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Article

Identification of a Novel apoB Variant in a Family Exhibiting Hypocholesterolemia: Mechanistic Insights

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Abstract

Familial hypobetalipoproteinemia (FHBL) is a rare autosomal codominant disorder. FHBL is often caused by a defect in apoB production that is required for lipoprotein formation. Here we identified three family members that exhibited very low circulating cholesterol levels. Analyses of their plasma lipid profiles revealed that the affected individuals have low levels of cholesterol and LDL-cholesterol (LDLc) lipoproteins, with no difference in lipoprotein particle size. Sequencing of the *APOB* gene revealed a single heterozygote deletion of an adenosine in exon 3 at the nucleotide position 1268 in all affected members. This deletion introduces a reading frame shift at glutamine 380 resulting in a stop codon at position 397. This C-terminally truncated apoB, called apoB9, is a variant spanning ~9% of the full-length protein. Upon expression of apoB9 in human hepatocyte IHH cells, the protein was detected intracellularly but it did not exit the endoplasmic reticulum and hence was not secreted into the media. Molecular modeling revealed that apoB9 lacks the βA- and βB-sheets that are required for lipid particle formation, which can explain the absence of apoB9 secretion and the low levels of plasma apoB in the affected family members, likely modifying their risk of developing CVD.

Keywords: *APOB;* rare variant; lipid metabolism; structure-function; plasma LDL cholesterol; apoB100 and apoB48 profiles

1. Introduction

Apolipoprotein B (apoB) is responsible for carrying lipids in plasma, including cholesterol, and is the primary apolipoprotein of chylomicrons, very low-density lipoproteins (VLDL), lipoprotein(a) [Lp(a)], intermediate-density lipoprotein (IDL), and low-density lipoprotein (LDL) particles. Its measurement is commonly used to detect the risk of atherosclerotic cardiovascular disease (CVD) [1,2]. Apolipoprotein B100 (apoB100), the major protein component of LDL, is a ligand for the LDL receptor (LDLR) [3]. Mutations in apoB100 or in LDLR cause familial hypercholesterolemia (FH), an

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autosomal dominant disease that is characterized by a marked increase in LDL-cholesterol (LDLc) and a higher risk of CVD [4]. However, apoB levels outperform those of LDLc as a biomarker for CVD [5-8]. Thus, apoB is the preferred primary indicator in clinical care to estimate the CVD risk attributable to apoB lipoproteins and the efficacy of lipid-lowering therapies to reduce this risk [6].

Proprotein convertase subtilisin-kexin 9 (PCSK9) is the 9th member of a family of secretory proteases implicated in various physiological and pathological functions [9,10]. The implication of PCSK9 in CVD became apparent when it was shown that gain-of-function (GOF) variants are associated with significantly higher LDLc levels [11]. The underlying mechanism was resolved when it was realized that in a non-enzymatic fashion the hepatocyte-derived circulating PCSK9 enhances the degradation of the liver LDLR in endosomes/lysosomes [12,13] leading to decreased clearance of apoB-containing lipoproteins and higher LDLc and apoB plasma levels [14].

The *APOB* gene is located on the short arm of human chromosome 2 (2p24.1) and spans 43 kb including 29 exons and 28 introns. It encodes a very large hydrophobic protein composed of 4563 amino acids (aa; including a 27 aa signal peptide) [3,15,16]. Recently, two cryo-electron microscopy structures of apoB100 on LDL were reported [17,18]. One of these revealed the detailed structure of apoB100 on LDL bound to the LDLR, including high-resolution structures of the interfaces between apoB100 and LDLR [17]. These data also led to a better structural understanding of known loss-of-function (LOF) mutations in either apoB100 or LDLR associated with high levels of LDLc and located at the LDL-LDLR interface [17]. Circulating human apoB consists of two isoforms: apoB100 derived from hepatocytes (primary source) and small intestine, as well as an intestinal-specific apoB48 resulting from mRNA editing. ApoB100 (aa 28-4563; molecular weight ~550 kDa) and apoB48 (aa 28-2179; molecular weight ~264 kDa) correspond to 100% and 48% of the molecular weight of full length apoB100 protein, respectively. ApoB100 is associated with VLDL, IDL and LDL, while apoB48 is found only in chylomicrons [19].

ApoB is an important marker for CVD risk since it is found in atherogenic lipoproteins particles like LDL. Elevated circulating apoB levels are directly associated with hyperlipidemia and atherosclerosis. Variants in *APOB* are the second most frequent (14%) cause of familial hypercholesterolemia (FH), whereas 67% and 2% are attributed to variants in the *LDLR* and *PCSK9* genes, respectively [11,20,21]. The most common variants in the *APOB* gene occurs in the LDLR-binding domain and affect its ability to bind to the LDLR and to reduce LDLc clearance from the blood. Indeed, lipoprotein particles with LOF variants of apoB cannot be effectively removed from the blood [22], resulting in very high levels of circulating LDLc and increasing the risk of atherosclerosis and heart attack. Treatment of FH patients involves a combination of cholesterol-lowering medications, such as statins and PSCK9 inhibitors, as well as lifestyle changes [23,24].

