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Interrogation of selected genes influencing serum LDL-Cholesterol levels in patients with well characterized NAFLD

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Abstract

Background—The clinical significance of rare mutations in LDL metabolism genes on nonalcoholic fatty liver disease (NAFLD) severity is not well understood.

Objective—To examine the significance of mutations in LDL metabolism genes including apolipoprotein B (*APOB*), proprotein convertase subtilisin kexin 9 (*PCSK9*) and LDL receptor (*LDLR*) in patients with NAFLD.

Methods—Patients with biopsy-confirmed NAFLD from the NASH Clinical Research Network studies were stratified into 3 groups of LDL-C (≤ 50 mg/dL, 130–150 mg/dL, ≥ 190 mg/dL) and then 120 (40 per group) were randomly selected from the strata. We examined the presence of mutations on LDL genes and analyzed its association with selected NAFLD-related features. Multivariable analyses were adjusted for age, race, gender and use of statins.

Results—Among 40 patients with LDL-C ≤ 50 mg/dL, 7 (18%) patients had heterozygous variants in *APOB* and 2 had heterozygous variants in *PCSK9* (5%). We also found heterozygous

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Specific author contributions

All authors made substantial contributions to the intellectual content of the paper and approved the final version of the manuscript.

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mutations in 3 (8%) patients with LDL-C ≥ 190 mg/dL; 2 and 1 located in *LDLR* and *APOE* genes, respectively. Compared to wild-type controls with LDL-C ≤ 50, *APOB* carriers displayed higher levels of alanine aminotransferase (85.86 ± 35.14 U/L vs 45.61 ± 20.84 U/L, Adj. P=0.002) and steatosis >66% (57% vs 24%, Adj. P=0.050). These associations remained statistically significant after excluding statin users. Other histological features of NAFLD severity were not different between wild-type controls and *APOB* mutation carriers.

Conclusion—Mutations in the *APOB* gene are common among NAFLD patients with very low LDL-C and may be associated with increased aminotransferase levels and steatosis severity.

Keywords

primary hypobetalipoproteinemia; hypercholesterolemia; apolipoprotein B gene; proprotein convertase subtilisin kexin 9 gene; low-density lipoprotein receptor gene; nonalcoholic fatty liver disease; rare genetic variants; next-generation sequencing

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) may develop and progress towards cirrhosis and hepatocellular carcinoma (HCC) as a result of a complex process in which many factors, including metabolic and genetic susceptibilities, are involved.[1–3] A growing body of evidence indicates that NAFLD predisposition and severity may be strongly modulated by the host genetic background, that along with environmental factors will determine different clinical phenotypes and patterns of disease progression.

Familial hypobetalipoproteinemia (FHBL) is caused by several rare mutations in genes affecting LDL-C metabolism, including apolipoprotein (apo) B (*APOB*), proprotein convertase subtilisin kexin 9 (*PCSK9*), and angiopoietin-like protein 3 (*ANGPTL3)* which result in low or absent levels of apoB, low-density lipoprotein cholesterol (LDL-C) (<50 mg/dl or below the 5th percentile) and triglycerides in plasma.[4] Although most of individuals with FHBL might be at lower risk of atherosclerosis and coronary heart disease (CHD), only those due to heterozygous hypobetalipoproteinemia, abetalipoproteinemia and apoB truncations may exhibit hepatic steatosis.[5] In patients with *APOB*-related FHBL, the intracellular increase of triglycerides in the liver is due to a lower secretion rate of apoB-100 very low-density lipoprotein (VLDL) from the liver, decreased production of apoB-100 LDL, increased catabolism of VLDL and extremely low secretion of the truncated apoB.[6–9] VLDL is a major triglyceride carrier in plasma, thus its decreased secretion from the liver would result in a significant reduction of triglyceride export from the liver, which may lead to the development of hepatic steatosis.[10] Interestingly, the increase accumulation of triglycerides causing fatty liver in FHBL seems to be independent of hepatic or peripheral insulin resistance or diabetes.[6]

It has been suggested that *APOB*-related FHBL is associated not only with hepatic steatosis but also with its severity, which may depend on the ability of the truncated apoB to become lipidated; shorter truncations seem to be associated with more severe steatosis.[7, 11–14]. However, it is believed that *APOB*-related FHBL is not associated with aggressive

histological phenotypes of NAFLD because there is only accumulation of triglycerides, not other lipotoxic mediators such as ceramides and free fatty acids, etc.[15]

It is now known that certain genetic variants such as the *PNPLA3* (patatin-like phospholipase domain–containing 3) rs738409-G[16], *TM6SF2* (transmembrane 6 superfamily member 2) rs58542926-T[17], *HSD17B13* (hydroxysteroid 17-beta dehydrogenase 13) rs6834314-G[18], *MBOAT7* (membrane bound O-acyltransferase domain containing 7) rs641738-T[19] and *GCKR* (glucokinase regulatory protein) rs1260326-T[20] may disturb certain pathways in hepatic lipid metabolism and are considered key determinants in the susceptibility to progress to nonalcoholic steatohepatitis and cirrhosis among NAFLD individuals. The risk allele frequency for each genetic variant is as follows: *PNPLA3* rs738409 (G-allele), 29%[16]; *HSD17B13* rs6834314 (G-allele), 21%[18]; *TM6SF2* rs58542926 (T-allele), 5%[21]; *MBOAT7* rs641738 (T-allele), 45%[22] and *GCKR* rs1260326 (T-allele), 31%[20], although it can greatly vary by race and ethnicity.

We recently reported a father-son pair who were each carriers of *APOB*-related FHBL and had aggressive NAFLD, including nonalcoholic steatohepatitis (NASH), cirrhosis, and HCC in the father.[23] Interestingly, those two patients also were carriers for *PNPLA3* rs738409-G allele, which is associated with an increased risk of NASH.[24] Thus, we hypothesized that double heterozygosity for *APOB* and *PNPLA3* rs738409 resulted in clinically significant NAFLD in our father-son case series. This led us to search for mutations in selected genes involved in LDL-C metabolism, its interactions with other genetic variants related to NAFLD and their impact on liver histology severity in patients with well characterized NAFLD who had a wide range of serum LDL-C levels.

MATERIALS AND METHODS

Study population

A total of 1511 adults with biopsy confirmed NAFLD, ≥ 18 years of age and both sexes were selected from the NASH Clinical Research Network (NASH CRN) studies, including NAFLD Adult Database 1, NAFLD Adult Database 2 (NCT01030484) and PIVENS (Pioglitazone vs Vitamin E vs Placebo for Treatment of Non-Diabetic Patients With Nonalcoholic Steatohepatitis) (NCT00063622) and FLINT (The Farnesoid X Receptor Ligand Obeticholic Acid in NASH Treatment) (NCT01265498) trials.[25–27] We first stratified the entire cohort into three groups of LDL-C levels (≤ 50 mg/dL, between 130 and 150 mg/dL and ≥ 190 mg/dL) and then 120 (40 patients per group) were randomly selected from the strata. The NASH CRN includes a multiethnic and multicenter US-based cohort consisting of 1697 patients with biopsy-proven NAFLD and absence of significant alcohol intake (defined as > 20g/day for men, > 10g/day women) who were prospectively enrolled and evaluated from 2002 through 2014. Given that a higher proportion of patients with NAFLD were taking lipid-lowering drugs, we did not exclude individuals who were on lipid-lowering medications. Details related with the NASH CRN observational study, including the subjects' demographic and anthropometric features, alcohol consumption and medical history, medications, including statin use at the time of study enrollment, lab tests, liver biopsy results, and complete inclusion and exclusion criteria have been published elsewhere.[25]

This study included three groups of patients with very low, middle and very high LDL-C levels, because different kinds of rare *APOB* mutations can have polar opposite effects on plasma LDL-C levels. FHBL variants affecting apoB protein integrity and stability produce very low LDL-C levels, while familial hypercholesterolemia-like variants occurring within the receptor-binding domain of apoB (such as R3500Q (R3527Q), among many others) result in very high LDL-C levels.[28] So a priori, there is a chance that blinded sequencing could find variants in either high or low extremes; in contrast, the middle of the LDL-C distribution should be completely blank for any rare mutations.

The study was approved by the institutional human investigation committee of each participating institution. Written informed consent was obtained from all participants.

Biochemical analysis and histological assessment

All laboratory, clinical and histological data analyzed in this study were obtained within 6 months of the liver biopsy. The following laboratory variables were included: serum alanine (ALT, U/L) and aspartate (AST, U/L) aminotransferase, triglyceride (mg/dL), total cholesterol (mg/dL), low-density lipoprotein cholesterol (LDL-C, mg/dL) high-density lipoprotein cholesterol (HDL-C, mg/dL) cholesterol, total bilirubin (mg/dL), fasting serum glucose (mg/dL), glycosylated hemoglobin (HbA1c in %) and insulin levels (mIU/L).

