Supplemental Materials

Identification of a gain-of-function *LIPC* variant as a novel cause of familial combined hypocholesterolemia

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Supplemental Figures S1-5

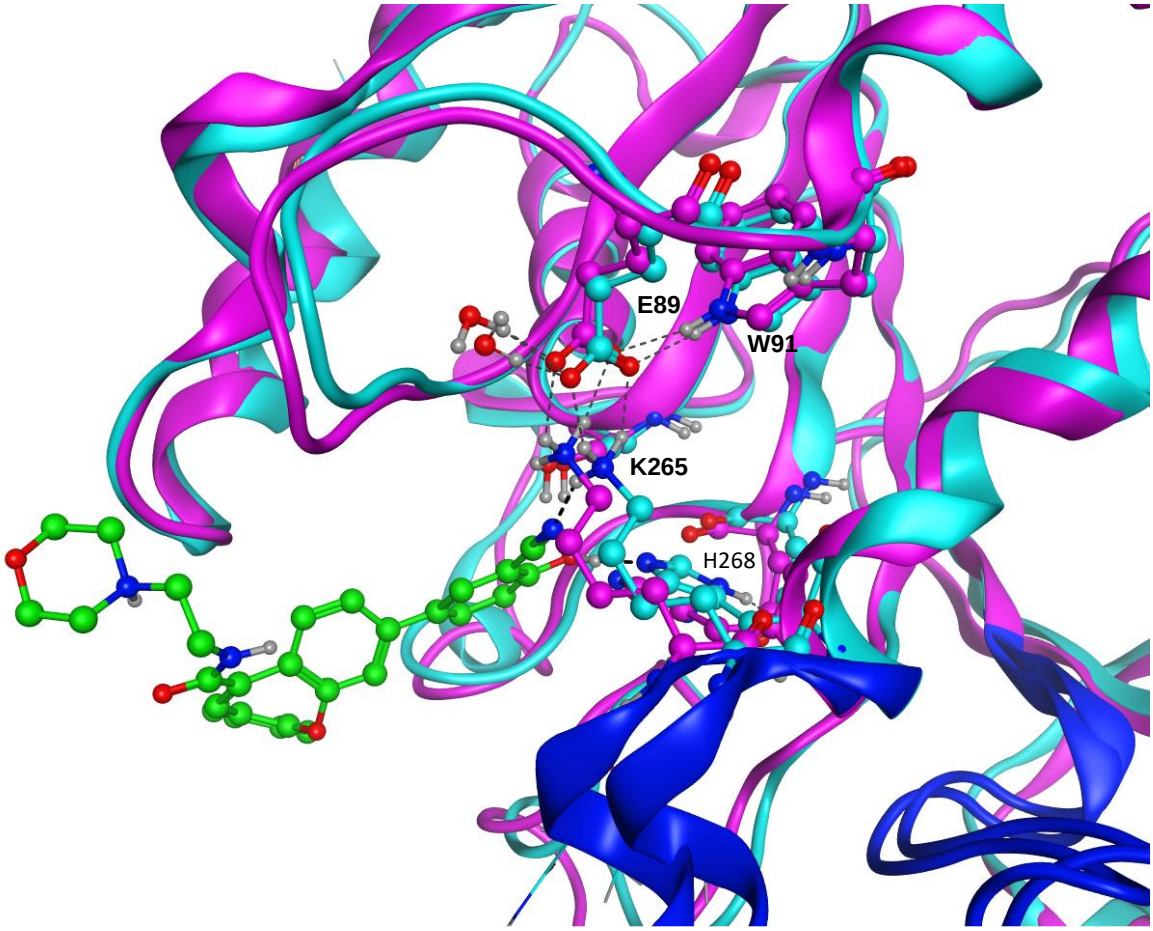
Supplemental Tables S1-2

Expanded Materials & Methods

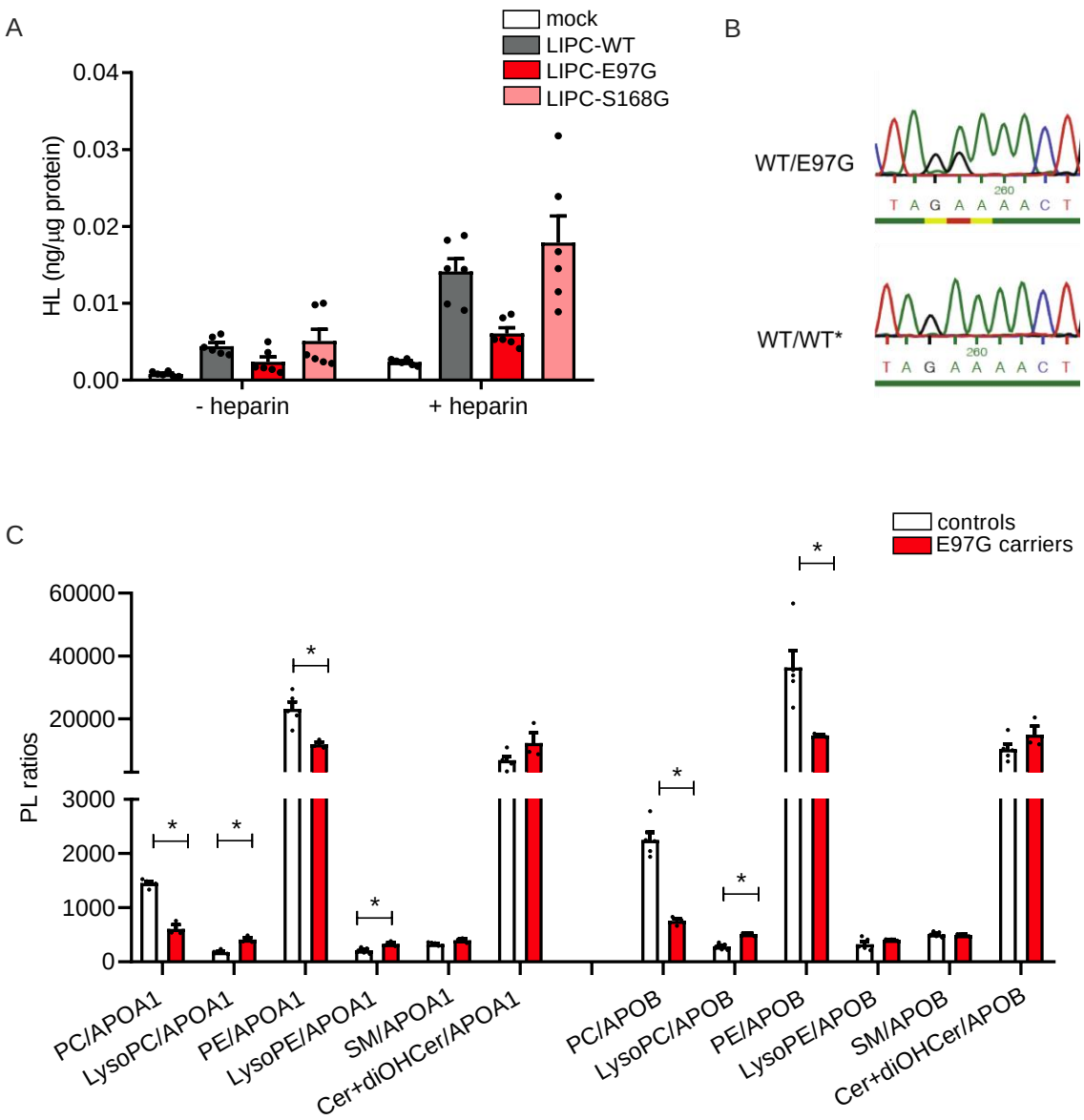
Abbreviations

APOA1	Apolipoprotein A1
APOB	Apolipoprotein B
APOE*3.Leiden	Apolipoprotein E*3.Leiden
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CO	Cholesteryl oleate
EC	Esterified cholesterol
FPLC	Fast protein liquid chromatography
IDL	Intermediate-density lipoprotein
IHH	Immortalized human hepatocytes
HDL-C	High-density lipoprotein cholesterol
HL	Hepatic lipase
HMGCOR	3-Hydroxy-3-Methylglutaryl-CoA Reductase
LDL-C	Low-density lipoprotein cholesterol
LIPC	Lipase C, hepatic type (Hepatic Lipase)
LPL	Lipoprotein lipase
PL	Phospholipids
PLA1	Phospholipase A1
TBG	Thyroxin-binding globulin
TG	Triglycerides
TGRL	Triglyceride-rich lipoprotein
TO	Triolein
VLDL	Very low-density lipoprotein
WGS	Whole genome sequencing

