Modulating energy metabolism: pathophysiological aspects and novel interventions

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PART 1

PATHOPHYSIOLOGICAL ASPECTS OF CARDIOMETABOLIC DISEASES

CHAPTER 2

Comprehensive (apo)lipoprotein profiling in patients with genetic hypertriglyceridemia using LC-MS and NMR spectroscopy

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ABSTRACT

Background

Mutations in genes encoding lipoprotein lipase (LPL) or its regulators can cause severe hypertriglyceridemia (HTG). Thus far, the effect of genetic HTG on the lipid profile has been mainly determined via conventional techniques.

Objective

To show detailed differences in the (apo)lipoprotein profile of patients with genetic HTG by combining LC-MS and NMR techniques.

Methods

Fasted serum from 7 patients with genetic HTG and 10 normolipidemic controls was used to measure the concentration of a spectrum of apolipoproteins by LC-MS, and to estimate the concentration and size of lipoprotein subclasses and class-specific lipid composition using NMR spectroscopy.

Results

Patients with genetic HTG compared to normolipidemic controls had higher levels of apoB48 (fold change [FC] 11.3, $P<0.001$), apoC-I (FC 1.5, $P<0.001$), apoC-II (FC 4.3, $P=0.007$), apoC-III (FC 3.4, $P<0.001$), and apoE (FC 4.3, $P<0.001$), without altered apoB100. In addition, patients with genetic HTG had higher concentrations of TG-rich lipoproteins (*i.e.*, chylomicrons and very-low-density lipoproteins [VLDL]; FC 3.0, P<0.001), but lower LDL (FC 0.4, P=0.001), of which medium and small-sized LDL particles appeared even absent. While the correlation coefficient between NMR and enzymatic analysis in normolipidemic controls was high, it was considerably reduced in patients with genetic HTG.

Conclusion

The lipoprotein profile of patients with genetic HTG is predominated with large lipoproteins (*i.e.*, chylomicrons, VLDL), explaining high levels of apoC-I, apoC-II, apoC-III and apoE, whereas small atherogenic LDL particles are absent. The presence of chylomicrons in patients with HTG weakens the accuracy of the NMR-based model as it was designed for normolipidemic fasted individuals.

INTRODUCTION

Hypertriglyceridemia (HTG) is a common lipid abnormality that has been identified as an independent risk factor for cardiovascular diseases (1-3). Severe HTG can be the result of various genetic mutations and can be monogenic or multifactorial in nature. The monogenic form of HTG, known as familial chylomicronemia syndrome (FCS), is an extremely rare disorder characterized by fasting chylomicronemia and severe HTG of ≥ 10 mmol/L (≥ 885 mg/dL) (4). FCS affects approximately one individual per 100,000 - 1,000,000 and typically presents early in life (5, 6). FCS is caused by homozygous, compound heterozygous or double heterozygous mutations in the gene encoding lipoprotein lipase (*LPL*) or in genes that encode LPL regulators, such as apolipoprotein (apo) C-II (*APOC2)*, apoA-V (*APOA5)*, glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (*GPIHBP1)*, and lipase maturation factor 1 *(LMF1)* (6). LPL is present on the capillary walls of metabolic tissues and is the rate-limiting enzyme to catalyze the hydrolysis of triglycerides (TGs) from TG-rich lipoproteins with subsequent uptake of the liberated fatty acids into these tissues (7). Dysfunctional LPL leads to severe HTG due to impaired clearance of TG-rich lipoproteins, *i.e.* chylomicrons and very-low-density lipoproteins (VLDL) (5). The most important health risk for patients with FCS is the development of recurrent hypertriglyceridemic acute pancreatitis, with a mortality rate of 5-6% (5). The remainder of genetic HTG is due to multifactorial chylomicronemia syndrome (MCS). Here, a heterozygous mutation in candidate genes in combination with a co-morbidity that is known to raise circulating triglycerides (TG) is causal to the severe HTG. Although MCS is also characterized with an increased risk on acute pancreatitis, the risk is notably lower compared to in patients with FCS (8). On the other hand, the cardiovascular disease risk in patients with FCS appears to be limited compared to the risk in patients with MCS (9-11).

To increase understanding of the metabolic phenotype of patients with genetic HTG, beyond measuring total concentrations of TGs, characterization of lipoproteins and lipoprotein class-specific lipid concentrations is of importance (12). Several previous studies characterized clinical manifestations and biochemical features of patients with genetic HTG, but were limited to conventional enzymatic measurements of lipid concentrations leading to uninterpretable results due to interference of the high triglyceride levels and missed broad assessment of (apo)lipoproteins (9, 11, 13, 14). Therefore, in this study we combined two approaches to characterize the lipoprotein profile in patients with genetic HTG in detail. First, we used multiplex liquid chromatography–mass spectrometry (LC-MS) to provide an overview of a broad spectrum of apolipoproteins that are specific for particular lipoproteins (15). Secondly, we applied nuclear magnetic resonance (NMR) spectroscopy to estimate lipoprotein

subclasses and class-specific lipid composition. By combining both modalities we provide a comprehensive overview of the circulating lipoprotein profile in patients with genetic HTG, compared to normolipidemic individuals.

METHODS

Study design

The blood samples used in this study were obtained as part of a previously conducted clinical trial aiming to investigate the effects of mirabegron on brown adipose tissue metabolism (clinical trial registration number: NCT03012113) that was conducted between June 2017 and June 2018 at the LUMC (16). In this study, ten Dutch Europid and ten Dutch South Asian participants were included, of whom the 10 Dutch Europid participants were included in the current study as normolipidemic controls. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC) and carried out according to the principles of the revised Declaration of Helsinki (17). By means of an ethical amendment, 7 Dutch patients with genetic HTG were later additionally recruited. Written informed consent was obtained from all volunteers prior to participation.

Participants

For patients, genetic HTG was confirmed based on the presence of a loss-of-function mutation (homozygous, double heterozygous, or heterozygous) in genes affecting LPL activity: *LPL*, *APOC2*, *APOA5*, *GPIHBP1*. For normolipidemic controls, the exclusion criteria included: (i) presence of any chronic endocrine or cardiovascular disease as determined by medical history and physical examination; (ii) the use of drugs that are known to influence the cardiovascular or endocrine system, or the use of drugs that are known to influence glucose or lipid metabolism; (iii) smoking or abuse of alcohol or drugs; (iv) excessive weight loss or exercise. Participants were instructed not to exercise 48 hours in advance of the study day, not to drink alcohol, tea or coffee 24 hours in advance of the study day and to eat a standardized meal the evening before the study day. An elaborated description of the study can be found elsewhere (16).

Laboratory analyses

Blood was drawn from the antecubital vein after a 10-hour overnight fast. For measurements in serum, blood was collected with Vacutainer® SST™ tubes. After a clotting time of at least 30 min, samples were centrifuged to obtain serum. For measurements in plasma, blood was collected with Vacutainer® Ethylene Diamine Tetra Acetic acid (EDTA) tubes that were promptly centrifuged at 4°C to obtain plasma. Serum and EDTA-plasma were aliquoted and stored at -80°C until batch-wise analyses.