In contrast, familial hypobetalipoproteinemia (FHBL) is characterized by abnormally low levels of circulating LDLc and apoB. FHBL results from mutations in the genes encoding Microsomal Triglyceride Transfer Protein (MTP) [25,26] or apoB, which are essential for producing and assembling lipoproteins [27]. LOF *APOB* mutations most often result from deletions or substitutions that cause premature termination of *APOB* mRNA translation, leading to truncated, non-functional proteins and low LDLc [28-30]. In humans, various truncated apoB forms have been reported, ranging in molecular weight from 9% (apoB9) to 89% (apoB89) of the full length apoB100 [29,31-33]. It has been shown that the C-terminal truncation of apoB affects the size of the resulting apoB-associated lipoproteins and their fate. Indeed a 10 % decrease in apoB's length results in a 13% decrease in the core density of lipoproteins, indicating that lipid recruitment by apoB is progressively reduced by its C-terminal truncation, which can affect protein stability [34-36]. The length of the C-terminal segment of apoB affects its secretion, as only truncated forms longer than 27% (apoB27) can be efficiently secreted into the plasma [37,38].

In the present study, we recently identified family members that did not present variants in either *LDLR* or *PCSK9* but that exhibited very low circulating cholesterol levels. Analyses of their plasma lipid profiles revealed that these subjects had exceedingly low levels of LDLc without affecting their lipoprotein particle size. Sequencing of the *APOB* gene in a control (unaffected sister)

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and three affected family members (father, son and daughter) revealed a single heterozygote deletion of an adenosine in *exon 3* at position 1268. This novel deletion introduces a reading frame shift at glutamine 380 resulting in a stop codon at aa position 397. Such C-terminally truncated apoB leads to a variant protein spanning ~9% (397 aa; herein called apoB9) of the full-length protein (aa 28-4563). Expression of apoB9 in human hepatocyte IHH cells, revealed an intracellular form with an apparent molecular size of ~60 kDa, which does not exit the endoplasmic reticulum (ER), and hence is not secreted into the media. These results suggest that the low levels of circulating apoB (-68%, estimated by proteomics) in the affected family members, likely modify their risk of developing CVD.

2. Materials and Methods

2.1. Cell Culture, Transfections

Native human hepatocyte IHH cells were grown in William's Medium (ThermoFisher, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS; GIBCO BRL) and 1% Penicillin-Streptomycin (Sigma, MA, USA). Cells were maintained at 37°C under 5% CO_2. Cells were seeded in 12 well plates at a density of $0.6x10^6$ cells per well and co-transfected with equimolar quantities of each plasmid using FuGene HD, according to the manufacturer's instructions (Promega, Madison, WI, USA).

2.3. Cell Treatments

Proteins were extracted in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% SDS, 1% Nonidet P40 and 0.25% Na deoxycholate) with a complete cocktail of protease inhibitors (Sigma, MA, USA). 30-50 mg proteins or 2 to 5 mL of plasma were analyzed by SDS-PAGE and transferred to PVDF (EMD Millipore,Toronto, ON, Canada) membranes. After blocking in 5% skim milk for 1h, membranes were incubated overnight with primary and secondary antibodies according to the manufacturer's recommendations: anti-V5 (Invitrogen, ON, Canada #46-0705,1/5000) and anti-HA (Abcam, Toronto, ON, Canada #ab128131 [1:5000]) and anti-human apoB (Sigma, MA, USA #A178467, [1:10000]). The antigen-antibody complexes were visualized using appropriate HRP conjugated secondary [1:10000] antibodies and an enhanced chemiluminescence kit (ECL; Amersham) [39,40].

2.2. Western Blot Analysis and Antibodies Used

Twenty four hours post-transfection, cells were washed in serum-free medium followed by an additional 24h with fresh media alone (non treated, NT) or media with protease inhibitors. the cell were then lysed in RIPA 1X buffer and analysed on SDS-PAGE: MG132: 1 mM, Lactacystin: 30 mM, Bafilomycin: 0.1 mM, Amonium Chloride: 10 mM, Chloroquine: 50 mM,).

2.4. Glycosidase Treatment.

Proteins (30 to 50 mg) were digested for 90 min at 37°C with endoglycosidase H (endo H; P0702L) or endoglycosidase F (endo F; P0705S) as recommended by the manufacturer (New England BioLabs, ON, Canada).

2.5. Plasma Collection and Circulating Cholesterol Measurement

Blood was collected from 16 h fasting patients and the plasma was obtained by centrifugation at 3000 x g for 15 min. Plasma total cholesterol (TC) and trigycerides (TG) were determined using the Infinity reagent (Thermo Fisher Scientific, ON, Canada). For lipoprotein profiles, 0.3 ml was analyzed by FPLC on a Superose 6 column (Pharmacia, Stockholm, Sweden) with a flow rate of 0.3 ml/min [41].

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2.6. lipoprotein Profiling and Lipoprotein Size Test.

Plasma from patients (20 mL) were subjected to High Performance Liquid Chromatography (HPLC), Triglyderides and cholesterol were then determined in each liprotein particle. The size of lipoproteins were determined using nuclear magnetic resonance (NMR) spectroscopy (LipoSEARCH, Gunma, Japan). The test measures the distinct NMR signals emitted by lipid methyl groups on different lipoprotein particles. The amplitudes of signals were used to calculate the size distribution of lipoprotein particles, e.g., VLDL, IDL, LDL, and HDL subfractions.