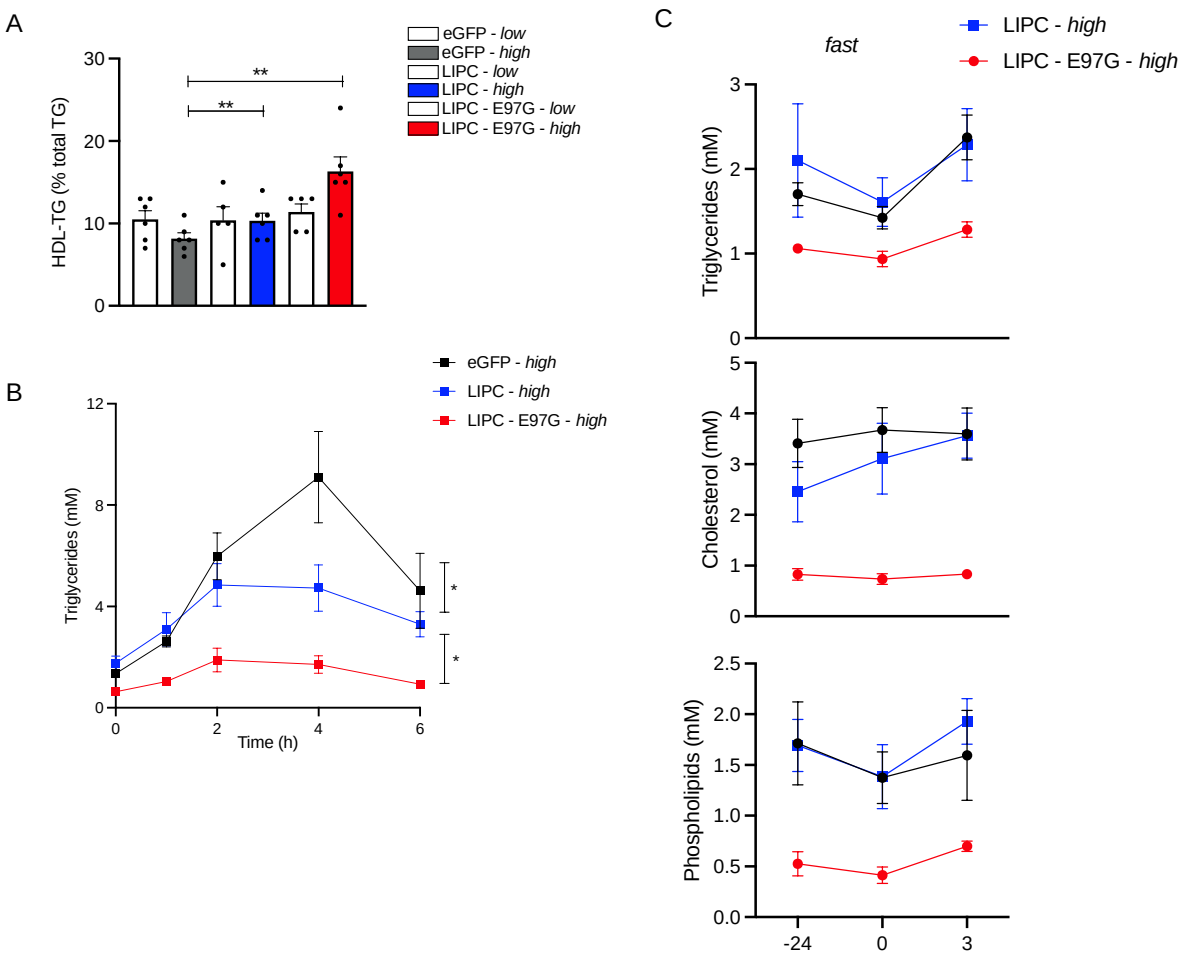
Supplemental Figures



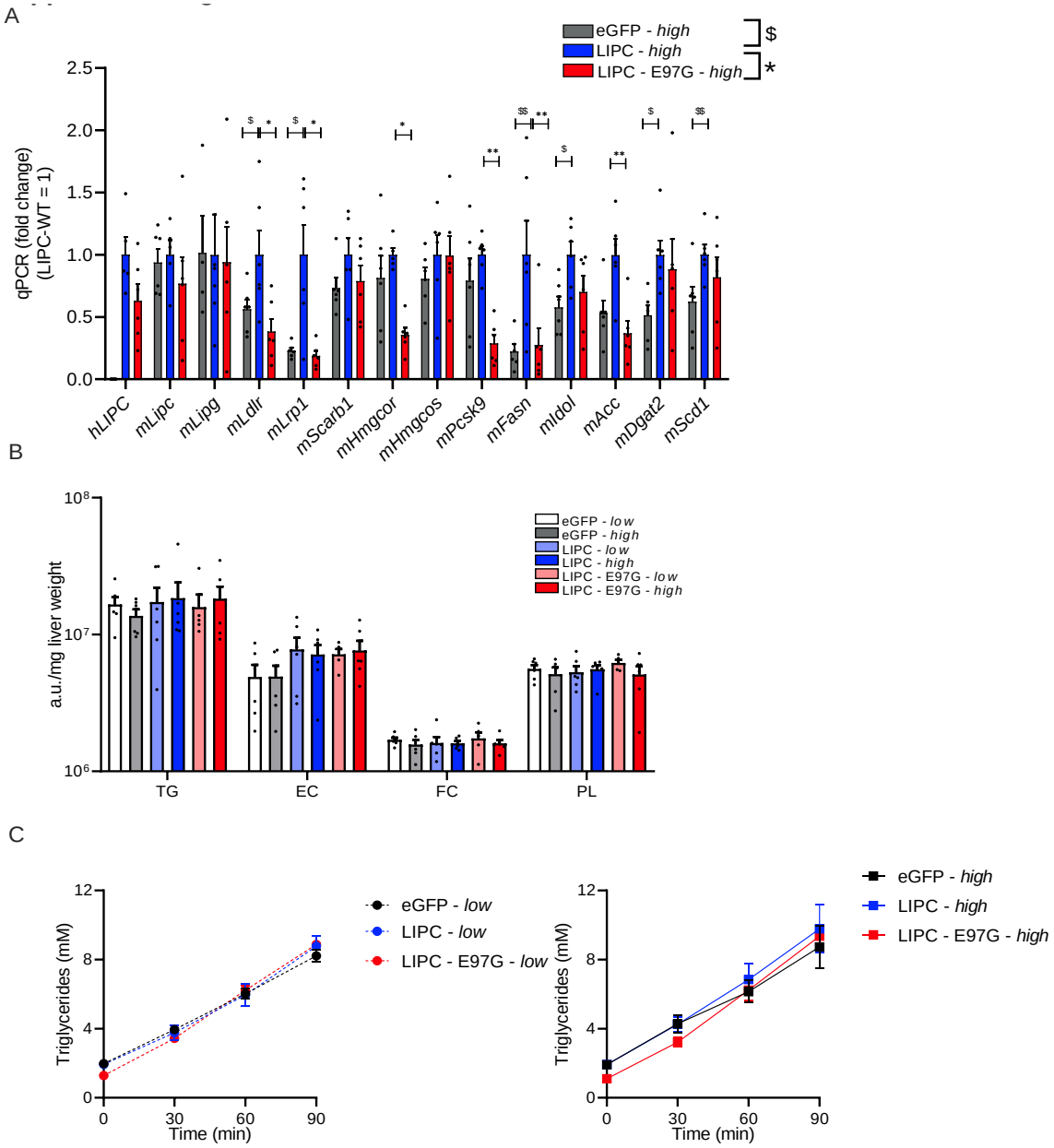
Supplemental Figure S1. Hepatic lipase homology model. A homology model for hepatic lipase (HL) based on a recently established crystal structure of lipoprotein lipase (LPL) was created (6OB0) [16]. Pairwise sequence alignments of HL and LPL show 45.9 % of identity. Superposition of the HL homology model (in purple) on the LPL crystal structure (6OB0, in cyan) in the vicinity of the salt bridge between E89 and K265 (labelling of LPL). The structure of the bound inhibitor co-crystallized with LPL is shown in green. The name of some residues is indicated for clarity. The dashed lines represent the interactions (salt bridge, hydrogen bonds) between the various residues. Residues of the lid domain are colored in blue. The importance of this structural motif and of the involved residues is supported by the fact that in the experimental x-ray structure of LPL, the ammonium group of the lateral chain of K265 is hydrogen-bonded (d((Nz)H…N)=1.96 Å ; NzHN valence angle :153°) with the nitrile group of the inhibitor bound in the LPL active site. In