Commercially available enzymatic kits were used to measure serum concentrations of TG, total cholesterol (Roche Diagnostics, the Netherlands) and HDL-cholesterol (HDL-C) (Roche Diagnostics). Enzymatic test results for serum total cholesterol, triglycerides and HDL-C are traceable to internationally recognized Reference Measurement Systems hosted by the Cholesterol Reference Method Laboratory Network of the Centers for Disease Control, Atlanta, Georgia, USA, guaranteeing their accuracy within allowable limits of measurement uncertainty.

Apolipoprotein measurements

Multiplex LC-MS was used for the quantification of total serum levels of apoB [apoB (tot)], apoB100, apo (a), apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE (18). ApoA-I, apoB and apo (a) tests results are traceable to the internationally endorsed WHO-IFCC Reference Measurement Systems for these measurements (11). In addition, plasma apoB48 levels were assessed using a commercially available ELISA kit (Fujifilm WAKO, Neuss, Germany) according manufactural instructions (genetic HTG: 5,000 dilution; normolipidemic controls: 1,000 dilution).

Quantification of lipoprotein (sub)class concentration and composition

Sample preparation

NMR spectroscopy was used to quantify lipoprotein (sub)class concentration and composition. The sample preparation was carried out to produce the buffer composition/pH and sample-to-buffer mixing ratio requirements for Bruker IVDr platform. Serum samples were thawed at room temperature. 110 µL of 75 mM disodium phosphate buffer in H_2O/D_2O (80/20) with a pH of 7.4 containing 6.15 mM NaN_3 and 4.64 mM sodium 3-[trimethylsilyl] d4-propionate (Cambridge Isotope Laboratories) was pipetted into a Ritter 96 well plate. Next, 110 µL of serum was added to the buffer and mixed by aspirating and dispensing three times. Using a modified Gilson 215 liquid handler, 190 µL of each sample was transferred into 3-mm NMR SampleJet tubes. Subsequently the tubes were closed by inserting POM balls into the caps and transferred to the SampleJet autosampler where they were kept at 6°C while queued for acquisition.

NMR experiments and processing

All proton nuclear magnetic resonance (^{1}H-NMR) experiments were acquired on a 600 MHz Bruker Avance Neo spectrometer (Bruker Corporation, Billerica, USA) equipped with a 5-mm triple resonance inverse (TCI) cryogenic probe head with Z-gradient system and automatic tuning and matching. A standard 3mm sample of 99.8% methanol-d4 (Bruker Biospin) was used for temperature calibration before the measurements (19). All experiments were recorded at 310 K. The duration of the π/2 pulses were automatically

calibrated for each individual sample using a homonuclear-gated nutation experiment on the locked and shimmed samples after automatic tuning and matching of the probe head (20). For water suppression, presaturation of the water resonance with an effective field of γB1 = 25 Hz was applied during the relaxation delay and the mixing time of the NOESY1D experiment (21). The NOESY1D experiment was recorded using the first increment of a NOESY pulse sequence (22), with a relaxation delay of 4 s and a mixing time of 10 ms 16 scans of 98,304 points covering a sweep width of 18,029 Hz were recorded after applying 4 dummy scans. The lipoprotein values were extracted from the NOESY1D serum spectra by submitting the data to the commercial Bruker IVDr (In-Vitro Diagnostics for research) Lipoprotein Subclass Analysis (B.I.-LISA) platform. With this approach, information was extracted about the total lipids in serum, as well as lipids (*i.e.*, total cholesterol, free cholesterol, phospholipids and triglycerides) and apolipoproteins (*i.e.*, apoA-I, apoA-II and apoB100) within the various lipoprotein subclasses.

Statistical analyses

Statistical analyses were performed with SPSS® Statistics (version 25, IBM® Corporation, Armonk, NY, USA). Nonparametric tests were conducted for all comparisons as the sample size was assumed too small for standard parametric tests. Baseline characteristics, serum lipids and plasma apolipoproteins between patients with genetic HTG *vs.* normolipidemic controls were compared with the Mann-Whitney U test for independent samples. Simple linear regression was used to test the relationship between serum levels of triglycerides, total cholesterol and HDL-cholesterol measured by NMR spectroscopy (dependent outcome) *vs.* enzymatic assays (independent outcome). Similarly, simple linear regression was used to test the relationship between apoB100, apoA-I and apoA-II measured using NMR (dependent outcome) *vs.* LC-MS (independent outcome). Hedges' g effect sizes were used to determine the difference between patients with genetic HTG and normolipidemic controls regarding lipid content of lipoproteins and lipoprotein subclasses as estimated with NMR spectroscopy. Effect sizes were calculated using the mean differences between the groups and the pooled standard deviation. Then, effects sizes were corrected for bias using Hedges' correction factor (23). 95% confidence intervals were calculated as: Hedges' g ± z-value * standard error of Hedges' g and were considered statistically significant if they did not include the value zero. For all other comparisons, a P-value ≤ 0.05 was considered statistically significant. All data are presented as median with interquartile range (IQR) or, only when indicated, as standardized mean difference with 95% confidence intervals (CI). Fold changes are calculated as: median value of patients with genetic HTG / median value of normolipidemic controls. All figures were prepared with Prism 9 for Windows (version 9.0.1, 2021, GraphPad Software, LLC, San Diego, California, USA).

RESULTS

Participant characteristics

In this study, we included 7 patients with genetic HTG (6 males, 1 female; 32 [IQR 8] years old; BMI: 24.0 [3.0] kg/m^2,) and 10 normolipidemic controls (all males; 23 [2] years old, BMI: 24.0 [3.0] kg/m$^{2)}$ (**Table 1**). Among patients with genetic HTG, we enrolled two patients with a homozygous mutation in *LPL*, one patient with a homozygous mutation in *GPIHBP1*, one patient with double heterozygous mutations in *APOC2* and *LPL,* one patient with a heterozygous mutation in *APOA5,* one patient with a heterozygous mutation in *APOC2*, and one patient with a heterozygous mutation in *APOA5* together with *APOE2/E2* (**Table 1**).

Table 1. Baseline characteristics and genetic variations of patients with genetic hypertriglyceridemia and normolipidemic controls

	Form of genetic HTG	Sex	Age, years	BMI, kg/m^2	Gene	Hetero-/ homozygous	Mutation
1	**LPL** deficiency	Male	28	24.9	*LPL*	Homozygous	c. 548A>G, p. Asp183Gly
2	**LPL** deficiency	Female	32	26.7	*LPL*	Homozygous	c. 548A>G, p. Asp183Gly
3	**GP1HBP** deficiency	Male	27	24.0	*GP1HBP*	Homozygous	Deletion
4	**ApoC-II** and **LPL** deficiency	Male	36	23.9	*APOC2, LPL*	Double heterozygous	*APOC2*: c. 245T>G *LPL*: c. 1279G>A
5	**ApoA-V** deficiency	Male	36	23.7	*APOA5*	Heterozygous	427delC
6	**ApoC-II** deficiency	Male	29	31.6	*APOC2*	Heterozygous	c. 245T>G
7	**ApoA-V** deficiency	Male	51	23.1	*APOA5, APOE*	Double heterozygous	*APOA5*: 161+5 SC>G *APOE*: E2/E2
	Normolipidemic controls (n=10)	Males (n=10)	23 [2]	22.1 [0.7]	-	-	-
	Patients with genetic HTG (n=7)	Males (n=6), Female (n=1)	32 [8]	24.0 [3.0]	-	-	-

Values are presented as median and interquartile range.
Apo, apolipoprotein; BMI, body mass index; GPIHBP1, glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; HTG, hypertriglyceridemia; LPL, lipoprotein lipase.