2.7. Lipidomics

Targeted lipidomics analysis was performed as described in the Agilent application note (https://www.agilent.com/search/?Ntt=RA44413.1612962963). Briefly, samples were extracted in triplicates with (1:10 v:v) extraction solvent butanol:MeOH (1:1) containing 1% (v:v) each: 10x diluted Equisplash internal standard mix (Sigma), 1 mg/ml cholesterol-d7, 1 mg/ml acetylcarnitine-d3 and 5 mg/ml docosahexaenoic acid-d5 (Cayman Chemical). The samples were sonicated for 5 mins and centrifuged 21000 x g for 1 min room temperature (23°C). 80 mL of each sample supernatants were used for the LC-MS analysis. UPLC-MRM-MS analyses were performed using Agilent 1290 LC with 16 mins gradient (A): 0.1% formic acid, 10 mM ammonium formate in 5:3:2 water: acetonitrile: 2-propanol, (B): 0.1% formic acid, 10 mM ammonium formate in 1:9:90 water: acetonitrile: 2-propanol at 400 mL/min flow rate using Zorbax eclipse plus C18 RRHD 100 x 2.1 mm 1.8 um column (Agilent) heated to 45°C. 1 uL injection volumes were used for each run. Data were acquired using Agilent 6495A mass spectrometer and were analyzed using MassHunter software (Agilent).

2.8. Proteomics

Targeted proteomics analysis was performed using PeptiQuant Plus Proteomics Kit (MRM Proteomics) as described previously [42,43]. Briefly, 10 ml of plasma samples in quadruplicates were supplemented with 1 ml of 1 M ABC, reduced with 1 ml of 0.1 M DTT for 30 mins at 37°C, alkylated with 1 ml of 0.3 M iodoacetamide for 30 mins at 37°C. Proteins were precipitated with addition of 90 ml acetonitrile, samples were centrifuged for 1 min at 21000 x g at room temperature (23°C), pellets were resuspended in 700 ml of 50 mM ABC, and proteins were digested with 70 ml of 1 mg/ml of trypsin (~1:10 enzyme:protein) (Worthington) overnight at 37°C. Trypsin digests were acidified with 7.7 ml formic acid and centrifuged for 1 min at 21,000 x g at room temperature. Aliquots of supernatants were supplemented with SIS peptide mix and analyzed by LC-MS. UPLC-MRM-MS analyses were performed using Sciex QTrap 6500+ triple quadrupole mass spectrometer supplemented with Shimadzu Nexera UPLC system. Chromatography was performed using 30 mins gradient 0-30% B (A): 0.1% formic acid, (B): 0.1% formic acid in acetonitrile at 200 mL/min flow rate using Zorbax eclipse plus C18 RRHD 150 x 2.1 mm 1.8 mm column (Agilent). MRM transitions for 16 apolipoproteins from the kit protein panel were used in unscheduled acquisition method. 10 ml of 11x diluted SIS peptide mixture from the kit was combined with 12 ml of 50x diluted NAT peptide mixture from the kit or 12 ml of plasma sample tryptic digests, 20 ml was injected for the analysis. Data were analyzed using Sciex Analyst software. Concentration of the SIS peptides were determined from the runs of NAT+SIS peptide mixtures using known NAT peptide concentrations, and concentration of the apolipoprotein peptides were determined from the runs of plasma sample digests+SIS peptide mixtures using calculated SIS peptide concentrations.

2.9. Apolipoprotein B Sequencing

Whole-genome sequencing was performed in all four family members to screen for relevant mutations across all protein-coding genes. PCR-free libraries were prepared from 1µg of genomic DNA using the KAPA HyperPrep library kit (Roche Diagnostics). Size distribution of the final libraries were assessed on high sensitivity DNA bionalyzer and libraries were quantified by quantitative PCR. Libraries were pooled equimolarly and sequenced on a lane of Novaseq S4 (300

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cycles) flowcell using paired-end reads (2 x 150 bp). Genomes were sequenced to an average depth of 39x. Reads were aligned to the human genome reference version, GRCh38, using Bowtie2 software in "no-discordant" alignment mode. Variant calling was performed using the Samtools "mpileup" command with default settings. Using annovar software, variant effects were annotated based on the database of non-synonymous SNP functional predictions (dbNSFP) which describes. Non-synonymous variants that were rare were prioritized, with rare being defined as genetic variants with allele frequencies less than 0.001 in all superpopulations within the genome aggregation database (gnomAd v4.1), a human repository of over 800,000 genome and exome sequences. Genetic variants were further filtered based on phenotypic cosegregation within the family.

2.10. Apolipoprotein B9 and B21 Models

PDB files of recently published cryoEM structures of apoB100 on LDL bound to the LDL receptor and legobody [17] (PDB 9BDT, 9BD1) were downloaded from RCSB PDB. The structures were visualized in UCSF ChimeraX software. The LDL receptor and legobody were removed and regions corresponding to apoB9 (aa 39-380; residues 28-38 are missing in the structure) or apoB21 (aa 39-965) were highlighted.

3. Results

3.1. Identification of a Hypabobetaliproteinema Family Presenting Low LDLc

A 68-year-old Caucasian man visited his primary care physician for a routine evaluation. His lipid profile demonstrated a very low LDLc level of 0.37 mmol/L (Table 1). The patient was referred to a specialty lipid clinic for further evaluation. His physical examination was essentially normal and revealed no evidence of hepatomegaly.

Cascade screening for low levels of LDLc was performed for first degree relatives (Table 1). A 39 year old daughter had an LDLc of 0.91 mmol/L and a 37 year old son had an LDLc of 0.44 mmol/L, both being below the 5th percentile. Both were asymptomatic and in good health. A 65-year-old sister had an LDLc of 2.69 mmol/L, was unaffected and served as a control.

Table 1. Age and levels of plasma lipid profile of family members.

	Daughter	Father	Sister	Son
Age (years)	39	68	65	37
		mmol/L		
Triglycerides	0.90	0.64	1.42	0.53
Cholesterol	2.52	1.56	4.65	2.22
HDLc	1.20	0.90	1.31	1.54
LDLc	0.91	0.37	2.69	0.44
non HDLc	1.32	0.66	3.34	0.68

3.2. Plasma LDLc and Apolipoproteins of Family Members

Quantification of the circulating levels of LDLc in fasted family members revealed that compared to the sister the LDLc levels of the father and son were about 6.5-fold lower, whereas the daughter exibited 3.2-fold lower levels (Table 1). Plasma Western blot analyses revealed reductions in the levels of apoB (-50%) and apoE (-60%) for the father compared to the sister (Figure 1A). We next used quantitative mass spectrometry to estimate the absolute levels of the plasma concentrations of apoB100, apoC2, apoC3, and apoE of the four family members (Figure 1B). Overall, the data showed that compared to those of the sister (black bar), the plasma levels of the above lipoproteins are significantly lower for the father, son and daugther.

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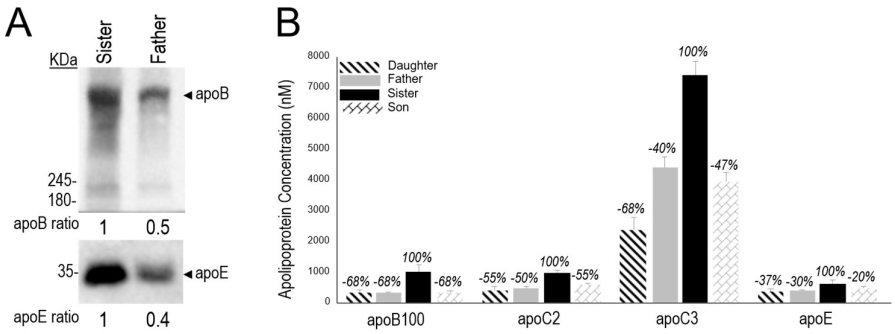

Figure 1. Levels of plasma LDLc and apolipoproteins of hypabobetaliproteinema family members: **(A)** Western blot analysis of plasma apoB and apoE. **(B)** Mass spectrometry analysis of circulating apoB100, apoC2, apoC3 and apoE.

3.3. Lipid Profile of Family Members

We next measured the relative abundance of various lipids in the plasma of all family members (Figure 2). Here also, except for lysophosphatidylcholine (LPC), the levels of cholesteryl ester (CE), non esterified cholesterol (COH), phosphatidylcholine (PC), sphingomyelin (SM) and triglycerides (TG) were all significantly lower in the plasma of the father, son and daughter compared to those of the sister (black bar).

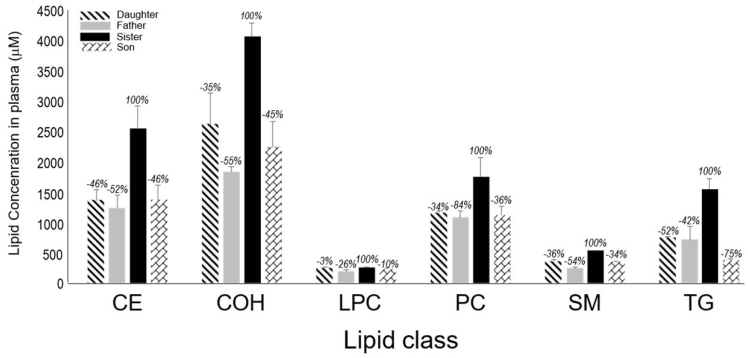

Figure 2. Relative abundance of lipids in plasma from different family members. CE: cholesteryl ester, COH: non esterified cholesterol , LPC: lysophosphatidylcholine: PC, phosphatidylcholine: SM: Sphingomyelin, TG: triglycerides.

The relative abundance of lipoprotein particles in the plasma of all subjects, was next analysed by Fast Protein Liquid Chromatography (FPLC) where we measured the levels of TG and cholesterol (Figure 3A, B). While all affected family members exhibited lower LDLc and VLDL triglycerides, we noted that the son has even lower VLDL levels ( ) compared to his sister or father (Figure 3A). Western blot analyses of apoB and apoE was also performed on fractions corresponding to VLDL and LDL eluting positions for the father and sister (Figure 3C, D).

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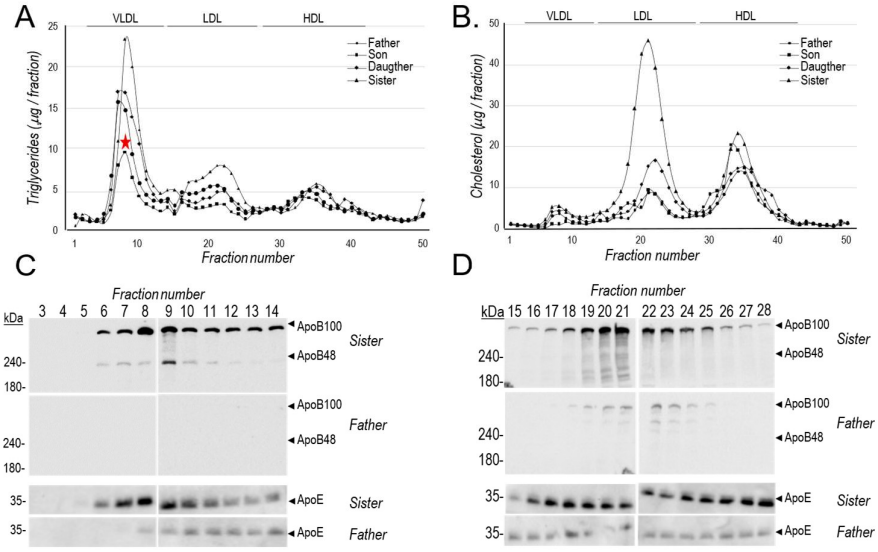