addition, it is worth noting that in the LPL and HL structures the carboxylate group of the glutamate residue appears maintained in a particular conformation through a hydrogen-bond interaction with the indole NH of the lateral chain of Trp91 (Trp99 in HL).



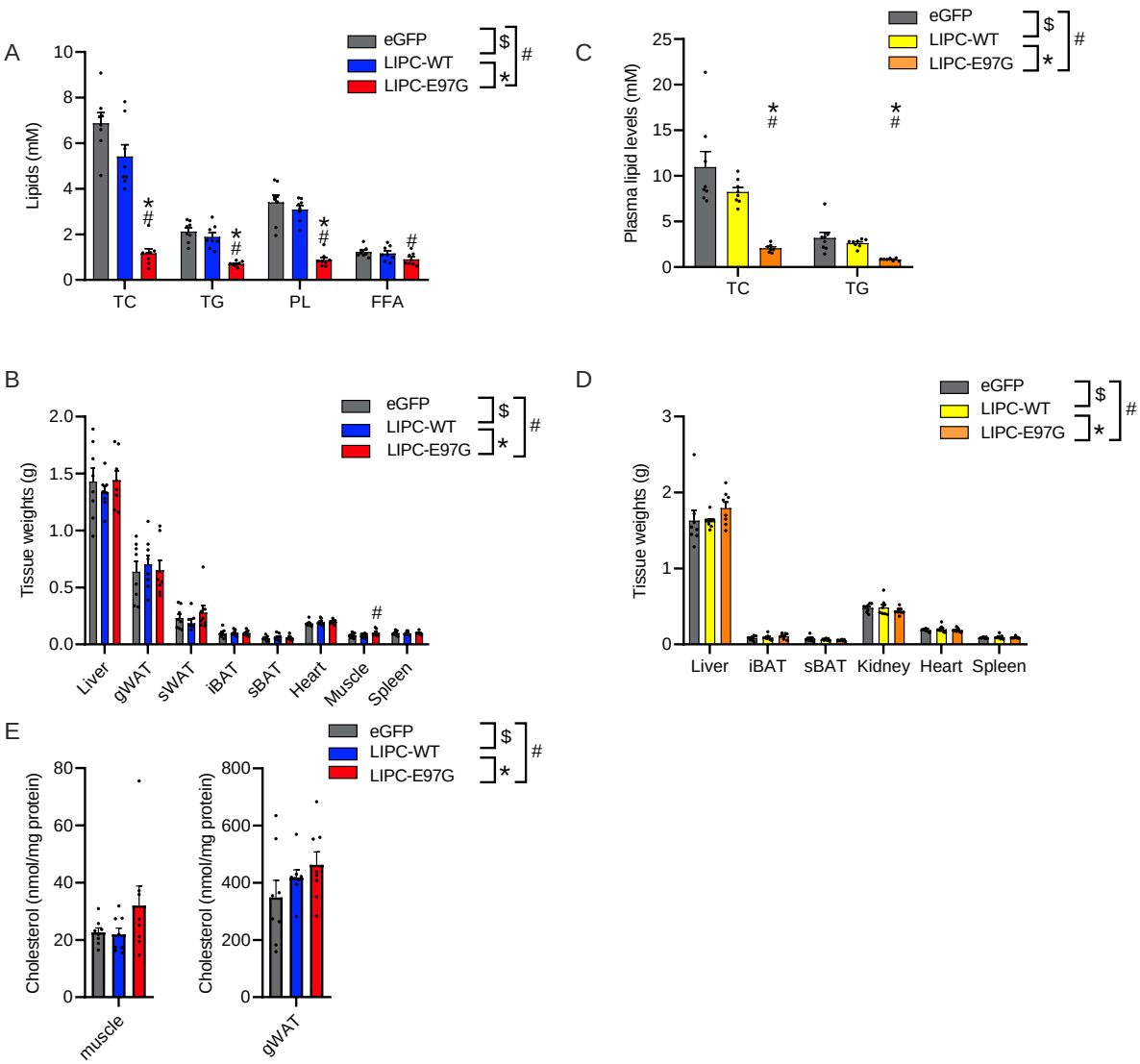
Supplemental Figure S2. A. Hepatic lipase (HL) concentrations in medium of immortalized human hepatocytes (IHH) with overexpression of wild-type LIPC (LIPC-WT), LIPC-E97G or LIPC-S168G, treated or not with heparin and as determined by ELISA. B. Sanger sequencing of DNA isolated from CRISPR-Cas9 generated IHH with heterozygous insertion of the E97G variant or a re-correction of the E97G variant to the wild-type allele (WT/WT*). C. Ratios of different phospholipids and lysophospholipids to plasma APOA1 or APOB concentrations in control individuals (n=5) or family members (n=3). PC = phosphatidylcholine; PE = phosphatidylethanolamine; SM = sphingomyelin; Cer = ceramides. Cell culture data are of 3 independent experiments with a technical duplicate. Statistical significance determined by Mann-Whitney tests, * $p<0.05$; ** $p<0.01$, *** $p<0.001$.