Increased serum triglyceride and total cholesterol levels in patients with genetic HTG

As expected, circulating TG concentration was higher in patients with genetic HTG compared to normolipidemic controls (16-fold; 13.9 [2.0] mmol/L *vs.* 0.9 [0.4] mmol/L, *P*<0.001 (**Figure 1**). In addition, total cholesterol concentration was higher in patients with genetic HTG compared to normolipidemic controls (1.5-fold; 5.7 [4.8] mmol/L *vs.* 3.8 [1.5] mmol/L, *P*=0.007), whereas HDL-cholesterol concentration was lower (0.4-fold; 0.5 [0.6] mmol/L *vs.* 1.2 [0.3] mmol/L, *P*=0.01). Consistently, patients with genetic HTG had a higher concentration of non-HDL-cholesterol (2-fold; 5.3 [4.9] mmol/L *vs.* 2.5 [1.3] mmol/L, *P*<0.001) and a higher ratio between total cholesterol and HDL-cholesterol compared to normolipidemic controls (4-fold; 12.1 [6.6] *vs.* 3.1 [0.8], *P*<0.001, not shown).

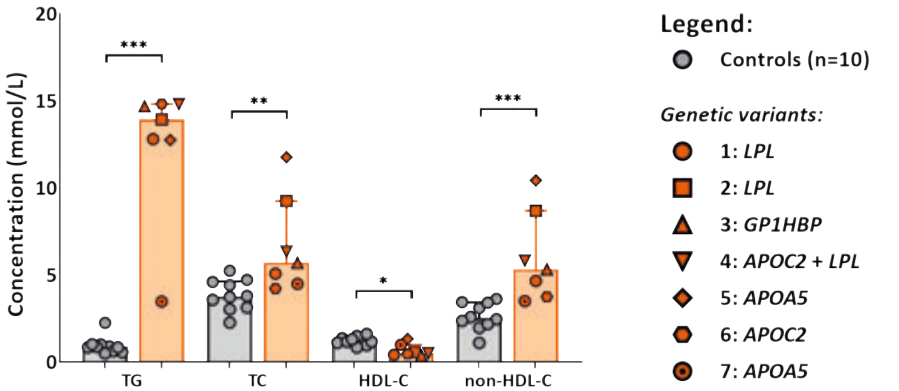

Figure 1. Serum triglyceride and cholesterol levels in patients with genetic hypertriglyceridemia vs. normolipidemic controls as measured with enzymatic assays.
Every shape illustrates a single patient with genetic hypertriglyceridemia with the genetic variant described in the legend. P-values are obtained from Mann-Whitney U test for independent groups. Values are median and interquartile range. ** P<0.01, ***P<0.001.
APO, gene encoding apolipoproteins; GPIHBP1, gene encoding glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; HDL-C, high-density lipoprotein-cholesterol; LPL, gene encoding lipoprotein lipase; non-HDL-C, total cholesterol minus HDL-cholesterol; TC, total cholesterol; TG, triglycerides.

Increased plasma apolipoproteins C-I, C-II, C-III, E and B48 in patients with genetic HTG

To further characterize the lipoprotein profile in patients with genetic HTG, we next measured circulating apolipoproteins using LC-MS. Patients with genetic HTG compared to normolipidemic controls had a similar total apoB (64.0 [27.1] mg/dL *vs.* 62.3 [28.8] mg/dL, *P*=0.96) and apoB100 concentration (55.2 [26.0] mg/dL *vs.* 60.4 [32.7] mg/dL, *P*=0.67) (**Figure 2A**; **Supplementary Table S1**). The concentration of apoB48 was higher in patients with genetic HTG (11-fold: 4.4 [7.6] mg/dL *vs.* 0.4 [0.3] mg/dL, *P*<0.001).

Patients with genetic HTG, compared to normolipidemic controls, also had a higher concentration of apoC-I (1.5-fold: 2.6 [2.0] mg/dL *vs.* 1.7 [0.5] mg/dL, *P*<0.001), apoC-II (4.3-fold: 7.7 [9.9] mg/dL *vs.* 1.8 [1.8] mg/dL, *P*=0.007), apoC-III (3.4-fold: 21.7 [16.7] mg/dL *vs.* 6.4 [4.3] mg/dL, *P*<0.001), and apoE (4.3-fold: 8.9 [7.1] mg/dL *vs.* 2.1 [1.1] mg/dL, *P*<0.001). In contrast, concentrations of apoA-I (90.5 [50.4] mg/dL *vs.* 114.2 [37.1] mg/dL, *P*=0.11), apoA-II (17.9 [12.1] mg/dL *vs.* 24.8 [5.2] mg/dL, *P*=0.23), and apoA-IV (25.3 [9.7] mg/dL *vs.* 17.6 [11.2] mg/dL, *P*=0.07), did not significantly differ between patients with genetic HTG compared to normolipidemic controls.

The ratio between apoB100 and apoA-I in patients with genetic HTG was comparable to the ratio in normolipidemic controls (0.7 [0.4] mg/dL *vs.* 0.5 [0.2] mg/dL, *P*=0.23; **Figure 2B**). The ratio between non-HDL-cholesterol and apoB100, as estimation of the cholesterol load per apoB100 carrying lipoprotein particle, was higher in patients with genetic HTG than in normolipidemic controls (9.5 [2.8] *vs.* 4.4 [0.4], *P*<0.001; **Figure 2B**).

Comparison of between NMR-based results *vs.* enzymatic assays and LC-MS

Through analysis of the NMR spectra we obtained a second estimation of lipoprotein lipid and apolipoprotein levels, although the model that was used to extract these values from the serum NMR spectra (Bruker B.I.-LISA) has not been validated for serum with chylomicrons. Since chylomicrons are most similar to VLDL particles, they probably contribute to 'VLDL' values, although estimation of other lipoprotein classes may be affected as well. In concurrence with the enzymatic assay, TG concentration was higher in patients with genetic HTG compared to normolipidemic controls (3.4 [2.8] mmol/L *vs.* 0.8 [0.3] mmol/L, *P*<0.001), whereas HDL-cholesterol concentration was lower in patients with genetic HTG (0.7 [0.3] mmol/L *vs.* 1.2 [0.5] mmol/L, *P*=0.001) (**Supplementary Figure S1**). In contrast to enzymatic analysis of total cholesterol, the model found no significant difference between patients with genetic HTG and normolipidemic controls for the total cholesterol concentration (2.3 [2.3] mmol/L *vs.* 3.2 [1.2] mmol/L, *P*=0.11). LDL-cholesterol, which could not be provided by simple enzymatic methods, was lower in patients with genetic HTG (0.2 [0.3] mmol/L *vs.* 1.7 [0.7] mmol/L, *P*<0.001). Similar as when measured with LC-MS, apoB100 did not differ between patients with genetic HTG and normolipidemic controls (52.3 [25.9] mg/dL *vs.* 54.3 [17.5] mg/dL, *P*=0.19). In contrast, apoA-I was lower in patients with genetic HTG compared to normolipidemic controls (83.0 [42.4] mg/dL *vs.* 123.0 [27.7] mg/dL, *P*=0.03), just as apoA-II (18.3 [10.5] mg/dL *vs.* 25.8 [3.1] mg/dL, *P*=0.007; **Supplementary Figure S1**). Notably, these differences in the levels of apoA-I and apoA-II between patients with genetic HTG and normolipidemic controls were not statistically significant when measured with LC-MS.

