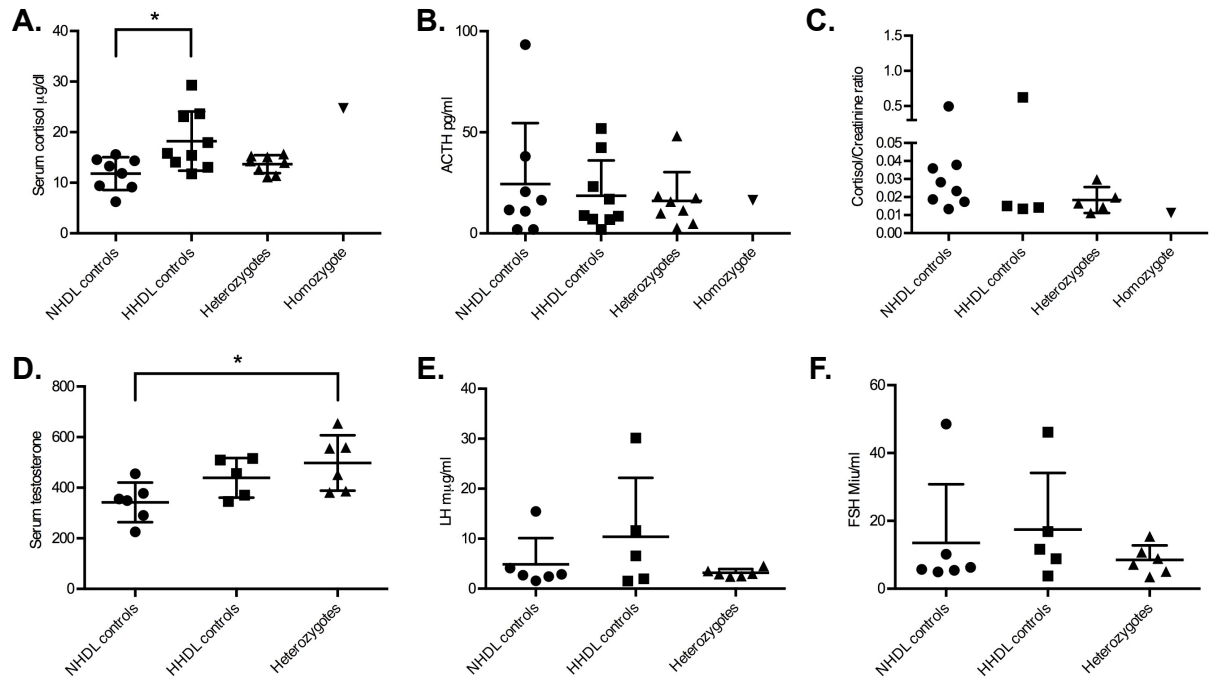


Fig. S6. Impact of *SCARB1* P376L on adrenal and gonadal steroidogenesis. **A.** Morning serum cortisol in carriers vs. controls. **B.** Morning plasma ACTH. **C.** Cortisol / creatinine ratio in 24-hour urine. **D.** Serum testosterone in males. **E.** Serum LH in males. **F.** Serum FSH in males. Bars represent mean values \pm S.D.. * $P < 0.05$, one-way ANOVA followed by Tukey's multiple comparisons test.

Fig. S7

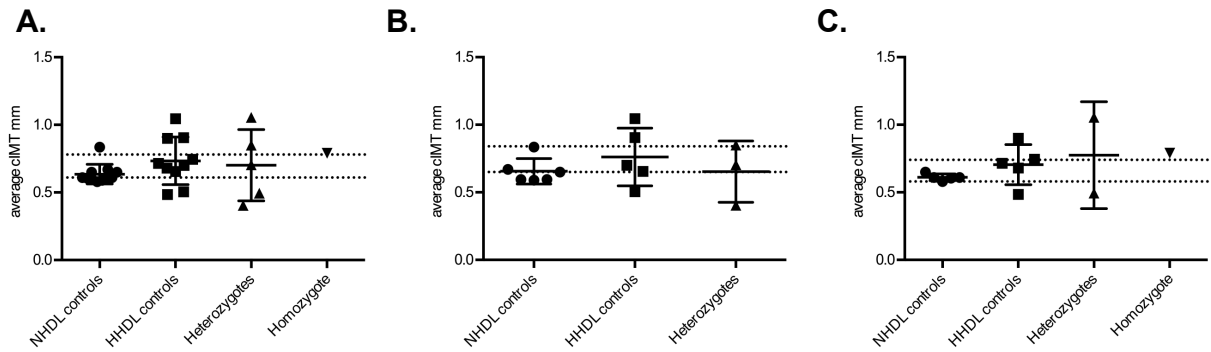


Fig. S7. Carotid intima media thickness (cIMT) in *SCARB1* P376L carriers vs. controls. A. cIMT for all subjects (male and female combined). **B & C.** cIMT results for males and females, respectively. Dotted lines represent the 25th and the 75th percentile values from the ARIC study. Bars represent mean values \pm S.D.. All data shows the average left / right cIMT.

Table S1. Association of *SCARB1* P376L with plasma lipid traits in global lipids genetics consortium exome array genotyping. The relationship between the frequency of P376L carriers and plasma lipid traits was measured in the Global Lipids Genetics Consortium cohort by genotyping of the variant on the Exome Array and using the Score test.

Trait	Number of subject included	Minor allele frequency	Beta (SE) in SD	<i>P</i> value (score test)
HDL-C	301025	0.00033	+0.57 (0.071)	1.41×10^{-15}
LDL-C	280551	0.00033	+0.065 (0.074)	0.381
TG	290277	0.00034	−0.052 (0.072)	0.474

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