Supplemental Figure S3. Over-expression of LIPC-E97G in APOE*3.Leiden.CETP mice. A. Percentage of HDL-TG to total TG plasma concentrations of APOE*3.Leiden.CETP mice over-expressing low or high doses of AAV-TBG-eGFP, AAV-TBG-LIPC and AAV-TBG-LIPC-E97G as determined using Nuclear Magnetic Resonance. B. Post-prandial lipemia. Plasma TG concentrations of APOE*3.Leiden.CETP mice over-expressing high doses of AAV-TBG-eGFP, AAV-TBG-LIPC and AAV-TBG-LIPC-E97G. C. Triglyceride, cholesterol and phospholipid concentrations in plasma of APOE*3.Leiden.CETP mice over-expressing high doses of AAV-TBG-eGFP, AAV-TBG-LIPC and AAV-TBG-LIPC-E97G in random-fed (*-24h*), 24h fasted (*0*) or 3h refed (*3h*) conditions. HDL-TG, high-density lipoprotein-triglycerides; TG, triglycerides. N=5/6 per group. A one-way ANOVA with Tukey's correction for multiple comparisons was used for statistical analysis, with a p-value cut-off at $p<0.05$. * $p<0.05$; ** $p<0.01$, *** $p<0.001$.



Supplemental Figure S4. Impact of over-expression of LIPC-E97G in APOE*3.Leiden.CETP mice on hepatic mRNA levels, hepatic lipid levels and VLDL secretion. A. Gene expression levels of different genes involved in lipoprotein metabolism in livers of APOE*3.Leiden.CETP mice over-expressing high doses of AAV-TBG-eGFP, AAV-TBG-LIPC and AAV-TBG-LIPC-E97G. B. Triglycerides (TG), esterified cholesterol (EC), free cholesterol (FC) and phospholipids (PL) in livers of APOE*3.Leiden.CETP mice over-expressing high doses of AAV-TBG-eGFP, AAV-TBG-LIPC and AAV-TBG-LIPC-E97G as measured using Nuclear Magnetic Resonance. C. Triglyceride secretion rates (signifying VLDL secretion) of APOE*3.Leiden.CETP mice over-expressing low (left figure) or high (right figure) doses of AAV-TBG-eGFP, AAV-TBG-LIPC and AAV-TBG-LIPC-E97G following tyloxapol injection. N=5/6 per group. A one-way ANOVA with Tukey's correction for multiple comparisons was used for statistical analysis, with a p-value cut-off at $p<0.05$. * $p<0.05$; ** $p<0.01$, *** $p<0.001$. Comparison LIPC-WT *vs* LIPC-E97G; $ $p<0.05$, $$ $p<0.01$ comparison eGFP *vs* LIPC-WT.



Supplemental Figure S5. A. Plasma lipid concentrations and B. tissue weights in APOE*3.Leiden.CETP mice over-expressing AAV-TBG-eGFP, AAV-TBG-LIPC, and AAV-TBG-LIPC-E97G injected with VLDL-like particles. C. Plasma lipid concentrations and D. tissue weights in APOE*3.Leiden.CETP mice over-expressing AAV-TBG-eGFP, AAV-TBG-LIPC, and AAV-TBG-LIPC-E97G injected with radiolabeled murine VLDL. E. Cholesterol levels in muscle and gWAT of APOE*3.Leiden.CETP mice over-expressing AAV-TBG-eGFP, AAV-TBG-LIPC, and AAV-TBG-LIPC-E97G injected with VLDL-like particles. N=8 per group. A one-way ANOVA with Tukey's correction for multiple comparisons was used for statistical analysis, with a p-value cut-off at $p<0.05$. * $p<0.05$ comparison LIPC-WT vs LIPC-E97G; $ $p<0.05$ comparison eGFP vs LIPC-WT, # $p<0.05$ comparison eGFP vs LIPC-E97G.

Supplemental Table S1. 1. Plasma lipid concentrations of the LIPC-E97G carriers. The total cholesterol, triglyceride, and LDL and HDL cholesterol levels (in mmol/L and mg/dL) and APOB plasma concentrations in g/L. In bold: values below the 5th percentile for age and sex determined for children between 7 and 18 years of age according to [43], for adults using https://www.lipidtools.com/en/perc/ according to [44] and for APOB according to the experience of the lab. To convert values for cholesterol to mg/dL, multiply by 38.67. To convert values for triglycerides to mg/dL, multiply by 88.57. NA = not available. **1.2.** Nuclear Magnetic Resonance Liposcale data of plasma of family members (n=3). **1.3.** Analysis of DNA from family members (n=2) for rare genetic variants using Dysliseq. **1.4.** Rare genetic variants in common between family members with familial combined hypocholesterolemia following whole genome sequencing (WGS) analysis. **1.5.** Raw lipidomics data of plasma of control individuals (n=5) or family members (n=3).

Supplemental Table S2. 1. Nuclear magnetic resonance lipoprotein analysis of plasma of APOE*3.Leiden.CETP mice over-expressing low or high doses of AAV-TBG-eGFP, AAV-TBG-LIPC and AAV-TBG-LIPC-E97G (n=5/6 per group). **2.2.** Nuclear magnetic resonance lipidomics data of liver of APOE*3.Leiden.CETP mice over-expressing low or high doses of AAV-TBG-eGFP, AAV-TBG-LIPC and AAV-TBG-LIPC-E97G (n=5/6 per group).

Expanded Materials & Methods

Lipid profiles

Fasting plasma lipid concentrations including total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), apolipoprotein A1 (APOA1), apolipoprotein B (APOB), total phospholipids and free cholesterol were determined using dedicated commercial kits on an Architect C16000 autoanalyzer (ABBOTT Diagnostics, Illinois, USA) or by spectrometry on EnSpire Multimode Plate Reader.

Whole genome sequencing

Whole genome sequencing (WGS) was performed by the Centre National de Recherche en Génomique Humaine (CNRGH, Institut de Biologie François Jacob, Evry, France). After a complete quality control, genomic DNA (1µg) was used to prepare a library for WGS, using the Illumina TruSeq DNA PCR-Free Library Preparation Kit, according to the manufacturer's instructions. After normalization and quality control, qualified libraries were sequenced on a NovaSeq6000 platform from Illumina (Illumina Inc., CA, USA), as paired-end 150 bp reads. Libraries were pooled in order to reach an average sequencing depth of 30x for each sample. Sequence quality parameters have been assessed throughout the sequencing run and standard bioinformatics analysis of sequencing data was based on the Illumina pipeline to generate FASTQ file for each sample. Raw sequence reads were aligned to the human reference genome (GRCh37) using BWA-MEM (version 0.7.5a)[45]. GATK 4.1.1.0 was used for indel realignment and base recalibration, following GATK DNA Best Practices[46] using the HaplotypeCaller tool and written to a VCF file, along with information such as genotype quality, strand bias and read depth for that SNV/Indel. Our analysis workflow focused on: -i) rare variants (minor allele frequency lower than 0.1% in the Genome Aggregation Consortium database (https://gnomad.broadinstitute.org)[47]), -ii) variants shared between patients with combined-hypocholesterolemia [II:8; II:9; IV:2; IV:3; IV:4; IV:5 (Figure 1)], -iii) variants absent in relatives with normal lipid levels [II:7 IV:1 (Figure 1)], -iv) variants predicted to affect protein coding (SO:0001818) or splicing (SO:0001568) sequence using SnpEff annotations (version 4.3[48]). Filtering was performed using bcftools and jvarkit[49,50]. Additional annotations related to pathogenicity prediction and conservation algorithms (SIFT pred ; Polyphen2 HDIV pred ; Polyphen2 HVAR pred ; LRT pred ; MutationTaster pred FATHMM pred ; MetaSVM pred ; MetaLR pred ; GERP++ RS ; CADD raw) were added using wANNOVAR[51].