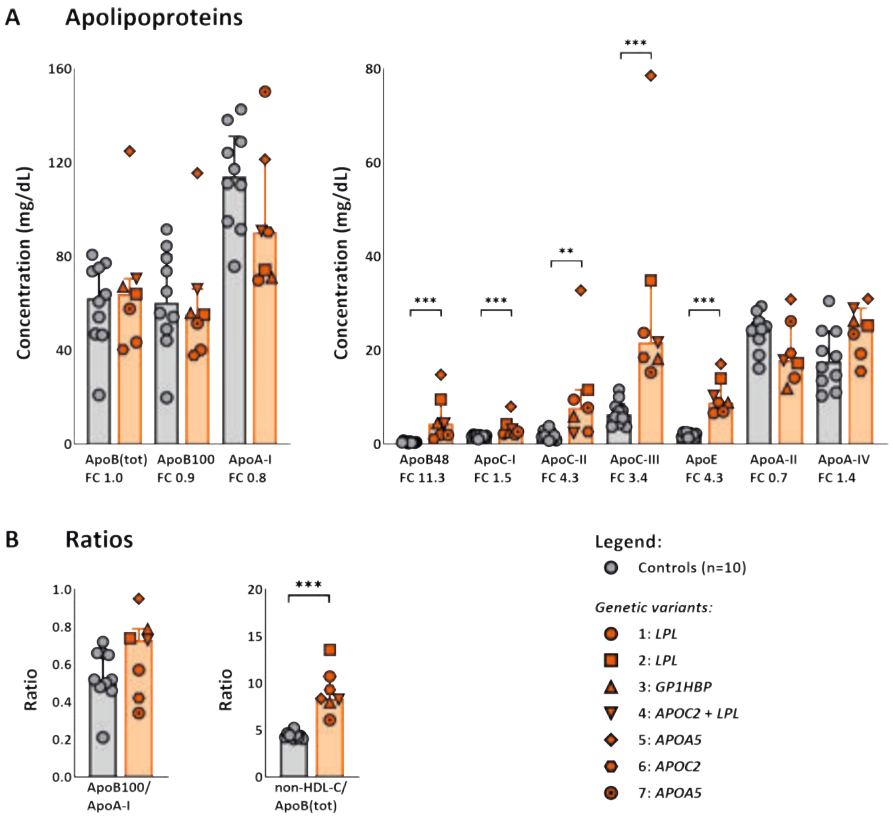

Figure 2. Serum apolipoprotein levels in patients with genetic hypertriglyceridemia vs. normolipidemic controls as measured with liquid chromatography–mass spectrometry or ELISA (apoB48).
Every shape illustrates a single patient with genetic hypertriglyceridemia with the genetic variant described in the legend. P-values are obtained from Mann-Whitney U test for independent groups. Values are median and interquartile range. ** P<0.01, ***P<0.001.
APO, gene encoding apolipoproteins; Apo, apolipoprotein; FC, fold change (based on median values and calculated as 'patients/controls'); GPIHBP1, gene encoding glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; LPL, gene encoding lipoprotein lipase; non-HDL-C, total cholesterol minus HDL-cholesterol.

To check whether the results obtained with NMR-based analysis method match the more established enzymatic assays and LC-MS methods, we conducted linear regression analyses between the concentrations estimated with NMR spectroscopy (as dependent outcome) and the concentrations measured with an enzymatic assay or LC-MS (as independent outcome). This analysis was carried out separately for normolipidemic controls and genetic HTG patients in order to expose differences in performance of the model for the two groups. For TG concentration, there was a strong correlation between the two methods in normolipidemic controls (R^2=0.986, *P*<0.001),

but not in patients with genetic HTG (R^2=0.055, *P*=0.61; **Figure 3A**). Similarly, for total cholesterol concentration, there was a strong correlation between the two methods in normolipidemic controls (R^2=0.818, *P*<0.001), but not in patients with genetic HTG (R^2=0.228, *P*=0.28; **Figure 3B**). For HDL-cholesterol concentration, the two methods did correlate both in normolipidemic controls (R^2=0.788, *P*<0.001) and patients with genetic HTG (R^2=0.682, *P*=0.02; **Figure 3C**). For apoB100, there was a strong correlation between the two methods in normolipidemic controls (R^2=0.852, *P*<0.001), but not in patients with genetic HTG (R^2=0.386, *P*=0.14; **Figure 3D**). For apoA-I, the two methods did correlate both in normolipidemic controls (R^2=0.691, *P*=0.003) and patients with genetic HTG (R^2=0.796, *P*=0.007; **Figure 3E**). For apoA-II, there was no correlation between the two methods in normolipidemic controls (R^2=0.088, *P*=0.41), while there was a correlation in patients with genetic HTG (R^2=0.606, *P*=0.04; **Figure 3F**).

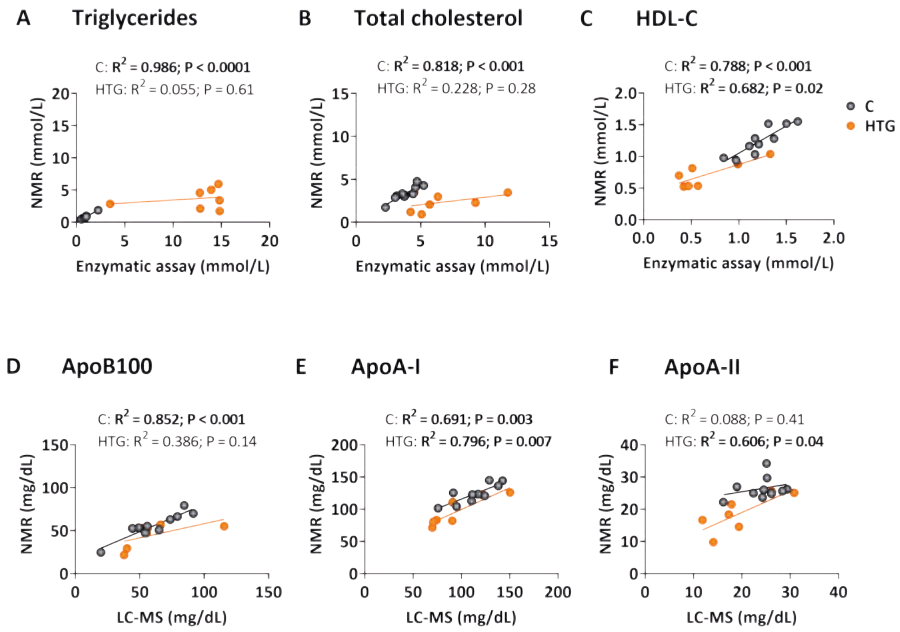

Figure 3. Association between lipids and apolipoproteins measured by NMR spectroscopy vs. enzymatic assays and LC-MS.