Steatosis, lobular inflammation, hepatocyte balloon degeneration, fibrosis and NAFLD activity scores, and the presence of NASH by pattern recognition were systematically assessed in a blinded fashion based on central pathology reading and according to the published NASH CRN Scoring System.[29]

Genetic analysis

The blood samples were maintained at 4°C until the plasma and serum were separated, aliquoted, and stored at −80°C. DNA samples corresponding to 120 patients with LDL-C levels ≤ 50 mg/dL (n=40), between 130 and 150 mg/dL (n=40) and ≥ 190 mg/dL were sent from the CRN consortium at a minimum concentration of 50 ng/μL per sample to Robarts Research Institute, Ontario, Canada for genetic testing. We used targeted next-generation sequencing to robustly characterize the genetic determinants influencing LDL-C levels in patients with extremely low and high, and middle levels of LDL-C. Patients were genotyped using LipidSeq, a targeted next-generation sequencing panel,[30] at the London Regional Genomics Centre using standard protocols (www.lrgc.ca). Sequencing was performed using an Illumina MiSeq personal sequencer (Illumina, San Diego CA, USA). CLC Bio Genomics Workbench (version 12.0; CLC Bio, Aarhus, Denmark) was used for the alignment of sequencing reads against the human reference genome (build hg19), the calling of variants, and the generation of variant call (VCF) and binary alignment (BAM) files. Rare variant analysis utilized several steps. Briefly, single-nucleotide variants were annotated using VarSeq® (version 2.1.1; Golden Helix, Inc., Bozeman MT, USA). Rare variants were defined as those having a minor allele frequency of ≤1% or missing from the Genome Aggregation Database (gnomAD; https://gnomad.broadinstitute.org/). Missense, nonsense, deletion, insertion, splice-acceptor, and splice-donor variants within candidate genes - *APOB*, *PCSK9*, *LDLR* and *APOE* - were retained for analysis. In silico prediction

algorithms were used to select variants with likely large phenotypic impacts: i.e. variants with a top 5th percentile Combined Annotation Dependent Depletion (CADD; http://cadd.gs.washington.edu/score) and predicted to be deleterious or damaging by at least one additional prediction tool, including Polymorphism Phenotyping version 2 (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant From Tolerant (SIFT; http://sift.jcvi.org/), or MutationTaster (http://www.mutationtaster.org/). Weighted genetic risk scores for LDL-C using common variants identified from genome-wide association studies were calculated for all patients as previously described.[31] This polygenic score has been used to detect patients at increased risk of coronary artery disease.[32]

We further genotyped all patients for the following SNPs: patatin-like phospholipase domain–containing 3 (*PNPLA3)* rs738409, transmembrane 6 superfamily member 2 (*TM6SF2)* rs58542926, *MBOAT7* rs64173, hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13)* rs6834314 and glucokinase regulatory protein (*GCKR)* rs1260326.

Statistical analysis

The baseline characteristics were summarized in percentages for categorical variables and mean ± standard deviation for continuous variables. Differences in the distribution of categorical variables including two groups were assessed by χ^2 test. The association between genetic mutations and categorical ordinal variables including three or more groups were assessed by the Jonckheere-Terpstra or Mantel-Haenszel trend tests. Differences between groups for continuous variables were assessed by *t* test or Mann-Whitney test when appropriate. Multivariable linear or logistic or ordinal regression models were used to determine association between presence of *APOB* mutations and selected baseline features. These analyses were adjusted for age, gender, race, and consumption of statins. Given that a higher proportion of wild-type controls with low LDL-C were taking statins at study enrollment, and this might have a significant impact on the distribution of certain baseline features (age, smoking status, prevalence of type 2 diabetes and hypertension), a sensitivity analysis was conducted by excluding those who did report statin therapy. Statistical analysis was performed using the Stata (Release 16. College Station, TX: StataCorp LLC) program.

RESULTS

Of the 1697 adults with biopsy proven NAFLD, 1557 were included after applying inclusion/exclusion criteria. Of them, 46 were excluded due to missing LDL-C values. Of the remaining 1511 participants with NAFLD, 47 (3%) had an LDL-C ≤ 50 mg/dL and 47 (3%) had an LDL-C ≥ 190 mg/dL. Figure 1 displays the flow of patients through the study and supplemental Table 1 shows the baseline characteristics of patients included in the whole cohort (n=1511) according to three different levels of LDL-C. The prevalence of definite steatohepatitis and advanced stages of fibrosis (bridging fibrosis and cirrhosis) was 65% and 23% in our selected cohort (n=120), respectively, and it did not significantly differ from the whole cohort (n=1511) (59% and 28%) and those patients that were excluded (n=1491) (55% and 26%) after our random selection.

Demographic and clinical characteristics of the selected cohort (n=120) are shown in Table 1. The proportion of patients taking statins was significantly higher among patients with

either very low (24, 60%) or very high (21, 53%) LDL-C levels. The frequency of type 2 diabetes was higher in patients with LDL-C levels ≤ 50 mg/dL (20, 60%) as compared to those between 130–150 mg/dL (14, 35%) or ≥ 190 mg/dL (11, 28%), respectively. The number of individuals who had never smoked was slightly higher among those with LDL-C levels ≤ 50 mg/dL (23, 59%) compared to those with middle (15, 38%) and very high (18, 45%) LDL-C levels subgroups. Since cholesterol-lowering therapy, particularly statins, are commonly prescribed in patients with very high risk of cardiovascular disease (older people, type 2 diabetes, smoking, etc.), it would be expected that patients enrolled in very low and very high LDL-C groups might be older and have higher prevalence of type 2 diabetes or smoking. There was a positive dose-dependent correlation between LDL-C and ALT levels even after controlling for age, gender, lipid-lowering medications, and alcohol consumption (Adj. correlation coefficient = 0.304, P<0.001). The remaining baseline features were similar across the three LDL-C subgroups.

Among 40 patients with LDL-C ≤ 50 mg/dL, a total of 8 (20%) participants had a heterozygous loss-of-function (LOF) mutation in either *APOB (n=6)* or *PCSK9 (n=1)* or both (n=1). No patient with *APOB* or *PCSK9* was taking statins at the time of study enrollment. Among those with LOF mutations in *APOB*, four individuals were heterozygous for frameshift deletions in exon 26 (c.10238delC (n=3), c.9115_9119del (n=1)), 2 patients were heterozygous for nonsense mutations in exon 26 (c.7472T>G, c.11330C>A) and one individual was heterozygous for a frameshift deletion in exon 7 (c.741_745del); all of these resulting in different premature truncations of apolipoprotein B. The most common mutation found in the *APOB* gene was the frameshift deletion in exon 26 (c.10238delC), seen in three carriers. Two individuals were found to be heterozygous either for a nonsynonymous substitution (c.137G>T) in *PCSK9* or frameshift deletion (c.202delG) in *PCSK9* exon 1, which result in a predicted LOF or premature truncation, respectively. Focusing on carriers of rare *APOB* variants, the 17.5% frequency (7/40) in this low LDL-C cohort compares to 0.0024% (6/250480) seen in the Genome Aggregation Database (gnomAD; https://gnomad.broadinstitute.org/); the risk ratio for carriers of these rare *APOB* variants among low LDL-C patients compared to controls was therefore 7300 (95% confidence interval 2568 to 202782; P<0.001).

Among patients with very high LDL-C (≥ 190 mg/dL) levels, 2 patients out of 40 (5%) were heterozygous for nonsynonymous substitutions in exon 6 (c.858C>A) and 3 (c.241C>T) of the *LDLR* gene, respectively; and 1 (2.5%) was heterozygous for an in-frame deletion in *APOE* exon 4 (c.496_498del), resulting in a known dysfunctional variant called p.Leu167del, which has been previously associated with hypercholesterolemia.[33]

The polygenic risk score was significantly higher in patients with very high (13.90 ± 2.09) levels of LDL-C as compared to those with middle (12.63 ± 1.97) levels of LDL-C, P=0.006. Among individuals with very low LDL-C levels, the mean level of polygenic risk score tended to be slightly higher than those with middle LDL-C levels (13.30 ± 1.55 vs 12.63 ± 1.97), although the difference was not statistically significant (P=0.124).

Associations of APOB mutations with NAFLD-related features

Table 2 displays the effect of *APOB* mutations on different NAFLD-related features among patients with very low LDL-C (≤ 50 mg/dL) levels. Patients carrying *APOB* mutations were more likely to be younger and consequently had lower frequency of type 2 diabetes and hypertension than those with wild-type *APOB*. However, these differences were no longer significant after controlling for the use of statins, age, gender, and race. Among patients with LDL-C ≤ 50 mg/dL, serum levels of triglycerides (58.14 ± 15.84 mg/dL vs 209.94 ± 199.09 mg/dL, adjusted P=0.037 after controlling for statins use and other confounders) were significantly lower among individuals with *APOB* variants than their controls. In addition, carriers of *APOB* mutations had increased levels of ALT (85.86 ± 35.24 U/L vs 45.61 ± 20.84 U/L, adjusted P=0.002 after controlling for statin use and other confounders) as compared with their controls. Regarding specific histologic findings, the severity of steatosis was higher in carriers of *APOB* mutations compared with controls (steatosis > 66%: 57% in *APOB* mutations vs 24% in wild-type controls, adjusted P=0.050 after controlling for statin use and other confounders). There were no statistically significant differences in other histological features, including fibrosis and presence of NASH, between carriers of *APOB* mutations and their wild-type controls.

Due to high frequency (24, 60%) of patients taking statins in the very low LDL-C (≤ 50 mg/dL) group, a separate analysis, excluding statin users, was conducted (Table 3). Thus, seven patients with *APOB* mutations were compared with 10 *APOB* wild-type controls. No statistically significant differences were observed for the frequency of type 2 diabetes (1 [14%] vs 3 [30%], P=0.452), hypertension (3 [43%] vs 5 [50%], P=0.772) and other clinical features between *APOB* variants and wild-type controls. Furthermore, serum levels of HbA1c (5.66 ± 1.10% vs 5.83 ± 1.45, P=0.795) and HOMA-IR (4.49 ± 2.10 vs 6.46 ± 7.67, P=0.523) were comparable between both groups, whereas ALT (85.86 ± 35.24 U/L vs 46.80 ± 27.86 U/L, P=0.022) and AST (58.14 ± 15.80 U/L vs 38.50 ± 15.15 U/L, P=0.021) levels remained significantly higher in carriers of *APOB* variants compared with their wild-type controls. As expected, serum triglycerides (56.14 ± 28.93 mg/dL vs 150.40 ± 116.23 mg/dL, P=0.033) was significantly lower among those carrying *APOB* variants versus wild-type controls. Finally, the severity of specific histological features did not significantly differ between *APOB* mutation carriers and controls, except for steatosis severity >66% (4 [57%] vs 1 [10%], P=0.036). Figure 2 displays mean levels of serum ALT (Panel A), AST (Panel B) and triglycerides (Panel C) as well as the proportion of patients with steatosis >66% (Panel D) amongst *APOB* mutations carriers and their controls.

Interestingly, the G allele of *PNPLA3* rs738409 variant was present in 6 (86%) and 9 (90%) patients with or without *APOB* mutations, respectively, which could explain partly the higher rates of definite NASH (4 [57%] vs 6 [60%]) and clinically significant fibrosis (3 [42%] vs 50 [50%]) seen in both groups, despite the fact that both groups showed a favorable metabolic phenotype. Furthermore, the proportion of patients with other NAFLD-related genetic variants (*TM6SF2* rs58542926-T, *HSD17B13* rs6834314-G, *MBOAT7* rs641738-T and *GCKR* rs1260326-T) associated with increased risk of NASH were fairly evenly distributed among *APOB* mutation carriers and their controls (Tables 3 and 4).

Case-based comparisons of baseline characteristics among carriers of APOB mutations.

Table 4 depicts individual baseline characteristics for those patients in whom *APOB* and/or *PCSK9* mutations were present. All patients (7 of 7) displayed histological features of severe NAFLD, including either a diagnosis of definite NASH or an NAS ≥ 4 or advanced fibrosis. Four (57%) of 7 patients also had a diagnosis of severe steatosis (>66%). Interestingly, most of them were very young (5 of 7 were ≤ 40 years old) and did not report a previous history of either type 2 diabetes (6 of 7) or hypertension (4 of 7). Five of 7 (71%) had ALT levels higher than 70 U/L and all had low levels of triglycerides, ranging between 18–94 mg/dL. A higher proportion of patients were carriers of SNPs with recognized association with NAFLD severity; *PNPLA3* rs738409-G allele (86%), *HSD17B13* rs6834314-G (71%), *MBOAT7* rs641738-T (57%) and *GCKR* rs1260326-T (57%). One of the patients with an *APOB* mutation also had a heterozygous *PCSK9* LOF variant which has also been associated with very low LDL-C in plasma, but not with steatosis. We additionally identified another patient with an isolated *PCSK9* LOF variant. This patient exhibited moderate steatosis along with moderate lobular inflammation and severe ballooning degeneration; he was neither diabetic, nor hypertensive, but was a carrier of 2 polymorphisms on *PNPLA3* (rs738409) and GCKR (rs1260326) gene, previously shown to be associated with the severity of NAFLD.

Case-based comparisons of baseline characteristics among carriers of LDLR or APOE mutations.

Among patients with very high LDL-C levels, we found two heterozygous individuals of loss-of-function *LDLR* missense mutations, namely p.Arg81Cys and p.Ser286Arg, that each disrupt receptor function leading to reduced hepatic LDL-C clearance and elevated plasma LDL-C, and one heterozygous patient for an in-frame deletion p.Leu167del variant in the *APOE* gene that is well known to be associated with hypercholesterolemia.[33] Table 5 shows individual baseline features for those patients carrying *LDLR* and *APOE* variants. Regarding histological features of NAFLD in these 3 patients, all of them had a diagnosis of definite NASH and 2 had advanced fibrosis. Two of 3 were on a statin at the time of study enrollment. The proportion of patients with NAFLD-related genetic variants linked to aggressive NASH was significantly higher for *PNPLA3* rs738409-G allele (100%), *HSD17B13* rs6834314-G (67%), *MBOAT7* rs641738-T (100%).

The genetic features as well as biological consequences of each mutation found in our study are shown in Table 6. Supplemental Table 2 and 3 display a comparative analysis of baseline features among patients with or without *LDLR* and *APOE* mutations.

DISCUSSION

In this study, we determined the frequency of rare gene mutations related with extremely low or high plasma LDL-C and assessed whether these rare genetic variants in *APOB*, *PCSK9*, *LDLR* and *APOE* were associated with increased severity of NAFLD as compared with their wild-type controls.

In our cohort of patients with very low LDL-C (≤ 50 mg/dL) levels and biopsy proven NAFLD, we identify 8 heterozygous carriers of *APOB* and *PCSK9* genetic variants. These individuals represent 20% of the whole population with LDL-C ≤ 50 mg/dL and 47% of those who did not report taking any lipid-lowering drug but also had very low LDL-C levels. The prevalence of carriers of such rare variants in this cohort exceeds that observed in the general population by several orders of magnitude. We further note that each of the 7 heterozygotes with *APOB* variants had not only extremely low LDL-C and triglyceride levels, but also histological evidence of severe NAFLD, including presence of definite NASH, or an NAS ≥ 4 or advanced fibrosis. Our results also suggest that, among heterozygotes for *APOB* mutations, advanced histological forms of NAFLD can be seen in very young adults (usually ≤ 40 years old), in the absence of well-known risk factors associated with NASH severity such as diabetes, hypertension or insulin resistance. Compared to individuals with very low LDL-C, patients carrying *APOB* genetic variants showed significantly higher levels of ALT, AST, and steatosis severity. However, no remarkable differences were observed for other histological features, including lobular inflammation, hepatocyte ballooning degeneration, and fibrosis between carriers of *APOB* variants and their wild-type controls. Therefore, based on our data, it is reasonable to consider that intrahepatic triglyceride accumulation due to *APOB* mutations per se may not be responsible for the aggressive histological phenotypes seen in these patients.[34]

Interestingly, most patients with heterozygous *APOB* and/or *PCSK9* mutations were coincidentally also carrying other deleterious genetic variants, including *PNPLA3* rs738409-G (88%), *HSD17B13* rs6834314-G (63%), *MBOAT7* rs641738-T (50%) and *GCKR* rs1260326-T (63%), which are associated with increased risk of steatosis and liver injury. We hypothesized that the co-inheritance of *APOB/PCSK9* variants with other hepatic steatosis-associated SNPs could explain the severe histological phenotypes seen among individuals carrying *APOB* or *PCSK9* mutations. These genetic variants may have multiple and diverse effects on metabolic pathways resulting in triglyceride accumulation, inflammation and fibrosis; thus, more severe histological phenotypes would be expected among patients carrying *APOB* and/or *PCSK9* genetic variants when other hepatic steatosis-associated SNPs are present.[6, 10, 16–18, 24, 35, 36]

Overall, our results are in alignment with previous studies reporting a positive association between *APOB* genetic variants[5, 37] or the use of apolipoprotein B inhibitors and fatty liver[38, 39]. It has also been suggested that carriers of *APOB*[5] or *PCSK9*[40] mutations are at substantially reduced risk of CHD. In our study, no patient carrying either an *APOB* or *PCSK9* mutation reported a history of cardiovascular disease. These findings suggest that identifying carriers with *APOB/PCSK9* mutations among patients with suspected primary hypobetalipoproteinemia is of paramount importance, given the divergent risks of CHD and fatty liver, so that efforts to ameliorate fatty liver progression may be prioritized.

APOB-related FHBL has been consistently associated with increased risk of hepatic steatosis, but little is known about the causal relationship between liver injury and *APOB* mutations. About 60 mutations have been reported to date in *APOB* causing FHBL, most are missense and frame-shift mutations specifying the production of truncated proteins. The length of apoB truncations seems to be responsible of the severity of the clinical phenotypes;

shorter truncations are associated with lower production/secretion and increased catabolism/clearance of ApoB-containing lipoprotein.[8] As a result, the transport of triglycerides from the liver through the VLDL export system is impaired resulting in hepatic accumulation of triglycerides and perhaps other lipid components of VLDL, including phospholipids, sphingolipids, and free cholesterol and fatty acids that have been implicated in the development and progression of NAFLD.[41] However, studies in mice with apoB truncations have shown a significant accumulation of triglycerides in the liver but no other lipids as cholesterol and phospholipid.[42, 43] Thus, whether free fatty acids, phospholipids, sphingolipids and cholesterol may accumulate in the liver of patients with *APOB*-related FHBL would require further confirmation.

Finally, it should be noted that interaction with other genes and environmental factors could also influence the phenotype *APOB*-related FHBL. A small number of case studies have reported that individuals with FHBL can develop cirrhosis and hepatocellular carcinoma.[44, 45] However, most of these studies did not explore the influence of other fatty liver-associated SNPs on the susceptibility to develop more aggressive clinical forms of NAFLD. These genetic variants in genes affecting lipid metabolism, oxidative stress and insulin resistance may act as predisposing factors to development of fatty liver and its progression. Our study provides interesting insights on potential interactions between *APOB* mutations and other fatty liver-related SNPs and the risk on NASH and fibrosis. In absence of insulin resistance, the liver damage observed among *APOB/PCSK9* carriers suggests that mechanisms involving other metabolic pathways might trigger the transition from simple steatosis to NASH and cirrhosis. Further large-scale studies might confirm the long-term effects of certain SNPs associated with fatty liver in patients with FHBL.

Although carriers of *APOB* mutations are at increased risk of fatty liver, the association between hepatic steatosis and *PCSK9* mutations is less clear. In fact, either loss of function mutations in the *PCSK9* gene or reduction of LDL-C following treatment with PCSK9 antibodies have not been linked with hepatic steatosis.[46–48] We note that both heterozygotes patients for *PCSK9* mutations had severe NAFLD, however one of them was also heterozygous for an *APOB* mutation, and furthermore the allele G of *PNPLA3* rs738409 variant was present in both individuals. Thus, the increased severity of NAFLD seen among carriers of LOF *PCSK9* mutations may be related to accumulation of other genetic variants linked to NAFLD severity.

Finally, we found that 3 patients with extremely high levels of LDL-C (≥190 mg/dL) had heterozygous mutations in *APOE* (1 patient) and *LDLR* (2 patients) genes. The extremely rare *APOE* mutation is an in-frame deletion of the leucine at residue 167 and has been reported in families with heritable hypercholesterolemia.[33] Two additional patients had more typical rare LOF heterozygous mutations in the *LDLR* gene, which accounts for > 90% of mutations seen in patients with familial hypercholesterolemia.[49] Mutations in the *LDLR* gene generally lead to decreased clearance of LDL-cholesterol from plasma and consequently increases in plasma total and LDL-cholesterol concentrations.[50] Although patients here with rare *APOE* and *LDLR* genetic variants had severe histological phenotypes of NAFLD, there is not clear pathobiological explanations to link these genetic mutations with NAFLD severity.

To our knowledge, very few studies have specifically examined the relationship between fatty liver severity and rare mutations in *APOB* gene in well-characterized NAFLD populations. In fact, this is the first study exploring the frequency of rare mutations in *APOB, PCSK9, LDLR* and *APOE* genes in adults with biopsy-confirmed NAFLD and extremely low or high levels of LDL-C.

Some study limitations need to be highlighted. We were not able to compare the effect of *APOB/PCSK9* variants stratified by other hepatic steatosis-associated SNPs (e.g., *PNPLA3* rs738409 allele G vs C) given the small number of patients with *APOB/PCSK9* mutations. Due to the cross-sectional study design, a further limitation was our inability to examine the effect of LDL-C related genetic variants on long-term hepatic and cardiovascular consequences, thus the long-term clinical impact of these genetic variants needs to be validated in future prospective studies. Our findings may only be applicable to non-Hispanic whites. Finally, we could not quantify the levels of free fatty acids, cholesterol, phospholipids, and sphingolipids in the liver.

In summary, our findings demonstrate the importance of using a next-generation sequencing platform to enable characterization of low-frequency and rare pathogenic genetic variants involved in the predisposition of fatty liver and/or cardiovascular disease. In individuals with very low LDL-C levels, we confirmed that about one-fifth of patients had rare *APOB* or *PCSK9* variants. Patients carrying *APOB* mutations associated with hypobetalipoproteinemia are at increased risk of moderate or severe steatosis, however, the risk of inflammation and fibrosis in these patients does not seem to be different as compared to their wild-type controls. Furthermore, our study generates the hypothesis that more severe clinical expression may reflect the joint effect of the *APOB/PCSK9* variants plus fatty liver-associated SNPs that have previously been associated with more aggressive histological phenotypes of NAFLD. Finally, in patients with extremely high levels of LDL-C, we identify two *LDLR* genetic variants associated with increased risk of cardiovascular disease. Taken all together, our findings highlight the importance of screening for highly informative rare and low-frequency genetic variants through genome-wide association studies and next generation sequencing approaches, with implications for associated liver disease among risk NAFLD individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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For full disclosure

Dr. Chalasani has ongoing paid consulting activities (or had in preceding 12 months) with NuSirt, AbbVie, Allergan (Tobira), Madrigal, La Jolla, Foresite labs, Galectin, Zydus, and Genentech. These consulting activities are generally in the areas of nonalcoholic fatty liver disease and drug hepatotoxicity. Dr. Chalasani receives research grant support from Exact Sciences, Intercept, and Galectin Therapeutics where his institution receives the funding.

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Dr. Vilar-Gomez and Liang have nothing to disclose.

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Abbreviations

NAFLD	Nonalcoholic fatty liver disease
HCC	hepatocellular carcinoma
PNPLA3	Patatin-like phospholipase domain-containing 3
HSD17B13	hydroxysteroid 17-β dehydrogenase 13
TM6SF2	transmembrane 6 superfamily member 2
MBOAT7	membrane bound O-acyltransferase domain-containing 7
GCKR	glucokinase regulatory protein gene
NASH	Nonalcoholic steatohepatitis
FHBL	Familial hypobetalipoproteinemia
CHD	coronary heart disease
LDL	Low-density lipoprotein
APOB	Apolipoprotein B
PCSK9	Proprotein convertase subtilisin kexin 9
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
HDL	High-density lipoprotein
BMI	Body mass index

Hb1Ac	Hemoglobin A1c
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
NAS	NAFLD activity score
LDLR	low-density lipoprotein receptor
APOE	Apolipoprotein E
LOF	loss-of-function

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HIGHLIGHTS

- Genetic mutations in the *APOB* gene are responsible of the majority of FHBL
- *APOB* mutations associate to hepatic steatosis
- Whether *APOB* mutations associate to hepatic inflammation and fibrosis is unclear
- *APOB* mutations associated with increased ALT levels and steatosis severity
- Severity of inflammation/fibrosis was not significantly increased in *APOB* mutations

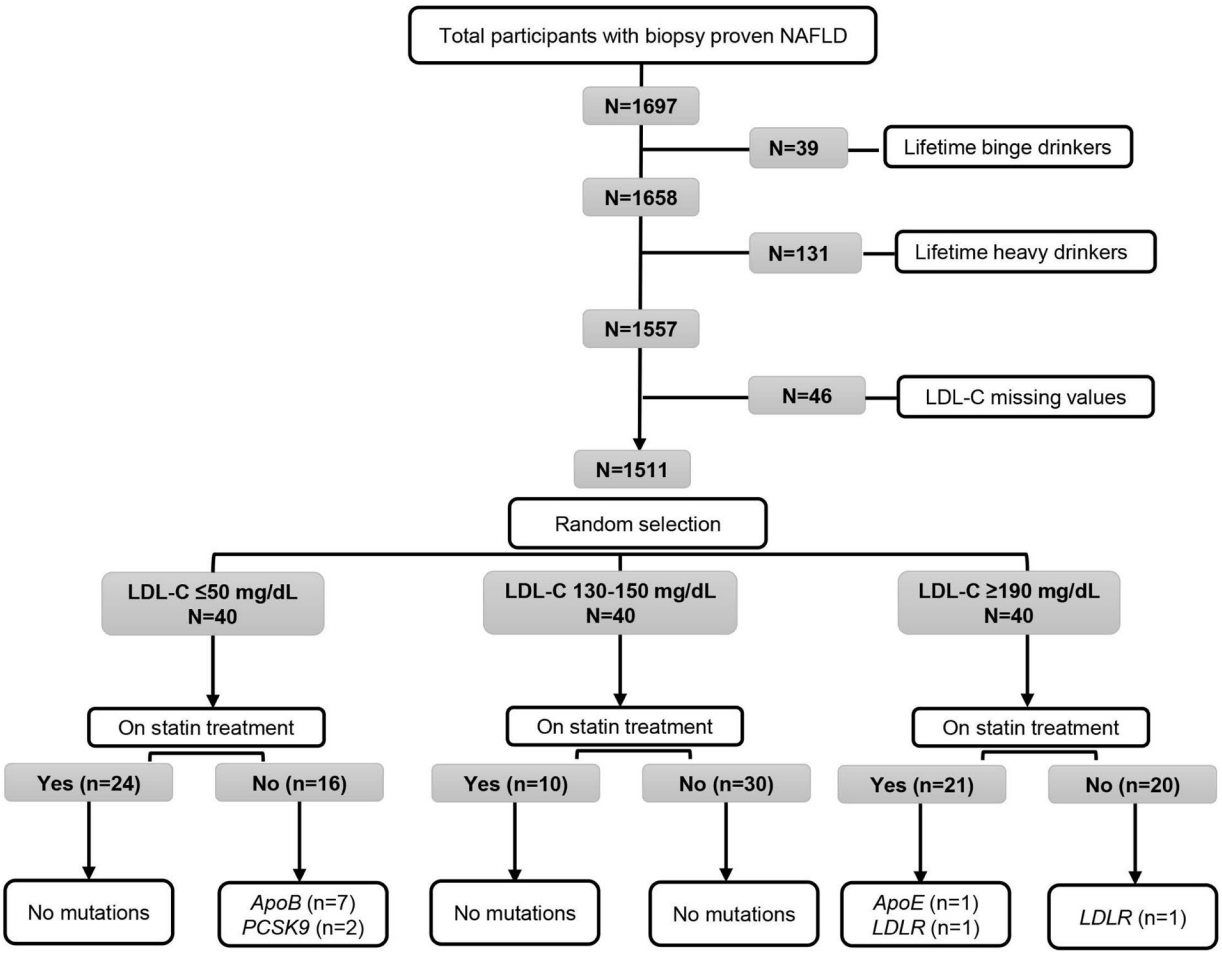
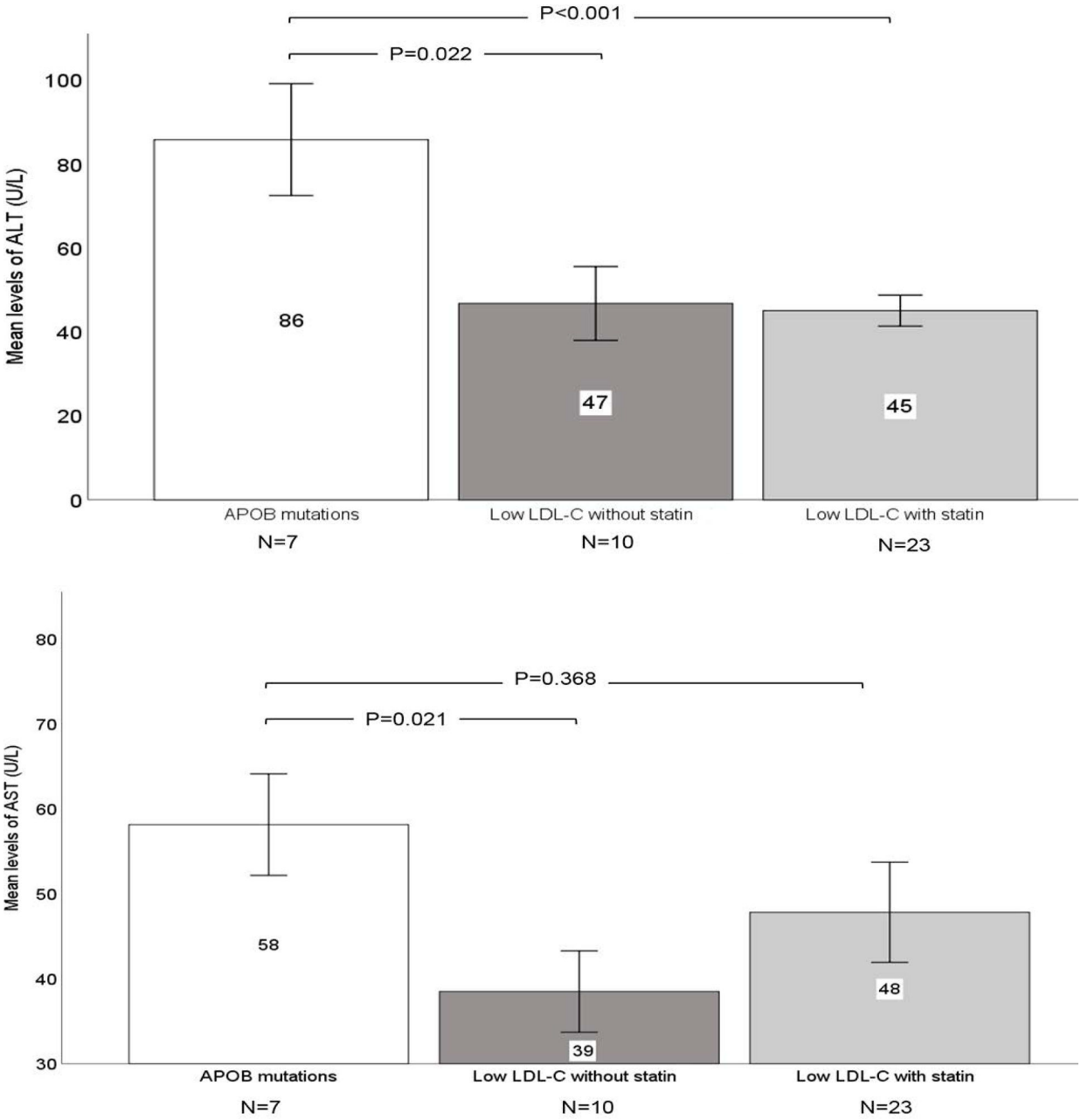


Figure 1.

Flow of patients through the study.

Abbreviations: LDL, low-density lipoprotein; **APOB**, apolipoprotein B; **PCSK9**, proprotein convertase subtilisin/kexin type 9; **LDLR**, low-density lipoprotein receptor; **APOE,** apolipoprotein E.



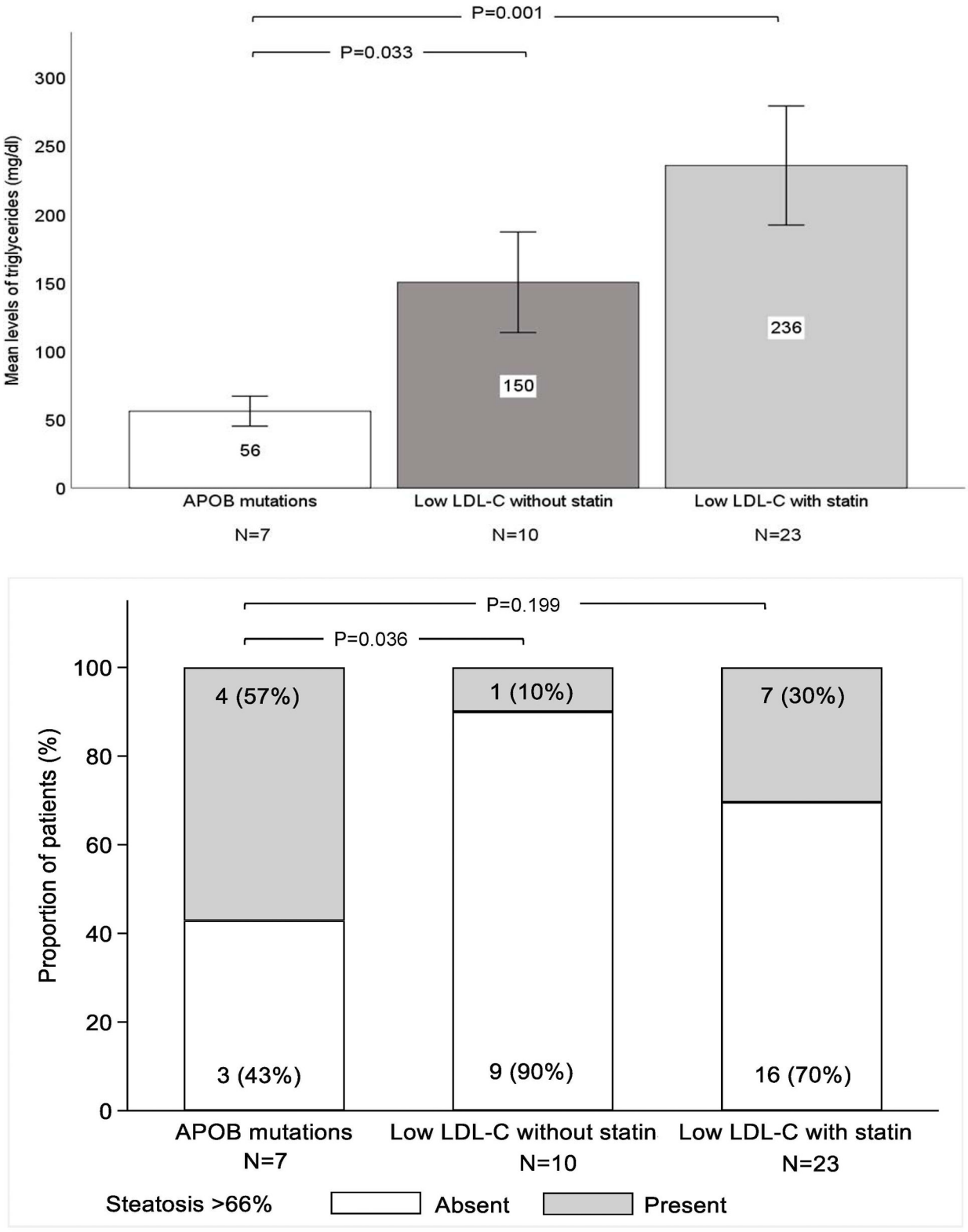


Figure 2.
Influence of *APOB* mutations on selected patients' characteristics.

Panel A: Mean levels of alanine aminotransferase.
Panel B: Mean levels of aspartate aminotransferase.
Panel C: Mean levels of serum triglycerides.
Panel D: Proportion of patients with hepatic steatosis higher than 66%.
Abbreviations: LDL, low-density lipoprotein; **APOB**, apolipoprotein B; **ALT,** alanine aminotransferase; **AST**, aspartate aminotransferase.
Error bars represent 95% confidence interval

Table 1.

Baseline features among patients with LDL-C levels of ≤50 mg/dL, 130–150 mg/dL and ≥190 mg/dL.

Variable	LDL-C ≤50 mg/dL	LDL-C 130–150 mg/dL	LDL-C ≥190 mg/dL	*P value*[*]	*P value*[†]
No. of patients	40	40	40	-	-
Age, years	50.22 ± 14.11	49.20 ± 12.69	49.15 ± 9.81	-	-
Gender (male), n (%)	17 (43)	15 (38)	11 (28)	-	-
Race, n (%)				-	-
White – Caucasian	32 (80)	30 (75)	31 (77.5)		
Black	1 (2.5)	1 (2.5)	2 (5)		
Asian	3 (7.5)	4 (10)	1 (2.5)		
American Indian/Alaska Native	0 (0)	0 (0)	2 (5)		
Hispanic	2 (5)	3 (7.5)	3 (7.5)		
More than 1	2 (5)	2 (5)	1 (2.5)		
Body mass index (kg/m^2)	34.99 ± 7.31	33.80 ± 5.37	33.63 ± 5.53	0.342	0.354
Type 2 diabetes mellitus, n (%)	20 (50)	14 (35)	11 (28)	0.509	0.058
Hypertension, n (%)	28 (70)	23 (58)	23 (58)	0.927	0.285
History of CVD, n (%) [≠]	4 (10)	2 (5)	3 (8)	0.858	0.779
Non-heavy drinkers, n (%)	15 (37)	14 (35)	12 (30)	0.928	0.508
Smoking status, n (%)					0.039
Never	23 (59)	15 (37.5)	18 (45)		
Current	15 (38.5)	22 (55)	15 (35)		
Former	1 (2.5)	3 (7.5)	8 (20)		
Statin intake, n (%)	24 (60)	10 (25)	21 (53)	-	-
Genetic background					
PNPLA3 rs738409, n (%)					
GG/GC genotypes	27 (68)	32 (80)	24 (60)	0.559	0.377
HSD17B13 rs6834314, n (%)				0.035	0.107
AA genotype	19 (47.5)	28 (70)	26 (65)		
AG genotype	17 (42.5)	12 (30)	12 (30)		
GG genotype	4 (10)	0 (0)	2 (5)		
TM6SF2 rs58542926, n (%)				0.973	0.083
TT/TC genotypes	11 (29)	13 (33)	5 (13)		
MBOAT7 rs641738, n (%)				0.215	0.405
TT/TC genotypes	29 (73)	22 (55)	24 (63)		
GCKR rs1260326, n (%)				0.751	0.040
TT/TC genotypes	31 (78)	29 (73)	21 (55)		
Polygenic risk score	13.30 ± 1.55	12.63 ± 1.97	13.90 ± 2.09	0.124	0.162
Polygenic risk score (percentiles)	60.05 ± 26.22	49.23 ± 29.11	68.38 ± 28.80	0.122	0.190
Lab panel					
Cholesterol (mg/dl)	114.38 ± 28.65	215.65 ± 15.70	289.05 ± 20.65	<0.001	<0.001
Triglycerides (mg/dl)	183.03 ± 180.72	170.48 ± 77.55	190.40 ± 65.61	0.725	0.700

Variable	LDL-C ≤50 mg/dL	LDL-C 130–150 mg/dL	LDL-C ≥190 mg/dL	*P value*[*]	*P value*[†]
HDL (mg/dl)	41.65 ± 12.89	43.35 ± 9.53	43.80 ± 9.91	0.867	0.455
LDL-C (mg/dl)	37.70 ± 9.24	139.18 ± 0.95	211.85 ± 16.70	<0.001	<0.001
ALT (U/L)	52.65 ± 28.06	79.25 ± 50.83	94.45 ± 80.59	0.011	0.003
AST (U/L)	47.30 ± 24.14	58.58 ± 45.70	59.58 ± 43.04	0.084	0.122
Glucose (mg/dl)	114.43 ± 35.69	117.48 ± 54.56	99.53 ± 19.60	0.686	0.057
HbA1c (%)	6.44 ± 1.38	6.31 ± 1.36	6.06 ± 0.79	0.812	0.163
Insulin (mIU/L)	29.84 ± 40.18	20.62 ± 12.00	22.06 ± 15.66	0.382	0.282
HOMA-IR	8.81 ± 11.02	6.23 ± 4.70	5.46 ± 4.71	0.402	0.093
Histology reports					
Steatosis, n (%)				0.274	0.756
<5%	1 (2.5)	0 (0)	0 (0)		
5–33%	14 (35)	18 (45)	17 (42.5)		
33–66%	13 (32.5)	15 (37.5)	12 (30)		
>66%	12 (30)	7 (17.5)	11 (27.5)		
Lobular inflammation, n (%)				0.661	0.344
No foci	0 (0)	0 (0)	1 (2.5)		
<2 foci/200x	23 (57.5)	21 (52.5)	18 (45)		
2–4 foci/200x	14 (35)	14 (35)	15 (37.5)		
>4 foci/200x	3 (7.5)	5 (12.5)	6 (15)		
Ballooning, n (%)				0.705	0.238
None	8 (20)	13 (32.5)	12 (30)		
Few	17 (42.5)	8 (20)	17 (42.5)		
Many	15 (37.5)	19 (47.5)	11 (27.5)		
Portal inflammation, n (%)				0.926	0.196
None	2 (5)	5 (12.5)	5 (12.5)		
Mild	28 (70)	23 (57.5)	28 (70)		
> Mild	10 (25)	12 (30)	7 (17.5)		
Fibrosis stages, n (%)				0.462	0.421
0	8 (20)	10 (25)	10 (25)		
1	12 (30)	8 (20)	12 (30)		
2	10 (25)	11 (27.5)	12 (30)		
3	8 (20)	7 (17.5)	5 (12.5)		
4	2 (5)	4 (10)	1 (2.5)		
Steatohepatitis, n (%)				0.662	0.342
None	5 (12.5)	9 (22.5)	10 (25)		
Borderline steatohepatitis	7 (17.5)	6 (15)	5 (12.5)		
Definite steatohepatitis	28 (70)	25 (62.5)	25 (62.5)		
NAS ≥4	32 (80)	29 (73)	26 (65)	0.389	0.130

Abbreviations: LDL-C, low-density lipoprotein cholesterol; **CVD**, cardiovascular disease; ***PNPLA3***, patatin-like phospholipase domain-containing protein 3 gene; ***HSD17B13***, hydroxysteroid 17-β dehydrogenase 13 gene; ***TM6SF2***, transmembrane 6 superfamily member 2 gene; ***MBOAT7***, membrane bound O-acyltransferase domain-containing 7 gene; ***GCKR***, glucokinase regulatory protein gene; **HDL**, high-density lipoprotein; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **HOMA-IR**, homeostatic model assessment of insulin resistance; **NAS**, NAFLD activity score.

[*] P values represent the comparison between low vs middle LDL groups.

[†] P values represent the comparison between low vs high LDL groups.

[‡] It includes coronary artery and cerebrovascular diseases.

P values were calculated while controlling for statin use, age, gender, and race. Multivariate linear or binary or ordinal regression models were used to explore associations between baseline covariates and LDL-C groups.

Continuous variables were expressed as mean ± standard deviation.

Table 2.

Baseline characteristics of patients with very low LDL-C (≤50 mg/dL) levels. Comparative analysis between patients with or without *APOB* mutations.

Variable	*APOB* mutations N=7	*APOB* wild-type N=33	*P value*[*]	*P value*[†]
Age, years	41.66 ± 10.30	52.04 ± 14.26	0.077	-
Gender (male), n (%)	2 (29)	15 (45)	0.412	-
Race, n (%)			0.481	-
White – Caucasian	6 (86)	28 (85)		
Black	0 (0)	1 (3)		
Asian	0 (0)	3 (9)		
More than 1	1 (14)	1 (3)		
Body mass index (kg/m^2)	38.05 ± 8.75	34.35 ± 6.96	0.229	0.165
Type 2 diabetes mellitus, n (%)	1 (14)	19 (58)	0.037	0.461
Hypertension, n (%)	3 (43)	25 (76)	0.084	0.772
History of CVD, n (%) [≠]	0 (0)	4 (12)	0.332	-
Non-heavy drinkers, n (%)	3 (43)	12 (36)	0.747	0.318
Smoking status, n (%)			0.021	0.994
Never	7 (100)	16 (50)		
Current	0 (0)	15 (47)		
Former	0 (0)	1 (3)		
Statin intake, n (%)	0 (0)	23 (70)	0.001	-
Genetic background				
PNPLA3 rs738409, n (%)			0.257	0.788
GG/GC genotypes	6 (86)	21 (64)		
HSD17B13 rs6834314, n (%)			0.311	0.086
AA genotype	2 (29)	17 (52)		
AG genotype	4 (57)	13 (39)		
GG genotype	1 (14)	3 (9)		
TM6SF2 rs58542926, n (%)			0.470	0.203
TT/TC genotypes	1 (17)	10 (31)		
MBOAT7 rs641738, n (%)			0.316	0.587
TT/TC genotypes	4 (57)	25 (76)		
GCKR rs1260326, n (%)			0.156	0.142
TT/TC genotypes	4 (57)	27 (82)		
Polygenic risk score	13.42 ± 2.15	13.27 ± 1.44	0.813	0.928
Polygenic risk score (percentiles)	63.43 ± 34.33	59.33 ± 24.78	0.713	0.956
Lab panel				
Cholesterol (mg/dl)	94.86 ± 25.90	118.52 ± 27.81	0.046	0.071
Triglycerides (mg/dl)	56.14 ± 28.93	209.94 ± 188.09	<0.001	0.037
HDL (mg/dl)	50.71 ± 21.94	39.73 ± 9.52	0.039	0.213
LDL-C (mg/dl)	32.86 ± 7.82	38.73 ± 9.30	0.129	0.238

Variable	*APOB* mutations N=7	*APOB* wild-type N=33	*P value*[*]	*P value*[†]
ALT (U/L)	85.86 ± 35.24	45.61 ± 20.84	<0.001	0.002
AST (U/L)	58.14 ± 15.80	45.00 ± 25.15	0.099	0.104
Glucose (mg/dl)	99.14 ± 25.58	117.67 ± 36.95	0.216	0.654
HbA1c (%)	5.66 ± 1.10	6.61 ± 1.39	0.076	0.785
Insulin (mIU/L)	19.23 ± 11.08	32.09 ± 43.76	0.148	0.952
HOMA-IR	4.49 ± 2.11	9.73 ± 11.93	0.024	0.717
Histology reports				
Steatosis, n (%)			0.197	0.047
<5%	0 (0)	1 (3)		
5–33%	2 (28.6)	12 (36.4)		
33–66%	1 (14.3)	12 (36.4)		
>66%	4 (57.1)	8 (24.2)		
Severe steatosis (>66%), n (%)	4 (57.1)	8 (24.2)	0.084	0.050
Lobular inflammation, n (%)			0.745	0.559
No foci	0 (0)	0 (0)		
<2 foci/200x	4 (57.1)	19 (57.6)		
2–4 foci/200x	3 (42.9)	11 (33.3)		
>4 foci/200x	0 (0)	3 (9.1)		
Ballooning, n (%)			0.215	0.350
None	2 (28.6)	6 (18.2)		
Few	4 (57.1)	13 (39.4)		
Many	1 (14.3)	14 (42.4)		
Portal inflammation, n (%)			0.747	0.833
None	1 (14.3)	1 (3)		
Mild	4 (57.1)	24 (72.7)		
> Mild	2 (28.6)	8 (24.3)		
Fibrosis stages, n (%)			0.943	0.901
0	2 (28.5)	6 (18.2)		
1	2 (28.6)	10 (30.3)		
2	1 (14.3)	9 (27.3)		
3	1 (14.3)	7 (21.2)		
4	1 (14.3)	1 (3)		
Steatohepatitis, n (%)			0.549	1.00
None	1 (14.3)	4 (12.1)		
Borderline steatohepatitis	2 (28.6)	5 (15.2)		
Definite steatohepatitis	4 (57.1)	24 (72.7)		
Definite steatohepatitis, n (%)	4 (57)	24 (73)	0.414	0.906
NAS ≥4	6 (86)	26 (79)	0.677	0.762

Abbreviations: LDL-C, low-density lipoprotein cholesterol; ***APOB***, apolipoprotein B gene; ***PCSK9***, proprotein convertase subtilisin/kexin type 9 gene; ***PNPLA3***, patatin-like phospholipase domain-containing protein 3 gene; ***HSD17B13,*** hydroxysteroid 17-β dehydrogenase 13 gene; ***TM6SF2***, transmembrane 6 superfamily member 2 gene; ***MBOAT7***, membrane bound O-acyltransferase domain-containing 7 gene; ***GCKR***, glucokinase

regulatory protein gene; **HDL**, high-density lipoprotein; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **HOMA-IR**, homeostatic model assessment of insulin resistance; **NAS**, NAFLD activity score.

[*] T test or Wilcoxon rank-sum test for continuous variables. Chi-square statistic test categorical variables. Mantel-*Haenszel* trend test for binary categorical variables with more than two groups and Jonckheere-Terpstra test for ordered alternatives.

[†] P values were calculated while controlling for statin use, age, gender and race. Multivariate linear or binary or ordinal regression models were used to explore associations between baseline covariates and presence of *APOB* mutations.

[‡] It includes coronary artery and cerebrovascular diseases.

Continuous variables were expressed as mean ± standard deviation.

Table 3.

Baseline features of patients with very low LDL-C (≤ 50 mg/dL) levels, excluding statin users. Comparative analysis between patients with or without *APOB* mutations.

Variable	*APOB* mutations N=7	*APOB* wild-type N=10	*P value* *
Age, years	41.65 ± 10.30	41.37 ± 10.67	0.956
Gender (male), n (%)	2 (29)	7 (70)	0.092
Race, n (%)			
White – Caucasian	6 (86)	9 (90)	0.787
Body mass index (kg/m^2)	38.05 ± 8.75	32.94 ± 6.35	0.182
Type 2 diabetes mellitus, n (%)	1 (14)	3 (30)	0.452
Hypertension, n (%)	3 (43)	5 (50)	0.772
History of CVD, n (%) †	0 (0)	0 (0)	1.00
Non-heavy drinkers, n (%)	3 (43)	2 (20)	0.309
Smoking status, n (%)			
Never	7 (100)	7 (70)	0.110
Polygenic risk score	13.42 ± 2.15	13.50 ± 1.35	0.934
Polygenic risk score (percentiles)	63.43 ± 34.33	62.70 ± 23.41	0.959
Genetic background			
PNPLA3, n (%) rs738409			0.787
GG/GC genotypes	6 (86)	9 (90)	
HSD17B13 rs6834314, n (%)			0.070
AA genotype	2 (28.6)	7 (70)	
AG genotype	4 (57.1)	3 (30)	
GG genotype	1 (14.3)	0 (0)	
TM6SF2 rs58542926, n (%)			0.182
TT/TC genotypes	1 (17)	5 (50)	
MBOAT7 rs641738, n (%)			0.585
TT/TC genotypes	4 (57)	7 (70)	
GCKR rs1260326, n (%)			0.116
TT/TC genotypes	4 (57)	9 (90)	
Lab panel			
Cholesterol (mg/dl)	94.86 ± 25.91	120.40 ± 24.89	0.058
Triglycerides (mg/dl)	56.14 ± 28.93	150.40 ± 116.23	0.033
HDL (mg/dl)	50.71 ± 21.94	43.00 ± 5.69	0.299
LDL-C (mg/dl)	32.86 ± 7.82	38.30 ± 9.53	0.233
ALT (U/L)	85.86 ± 35.24	46.80 ± 27.86	0.022
AST (U/L)	58.14 ± 15.80	38.50 ± 15.15	0.021
Glucose (mg/dl)	99.14 ± 25.58	107.00 ± 30.69	0.587
HbA1c (%)	5.66 ± 1.10	5.83 ± 1.45	0.795
Insulin (mIU/L)	19.23 ± 11.08	20.44 ± 17.59	0.875
HOMA-IR	4.49 ± 2.10	6.46 ± 7.67	0.523

Variable	*APOB* mutations N=7	*APOB* wild-type N=10	*P value* *
Histology reports			
Steatosis, n (%)			0.151
<5%	0 (0)	0 (0)	
5–33%	2 (28.6)	4 (40)	
33–66%	1 (14.3)	5 (50)	
>66%	4 (57.1)	1 (10)	
Lobular inflammation, n (%)			0.499
No foci	0 (0)	0 (0)	
<2 foci/200x	4 (57.1)	4 (40)	
2–4 foci/200x	3 (42.9)	6 (60)	
>4 foci/200x	0 (0)	0 (0)	
Ballooning, n (%)			0.352
None	2 (28.6)	2 (20)	
Few	4 (57.1)	4 (40)	
Many	1 (14.3)	4 (40)	
Portal inflammation, n (%)			0.855
None	1 (14.3)	1 (10)	
Mild	4 (57.1)	6 (60)	
> Mild	2 (28.6)	3 (30)	
Fibrosis stages, n (%)			0.786
0	2 (28.6)	3 (30)	
1	2 (28.5)	2 (20)	
2	1 (14.3)	3 (30)	
3	1 (14.3)	2 (20)	
4	1 (14.3)	0 (0)	
Steatohepatitis, n (%)			0.942
None	1 (14.3)	2 (20)	
Borderline steatohepatitis	2 (28.6)	2 (20)	
Definite steatohepatitis	4 (57.1)	6 (60)	
Definite steatohepatitis, n (%)	4 (57)	6 (60)	0.906
NAS ≥4	6 (86)	8 (80)	0.761

Abbreviations: LDL-C, low-density lipoprotein cholesterol; ***APOB***, apolipoprotein B gene; ***PCSK9***, proprotein convertase subtilisin/kexin type 9 gene; ***PNPLA3***, patatin-like phospholipase domain-containing protein 3 gene; ***HSD17B13***, hydroxysteroid 17-β dehydrogenase 13 gene; ***TM6SF2***, transmembrane 6 superfamily member 2 gene; ***MBOAT7***, membrane bound O-acyltransferase domain-containing 7 gene; ***GCKR***, glucokinase regulatory protein gene; **HDL**, high-density lipoprotein; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **HOMA-IR**, homeostatic model assessment of insulin resistance; **NAS**, NAFLD activity score.

*T test or Wilcoxon rank-sum test for continuous variables. Chi-square statistic test categorical variables. Mantel-*Haenszel* trend test for binary categorical variables with more than two groups and Jonckheere-Terpstra test for ordered alternatives.

†It includes coronary artery and cerebrovascular diseases.

Continuous variables were expressed as mean ± standard deviation.

Table 4.

Case-based comparisons of baseline characteristics of patients with variants associated with low LDL-C levels.

Variable	Patients with *APOB* and/or *PCSK9* mutations							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Age	31.8	36.7	31.9	39.9	54.1	57.7	39.5	47.5
Gender	male	female	female	female	female	female	male	male
Body mass index (kg/m^2)	32.11	38.88	33.08	30.41	49.69	50.76	31.45	41.72
Waist (cm)	106.42	105.79	124.46	109.22	122.60	-	102.50	125.73
Race/ethnicity	More than 1	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
Type 2 diabetes mellitus	no	no	yes	no	no	no	no	no
Hypertension	yes	no	no	no	yes	yes	no	no
History of CVD	no	no	no	no	no	no	no	no
Smoking status	never	never	never	never	never	never	never	never
Non-heavy alcohol intake	yes	no	no	no	no	yes	yes	no
Genetic background								
cDNA change								
APOB mutations	c.10238delC	c.10238delC	c.10238delC	c.741_745del	c.9115_9119del	c.11330C>A	c.7472T>G	-
PCSK9 mutations	-	-	-	-	c.137G>T	-	-	c.202delG
Polygenic risk score	16	15	15	12	12	14	10	14
Polygenic risk score (PCT)	99	92	92	37	37	75	12	75
PNPLA3 rs738409	GG	CG	CG	CC	CG	CG	CG	CG
HSD17B13 rs6834314	AG	AA	GG	AG	AG	AA	AG	AA
TM6SF2 rs58542926	CC	TC	CC	CC	CC	CC	CC	CC
MBOAT7 rs641738	CC	CC	TC	CC	TC	TT	TC	CC
GCKR rs1260326	TC	TC	TT	TC	CC	CC	CC	TC
Lab panel								
LDL-C (mg/dl)	31	42	33	24	41	37	22	46
HDL (mg/dl)	38	92	34	33	62	60	36	44
Cholesterol (mg/dl)	88	138	81	74	113	107	63	105
Triglycerides (mg/dl)	94	18	70	86	48	52	25	74
HbA1c (%)	5.5	4.9	7.8	5.1	6.4	4.6	5.3	5.6

Variable	Patients with *APOB* and/or *PCSK9* mutations							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
HOMA-IR	5.68	1.46	3.84	4.30	7.68	5.89	2.62	2.56
ALT (U/L)	138	121	53	88	88	38	75	49
AST (U/L)	67	63	58	85	52	46	36	28
Liver histology								
Steatosis	>66%	5–33%	>66%	>66%	>66%	5–33%	33–66%	33–66%
Lobular inflammation	2–4 foci per 200 × field	< 2 foci per 200 × field	<2 foci per 200 × field	2–4 foci per 200 × field	<2 foci per 200 × field	< 2 foci per 200 × field	2–4 foci per 200 × field	2–4 foci per 200 × field
Ballooning	Few	Many	Few	None	Few	None	Few	Many
NAS	6	4	5	5	5	2	5	6
Fibrosis	0	0	2	3	1	4	1	0
Steatohepatitis diagnosis	Borderline	Definite	Definite	Borderline	Definite	Not steatohepatitis	Definite	Definite
NAS ≥4	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes

Abbreviations: LDL-C, low-density lipoprotein cholesterol; ***APOB***, apolipoprotein B gene; ***PCSK9***, proprotein convertase subtilisin/kexin type 9 gene; ***PNPLA3***, patatin-like phospholipase domain-containing protein 3 gene; ***HSD17B13***, 17-beta hydroxysteroid dehydrogenase 13 gene; ***TM6SF2***, transmembrane 6, superfamily member 2 gene; ***MBOAT7***, membrane bound O-acyltransferase domain containing 7 gene; ***GCKR***, glucokinase regulatory protein gene; **HDL**, high-density lipoprotein; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **HOMA-IR**, homeostatic model assessment of insulin resistance; **NAS**, NAFLD activity score; **PCT**, percentiles.

Table 5.

Case-based comparisons of baseline characteristics of *APOE* and *LDLR* mutation carriers.

Variable	Patients with *LDLR* or *APOE* mutations		
	1	**2**	**3**
Age (years)	51.5	34.3	54.7
Gender	female	female	female
BMI (kg/m^2)	35.56	40.22	29.81
Waist (cm)	108.00	109.22	94.36
Race/ethnicity	Caucasian	More than 1	Caucasian
Type 2 diabetes mellitus	no	no	no
Hypertension	no	yes	no
History of CVD	no	no	no
Smoking status	never	never	never
Statin intake	no	yes	yes
Non-heavy alcohol intake	no	no	yes
Genetic background			
Polygenic risk score	16	11	16
Gene mutated	*LDLR*	*LDLR*	*APOE*
cDNA change	c.858C>A	c.241 C>T	c.496_498del
PNPLA3 rs738409	CG	GG	CG
HSD17B13 rs6834314	AG	AA	AG
TM6SF2 rs58542926	CC	CC	CC
MBOAT7 rs641738	TC	TC	TC
GCKR rs1260326	CC	TC	CC
Lab panel			
LDL-C (mg/dl)	279	139	209
HDL (mg/dl)	42	52	44
Cholesterol (mg/dl)	354	220	274
Triglycerides (mg/dl)	165	145	107
HbA1c (%)	6.1	5.7	5.6
HOMA-IR	5.29	2.44	2.45
ALT (U/L)	48	103	95
AST (U/L)	36	65	87
Liver histology			
Steatosis	>66%	5–33%	<33%
Lobular inflammation	2–4 foci per 200 × field	2–4 foci per 200 × field	<2 foci per 200 × field
Ballooning	Many	Many	Few
NAS	7	6	3
Fibrosis	1	3	3
Steatohepatitis diagnosis	Definite	Definite	Definite
NAS ≥4	Yes	Yes	No

Abbreviations: LDL-C, low-density lipoprotein cholesterol; **APOE**, apolipoprotein E gene; **LDLR**, low-density lipoprotein receptor gene; **BMI**, body mass index; **PNPLA3**, patatin-like phospholipase domain-containing protein 3 gene; **HSD17B13**, 17-beta hydroxysteroid dehydrogenase 13 gene; **TM6SF2**, transmembrane 6, superfamily member 2 gene; MBOAT7, membrane bound O-acyltransferase domain containing 7 gene; **GCKR**, glucokinase regulatory protein gene; **HDL**, high-density lipoprotein; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **HOMA-IR**, homeostatic model assessment of insulin resistance; **NAS**, NAFLD activity score.

Table 6.

APOB, PCSK9, LDLR and *APOE* mutations.

Gene symbol	Chromosome:position	Exon	cDNA change	Protein change	Zygosity	Mutation type	Molecular consequence	Number of carriers
APOB	2:21258529_21258533	7	c.741_745del	p.Y247fs	Heterozygous	Frameshift deletion	Premature truncation by 95%	1
APOB	2:21232268	26	c.7472T>G	p.L2491X	Heterozygous	Nonsense	Premature truncation by 45%	1
APOB	2:21230621_21230625	26	c.9115_9119del	p.F3039fs	Heterozygous	Frameshift deletion	Premature truncation by 33%	1
APOB	2:21229502	26	c.10238delC	p.T3413fs	Heterozygous	Frameshift deletion	Premature truncation by 25%	3
APOB	2:21228410	26	c.11330C>A	p.S3777X	Heterozygous	Nonsense	Premature truncation by 17%	1
PCSK9	1:55505647	1	c.137G>T	p.R46L	Heterozygous	Nonsynonymous	Missense mutation (loss of function)	1
PCSK9	1:55505712	1	c.202delG	p.A68fs	Heterozygous	Frameshift deletion	Premature truncation by 90%	1
LDLR	19:11218108	6	c.858C>A	p.S286R	Heterozygous	Nonsynonymous	Missense mutation (loss of function)	1
LDLR	19:11213390	3	c.241C>T	p.R81C	Heterozygous	Nonsynonymous	Missense mutation (loss of function)	1
APOE	19:45412049_45412051	4	c.496_498del	p.Leu167del	Heterozygous	In-frame deletion	Small deletion mutant; known dysfunctional variant	1

Abbreviations: ***APOB***, apolipoprotein B gene; ***PCSK9***, proprotein convertase subtilisin kexin type 9 gene; ***LDLR***, low density lipoprotein receptor gene; ***APOE***; apolipoprotein E gene.