Polygenic risk score

To study the polygenic cause of primary HBL, we used a compilation of 12 SNPs for patients from the family and controls (n=856 subjects) as developed by Talmud *et al.* [52]. Briefly, the genotypes of 12 SNPs were extracted from whole-genome sequencing data and the polygenic risk score was calculated as previously described [8].

Human Plasma Lipoprotein Analysis by nuclear magnetic resonance

1H-NMR analysis was conducted at Biosfer Teslab (Reus, Spain). 200 µL of plasma was diluted with 50 µL of deuterated water (D2O) and 300 µL of 50 mM phosphate buffer solution (PBS) at pH 7.4. 1H-NMR spectra were recorded at 306 K on a Bruker Avance III 600 spectrometer operating at a proton frequency of 600.20 MHz (14.1 T). We used the advanced lipoprotein profile Liposcale® test (IVD-CE) to obtain the lipid composition, particle concentration and size of the mean lipoprotein classes and subclasses as previously described [53].

Lipid extraction

For lipidomics analyses, phospholipids and sphingolipids were extracted according to the method of Bligh and Dyer as described by Reis A. *et al.* [54] Briefly, plasma samples (200 µL) were diluted with saline (800 µL) and spiked with 20 µL of Splash® Lipidomix® and 4 µL of Ceramide Lipidomix®. Total lipids were extracted with 3750 µL of chloroform/methanol 2/1 for 30 min, chloroform (1250 µL) for 30 min and distilled water (1250 µL) was finally added to induce phase separation. After centrifugation (10000g, 10 min, 4°C) the organic phase was collected. The remaining aqueous phase was acidified with hydrochloric acid 3 mol/L (40 µL) and further extracted with chloroform (3000 µL) for 30 min. After centrifugation (10000g, 10 min, 4°C) the organic phase was collected and combined to the previous one. Pooled organic phases were washed with 3000 µL of authentic upper phase of Chloroform/Methanol/distilled water (96.7/93.3/90). Organic phase was collected and evaporated under vacuum. Extracts were finally dissolved with 200 µL of Chloroform/Methanol/distilled water 60/30/4.5.

Fatty acids analysis was conducted using 20 µL of plasma. Samples were mixed with 1200 µL of BHT in ethanol (50 mg/L) and 25 µL of a fatty acids internal standard mix containing 1146 ng of myristic acid-d3, 4973 ng of palmitic acid-d3, 3703 ng of stearic acid-d3, 3174 ng of linoleic acid d4, 45.8 ng of arachidic acid-d3, 1632 ng of arachidonic acid-d8, 47.6 ng of behenic acid-d3, 476.1 ng of DHA-d5, 22.9 ng of Lignoceric-d4, and 17.6 ng of cerotic acid-

d4. Samples were further hydrolyzed with potassium hydroxide (60 µL, 10 M) for 50 min at 56 °C. Total fatty acids were extracted with HCl 1.2 M (1 mL) and hexane (3 mL), and derivatized as penta-fluorobenzyl esters as previously described[55]. Total fatty acids were analyzed on a 7890A GC system coupled to a 5975C mass selective detector operating in negative chemical ionization mode (Agilent Technologies) as previously described [55]. Phospholipids and ceramides were analyzed on a 1200 6460-QqQ LC-MS/MS system equipped with an ESI source (Agilent technologies) as previously described [56,57]. Splash® Lipidomix® (P/N 330707) and Ceramide Lipidomix® (P/N 330713X) mass spectrometry standards were obtained from Avanti Polar Lipids (Coger SAS, Paris, France) and were used as deuterated internal standards. Saturated and unsaturated fatty acids used as internal standards were purchased from CDN Isotopes (Cluzeau Info Lab, Sainte Foy La Grande, France) and Cayman (Bertin Pharma, Montigny-le-Bretonneux, France) respectively. Chemicals of the highest grade available were from Sigma Aldrich (Saint-Quentin Fallavier, France). LC-MS/MS quality grade solvents were purchased from Fischer Scientific (Illkirch, France).

In silico hepatic lipase molecular modeling

Preparation of the protein structures

Two experimental crystallographic structures corresponding respectively to an apo form (6OAU, 2.48 Å)[16] and an inhibitor bound complex (6OB0, 2.81 Å) [16] of wild type Lipoprotein Lipase (LPL) were extracted from the protein data bank (PDB) [58] and used as models and templates for homology modeling. Without any experimental structure for HL being available, we used these two forms of LPL as templates since the LPL enzyme has been shown to share significant sequence similarities with HL [59]. The MOE program (Molecular Operating Environment (MOE), 2019.01; Chemical Computing Group ULC, 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2020) was used to prepare the protein structures (addition of hydrogen atoms, control of the ionization state of the amino acid residues (through Protonate 3D, available in MOE) and the energy minimizations. For the latter, the AMBER10:EHT force field implemented in MOE was used with a gradient of 0.1 kcal/mol/Å^2. It is worth noting that the water molecules of the structures have been conserved to generate the models. Indeed, the pivotal role of water in mediating interactions from a structural and functional perspective is well documented for several proteins [60]. Once the protein structures had been energy minimized, the surroundings of the E97 residue were explored through the MOE graphical interface and the apo and complexed structures of LPL were aligned.

Homology Modeling

The amino acid sequences of the HL and LPL proteins were extracted from the Uniprot-server (www.expasy.org)[61]. The closest homology of the target sequences was identified using the BLAST programme [62]. As specified above, two crystal structures of LPL were used as templates to build the three-dimensional (3D) models of HL: 6OAU (apo) and 6OB0 (an inhibitor complexed structure). Pairwise sequence alignments were carried out to align the target and the template sequence. The 3D homology models were built through the SWISS-MODEL server [63]. The quality of the models was verified through the GMQE and QMEAN scores used on SWISS-MODEL. The GCME score allows to assess the accuracy of the tertiary structure of the homology models generated whereas the QMEAN, based on four terms, provides both global (i.e. for the entire protein structure) and local (i.e. per residue) absolute quality estimates [64]. The 3D-models generated by SWISS-MODEL were then downloaded and further analyzed with the MOE program. The E97G mutants were built in MOE from the two wild type models, their energy being minimized with the same methodology as the other structures investigated (AMBER10 :EHT force field, gradient of 0.1)

Cell culture

Basic culture

Immortalized human hepatocytes (IHH) were a kind gift from Folkert Kuipers (UMCG, Groningen, the Netherlands) [65]. IHH were maintained in collagen-coated TPP® tissue culture flasks in Williams's E (Gibco™, ThermoFisher Scientific, #12551032) supplemented with 10% decomplemented FBS (Corning™), 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (complete culture medium). Once 80-90 % confluence was reached, cells were detached with trypsin-EDTA (Sigma Aldrich, Saint Quentin Fallavier, France) and diluted for maintenance or seeded for the protocols as described below.