Top panels: linear regression between the measurement of triglycerides (A), total cholesterol (B) and HDL cholesterol (C) as measured with nuclear magnetic resonance (NMR) spectroscopy vs. an enzymatic assay. Bottom panels: linear regression between the measurement of apoB100 (D), apoA-I (E) and apoA-II (F) as measured with nuclear magnetic resonance (NMR) spectroscopy vs. liquid chromatography–mass spectrometry (LC-MS).

Apo, apolipoprotein; C, control group; HTG, hypertriglyceridemia; HDL-C, high-density lipoprotein-cholesterol; LC-MS, liquid chromatography–mass spectrometry; NMR, nuclear magnetic resonance; R^2, explained variance.

Large lipoproteins predominate in plasma of patients with genetic HTG

Next, we aimed to quantify the lipid composition of the lipoprotein subclasses using NMR spectroscopy. Patients with genetic HTG compared to normolipidemic controls had a higher chylomicron/VLDL particle concentration (327 [256] mmol/L *vs.* 110 [53] mmol/L, *P*<0.001) and higher IDL particle concentration (117 [92] mmol/L *vs.* 13 [25] mmol/L, *P*=0.007), while the LDL particle concentration was markedly lower (356 [157] mmol/L *vs.* 798 [268] mmol/L, *P*=0.001; **Figure 4A**). Further specification of the LDL subfractions showed that patients with genetic HTG, compared to normolipidemic controls, had a lower concentration of the smaller and denser LDL particles, namely LDL-3 (L; 32 [55] mmol/L *vs.* 109 [86] mmol/L, *P*=0.005), LDL-4 (M; 0.0 [0.0] mmol/L *vs.* 82 [56] mmol/L, *P*=0.001), and LDL-5 (S; 0.0 [0.0] mmol/L *vs.* 111 [91] mmol/L, *P*<0.001; **Figure 4B**). Of note, LDL-6 (XS) appeared similar between patients with genetic HTG and normolipidemic controls (248.1 [186.1] mmol/L vs. 225.5 [107.0] mmol/L, *P*=0.81; **Figure 4B**).

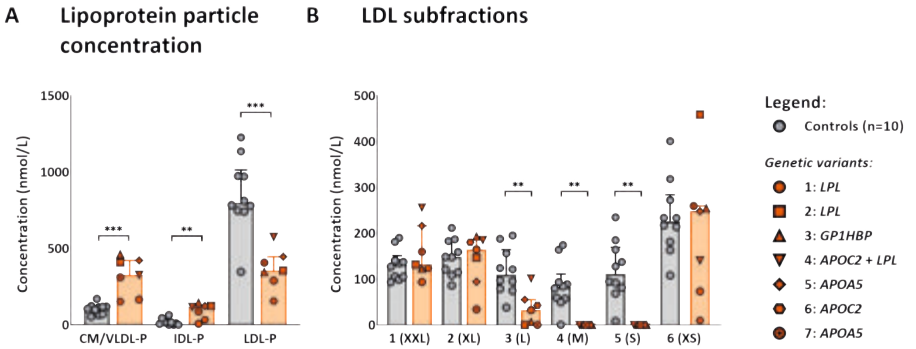

Figure 4. Lipoprotein particle concentration (A) and concentration of the different subfractions of the low-density-lipoprotein- (LDL-) particle based on size (B) in patients with genetic hypertriglyceridemia vs. normolipidemic controls as measured with nuclear magnetic resonance (NMR) spectroscopy.
Every shape illustrates a single patient with genetic hypertriglyceridemia with the genetic variant described in the legend. P-values are obtained from Mann-Whitney U test for independent groups. Values are median and interquartile range. ** P<0.01, ***P<0.001.
APO, gene encoding apolipoproteins; CM, chylomicron; GPIHBP1, gene encoding glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; IDL-P, intermediate-density lipoprotein particle; LDL-P, low-density lipoprotein particle; LPL, gene encoding lipoprotein lipase; VLDL-P, very-low-density lipoprotein particle.

We also estimated class-specific lipid composition and calculated standardized mean differences with 95% confidence intervals between patients with genetic HTG and normolipidemic controls. TG levels were higher for all lipoprotein classes in patients with genetic HTG, explicitly IDL (+2.3 [1.1 to 3.6]), LDL (+1.3 [0.3 to 2.4]), HDL (+3.8 [2.2 to 5.4]); **Supplementary Figure S2A**). In addition, patients with genetic HTG had higher chylomicron/VLDL-cholesterol (+2.6 [1.3 to 3.9]) and IDL-cholesterol (+2.0[0.9 to 3.2]),

but lower LDL-cholesterol (-2.7 [-4.0 to -1.4]) and HDL-cholesterol (-2.3 [-3.6 to -1.1]). Further division of the lipoprotein classes into subclasses showed that patients with genetic HTG had similar TGs, cholesterol, and phospholipids carried by LDL-1 (XXL) and LDL-2 (XL) (**Supplementary Figure S2C**). In HDL subclasses, patients with genetic HTG had higher TGs in all subclasses, but lower cholesterol and phospholipids in HDL-2 (M), HDL-3 (S) and HDL-4 (XS) (**Supplementary Figure S2D**).

Lipid levels associated with VLDL are particularly challenging for the analysis platform because of the similarity between VLDL and chylomicrons. Nevertheless, TG and cholesterol levels were determined to be higher in chylomicrons/VLDL in patients with genetic HTG (+2.5[CI 1.2 to 3.7] and +2.6[1.3 to 3.9], respectively). When we focus on the subclasses, chylomicrons are more likely to be categorized as extremely large VLDL particles. Thus, we can conclude that patients with genetic HTG have higher TGs, cholesterol, and phospholipids carried by chylomicrons/VLDL-1 (XL), VLDL-2 (L), VLDL-3 (M) and VLDL-4 (S) (**Supplementary Figure S2B**).

DISCUSSION

The purpose of this study was to obtain a comprehensive overview of the circulating (apo)lipoprotein profile in patients with genetic HTG using two modalities, *i.e.* LC-MS and NMR spectroscopy. We show that, in addition to apoB48, a chylomicron-specific apolipoprotein, also the levels of the exchangeable apolipoproteins apoC-I, apoC-II, apoC-III and apoE, are higher in patients with genetic HTG compared to normolipidemic controls, probably as constituents of accumulating chylomicrons and VLDL. On the other hand, although the total apoB100 level was similar between patients and normolipidemic controls, patients with genetic HTG had lower numbers of the smaller apoB100 carrying LDL particles compared to normolipidemic controls and this difference becomes stronger the smaller the lipoproteins get. Together, this supports the notion that dysfunctional LPL leads to accumulation of large TG-rich lipoproteins, whereas it prevents the formation of the small atherogenic lipoprotein remnant particles, probably limiting the cardiovascular disease risk in patients with genetic HTG. This is further corroborated by a trend towards lower apo (a) levels between patients with genetic HTG and normolipidemic controls.