Figure 3. FPLC analyses: **(A, B)** FPLC analysis of total triglycerides (**A**) and cholesterol (**B**) levels in the plasma of all fasting subjects. The elution positions of VLDL, LDL and HDL are indicated. Western blot analyses: apoB and apoE in fractions corresponding to **(C)** VLDL (fractions 3 to 14) and **(D)** LDL (fractions 15 to 28) of the father and sister.

In order to obtain a more detailed lipoprotein profiling we solicited the help of a commercial LipoSEARCH service, consisting of a high resolution sensitive gel-filtration High Performance Liquid Chromatography (HPLC) method, which analyzes the major classes of lipoproteins including chylomicrons (CM), VLDL, LDL and HDL) for cholesterol and TG levels of lipoprotein components contained in 20 subclasses, providing a complete set of profiling data (https://www.ibl-japan.co.jp/en/business/diagnosis/inspection/) of lipids in plasma.

The data confirmed our FPLC comparisons between the father and his sister (Figure 3) and revealed a ~ 50-80% reduction in the levels of father's TG (Figure 4A) and cholesterol (Figure 4B) within CM, VLDL and LDL lipoprotein particles, but much less reductions in HDL particles (Figure 4). Interestingly, these lipid reductions had no impact on the size of the lipoprotein particles (Figure 4C).

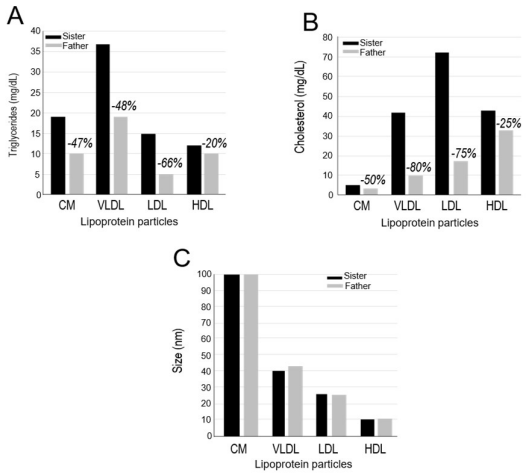

Figure 4. Lipid profiling and size of lipoprotein particles: Plasma lipoproteins from the sister (control) and father (subject) were separated by high resolution HPLC. The Triglycerides **(A)** and cholesterol **(B)** levels were then

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measured in each lipoprotein particle. **(C)** Nuclear magnetic resonance (NMR) spectroscopy was used to determine the size of the lipoprotein particles.

We conclude that all the above analyses converge towards the same conlusion that compared to the sister, the three other family members exhibit much lower cholesterol and TG levels in all lipoprotein particles, except for HDL particles, which exhibit a milder phenotype. In addition, we also observed a reduction in the levels of various lipoproteins including apoE, apoC2 and apoC3 and more profoundly for apoB100 (Figure 1B). Interestingly, compared to the sister, after fasting we could not detect apoB100 or apoB48 in the father's VLDL particles (Figure 3C), but smaller amounts of apoB100 were detected in the LDL fractions of the father (Figure 3D), likely reflecting low levels of circulating apoB100.

3.4. Genomic Sequencing – Identification of an APOB Single Nucleotide Heterozygote Deletion

The overall phenotype of the affected family members clearly reflected a possible link to genes implicated in the observed large reduction of lipoproteins including apoB100, apoC2, apoC3 and apoE, as well as >50% reductions in the levels of CM, VLDL and LDL lipoprotein particles. To explore the possibility that these variations may be related to lipid regulation or synthesis, we performed DNA sequencing of all family members that revealed no mutations in *LDLR*, *MTP*, protein disulfide isomerase (*PDI*), *APOE*, *APOC3 or APOC2*. However, the son exhibited a rare coding variant V12I in *APOC2* (rs150887575; APOC2:NM_000483:exon2:c.G34A:p.V12I; gnoMAD MAF=0.0003) within the signal peptide, which is absent from the father, daughter and sister. Thus, co-segregating changes in any of these genes could not explain the observed phenotype. In addition, the low LDLc and TG levels observed in the affected family members, are not a consequence of mutation(s) in the exomes or genes encoding the proprotein convertase *PCSK9*, a potent LDLR modulator [44,45] or *PCSK7* that regulates VLDL levels *via* apoB modulation [46,47].

In contrast, while the above results were confirmed upon whole exome sequencing of all family members, a clear single heterozygote variant was identified in all three affected family members but not in the sister's exomes. Here, a heterozygous frameshift deletion (hg38, chr2: 21032565-CT>C; APOB:NM_000384:exon10:c.1140delA:p.Q380fs) in *APOB* co-segregates with all three affected individuals (father, son, daughter) and is absent from the unaffected sister. Notably, Gln_{380} is conserved among vertebrates, and the observed variant is absent from gnomAD, with only a single missense carrier (p.Gln380Lys; MAF=$6.20x10^{-7}$) reported in gnomAD4.1.0 affecting the same APOB residue out of 806,980 sequenced individuals (https://gnomad.broadinstitute.org/gene/ENSG00000084674?dataset=gnomad_r4).

This conclusion was confirmed by Sanger sequencing, where the sister's control sequence TTTACA**A**GCC, has a single nucleotide Adenine (A) deletion in the sequence of the three affected members, resulting in a truncated TTTACA-GCC sequence (Figure 5A). Such deletion results in a A381P aa variant followed by a new 17 aa peptide and an early termination codon after Gly_{397} (Figure 5B). Because this results in a variant protein spanning ~9% (aa 28-397; apoB9) of the full-length protein (aa 28-4563), we propose to call it apoB9.

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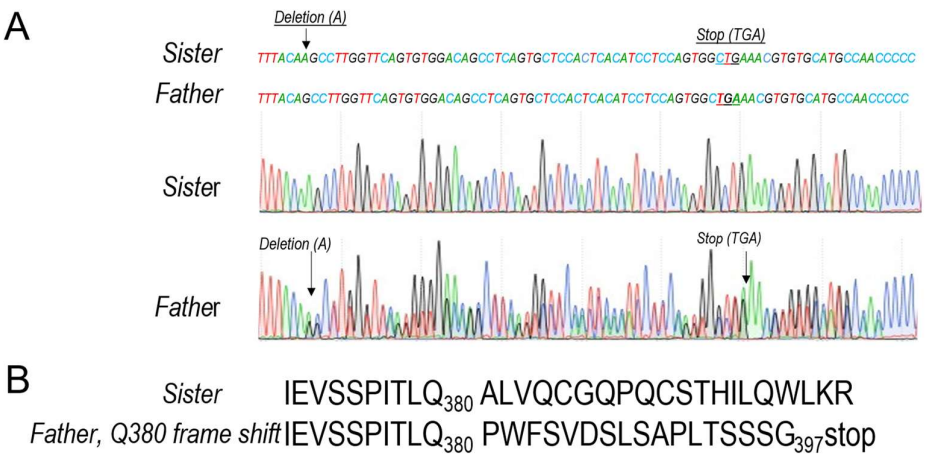

Figure 5. *APOB* gene sequencing: **(A)** Electropherograms showing a partial sequencing of the exon 3 of *APOB* gene in the control (sister) and the father (Q380 frame shift). The lower panel shows the corresponding sequence electropherograms from a normal control (top) and the proband (bottom). The putative deleted nucleotide is indicated by a star. **(B)** The single nucleotide deletion in exon 3 resulted in the substitution of an alanine to proline substitution at position 381 (A381P) just following Gln_{380}, and a frame shift premature stop codon at amino acid position 397, after the new 17 aa extended peptide (381-397).

Thus, the heterozygote single nucleotide deletion in the *APOB* gene observed in the three affected family members rationalizes the lipoprotein and lipid profile data. However, we also noted that the son had additionally a single nucleotide variant that leads to a V12I variation in the signal peptide. In that context, LOF mutations in the *APOC2* gene lead to severe, inherited hypertriglyceridemia and chylomicronemia [48] due to the inability to activate lipoprotein lipase (LPL), a key enzyme in triglyceride clearance [49]. Thus, we presume that the V12I variant would result in a GOF in accord with the lowest VLDL triglyceride levels as observed in the son compared to the rest of the family (Figure 3A).

3.5. ApoB9 Remains Within the Endoplasmic Reticulum and is not Secreted

We next aimed to define the possible fate of human apoB9 in hepatocytes. Thus, we generated a cDNA construct coding for apoB21 (21% of full-length protein) doubly tagged with 3xHA just after the signal peptide (26 aa) and with V5 at the C-terminus and cloned in a pIRES-EGFP vector under the control of a CMV promotor, as reported [46]. To mimic the apoB frame shift observed in affected family members, a single nucleotide deletion was introduced in apoB21 to generate a truncated TTTACA-GCC sequence that would result in an apoB9 protein ending with 17 aa following Gln_{380} (Figure 6A).

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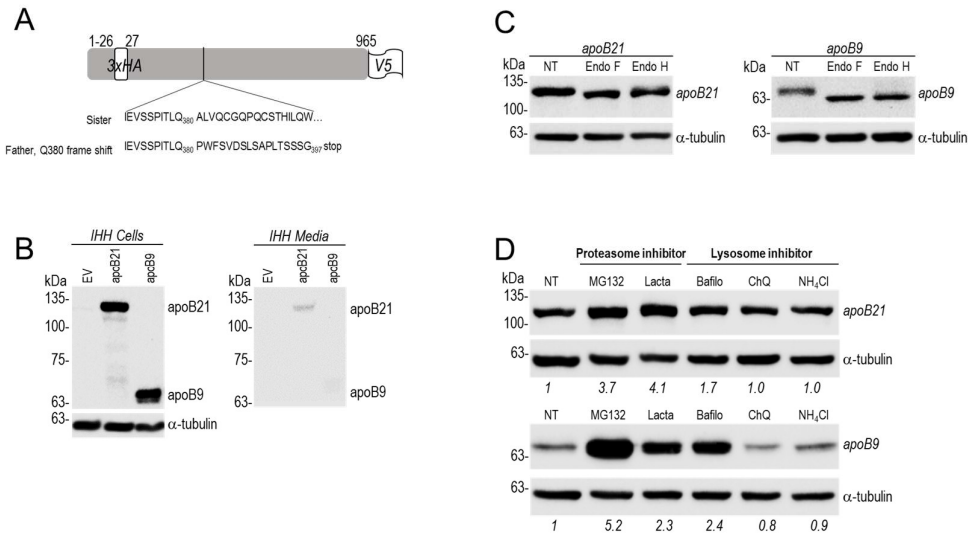

Figure 6. apoB expression in IHH naïve cells. **(A)** schematic representation of HA/V5 doubly tagged human apoB 21 (21%), as well as the apoB9 (9%) resulting from an early stop codon at position 397. (B) IHH cells were transiently transfected with an empty vector (EV) or vectors encoding indicated proteins, the proteins were then analysed in cell extracts and media using the N-terminal tag (HA tag). **(C)** Glycosidase treatment, Proteins (30 to 50 µg) from IHH cells expressing apoB21 (left panel) or apoB9 (right panel) were digested for 90 min at 37°C with endoglycosidase H (endo H) or endoglycosidase F (endo F) and analysed by western blot **(D)**. IHH transiently expressing apoB21 or apoB9 were treated for 24h with indicated protease inhibitors (MG132: 1 µM, Lactacystin (Lacta): 30 µM, Bafilomycin (Bafilo): 0.1 µM, NH_4Cl: 10 mM, Chloroquine (ChQ): 50 µM). NT = non-treated cells. The cells extracts were then analysed by Western blot using HA tag. Quantification of immunoreactive proteins was performed using Image Lab software (Bio-Rad).

We thus analyzed in human hepatocyte IHH cells the expression of cDNAs encoding apoB21 or apoB9 compared to a control empty vector (EV). Western blot data using an HA-tag revealed that while intracellularly both proteins are similarly visible on the gel, only a fraction of apoB21 was secreted into the medium, but apoB9 was absent (Figure 6B). The latter is clearly visible in cells migrating with an apparent molecular size of ~60 kDa.

We hypothesized that the exit of apoB21 and apoB9 from the ER may be differentially modulated. To support this, we tested the sensitivity of cell extracts containing these proteins to digestion by endoglycosidase H (endo H) and PNGase F (endo F). Endo H cleaves high-mannose and hybrid glycans in the ER but not complex glycans found in the Golgi apparatus. In contrast, endo F cleaves all types of N-linked glycans, including high-mannose, hybrid, and complex glycans, making it suitable for a broader range of deglycosylation [50]. Using these criteria, endo H resistant forms are mature N-glycosylated proteins and hence reflect the forms that exited the ER. In contrast endo H sensitive forms likely represent proteins that are still in the ER. In both cases endo F treatment would result in non-glycosylated proteins of similar molecular size. The data revealed that intracellular apoB9 is sensitive to endo H digestion but not apoB21 (Figure 6C). This indicates that difference from apoB21, the apoB9 isoform resides in the ER and hence is not secreted.

It is well known that a fraction of non-lipidated apoB100 is degraded by the proteasome, to regulate the amount of secretion of newly synthesized apoB100. The latter is regulated by the proteasome through an ER-associated degradation (ERAD) pathway where misfolded or improperly assembled apoB100 is exported from the ER into the cytosol, polyubiquitinated, and subsequently degraded by the ubiquitin-proteasome system (UPS). This degradation is a key quality control mechanism that impacts VLDL particle secretion and hepatic TG production [51]. Factors like MTP inhibition or insufficient lipid availability can lead to apoB100 misfolding and increased proteasomal degradation [52].

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Accordingly, IHH cells expressing apoB21 or apoB9 were treated for 24h with proteasome inhibitors (MG132 and Lactacystin) or alkalinizing agents (Bafilomycin, NH_4Cl or Chloroquine). Western blot analysis of the intracellular apoB proteins revealed that the levels of both proteins are not affected by NH_4Cl or Chloroquine, but are significantly higher in presence of proteasome inhibitors, especially with MG132 for apoB9 (Figure 6D). Overall, our data suggest that both proteins are partially degraded by the proteasome, but that only apoB9 is not secreted and likely resides in the ER. These results rationalize the low levels of plasma apoB (-68%, estimated by proteomics) in the affected family members who are heterozygote for apoB9 (Figure 1B).

3.6. Molecular Models of apoB9 and apoB21

ApoB9 contains residues 28-380 of mature apoB100 and includes only the β-barrel and the first three helices from the α-solenoid region within the N-terminal βα1 domain of apoB100 (Figure 7). Importantly, apoB9 lacks the βA- and βB-sheets underlying the β-barrel and α-solenoid that are required for lipid particle formation [3,53,54]. In contrast, apoB21 contains the βA-sheet and part of the βB-sheet and can form lipid particles, although mostly as unstable aggregates [54]. The availability of lipids and apoB lipid-binding ability affects its secretion efficiency [55,56]. This is consistent with our observation that apoB9 was retained in the ER, whereas apoB21 was secreted.

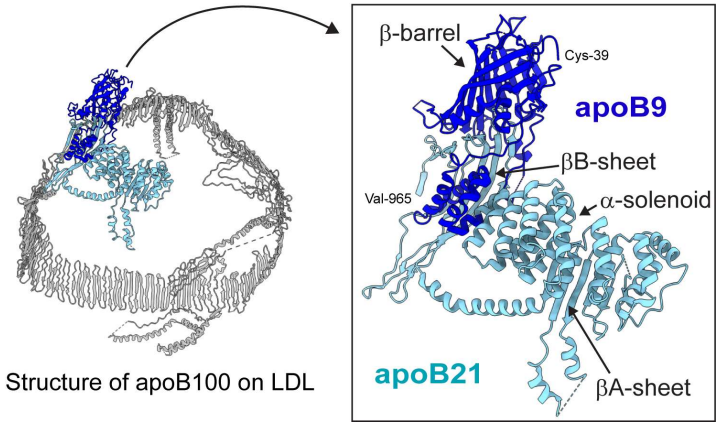

Figure 7. CryoEM structure of apoB100 on LDL (PDB 9BDT). The inset shows apoB21 (residues 28-965; based on PDB 9BD1), with the apoB9 region (residues 28-380) highlighted in dark blue. Note that residues 28-38 are missing in the structure.

4. Discussion

Mutations in the *APOB* gene result in defective forms of the apoB100 protein that is crucial for transporting cholesterol on lipoproteins particles. This leads to the accumulation of LDLc in the bloodstream, thereby increasing the risk of premature CVD. So far, more than 4661 germ line *APOB* gene variants were reported, most of which are missense variants (2686; 58%) or result in frameshift mutations (117; 0.03%) (https://www.ncbi.nlm.nih.gov/clinvar/?term=%22apoB%22%5BGENE%5D&redir=gene). The *APOB* gene comprises 29 exons and the pathogenic mutations are distributed throughout the coding region. Most of these variants are in the C-terminal domain essentially in the last 4 exons of the *APOB* gene [57]. However, heterozygote *APOB* gene truncations that cause FHBL, result in an apoB protein that is too short to function properly, leading to low levels of circulating LDLc and are likely protective against CVD.

Familial hypobetalipoproteinemia (FHBL) is an autosomal co-dominant disorder, defined by plasma concentrations of total cholesterol, low density lipoprotein cholesterol LDL-C and apolipoproteinB100 below the 5th percentile. The frequency of apoB truncations is reported to be 1:3000. FHBL can be caused by mutations in the two differents genes: (1) the gene coding for the MTP [25,26] which is essential for the assembly of apolipoprotein B (apoB)-containing lipoproteins

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including chylomicrons and VLDL. Its primary role is to facilitate the transfer of lipids, particularly triglycerides, into the lumen of the ER, which is necessary for the proper folding and secretion of apoB-lipoprotein particles. Without MTP, apoB proteins are not efficiently secreted from the liver and intestine. (2) The *APOB* gene encodes the essential protein that forms the structural apoB-containing lipoproteins, such as VLDL and LDL. Its role is to mediate the assembly and secretion of these particles from the liver and small intestine by providing the structural framework and a single copy of apoB on each lipoprotein particle. During this process, apoB acts as a docking site for lipids, which are transferred to it by proteins to build the final lipoprotein particle.

LOF *APOB* mutations most often result from deletions or substitutions that cause premature termination of *APOB* mRNA translation, leading to truncated, non-functional proteins and low LDLc [28-30]. In human, various truncated apoB forms were reported, ranging in molecular weight from 9% (apoB9) and 89% (apoB89) of the full length apoB100 have been described [29,31-33]. It has been shown that the C-terminal truncation of apoB affects the size of the resulting apoB-associated lipoproteins and their fate and that lipid recruitment by apoB is progressively reduced by its C-terminal truncation resulting in reduced protein stability [34-36]. Only truncated forms longer than 27% (apoB27) were observed to be secreted into the plasma [37,38].

Herein, we present evidence for the existence of an apoB9 form in the genome of three family members that exhibit ~70% lower levels of circulating apoB100 and of total and LDLc. We rationalized such low levels by the inability of apoB9 to be secreted from hepatocytes (Figure 6B), its retention in the ER (Figure 6C) and its degradation by the proteasome (Figure 6D).

We thus, wished to provide a structural rationale for our observations. Since apoB9 lacks the critical lipid-binding βA- and βB-sheets (Figure 7), it likely has poor lipid affinity and cannot form lipid particles. The secretion efficiency of truncated apoB forms correlates with their length and lipid-binding capacity [55,56]. For example, apoB18 (aa 28-808) [36]) and apoB17.6 (aa 28-827), which contain the βA- but not the βB-sheet, are secreted from cultured cells but at a much lower rate than apoB20.5 or apoB21.08 (aa 28-958 and aa 22-983, respectively) [54]. Notably, secretion of monodisperse and stable particles in cell culture requires the complete βα1 domain (apoB22, aa 28-1027) [54], which is still in large part in apoB21 (aa 28-965; Figure 7), likely rationalizing its low secretion levels (Figure 6B). Altogether, these findings explain why apoB9 is retained in the ER, whereas apoB21 is secreted.

The novel frameshift mutation which we identified in apoB segregates in a dominant manner and due to lifelong reductions in atherogenic proteins, familial hypolipoprotein patients exhibiting such rare protein-truncating variants in the *APOB* gene have a 72% reduced risk for coronary heart disease (CHD) [58]. The principal clinical concern in these patients is a risk for hepatic steatosis, which affects a small minority of individuals [59]. Some cases of severe liver disease [60], cirrhosis [61] and hepatocellular carcinoma [62] have been reported. Genetic testing is important to determine whether these patients require long term follow up for liver disease compared to PCSK9 loss of function carriers who do not demonstrate any adverse liver complications.

These data suggested that the affected family members identified in this study could be protected against the development of atherosclerosis and various aspects of CVDs. In fact, research has shown that apoB is a stronger predictor of cardiovascular events, such as heart attacks and strokes, than LDLc [5-8].

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