Over-expression of LIPC-WT, LIPC-E97G and LIPC-S168G in IHH

A pcDNA3 plasmid containing wild-type HL was kindly provided Gilles Lambert (l'Université de la Réunion, France). The E97G and S168G variants were introduced using the Q5 site directed mutagenesis kit (New England Biolabs, #E0554S) using the manufacturer's protocol and using 20 ng plasmid DNA and the following primers. E97G; forward GGCGTGCTAGgAAACTGGATCTG, reverse GTCCACCGACCACCCGTG). S168G; forward AATTGGGTACgGCCTGGGTGC, reverse AGGTGAACATGGCTTCGAG.

Colonies growing in ampicillin-containing LB-Agar plates were picked and grown during 8h at 37°C in LB containing 100 µg/mL ampicillin. After 8h, bacterial colonies were spun down at 11.000*g for 1 min and plasmid DNA was isolated using the NucleoSpin® Plasmid kit (Macherey Nagel, # 740588.50). Plasmid DNA was sent for Sanger Sequencing (Eurofins Genomics) using the BGH-rev primer to verify insertion of variants. Positive colonies for each variant were then amplified 100 mL LB overnight at 37°C. After a spin down at 4500*g, plasmid DNA was isolated from bacterial pellets using the NucleoBond® Xtra Midi EF (Macherey Nagel, #740420.50) and re-sequenced.

For over-expression studies, IHH were seeded $0.25*10^6$/mL in collagen-coated 24-wells or 6-wells plates (TPP®). The next day, the regular culture medium was replaced by culture medium (Williams's E supplemented with 10% decomplemented FBS, 2 mM glutamine) without antibiotics. 0.25 µg (per 24-well) or 2.5 µg (per 6-well) of LIPC-WT, LIPC-E97G and LIPC-S168G containing plasmids were diluted in Opti-MEM (Gibco™, ThermoFisher Scientific) and Lipofectamine LTX plus reagent (Invitrogen™, ThermoFisher Scientific, #15338030) was added according to manufacturer's protocol. Lipofectamine LTX reagent was next diluted in Opti-MEM, combined in a 1:1 ratio with the diluted plasmid, incubated for 15 min at room temperature and dropwise added to the cell culture media.

After 24h of incubation, medium was replaced with complete culture medium with or without 20 IU/mL of heparin and incubated for another 24h. Medium was removed and spun down at 1200 rpm for 5 minutes to pellet dead cells. Cells were washed twice with PBS and stored in -20°C until RNA or protein isolation. Medium from cells incubated with 20 IU/mL were used for phospholipase and TG lipase activity measurements.

CRISPR-Cas9 insertion of E97G variant

To insert the c.290A>G p.(Glu97Gly) variant into immortalized human hepatocytes (IHH), we first determined optimal guide RNAs using the IDT Alt-R® CRISPR-Cas9 guide RNA tool and CRISPOR (http://crispor.tefor.net/)[66]. With these tools, we identified and ordered two possible crRNAs, guide 1 TGGACGGCGTGCTAGAAAAC and guide 2 GCGTGCTAGAAAACTGGATC (IDT). To determine the guide efficiency, we transfected the crRNAs together with Alt-R® CRISPR-Cas9 tracrRNA ATTO™ 550, (IDT, #1075927) and Alt-R® S.p. Cas9 Nuclease V3 (IDT, # 1081058) into IHH. We first seeded IHH in 6-wells plates at a density of 150.000 cells/mL. The next day, we combined the crRNAs and the Alt-R® CRISPR-Cas9 tracrRNA in a 1:1 ratio to obtain a 100 µM solution and incubated at 95ºC for 5 minutes to anneal the two components (gRNA). We next diluted the Alt-R® S.p.

Cas9 Nuclease V3 in Opti-MEM (Gibco) to obtain a 1µM solution and combined 24 µL of diluted Cas9 and 24 µL cRNA/tracrRNA in 100 µL Opti-MEM. After brief vortexing, we added 12.5 µL of Cas9 plus reagent (Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent, # CMAX00001, ThermoScientific) and incubated for 5 min at room temperature. Following the dilution of 7.5 µL of Lipofectamine CRISPRMAX solution with 115 µL of Opti-MEM, the diluted transfection reagent was added to the ribonucleoprotein (RNP) complex and incubated for 20 min at room temperature. After refreshment of the cell culture medium with antibiotics-free medium, the Lipofectamine-RNP complex was added dropwise to the cell culture medium. Cells were incubated overnight and then harvested for DNA extraction using QuickExtract solution (Lucigen, #QE09050).

For DNA extraction, IHH were washed twice with PBS and QuickExtract solution was added (250 µL/ well of a 6-wells plate). Lysed cells were placed overnight in -80 °C and subsequently placed in a 96-wells plate for extraction using a thermocycler: 65°C for 15 min, 68°C for 15 min and 98°C for 10 min. After DNA extraction, a PCR was performed to amplify the corresponding region in *LIPC* using the following primers: forward TGGAGACATTTTTGGTTTTCAA, reverse GCCTTCTCATTCCTGTGAGC (product length 587 bp). PCR was performed using Taq polymerase (Qiagen, #201203) according to manufacturer's protocol. To identify the presence of non-homologous end joining (NHEJ) and homology direct repair (HDR) event, PCR was treated with a T7 endonuclease I (New England Biolabs, #M0302S) that recognizes non-perfectly matched DNA. For this, 5 µL PCR product was placed in a new 96-wells PCR plate to denature and form heteroduplexes using the following program in a thermocycler: 95°C - 5min; 85°C (-2°C/sec) - 15sec; 25°C (-0,1°C/sec) - 30sec; 4°C – α. Next, 0.25 µL T7 endonuclease was mixed with 1 µL 10x NEB buffer and 3.75 µL H_2O, added to the 5 µL denatured PCR product and incubated for 30 min at 37°C. Products were migrated on a 4150 TapeStation (Agilent) to determine NHEJ and HDR events. In our hands, more efficient cleavage was obtained with guide RNA 1.

In order to introduce the c.290A>G p.(Glu97Gly) variant we thus used guide RNA 1 in combination with a donor DNA complementary to the DNA of interest that was Alexa488 tagged and had 2x PTO (*) modifications at both 5' and 3' ends: (Alexa488) C*C*CTCCCTCTGTCCCCTCCTCAAGTGGACGGCGTCCTAGGAAATTGGATTTGGC AGATGGTGGCCGCGCTGAAGTCTC*A*G (Eurofins Genomics). For transfections, the above protocols were used with the addition of 6 µL of 1µg/µL in OptiMEM diluted Donor. Instead of harvesting the cells 24h after transfection for DNA extraction, cells were trypsinized (IHH) and ATTO550 and Alexa488-positive cells were sorted into 96-well plates to obtain

clonal populations using a FACSMelody™ (BD Biosciences). IHH were treated for 1 week following cell sorting with Rock1 inhibitor (1/1000) to promote cell survival. When colonies had significantly grown (approx. 4-5 weeks), wells were duplicated into 2x new 96-wells plates of which one was used for cell freezing and one for DNA extraction and T7 NHEJ and HDR analysis as described above (50 µL of QuickExtract solution was used for a confluent well in a 96-well plate). Instead of TapeStation, a 1.5% agarose DNA gel was used to determine the presence of NHEJ or HDR events.