In line with previous reports (14, 24), we show that patients with genetic HTG have high circulating apoB48. As a single apoB48 molecule is present per chylomicron particle (25), the number of chylomicron particles is on average 11-fold higher in patients with genetic HTG. Chylomicron particles transport dietary lipids from the small intestine to peripheral tissues. They consist of >90% triglycerides, resulting in their ultra-low dense

and large sized appearance (25). LPL is crucial for the hydrolysis of the triglycerides from chylomicrons to ensure that the smaller-sized chylomicron remnants can pass the endothelial fenestrae of the liver of approx. 100 nm (26). On the other hand, VLDL, IDL and LDL particles have a single molecule of apoB100 (15). The comparable apoB100 levels in patients with genetic HTG and normolipidemic controls suggests that production and uptake of apoB100-carrying lipoprotein particles by the liver overall is intact. However, these data should be interpreted with caution as NMR spectroscopy demonstrated higher combined chylomicron/VLDL as well as IDL concentrations and lower LDL concentrations in patients with genetic HTG compared to normolipidemic controls. This effect can be explained by impaired LPL-mediated lipolytic processing of VLDL resulting in a lower concentration of the lipolytic end product LDL (27).

In addition, we show that the concentration of the exchangeable apolipoproteins, *i.e.* apoC-I, apoC-II, apoC-III and apoE, is higher in patients with genetic HTG compared to normolipidemic controls. TG-rich lipoproteins acquire these liver-derived apolipoproteins during their secretion of the liver (*i.e.*, VLDL) and/or from the circulation (*i.e.*, chylomicrons), to regulate LPL-mediated lipolysis and the formation of lipoprotein remnants (28, 29). A likely determinant for the increase in the circulating apoC-I, apoC-II, apoC-III and apoE is the relative increase in surface area of the TG-rich lipoproteins in patients with genetic HTG, that simply can harbor more apolipoproteins. Nevertheless, in patients with genetic HTG *vs.* normolipidemic controls the relative enrichment of apoC-II (4.3-fold) is bigger than that of apoC-I (1.5-fold) or apoC-III (3.4-fold), suggesting that the available surface area is not the only determinant for the increase in the exchangeable apolipoproteins. It is likely that decreased lipoprotein surface curvature of larger lipoproteins also play a role, as we previously observed that the ratio of apoE/apoC acquired by recombinant triglyceride-rich lipoproteins after incubation with serum highly depends on particle size (30).

The exchangeable apolipoproteins have specific functions in lipoprotein metabolism. Whereas apoC-II acts as cofactor in the activation of LPL, LPL activity is inhibited by apoC-I (31) and apoC-III (32-35). Moreover, apoC-I and apoC-III both inhibit the uptake of lipoprotein remnants by the liver (32), whereas apoE mediates the uptake of lipoprotein remnants by the liver via the LDL receptor and LDL receptor-related protein-1 (LRP1) (28). Interestingly, consistent with our results, increased apoC-II is associated with hypertriglyceridemia (36), and chylomicrons are shown to contain relatively more apoC-II than VLDL particles in postprandial state (37). The relatively high apoC-II and apoE levels in patients with genetic HTG compared with apoC-I and apoC-III levels may be an attempt of the body to compensate for impaired LPL activity by stimulating residual LPL activity and enhancing hepatic uptake of triglyceride-rich lipoproteins.

In this study, we included four patients with FCS due to homozygous mutations in *LPL* (n=2) or *GPIHBP1* (n=1), or due to double heterozygous mutations in *APOC2* and *LPL* (n=1). In addition, we included three patients with MCS, due to heterozygous mutations in *APOA5* (n=2) or *APOC2* (n=1). The two patients with a homozygous *LPL* mutation had the highest non-HDL-cholesterol/apoB ratio. In line with this, LDL-cholesterol was previously shown to be lower in patients with a *LPL* mutation compared to other forms of FCS (14), together hinting towards a higher cholesterol content particularly in larger TG-rich lipoproteins in patients with an *LPL* mutation. Interestingly, the patient with MCS due to a mutation in *APOA5* (patient with genetic HTG number 5) showed the highest levels of apoB48, apoB100, apoC-I, apoC-II, apoC-III and apoE. In addition, this patient had the highest cholesterol levels, whereas TG levels were comparable with the other patients with genetic HTG. Though only a single observation, it is tempting to speculate that this mutation specifically increases the number of circulating concentrations of TG-rich lipoproteins, whereas the lipoprotein particles itself are not larger in size. Such a conclusion would be in line with the proposed role for apoA-V in lipoprotein remnant uptake by proteoglycans and/or lipoprotein receptors (38). Nevertheless, biochemical differences between the different mutations affecting LPL function are subtle and should be confirmed using a larger sample size.

We used NMR spectroscopy to estimate lipoprotein subclasses and class-specific lipid composition. NMR spectroscopy uses the distinctive magnetic susceptibility of lipoprotein subclasses that result from their chemical composition and size, to obtain a unique NMR spectrum that predicts lipoprotein particle concentration (39, 40). However, the prediction of the lipoprotein subclasses and their lipid composition is dependent on the calibration with reference methods, by the use of a machine learning model that assumes that the complex signals generated by the lipoprotein in the NMR spectrum behave similarly as in fasted normolipidemic individuals (40). Indeed, for our normolipidemic controls, we could verify that NMR-derived TG, total cholesterol and HDL-cholesterol levels correlate well with the values measured using an enzymatic assay. For our patients with HTG, both methods showed that TG levels were elevated and HDL-cholesterol were reduced, however, we found that TG and total cholesterol levels in patients with genetic HTG as measured with NMR spectroscopy did not show a significant correlation with the direct enzymatic measurement of these lipids. Moreover, the counterintuitive finding that the concentration of LDL-6 (XS) was similar between patients with genetic HTG and normolipidemic controls can probably be explained by misclassification of this subfraction in the analysis model, leading to the inclusion of large HDL particles within this subfraction. For the hyperlipidemic samples of patients with genetic HTG, we hypothesize that the high concentrations of exceptionally large chylomicron particles disturb the machine learning model, due

to their large deviation from the norm and the absence of chylomicrons in the B.I.-LISA model. The same applies to the B.I.-LISA-derived apolipoprotein concentrations. Therefore, we propose that an adjusted or dedicated machine learning model should be developed in order to achieve a more accurate prediction of the lipoprotein (sub) fraction concentrations and their lipid composition for samples of patients with genetic HTG using NMR spectroscopy.

