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Novel protein-truncating variant in the *APOB* gene may protect from coronary artery disease and adverse cardiovascular events

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ABSTRACT

Background and aims: Genetic testing is still rarely used for the diagnosis of dyslipidemia, even though gene variants determining plasma lipids levels are not uncommon.
Methods: Starting from a a pilot-analysis of targeted Next Generation Sequencing (NGS) of 5 genes related to familial hypercholesterolemia (*LDLR*, *APOB*, *PCSK9*, *HMGCR*, *APOE*) within a cardiovascular cohort in subjects with extreme plasma concentrations of low-density lipoprotein (LDL) cholesterol, we discovered and characterized a novel point mutation in the *APOB* gene, which was associated with very low levels of apolipoprotein B (ApoB) and LDL cholesterol.
Results: *APOB* c.6943 G > T induces a premature stop codon at the level of exon 26 in the *APOB* gene and generates a protein which has the 51% of the mass of the wild type ApoB-100 (ApoB-51), with a truncation at the level of residue 2315. The premature stop codon occurs after the one needed for the synthesis of ApoB-48, allowing chylomicron production at intestinal level and thus avoiding potential nutritional impairments. The heterozygous carrier of APOB c.6943G > T, despite a very high-risk profile encompassing all the traditional risk factors except for dyslipidemia, had normal coronary arteries by angiography and did not report any major adverse cardiovascular event during a 20-years follow-up, thereby obtaining advantage from the gene variant as regards protection against atherosclerosis, apparently without any metabolic retaliation.
Conclusions: Our data support the use of targeted NGS in well-characterized clinical settings, as well as they indicate that.a partial block of ApoB production may be well tolerated and improve cardiovascular outcomes.

Introduction

Although gene variants influencing plasma lipids and, consequently, cardiovascular risk are not rare (e.g. familial hypercholesterolemia (FH) is recognized as the most common genetic cause of CVD), genetic testing is still underused and these conditions remain often underdiagnosed [1].

Changes in the DNA sequence of the crucial genes regulating cholesterol homeostasis, like LDL-receptor (*LDLR*) and apolipoprotein B (*APOB*), are usually considered for the issue of hypercholesterolemia, but according to the type of mutation they can cause either high or low levels of LDL cholesterol.

Familial hypobetalipoproteinemia (FHBL) is an autosomal codominant genetic disorder, which is mainly due to nonsense and/or missense variants affecting *APOB* gene. However, FHBL is also genetically heterogeneous and other genes may be involved, like proprotein convertase subtilisin/kexin 9 (*PCSK9*) [2,3] and angiopoietin-like 3 protein (*ANGPTL3*) [4–7]. FHBL is characterized by very low plasma concentration of apolipoprotein B (ApoB) and

Abbreviations: ApoB, Apolipoprotein B; CAD, Coronary Artery Disease; NGS, Next Generation Sequencing.

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LDL cholesterol, while its clinical manifestations vary according to the number and the severity of the carried mutations. Subjects with heterozygous FHBL may be asymptomatic or at most with mild hepatic steatosis, while those with severe biallelic mutations had potentially severe health problems, like advanced nonalcoholic fatty liver disease, gastrointestinal and neurological abnormalities, and malnutrition with deficiency of fat-soluble vitamins [4].

In the present work, within a pilot-analysis of targeted next-generation sequencing (NGS) of 5 genes related to FH (*LDLR*, *APOB*, *PCSK9*, *HMGCR*, and *APOE*) in a subsample of subjects within the cardiovascular cohort of the Verona Heart Study (VHS) with extreme plasma levels of LDL cholesterol, we discovered and characterized a novel point mutation in the *APOB* gene generating the truncated isoform ApoB-51, which was associated with both very low LDL levels and striking protection from coronary artery disease (CAD).

Materials and methods

This study was performed within the framework of VHS, a regional observational survey aimed to look for CAD risk factors in subjects with angiographic documentation of their coronary vessels [8,9]. The study was approved by the Ethic Committee of our Institution (Azienda Ospedaliera Universitaria Integrata, Verona). A written informed consent was obtained from all the participants.

In a NGS-pilot analysis we selected 24 subjects not taking any lipid-lowering drugs at time of enrollment who had either very high ($\geq$190 mg/dL) or either very low ($\leq$60 mg/dL) values of LDL cholesterol plasma levels (threshold values were arbitrarily defined). In the present work, we addressed our interest on the latter group.

Samples of venous blood were drawn from each subject, after an overnight fast, at the time of enrolment. Serum lipids and apolipoproteins were determined as previously described [8,9]. [10,11].

Genomic DNA was purified from whole blood using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). After quantity and quality check, target enrichment and library preparation were carried out through the NebNEXT Direct Custom Panel (*New England Biolabs, Ipswich, MA, USA*). DNA libraries were sequenced on Illumina NextSeq500 (Illumina, San Diego, CA, USA). The reads were mapped to the hg38 reference genome using BWA-mem 0.7.15. The alignment files were processed with fgbio 0.4.0 and Picard 2.17.6. The variant calling procedure was performed with the GATK HaplotypeCaller algorithm and only the genetic variants presenting an estimated Minor Allele Frequency (MAF) lower than 5% were selected and their possible pathogenetic impact was predicted according to five tools: SIFT, PolyPhen-2 HVAR, MutationTaster, MutationAssessor and FATHMM. *APOB* variant classification was referred to NM_000384.3 and NP_000375.3.

APOB c.6943 G > T variant was validated by Sanger sequencing (Forward primer 5'-CTTCATACCTCTCGATTAAC-3', Reverse primer 5'-GAATATAGACATCCAGCACC-3).

The effect of *APOB* c.6943 G > T variant was visually represented on the low-resolution model of ApoB kindly provided by Prof. Ruth Prassl [12]. ApoB-48 and ApoB-51 three-dimensional structures were predicted with AlphaFold [13], modelling the first, the second and a part of the third domain of ApoB-100, separately because of limitations in the number of residues to be modeled. The best model for each domain was selected according to the predicted local distance difference test (pLDDT). The putative low-resolution full-length structure was hypothesized by joining the N-terminal and the C-terminal ends of consecutive domains with UCSF Chimera 1.14.0. The three-dimensional structures were visualized and edited through PyMOL Molecular Graphics System, 2.4.1, Schrödinger, LLC.

Results

In the 6 subjects with very low LDL cholesterol plasma levels ($\leq$60 mg/dL), among the 5 genes which were sequenced, only one *APOB* rare gene variant was found, while no relevant mutations were observed in *LDLR*, *PCSK9*, *HMGCR*, and *APOE*. More precisely, we identified a heterozygous point mutation: *APOB* c.6943 G > T, inducing a premature stop codon at the level of exon 26 in the *APOB* gene and generating a protein which has the 51% of the mass of the wild type ApoB-100 (named ApoB-51), with a truncation at the level of residue 2315.

It is worth noting that the subject carrying this heterozygous point mutation was CAD-free in spite of an apparent very high-risk cardiovascular profile.

A 43-year-old Caucasian man was admitted to our Internal Medicine Unit because of congestive heart failure in dilated cardiomyopathy. The family history was scanty (no data on parents, one sister reported without health problems) while his personal history was characterized by several risk factor for cardiovascular disease. He was obese, active heavy smoker, and heavy-drinker. He had a history of arterial hypertension since about 10 years, for which he was not taking any regular therapy, and previous finding of impaired fasting glucose. On the other hand, his laboratory profile of plasma lipids showed very low levels of cholesterol and low triglycerides levels, normal levels of ApoA-I, very low levels of ApoB and Lp(a), low levels of ApoC-III and ApoE (Table 1). Abdominal ultrasound showed mild hepatomegaly and hepatic steatosis. Liver function tests were substantially in normal ranges. Taking into account the multiple risk factors for atherosclerosis (with the notable exception of dyslipidemia) defining a high-risk profile for ischemic heart disease, a coronary angiography was performed, but epicardial coronary vessels appeared normal without any atherosclerotic stenosis. Finally, he was discharged with a diagnosis of acute heart failure in dilated cardiomyopathy, which was probably related to the high alcohol intake. Drug therapy with loop diuretics and ACE-inhibitors was prescribed and it was recommended to stop both smoking habit and alcohol consumption. After discharge, the patient was followed by telephone calls and review of medical records. After a 20-years follow-up, although he continued smoking, no major adverse cardiovascular events and/or related hospitalizations were reported.

The presence of the *APOB* 6943 G > T variant in heterozygosis was verified by the double peaks shown in the electropherogram resulting from the Sanger sequencing (Fig. 1A), confirming the existence of the stop codon TAG, which had never been reported so far. Conversely, in this position the G > A substitution was previously reported, leading to the glutamate to lysine substitution (Glu2315Lys, rs1223711938) [dbSNP, https://www.ncbi.nlm.nih.gov/snp/accessed in March 05, 2022].

The three-dimensional model of ApoB-51 (Fig. 1B) obtained with AlphaFold [13] presented an average pLDDT of 83.75 and revealed a secondary structure composition consistent with the information present in literature [14 12]. Our model allowed to visually assess the structural impact of the truncation due to the presence of the *APOB* 6943 G > T variant generating ApoB-51, also in relation to the size of the intestinal isoform ApoB-48 (Fig. 1B). Indeed, it can be vividly appreciated that the variant truncates about half of the translated protein (grey region in Fig. 1B), with possible consequences in the structural/functional properties of the protein.

Discussion

In this study, we characherized a novel *APOB* point mutation, *APOB* c.6943 G > T, codifying for an ApoB truncating isoform (ApoB-

Table 1
Clinical and laboratory characteristics of the heterozygous carrier of the truncating variant *APOB* c.6943G > T.

Ancestry	Caucasian
Sex	Male
Age	43 years
Coronary artery disease status (angiographically demonstrated)	CAD-free
Body Mass Index	36.0 kg/m^2
Smoke	60 cigarettes/day
Alcohol intake	6 alcohol units/day
Hypertension	Yes
Diabetes	No
Glucose	115 mg/dL
Total Cholesterol	88 mg/dL
HDL-Cholesterol	32 mg/dL
LDL-Cholesterol	44 mg/dL
Triglyceride	75 mg/dL
ApoA-I	0.98 g/L
ApoB	0.30 g/L
ApoC-III	3.91 mg/dL
ApoE	0.024 g/L
Lp (a)	<28 mg/L
Creatinine	0.67 mg/dL
AST	23 U/L
ALT	24 U/L
GGT	22 U/L
Bilirubin	1.17 mg/dL
Albumin	37.6 g/dL
Fibrinogen	3.68 g/L
PT	1.09
aPTT	1.11

51) and apparently conferring protection from CAD. It was clinically impressive to find such mutation in a subject without any sign of atherosclerosis at coronary vessels and with a long follow-up free from cardiovascular events in spite of a high-risk cardiovascular profile.

APOB gene codifies for ApoB, which is a structural component of lipoproteins and is the key ligand for the LDL receptor. ApoB occurs in plasma as two main isoforms, ApoB-48 and ApoB-100, by means of post-transcriptional regulation [15]. The former is the main apolipoprotein of chylomicrons, while the latter is the main apolipoprotein of LDLs. ApoB-100 represents the full-length transcript containing 4536 amino acids, while ApoB-48 [16] represents the amino-terminal 2152 amino acids of ApoB-100. ApoB-100 contains within the carboxy-terminus the domain required for interaction with the LDL receptor, as well as an unpaired cysteine residue mediating a covalent interaction with apo(a) generating Lp(a). ApoB-48 lacks these domains, thus having important differences in functional properties [17].

Mutations in *APOB* gene may have very heterogeneous functional consequences, leading to either hypercholesterolemia like FH (typically due to missense variants within the LDLR binding domain) or hypocholesterolemia like FHBL (typically due to mutations affecting the structural integrity of ApoB) [18]. *APOB* rare truncating gene variants causing FHBL have been recently shown to confer protection against CVD, according with reduced plasma levels of LDL cholesterol and triglyceride [19]. An association with psychiatric disorders has been also proposed [20].

From a biochemical point of view, ApoB three-dimensional structure characterization is still a challenge, mainly because its size and its poor solubility in aqueous environment [21]. However, both low-resolution experimental results and computational predictions are consistent in suggesting a structural model composed by five domains [14,22]:

NH_2- $\beta\alpha_1$ - β_1 - α_2 - β_2 - α_3 —COOH

The alternating α-helix and β-strand regions reflects an amphipathic nature which is essential for the association with the lipid core of lipoproteins. The N-terminal domain plays an essential role in the initial assembly of chylomicrons and VLDLs [23]. *In vitro* studies showed an association of ApoB length with both lipoprotein level and lipoprotein lipid content [24,25].

The premature stop codon generated by the variant *APOB* 6943 G > T induces an interruption of protein synthesis at about the half of α_2 domain as shown in Fig. 1B. The truncation specifically occurs at residue 2315, blocking the formation of a part of the α_2 domain and the totality of β_2 and α_3 domains, which account for approximately the 50% of the entire ApoB (ApoB-51). The N-terminal $\beta\alpha_1$ and β_1 domains are preserved, allowing the correct ApoB-containing lipoproteins secretion [26]. Chylomicrons can still be produced because this variant occurs after the natural stop codon, inducing a truncation at the level of residue 2179 and leading to the synthesis of ApoB-48 [27]. On the other hand, considering VLDL production, it is likely that the truncated ApoB-51 will generate lipoproteins with smaller size and lower lipid content. Speculatively, the ApoB-51 variant may have a functional impact similar to the ones observed by Parhofer et al. [28], especially the truncated protein ApoB-54.8, which has reduced VLDL secretion rates and minimal conversion rates to IDLs and LDLs [28]. Our data are also consistent with the findings of a pioneristic work identifying an APOB truncating variant leading to isoform ApoB-50 in a subject with normotriglyceridemic abetalipoproteinemia. In the homozygous carrier of this variant, fat absorbtion and the synthesis of chylomicrons were normal [29]. Another truncated isoform, ApoB-48.4, has been associated with hypobetalipoproteinemia with a normal fat absorption, as expected for isoforms with a length similar to that of apo B-48 [30].

Notably, the subject with heterozygous carriership of *APOB* c.6943 G > T mutation seems to obtain advantage from the gene variant as regards protection against atherosclerosis, apparently without any metabolic retaliation. Although the patient did not repeat coronary angiography during the follow-up and we cannot exclude the development of subsequent atherosclerotic plaques, the 20 years-long follow-up without major adverse cardiovascular

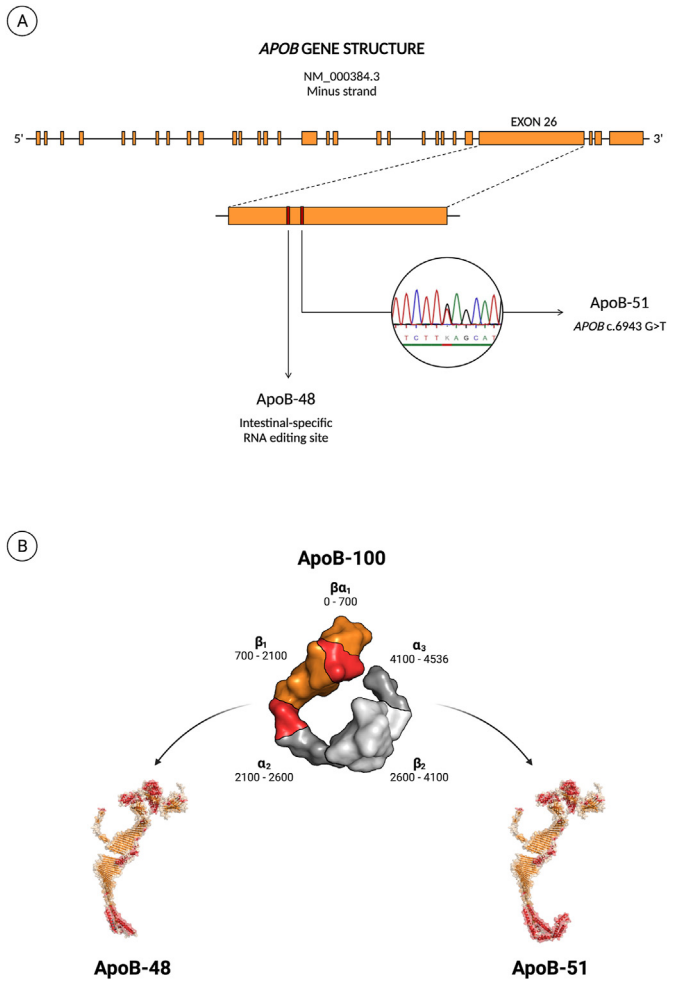

Fig. 1. Schematic representation of *APOB* genetic and structural features.
(A) *APOB* gene architecture. The variant *APOB* c.6943 G > T occurs in heterozygosis at the level of exon 26 as shown by the electropherogram resulting from Sanger sequencing. The double peak indicated by K illustrates the presence of both guanine and thymine. (B) Graphical representation of the variant *APOB* p. Glu2315Ter obtained by editing of the low-resolution model of ApoB-100 provided by Prof. Ruth Prassl [12]. The part of the protein successfully translated (as it occurs before the premature truncation, generating the mutant ApoB-51) is represented by the orange-red region, while the missing regions are shown in grey. The models of ApoB-51 and ApoB-48 were colored according to the secondary structure composition, with α-helixes and β-sheets in red and orange, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

events *per se* supports the hypothesis of a protective role of ApoB-51 against CVD. This favorable condition may be due to specific characteristics of this mutation, which could be considered benign for two main reasons. First, it occurs in heterozygosis, allowing a residual production of ApoB and thus limiting the possible detrimental effects related to a complete block of ApoB production. Second, the premature stop codon occurs after the one needed for the synthesis of ApoB-48, allowing chylomicron production at intestinal level and thus avoiding potential nutritional impairments. Speculatively, from a ApoB-lowering therapeutic point of view [4,27], this variant emphasizes that a block of ApoB production, which is not complete and allows the synthesis of ApoB-48, could be well tolerated without major harmful side effects.

Our study has limitations which should be acknowledged, like the use of a restricted gene panel which could reduce the capacity to detect other significant variants in modulating plasma lipids and cardiovascular risk, like in *ANGPTL3* and *APOC3* [31]. Nonetheless, our work suggests that targeted NGS of appropriately selected genes in accurately phenotyped subjects and in well characterized clinical settings may lead to potentially valuable findings.

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Authors’ contribution

NM and OO designed the study. NM and AC supervised the study. GM, NO, SU, AC, and AV performed the experiments and collected the data. SF, FP, DG and GM contributed to the data analysis. NM, AC, SU, GM, and NO contributed to the manuscript writing and data interpretation. All authors: drafted the article and approved the final version for submission.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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