For clones for which NHEJ or HDR events were suspected, a new PCR for the corresponding *LIPC* region was performed and PCR products were sent to Eurofins Genomics for Sanger Sequencing. Finally, we identified 1 clone in IHH with the correct variant (Figure S2). To revert the c.290A>G p.(E97G) variant back to the wild-type allele (designated as WT/WT*), we treated our LIPC-E97G clone as described above. Following clonal amplification, DNA from colonies was isolated and a PCR was performed, as described above, and PCR products were sent directly for Sanger Sequencing. One colony was found in which the E97G variant was reverted to its wild-type allele (Figure S2).

Mouse studies

All mouse studies were performed using APOE*3.Leiden.CETP mice that have a humanized plasma lipid profile [12] and that were bred at Leiden University Medical Centre, Leiden, the Netherlands). All mouse experiments were approved by the ethics committee of Pays de la Loire (France, 006) and the Ministère de l'enseignement supérieur de la recherche et de l'innovation (France) (APAFIS 26862) or the Central Committee on Animal Experimentation of the Netherlands (AVD11600202010187) and Animal Welfare Body of the Leiden University Medical Center. The protocols were written prospectively. Around the age of 10 weeks male mice were placed on a diet containing 0.5% cholesterol and 15% cocoa butter (Ssniff, #S8854-E035 EF 4021-04T) for 3 (initial experiment and VLDL-like particles)-5 (murine VLDL) weeks, after which a small blood sample (25 µL) in an EDTA-containing tube was taken and spun down at 6000 rpm for 5 minutes to obtain plasma. Plasma cholesterol (Sobioda, #W1306.139 or Roche Diagnostics, #11489232216) and TG (Sobioda, #WTRIG1000 or Roche Diagnostics, #10166588130) levels were measured. Mice were then randomized over 3-6 groups based on plasma cholesterol and TG levels and body weight using the RandoMice tool [67]. Mice were injected intravenously with $3*10^{10}$ or $3*10^{11}$ GCs of adeno-associated viruses (AAV8) under the TBG promoter and containing either eGFP, wild-type LIPC or LIPC-E97G (Vector Biolabs). Mice were monitored weekly for changes in plasma

cholesterol and TG levels and body weight. Four weeks after injection, experiments to phenotypically characterize were started as described below, each with at least a one-week interval or a final clearance experiment (VLDL-like particles or murine VLDL) was performed. Primary outcomes were defined as a change in plasma cholesterol levels in the initial study and glycerol tri[^{3}H]oleate and [^{14}C]cholesteryl oleate tissue uptake in the clearance studies. Secondary outcomes were changes in plasma phospholipase and TG lipase activity in the initial study and plasma clearance in the clearance studies. To assure blinding, mice maintained their initial numbers during the experiments and only the person that performed the i.v. injections was aware of the group attributions. Power calculations were not performed for the initial, exploratory study, but the results from this study and previous studies conducted at the LUMC, Leiden (NL) were used to perform power calculations for the clearance studies. Only male mice were used for the described studies due to their more moderate plasma cholesterol levels compared to female APOE*3.Leiden.CETP mice. Female APOE*3.Leiden.CETP mice were used as the source of VLDL for the clearance experiment with murine VLDL. For all statistical analyses, a one-way ANOVA with Tukey's correction for multiple comparisons was used, with a two-sided p-value cut-off at $p<0.05$.

Post-heparin lipase activity

To measure TG and phospholipase A1 (PLA1) activity levels in mouse plasma, mice were fasted for 4h after which a blood sample (25 µL) was taken (pre-heparin). Next, all mice were intravenously injected with 300 IU of heparin/kg diluted in 0.9% NaCl. 10 minutes after injection, a second blood sample was taken (post-heparin). TG and PLA1 activity levels were measured in pre- and post-heparin blood as described below.

Murine Plasma Lipoprotein Analysis by NMR

Advanced lipoprotein testing was performed with Liposcale, a validated 2-dimensional ^{1}H-nuclear magnetic resonance spectroscopy, at Biosfer-Teslab (Reus) [53]. For the 1H-NMR analysis, 50 µL of murine plasma were added to a standardized 150 µL human plasma standardized buffer and diluted with 50 µl deuterated water (D2O) and 300 µL of 50 mM phosphate buffer solution (PBS) at pH 7.4. 1H-NMR spectra were recorded at 306 K on a Bruker Avance III 600 spectrometer operating at a proton frequency of 600.20 MHz (14.1 T).

Post-prandial lipemia

For post-prandial lipemia experiments, mice were fasted overnight and an initial blood sample (25 µL) was taken (T0). Next, mice received an oral gavage of 300 µL of olive oil (Lessieur). Blood samples were taken 1h, 2h, 4h and 6h after oral gavage. Plasma triglyceride concentrations were measured in 2 µL of plasma using Triglyceride liquicolor Mono (Human via Instruchemie, the Netherlands).

Fasting/refeeding

For fasting/refeeding experiments, an initial blood sample (25 µL) was taken at a random-fed condition, after which the mice were fasted for 24h. After 24h, a blood sample (25 µL) was taken and mice were again given free access to food. 3h after re-feeding, a blood sample (25 µL) was taken. Plasma cholesterol (Sobioda) and TG (Human, via Instruchemie) concentrations were measured in 2 µL of plasma.

Liver Lipids NMR

Lipophilic extracts were obtained from the murine liver tissue using the BUME method [68] with slight modifications[69]. Three extraction cycles were performed in order to maximize lipid extraction. In the first cycle, 500 µL of a butanol:methanol (3:1) mix, 500 µL of a di-isopropyl ether:ethyl acetate (3:1) mix and 500 µL of D_2O were added. In the second and the third cycle, only 500 µL of a di-isorpopil ether:ethyl acetate (3:1) mix was added. Lipophilic extracts were recovered and completely dried in Speedvac until evaporation of organic solvents and frozen at -80ºC until ^{1}H-NMR analysis. Lipid extracts were reconstituted in a solution of $CDCl_3$:CD_3OD: D_2O (16:7:1, v/v/v) containing Tetramethylsilane (TMS) as a chemical shift reference and were transferred into 5-mm NMR glass tubes. ^{1}H-NMR spectra were recorded at 286 K on a Bruker Avance III 600 spectrometer operating at a proton frequency of 600.20 MHz (14.1 T). A 90° pulse with water pre-saturation sequence (ZGPR) was used for lipophilic extracts.

VLDL-TG secretion

For VLD-TG secretion measurements, mice were fasted overnight and an initial blood sample (25 µL) was taken (T0). Mice were then intravenously injected using tyloxapol (500 mg/kg) and blood samples (25 µL) were taken 30 minutes, 60 minutes and 90 minutes after injection. Plasma TG concentrations (Human, via Instruchemie) were measured in 2 µL of plasma.

D7 cholesterol

Three days before sacrifice, mice were anesthetized with isoflurane and received 0,3 mg of cholesterol-D7 in 100 µL intralipid by intra penile injection and were placed in individual cage for 3 days. Faeces production was harvested every 24h for 3 days. The third day mice were fasted for 3 hours and anesthetized with a xylazine/ketamine solution (10/80 mg/kg, i.p. injection). Blood, faecal and hepatic samples were harvested and D7 cholesterol content was analyzed by GC-MS. A lipid extraction was performed following 3 steps: a first chloroform methanol extraction, a saponification, and a second lipid extraction using cyclohexane. Samples were derived using MSTFA. Cholesterol and D7-cholesterol content in plasma, hepatic and fecal samples were further analyzed by gas chromatography–mass spectrometry.

VLDL-like emulsion particle labelling

Radiolabelled VLDL-like emulsion particles were prepared as previously described [70] (Briefly, 100 mg of lipids (triolein, egg yolk phosphatidylcholine, lysophosphatidylcholine, cholesteryl-oleate and cholesterol) were mixed with glycerol tri[^{3}H]oleate and [^{14}C]cholesteryl-oleate (GE Healthcare, Little Chalfont, UK) and sonicated. The emulsion was fractionated by consecutive density gradient ultracentrifugation (Beckman, California, USA) to yield VLDL-like particles with a diameter of ~80 nm.

Murine VLDL particle labelling

4 hr fasted blood samples were collected from female APOE*3.Leiden.CETP mice that were fed a diet containing 0.15% cholesterol and 15% cocoa butter (Sniff #S8854-E022 Diet T). Labelling of lipoproteins was performed by incubating serum with liposomes containing glycerol tri[^{3}H]oleate and [^{14}C]cholesteryl oleate and EDTA for 24 hrs at 37°C. Labelled VLDL was subsequently isolated by density gradient ultracentrifugation.

TGRL clearance experiments

All animals were fasted for 4 hr, after which they were injected with either 200 µL radiolabeled VLDL-like particles (1 mg TG) or murine VLDL (0.2 mg TG). Lipid clearance was determined using plasma taken at 2, 5 and 10 min following injection. In the experiment with murine VLDL an additional blood sample was taken at 15 min after injection. Plasma volumes were estimated as 0.04706 x body weight (g), and used to calculate the total amount of ^{3}H and ^{14}C activity in plasma as percentage of the injected dose. After the last blood draw, mice were killed and perfused via the heart with ice-cold PBS containing 50 IU/mL heparin or ice-cold

PBS, respectively. Multiple organs were collected, weighed and solubilized in Solvable (Perkin Elmer) overnight. ^{3}H and ^{14}C radioactivity was determined via liquid scintillation counting. Uptake of radioactivity derived from VLDL-like particles was calculated as % uptake of the injected radiolabel per gram tissue.

Liver, muscle and white adipose tissue cholesterol content.

Lipids were extracted according to a modified method of Bligh and Dyer. Briefly, liver, muscle and white adipose tissue samples (approx. 50 mg) were homogenized in methanol (10 µL/mg). 1800 µL chloroform/methanol 3/1 was added to 45 µL homogenate. The organic phase was collected after vortexing and centrifugation (20000g, 15 min) and dried under nitrogen. Extracts were reconstituted in 2% Triton X-100 in chloroform and dried again. Finally, extracts were reconstituted in water and cholesterol levels were determined (Roche Diagnostics, #11489232216).

Molecular biology

hLIPC ELISA

For measurements of hLIPC quantities in cell culture media or pre- and post-heparin mouse plasma, the HTGL serum ELISA from IBL International Diagnostics (#30131802) was used according to manufacturer's protocol. Medium samples were diluted 2x in EIA buffer prior to measurements. Pre-heparin plasma was diluted 20x and post-heparin plasma was diluted 750x in EIA buffer.

Protein extractions

Proteins were isolated using modified RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% v/v NP-40, 0.1% SDS, 1/100 diluted protease inhibitors (P8340, Sigma)). For IHH, cells were washed with PBS and 200 µL (for a 24-wells plate) or 350 µL (for a 6-wells plate) of modified RIPA was added to the wells. Lysis buffer was left to sit for 5 min and was followed by scraping of the wells and transfer into an Eppendorf tube. Cell lysates were incubated for 20 minutes on ice before spinning down at 13300 rpm for 10 min. Supernatants were transferred to a new Eppendorf tube and protein contents were quantified using the Pierce BCA assay (ThermoScientific, #23225). For HLCs, protocol was essentially the same with the exception that cell lysates were sonicated 2x 10 seconds on ice following scraping.

PLA1 activity measurement

Phospholipase A1 (PLA1) activity was measured using the Enzchek phospholipase A1 kit according to manufacturer's instructions (Molecular Probes, ThermoScientific #E10219). First, a standard curve of Lecithase A1 was prepared (0-10 U/mL) in 1x PLA1 assay buffer. Next, 50 µL of non-diluted cell culture supernatant or 1 µL of plasma diluted in 49 µL 1x PLA1 assay buffer were pipetted into a black 96-wells plate. A lipid mixture was prepared by combining 10 mM dioleoylphosphatidylcholine, 10 mM dioleoylphosphatidylglycerol and 2 mM PLA1 substrate in a 1:1:1 ratio. Then, the lipid mixture was diluted in 1x PLA1 assay buffer at a 1/100 dilution (e.g. 50 µL of lipid substrate in 5 mL 1x PLA1 assay buffer) by slowly adding the lipid mixture to a small beaker containing the 1x PLA1 assay buffer and a magnetic stir bar, situated on a magnetic stirrer. 50 µL of the substrate-liposome mixture was added to each well to start the reaction. 96-wells plate was incubated for 30 minutes at 37°C with readings every minute at 485 nm excitation and 515 nm emission at a Varioskan (ThermoFisher). Measurements were performed in the presence and absence of 1 M NaCl. Lipase activity was determined by subtracting the no-PLA1 control and by calculation from the standard curve.

TG lipase activity measurement

To determine TG lipase activity, the EnzChek lipase fluorescent substrate was used as described previously (Molecular Probes, ThermoFisher Scientific, E33955)[71]. Briefly, 50 µL of cell culture supernatant or 1 µL of plasma diluted in 49 µL H_2O was combined with 25 µL 4x Assay Buffer (0.6 M NaCl, 80 mM Tris–HCl pH 8.0, and 6% fatty acid–free BSA) in a black 96-wells plate. EnzChek lipase substrate was dissolved in 100 µL DMSO to obtain a 1 mM solution. A substrate solution was next prepared containing 2.48 µM EnzChek lipase fluorescent substrate in 0.05% 3-(N,N-Dimethylmyristylammonio) propanesulfonate zwittergent detergent (Acros Organics, 427740250). 25 µL of Enzchek lipase substrate solution was added to each well, and the plate was incubated at 37 °C for 30 min and read every minute at 485 nm excitation/515 emission at a Varioskan (ThermoScientific). Measurements were performed in the presence and absence of 1 M NaCl, to inhibit lipoprotein and endothelial TG lipase activity. Relative lipase activity was calculated by subtracting background (calculated by reading fluorescence of a sample with no LPL) and then calculating the slope of the curve between fluorescence at 30 minutes and 0 minutes.