This work is not without limitations. Inherent to the rare nature of genetic HTG with mutations in *LPL* or genes encoding for its regulators, the sample size of the current study is small, hindering statistical comparisons between the individual mutations. Difficulties in recruiting patients with genetic HTG additionally resulted in poorly matched groups, wherein patients with genetic HTG appeared to be somewhat older and had a slightly higher BMI compared to the normolipidemic controls. Nonetheless, in the figures and tables we show all individual datapoints and illustrate therewith that differences between each patient with genetic HTG and the normolipidemic controls were consistent regardless of age and/or BMI. Lastly, as discussed in the previous paragraph, our results obtained with NMR spectroscopy should be interpreted with caution. Yet, the results of the current study are still of interest as a lead for future research into the importance of lipoprotein subfractions.

In conclusion, by assessing a broad spectrum of apolipoproteins using LC-MS in combination with the prediction of lipoprotein subclasses and their composition using NMR spectroscopy, we show that patients with genetic HTG accumulate large TG-enriched lipoproteins (*i.e.*, chylomicrons, VLDL, IDL), which can explain high levels of the exchangeable apolipoproteins apoC-I, apoC-II, apoC-III and apoE, whereas the formation of the small atherogenic LDL particles is limited.

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SUPPLEMENTAL DATA

Table S1. Apolipoprotein concentration in patients with genetic hypertriglyceridemia vs. normolipidemic controls.

			1	2	3	4	5	6	7
	Normolipidemic controls (n=10)	**Patients with genetic HTG (n=7)**	***LPL* c. 548A>G. p. Asp183Gly**	***LPL* c. 548A>G. p. Asp183Gly**	***GP1HBP* deletion**	***APOC2* c.245T>G and *LPL* c.1279G>A**	***APOA5* 427delC**	***APOC2* c.245 T>G**	***APOA5* 161+5 SC>G and E2/E2**
ApoB100 mg/dL	60.4 [32.7]	55.2 [26.0]	40.1	55.2	55.9	66.1	115.5	37.8	51.4
ApoB (tot) mg/dL	62.3 [28.8]	64.0 [27.1]	43.4	64.0	67.1	70.5	124.8	40.3	57.7
ApoB48 mg/dL	0.4 [0.3]	4.4 [7.6]***	2.0	9.5	4.4	4.4	14.8	1.1	1.9
ApoC-I mg/dL	1.7 [0.5]	2.6 [2.0]***	2.2	4.2	2.5	3.1	8.0	2.0	2.6
ApoC-II mg/dL	1.8 [1.8]	7.7 [9.0]**	9.5	11.6	5.9	2.3	32.8	2.6	7.7
ApoC-III mg/dL	6.4 [4.3]	21.7 [16.7]***	23.7	34.9	18.2	21.7	78.5	18.5	15.3
ApoE mg/dL	2.1 [1.1]	8.9 [7.1]***	6.6	14.0	8.9	10.3	17.1	8.9	6.9
ApoA-I mg/dL	114.2 [37.1]	90.5 [50.4]	69.8	74.2	71.0	90.9	121.3	90.5	150.2
ApoA-II mg/dL	24.8 [5.2]	17.9 [12.1]	14.1	17.3	11.9	17.9	30.8	19.4	26.2
ApoA-IV mg/dL	17.6 [11.2]	25.3 [9.7]	19.2	25.3	26.3	28.9	31.0	15.5	23.5
Apo (a) nmol/dL	10.0 [23.8]	5.4 [8.4]	2.0	4.1	9.1	19.6	5.4	5.2	12.5
ApoB100 /Apo A-I	0.5 [0.2]	0.7 [0.4]	0.6	0.7	0.8	0.7	1.0	0.4	0.3

Patients with genetic hypertriglyceridemia (HTG) and normolipidemic controls are compared with independent-samples Mann-Whitney U test. Values are presented as median and interquartile range. **P<0.01, ***P<0.001.

Apo, apolipoprotein; GPIHBP1, glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; LPL, lipoprotein lipase.

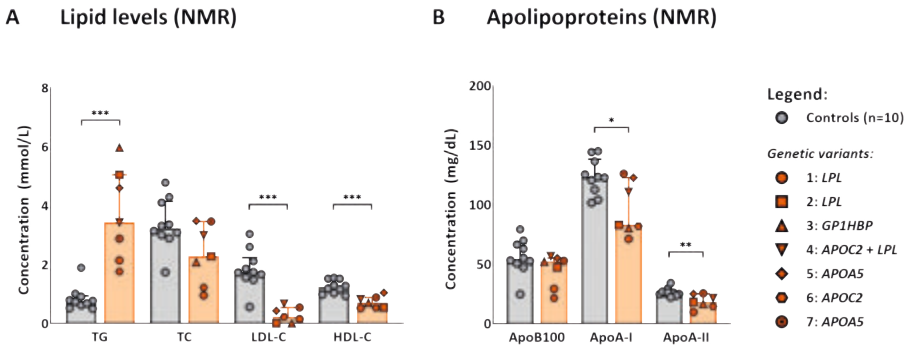
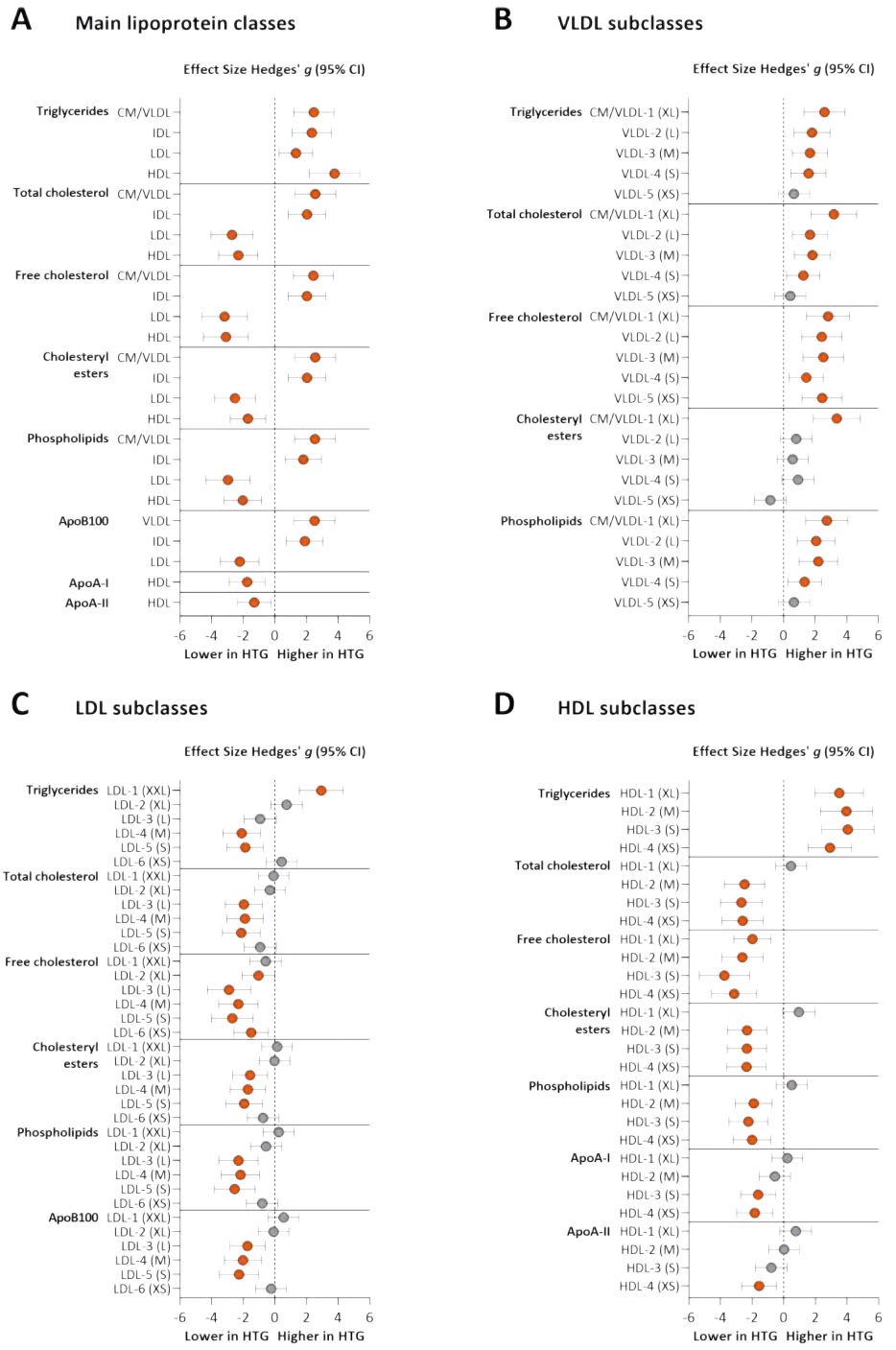


Figure S1. Serum lipid and apolipoprotein levels in patients with genetic hypertriglyceridemia vs. normolipidemic controls as measured with nuclear magnetic resonance (NMR) spectroscopy.
Every shape illustrates a single patient with genetic hypertriglyceridemia with the genetic variant described in the legend. P-values are obtained from Mann-Whitney U test for independent groups. Values are median and interquartile range. ** P<0.01, ***P<0.001.
Apo, apolipoprotein; APO, gene encoding apolipoproteins; GPIHBP1, gene encoding glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; LPL, gene encoding lipoprotein lipase; NMR, nuclear magnetic resonance; TC, total cholesterol; TG, triglyceride.


A
Main lipoprotein classes
Effect Size Hedges' g (95% CI)
Triglycerides CM/VLDL
IDL
LDL
HDL
Total cholesterol CM/VLDL
IDL
LDL
HDL
Free cholesterol CM/VLDL
IDL
LDL
HDL
Cholesteryl esters CM/VLDL
IDL
LDL
HDL
Phospholipids CM/VLDL
IDL
LDL
HDL
ApoB100 VLDL
IDL
LDL
ApoA-I HDL
ApoA-II HDL
-6 -4 -2 0 2 4 6
Lower in HTG Higher in HTG
B
VLDL subclasses
Effect Size Hedges' g (95% CI)
Triglycerides CM/VLDL-1 (XL)
VLDL-2 (L)
VLDL-3 (M)
VLDL-4 (S)
VLDL-5 (XS)
Total cholesterol CM/VLDL-1 (XL)
VLDL-2 (L)
VLDL-3 (M)
VLDL-4 (S)
VLDL-5 (XS)
Free cholesterol CM/VLDL-1 (XL)
VLDL-2 (L)
VLDL-3 (M)
VLDL-4 (S)
VLDL-5 (XS)
Cholesteryl esters CM/VLDL-1 (XL)
VLDL-2 (L)
VLDL-3 (M)
VLDL-4 (S)
VLDL-5 (XS)
Phospholipids CM/VLDL-1 (XL)
VLDL-2 (L)
VLDL-3 (M)
VLDL-4 (S)
VLDL-5 (XS)
-6 -4 -2 0 2 4 6
Lower in HTG Higher in HTG
C
LDL subclasses
Effect Size Hedges' g (95% CI)
Triglycerides LDL-1 (XXL)
LDL-2 (XL)
LDL-3 (L)
LDL-4 (M)
LDL-5 (S)
LDL-6 (XS)
Total cholesterol LDL-1 (XXL)
LDL-2 (XL)
LDL-3 (L)
LDL-4 (M)
LDL-5 (S)
LDL-6 (XS)
Free cholesterol LDL-1 (XXL)
LDL-2 (XL)
LDL-3 (L)
LDL-4 (M)
LDL-5 (S)
LDL-6 (XS)
Cholesteryl esters LDL-1 (XXL)
LDL-2 (XL)
LDL-3 (L)
LDL-4 (M)
LDL-5 (S)
LDL-6 (XS)
Phospholipids LDL-1 (XXL)
LDL-2 (XL)
LDL-3 (L)
LDL-4 (M)
LDL-5 (S)
LDL-6 (XS)
ApoB100 LDL-1 (XXL)
LDL-2 (XL)
LDL-3 (L)
LDL-4 (M)
LDL-5 (S)
LDL-6 (XS)
-6 -4 -2 0 2 4 6
Lower in HTG Higher in HTG
D
HDL subclasses
Effect Size Hedges' g (95% CI)
Triglycerides HDL-1 (XL)
HDL-2 (M)
HDL-3 (S)
HDL-4 (XS)
Total cholesterol HDL-1 (XL)
HDL-2 (M)
HDL-3 (S)
HDL-4 (XS)
Free cholesterol HDL-1 (XL)
HDL-2 (M)
HDL-3 (S)
HDL-4 (XS)
Cholesteryl esters HDL-1 (XL)
HDL-2 (M)
HDL-3 (S)
HDL-4 (XS)
Phospholipids HDL-1 (XL)
HDL-2 (M)
HDL-3 (S)
HDL-4 (XS)
ApoA-I HDL-1 (XL)
HDL-2 (M)
HDL-3 (S)
HDL-4 (XS)
ApoA-II HDL-1 (XL)
HDL-2 (M)
HDL-3 (S)
HDL-4 (XS)
-6 -4 -2 0 2 4 6
Lower in HTG Higher in HTG

◀—— **Figure S2.** Lipoprotein content per distinct lipoprotein class (A), VLDL subclass (B), LDL subclass (C), and HDL subclass (D) in patients with genetic hypertriglyceridemia vs. normolipidemic controls as measured with nuclear magnetic resonance (NMR) spectroscopy.
Hedges' g effect sizes were used to determine the difference between patients with genetic hypertriglyceridemia (HTG) and normolipidemic controls. Values are standardized effect sized and 95% confidence intervals. Datapoints on the left side of the dashed line indicate that the concentration is lower in patients with genetic HTG, datapoints on the right side of the dashed line indicate that the concentration is higher in patients with genetic HTG. Orange color indicates that the difference between patients with genetic HTG and normolipidemic controls is statistically significant, grey color indicates not significant.
CI, confidence interval; CM, chylomicron; HDL, high-density lipoprotein; HTG, hypertriglyceridemia; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein.